INVESTIGATION OF INFLAMMATION ASSOCIATED CYP2E1 AND CYP1A1 EXPRESSION IN THE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS MOUSE MODEL OF MULTIPLE SCLEROSIS

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ABSTRACT

INVESTIGATION OF INFLAMMATION ASSOCIATED CYP2E1 AND CYP1A1 EXPRESSION IN THE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS MOUSE MODEL OF MULTIPLE SCLEROSIS

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Multiple sclerosis (MS) is an autoimmune neurodegenerative disease which affects brain and spinal cord. Myelin which wraps around the axon and its progenitor cells, oligodendrocytes, are destroyed in this disease. This results in the loss of signal transmission which leads to axonal, and eventually neuronal loss. There are various symptoms of the disease such as unstable feelings, fatigue, visual disability, muscle spasms, and walking difficulties. The etiology of the disease is still in its infancy; however, genetic, environmental factors, and abnormal immune responses are known to play a role in the development of multiple sclerosis.

Cytochrome P450, one of the members of monooxygenases, are the enzymes predominantly localized in the liver but also in lung, brain, and kidney. They have an essential role in the metabolism of drugs, other xenobiotics, and endogenous substrates and activation of the procarcinogens. CYP2E1 is the second highly expressed CYP450 enzyme in liver and is also found in brain. CYP1A1 is mainly localized in lung but also in the brain and liver. The expression of CYP450 enzymes

varies under inflammatory conditions. Accordingly, the bioavailability of drugs and other exogenous and endogenous substrates that are metabolized by these enzymes will be equally affected. Therefore, as multiple sclerosis is an inflammatory neurodegenerative disorder, unique expression levels of these enzymes need to be examined in these conditions. The possible change in gene (mRNA) and protein expression of CYP2E1 and CYP1A1 enzymes in liver and brain in the case of MS has not been investigated before.

In this study, female C57BL/6 mouse model for experimental autoimmune encephalomyelitis (EAE - MS Model) was used to investigate the effects of EAE on mRNA and protein levels of CYP2E1 and CYP1A1 by qPCR and Western blotting techniques, respectively. The mRNA and the protein expression of the control group were equilibrated to 1.00 fold and the relative expression of the EAE group were estimated. The results of this study illustrate that the EAE immunization was not significantly affecting the mRNA and the protein levels of CYP2E1 and CYP1A1 in mice liver and brain. Nevertheless, protein expression of CYP2E1 is slightly higher in the EAE group compared to the control group. Moreover, CYP2E1 and CYP1A1 mRNA expressions were increased slightly in the liver of the EAE group and CYP2E1 mRNA expression was decreased slightly in the brain of EAE group compared to the control group, but the results were not statistically significant.

Keywords: Multiple Sclerosis (MS), CYP2E1, CYP1A1, Experimental autoimmune encephalomyelitis (EAE), Brain, Liver, Mouse, Inflammation

ÖZ

İNFLAMASYONLA İLİŞKİLİ CYP2E1 VE CYP1A1 EKSPRESYONUNUN DENEYSEL OTOİMMÜN ENSEFALOMYELİT MULTİPL SKLEROZ FARE MODELİNDE ARAŞTIRILMASI

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Multipl skleroz (MS), beyni ve omuriliği etkileyen otoimmün nörodejeneratif bir hastalıktır. Bu hastalıkta aksonun etrafını saran miyelin kılıf ve onun progenitör hücresi olan oligodendrosit hücreleri zarar görür. Bu, sinyal iletiminin yavaşlamasına veya durmasına ve önce aksonal kayba sonrasında da nöronal kayba yol açar. Duygu dengesizliği, yorgunluk, görme bozukluğu, kas spazmları ve yürüme güçlüğü hastalığın belirtileri arasındadır. Hastalığın etyopatogenezi henüz çözülememiştir ancak multipl skleroz gelişiminde ve oluşumunda genetik ve çevresel faktörlerin ve anormal bağışıklık yanıtlarının rol oynadığı bilinmektedir.

Sitokrom P450 monooksijenazlar çoğunlukla karaciğerde bulunurlar ancak akciğer, beyin ve böbrekte de bulunmaktadırlar. İlaçların ve diğer ksenobiyotiklerin, endojen moleküllerin metabolizmasında ve karsinojenlerin biyotranformasyonuda önemli görevleri vardır. Karaciğerde en çok ekspres edilen ikinci CYP ailesi üyesi enzim CYP2E1 enzimidir. Bu enzim ayrıca beyinde de ekspres edilmektedir. CYP1A1 ise beyinde ve karaciğerde ekspres edilmektedir. İnflamasyon durumunda CYP ailesi enzimlerinin ekspresyonları değişiklik göstermektedirler. Dolayısıyla, bu CYP enzimleri ile metabolize olan ilaçların, ksenobiyotiklerin ve endojenik moleküllerin

biyoverimliliği de etkilenmektedirler. Bu yüzden CYP enzimlerinin ekpresyonlarının nörodejeneratif inflamatuvar bir hastalık olan MS durumunda incelenmesi gerekmektedir. CYP2E1 ve CYP1A1 enzimlerinin gen ifade (mRNA) ve protein düzeyleri multipl skleroz hastalığı durumunda daha önce incelenmemiştir.

Bu çalışmada dişi C57BL/6 farelerde deneysel otoimmün ensefalomiyelit (DOE- MS Modeli) modeli oluşturularak multipl skleroz hastalığının karaciğerdeki ve beyindeki CYP2E1 ve CYP1A1 enzimlerinin mRNA ve protein ekpresyonları üzerindeki etkisi qPCR ve Western blot teknikleri ile incelenmiştir. Kontrol grubunun mRNA ve protein ekspresyonları 1.00 kat kabul edilerek DOE grubunun ekspresyonları hesaplanmıştır. Bu çalışmanın sonuçlarına göre, DOE immünizasyonu, fare beynindeki ve karaciğerindeki CYP2E1 ve CYP1A1 enzimlerinin gen ifadesini ve protein seviyesini önemli ölçüde değiştirmemiştir. Ancak karaciğerdeki CYP2E1 protein seviyesi önemli ölçüde olmasa da artmıştır. Ayrıca CYP2E1 ve CYP1A1 mRNA seviyesi de karaciğerde artmıştır ancak önemli bir ölçüde değildir.

Anahtar Kelimeler: Multipl Skleroz (MS), CYP2E1, CYP1A1, Deneysel Otoimmün Ensefalomyelit (DOE), Beyin, Karaciğer, Fare, İnflamasyon

This work is dedicated to my family.

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LIST OF ABBREVIATIONS

ABBREVIATIONS

AhR	Aryl Hydrocarbon Receptor
AP	Alkaline phosphatase
APS	Ammonium per sulfate
BCA	Bicinchoninic acid
BCIP	5-bromo 4-chloro 3-indoyl phosphate
bp	Base pair
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EROD	ethoxyresorufin-O-deethylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA

HS	High spin
LPS	Lipopolysaccharide
LS	Low spin
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger RNA
MS	Multiple sclerosis
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NBT	Nitrotetrazolium blue chloride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
PXR	Pregnane X receptor
qPCR	Quantitative polymerase chain reaction
rpm	Revolutions per minute
RNA	Ribonucleic acid
RRMS	Relapsing-remitting multiple sclerosis
ROS	Reactive oxygen species
SDB	Sample dilution buffer
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline

TBST	Tris-buffered saline and Tween 20
TEMED	Tetramethylethylenediamine
TNFα	Tumor necrosis factor α

LIST OF SYMBOLS

SYMBOLS	
^{o} C	Centigrade degree
g	Gravitational force
kDa	Kilo Dalton
µ	Micro

CHAPTER 1

INTRODUCTION

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune neurodegenerative disease which affects the central nervous system. MS is characterized by inflammation, demyelination, and axonal and neuronal loss. Myelin which wraps around the axon and its progenitor cell, oligodendrocyte, are destroyed. This results in the loss of signal transmission which leads to axonal, and eventually neuronal loss. The etiology of the disease has not been solved yet; however, genetic and environmental factors, and abnormal immune responses are known to play a role in the development of multiple sclerosis (Ortiz et al., 2013).

There are four different forms of MS: relapsing remitting MS (RRMS), secondary progressive (SP-MS), primary progressive (PP-MS), and progressive relapsing MS (PR-MS). The most common form, RRMS, shows attacks (relapses) of various neurologic symptoms, unstable feelings, fatigue, visual disability, muscle spasms, walking difficulties, and balance problems (Signorile et al., 2020, Tobore, 2019). This relapse period is followed by a partial or complete recovery which is called remission (Signorile et al., 2020). 80 % of RRMS progress into SP-MS, in which initial relapsing-remitting is followed by worse neurological symptoms. PRMS is a combination of progression and relapse, in which the neurologic problems accumulate independently from relapses. The last form, PPMS, is characterized by the accumulation of disabilities from the onset of the disease (Lublin et al., 1996).

MS usually affects people between the ages of 20 and 45 years. However, children are also known to be diagnosed with MS (Renoux et al., 2007, Compston & Coles,

2008). Women have been diagnosed with MS more than men (2:1 or 3:1 ratio) (Renoux et al., 2007, Orton et al., 2006). There are different hypothesizes regarding multiple sclerosis pathogenesis. One of them suggests that neuroinflammation, neurodegeneration and myelin damage are caused by the increased migration of the autoreactive T cells and B cells through the blood brain barrier (Fig 1.1) (Compston and Coles, 2008; Fletcher et al., 2010; Trapp and Nave, 2008). Another hypothesis suggests that primary oligodendrocyte dysfunction is the primary cause of the pathogenesis of the diseases (Barnett and Prineas, 2004).

Moreover, despite the underlying mechanisms of multiple sclerosis have not been revealed yet, studies suggest that oxidative stress, mitochondria dysfunction, thyroid dysfunction and sex hormones play an essential role in multiple sclerosis pathology. Also, variation in human leukocyte antigen (HLA) system was suggested as one of the genetic contributor to MS.

Figure 1.1 illustrates the MS inflammation (Ohl et al., 2016). In periphery lymphnodes, dendritic cells activates T cells, and trigger them to differentiate into Th1 and Th17 cells by releasing cytokines such as IL-6, IL23, and IL-12. Regulatory T cells (Treg) cells control activation of T cells. However, in MS this regulation is disturbed. Th1 and Th17 cells migrate and pass through the blood-brain barrier into CNS. These T cells are reactivated in CNS by antigen-presenting cells (APCs). In CNS, although there are Treg cells, T cells are activated out of control in MS. As a result, effector T cells are spread and lead to inflammation. Macrophages, microglia, and astrocytes release reactive oxygen species (ROS). Both MS and animal studies showed that oxidative stress resulted from excessive reactive oxygen species (ROS) triggers demyelination and axonal damage.Moreover, previous researches show that oxidative stress participated in the immune cell activation (especially T cells), loss of blood brain barrier integrity, T-cell migration and infiltration to central nerval system, and elevation of cytokine expression (Signorile et al., 2020).

Experimental autoimmune encephalomyelitis model presents similar clinical and pathological features with multiple sclerosis (Fletcher et al., 2010). It has been

widely used as model for MS. EAE is formed by immunization with one of the several different myelin antigens such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), myelin-associated glycoprotein (MAG), myelin-associated oligodendrocytic basic protein (MOBP) (Glatigny & Bettelli, 2018). Different mouse strains can be used to induce EAE, such as C57BL/6, C3H.SW, SJL/J, and PL/J (Glatigny & Bettelli, 2018). The use of these different antigens and mouse strains results in different types of EAE (primer progressive EAE, secondary progressive EAE) (Glatigny & Bettelli, 2018). EAE model has been utilized to seek explanation for the MS progress and to test and approve several therapeutics for MS, including glatiramer acetate (GA), mitoxantrone, natalizumab, and fingolimod, teriflunomide, and dimethyl fumarate [DMF] (Glatigny & Bettelli, 2018, Fletcher et al., 2010).

