Pharmaco-molecular assessment of the effects of anandamide and its antagonists on hippocampal tissue in Wistar albino rats

I. YILMAZ[1], N. KARAARSLAN[2], D. YASAR SIRIN[3], H. OZBEK[1]

[1]Department of Medical Pharmacology, Istanbul Medipol University School of Medicine, Turkey
[2]Department of Neurosurgery, Namik Kemal University School of Medicine, Turkey
[3]Department of Molecular Biology and Genetics, Namik Kemal University Faculty of Arts and Sciences, Turkey

Numan Karaarslan, Duygu Yasar Sirin and Hanefi Ozbek are co-authors

Abstract. – OBJECTIVE: The members of the matrix metalloproteinase (MMP) family and cannabinoids (CBs) are reportedly associated with hippocampus-dependent memory functions. However, the effects of endogenously formed CBs on hippocampal long-term potentiation remain unknown. The present study aimed to investigate the changes in the gene and protein expression levels of matrix metallopeptidase 9 (MMP-9), phosphatase and tensin homolog (PTEN), and NOTCH receptor 1 (NOTCH1) in rat hippocampal tissues treated with anandamide (AEA), AM251, 6-iodopravadolin (AM630), and N-[4-{[(3,4-Dimethyl-5-isoxazolyl)amino]sulfonyl}phenyl] (ML193).

MATERIALS AND METHODS: The subjects were divided into 10 groups (n = five per group). The pharmaceuticals were administered via intraperitoneal injection once a day for seven days, except for the control group. The resected hippocampal tissues were then evaluated using a quantitative real-time polymerase chain reaction (RT-qPCR) and Western blot analysis. The data obtained were statistically analyzed, and p < 0.01 was considered statistically significant.

RESULTS: Contrary to the literature, the changes in MMP-9 expression were not statistically significant, but the changes in PTEN and NOTCH1 were. The findings of this in vivo experimental study revealed that the agonists and antagonists acting on the CB system have significant molecular effects on hippocampal tissue.

CONCLUSIONS: The changes in gene and protein expressions may be one of the reasons for the neurodegenerative processes observed in patients using these agonists and antagonists, whose effects on the CB system have not been fully explained yet. Our study can contribute to the literature as it is the first study investigating the MMP-9, PTEN and NOTCH1 gene and protein expression.

Key Words:
Anandamide, Hippocampus, MMP-9, NOTCH1, PTEN.

Abbreviations

AEA = N-arachidonoylethanolamine also known as anandamide (AEA); AM630 = 6-iodopravadolin; CB = cannabinoid; CBR = cannabinoid receptors; eCBs = endocannabinoid system endocannabinoid system; CB receptors (CBRs) GPR55 = G protein-coupled receptor 55; LTP = long - term potentiation; MF-CA3 = mossy fiber-CA3; MMP = matrix metalloproteinase; NOTCH1 = NOTCH receptor 1; PTEN = phosphatase and tensin homolog.

Introduction

The hippocampus plays a significant role in the formation of physiological events associated with learning and memory[1]. Various physiological events, such as neurogenesis, synaptic plasticity, and emotional states, are largely associated with hippocampal tissues; the cannabinoid (CB) system (CBs) is involved in the regulation of these events[2].

The endocannabinoid system (eCBs) acts on CB receptors (CBRs) and regulates various aspects of human physiological, behavioral, immunological, and metabolic functions[2]. The eCBs affects the neuromodulators involved in the regulation of neuroendocrine and metabolic functions, as well as the pathogenesis of cardiovascular diseases and obesity[3].

Corresponding Author: Ibrahim Yilmaz, Ph.D; e-mail: ibrahimyilmaz77@yahoo.com

The eCBs is known to be involved in many physiopathological processes, including atherosclerosis[4], obesity-related hypertriglyceridemia, and glucose metabolism of the liver[5].

Moreover, scholars[5] suggest that the eCBs is involved in the pathogenesis of pain, inflammation, and glaucoma. Due to its antiproliferative effect on tumor cells, it has led to investigations in glioblastoma and cancer treatments[6], as well as hypertension, heart failure, obesity, diabetes mellitus, metabolic syndrome, starvation, chronic stress, depression, and other psychiatric disorders[7]. The eCBs is synthesized "on-demand" from long-chain polyunsaturated fatty acids and acts on cells in a paracrine or autocrine manner[8]. This system plays a role in the physiological or physiopathological processes of many diseases, such as Alzheimer's, Parkinson's, multiple sclerosis, epilepsy, hypertension, and hypercortisolism[9], and is located in different parts of the brain, especially the hippocampus[10]. Almeida et al[11] show the expression of eCBs in several regions of the brain involved in fear response, including the thalamus, cortex, amygdala, and the hippocampus.

Hippocampal dysfunction has been suggested as the underlying reason for age-related neurodegenerative diseases that affect cognitive functions. Therefore, it is vital to investigate the role of new genes whose expressions have been changed in the physiology and pathology of the hippocampus[12,13].

Proteolytic activity mediated by many complex systems of protease, including members of the matrix metalloproteinase (MMP) family, plays a pivotal role in the mechanisms of hippocampal synaptic plasticity. Furthermore, MMPs are involved in long-term synaptic plasticity, learning, and memory[12,13].

MMP-9 plays a significant role in long-term potentiation (LTP) maintenance in the Schaffer collateral-CA1 pathway and in the acquisition of hippocampus-dependent memory, and changes in MMP-9 levels are therefore a determinant of neurodegenerative processes[14]. Wiera et al[15] have reported that MMP blockades disrupt LTP maintenance in the mossy fiber-CA3 (MF-CA3) projection in which LTP induction and expression are largely presynaptic, and LTP induction is correlated with increased MMP-9 expression.

Anandamide (AEA), known as N-arachidonoyl ethanolamine, is a cannabinoid (CB) neurotransmitter derived from arachidonic acid in the brain and can be found in the central and peripheral nervous systems. Two subtypes of CBRs – cannabinoid-1 receptor (CB1R) and cannabinoid-2 receptor (CB2R) – are located in the central and peripheral nervous systems[16].

The CB1R antagonist AM251, the CB2R antagonist 6-iodopravadoline (AM630), and the G protein-coupled receptor 55 (GPR55) antagonist N-(4-{[(3,4-Dimethyl-5-isoxazolyl)amino] sulfonyl}phenyl) (ML193) are known to be potent and selective antagonists in the brain[17]. This is because the CB1R antagonists damage cognition and prevent the synaptic transmission of LTP. Colangeli et al[18] have reported that CBs are associated with hippocampus-dependent neurodegenerative processes. However, studies on the effect of endogenously formed CBs on hippocampal LTP have failed to provide clarity[19], and no studies have yet investigated the effects of AEA, AM251, AM630, and ML193.

