Endocannabinoids as Amyloid-Beta (Aβ) Aggregation Inhibitors

By

Marzie Khavandi

A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Science

in

Pharmacy

Waterloo, Ontario, Canada, 2022

Author's Declaration

I hereby declare that I am the sole author of this thesis. This is the true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

The endocannabinoid system, including endogenous cannabinoids and their corresponding receptors, has received extensive attention in the last few years for their neuroprotective effect in the central nervous system. The regulation and metabolism of these molecules are potential therapeutic targets for neurodegenerative diseases such as Alzheimer's disease, which is characterized by Aβ aggregation-induced cell toxicity, inflammation, tau phosphorylation, disruption of neurotransmitters pathways, mitochondrial dysfunction, and oxidative stress.

The endocannabinoids, such as 2-AG, AEA, NADA, noladin, OAE, and their main metabolite, arachidonic acid, may be involved in the multiple neuroprotective effects, including excitotoxicity attenuation, oxidative stress reduction, and inflammation prevention through CB1, CB2 receptors as well as other possible pathways, including inhibition of Aβ oligomer formation via interactions with these toxic peptides. However, the interactions of endocannabinoids with Aβ species and their mechanisms have not been fully explored.

Therefore, we hypothesized that endocannabinoids might reduce amyloid β-protein deposition and inhibit neuronal cell death through CB1 or other possible pathways. *In vitro* experiments, including cell studies using two cell lines (HTT22 and CB1-CHO), ThT based kinetic assay, and TEM studies were used to determine the effects of above mentioned five endocannabinoids, arachidonic acid, and a CB1 antagonist to understand the role of endocannabinoid ligands and pathways involved in Aβ-induced neurotoxicity. The results of this study on HT22 cells showed that some, but not all of the endocannabinoids were able to exhibit neuroprotective effects against Aβ-induced toxicity. However, AM251, as a CB1 receptor antagonist, could not reverse this neuroprotection. On the other hand, AM251 was able to inhibit the protective effects of some, but not all, of the endocannabinoids in CB1-CHO cells.

Acknowledgments

I would like to express my gratitude to my supervisor, Professor Mike Beazely, and my co-supervisor, Professor Praveen Nekkar Rao who encouraged and supported me with their knowledge and attitude throughout this journey. Thank you Mike, for always believing in me and providing this outstanding opportunity to grow. I am truly blessed to have you as my mentor. I would like to thank Dr. Nekkar for his guidance, understanding, and valuable feedback. I would like to extend my acknowledgment to my committee members, Dr. John Mielke, who gave me this wealthy opportunity to start a new pathway, and Dr. Tejal Patel, for her advice and support over the past two years.

I would also like to thank my student colleagues who helped, especially acknowledge Morgan Robinson and Arash Shakeri, for their guidance and support during this project and for mentoring me along the way.

I would like to thank my family for their unwavering faith and support and my friends for not letting me walk this journey alone.

Finally, I thank myself, who accepts all my shortcomings and makes everything possible.

Dedication

I would like to dedicate this thesis to the 176 beautiful souls on flight PS752 as well as those who fight and fly through their lives to infinity and beyond.

Table of Contents

Author's Declaration ..... **ii**

Abstract ..... **iii**

Acknowledgments ..... **iv**

Dedication ..... **v**

List of Figures ..... **ix**

List of Tables ..... **xi**

List of Abbreviations ..... **xii**

Chapter 1: Introduction 1

1.1 The Endocannabinoid system 1

1.2 Cannabinoid Ligands 5

1.3 Endocannabinoids 6

1.4 Endocannabinoids: Synthesis & Degradation 9

1.5 Alzheimer's disease (AD) 11

1.6 Role of Aβ in AD 13

1.7 Aβ Cascade 15

1.8 Endocannabinoids and Alzheimer's Disease 17

Chapter 2: Hypotheses and Objectives 21

2.1 Study Objectives 21

2.2 Hypotheses 22

Chapter 3: Material & Methods 23

3.1 Biological Screening 23

3.1.1 MTT Cell viability assay 23

3.2 Aβ preparation 24

3.1.3 Statistics 25

3.3 Western Blot 25

3.4 Thioflavin-T (ThT) based Aβ Aggregation Kinetic Studies 26

3.5 Transmission electron microscopy (TEM) studies 30

Chapter 4: Results 31

4.1 MTT Test 31

4.1.1 HT22 Cell Viability 31

4.2 CHO Cells Expressing CB1 Receptor 40

4.3 CB1-CHO Cell Viability 41

4.4 ThT based Aβ Aggregation Kinetic assay 49

4.3 Transmission electron microscopy (TEM) data **..... 53**

Chapter 5: Discussion 57

Chapter 6: Conclusion and Future Directions 64

6.1 Conclusion 65

6.2 Future Directions 66

References 68

List of Figures

Figure 1. Endocannabinoids signaling 4

Figure 2. Endocannabinoids synthesis and degradation. 11

Figure 3. Pathology of multifactorial Alzheimer's disease 13

Figure 4. Formation of plaques from Aβ monomers 15

Figure 5. Amyloid-beta cascade..... 16

Figure 6. Principle of ThT assay 28

Figure 7. Schematic view of (ThT) based Aβ aggregation kinetic 29

Figure 8. Effect of 2-AG and AEA and their vehicles on HT22 Cells 31

Figure 9. Effect of NADA, noladin, OAE and AA and their vehicle on HT22 Cells.. 32

Figure 10. Aβ42 (5 μM) toxicity on HT22 Cells 33

Figure 11. The protective effects of 2-AG aginst Aβ42 (5μM) on HT22 cells 34

Figure 12. Effect of AEA against Aβ42 (5μM) on HT22 cells 34

Figure 13. Effect of AEA with AM251 (5μM) against Aβ42 (5 μM) on HT22 cells..... 35

Figure 14. Effect of NADA against Aβ42 (5 μM) on HT22 cells 35

Figure 15. Effect of noaldin against Aβ42 (5 μM) on HT22 cells..... 36

Figure 16. Effect of noaldin with AM251 (5 μM) against Aβ42 (5 μM) on HT22 cells..... 36

Figure 17. Effect of OAE against Aβ42 (5 μM) on HT22 cells 37

Figure 18. Effect of OAE with AM251 (5 μM) against Aβ42(5 μM) on HT22 cells..... 37

Figure 19. Effect of AA against Aβ42 (5μM) on HT22 cells. 38

Figure 20. Effect of AAwith AM251 (5 μM) against Aβ42(5 μM) on HT22 cells 38

Figure 21. CB1-CHO cells Western blots analysis..... 40

Figure 22. Aβ42 (5 μM) toxicity on CB1-CHO cells. 42

Figure 23. Effect of endocannabinoids and their vehicle on CB1-CHO Cells. 43

Figure 24. Effect of endocannabinoids against Aβ42 toxicity on CB1-CHO cells 44

Figure 25. Effect of noladin ether against Aβ42 (5 μM) toxicity on CB1-CHO cells..... 45