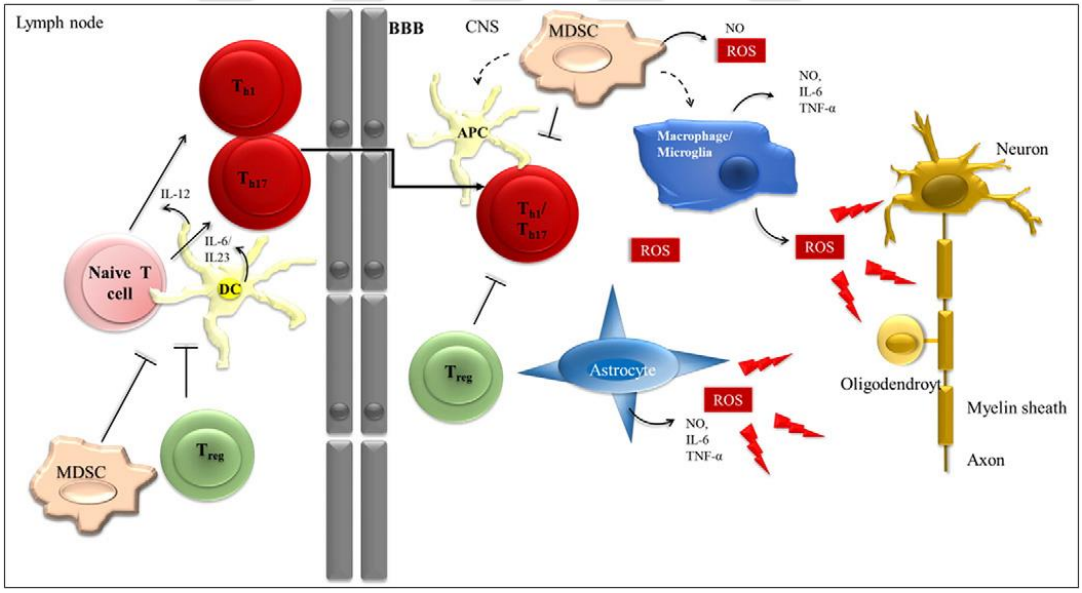

Figure 1.1 MS inflammation (Ohl et al., 2016)

1.2 Cytochrome P450s

Cytochrome P450s, one of the members of monooxygenases, are the superfamily of enzymes localized in almost all tissues including predominantly in liver but also in lung, brain, small intestine, and kidney (Pelkonen et al., 2008). They are found as membrane bound protein in the inner mitochondrial membrane and in endoplasmic reticulum (Mescher & Haarmann-Stemmann, 2018). They present central iron in their active site, which binds to cystaine thiolate (Zhao et al., 2021). That is why they are classified as heme-thiolate protein. Their tertiary structure are usually composed of 12 α-helices (A–L) and 4 β-sheets (β 1– β 4). However, there are differences in the precise position of these structural elements (Zhao et al., 2021).

They are composed of approximately 500 amino acid residues and a single heme prosthetic group in their active site (Esteves et al., 2021). There are 57 functional genes and 58 pseudogenes of CYP in human, whereas in mice, 102 functional genes and 88 pseudogenes of CYP are present (Nelson et al., 2004). In human, there are 18 families and 44 subfamilies of CYPs (Zanger & Schwab, 2013), where members of the same family and subfamily share at least 40% and 55 % amino acid sequence identity respectively (Esteves et al., 2021). The number "450" in the name of the superfamily stands for a maximum absorption wavelength of the reduced CYPs when compounded with carbon monoxide at 450nm (Omura & Sato, 1964). As CYP450 enzymes contain many enzymes in order to prevent confusing nomenclature, in 1987, CYP 450 nomenclature was recommended by Nebert et al., 1987. This nomenclature starts with cytochrome P450 abbreviation, CYP followed by a number for the CYP family (e.g., CYP2, and CYP1), a capital letter for the subfamily (e.g., CYP2E, and CYP1A) and another number for the individual enzyme (e.g., CYP2E1, and CYP1A1) (Nebert et al., 1987).

CYPs have an essential role in the metabolism of drugs, other xenobiotics, and endogenous substrates, and activation of the procarcinogens. They are classified as phase 1 oxidative enzymes, and they metabolize most of therapeutic drugs. Although there are many families in CYPs, only CYP 1, 2 and 3 families participate in

metabolism of majority of the xenobiotics which covers 70-80% of therapeutic drugs. Others, join biosynthesis of steroid hormones, prostaglandins, bile acids, and other endogenous substrates (Zanger & Schwab, 2013).

CYP450 enzymes catalyse aliphatic, aromatic and N-hydroxylation, aromatic epoxidation, N-, O- and S-dealkylation, N- and S-oxidation, oxidative deamination, dehalogenation, dehydrogenation; dehydration, C-C bond cleavage, isomerization, reduction, and esterase (Esteves et al., 2021).

In biological system, ferric iron (Fe^{3+}) usually exists in low-spin state, LS, (S=1/2) and high-spin state, HS, (S=5/2). Also, in hemoproteins, Fe^{3+} at six-fold coordination is generally found at LS and at five-fold coordination is at HS (Segall et al., 1998). LS is stabilized in the presence of a water molecule at the sixth position of the heme iron. Figure 1.2 illustrates the monooxygenase reaction by CYPs (Guengerich, 2018). Monooxygenase is insertion of one oxygen atom into a substrate. CYPs use molecular oxygen to insert an oxygen atom into a substrate and release one water molecule. The required electrons are provided by NADPH-P450 reductase (Meunier et al., 2004). Initially, binding of substrate leads to the displacement of low-spin state stabilizing water and changes in the coordination state of the Fe^{3+} from six-fold to five-fold; therefore, Fe^{3+} is shifted to the high-spin state. Then, NADPH-P450 reductase transfers one electron to Fe^{3+} via NADH or NADPH and reduces Fe^{3+} into Fe^{2+} (ferrous iron). As a result, the affinity of CYPs for diatomic gases is increased. Then, molecular oxygen binds to CYP-substrate complex and forms a relatively stable structure. NADPH-P450 reductase transfers an electron to this structure and form negatively charged $Fe^{3+}O^{2-}RH$. Then¸ two protons are added one by one to terminal oxygen of the structure. Then, O-O bond is broken and H_2O is released. Highly reactive species, which is formed in the previous step, take a hydrogen atom from the substrate. Finally, the substrate is released, and the enzyme is stabilized by a water molecule which binds to the sixth ligand position of the heme iron (Meunier et al., 2004; Gilardi & Di Nardo, 2016; Guengerich, 2001; Segall et al., 1998).

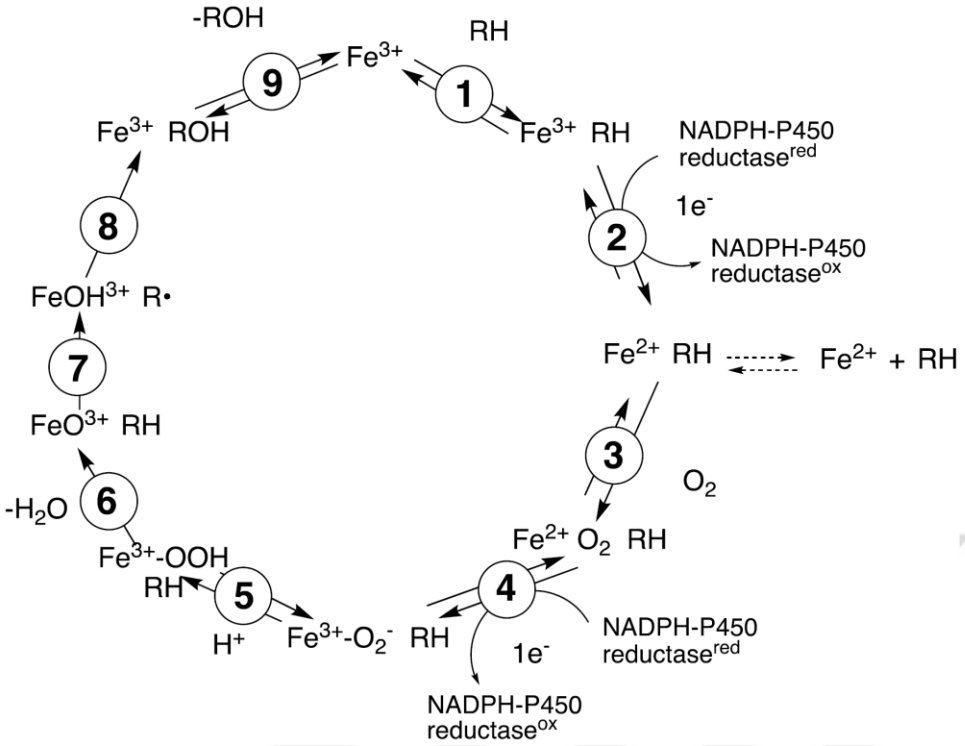

Figure 1.2 Monooxygenation reaction cycle by CYP450 (Guengerich, 2018).

CYP450 expression and activity are affected and regulated by various factors; genetic, environmental, and physiological factors, such as altered DNA methylation, microRNA (miR) regulation, age, sex, hormone levels, xenobiotics, inflammation, infection, and cancer (Esteves et al., 2021). These factors regulate CYPs' activity and expression through stabilization of mRNA and protein and inhibiting or mediating protein degradation via different ways (Esteves et al., 2021). Xenobiotics can mediate the expression of CYPs through receptors via xenobiotic-receptor dependent mechanisms such as the aryl hydrocarbon receptor (AhR), the pregnane nuclear receptor (PXR), and the constitutive androstane receptor (CAR); they mediate the expression of CYP1A1, CYP2A6 and CYP3A4, respectively (Esteves et al., 2021).

Prior studies showed that in the case of inflammation and infection, CYP450 family enzymes are regulated differentially (Renton, 2001; Morgan et al., 2002). Downregulation of protein levels and activity of several CYP450 enzymes

(CYP1A1/2, CYP2B1/2, CYP2D5, and CYP3A) have been showen by lipopolysaccharide (LPS) injection to rat, which triggers an increase of pro-inflammatory cytokines, a characteristic of potent acute phase response (Barker et al., 1992; Barker et al., 1994; Pan et al., 2000; Renton and Nicholson,2000; Morgan, 2001; Nicholson and Renton, 2001; Renton, 2001; Morgan et al., 2002; Nicholson and Renton, 2002; Garcia Del Busto Cano and Renton, 2003).

1.2.1 CYP2E1

CYP2E1 enzyme, Cytochrome P450 family 2 subfamily E member 1, is encoded on chromosome 10q26.3. CYP2E1 protein, 56.8 kDa, is expressed in liver, brain, kidney and lung (Yu et al., 2021, Farin & Omiecinski, 1993). This enzyme is located in the different compartments of the cell, mainly endoplasmic reticulum and mitochondria, and golgi apparatus. Catalytic activity of CYP2E1 is similar (no large differences) among humans, rats, rabbits, and mice. This suggests that it has an integral role in mammals. Also, there is no polymorphism causing function or catalytic activity change in all those groups (Gonzalez, 2006). Human and mouse CYP2E1 have homology between them; 79.4 % and 78.1% DNA and protein identity, respectively (Cui et al., 2012). This enzyme constitutes 7% of the total CYP450 enzymes in liver. CYP2E1 level is less in the brain than in the liver. Similarly, total CYP450 enzymes in the brain are much lower than in the liver (Başaran et al., 2012).

CYP2E1, which is mainly involved in ethanol metabolism, is participated in detoxification and activation of small and hydrophobic exogenous and endogenous substrates. Benzene, nitrosamines, chloroform, carbon tetrachloride, fatty acids, ketones, and some drugs such as acetaminophen, halothane and chlorzoxazone are the examples of its substrates (García-Suástegui et al., 2017). As CYP2E1 participates in the metabolism and activation of many toxicologically important compounds, it has been subject to much research.

Figure 1.3 shows the 3D structure of human CYP2E1. The active site of the enzyme is the smallest active site among the CYP450 enzymes. This is consistent with the small-sized substrates of the CYP2E1 enzyme, such as acetaminophen (151 Da), chlorzoxazone (170 Da), and ethanol (40Da) (Porubsky et al., 2008). The CYP2E1 structure is composed of 12 major α-helices (A–L) and 4 β-sheets (β 1– β 4) (Porubsky et al., 2008).

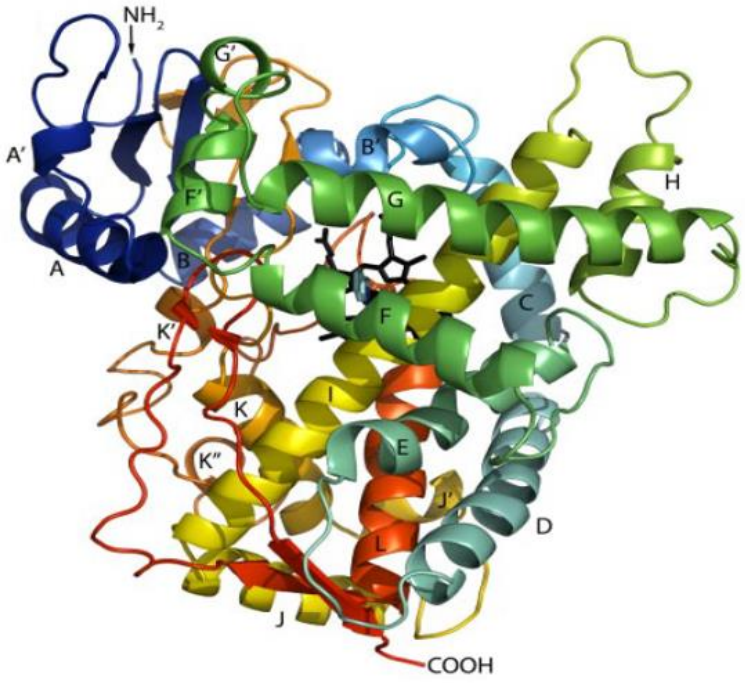

Figure 1.3 3D structure model of CYP2E1 (Porubsky et al., 2008).