The present study aimed to investigate the changes in the gene and protein expression levels of MMP-9, phosphatase and tensin homolog (PTEN), and NOTCH receptor 1 (NOTCH1) in rats treated with AEA, AM251, AM630, and ML193. The PTEN gene codes the PTEN enzyme, which acts as a dual-specificity protein phosphatase that modifies proteins and fats (lipids) by removing phosphate groups. PTEN dephosphorylates the tyrosine-phosphorylated focal adhesion kinase and inhibits cell migration, integrin-mediated cell spreading, and focal adhesion formation. PTEN can also inhibit MMP-9 through the regulation of NF-kB, and it plays a role as a key modulator of the AKT-mTOR signaling pathway in controlling neuron integration during adult neurogenesis, including correcting neuron positioning, dendritic development, and synapse formation.

Notch signaling is an evolutionarily conserved intercellular signaling pathway that regulates interactions between adjacent cells. NOTCH1 signaling is known to have a pivotal role in brain and memory development, and the MMP-9 protein has a significant influence on NOTCH1 activity. The present study investigated the main role played by the proteins involved in neurodegenerative diseases. It may be possible to not only clarify the probable association between the inhibition of GPR55 and the consecutive changes in the level of MMP-9, previously reported to have several implications at the molecular level in hippocampal tissue, but also reveal the eventual MMP-9 levels in response to the induction or inhibition of different CBRs.

Materials and Methods

Animal Experiments

This randomized, double-blind, in vivo experimental study was performed with the approval of the Local Ethics Committee for Animals. The experiments were carried out in the Istanbul Medipol University Medical Research Center laboratory.

Live mammalian subjects were fed using standard food pellets. The subjects were kept for periods of 12 h in the dark and 12 h in the light. The pharmaceuticals were administered via intraperitoneal injection once a day for seven days.

Preparation of Drugs

All pharmaceuticals were prepared using appropriate diluents in accordance with the manufacturer's protocols. In determining drug concentrations, the specified doses in previous *in vivo* studies were used.

Accordingly, AEA (Catalog No. A0580, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was dissolved in a physiological saline solution to reach a final concentration of 6 mg/ml[20]; AM251 (Catalog No. A6226, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was dissolved in a physiological saline solution to reach a final concentration of 1 mg/ml[21]; AM630 (Catalog No. SML0327, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was dissolved in saline solution to reach a final concentration of 1.25 mg/ml[22]; and ML193 (Catalog No. SML1340, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was dissolved in 0.05% dimethyl sulfoxide (Catalog No. DMS555, BioShop, Canada, Inc., Canada) to reach a final concentration of 5 mg/ml[23]. Stock solutions were stored at 4°C.

Fifty Wistar albino male rats with an average weight of 310 g aged 10 weeks were divided into 10 groups. Group 1 was the control group where no drug application was performed; groups 2 and 3 were sham and diluent control groups, respectively. Daily intraperitoneal medication was applied to the subjects in groups 4-10 for one week. The application, sample number, daily dosage, and application volumes are provided in Table I.

Dissection of Hippocampal Tissues

At the end of one week of applications described in Table I, the subjects were administered Isoflurane-USP 100 ml® (Adeka, Maslak, Istanbul), an inhalation anesthetic, and then, decapitated under general anesthesia using a rodent decapitator (Decapitator Catalog No. 253030599999900006514000001, Remer, Kavacik, Istanbul). A light source (CL 6000 LED®, Zeiss™, Göteborg, Sweden) was used throughout the process. The subjects' heads were taken to the operation site, and the skin and subcutaneous tissue were excised to uncover the osseous tissue. The anatomically uncovered osseous tissues were incised using iris scissors along the caudolateral border of the interparietal bone. Frontal, parietal, and interparietal bone tissues covering the dorsal surface of the cerebral tissue were excised. The cerebral tissue was carefully detached from the surrounding meninges. The cerebral tissue was extracted without damaging the adjacent osseous structures, and the excised cerebral tissue was immediately placed on a frosted.

The cerebellum, pons, and medulla were dissected from the cerebral cortex with a dissector, and the tissues were then detached from the cerebral cortical structures with a surgical steel scalpel (No. 15 blade). The interhemispheric fissure, also known as the medial longitudinal fissure, was incised with the scalpel. The two cerebral hemispheres were thus separated from each other. The left cerebral hemisphere was dissected using

Table I. Group number, application, sample number, daily dosage, and application volume.

Group	Application	N	Daily dosage	Application volume
1	Control	5	No application	-
2	Sham	5	0.9% NaCl (SF)	200 µL
3	Diluent control	5	0.05% DMSO	200 µL
4	AEA	5	0.17 mg/ml	200 µL
5	AM251	5	0.05 mg/ml	200 µL
6	AM630	5	0.17 mg/ml	200 µL
7	ML193	5	0.7 µg/ml	200 µL
8	AEA + AM251	5	0.17 mg/ml + 0.05 mg/ml	200 µL
9	AEA + AM630	5	0.17 mg/ml + 0.17 mg/ml	200 µL
10	AEA + ML193	5	0.17 mg/ml + 0.7 µg/ml	200 µL

small curved forceps, a scalpel, and a dissector. The same procedure was performed on the right cerebral hemisphere, and bilateral hippocampal tissues were obtained[24]. The excised hippocampal tissues were placed in tubes (Figure 1), and the extracted tissues were irrigated in 0.15 ml cold (+4°C) potassium chloride and dried with blotting paper.

Quantitative Real Time-Polymerase Chain Reaction (RT-PCR)

The tissues were then weighed and registered. Half of the extracted hippocampal tissue was used for RNA isolation and the other half for protein isolation. The RNA was extracted from the tissues using a PureLink RNA Mini Kit (Thermo Fisher Scientific, Catalog No. 121830A, Waltham, MA, USA). The tissues were transferred to the lysis buffer (included in the kit) and fully disintegrated mechanically using a rotator. The RNA was isolated in accordance with the manufacturer's instructions, and the extracted RNA was reverse transcribed using a high capacity cDNA RT Kit (Thermo Fisher Scientific, Catalog No. 4368814, Waltham, MA, USA) to obtain cDNA. The reaction conditions were 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C. The changes in MMP-9, NOTCH1, and PTEN expressions were determined with qPCR, which was performed on an Applied Biosystems 7300/7500 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). To determine the gene expression profiles, all genes were amplified using TaqMan Gene Expression Assays MMP-9 (Cat#4331182, Mmp9. Rn00579162_m1, RefSeq NM_031055.1), NOTCH1 (Cat#4331182, Notch1. Rn01758633_m1, RefSeq NM_001105721.1), and PTEN (Cat#4331182, PTEN.Rn00477208_m1, RefSeq NM_031606.1) from Thermo Fisher Scientific, Waltham, MA USA. β-actin TaqMan Gene Expression Assay (Cat#4331182, Actb. Rn00667869_m1, RefSeq NM_031144.3, Thermo Fisher Scientific, Waltham, MA USA) was used for the quantification of gene expression.