Figure 26. Effect of noladin with AM251 (5 μM) against Aβ42 (5 μM) on CB1-CHO 46

Figure 27. Effect of OAE against Aβ42 (5 μM) on CB1-CHO cells..... 46

Figure 28. Effect of OAE with AM251 (5 μM) against Aβ42 (5 μM) on CB1-CHO 47

Figure 29. Effect of AA against Aβ42 (5 μM) on CB1-CHO cells 47

Figure 30. Effect of AA with AM251 (5 μM) against Aβ42 (5 μM) on CB1-CHO..... 48

Figure 31. ThT-monitored 24 h kinetics of Aβ42 49

Figure 32. ThT-monitored 24h kinetics of Aβ42 (5 μM) in the presence of 2-AG 50

Figure 33. ThT- monitored 24h kinetics of Aβ42 (5 μM) in the presence of AEA 50

Figure 34. ThT-monitored 24h kinetics of Aβ42 (5 μM) in the presence of Noladin 51

Figure 35. ThT-monitored 24h kinetics of Aβ42 (5 μM) in the presence of OAE 51

Figure 36. ThT-monitored 24h kinetics of Aβ42 (5 μM) in the presence of AA 52

Figure 37. Morphology of aggregate species of Aβ42 53

Figure 38. Morphology Aβ42 in the presence of 2AG 54

Figure 39. Morphology Aβ42 in the presence of AEA..... 54

Figure 40. Morphology Aβ42 in the presence of noladin. 55

Figure 41. Morphology Aβ42 in the presence of OAE. 56

List of Tables

Table 1. Endocannabinoids structure & molecular 9

Table 2. Reagents and assay protocol for the ThT-based Aβ aggregation..... 29

Table 3. Summary of endocannabinoids'effect on cell viability of HT22 cells 39

Table 4. Summary of endocannabinoids'effect on cell viability of CB1-CHO.. 48

Table 5. Inhibition percentage of endocannabinoids in ThT aggregation kinetics assay 52

List of Abbreviations

2-AG: 2-Arachidonoyl glycerol

AA: Arachidonic Acid

Abn-CBD: Abnormal Cannabidiol

AChE: Acetylcholinesterase

ACN: Acetonitrile

AD: Alzheimer's Disease

AEA: *N*- Arachidonoyl Ethanolamine

AICD: Amyloid Intracellular Domain

ANOVA: Analysis Of Variance

APOE: Apolipoprotein E

APP: Amyloid Precursor Protein

APS: Extracellular Amyloid Plaques

Aβ: Amyloid-Beta

AβN3pE: N-terminally truncated Aβ with a pyroglutamate modification

BACE: β-site APP Cleaving Enzyme

BBB: Blood Brain Barrier

BDNF: Brain Derived Neurotrophic Factor

CAA: Cerebral Amyloid Angiopathy

Cation Channel, Vanilloid Type 1

CBD: Cannabidiol

CBN: Cannabinol

ChAT: Choline Acetyltransferase

ChEIs: Cholinesterase Inhibitors

CNS: Central Nervous System

CPM: Cycles Per Minute

CSF: Cerebrospinal Fluid

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

DSE: Depolarisation suppression of excitation

DSI: Depolarization Suppresses Inhibition

EC: Endocannabinoid

Endocannabinoids: Endocannabinoid system

FAAH: Fatty Acid Amide Hydrolase

FBS: Fetal Bovine Serum

GFs: Growth Factors

GPCR: G Protein Coupled Receptor

HFIP: 1,1,3,3,3-Hexafluoro-2-propanol

IC_{50}: Half-Maximal Inhibitory Concentration

MAGL: Monoacylglycerol lipase

MAPK- Mitogen-activated protein kinase

MB: Methylene blue

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5- Diphenyltetrazolium Bromide

NADA : Arachidonoyl Dopamine

NFTs: Neurofibrillary Tangles

NMDA: N-Methyl-D-Aspartate

Noladin Ether: 2-Arachidonoyl Glyceryl Ether

OAE: O-Arachidonoyl Ethanolamine

P-Aβ: Pyroglutamylated Amyloid Beta

PBS: Phosphate Buffered Saline

PHF: Paired Helical Filament

PKC: Protein Kinase C

PPARs : Peroxisome proliferator-activated receptors

ROS: Reactive oxygen species

SDS: Sodium dodecyl sulfate

RTK: Receptor Tyrosine Kinase

TEM: Transmission Electron Microscopy

THC: Tetrahydrocannabinol

TRPV1: Transient Receptor Potential

TrkB: tropomyosin-receptor-kinas

Chapter 1: Introduction

1.1 The Endocannabinoid system

The endocannabinoid system, an endogenous signalling system with complex roles, consists of ligands known as endocannabinoids and cannabinoid receptors such as CB1 and CB2 [1-3]. Endocannabinoids are polyunsaturated fatty acid-based lipid molecules, and cannabinoid receptors are G-protein-coupled receptors (GPCR) distributed in the central and peripheral nervous systems [4]. The CB1 receptor is primarily distributed in the cingulate gyrus, hippocampus, cerebellum, cortex, and basal ganglia and is the most frequent cannabinoid receptor found in the mammalian brain; however, it can be found in the peripheral nervous system and immune cells as well [5, 6]. There are moderate concentrations of receptors in the forebrain, amygdala, nucleus accumbens, and hypothalamus, and a lower density of receptors are found in the midbrain, medulla, pons, and thalamus [5]. Activation of CB1 receptors in the central nervous system (CNS) and their location on GABAergic and glutamatergic synapses are responsible for cannabinoids' psychoactive effects [7].

CB2 receptors are mostly found on immune cells, especially in B cells and natural killer cells [7, 8]. However, CB2 receptors have recently been found in the CNS, specifically in microglia, the brain's "immune cells", albeit at low levels [7, 8]. The CB2 receptor plays a vital role in immunologic processes and is also found in bone, GI, and reproductive systems [8, 9]. Activation of CB1/CB2 receptors by endocannabinoids leads to neuroprotection through reducing excitatory neurotransmitter release at the presynaptic neurons, as well as excitatory transmission reduction at the postsynaptic sites through CB1 receptor through coupling with NR1 subunits of

the N-methyl-D-aspartate receptor (NMDAr) (Figure 1) [10]. Additionally, other pathways could be involved in neuroprotection, such as modulation of G protein-coupled chemokine receptor CXCR4 or the activation of peroxisome proliferator-activated receptors (PPARs), resulting in a reduction of inflammatory responses [10]. PPARs are a sub-family of nuclear receptors that modulate gene expression, regulate metabolism and energy homeostasis, and are involved in cell differentiation [3]. These mechanisms suggest that endocannabinoids are able to activate multiple pathways for neuroprotective responses.

The endocannabinoid system is also thought to play a crucial role in synaptic transmission. Endocannabinoids can modulate various receptors, including voltage-gated ion channels such as Ca^{+2} channels, Na^{+} channels, multiple types of K^{+} channels, or ligand-gated ion channels such as glycine and nicotinic acetylcholine receptors [11]. Additionally, endocannabinoids have modulatory effects on other ion-transporting membrane proteins such as transient potential receptor-class channels, gap junctions, and transporters for neurotransmitters. There is also evidence that endocannabinoids can alter the function of neurotransmitters and neuropeptides by direct action [11].