CYP2E1 has a role in the generation of ROS; superoxide, hydrogen peroxide, hydroxyl radical, hydroxyl ion, and nitric oxide (Figure 1.4). ROS have unpaired electrons on the outer orbital, and it leads to oxidative stress. This results in protein denaturation, enzyme inactivation, damage to nucleic acids, and cell death (Leung & Nieto, 2013). Previous studies showed that ROS participated in many diseases such as cerebral ischemia-reperfusion injury, atherosclerosis, diabetes, neurodegenerative diseases including Parkinson's disease and Alzheimer's disease, vitamin deficiency,

drug toxicity, inflammation, alcohol induced liver injury (Caro & Cederbaum, 2004). Previous studies showed that the toxicity caused by xenobiotic compounds was increased by the induction of CYP2E1, and the toxicity was decreased in inhibition or knockout of CYP2E1 in mice (Lee et al., 1996).

CYP2E1 is regulated through various ways; transcriptional, pretranslational, translational, and posttranslational (Song & Cederbaum, 1996). Several different pathophysiological conditions such as diabetes, obesity, ethanol intoxication affect the expression and protein level of CYP2E1 (Song & Cederbaum, 1996). Downregulation in the activity and protein levels of CYP2E1 was observed in a study by Renton and Nicholson in 2000, in which cytokines and/or lipopolysaccharides (LPS), which trigger an increase of pro-inflammatory cytokines, were administrated through intraperitoneal injection (Renton and Nicholson, 2000). Abdulla, D. and her colleagues, 2006 observed a significant increase in the mRNA expression and a significant decrease in the activity of CYP2E1 in the liver of rat, 24 hours after LPS administration. Both intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) injection separately showed the same result (Abdulla et al., 2006). Moreover, they observed a significant decrease in CYP2E1 protein level in the liver 24 hours after i.c.v. injection of LPS, and no change in i.p. injection. In addition, the upregulation of CYP2E1 expression was observed in the brain after LPS injection in both *in vivo* and *in vitro* studies (Tindberg et al., 1996). Another study on rat hepatoma cell line showed that the expression and activity of CYP2E1 were downregulated after administration of pro-inflammatory cytokines (Hakkola et al., 2003).

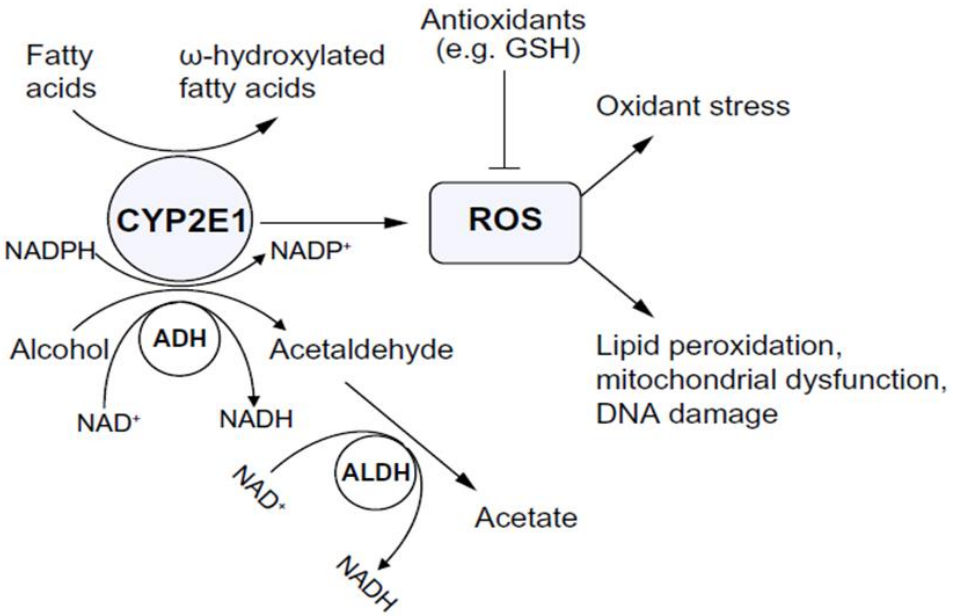

Figure 1.4 Representative figure for metabolism of alcohol and fatty acids by CYP2E1 and ROS production (Leung & Nieto, 2013).

1.2.2 CYP1A1

CYP1A1, Cytochrome P450 family 1 subfamily A member 1, is encoded on chromosome 15q24.1 (Zanger & Schwab, 2013). Figure 1.5 shows the 3D structure model of human CYP1A1 (Walsh et al., 2013). It is expressed in lung, liver, brain and small intestine (Stiborová et al., 2005, Lang et al., 2019, Kaye et al., 2016). In the brain, CYP1A1 expression presents regional and cellular heterogeneity. It is mainly expressed in cerebral cortex neurons, cerebellum (Purkinje and granule cells) and hippocampus (pyramidal neurons of CA1, CA2, and CA3 subfields) (Chinta et al., 2005). CYP1A1 is one of the extensively studied CYP isoforms in human brain (Dutheil et al., 2008). It is involved in the metabolism of xenobiotics and procarcinogens including by-products of the petroleum industry, combustion-engine exhaust, cigarette smoke and charcoal-grilled food (Lang et al., 2019). Moreover, it has a role in the metabolism of endogenous substrates such as 17β estradiol,

melatonin, arachidonic acid and eicosapentoic acid (Badal & Delgoda, 2014). CYP1A1 catalyses the hydroxylation, epoxidation, *N*-hydroxylation and *O*-demethylation, and nitroreductions (Mescher & Haarmann-Stemmann, 2018).

The expression of CYP1A1 is altered by tissue-specific factors, hormones, xenobiotics, and pathophysiological mechanisms (Walsh et al., 2013). CYP1A1 expression can be induced by xenobiotics via xenobiotic receptor-dependent mechanisms. Aryl hydrocarbon receptor (AhR) is an example of the receptor which mediates CYP1A1 expression in the presence of the ligands such as dioxins and benzopyrenes, (Esteves et al., 2021, Lang et al., 2019). AhR participates in the immune system's development and function. CYP1A1 is used as an indicator of AhR activation. Previous studies showed that the activation of AhR has a role as EAE protector by enhancing Th17 or Treg differentiation (Peres et al., 2017). Moreover, constitutive androstane receptor (CAR) regulates expression of CYP1A1 (Lang et al., 2019). TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) mediates the mRNA expression of CYP1A1 in rat hepatocytes. Other than xenobiotics, endogenous substrates such as FICZ (6-Formylindolo[3,2-b]carbazole) derived from tryptophan regulates CYP1A1 expression (Lang et al., 2019).

Previous studies showed that CYP1A1 protein and mRNA expression were downregulated in the rat liver in the case of central nervous system inflammation triggered by i.c.v. injection of LPS (Abdulla et al., 2005, Shimamoto et al., 1998). Also, in another study, CYP1A1 activity decreased and protein level did not significantly change in rat astrocyte cell culture treated with LPS (Nicholson & Renton, 1999). CYP1A1 mRNA expression is downregulated by proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and lipopolysaccharides (LPS) (Ke et al., 2001). IL-1 treatment to rat hepatocyte cells decreased the mRNA expression of CYP1A1 (Barker et al., 1992). Additionally, CYP1A1 expression is downregulated in the interleukin-6 (IL-6) treated human hepatoma cells. Moreover, a study on primary human monocytes illustrated that LPS inhibited the FICZ-mediated CYP1A1 mRNA expression (Peres et al., 2017). In

additon, TCDD mediated CYP1A1 mRNA expression was inhibitted by proinflammatory cytokines (IL-1β and TNF-α) in hepatocyte (Peres et al., 2017).

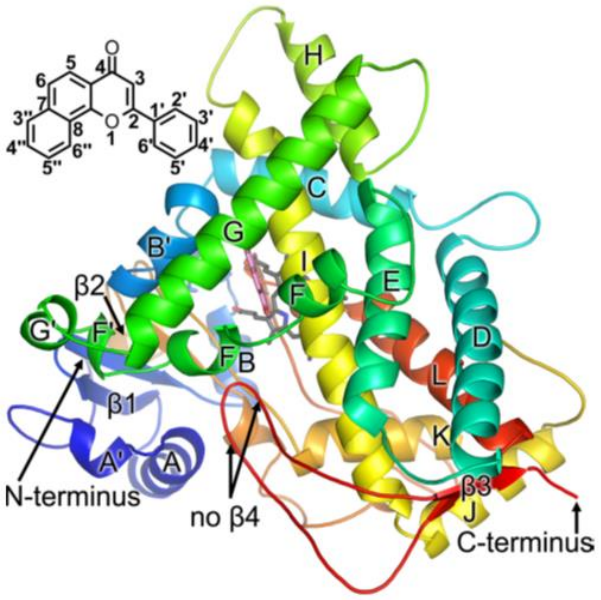

Figure 1.5 Human 3D structure model of CYP1A1 with alpha-naphthoflavone (Walsh et al., 2013).

1.3 Aim of the Study

Multiple sclerosis is an inflammatory-neurodegenerative disease which affects central nervous system. Cytochrome P450 superfamily involves in the metabolism of xenobiotics including majority of the therapeutic drugs, endogenous substrates, and activation of procarcinogens. The expression of CYP450 enzymes varies under inflammatory conditions. Accordingly, the bioavailability of drugs and other exogenous and endogenous substrates that are metabolized by these enzymes will be equally affected. CYP2E1 is involved in the metabolisms of many toxicologically important substrates, drugs, fatty acids and procarcinogens. CYP1A1 is involved in the metabolism of xenobiotics and activation of many procarcinogens. Both enzymes are highly regulated by their own substrates, other compounds, and inflammation. Therefore, since multiple sclerosis is an inflammatory neurodegenerative disorder, mRNA and protein levels of these enzymes need to be examined in this condition. The change in gene and protein expression of CYP2E1 and CYP1A1 enzymes in the liver and brain in the case of MS has not been investigated before.

In this study, the effects of multiple sclerosis on expression of CYP2E1 and CYP1A1 gene and protein in mouse liver and brain were investigated by utilizing molecular and biochemical approaches in a female EAE - MS mouse model. To the best of our knowledge, this is the first study investigating the effects of MS on CYP2E1 and CYP1A1 mRNA and protein expression at the brain and liver levels. Moreover, the results of this research may provide a new point of view related to drug metabolizing-inflammation related CYP enzymes for the development of new MS therapy.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Materials

BCIP®/NBT liquid substrate (B1911), bovine serum albumin (BSA; A7511), 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris; T1378), ammonium persulfate (APS; A-3678), bromophenol blue (B5525), glycerol (G5516), glycine (G-7126), ß-mercaptoethanol (M6250), hydrochloric acid 37% (HCl; 07101), methanol (34885), ethanol (24105), sodium dodecyl sulfate (SDS; L4390) and tween 20 (P1379) were purchased from Sigma-Aldrich Chemical Company, Saint Louis, Missouri, USA.

Ethylenediaminetetraacetic acid (EDTA; A5097) was acquired from Applichem GmbH, Germany.

Mod LabDiet® Laboratory with no added vitamin D pellet (1817422) was obtained from TestDiet, Saint Louis, Missouri, USA.

Hooke KitTM MOG35-55/CFA emulsion with pertussis toxin (EK-2110) was purchased from Hooke Laboratories, Lawrence, Missouri, USA.

Potassium dihydrogen phosphate (KH2PO4; 04871), di-potassium hydrogen phosphate (K2HPO4; 05101), sodium hydroxide (06462), sodium chloride (NaCl; 1.06400), sodium hydroxide (NaOH; 06462) were the products of E. Merck, Darmstadt, Germany.

GeneJet RNA purification kit (K0732), T-PER tissue protein extraction reagent (78510), HaltTM protease inhibitor cocktail (87786), PageRuler plus prestained protein ladder (26619), PierceTM BCA protein assay kit (23225), and RevertAid first strand cDNA synthesis kit (K1622) were purchased from Thermo Fisher Scientific, Waltham, Massachusetts, USA.

50 bp DNA ladder (N3236S) and DNase I (M0303S) were the product of the New England Biolabs, Ipswich, Massachusetts, USA

Non-fat dry milk (170-6404), tetra methyl ethylene diamine (TEMED; 161-0801), TGX Stain-FreeTM FastCastTM acrylamide kit 10% (161-0183), 10X TGS buffer (161-0732) were the products of Bio-Rad Laboratories, Richmond, California, USA.