Western Blotting

For the total protein isolation, the resected hippocampal tissues were weighed and placed in encoded Eppendorf tubes containing a Triton X-100 (Sigma-Aldrich, Cat#10789704001, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) protease inhibitor cocktail (Roche, Cat#04693159001, Taufkirchen, Germany). The tissues were mechanically degraded, and protein lysates were obtained.

The protein lysates were processed using a western blot test to reveal the expressions of MMP-9, NOTCH1, and PTEN. The total pro-

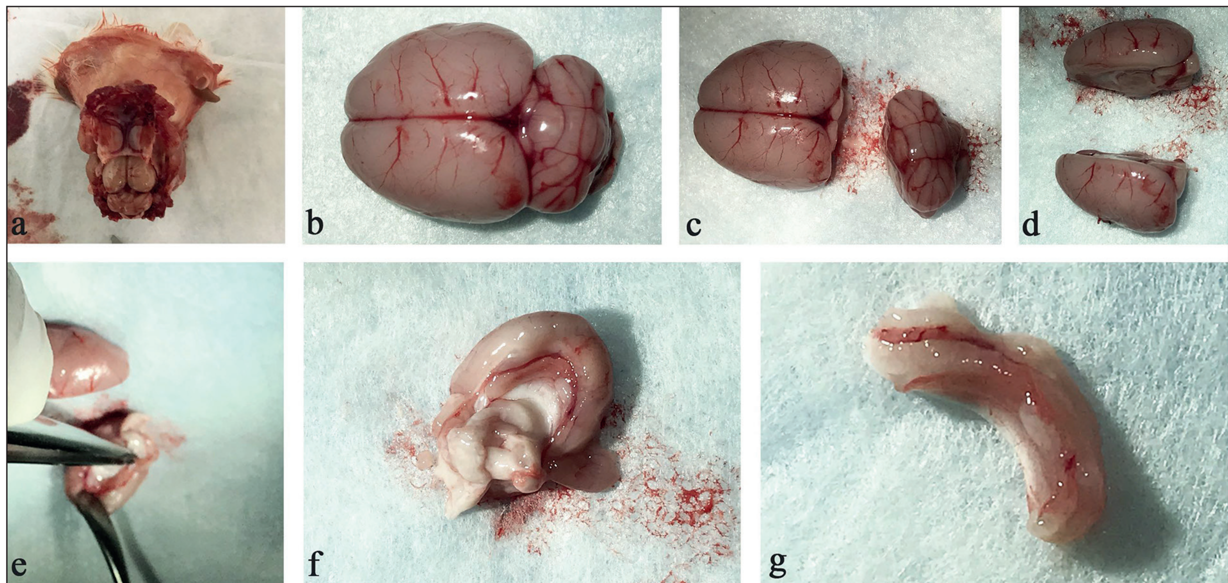

Figure 1. A, The cranial location of the cerebral tissue after the excision of the skin, subcutaneous tissue, and osseous tissue covering the cranium. **B,** Excised brain tissue on a frosted plate. **C,** Two cerebral hemispheres obtained through the excision of the cerebellum, pons, and medulla. **D,** and **E,** Medial surface of the right cerebral hemisphere extracted after the incision of the interhemispheric fissure and the dissection of the corpus callosum and hippocampal tissue in the right cerebral hemisphere. **F,** Hippocampal tissue in the right cerebral hemisphere. **G,** Excised right cerebral hippocampal tissue.

tein amount was determined with a Bradford protein assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the samples, each of which contained 100 mg of protein. Using iBlot, the samples were transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Cat#IB401001, Waltham, MA, USA) to perform immunoblotting. The PVDF membranes were blotted with an MMP-9 monoclonal antibody (Thermo Fisher Scientific, Cat#MA5-15886, Waltham, MA, USA) at a dilution of 1:500, a NOTCH1 polyclonal antibody (Thermo Fisher Scientific, Cat#PA5-23181, Waltham, MA, USA) at a dilution of 5mg/ml final concentration, a phospho-PTEN (Ser380) polyclonal antibody (Thermo Fisher Scientific, Cat#PA5-17826, Waltham, MA, USA) at a dilution of 1:1000, and a monoclonal β-actin antibody (Thermo Fisher Scientific, Waltham, Cat#MA1-140) at a dilution of 1:5000. Immunoblotting was performed using a Western Breeze chemiluminescence kit (Thermo Fisher Scientific, Cat#WB7104, Waltham, MA, USA) according to the manufacturer's instructions. The protein bands transferred to an X-ray film (Thermo Fisher Scientific, Cat#34090, Waltham, MA, USA) were analyzed using ImageJ software, and the specific amount of protein in each sample was determined.

Statistical Analysis

Non-parametric tests were used to analyze the data, as most of the variables did not meet the assumption of normality and the number of subjects per group (n = 5) was low. The relative quantification (RQ) and Western blot values in the experimental and control groups were compared using a Kruskal-Wallis test. A Mann-Whitney U test was used to show the differences between independent groups. Statistical analyses were performed using IBM SPSS 22 statistical software (IBM Inc., Armonk, NY, USA). The significance level was set at $\alpha = 0.01$.

Results

RT-qPCR and Western blot assays were performed for all groups (i.e., 100 samples in total). MMP-9, PTEN, and NOTCH1 gene expressions were normalized using β-actin, an internal control gene. Group 1 served as the control group, and changes in the gene expressions (RT-qPCR) and protein expressions (Western blot) of the experimental groups are presented as a fold change relative to group 1. For the RT-qPCR assays, the RQ value was set to one in group 1, and the gene expression level in this group was considered to be 100%, as shown in Table II.

Table II. RQ values of the study groups.

Group	MMP9	NOTCH1	PTEN
1	1	1	1
2	1.25	1.26	0.80
3	1.50	1.21	1.04
4	1.01	1.51	0.66
5	1.36	1.36	0.77
6	1.63	1.13	1.32
7	0.93	1.24	0.79
8	1.01	1.23	2.75
9	1.08	1.36	1.20
10	1.65	1.75	1.65

For the Western blot assays, the protein bands were quantified using ImageJ software, and the protein expression in group 1 was set to be 1 – that is, 100%, as shown in Table III.

The Western blot tests were repeated at least three times for each sample. An illustrative Western blot figure from group 1 and a graph showing the protein expression fold change in all groups are shown in Figure 2. Illustrative Western blots for all groups are given in the **Supplementary Data.**

An analysis of the RQ values in the control and study groups revealed that there were no significant differences in the MMP-9 ($\chi^2 = 10.848$, $p > 0.01$.), NOTCH1 ($\chi^2 = 8.771$, $p > 0.01$), and PTEN ($\chi^2 = 7.727$, $p > 0.01$) values. However, the Western blot results revealed significant differences in the PTEN values ($\chi^2 = 25.508$, $p < 0.01$); the PTEN values of group 1 were higher than those of groups 2, 3, and 4. Moreover, the values of group 4 were

Table III. Protein expression values (r).