Endocannabinoids mediate depolarization-induced suppression of inhibition (DSI) and depolarisation-induced suppression of excitation (DSE), forms of short-term synaptic plasticity in addition to long-term depression (eCB-LTD) at both excitatory and inhibitory synapses [12]. Endocannabinoids have been reported to act as retrograde messengers [6]. Through retrograde endocannabinoid signalling, postsynaptic activity triggers the production of endocannabinoids that move backward across the synapse, bind to presynaptic CB1 receptors, and suppress neurotransmitter release [12, 13]. Endocannabinoids are also able to induce depolarization on presynaptic cells, which can suppress inhibition (depolarization-induced suppression of inhibition,

DSI) through their effects on GABAergic neurons. Moreover, endocannabinoids could mediate neuroprotection via depolarization-induced suppression of excitation (DSE) by acting on glutamatergic neurons [6, 7, 14]. The inhibitory effects of CB1 receptor stimulation on GABA release, glutamate, acetylcholine, and noradrenaline are well documented [15]. In terms of long-term potentiation (LTP), by releasing endocannabinoids from postsynaptic neurons, an elevation of intracellular Ca^{2+} in astrocytes causes glutamate release, which activates mGluR1 receptors in presynaptic neurons, leads to persistent synaptic potentiation of neurotransmitter release. Glutamate release and NO release from postsynaptic neurons activate both mGluR1 and PKC in presynaptic neurons, resulting in long-term potentiation [16].

In addition to the endocannabinoid's multi-functions, they are believed to be involved with cell death/survival. The endocannabinoid system in hippocampal and cortical neurons is capable of responding to a number of toxic insults, including excitotoxicity, ischemia, and oxidative damage that result in apoptosis [17]. Neuronal cell death and excitotoxicity may be triggered as intracellular calcium increases in response to NMDA receptor activation. The accumulation of intracellular Ca^{2+} leads to endocannabinoid production, CB1 receptor stimulation, and a DSE-like presynaptic inhibitory effect on glutamatergic transmission with neuroprotective effects [13].

As CB1 receptors have a noticeable effect on second messenger signal transduction pathways, the endocannabinoid system plays an essential role in synaptic remodelling, neuronal differentiation, and neuronal survival [18]. Therefore, it seems that the endocannabinoid system plays a regulatory role in cognition and learning via its involvement in short-term and long-term synaptic plasticity [19].

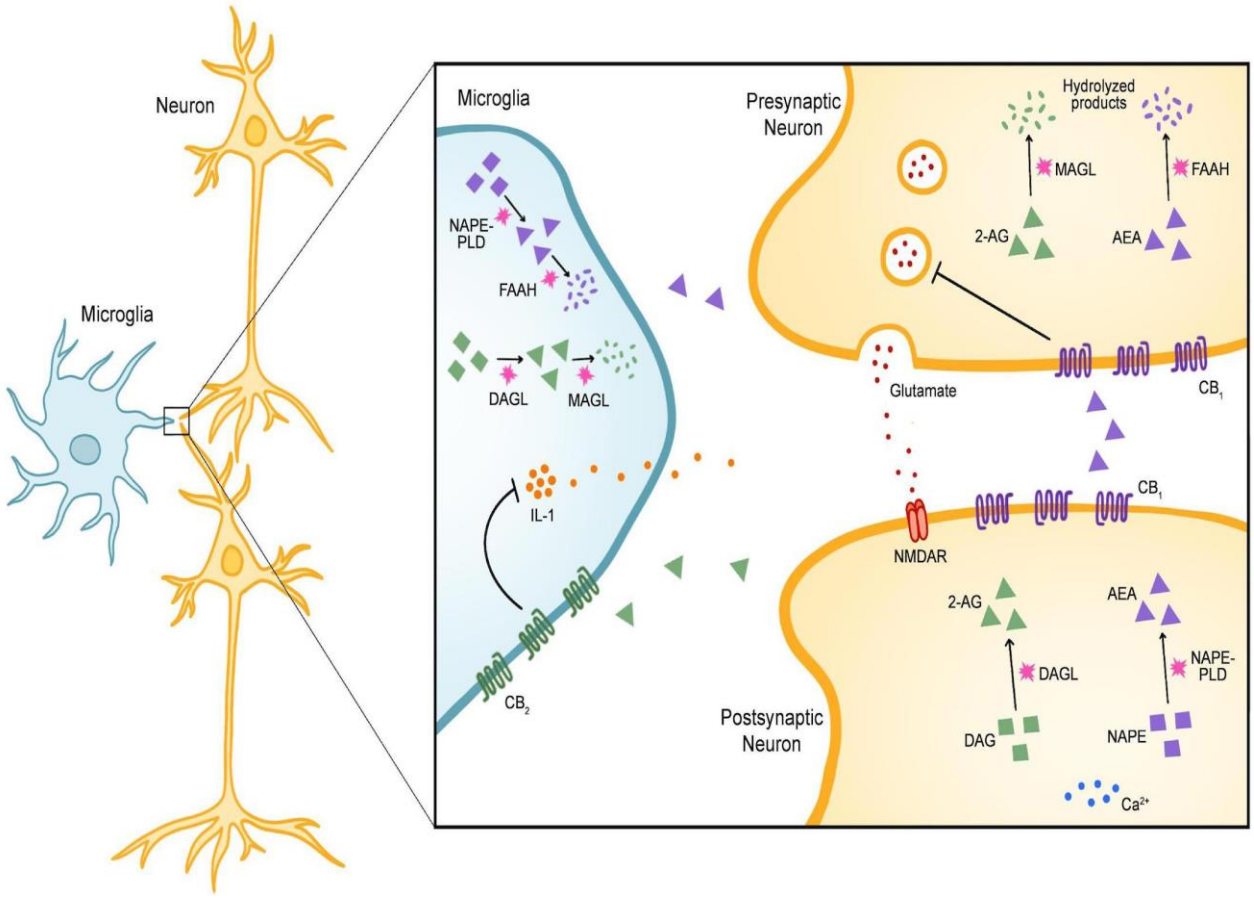


Figure 1. Endocannabinoids such as 2-AG and AEA are synthesized from arachidonic acid (AA) released by post-synaptic neurons and act in retrograde signaling at presynaptic neuron via binding to cannabinoid receptors (CB1 and CB2). Endocannabinoids can modulate various receptors, including voltage-gated ion channels such as Ca^{+2} channels, Na^{+} channels, multiple types of K^{+} channels, or ligand-gated ion channels such as glycine and nicotinic acetylcholine receptors to decrease neurotransmitter release. Additionally, endocannabinoids have modulatory effects on other ion-transporting membrane proteins such as transient potential receptor-class channels, gap junctions, and transporters for neurotransmitters [20].

1.2 Cannabinoid Ligands

Cannabinoids are structurally diverse lipophilic molecules that bind to cannabinoid receptors [21]. There are three categories of cannabinoids: phytocannabinoids, synthetic cannabinoids, and endocannabinoids [21]. Endocannabinoids are synthesized from lipid precursors within plasma membranes by Ca^{2+} and G protein-dependent processes. Endocannabinoids exhibit their effects not only by binding to CB receptors but also through the modulation of voltage-gated ion channels, including Ca^{2+} channels, Na^{+} channels, various types of K^{+} channels, and ligand-gated ion channels such as serotonin type 3, nicotinic acetylcholine, and glycine receptors. It is likely that these effects of endocannabinoids are the result of their lipophilic structures, although the mechanisms are not clear at this point [11].

Phytocannabinoids are derived from a medicinal plant (*Cannabis Sativa* L.) that has more than 60 bioactive derivatives, including tetrahydrocannabinol (THC) and non-psychoactive components such as cannabidiol (CBD) and many others [22]. Phytocannabinoids have been widely recognized for their potential therapeutic and clinical applications in pharmaceutical and medical fields, leading to the development of synthetic analogs, which are called synthetic cannabinoids [21].