FastStart universal SYBR® green master mix (04913850001) was purchased from Roche Diagnostics Gmbh, Germany

Goat anti-rabbit alkaline phosphatase conjugated secondary antibody (ab6722), CYP2E1 (ab84598) and recombinant anti-GAPDH antibody (ab181602) were the product of Abcam, Cambridge, United Kingdom.

The CY1A1 (sc-20772) antibody was purchased from Santa Cruz (Santa Cruz, CA).

Anti-GAPDH antibody (TA890003) was purchased from Origene, Rockville, MD, USA.

Primers for qPCR were made by Sentebiolab Ankara, Turkey.

In this study, only analytical grade and the highest grade of pure chemicals were used.

2.2 Animal Studies

The present thesis work is a continuation of the study of Dr. Emre Evin's Ph.D. Thesis, therefore, some procedures used present similarities (Evin E., 2021). All procedures of animal studies of this thesis work were approved by Bilkent University Animal Experimentation Ethics Committee as given in Appendices A and B. 10-12 weeks old female C57BL/6 mice weighing 20-25 g were used to construct an EAE model. The mice model were produced and housed at the Animal Experimental Unit in Bilkent University. Mice were randomly assorted into two groups as given in Table 2.1 and placed in individually ventilated cages.

Table 2.1 Experimental groups of the female C57BL/6 mice.

Groups	Number of Mice
Control	6
EAE	12

According to the study of Bachmanov et al., 2002, it was reported that daily food and water intake of C57BL/6 mice were approximately 4 g and 6 mL, respectively (Bachmanov et al., 2002).

2.2.1 Experimental Autoimmune Encephalomyelitis (EAE) Immunization

C57BL/6J (10-12 weeks old) mice were immunized with Hooke Labs' Kit (Hooke Labs Inc., #EK-2110), the manufacturer's instructions were followed. MOG_{35-55}/CFA emulsion (myelin oligodendrocyte glycoprotein/complete Freund's adjuvant) was injected to the mice from lower and upper back (100 µL to each site). After 2 hours and 24 hours, mice were injected with pertussis toxin (80 ng in 100µL PBS/animal) intraperitoneally. Both groups were treated in the same way, except control group which was injected with phosphate-buffered saline (PBS) instead of MOG_{35-55} peptide. Mice were monitored individually (ear tags were used). After immunization, mice were observed on a daily basis for 30 days. The clinical score of the disease is assigned by considering the manufacturer's scoring chart (Table 2.2). After 30 days of the EAE immunization, under anesthesia, the mice were sacrificed by perfusion with PBS. The liver and brain organs were isolated and stored at -80 ºC until further analysis.

Table 2.2 Clinical Observations and mouse EAE scoring.

Score	***Clinical Observation***
0	The tail has tension and is erect.
0.5	The tip of the tail is limp.
1	The tail is limp
1.5	The tail is limp, and the hind leg is inhibited.
2	The tail is limp, and the hind legs are weak.
2.5	The tail is limp, and the mouse is dragging hind legs.
3	The tail is limp, and the hind legs are completely paralyzed.
3.5	In addition to score 3, when the mouse is placed on its side, it cannot right itself.
4	In addition to score 3.5, there is partial front leg paralysis.
4.5	In addition to score 4, the mouse is not alert.
5	Death

2.3 Total Protein Extraction

The liver and brain organs were taken after the perfusion of mice with PBS solution. The organs were homogenized by grinding via the cryogenic grinding method with liquid nitrogen. Pestle and mortar were used as grinding tools. After homogenization, 1% protease inhibitor (Halt™ Protease Inhibitor Cocktail) was added to the T-PER™ tissue protein extraction reagent. Approximately 50 mg of the homogenate was weighed into a microcentrifuge tube and 500 µL of the T-PER™ reagent was added. Then, the sample (homogenate-reagent mixture) was centrifuged at 10,000 x g for 5 minutes. The supernatant which contains total protein was taken and stored at -80 ºC until use.

2.4 Determination of Protein Concentration

Total protein concentration of the mice liver and brain samples were determined via Pierce™ BCA Protein Assay Kit. This kit utilizes the reduction of Cu^{+2} to Cu^{+1} by protein in alkaline medium. This Cu^{+1} ion chelates with two molecules of bicinchoninic acid (BCA), which results in a color change to purple color. The absorbance of purple-colored product can be measured by spectrophotometer at 562 nm. The protein concentration of the sample is proportional to the absorbance.

The protocol which is suggested in kit's manual was followed. Bovine serum albumin (BSA) was used as the standard and was diluted to different concentrations (25, 125, 250, 500, 750, 1000, 1500, 2000 µg/mL). All the measurements, including blank, standards, and the samples, were performed as duplicates. 25µL of standards and the samples were loaded into 96 well plates. Working reagent was prepared with the ratio reagent A:B, 50:1. 200 µL of working reagent was added into each well and plate was placed on a plate shaker for 30 seconds. Then, plate was covered with aluminium foil and incubated at 37 ºC for 30 minutes. After incubation, absorbance was read at 562 nm in the microplate spectrophotometer (Multiskan™ GO, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Then blank absorbance was subtracted from each of the other measurements, and standard curve was plotted for standard's absorbance vs its concentration (µg/mL). This plot was used to determine the concentration of the samples.

2.5 Determination of Protein Expressions by Western Blotting Technique

Effects of EAE immunization on protein expression of CYP2E1 and CYP1A1 in the mice liver and brain were analyzed by the Western blot method (Towbin et al., 1979). GAPDH was used as a loading control. Initially, proteins were separated according to their size by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). For that purpose, 10 % separating gel and 4% stacking gel in discontinuous buffer system was utilized. SDS is a detergent which denatures the

proteins and makes them negatively charged proportional to their polypeptide chain length.

Initially, the gel was prepared according to Table 2.3. 4250 µL of 10 % separating gel was poured into the 1.0 mm space between glass plates on the casting stand. Immediately, 1000 µL of isopropanol was added in order to remove air bubbles and prevent oxygen contact, which causes inhibition of polymerization. After polymerization occur, the isopropanol was discarded and 1500 µL of stacking gel solution poured onto the separating gel. Immediately, the comb was placed (15 well comb for liver samples and 10 well comb for brain samples). Gel was stored in 1X electrophoresis running buffer (1X ERB) at 4 ℃ until its use.

The samples were diluted with dH_2O according to the following formula to get 2 mg/mL and 4 mg/mL final protein concentration of brain and liver respectively;

$$m_1 x V_1 = m_2 x 200$$

$$x = 200 - V_1 - 50$$

m_1 is the concentration of the sample and V_1 is the volume of sample to be taken to prepare 200 µL sample with m_2 (either 2mg/mL or 4mg/mL) concentration. x is the volume of water to be added. 50 µL of 4X sample dilution buffer was added to each sample. Sample dilution buffer (pH 6.8) contains 0.25M Tris-HCl, 8% SDS, 40% glycerol, 20 % β-mercaptoethanol, and 0.01% bromophenol blue. Then, the samples incubated in heat block at 100℃ for 3 minutes. The comb removed from the gel and wells were gently cleaned up with syringe. Samples were loaded into the wells (20 µg of sample loaded for liver CYP2E1, and brain CYP1A1 and CYP2E1, and 40 µg of sample loaded for liver CYP1A1). 1 µL of protein ladder was loaded as the marker. After loading the sample, the gel running module was placed into the tank which filled with 1X ERB. Then, the tank was connected to the Bio-Rad power supply, and electrophoresis was run at 90 V at stacking gel and 120 V at separating gel.

Table 2.3 Gel casting components and their volumes in µl for one gel.

Components	*Separating Gel 10 %*	*Stacking Gel 4%*
Gel Solution	2500	325
dH_2O	3010	1525
Separating Buffer	1875	-
Stacking Buffer	-	625
10 % SDS	75	25
10% APS	37.5	12.5
TEMED	7.5	5

The gel was removed from the running module and washed with transfer buffer (25 mM Tris, 192 mM Glycine) for 10 minutes. The PVDF membrane was cut 5 cm x 9 cm in size and immersed in 100% methanol for 1 minutes to prewet the membrane. Then the membrane was equilibrated in transfer buffer for 5 minutes. The gel and the PVDF membrane were put in sandwich, as shown in Figure 2.1. The transfer sandwich was placed in module of The Mini Trans-Blot® cell. The electrode module was placed in tank which is filled with transfer buffer. The lid of the tank was placed and plugged into the Bio-Rad power supply. The transfer was carried out at a constant 100 volt for 90 minutes.

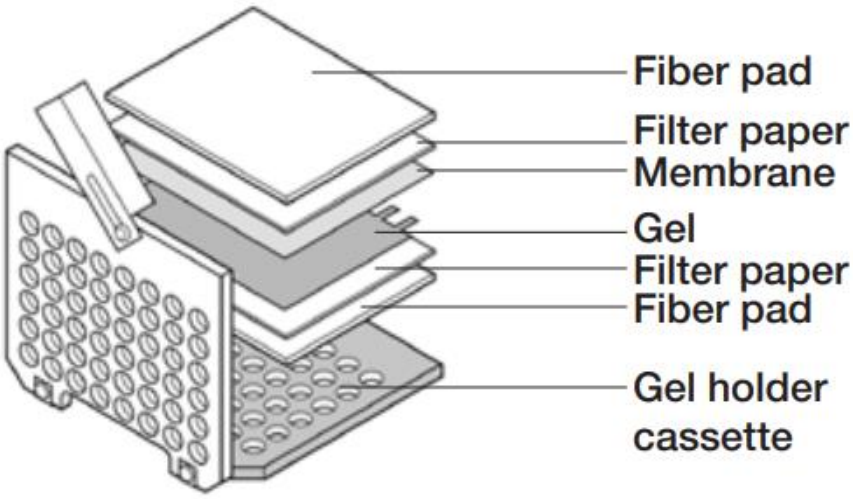

Figure 2.1 Preparation of western blot sandwich (Bio-Rad, 2021).

After the transfer was completed, the membrane was washed with TBST (20 mM Tris-HCl pH 7.4, 500 mM NaCl) for 10 minutes. Then, the membrane was incubated with blocking solution (5% non-fat dry milk in TBS) at room temperature for an hour on a shaker. Then, the membrane was cut into two pieces through corresponding ladder, and incubated with primary antibody of interest and GAPDH (housekeeping gene) primary antibody for 16 hours in cold room (+4°C) on a shaker. The primary and the secondary antibodies and their dilutions are given in Table 2.4. Next, the membranes were washed with TBST for 3x5 minutes at room temperature. After that, the membranes were incubated with secondary antibody for an hour at room temperature. Again, the membranes were washed with TBST for 3x5 minutes. As a last step, the membranes were incubated with the BCIP®/NBT alkaline phosphatase substrate, which is prepared according to the Table 2.5. First, Solution A was prepared, and its pH was arranged to 9.55 with saturated Tris. Then, its volume completed to 40mL. Then, 136 µL of Solution C was added onto Solution A, and then, 268 µL of Solution B was added onto mixture. Then, the membranes were incubated with this mixture of the solutions. Finally, after seeing bands the

membranes were washed with dH_2O and immediately covered with aluminium foil to increase the density of the bands. Chemidoc XRS + (Bio-Rad, USA) was used to take images. The band intensities were analyzed by Image Lab visualization software developed by NIH.

Table 2.4 Primary and secondary antibody dilutions.

	1st Antibody	*2nd Antibody*
Protein (Liver)		
CYP2E1	1/1000	1/2000
GAPDH	1/2000	1/2000
CYP1A1	1/100	1/2000
Protein (Brain)		
CYP2E1	1/500	1/2000
CYP1A1	1/100	1/2000
GAPDH	1/2000	1/2000

Table 2.5 Preparation of BCIP®/NBT alkaline phosphatase substrate.