Group	PTEN	MMP9	NOTCH1
1	1.00	1.00	1.00
2	0.17	1.00	0.35
3	0.41	4.25	2.87
4	0.11	1.33	1.56
5	0.32	1.36	4.87
6	0.50	2.39	2.69
7	0.70	1.76	2.59
8	0.62	1.92	1.93
9	0.93	5.51	8.10
10	0.82	3.19	2.38

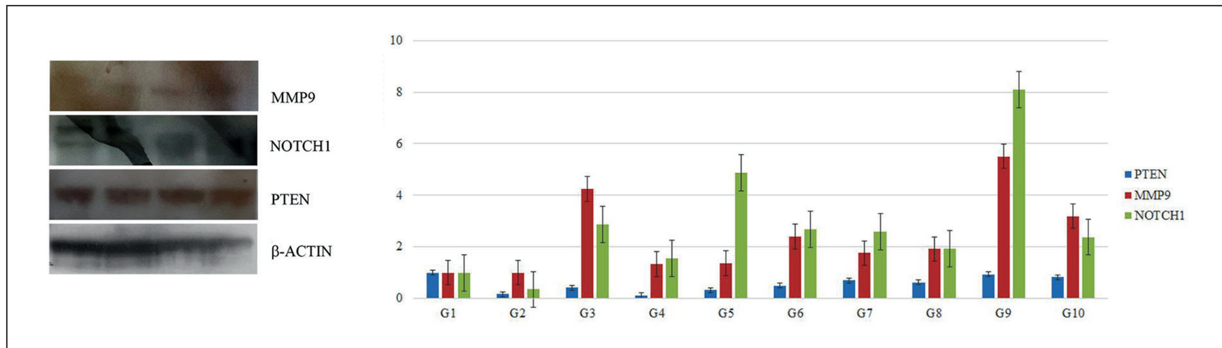

Figure 2. Western blot analysis. In the study, a western blot analysis was performed at least three times for each of 10 experimental groups, with five samples from each group. Illustrative Western blot figures are presented from group 1, and a graph showing the protein expression fold change in all groups is given. Each row shows the immunoblot results for MMP-9, NOTCH1, PTEN, and b-ACTIN.

lower than those of groups 6, 8, and 9 ($p < 0.01$).

There were also significant differences in NOTCH1 values ($\chi^2 = 29.179$, $p < 0.01$). The NOTCH1 values of group 9 were higher than those of groups 1, 3, 4, 7, 8, and 10; the values of group 5 were higher than those of groups 1, 7, 8, and 10; and the values of group 2 were lower than those of groups 3, 4, 5, 9, and 10. No significant differences were observed in the MMP-9 values ($\chi^2 = 15.472$, $p > 0.01$).

Discussion

Clinical manifestations resulting from a lifetime of neurodegeneration constitute the group of neurodegenerative diseases, such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, and Huntington's disease. Neurodegeneration can be defined as the progressive loss of structure or function of neurons.

While neurodegenerative diseases such as Alzheimer's and Parkinson's often occur due to aging, neurodegenerative diseases such as amyotrophic lateral sclerosis and Huntington's disease can occur at young ages. In other words, neurodegeneration is associated with both aging and genetic factors[25]. Dementia, a symptom of neurodegenerative diseases, is the destruction of mental and social abilities, especially memory, to the extent that it affects one's daily life activities. Alzheimer's disease is the most common disease in the primary degenerative dementia group with progressive cognitive disorders[25,26].

In the macroscopic examination of the brains of Alzheimer's patients, atrophy in the cerebral cortex and hippocampus, enlargement of the sulcus, reduction in frontotemporal areas and para-hippocampal gyrus, and ventricular enlargement due to tissue loss are observations[27].

Alzheimer's disease can be classified as early, moderate, or severe. In the early stage, clinical symptoms include amnesia, aphasia, apraxia, and agnosia. Episodic memory impairment and disorientation are important main symptoms, and episodic memory is related to the deterioration of the hippocampus tissue[28-30].

The hippocampus stores memories, and the amygdala functions by regulating emotional memories[31]. The amygdala performs rapid and overall processing while scanning whether the sensory stimulus matches an experience in an emotional memory. Therefore, not only hippocampus tissue damage but also amygdala damage may be important in regulating emotional memories[31].

Typical symptoms of Parkinson's disease include bradykinesia, postural instability, rigidity, and resting tremors. The main histopathological features of Parkinson's disease are the loss of dopaminergic neurons from the substantia nigra to the basal ganglia and the accumulation of so-called Lewy bodies in the cytoplasm of surviving neurons[30].

The eCBs plays a role in the regulation of brain development, the release of neurotransmitters, synaptic plasticity, and cytokine release from microglia by cell, tissue, organ, and organism homeostasis. As a result, the eCBs plays a role in many neurological disorders, such as the aforementioned Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, and Huntington's diseases[32,33].

The microtubule-associated tau protein is a distinguishing feature of many neurodegenerative

diseases, such as Alzheimer's disease and chronic traumatic encephalopathy[34-37]. It is a tauopathy associated with epilepsy that contributes to the acceleration of cognitive regression in temporal lobe epilepsy and has diagnostic and therapeutic consequences[34-37].

The most common adult epilepsy is mesial temporal sclerosis epilepsy, which is caused by hippocampal sclerosis and is associated with a high prevalence of cognitive impairment.

The potential efficacy of CB use in children with Dravet Syndrome and Lennox-Gastaut Syndrome has been investigated regarding epilepsy, where a neurodegenerative disease relationship has been suggested[35-38].

To reveal the mechanisms of occurrence of these diseases and to develop new treatments by creating experimental models that will make it possible to study these diseases in experimental animals.

Animal experiments and clinical studies have shown that cannabis-based therapy is beneficial in the treatment of different diseases, but further studies are still needed to investigate the efficacy and safety of such therapeutics[39]. The present study is aimed at investigating changes in the gene and protein expression levels of MMP-9, PTEN, and NOTCH1 in rats treated with AEA, AM251, AM630, and ML193. Furthermore, the main role played of proteins involved in neurodegenerative diseases was investigated.

LTP is effective in learning and memory, and the hippocampus plays an important role in transforming short-term memory into long-term memory in the neocortex. In this process, the hippocampus performs a critical function by providing the first inputs required for long-term memory, and then converts them into long-term memory by creating and strengthening the synaptic connections necessary for sustainable long-term retention[40].

The mechanism related to hippocampal memory and the affecting factors have been investigated in studies on protective and therapeutic methods for neurodegenerative diseases[41]. Molecular and pharmaco-molecular studies on gene expressions and proteins involved in hippocampal tissues have therefore gained popularity. The literature also indicates that neural progenitor cells can be affected by both extrinsic factors, such as growth factors, cytokines, and MMPs, and intrinsic factors, such as transcription factors and regulators of signaling pathways[42].