Synthetic cannabinoids are chemically synthesized to imitate the effects of phytocannabinoids, including THC analogs or CB receptor-selective agonists. It has been a challenge for pharmaceutical industries to design synthetic cannabinoids that retain the biological activity of natural cannabinoids with less psychoactive side effects [21]. The most successful examples of phytocannabinoid-related drugs that have been commercially available are Cesamet® (nabilone, a synthetic THC-like cannabinoid) and Marinol® (dronabinol, a synthetic THC). They

are approved by the US Food and Drug Administration (FDA) and Health Canada for the treatment of nausea and vomiting induced by chemotherapy [23].

1.3 Endocannabinoids

Endocannabinoids contain amides and esters with a long chain of polyunsaturated fatty acids and are considered as a class of signalling lipids [11]. These endogenous ligands are directly synthesized from membrane phospholipids "on demand". Their production is mainly thought to be "use-dependent" in response to neuronal activity. There is no intracellular storage for these fatty acid derivatives, and they do not release through vesicles into the synapses [19]. N-Arachidonoyl ethanolamide (AEA), also known as anandamide, and 2-arachidonoyl glycerol (2-AG) are the most studied endogenous cannabinoid ligands (Table 1) [19, 24]. Additionally, other endogenous cannabinoids have been identified, including arachidonoyl dopamine (NADA), 2-arachidonoyl glyceryl ether (Noladin ether or 2-AG ether), and O-arachidonoyl ethanolamine (Virodhamine) (Table 1) [24-26].

- **2-AG** is considered as a natural ligand at the CB1 receptor [27]. It has been reported that lipid rafts modulate the activity of GPCRs associated with these cholesterol-rich membrane microdomains and provide an organized platform for signalling complexes. CB1 receptors have been shown to localize within lipid rafts. 2-AG is concentrated in CB1 rich lipid rafts in the dorsal ganglion. Evidence shows that lipid raft disruption causing a noticeable increase in 2-AG synthesis as well as the binding activity of CB1 receptors [28]. 2-AG is present at relatively high levels in the central nervous system, roughly 100 times greater than anandamide, which is present often in low amounts [29].

- **AEA** is a well-studied endogenous cannabinoid first found in the brain and later in many other organs and fluids. AEA binds to CB receptors; as a partial agonist of CB1 and weak partial agonist/antagonist of CB2. It is also a full agonist for transient receptor potential cation channel, vanilloid type 1 (TRPV1). AEA seems to be distributed regularly in the plasma membrane. Evidence shows that lipid raft disruption fails to alter AEA metabolism [4, 24].

2-AG and anandamide are formed from arachidonic-acid containing glycerophospholipids [30]. They are formed locally from membrane phospholipids in response to intracellular calcium elevations, released immediately from the cell, and after rapid, selective reuptake, are hydrolyzed intracellularly [21, 30]. Over the last few years, several AA derivatives with cannabimimetic properties have been detected, such as NADA, noladin ether, and OAE [24].

- **NADA** was identified as an endogenous ligand for CB1 and transient receptor potential cation channel (TRPV1), while the low affinity to CB2 receptors has also been reported [31]. NADA can be found predominantly in the striatum, hippocampus, cerebellum, and dorsal root ganglia in the central nervous system and is proposed to play a role in neuronal pain and inflammation [29, 31]. NADA has been reported to play a role in neuronal pain and inflammation by its presence in the striatum, hippocampus, cerebellum, and dorsal root ganglia [29, 31, 32].

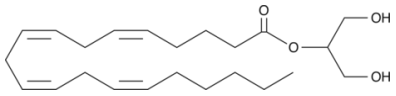
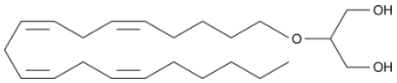
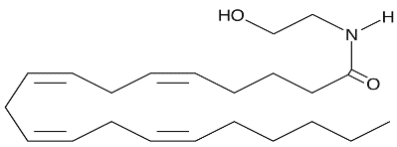
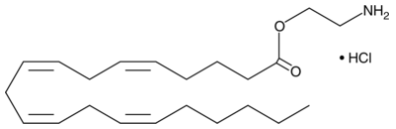
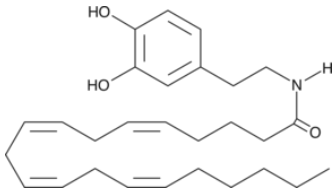
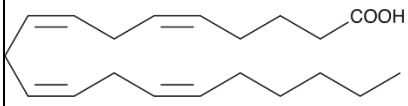
- **Noladin** ether is able to bind to CB1 receptors and weakly to CB2 receptors, along with affecting AEA [24]. Noladin ether interacting with PPARα and the orphan GPR55 receptor indicates that non-CBRs can be common targets for several endocannabinoids. Also, the selectivity of noladin ether for CB1 receptors implicates other possible pathways for the

effects of this endocannabinoid. However, this molecule's biosynthetic and catabolic enzymes are not known [24, 33].

- **OAE** has been shown to act as a partial agonist of CB1 and a full agonist of CB2r *in vitro*. In contrast, it can act as a CB1 antagonist *in vivo* and a weak inhibitor of AEA uptake [24]. OEA binds to peroxisome proliferator-activated receptors (PPARs) with high affinity and initiates transcription of genes involving lipid metabolism. Like noladin ether, the biosynthetic and catabolic enzymes of this molecule are not known [3, 24].

As endocannabinoids are all arachidonic acid derivatives, the function of the endocannabinoid system seems to be dependent on AA. Arachidonic acid is needed for biosynthesizing of endocannabinoids [24]. According to several preclinical studies, AA can increase serum levels of AEA and 2-AG. It has been reported that an excessive level of AA administered chronically may lead to excessive levels of endocannabinoids. Consequently, this may lead to desensitization and downregulation of CB1 and CB2 receptors [1, 3, 24]. To elucidate the mechanism of action of endocannabinoids, CB receptor antagonists seem a reasonable option to examine the effect of endocannabinoids on their receptors. AM251 is a CB1 antagonist that is considered as an inverse agonist. An inverse agonist reduces constitutive activity by preferentially binding to a receptor's inactive state, reducing signalling levels [32]. It has been reported that hippocampal infusion of AM251 could significantly cause cognitive behavioural impairment, induce forgetting of fear memory and prevent memory updating [34, 35].

Table 1. Endocannabinoids and Endocannabinoids-like compounds, their structure & molecular targets [24]

Bioactive Lipids Structure	Target Receptors	Endocannabinoids structure	Target Receptors
2-AG  2-Arachidonoyl Glycerol	CB1 CB2 TRPV1	Noladin  2-Arachidonyl Glycerol ether	CB1 CB2 PPARα
AEA  Arachidonoyl Ethanolamide	CB1 CB2 TRPV1 PPARα PPAR	OAE  O-Arachidonoyl Ethanolamine(hydrochloride)	CB1 CB2 PPARα
NADA  N-Arachidonoyl Dopamine	CB1 TRPV1 PPARγ	AA  Arachidonic Acid	

1.4 Endocannabinoids: Synthesis & Degradation

As mentioned earlier, endocannabinoids are lipophilic; therefore, they cannot be stored in vesicles like other neurotransmitters. Instead, they are produced on demand by receptor-stimulated cleavage of lipid precursors [36]. As a result, the endocannabinoid signalling is tightly regulated by their synthesis/degradation and release/uptake (Figure 2). Several different stimuli are

involved, including membrane depolarization, an increase in intracellular Ca^{2+}, and the activation of complex enzymatic machinery, which results in membrane phospholipid cleavage and subsequent endocannabinoid synthesis [36, 37]. It is important to note that different enzymes are involved in synthesizing distinct endocannabinoids, suggesting that endocannabinoids act independently under different circumstances [38].