Solution A	
1.5 M Tris-HCl Buffer (pH: 8.8)	2670 µL
1 M NaCl	4000 µl
Diethanolamine	96 µL
Magnezium Cloride	820 µL
Zinc Cloride	40 µL
Nitrotetrazolium Blue Chloride (NBT)	12.2 mg
Solution B	
Phenazine Methosulfate	2mg
dH_2O	1000 µL
Solution C	
5-bromo 4-chloro 3-indoyl phosphate (BCIP)	5.44 mg
N-N-dimethylformamide	136 µL

2.6 Determination of mRNA Expression

2.6.1 Isolation of Total RNA From Tissue Homogenates

The liver and brain organs were taken after perfusion of mice with PBS solution. The organs were homogenized by grinding via the cryogenic grinding method with liquid nitrogen. Pestle and mortar were used as grinding tools. GeneJet RNA purification kit was used to isolate total RNA, and the manufacturer's protocol was followed. Approximately 30 mg of each sample was taken into a 2 mL centrifuge tube. 300 µL of lysis buffer was added, and the lysate was passed through a 20-gauge sterile syringe. 600µL of proteinase K was added and vortexed thoroughly. The mixture was incubated at room temperature for 10 minutes, then, the lysate was centrifuged for 10 minutes at 12,000 x g. The supernatant was transferred into a new 2 mL centrifuge tube, and 450 µL absolute ethanol was added and mixed by pipetting. The lysate was transferred into the RNA purification column and centrifuged at 12,000 x g for 1 minute. The purification column was placed into a new 2 mL collection tube, and washed with 700 µL of Wash Buffer 1 through 12,000 x g centrifuge for 1 minute. The flow-through was discarded, and the purification column was placed back into the collection tube, and washed with 600 µL of Wash Buffer 2 through 12,000 x g centrifuge for 1 minute. The flow-through was discarded, and the purification column was placed back into the collection tube, and again washed with 250 µL of Wash Buffer 2 through 12,000 x g centrifuge for 1 minute. Then, the purification column was placed into a new sterile 1.5mL centrifuge tube, and 100 µL of nuclease-free water was added. Finally, RNA was eluted through 12,000x g centrifuge for 1 minute. The purification column was discarded, and purified RNA was stored at -80°C until use.

Isolated RNA concentration was quantified by measuring the absorbance at 260 nm and the purity was assessed by the 260/280 nm ratio by BioDrop µLite + (Biochrom, United Kingdom) spectrophotometer.

2.6.2 Genomic DNA (gDNA) Removal from RNA

RNase-free DNase I was used to remove gDNA and prevent PCR amplification of the gDNA template. Isolated RNA (10 µg), 1X DNase I reaction buffer (10 µL), and Dnase I (1 µL) were mixed. The total volume of the mixture was completed to 100 µL with nuclease-free water. The mixture was incubated at 37 ºC for 10 minutes. DNase I was inactivated, prior to cDNA synthesis, by adding 1 µl of 0.5 M EDTA and incubating at 75 ºC for 10 minutes.

2.6.3 cDNA Synthesis

RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used for reverse transcription of RNA to cDNA. 1 µg of total isolated RNA was taken into PCR tube, and 1 µL of oligo dT primer was added. The final volume of the mixture was completed to 10 µL with nuclease-free distilled water. Then, the solution was mixed gently and spun down by microcentrifuge. The mixture was incubated at 70°C for 5 minutes. After that, 5 µL of 5X reaction buffer, 0.5 µL of RiboLock (RNase Inhibitor), 0.5 µL of reverse transcriptase, 2.5 µL of 10 mM dNTP, and 6.5 µL of nuclease-free distilled water were added. The mixture was mixed gently and spun down by microcentrifuge. Then, it was incubated at 42 °C for 1 hour. Finally, it was kept at 70°C for 10 minutes in order to stop the reaction and chilled on ice. Synthesized cDNA was stored at -20°C for further use.

2.6.4 Quantitative Real-Time PCR

The expressions of CYP2E1 and CYP1A1 genes in the liver and brain were analyzed by quantitative Real-Time PCR (qPCR) using Corbett Rotor-Gene 6000 (Corbett life Science, PO Box 435, Concorde, NSW 2137). The 25 µL of final reaction mixture containing 100 ng cDNA, 0.5 mM reverse and forward primers, and FastStart Universal SYBR Green Master Mix (Roche, Basel, Switzerland) and RNase free

distilled water. To detect any contamination, no template control (NTC) was used. As an internal standard, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene was used. The DNA amplification carried out in a reaction mixture containing reverse and forward primers with specific nucleotide sequences used for CYP2E1, CYP1A1 and GAPDH genes were given in Table 2.6. The qPCR program consisted of the following cycling profile; initial melting at 95 °C for 5 minutes, amplification and quantification program repeated 50 times containing melting at 95 °C for 20 seconds, annealing at 60-63 °C (depending on the gene) for 30 seconds and extension at 72 °C for 20 seconds with a single fluorescent measurement. The melting curve was used to confirm expected product amplification without any non-specific products. Quantities of specific mRNAs in the sample were measured according to the corresponding gene and relative standard curve method. The results obtained using Corbett Rotor-Gene 600 quantitation software were normalized with internal standard GAPDH, and the Livak method was used to determine relative mRNA expression in different tissues by using C_t values (Livak et al., 2001).

2.7 Statistical Analysis

The GraphPad Prism version 9 statical software tool for Windows was used to perform statical analysis. The student's t-test was used for the comparison of two groups (control and EAE). All results were given as means with their Standard Deviation (mean ± SD), and the level for significance was chosen as $p<0.05$.

Table 2.6 Primer sequence of the genes.

GENE	**Forward Primer (5’ -> 3’)**	**Reverse Primer (3’ -> 5’)**	**Product Size (bp)**	**Annealing Temperature (°C)**
GAPDH	GTGATGGGTGTGAACCACGAG	CATGAGCCCTTCCACAATGC	132	60
CYP2E1	GGAGCTCAAAAAGACCAAAGGCCAG	CGTAATCGAAGCGTTTGTTGAAGAG	107	62
CYP1A1	TTGGAGCTGGGTTTGACACAGTCAC	CTCCAGAATGAAAGGCCTCCAGATAG	62	63

CHAPTER 3

RESULTS

3.1 Animal Studies

This study is a continuation of the work of the doctorate thesis of Dr. Emre Evin, therefore, some parts of the results (Section 3.1) include information from the previous study (Evin, 2021).

3.1.1 Clinical Scoring and Observation

In this study, there were 2 groups of mice, as given previously in "Methods" Table 2.1. Mice in EAE group were injected with the MOG35-55 peptide emulsion. Clinical scoring was carried out on a daily basis, starting from the immunization day as described before in "Methods" Table 2.2. This clinical scoring record was used to determine whether EAE model successful or not (Evin E., 2021).

3.2 Protein Concentration of Mouse Liver and Brain

Total protein concentration of the mice liver and brain samples were determined via Pierce™ BCA Protein Assay Kit and the protocol suggested in kit's manual was followed. Bovine serum albumin, at different concentration (25, 125, 250, 500, 750, 1000, 1500, 2000 µg/mL), was used as the standard. All the measurements, including blank, standards, and the samples, were performed as duplicates. Total protein concentration in liver and brain homogenate of the mice were given in Figure 3.1.

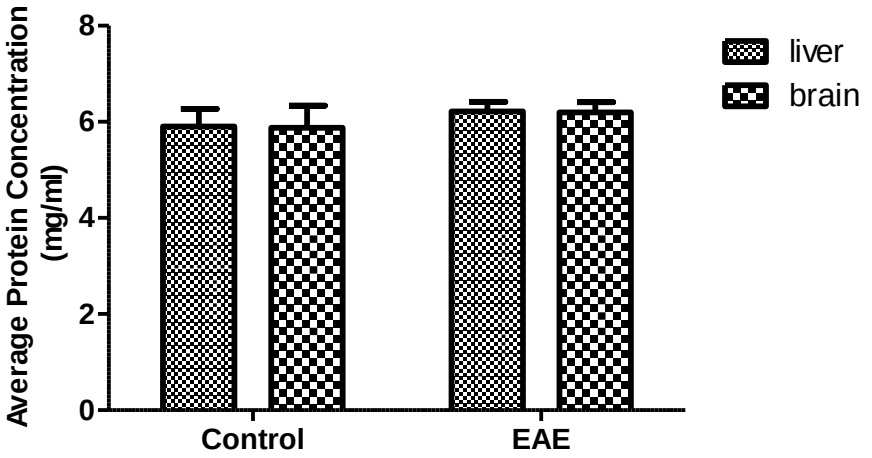

Figure 3.1 Protein Concentrations in mouse liver and brain homogenate.

3.3 Effects of EAE Immunization on Protein Expressions

Effects of EAE immunization on CYP2E1 and CYP1A1 protein expressions of female C57BL/6 mice in liver and brain were determined by Western blotting technique through specific antibodies. GAPDH was used as the protein loading control. GAPDH was detected via rabbit monoclonal anti-GAPDH primary antibody and anti-rabbit alkaline phosphatase-conjugated secondary antibody. Alkaline phosphatase substrate solution was prepared as explained in "Methods" 2.5. The band intensity was measured by Image Lab software as a relative peak area (RPA). The RPA of control group was roughly equilibrated to 1.00 and relative protein expression of the EAE group was estimated. Protein expression of the groups was compared by t-test. The quantifications were expressed as the mean ± SD of the relative protein expression from three independent experiments and the level of significance was chosen as $p<0.05$.

3.3.1 Effects of EAE Immunization on CYP2E1 Protein Expressions in the Mouse Liver

CYP2E1 (56 kDa) protein expression was determined by the Western blotting technique. 20 µg protein was loaded into each well of the polyacrylamide gel. Rabbit polyclonal anti-CYP2E1 primary antibody (1:1000 dilution) and alkaline phosphatase (AP) conjugated secondary anti-rabbit antibody (1:2000 dilution) were used to detect CYP2E1 protein (Figure 3.2/A). Images of the bands were taken via Chemidoc XRS + (Bio-Rad, USA), and intensities of the bands were measured via Image Lab software. Figure 3.2/ B shows the relative CYP2E1 protein expression in the mouse liver. Although, CYP2E1 expression in EAE immunized group is higher than control group, it was not statistically significant (p= 0.2, p> 0.05).

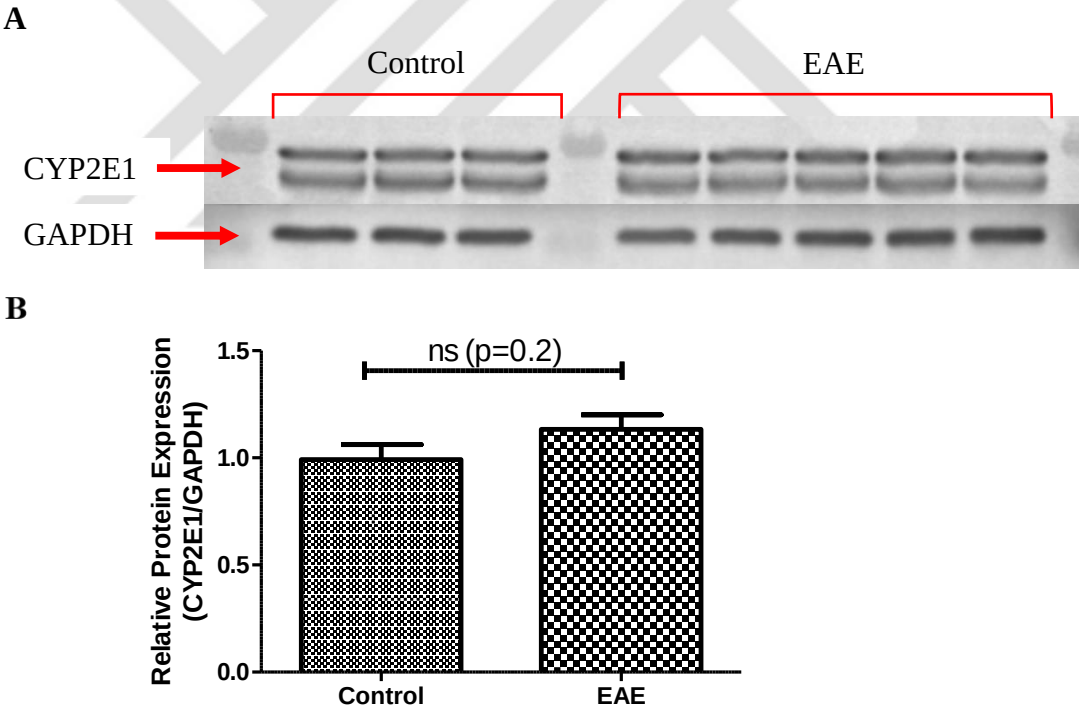

Figure 3.2 Effects of EAE immunization on CYP2E1 protein expression in mouse liver. **A)** Representative immunoblot of liver CYP2E1 protein in Control (C), and EAE Group. **B)** Comparision of CYP2E1 protein expression in the two groups. p=0.2, p>0.05.

3.3.2 Effects of EAE Immunization on CYP1A1 Protein Expressions in the Mouse Liver

CYP1A1 (56 kDa) protein expression was determined by the Western blotting technique. 40 µg protein was loaded into each well of the polyacrylamide gel. Rabbit polyclonal anti-CYP1A1 primary antibody (1:100 dilution) and alkaline phosphatase (AP) conjugated secondary anti-rabbit antibody (1:2000 dilution) were used to detect CYP1A1 protein (Figure 3.3/A). Image of the bands was taken via Chemidoc XRS + (Bio-Rad, USA), and intensities of the bands were measured via Image Lab software. Figure 3.3/ B shows the relative CYP1A1 protein expression in the mouse liver. There was no statistically significant change between the two groups, p=0.71, p>0.05.