PTEN independently modulates the functional and structural features of hippocampal neurons and plays a key role in the mechanisms of synaptic plasticity. However, LTP is insufficient when PTEN is removed or downregulated in hippocampal neurons, and behavioral abnormalities also arise – neurodegenerative pathologies have been reported to develop due to the suppression or down-regulation of PTEN in hippocampal neurons[43].

PTEN, a bidirectional phosphatase enzyme that influences many proteins, negatively regulates PI3K and dephosphorylates phosphatidylinositol 3.4.5-triphosphate to phosphatidylinositol 4.5-bisphosphate[44]. A loss of PTEN is directly associated with an increase in Akt activity[45]. In the present study, PTEN protein expression increased in the AM630-treated group (group 6), and ligands in CB receptor inhibitors increased in groups 8, 9, and 10. The increase in protein expression was statistically significant ($p < 0.01$).

The NOTCH1 signaling pathway – a highly conserved pathway that regulates cell fate – is a cell signaling system present in most multicellular organisms. Mammals possess four different notch receptors (NOTCH1, NOTCH2, NOTCH3, and NOTCH4). The NOTCH receptor is a single-pass transmembrane receptor protein, and the NOTCH pathway is involved in the regulation of the characteristic functions of cells and in controlling the stem and precursor cell layers[46]. The NOTCH1 signaling pathway is an evolutionarily conserved signaling mechanism that regulates development and is involved in plasticity-related processes, including changes in neurite structure and the preservation of neural stem cells[47]. NOTCH1 proteins have been reported to interact with presenilins and with β-amyloid precursor proteins, and may therefore play a role in familial and sporadic Alzheimer's disease[47]. Null heterozygous mutations in NOTCH1 may provoke significant impairments in spatial learning and memory without affecting other forms of learning, motor control, or exploratory activity[47]. The authors have previously suggested that a decrease in NOTCH1 signaling may lead to neurodegeneration and that abnormalities in NOTCH-dependent transcription may be associated with Alzheimer's disease and Alagille and Cadasil syndromes[47,48]. In the present study, the NOTCH1 protein increased in the AM251-treated group (group 5) and the AEA + AM630-treated group (group 9) ($p < 0.01$).

MMP expression may increase due to the influence of factors manipulating the mechanism of gene transcription during the remodeling of

tissues under various physiological and pathological conditions[49]. Similarly, to the progression of highly specific and potent orthosteric ligands, as well as the development of allosteric ligands, the eCBs has come to the forefront as a modulator of many physiological processes. As such, it has attracted interest from researchers who have investigated the role of the eCBs and medical cannabis in human physiology through pharmaceutical chemistry and pharmaco-molecular laboratories[50]. In the present study, the changes in MMP-9, PTEN, and NOTCH1 gene protein expressions were evaluated in live mammalian subjects treated with AEA agonists and antagonists. Salaga et al[51] conducted research in which a CB2-selective antagonist AM630 was used, and CB2R was distributed in the central and peripheral tissues, including immunocytes. Fowler et al[52] reported that there were no compounds selectively inhibiting AEA synthesis and that the mechanisms obstructing the release and uptake of AEA were not fully elucidated. The authors emphasized that the selective agonists and antagonists of CB1R and CB2R were well described. In their study, the modulation of the ECB system was stated to produce neuroprotective effects[52]. Aguayo et al[53] tested the hypothesis that MMP-9, an enzyme that breaks down ECM components and synaptic proteins, such as β-dystroglycan (β-DG43), changed its activities and distribution in the rat hippocampus during an acute stress response. In that study, after 24 h of stress, MMP-9 net activity increased in the somatic area. That is to say, in the stratum pyramidal and granular cell layers and in the synaptic area, especially in the *stratum radiatum* and molecular layer of the hippocampus, MMP-9 enriched the hippocampal synapto-neurosome fractions without altering their potential enzymatic activity[53].

Basavarajappa et al[54] investigated the effects of pharmaceuticals on neurodegenerative processes and reported that many exogenous and endogenous CBs, such as AEA and 2-AG, demonstrated an important role in hippocampal memory processes in rodents. However, the mechanisms through which endogenous AEA regulates these processes remain unknown. The authors noted that the acute administration of URB597 increased the effects of AEA without changing the levels of 2-AG or CB1R in the hippocampus and neocortex and that, in hippocampal slices, URB597 damaged LTP in CB1R WT, but not in the knockout (KO) offspring. In that study, URB597 increased extracellular-signal-regulated kinase (ERK) phosphorylation in WT without affecting ERK levels in WT or KO mice. The authors indicated that pharmacologically increased AEA damaged LTP, learning, and memory and inhibited Ca^{2+}/calmodulin kinases-IV and cyclic amp-response element binding protein phosphorylation through the activation of CB1Rs. The study showed the disruptive effects of the pharmacological elevation of AEA beyond normal concentrations on underlying physiological responses[54].

Terranova et al[55] reported that delta 9-tetrahydrocannabinol and the synthetic CB agonist HU-210(-)-11-OH-delta8-tetrahydrocannabinol-dimethylheptyl prohibited LTP induction in rat hippocampal slices.

Wiera et al[14] noted that LTP was commonly understood to be a memory substrate, and, in the hippocampal CA3-CA1 pathway, different forms of LTP hinged on NMDA receptors (nmdaLTP) or L-type voltage-gated calcium channels (vdccLTP). In their study, the regulation of different LTP forms by different MMPs in mice hippocampal slices was investigated. The authors indicated that MMP-3 inhibition or KO damaged late-phase LTP in the CA3-CA1 pathway, as well as the MMP-9 blockade, reduced late-phase LTP[14].

Both early- and late-phase LTP were observed to be damaged when MMP-3 and MMP-9 were disrupted[14]. Immunoblotting, in situ zymography, and immunofluorescence results revealed that LTP induction was correlated with an increase in MMP-3 expression and activity in the CA1 stratum radiatum[14].

The findings of the study showed that the activation of perisynaptic MMP-3 supported L-type channel-dependent LTP in the CA1 region, while NMDA LTP hinged on MMP-9. The authors stated that the underlying molecular signaling pathways in the hippocampal tissue were not well understood. The obtained results showed that different MMPs may behave as molecular switches for some types of LTP[14].

Szepezi et al[56] reported that chemically induced LTP (cLTP) induction in dissolved hippocampal cultures increased MMP-9 activity, which controls the formation of spine head protrusions (SHP). The authors indicated that auto-active recombinant MMP-9 promoted the formation of SHPs in organotypic hippocampal slices and that blocking MMP activity or microtubule dynamics eliminated the appearance of SHPs[56]. The results of the study demonstrated that MMP-9 plays a strong functional role in the formation of SHPs

and in the control of postsynaptic receptor distribution on the cLTP.