Endocannabinoids are mainly biosynthesized by N-acylphosphatidylethanolamines (NAPE) through a phosphodiesterase of the phospholipase D-type (PLD) [37, 38]. Following synthesis, endocannabinoids can either be released into extracellular space or move directly within cell membranes to activate cannabinoid receptors. Various degradation processes limit endocannabinoid signalling, including their uptake from extracellular space into cells as well as enzymatic catabolism mediated by specific intracellular enzymes [39]. Fatty acid amide hydrolase (FAAH) is the primary enzyme responsible for the degradation of endocannabinoids [37, 39]. An interesting aspect of endocannabinoid activity is the rapid induction of their synthesis, receptor activation, and degradation (Figure 2).

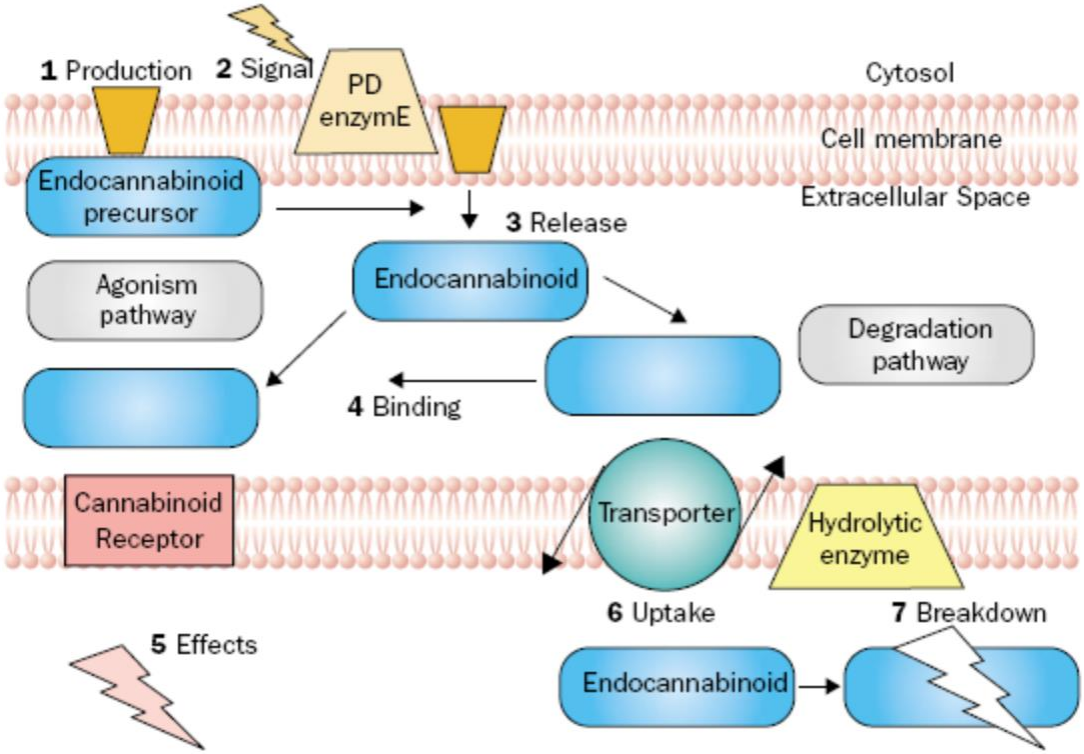


Figure 2. Endocannabinoids synthesis and degradation are formed via multiple biosynthetic pathways. These are produced from membranous fatty-acid precursors through the phosphodiesterase enzymes activity, including phospholipase D (anandamide) and phospholipase C (2-AG). This process starts by cellular stimulation resulting in endocannabinoid release. Then they can either bind to CB receptor or be degraded. Degradation of the endocannabinoids occurs by reuptake through diffusion facilitated transport molecules. Then, they catalyzed into arachidonic acid and ethanolamine by fatty-acid-amide hydrolase [37].

1.5 Alzheimer's disease (AD)

Among the neurodegenerative disorders, Alzheimer's disease is one of the most financially draining diseases in the health care system that could be called a significant health threat in the elderly population [40, 41]. This disease is classified as the most common form of dementia, which is associated with memory and cognition impairment [42]. Currently, 47 million people are affected by dementia worldwide [42, 43]. Alzheimer's disease is increasingly prevalent and is one

of the leading sources of morbidity and mortality in older adults, with an estimated lifetime risk of nearly 1 in 5 for women and 1 in 10 for men [44, 45]. The behavioural symptoms of AD, such as mood disruption and learning and language impairment, are related to progressive degeneration of the cortical and hippocampal neurons [46]. Various environmental and genetic factors have been shown to enhance AD risk as well as other factors such as age, head injuries, vascular diseases, and infections [47]. However, understanding the contribution of these risk factors to the etiology of AD is an enduring process [48]. Even though treatments including cholinesterase inhibitors (donepezil, rivastigmine, and galantamine) and NMDA antagonists (memantine) can alleviate some symptoms, there is no cure for this disease, and it progresses inevitably [49, 50]. Recently, the US Food and Drug Administration (FDA) has approved Aducanumab®, a recombinant monoclonal antibody directed against amyloid-beta (Aβ) for mild AD treatment [50]. In light of the FDA's previous recommendation against the approval of aducanumab and the fact that reducing (Aβ) plaques isn't yet established to provide clinical benefit, the approval of this medication has generated considerable controversy [51-53]. Post approval trials are required to verify the clinical benefits, lack of clarity in the clinical trials, adverse effects, and the monitoring requirements [50, 53]. The major difficulty in recognizing AD's etiology as a multifactorial disease and introducing safe and efficacious treatments for the disease is a significant challenge in neuroscience and medical research. Thus, there is a growing body of research worldwide toward understanding the effect and mechanism of novel AD treatment.