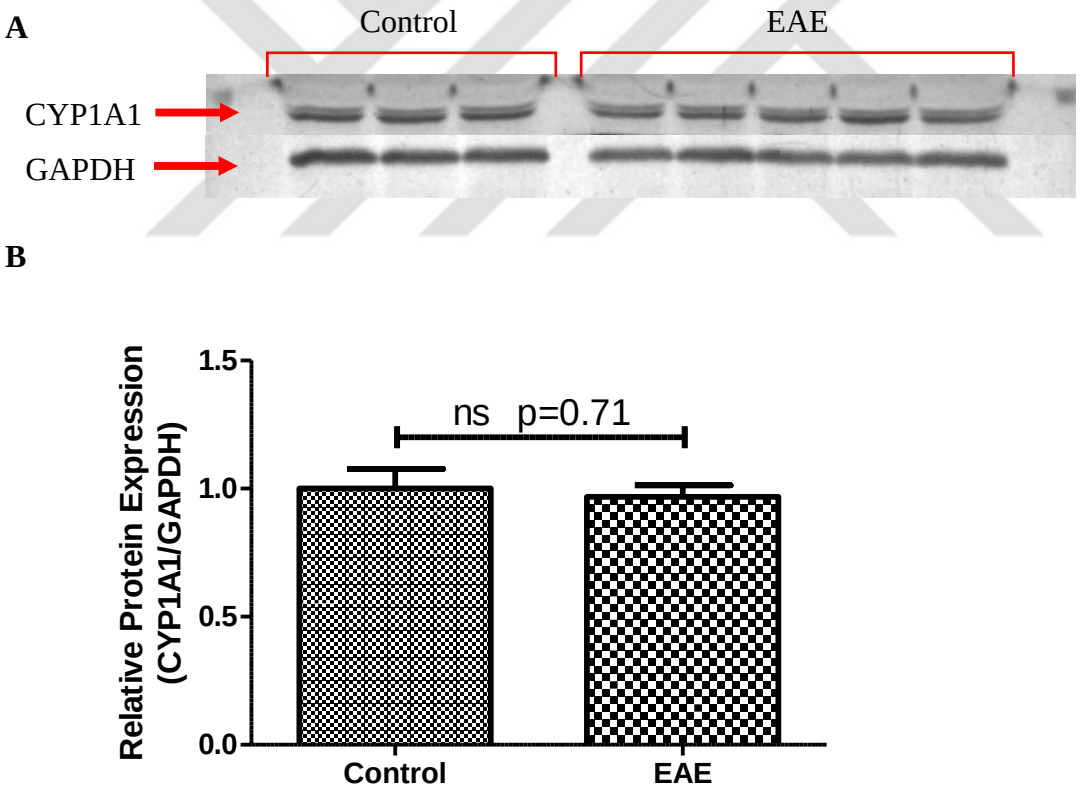

Figure 3.3 Effects of EAE immunization on CYP1A1 protein expression in mouse liver. **A)** Representative immunoblot of liver CYP1A1 protein in Control (C), and EAE Group. **B)** Comparision of CYP1A1 protein expression in the two groups. p=0.71, p>0.05.

3.3.3 Effects of EAE Immunization on CYP2E1 Protein Expressions in the Mouse Brain

CYP2E1 (56 kDa) protein expression was determined by the Western blotting technique. 40 µg protein was loaded into each well of the polyacrylamide gel. Rabbit polyclonal anti-CYP2E1 primary antibody (1:500 dilution) and alkaline phosphatase (AP) conjugated secondary anti-rabbit antibody (1:2000 dilution) were used to detect CYP2E1 protein (Figure 3.4/A). Images of the bands was taken via Chemidoc XRS+ (Bio-Rad, USA), and intensities of the bands were measured via Image Lab software. Figure 3.4/ B shows the relative CYP2E1 protein expression in the mouse brain. There was no significant difference between groups, $p>0.05$ ($p=0.89$).

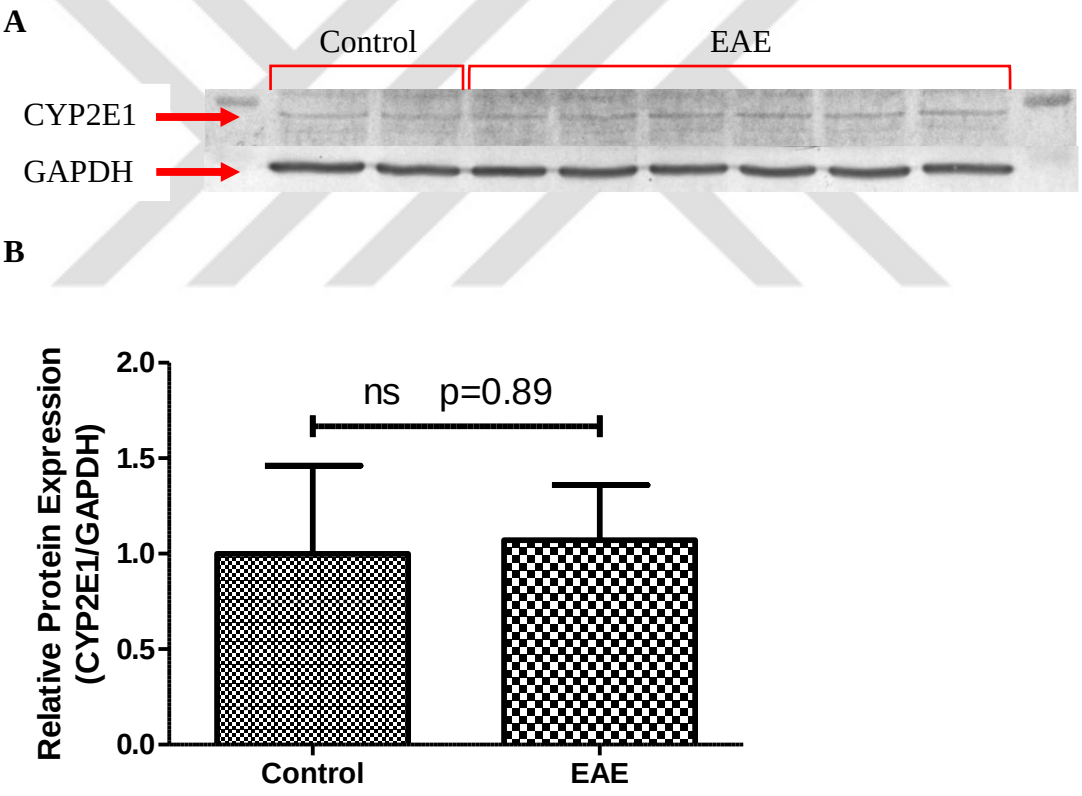

Figure 3.4 Effects of EAE immunization on CYP2E1 protein expression in mouse brain. **A)** Representative immunoblot of brain CYP2E1 protein in Control (C), and EAE Group. **B)** Comparision of CYP2E1 protein expression in the two groups. $p=0.89$, $p>0.05$.

3.3.4 Effects of EAE Immunization on CYP1A1 Protein Expressions in the Mouse Brain

CYP1A1 (56 kDa) protein expression was determined by the Western blotting technique. 40 µg protein was loaded into each well of the polyacrylamide gel. Rabbit polyclonal anti-CYP1A1 primary antibody (1:100 dilution) and alkaline phosphatase (AP) conjugated secondary anti-rabbit antibody (1:2000 dilution) were used to detect CYP1A1 protein (Figure 3.5/A). Image of the bands was taken via Chemidoc XRS+ (Bio-Rad, USA), and intensities of the bands were measured via Image Lab software. Figure 3.5/ B shows the relative CYP1A1 protein expression in the brain. There was no statistically significant change between the two groups, $p>0.05$ ($p=0.64$).

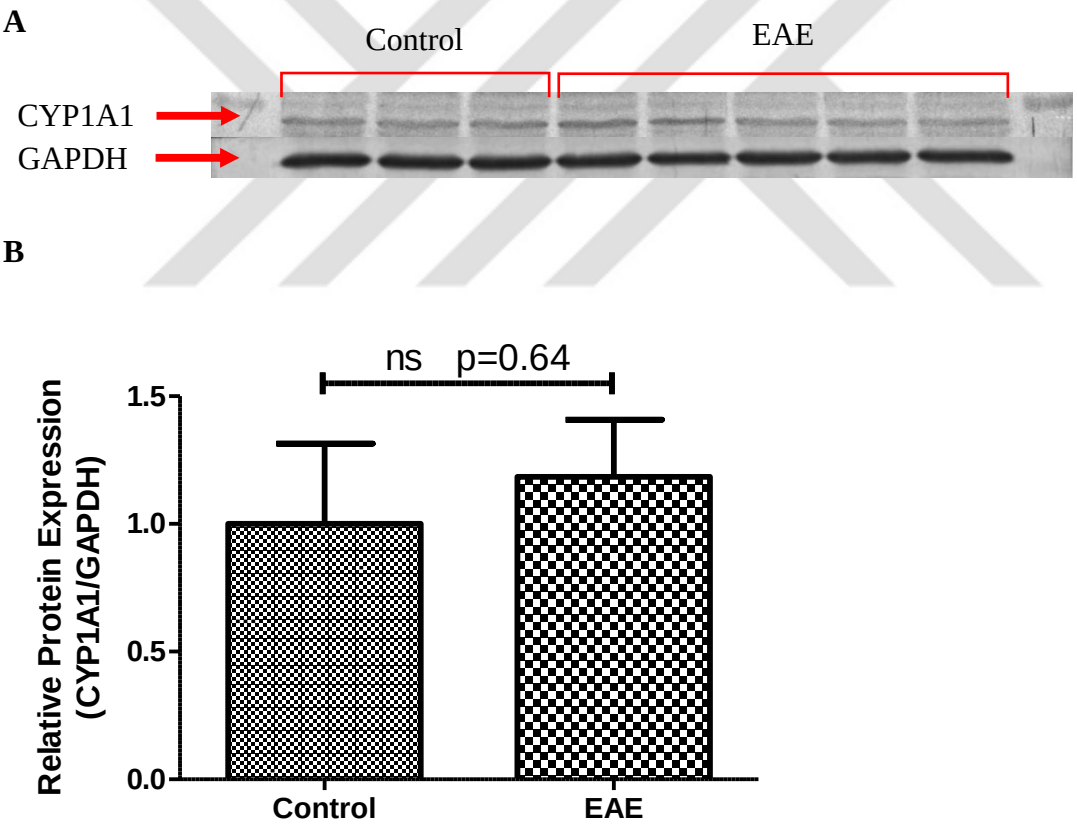

Figure 3.5 Effects of EAE immunization on CYP1A1 protein expression in mouse brain. **A)** Representative immunoblot of brain CYP1A1 protein in Control (C), and EAE Group. **B)** Comparision of CYP2E1 protein expression in the two groups. $p=0.64$, $p>0.05$.

3.4 Effects of EAE Immunization on mRNA Expression

3.4.1 Effects of EAE Immunization on CYP2E1 mRNA Expression in Mouse Liver

The CYP2E1 mRNA expression in the liver was determined by quantitative real-time PCR (qPCR). As an internal standard GAPDH gene was used. Specific primers and annealing temperatures for CYP2E1 and GAPDH genes were used as described before in Table 2.6. Relative CYP2E1 mRNA expression was analyzed by the Livak method. Figure 3.6 shows relative mRNA expression of CYP2E1 in the liver of control group and EAE mice. Although there was an increase in CYP2E1 mRNA expression in EAE group compared to control, it was not significant (p= 0.08, p> 0.05).

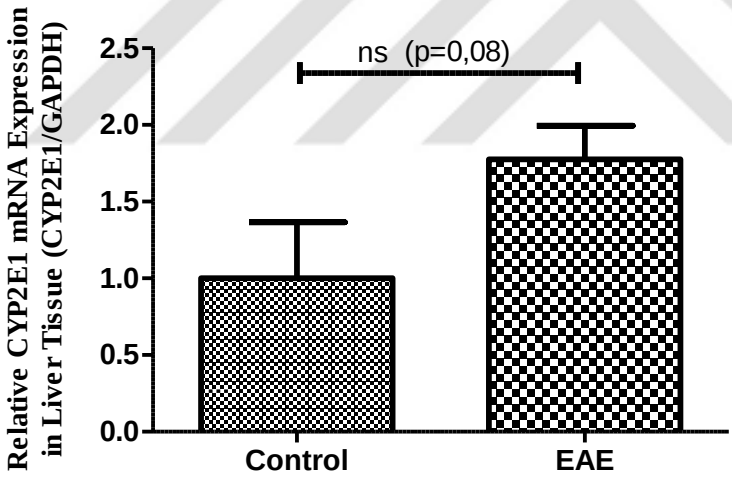

Figure 3.6 Effect of EAE immunization on CYP2E1 mRNA expression in the liver of mice. mRNA expression changes were determined by qPCR.