Dziembowska et al[57] reported that MMP-9 had a significant role in the regulation of synaptic plasticity and that MMP-9 mRNA was carried to dendrites for local translation and protein release. The authors suggested that locally secreted MMP-9 may contribute to the structural and functional plasticity of the activated synapses[57].

NOTCH receptors transduce extracellular signals at the cell surface and change the gene expression pattern of cells. NOTCH1 is one of the receptors that regulates PTEN expression and the activity of the PI3K-AKT signaling pathway[58].

The relationship between MMP-9 and NOTCH1 signaling has been demonstrated, and it is well known that an increase in MMP-9 expression results in the strong activation of NOTCH1 signaling[59]. Moreover, PTEN down-regulates MMP-9 in response to TNF-alpha through the transcription factor NF-kappaB and activation protein-1[60].

Wiera et al[15] indicated that exogenous protease could repair LTP in mice, whereas, in the wild-type, excess MMP-9 damaged LTP. The authors suggested that LTP maintenance in the MF-CA3 pathway may result in altered MMP-9 levels that may be harmful for cognitive processes, as observed in some neuropathologies[15].

Brzdąk et al[61] noted that MMP-NMDAR correlations were not fully elucidated. Their study investigated the involvement of MMP subtypes in E-S plasticity and NMDAR function in mouse hippocampal acute brain slices. The authors emphasized that the temporal necessity for MMP-3/NMDAR activity in E-S potentiation in the CA1 region mostly coincided, and MMP-3, but not MMP-2/9, activity was vital for the functional acquisition of NMDARs following LTP induction[61].

Tsilibary et al[62] suggested that there were indications implicating MMPs in major neuropsychiatric disorders, probably by generating synaptic aberrations. MMP-9, NOTCH1, and PTEN signaling are intersecting signal pathways that are associated with brain development and the hippocampus and have been the subject of many studies that have investigated drug dependence or neurological diseases[63]. In this study, both the agonist and antagonist drugs that were applied to the subjects resulted in changes in MMP-9 gene expression, but the changes observed were not statistically significant ($p > 0.01$). The present study is the first in vivo research seeking to investigate the effects of anandamide and its antagonists, which have been shown to cross the blood-brain barrier[64] on MMP-9, NOTCH1, and PTEN gene and protein expressions.

Group 1 served as the control group during the Western blot and RT-qPCR assays and included untreated samples. Groups 2 and 3 were treated with the drug diluents that did not provoke any significant changes in gene and protein expression. Changes in MMP-9, NOTCH1, and PTEN gene expressions were evaluated with RT-qPCR assays, and the observed changes were not considered statistically significant. The Western blot results revealed that the changes in MMP expression were not statistically significant and that the NOTCH1 protein increased in group 5, which was treated with AM251, and in group 9, which was treated with AEA + AM630. The increase in PTEN protein expression was statistically significant in group 6, which was treated with AM630, and in groups 8, 9, and 10, all of which were treated with ligands and CBR inhibitors.

A week of drug application did not change gene expression but did cause an acute increase in the amount of protein expression, and the hippocampal cells swiftly responded to the applied drugs. The applications did not change the MMP-9 gene and protein levels, yet the expression of NOTCH1-a signal pathway activated by MMP-9-increased, and the observed NOTCH1 activation was independent of MMP-9. The increase in PTEN expression in groups 6, 8, 9, and 10 was expected to suppress MMP-9 expression in the same groups, but MMP-9 expression remained unchanged.

The present study used rat hippocampal tissue for the analyses, and assays were performed using a very limited number of samples. The literature indicates that the sensitivity of human and animal tissues is different**.** Accordingly, the results obtained in these studies may differ from the results obtained with human samples and may be misleading, which is the primary limitation of this study.

Conclusions

This is the first study in which AEA, AM251, AM630, and ML193 were evaluated together in rat hippocampal tissues. The observed changes in MMP-9 gene expression following the administration of AEA – a potent endogenous agonist of CB1R and CB2R – and its antagonists were statistically insignificant. PTEN expression levels were increased with the administration of only AM630,

a potent and selective inverse agonist for CB2R in rat hippocampal tissue. PTEN expression levels were also increased with the combined application of AEA; AM251, an inverse agonist at the CB1R; AM630, a CB2R antagonist; and ML193, a GPR55 antagonist. An increase in NOTCH1 protein was observed following the administration of only AM251, an inverse agonist at the CB1R, and the concomitant administration of AEA and AM630, a CB2R antagonist. These results suggest that systemic manipulations of eCBs may alter neurodegenerative disorders associated with memory loss. This is an important aspect of the current research. Medication that can act on the CB system should be developed for the treatment of diseases in hippocampal tissue, and further researches that include many more subjects should be performed to provide further insight into the efficacy of these pharmaceuticals on MMP-9, PTEN, and NOTCH1 signaling pathways.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

The authors thank the Medical Research Center laboratory (Istanbul Medipol University) employees for all support and assistance

Availability of Data

The data and materials generated/analyzed in the present study are available from the corresponding author upon request.

Ethical Approval

The entire experimental procedure was approved by the Istanbul Medipol University Local Ethics Committee for Animals (Permission of Live Mammal Usage for Experiments No. 38828770-604.01.01-E.10835).

Authors' Contribution

I.Y., N.K., and D.Y.S. designed the study and the experiments. I.Y. and N.K. performed the intraperitoneal injections on rats. I.Y. and N.K. resected hippocampal tissues. I.Y., N.K., and D.Y.S. worked on the experiments and the molecular analysis of the tissues. I.Y. collected the data. I.Y., N.K., and H.O. carried out the statistical analysis. All the authors read and approved the final manuscript.