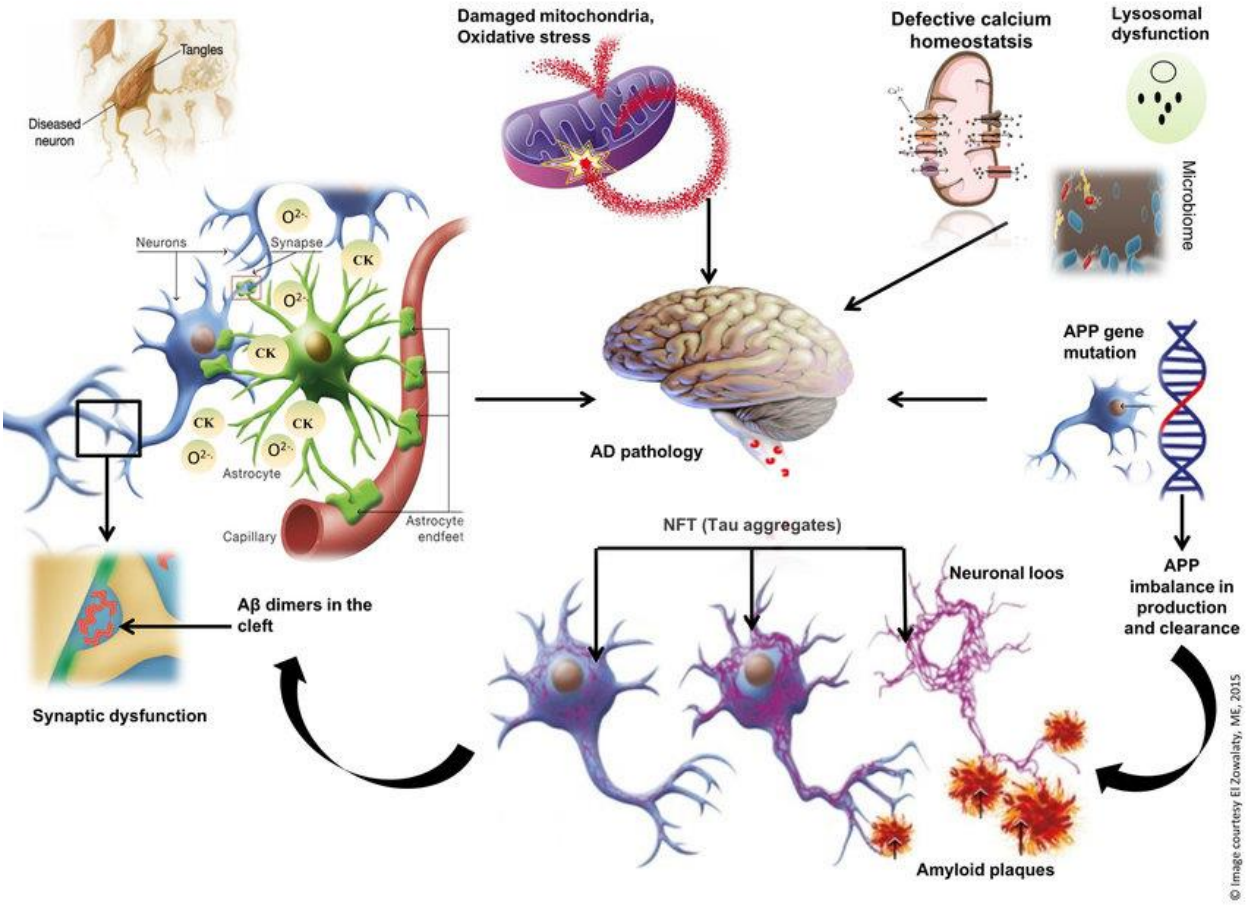


Figure 3. Pathology of multifactorial Alzheimer's disease including amyloid-β (Aβ) formation, extracellular amyloid, formation of Tau aggregates and neurofibrillary tangles (NFTs), neuronal loss, synaptic dysfunction, activation of astrocytes, the release of various cytokines (CK), and microglia generation of superoxide radicals, the loss of Ca^{2+} homeostasis leading to the excitotoxic activity [54].

1.6 Role of Aβ in AD

AD's pathogenesis is complex (Figure 3) and includes two main hypotheses proposed as a cause for AD, the cholinergic and amyloid cascade hypotheses [47]. The general hypotheses about AD pathophysiology involve Aβ peptides. There is also evidence that tau, a microtubule-

associated protein that assists in microtubule assembly and stabilization, was hyperphosphorylated in AD [55]. The pathological accumulation of Aβ and phosphorylated tau happens in a successive process; small numbers of monomers first aggregate into oligomers intraneuronally, which then keep aggregating into the fibrils detected in amyloid plaques and neurofibrillary tangles (NFTs) [56]. Several studies have reported an association between neocortical NFTs and cognitive impairment [55].

Aβ peptides are produced by the amyloid precursor protein (APP) gene and cleaved by beta-secretase and gamma-secretase. Mutations in the gamma-secretase complex lead to Aβ -production, or more neurotoxic forms of Aβ [57]. Experimental studies documented that small aggregates of Aβ peptides called oligomers and larger aggregates called fibrils lead to neurotoxicity (Figure 4).

Clinical diagnosis of AD involves neuropsychological and postmortem neuropathological assessments to detect amyloid plaques and tau pathology [55, 56, 58]. As tau becomes hyperphosphorylated in AD, it forms paired helical filaments (PHF) tau, a primary component of neurofibrillary tangles in the neuronal cytoplasm. The accumulation of this altered protein has been reported toxic to neurons in experimental models [59]. As AD progresses, it is believed that pathologic forms of tau spread between neurons, allowing distinct progression across neuronal regions [55, 56]. The AD pathogenesis involves other overlapping pathways as well. For example, the human apolipoprotein E (APOE) gene is a pleiotropic lipoprotein that affects several cellular processes that may increase AD risk by impairing Aβ clearance [60].

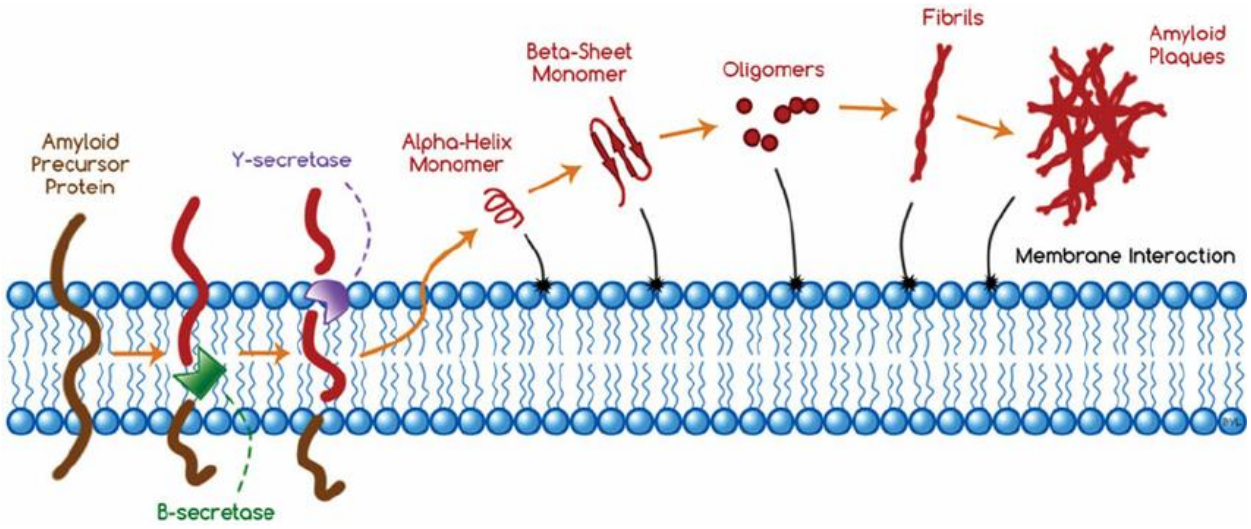


Figure 4. Formation of plaques from Aβ monomers [61]

1.7 Aβ Cascade

In addition to biochemical markers of AD mentioned above, such as amyloid plaques and tau phosphorylation, disruption of neurotransmitter pathways, mitochondrial dysfunction, and inflammation, oxidative stress seem to have a significant role. [62]. AD is characterized by the pathological accumulation of Aβ into extracellular plaque in the brain, the vasculature (known as cerebral amyloid angiopathy [CAA]) through the APP degradation (Figure 5) and atypically phosphorylated tau that accumulates intraneuronally forming neurofibrillary tangles (NFTs) [55]. Aβ peptides containing 42 amino acids form Aβ plaques due to increased Aβ42 production leading to Aβ aggregation. Aβ oligomers are the most neurotoxic species in AD as these species correlate much better with cognitive symptoms than the presence of plaques or NFTs [58]. The existence and amount of different Aβ species are important since each species has a special aggregation rate that could form distinctive aggregated species with different toxicity [55]. The most plentiful

forms of Aβ are Aβ1–40 and Aβ1-42. However, other important Aβ species include Aβ1–38, Aβ1–43, and Aβ with post-translational modifications such as AβN3pE (N-terminally truncated Aβ with a pyroglutamate modification), pAβ (Aβ with phosphorylated serine at position 8 or 26), and Aβ5-x (N-terminally truncated Aβ) have been observed [58].