3.4.2 Effects of EAE Immunization on CYP1A1 mRNA Expression in Mouse Liver

The CYP1A1 mRNA expression in the liver was determined by quantitative real-time PCR (qPCR). GAPDH gene was used as an internal standard. Specific primers and annealing temperatures for CYP1A1 and GAPDH genes were used (Table 2.6). Relative CYP1A1 mRNA expression was analyzed by the Livak method. Figure 3.7 shows relative mRNA expression of CYP1A1 in the liver of control group and EAE mice. Although there was an increase in CYP1A1 mRNA expression in EAE group compared to control, it was not significant ($p= 0.23$, $p> 0.05$).

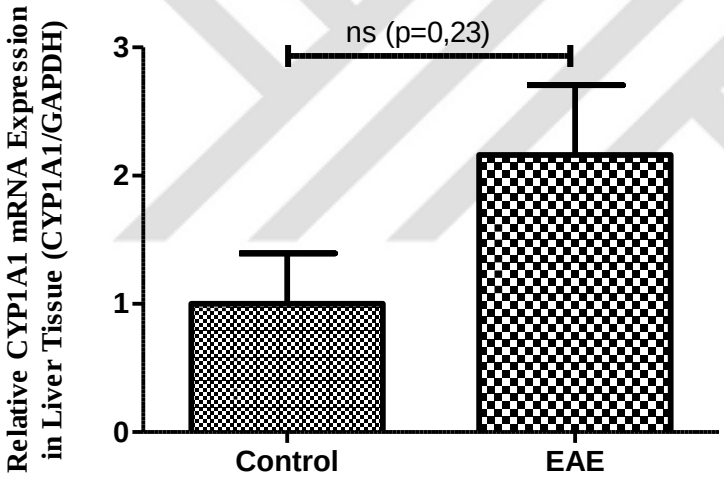

Figure 3.7 Effect of EAE immunization on CYP1A1 mRNA expression in the liver of mice. mRNA expression changes were determined by qPCR.

3.4.3 Effects of EAE Immunization on CYP2E1 mRNA Expression in Mouse Brain

The CYP2E1 mRNA expression in the brain was determined by quantitative real-time PCR (qPCR). GAPDH gene was used as an internal standard. Specific primers and annealing temperatures for CYP2E1 and GAPDH genes were used (Table 2.6). Relative CYP2E1 mRNA expression was analyzed by the Livak method. Figure 3.8 shows relative mRNA expression of CYP2E1 in the brain of control group and EAE mice. Although there was a decrease in CYP2E1 mRNA expression in EAE group compared to control, it was not significant ($p= 0.11$, $p> 0.05$).

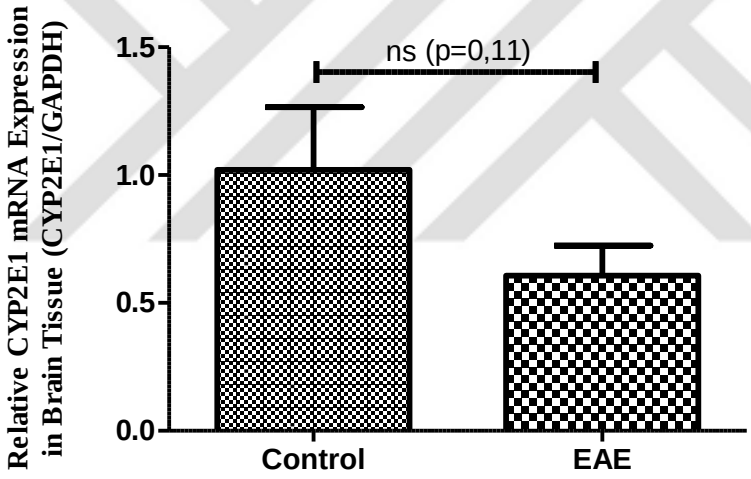

Figure 3.8 Effect of EAE immunization on CYP2E1 mRNA expression in the brain of mice. mRNA expression changes were determined by qPCR.

3.4.4 Effects of EAE Immunization on CYP1A1 mRNA Expression in Mouse Brain

The CYP1A1 mRNA expression in the brain could not be determined by quantitative real-time PCR (qPCR). Although GAPDH gene was detected in the brain, we could not detect CYP1A1 gene. GAPDH was used as an internal standard. Specific primers and annealing temperatures for CYP1A1 and GAPDH genes were used (Table 2.6). Figure 3.9 shows the amplification curve and melt curve for GAPDH gene.

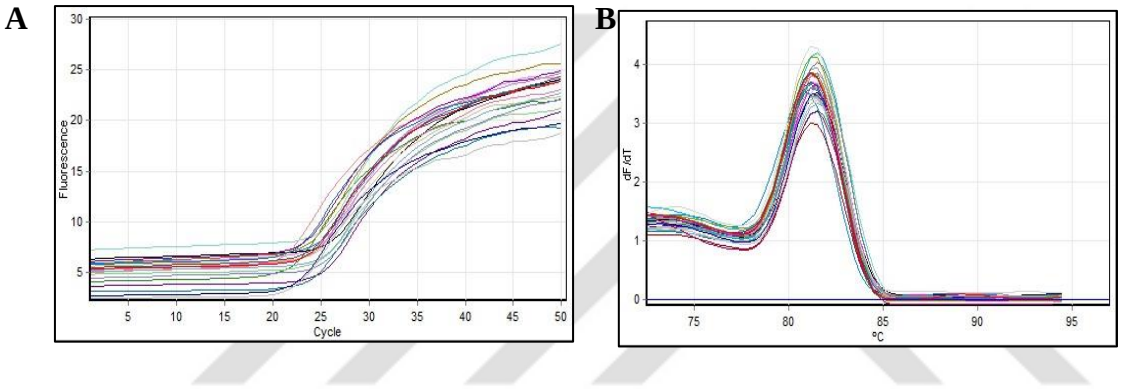

Figure 3.9 Amplification curve and melt curve for GAPDH gene in the brain of mice. A) Amplification curve of qPCR of GAPDH gene showing fluorescence accumulation at each cycle. B) Melting curve of qPCR of GAPDH gene showing fluorescence emission change versus temperature.

CHAPTER 4

DISCUSSION

Multiple sclerosis is an autoimmune, inflammatory neurodegenerative disease. It is the severe disease which affects many young people. The underlying mechanism of the disease have not been solved yet. However, studies showed that MS is complex disease, many factors participated in its pathology. Genetic factors, environmental factors such as low levels of sun exposure, Epstein-Barr virus infection, air pollutants, cigarettes, and toxins have a role in the MS formation (Compston & Coles, 2008). The therapeutic agents used for the treatment of MS, only slow down the process, there is not any treatment to eradicate the disease.

Cytochrome P450 (CYP) is a superfamily of heme containing monooxygenase enzymes. They are integral enzymes for the metabolism of endogenous and exogenous substrates. Endogenous substrates of CYPs are fatty acids, cholesterol, steroids, Vitamin D, arachidonic acids and bile acids. Exogenous substrates of the CYPs are ethanol, drugs including acetaminophen (APAP) and others, polycyclic aromatic hydrocarbons (PAH), aromatic amines, nitrosamines, and many others. Infection and inflammation alter the expression of CYPs as well as bioavailability of substrates metabolized by these enzymes (Renton, 2001). Change in CYP levels will alter the metabolism and activation of its substrate. Decreased level of CYP results in the increased amount of drugs, xenobiotics and endogenic substrates metabolized by the CYP. This may lead dose-dependent drug toxicity (Morgan, 1997). Also, increased level of CYP results in the decreased level of substrates such as drugs which leads to inadequate dosage and treatment. (McDonnell & Dang, 2013). Previous studies showed that i.c.v. injection of lipopolysaccharide (LPS) which mediates and increases pro-inflammatory cytokines, downregulates total CYPs levels (Renton and Nicholson, 2000). Moreover, CNS inflammation activates hepatic acute phase signalling, which shows activation of inflammatory response

(Abdulla et al., 2005). Inflammatory response in the brain includes microglial activation and monocytes recruitment into the brain. Then, macrophages, microglia and astrocytes are activated, and they release cytokines which are TNFα, IL-1β and IL-6 (Nicholson & Renton, 2001). Inflammatory response in the brain also includes cell infiltration and tissue damage. TNF and other cytokines are suggested to be part of the inflammation in the brain during multiple sclerosis (Renton et al., 1999). Moreover, immune responses in the brain or ischemic injury are suggested to alter CYPs. This makes important to consider expression of CYPs in the multiple sclerosis.

CYP2E1 is one of the members of the CYP family and it is found in liver, brain, and small intestine. CYP2E1 is mainly known as its role in ethanol metabolism. However, it also joins metabolism of fatty acids, nicotinamides and procarcinogen. CYP2E1 joins the metabolism of several drugs, including MS drugs such as Dalfampridine. CYP2E1 is a highly studied enzyme because of its broad categories of substrates. Moreover, CYP2E1 produces more reactive oxygen species compared to other CYPs, which is detrimental to the cell if it is at a high level (Tang et al., 2019).

CYP1A1 was detected in lung, liver, brain and small intestine (Stiborová et al., 2005, Lang et al., 2019, Kaye et al., 2016). CYP1A1 has a vital role in the bioactivation of polycyclic and polyaromatic hydrocarbons, heterocyclic amines, nitrosamines and arylamines, whose metabolites bind to DNA and cause damage (Lang et al., 2019). These substrates are found widely in our environments such as in petroleum byproducts, cigarate smoke, combustion-engine exhaust, charcoal-grilled food (Lang et al., 2019). CYP1A1 also participates in the metabolism of endogenous substrates such as melatonin and estradiol (Dutheil et al., 2008). The mRNA and protein expression of CYP2E1 and CYP1A1 are affected by various factors. One of the factors which change the mRNA and protein levels of CYP2E1 and CYP1A1 is inflammation and inflammatory cytokines. Previous studies showed that in the case of the brain inflammation, there are significant decrease in the protein and mRNA level of CYP1A1, CYP2E and several other CYPs in the liver (Renton, 2004). They

also found out that this decrease was not caused by the inflammation response in the liver. They were certain that this downregulation was result of brain signaling mechanism (Renton, 2004). To the best of our knowledge, there is no study investigating the mRNA and protein level of CYP2E1 and CYP1A1 in the brain and liver by using MS mouse model. Therefore, investigating these two inflammatory related enzymes (CYP2E1 and CYP1A1) by utilizing molecular and biochemical approaches in the liver and brain of the EAE mouse model may fill the gap in the literature.

In this study, protein and mRNA levels of CYP2E1 and CYP1A1 enzymes were investigated via Western blotting technique and qPCR, respectively. There was not significant change in CYP2E1 protein level in the liver. However, in EAE group CYP2E1 level was slighltly higher. In contrast to this result, CYP2E1 protein level in liver was significantly decreased 24h after i.c.v. injection of LPS to male Sprague-Dawley rats (Abdulla et al., 2006). Similar to liver result, we did not observe significant change in CYP2E1 in the brain. However, previous *in vivo* studies on gerbils and rats found out that ischemic injury resulted in increased CYP2E1 protein level in the brain (Tindberg., 1996). *In vitro* glial cell culture study showed that CYP2E1 level was increased after LPS treatment (Tindberg & Ingelman-Sundberg, 1996; Tindberg et al., 1996). CYP2E1 mRNA level was increased slightly in the liver and decreased slightly in the brain of EAE compared to control group, however the changes were not significant. Similarly, i.c.v LPS injection to rat mediated the CYP2E1 mRNA expression in the liver significantly (Abdulla et al., 2006). However, there are controversial results in literature. Studies on Fao rat hepatoma cell line showed that interleukin-1 (IL-1), IL-6 and TNF-α treatment downregulates the CYP2E1 mRNA expression after 24 hours and downregulated the protein levels after 72 hours of treatment (Hakkola et al., 2003). The author pointed out that there are differences between Fao cells and rat liver regarding CYP2E1 regulation (Hakkola et al., 2003). LPS injection (intraperitoneally) to C57BL/6N mice significantly down-regulates the CYP2E1 mRNA in the liver (Richardson & Morgan, 2005). However, they did not observe any significant change for CYP2E1

protein level. These differences support the Aitken et al. 2006 who suggested that different models of infection and inflammation may be differentially regulate CYP (Aitken et al., 2006).