References

1) Zhang JC, Zhang WJ, Zhao Q, Chen BN, Wang X, Luo YP, Zhang FX. Adiponectin improves isoflurane-induced cognitive dysfunction in elderly rats via inhibiting p38-MAPK signal pathway in hippocampus. Eur Rev Med Pharmacol Sci 2019; 23: 171-176.
2) Joshi N, Onaivi ES. Endocannabinoid system components: overview and tissue distribution. Adv Exp Med Biol 2019; 1162: 1-12.
3) Szczepanska-Sadowska E, Cudnoch-Jedrzejewska A, Ufnal M, Zera T. Brain and cardiovascular diseases: common neurogenic background of cardiovascular, metabolic and inflammatory diseases. J Physiol Pharmacol 2010; 61: 509-521.
4) Piscitelli F, Silvestri C. Role of the endocannabinoidome in human and mouse atherosclerosis. Curr Pharm Des 2019; 25: 3147-3164.
5) Bazwinsky-Wutschke I, Zipprich A, Dehghani F. Endocannabinoid system in hepatic glucose metabolism, fatty liver disease, and cirrhosis. Int J Mol Sci 2019; 20: 2516.
6) Ellert-Miklaszewska A, Ciechomska IA, Kaminska B. Cannabinoid Signaling in Glioma Cells. Adv Exp Med Biol 2020; 1202: 223-241.
7) García-Baos A, Alegre-Zurano L, Cantacorps L, Martín-Sánchez A, Valverde O. Role of cannabinoids in alcohol-induced neuroinflammation. Prog Neuropsychopharmacol Biol Psychiatry 2021; 104: 110054.
8) Horn H, Böhme B, Dietrich L, Koch M. Endocannabinoids in body weight control. Pharmaceuticals (Basel) 2018; 11: 55.
9) Maurya N, Velmurugan BK. Therapeutic applications of cannabinoids. Chem Biol Interact 2018; 293: 77-88.
10) Çakir M, Tekin S, Doğanyiğit Z, Erden Y, Soytürk M, Çiğremiđ Y, Sandal S. Cannabinoid type 2 receptor agonist JWH-133, attenuates Okadaic acid induced spatial memory impairment and neurodegeneration in rats. Life Sci 2019; 217: 25-33.
11) Almeida V, Levin R, Peres FF, Suiama MA, Vendramini AM, Santos CM, Silva ND, Zuardi AW, Hallak JEC, Crippa JA, Abílio VC. Role of the endocannabinoid and endovanilloid systems in an animal model of schizophrenia-related emotional processing/cognitive deficit. Neuropharmacology 2019; 155: 44-53.
12) Padamsey Z, Mcguinness L, Bardo SJ, Reinhart M, Tong R, Hedegaard A, Hart ML, Emptage NJ. Activity-dependent exocytosis of lysosomes regulates the structural plasticity of dendritic spines. Neuron 2017; 93: 132-146.
13) Xiong L, Duan L, Xu W, Wang Z. Nerve growth factor metabolic dysfunction contributes to sevoflurane-induced cholinergic degeneration and cognitive impairments. Brain Res 2019; 1707: 107-116.
14) Wiera G, Nowak D, Van Hove I, Dziegiel P, Moons L, Mozrzymas JW. Mechanisms of NMDA receptor- and voltage-gated L-Type calcium channel-dependent hippocampal LTP critically rely on proteolysis that is mediated by distinct metalloproteinases. J Neurosci 2017; 37: 1240-1256.
15) Wiera G, Wozniak G, Bajor M, Kaczmarek L, Mozrzymas JW. Maintenance of long-term potentiation in

hippocampal mossy fiber—CA3 pathway requires fine-tuned MMP-9 proteolytic activity. Hippocampus 2013; 23: 529-543.

16) Ulugol A. The endocannabinoid system as a potential therapeutic target for pain modulation. Balkan Med J 2014; 31: 115-120.

17) Bilir KA, Anli G, Ozkan E, Gunduz O, Ulugol A. Involvement of spinal cannabinoid receptors in the antipruritic effects of WIN 55,212-2, a cannabinoid receptor agonist. Clin Exp Dermatol 2018; 43: 553-558.

18) Colangeli R, Pierucci M, Benigno A, Campiani G, Butini S, Di Giovanni G. The FAAH inhibitor URB597 suppresses hippocampal maximal dentate after discharges and restores seizure-induced impairment of short- and long-term synaptic plasticity. Sci Rep 2017; 7: 11152.

19) Silva-Cruz A, Carlstrom M, Ribeiro JA, Sebastião AM. Dual influence of endocannabinoids on long-term potentiation of synaptic transmission. Front Pharmacol 2017; 8: 921.

20) Walentiny DM, Gamage TF, Warner JA, Nguyen TK, Grainger DB, Wiley JL, Vann RE. The endogenous cannabinoid anandamide shares discriminative stimulus effects with Δ9-tetrahydrocannabinol in fatty acid amide hydrolase knockout mice. Eur J Pharmacol 2011; 656: 63-67.

21) Bialuk I, Winnicka MM. AM251, cannabinoids receptors ligand, improves recognition memory in rats. Pharmacol Rep 2011; 63: 670-679.

22) Ross RA, Brockie HC, Stevenson LA, Murphy VL, Templeton F, Makriyannis A, Pertwee RG. Agonist-inverse agonist characterization at CB1 and CB2 cannabinoid receptors of L759633, L759656 and AM630. Br J Pharmacol 1999; 126: 665-672.

23) Rahimi A, Hajizadeh Moghaddam A, Roohbakhsh A. Central administration of GPR55 receptor agonist and antagonist modulates anxiety related behaviors in rats. Fundam Clin Pharmacol 2015; 29: 185-190.

24) Yilmaz I, Karaarslan N, Ozbek H. Practical performance of hippocampal tissue resection in rats in pharmacomolecular research. Turk Neurosurg 2020 Sep 29. doi: 10.5137/1019-5149.JTN.31730-20.1 [Online Publishing].

25) Hussain R, Zubair H, Pursell S, Shahab M. Neurodegenerative diseases: regenerative mechanisms and novel therapeutic approaches. Brain Sci 2018; 8: 177.

26) Duong S, Patel T, Chang F. Dementia: what pharmacists need to know. Can Pharm J 2017; 150: 118-129.

27) Alves L, Correia AS, Miguel R, Alegria P, Bugalho P. Alzheimer's disease: a clinical practice-oriented review. Front Neurol 2012; 3: 63.

28) Erkkinen MG, Kim MO, Geschwind MD. Clinical neurology and epidemiology of the major neurodegenerative diseases. Cold Spring Harb Perspect Biol. 2018; 10: a033118.

29) Apostolova LG. Alzheimer disease. Continuum (Minneap Minn) 2016; 22: 419-434.

30) Wang XX, Zhang B, Xia R, Jia QY. Inflammation, apoptosis and autophagy as critical players in vascular dementia. Eur Rev Med Pharmacol Sci 2020; 24: 9601-9614.

31) Bechara A, Tranel D, Damasio H, Adolphs R, Rockland C, Damasio AR. Double dissociation of conditioning and declarative knowledge relative to the amygdala and hippocampus in humans. Science 1995; 269: 1115-1118.

32) Mhyre TR, Boyd JT, Hamill RW, Maguire-Zeiss KA. Parkinson's disease. Subcell Biochem 2012; 65: 389-455.

33) Estrada JA, Contreras I. Endocannabinoid receptors in the CNS: Potential drug targets for the prevention and treatment of neurologic and psychiatric disorders. Curr Neuropharmacol 2020; 18: 769-787.

34) Cristino L, Bisogno T, Di Marzo V. Cannabinoids and the expanded endocannabinoid system in neurological disorders. Nat Rev Neurol 2020; 16: 9-29.

35) Tai XY, Koepp M, Duncan JS, Fox N, Thompson P, Baxendale S, Liu JY, Reeves C, Michalak Z, Thom M. Hyperphosphorylated tau in patients with refractory epilepsy correlates with cognitive decline: a study of temporal lobe resections. Brain 2016; 139: 2441-2455.