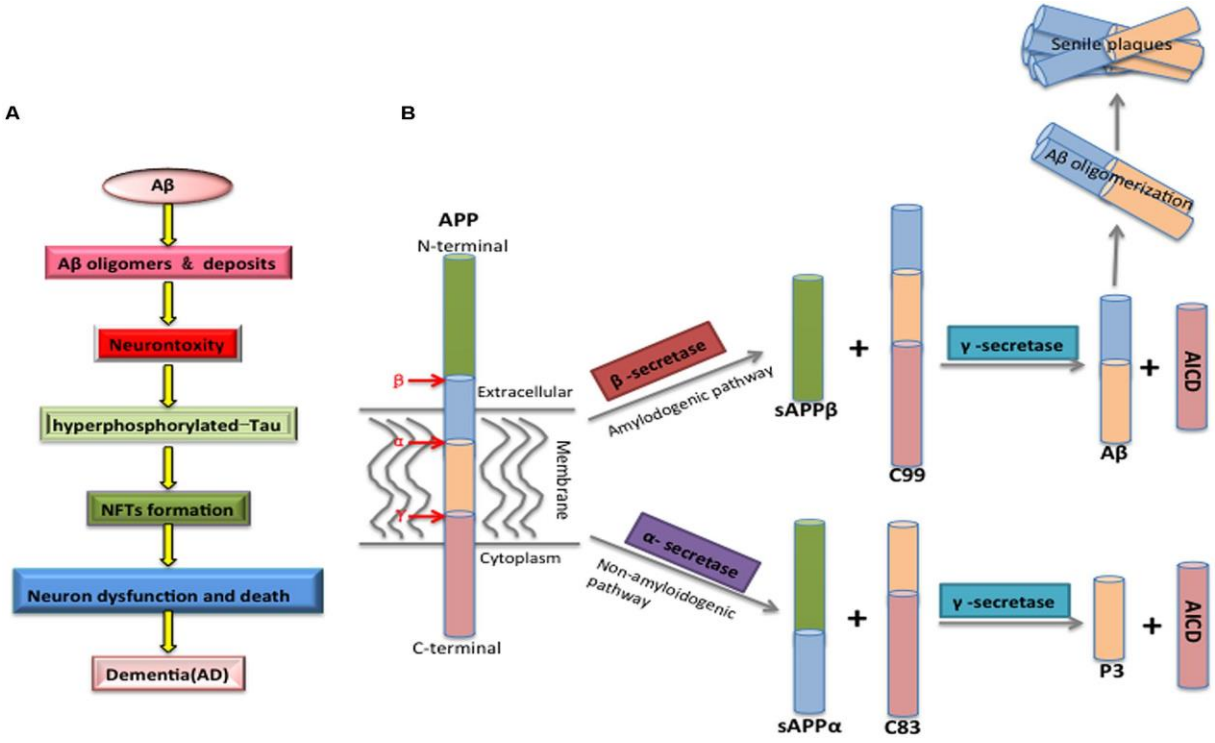


Figure 5. Amyloid-beta cascade: Production of Aβ peptide by APP cleavage from beta and gamma secretases. The oligomerization of Aβ gradually forms fibrils, and senile plaques alter the kinase/phosphatase activity, resulting in Tau protein hyperphosphorylated. The formation of neurofibrillary tangles (NFTs) consequently leads to synaptic and neuronal dysfunction of AD. APP is a transmembrane glycoprotein processed through either an amyloidogenic or non-amyloidogenic pathway. Through the amyloidogenic pathway: APP is cleaved by β-secretase that generate the soluble β-APP fragments (sAPPβ) and C-terminal β fragment (CTFβ, C99), and C99 is further cleaved via γ-secretase, producing APP intracellular domain (AICD) and Aβ. The non-amyloidogenic pathway is a distinctive way to prevent Aβ aggregation. First APP is recognized by α-secretase via Aβ domain, then produce soluble α-APP fragments (sAPPα) and C-terminal fragment α (CTFα, C83), which then cleaved through the γ-secretase, resulting in non-toxic P3 and AICD fragment [63]

1.8 Endocannabinoids and Alzheimer's Disease

Cognitive deficits in AD patients correlate with cerebral disturbances, primarily in the frontal cortex and hippocampal region, areas that are rich in CB1Rs [64]. Numerous studies have identified neuroprotective roles of the endocannabinoid system against excitotoxicity, oxidative stress, and inflammation, all pathological hallmarks of AD [17]. Endocannabinoids in the mouse hippocampus and frontal cortex decreases significantly with age, supporting the limited number of studies showing decreased CB1R density in aged animals [64]. Neuroprotective effects of endocannabinoids have been observed under various conditions [65]. According to most recent studies, endocannabinoids such as 2-AG have shown neuroprotection ability against excitotoxicity as well as involvement in the inhibition of presynaptic glutamate release as calcium increases intracellularly [18, 19, 65]. Different cannabinoid agonists were found to activate two MAP kinases, ERK1/2 and p38 MAPK, that are known to regulate neuronal survival and death. There is also a correlation between their potency in triggering these responses and their affinity for cannabinoid receptor subtypes [66].

Increased brain levels of pro-inflammatory cytokine TNF-α have been implicated in causing neurotoxicity and contributing to the sustained inflammatory processes associated with neurodegenerative disorders such as Alzheimer's disease in several clinical and preclinical studies. In one study, the prolonged exposure of HT22 cells to TNF-α causes apoptotic cell death in these hippocampal cells. Data from this study indicate that CB receptor activation reverses the TNF-α-mediated neurotoxicity through the microglia and astrocytes activation. This study also reported that CB1 activation inhibits TNF-α production and diminishes neuroinflammation [66].

Another study on HT22 cells demonstrated that upregulating CB1 expression could increase cell viability and reduce the apoptotic rate of HT22 cells exposed to Aβ42. It is reported that the neuroprotection might depend on the activation of protein kinase C (PKC) [67]. PKC is a group of serine/threonine kinases involved in neural damage, including ischemic and inflammatory damage. Also, PKC activities and expression were reduced in AD brains in several other studies [67, 68].

Several studies have identified neuroprotective roles for endocannabinoids in AD, including protection against apoptosis-induced cell death [17, 69]. A better understanding of the apoptotic pathway triggered by Aβ can contribute to the development of therapeutic strategies that can counteract Aβ toxicity and preserve cell viability. According to previous research, lysosomal membrane permeabilization plays an important role in Aβ-induced apoptosis [70]. Endocannabinoids stabilize the lysosomes by inhibiting the Aβ-induced up-regulation of the tumour suppressor protein, p53, and its interaction with the lysosomal membrane [17, 70]. Furthermore, CB1 receptors are able to stabilize lysosomes against Aβ toxicity, highlighting the role of these receptors. Since permeabilization of the lysosomal membrane negatively affects cell viability, stabilizing lysosomes with endocannabinoids may represent a different role by which these lipid modulators confer neuroprotection [17].