Moreover, there was not significant change in liver CYP1A1 protein level in the EAE group compared to control group. However, it was reported that CYP1A1 protein level was downregulated in i.c.v. LPS injected rat liver (Abdulla et al., 2005, Shimamoto et al., 1998). The authors observed that the downregulation of CYPs, including CYP1A1, was not results of liver inflammation, instead it was the results of brain signaling triggered by inflammation in the brain (Nicholson & Renton, 1999, Shimamoto et al., 1998). Moreover, in previous study, LPS treatment (i.c.v.) and EAE was compared for their effects on several CYPs. Ethoxycoumarin O-deethylation (ECOD) activity is an indicator of CYP1A and CYP2C activity. ECOD activity in the brain considerably decreased in two models (Monshouwer et al., 2000). While EAE did not affect the CYP1A activity and content in the liver, LPS treatment (i.c.v.) decreased the CYP1A activity and content significantly in the liver. The authors suggested that these different effects of the models might be caused by higher brain and spinal cord TNF levels in the LPS treated model compared to EAE model. Moreover, although they detect high level of TNF in the LPS treated model serum, they could not detect TNF in the serum of the EAE model. They suggested that this could be explanation of why they did not find any effects of EAE in the liver (Monshouwer et al., 2000).

In the brain, CYP1A1 level was not change between the two groups (EAE and control). *In vivo* studies on LPS treated rat astrocyte cell culture showed decreased CYP1A1 activity and no significant change in protein level (Nicholson & Renton, 1999). In contrast, *in vitro* glial cell culture study showed that CYP1A1 protein level was reduced after LPS, IL-1, and IL-6 treatment (Tindberg & Ingelman-Sundberg, 1996; Tindberg et al., 1996). LPS injection (10 mg) into the lateral ventricle of the rat brain resulted in the decrease of CYP1A1 activity, which was measured by

ethoxyresorufin-O-deethylase (EROD) activity, and protein level in the brain (Renton et al., 1999). In the brain region where CYP1A1 decreased, increase in the level of TNF-α and IL-1 were observed. Authors claimed this is an indicator of the CYP1A regulation through cytokines during LPS induced inflammation. In addition, rat astrocyte cell culture studies showed that Dibenz[a,h]anthracene (DBA), which is an aromatic hydrocarbon, treatment significantly increased CYP1A (CYP1A1 & CYP1A2) activity and protein level (Nicholson & Renton, 1999). Escherichia coli lipopolysaccharide treatment with Dibenz[a,h]anthracene (DBA) decreased the CYP1A activity to half of control group (only DBA treatment). However, LPS treatment with DBA did not significantly change CYP1A protein level (Nicholson & Renton, 1999). The authors also observed that cytokine release is an integral part of the LPS-induced downregulation of CYP1A activity.

Moreover, even though it was not significant, in this study there was a slight increase in CYP1A1 mRNA expression in the liver of EAE group compared to control group. Similar to this result, two different studies showed that Listeria monocytogenes administration, is used to model bacterial meningitis, CYP1A level and activity are regulated time-dependently (Armstrong and Renton, 1993, Garcia Del Busto Cano & Renton, 2003). CYP1A mRNA level initially increased significantly, then decreased to initial level in the liver of L. monocytogenes infection model. Moreover, CYP1A activity followed a similar pattern. It is increased significantly, then decreased significantly. However, these two studies found controversial results for protein level in the liver. They either found similar pattern for protein or did not observe any changes by CNS bacterial infection (Armstrong and Renton, 1993, Garcia Del Busto Cano & Renton, 2003). In addition, we could not detect CYP1A1 mRNA expression in the brain. In previous studies, CYP1A1 was detected at a very low level in the rat brain (Morse et al., 1998). Moreover, in brain total CYPs level is very low, and their activity is 1-10 % of liver CYPs activity (Geng & Strobel, 1997). These can be an explanation why we could not detect CYP1A1 mRNA in brain.

CHAPTER 5

CONCLUSION

Multiple sclerosis is one of the life quality lowering diseases which usually affects young adults. The mechanisms which lead the disease have not been understood yet. However, many different factors have been shown to have a role in the development and prognosis of the disease. Myelin and oligodendrocyte damage, axonal loss and neuron loss, and inflammation are the characteristics of this disease. This is the main reason why MS is classified as autoimmune-neurodegenerative disease. CYP2E1 and CYP1A1 are member of CYP 450 super family. Both enzymes have integral role in the body. CYP2E1 participates in ethanol, benzene, nitrosamines, chloroform, carbon tetrachloride, fatty acids, ketones, and some drugs. Moreover, CYP2E1 produces the highest amount of reactive oxygen species (ROS) among other CYPs. ROS is participated in signal transduction as a second messengers, and many disease initiation and progressions such as Parkinson's disease and Alzheimer's disease. CYP1A1 is involved in the metabolism of arachidonic acid, 17β estradiol, melatonin, polycyclic and polyaromatic hydrocarbons, nitrosamines, and arylamines. Both enzymes convert some of their substrates into more toxic compounds.

In this study, we used female C57BL/6 mouse for model experimental autoimmune encephalomyelitis (EAE - MS Model). We investigated the effects of EAE immunization on mRNA and protein levels of CYP2E1 and CYP1A1 by qPCR and Western blotting techniques, respectively. Relative mRNA and the protein expression of the EAE group to the control group were estimated. Since these two enzymes participated in the metabolism of a broad category of substrates and their mRNA and protein levels are altered in inflammatory conditions, investigating their levels in the case of MS is important. In the case of any alteration in these enzymes, the level of their substrates in the blood and tissues will be altered as well. These

changes may result in altered physiology because of endogenous substrates, and altered drug/xenobiotic response. The results of this study may help us to set a drug dosage, find a pathway for the disease etiopathogenesis and progress, and find a new therapeutic way. According to our results, MS does not significantly change protein levels of CYP2E1 and CYP1A1 in the liver ($p= 0.2$, $p=0.71$,respectively) and in the brain ($p=0.89$, $p=0.64$, respectively). Moreover, our results suggested that MS does not significantly change mRNA levels of CYP2E1 and CYP1A1 in the liver ($p= 0.08$, $p= 0.23$, respectively) and in the brain ($p= 0.11$, expression was not detected, respectively). Although this result suggested that CYP2E1 and CYP1A1 are not affected by MS, we did not check the activity of these enzymes. Maybe their activities are affected by MS. CYP1A1 is regulated time-dependently in the case of inflammation. Therefore, it can be possible that at different stages of the disease CYP1A1 mRNA and protein levels are significantly changed.

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APPENDICES

A. Animal Experimentation Ethics Committee Approval Document

Bilkent Üniversitesi

BİLKENT ÜNİVERSİTESİ HAYVAN DENEYLERİ ETİK KURUL KARARI

TOPLANTI TARİHİ : 27.9.2019
TOPLANTI NO : 7
DOSYA NO : 33
KARAR NO : 2019/33

Bilkent Üniversitesi öğretim üyelerinden Prof.Dr. Orhan Adalı'nın proje yürütücüsü olduğu "Vitamin D, Vitamin D Reseptörü ve Vitamin D Metabolizmasında Rol Alan CYP450 İzozimlerinin Hayvan Modelinde Multipl Skleroz (MS) Hastalığının Patofizyolojisine Etkilerinin Moleküler Düzeyde Araştırılması" başlıklı araştırma deney protokolü Bilkent Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu'nda incelenmiş, yapılan inceleme sonucunda çalışmanın Bilkent Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu Yönergesi'ne göre uygun bulunarak onaylanmasına katılan üyelerin oy birliği ile karar verilmiştir.

Michelle Adams (Başkan)

Ergin Atalar (Üye)

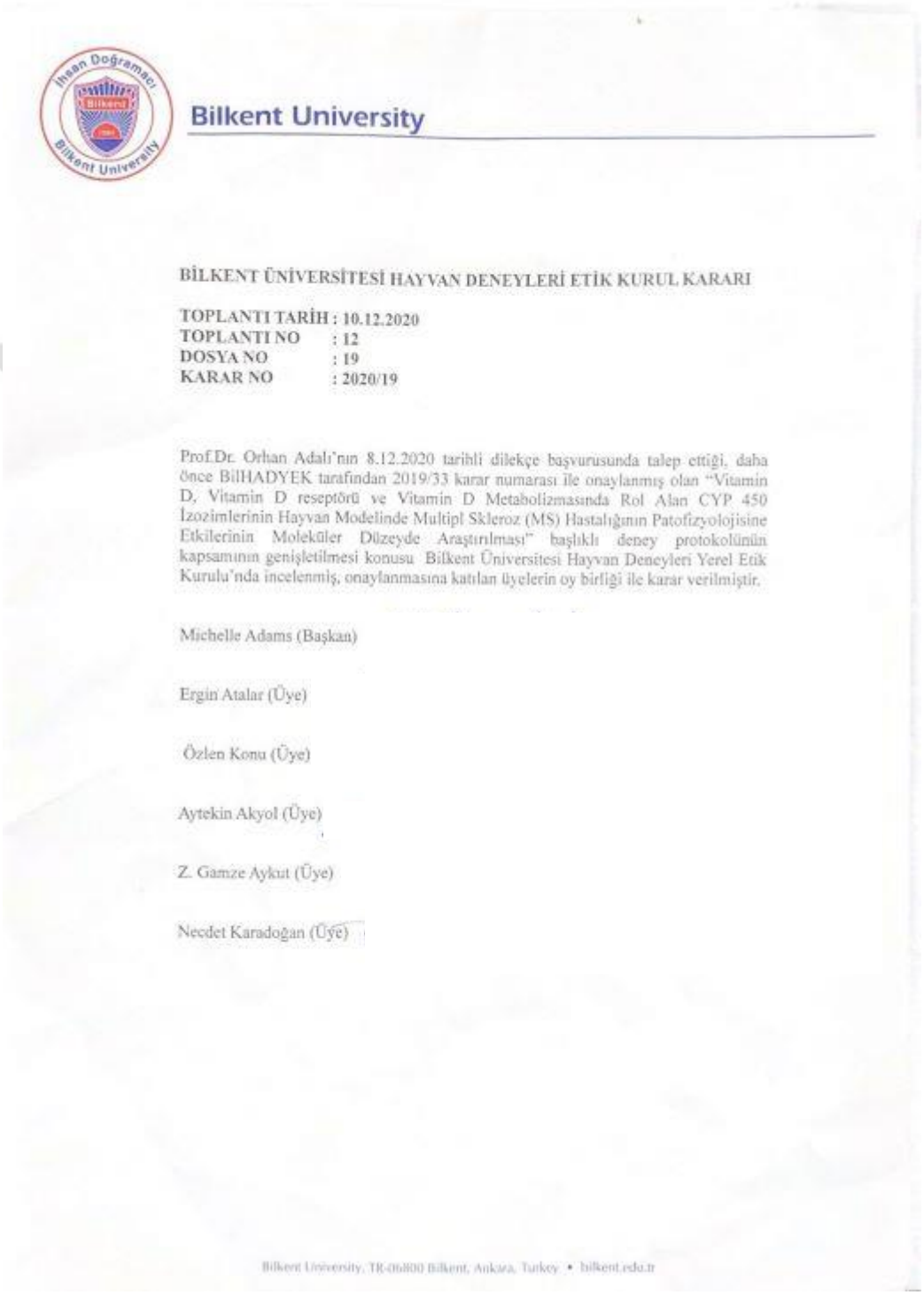
Özden Konu (Üye)

Aytekin Akyol (Üye)

Gamze Aykut (Üye)

Necdet Karadoğan (Üye)

B. Animal Experimentation Ethics Committee Approval Document



Bilkent University

BİLKENT ÜNİVERSİTESİ HAYVAN DENEYLERİ ETİK KURUL KARARI

TOPLANTI TARİH : 10.12.2020
TOPLANTI NO : 12
DOSYA NO : 19
KARAR NO : 2020/19

Prof.Dr. Orhan Adalı'nın 8.12.2020 tarihli dilekçe başvurusunda talep ettiği, daha önce BilHADYEK tarafından 2019/33 karar numarası ile onaylanmış olan "Vitamin D, Vitamin D reseptörü ve Vitamin D Metabolizmasında Rol Alan CYP 450 İzozimlerinin Hayvan Modelinde Multipl Skleroz (MS) Hastalığının Patofizyolojisine Etkilerinin Moleküler Düzeyde Araştırılması" başlıklı deney protokolünün kapsamının genişletilmesi konusu Bilkent Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu'nda incelenmiş, onaylanmasına katılan üyelerin oy birliği ile karar verilmiştir.

Michelle Adams (Başkan)

Ergin Atalar (Üye)

Özlen Konu (Üye)

Aytekin Akyol (Üye)

Z. Gamze Aykut (Üye)

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