36) Bernasconi N. Is epilepsy a curable neurodegenerative disease? Brain 2016; 139: 2336-2337.

37) Gaston TE, Szaflarski JP. Cannabis for the treatment of epilepsy: an update. Curr Neurol Neurosci Rep 2018; 18: 73.

38) Morano A, Fanella M, Albini M, Cifelli P, Palma E, Giallonardo AT, Di Bonaventura C. Cannabinoids in the treatment of epilepsy: current status and future prospects. Neuropsychiatr Dis Treat 2020; 16: 381-396.

39) Barrie N, Kuruppu V, Manolios E, Ali M, Moghaddam M, Manolios N. Endocannabinoids in arthritis: current views and perspective. Int J Rheum Dis 2017; 20: 789-797.

40) Moscarello JM, Maren S. Flexibility in the face or fear: Hippocampal-prefrontal regulation of fear and avoidance. Curr Opin Behav Sci 2018; 19: 44-49.

41) Zhao C, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. Cell 2008; 132: 645-660.

42) Gouignard N, Andrieu C, Theveneau E. Neural crest delamination and migration: looking forward to the next 150 years. Genesis 2018; 56: e23107.

43) Ogino M, Ichimura M, Nakano N, Minami A, Kitagishi Y, Matsuda S. Roles of PTEN with DNA repair in Parkinson's Disease. Int J Mol Sci 2016; 17: 954.

44) Tamura M, Gu J, Matsumoto K, Aota S, Parsons R, Yamada KM. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science 1998; 280: 1614-1617.

45) Stambolic V, Suzuki A, De La Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW. Negative regulation of PKB/Akt-de-

pendent cell survival by the tumor suppressor PTEN. Cell 1998; 95: 29-39.

46) Chillakuri CR, Sheppard D, Lea SM, Handford PA. Notch receptor-ligand binding and activation: insights from molecular studies. Semin Cell Dev Biol 2012; 23: 421-428.

47) Costa RM, Honjo T, Silva AJ. Learning and memory deficits in Notch mutant mice. Curr Biol 2003; 13: 1348-1354.

48) Sui Y, Zhang Y, Dong C, Xu B, Sun X. The small molecular CCR3 antagonist YM344031 attenuates neurodegenerative pathologies and improves learning and memory performance in a mouse model of Alzheimer's disease. Brain Res 2019; 1719: 1-10.

49) Karaarslan N, Gurbuz MS, Caliskan T, Ayan E, Aker FV, Berkman MZ. The effect of matrix metalloproteinase-3 on the prognosis and biological behaviour of meningiomas. Turk Neurosurg 2016; 26: 678-683.

50) Console-Bram LM, Zhao P, Abood ME. Protocols and good operating practices in the study of cannabinoid receptors. Methods Enzymol 2017; 593: 23-42.

51) Salaga M, Zatorski H, ZieliĐska M, Mosinska P, Timmermans JP, Kordek R, Storr M, Fichna J. Highly selective CB2 receptor agonist A836339 has gastroprotective effect on experimentally induced gastric ulcers in mice. Naunyn-Schmiedeberg's Arch Pharmacol 2017; 390: 1015-1027.

52) Fowler CJ, Holt S, Nilsson O, Jonsson KO, Tiger G, Jacobsson SO. The endocannabinoid signaling system: pharmacological and therapeutic aspects. Pharmacol Biochem Behav 2005; 81: 248-262.

53) Aguayo FI, Pacheco AA, García-Rojo GJ, Pizarro-Bauerle JA, Doberti AV, Tejos M, García-Pérez MA, Rojas PS, Fiedler JL. Matrix metalloproteinase 9 displays a particular time response to acute stress: cariation in its levels and activity distribution in rat hippocampus. ACS Chem Neurosci 2018; 9: 945-956.

54) Basavarajappa Bs, Nagre Nn, Xie S, Subbanna S. Elevation of endogenous anandamide impairs LTP, learning, and memory through CB1 receptor signaling in mice. Hippocampus 2014; 24: 808-818.

55) Terranova JP, Michaud JC, Le Fur G, Soubrié P. Inhibition of long-term potentiation in rat hippocampal slices by anandamide and WIN55212-2: reversal by SR141716 A, a selective antagonist of CB1 cannabinoid receptors. Naunyn-Schmiedeberg's Arch Pharmacol 1995; 352: 576-579.

56) Szepesi Z, Bijata M, Ruszczycki B, Kaczmarek L, Wlodarczyk J. Matrix metalloproteinases regulate the formation of dendritic spine head protrusions during chemically induced long-term potentiation. PLOS One 2013; 8: e63314.

57) Dziembowska M, Milek J, Janusz A, Rejmak E, Romanowska E, Gorkiewicz T, Tiron A, Bramham CR, Kaczmarek L. Activity-dependent local translation of matrix metalloproteinase-9. J Neurosci 2012; 32: 14538-14547.

58) Grabher C, Von Boehmer H, Look AT. Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukaemia. Nat Rev Cancer 2006; 6: 347-359.

59) Fazio C, Piazzi G, Vitaglione P, Fogliano V, Munarini A, Prossomariti A, Milazzo M, D'angelo L, Napolitano M, Chieco P, Belluzzi A, Bazzoli F, Ricciardiello L. Inflammation increases NOTCH1 activity via MMP9 and is counteracted by eicosapentaenoic acid-free fatty acid in colon cancer cells. Sci Rep 2016; 6: 20670.

60) Moon SK, Kim HM, Kim CH. PTEN induces G1 cell cycle arrest and inhibits MMP-9 expression via the regulation of NF-kB and AP-1 in vascular smooth muscle cells. Arch Biochem Biophys 2004; 421: 267-276.

61) Brzdak P, Wlodarczyk J, Mozrzymas JW, Wojtowicz T. Matrix metalloprotease 3 activity supports hippocampal EPSP-to-spike plasticity following patterned neuronal activity via the regulation of NMDAR function and calcium flux. Mol Neurobiol 2017; 54: 804-816.

62) Tsilibary E, Tzinia A, Radenovic L, Stamenkovic V, Lebitko T, Mucha M, Pawlak R, Frischknecht R, Kaczmarek L. Neural ECM proteases in learning and synaptic plasticity. Prog Brain Res 2014; 214: 135-157.

63) Tanveer R, Gowran A, Noonan J, Keating SE, Bowie AG, Campbell VA. The endocannabinoid, anandamide, augments Notch-1 signaling in cultured cortical neurons exposed to amyloid-β and in the cortex of aged rats. J Biol Chem 2012; 287: 34709-34721.

64) Calapai F, Cardia L, Sorbara EE, Navarra M, Gangemi S, Calapai G, Mannucci C. cannabinoids, blood-brain barrier, and brain disposition. Pharmaceutics 2020; 12: 265.