Additionally, the endocannabinoid system has been reported to target inflammatory neurodegenerative processes [6]. Microglial cell activation, and macrophage presence around amyloid plaques are critical elements of the inflammatory response, and both cell type activities are suppressed by CB2 receptor activation. Moreover, both CB1 and CB2 receptors are up-

regulated to reduce neuroinflammation, and CB2 receptors may reduce inflammatory cytokines activated by microglia [6, 8].

An additional contribution of endocannabinoids in the inflammatory process is fatty acid amide hydrolase (FAAH) upregulation within amyloid plaque formations, resulting in excessive arachidonic acid production. FAAH inhibitors reduce Aβ protein deposition by increasing the level of endocannabinoids indirectly [71]. Other studies showed that endocannabinoids reduced Aβ - induced cell toxicity and memory loss in rodents [72]. Studies have demonstrated that cannabinoid receptor agonists such as AEA and noladin ether also protect neurons from Aβ-induced neurotoxicity [64].

Furthermore, the antioxidant properties of endocannabinoids reduce lipid peroxidation, which may further reduce Aβ-induced neuronal cell death through scavenging ROS [73]. Endocannabinoids are produced in more significant quantities in cells lacking CB1 receptors since such cells are more vulnerable to neuronal damage. For instance, some other studies demonstrated that Aβ-induced hippocampal degeneration and cognitive impairment resulting in 2-AG increased level, reflect the attempt of the endocannabinoid system to provide neuroprotection against Aβ-induced neuronal toxicity [17, 72, 74].

Moreover, *in vitro* and *in vivo* studies suggest that strengthening endogenous cannabinoid signalling may exert neuroprotective effects against Aβ-induced microglial activation, resulting in neurotoxicity and rescuing AD-like pathology, learning, and memory impairments in animal models of AD [69]. Similarly, treatment with non-selective CB receptor agonists prevented the inflammatory profile and excitotoxic glutamate release and neuronal damage in hippocampal slices induced by Aβ [75]. Translational studies have reported that administration of an

endocannabinoid cellular reuptake inhibitor (VDM11) reduces hippocampal damage and may be able to help restore neurogenesis and cognition in AD [76, 77].

Several studies indicated that endocannabinoids modulate memory-related processes through CB1 receptors expressed on hippocampal GABAergic neurons and astrocytes. However, the exact mechanism(s) by which endocannabinoids do this remains unknown [78]. A recent study has shown that endocannabinoids regulate synaptic plasticity and memory via postsynaptic CB1 receptors, which modulate the activity of hyperpolarization-activated cyclic nucleotide-gated channels (HCN), responsible for dendritic excitability. The CB1R-HCN pathway is associated with dendritic integration of excitatory inputs, long-term potentiation, and spatial memory formation [78, 79].

Endocannabinoids and exogenously administered CB1 receptor agonists seem to hold promise in treating several neurodegenerative/neuroinflammatory disorders, including ischemia, seizures, multiple sclerosis, Huntington's and Parkinson's diseases [72, 80-82]. The widespread distribution of endocannabinoids and their role across the CNS and CB1 receptor expression warrants additional investigation. Due to the involvement of endocannabinoids in many physiological and cellular mechanisms, they are an emerging target for drug discovery. Although research in the last decade has revealed several secrets of the endocannabinoids, understanding the endocannabinoids' ability to protect neurons from harmful insults remains limited; thus, further research is required. Therefore, pharmacological modulation of the endocannabinoids is a viable target that could pave the way for therapeutic intervention in a wide range of diseases

Chapter 2: Hypotheses and Objectives

2.1 Study Objectives

According to the known structure-activity relationships (SAR) of endocannabinoids, their pharmacological activities are highly correlated with their chemical structure and molecular shape [83]. There are two main chemical groups in the endocannabinoids structure, including a polar ethanolamide group and a hydrophobic arachidonic chain [30, 83]. The lipophilic nature of their structure can provide excellent blood-brain barrier (BBB) permeability that makes them a potential therapeutics for CNS diseases such as AD [30]. In this regard, this project aimed to determine the role of endocannabinoids as Aβ42 aggregation inhibitors, with a goal of developing potential therapeutic interventions that prevent the formation of toxic soluble Aβ oligomers and Aβ fibrils, especially targeting the early stages of Aβ aggregation, such as stabilizing Aβ dimers, trimers, and tetramers in the amyloid cascade.

The main objectives of this thesis were to i) determine the ability of endocannabinoids to prevent Aβ42 aggregation and ii) investigate the role of cannabinoid receptors (CB1r) in endocannabinoid-mediated neuroprotection. To achieve this, five endocannabinoids, including 2-AG, AEA, NADA, noladin ether, and OAE, were evaluated to assess their inhibition against Aβ42-induced cell toxicity in mice hippocampal neuron cell lines (HT22). Also, because these endocannabinoids are all derived from arachidonic acid, we used this compound as a control. We used AM251 as a CB1 receptor antagonist to determine if it could reverse the endocannabinoids effects against the Aβ peptide. After that, we aimed to determine whether the CB1 receptor was involved in protection mediated by endocannabinoids, using Chinese hamster ovary (CHO) cells stably transfected and expressing the human CB1 receptor (CB1-CHO). The secondary goal of this study was to provide preliminary evidence for the interaction of endocannabinoids with the

Aβ oligomerization process by biophysical methods, including fluorescence-based aggregation kinetics assays and by transmission electron microscopy (TEM).

2.2 Hypotheses

In accordance with the aims, the following was hypothesized:

1. Based on previous reports, including the use of Aβ42 in our lab [57], I anticipate that treatment with Aβ42 (5 µM) will decrease cell viability.
2. Based on the literature, I predict that endocannabinoids will not be toxic to the cells. Instead, they will show neuroprotective effects against Aβ42 [17].
3. In light of the classical mechanisms of CB1 receptor agonists, I anticipated that endocannabinoids would be neuroprotective in HT22 cells as a neuronal cell line.
4. Based on the previously stated hypotheses, I would observe the protective ability of endocannabinoids against Aβ peptides through the CB1 receptor in the CHO cells, which express the human CB1 receptor.
5. Based on the above hypotheses, I anticipated that endocannabinoids' effect on preventing Aβ induced toxicity could be reversed through the CB1 antagonist (AM251) in HT22 and CB1-CHO cells.
6. CB1 receptors in HT22 (mouse CB1 receptor) and CB1-CHO (human CB1 receptor) will have a different level of CB1 receptor expression. Given that, I expect that endocannabinoids' effect on preventing Aβ-induced toxicity, the CB1 receptor antagonist will exhibit different results in these two cell lines.

Chapter 3: Material & Methods

3.1 Biological Screening

3.1.1 MTT Cell viability assay

A cell-based assay was used to display a reduction in Aβ42 (>97%, rPeptide company, Bogart, GA, USA)-mediated toxicity by five Endocannabinoids' and Arachidonic acid (>98%, Cayman Chemical Company, USA) *in-vitro*. CB1 antagonist (AM251) (>98%, Cayman Chemical Company, USA) was used to assess the endocannabinoids effects through the CB1 receptors. Both cell lines were cultured in DMEM and HAM's F12 (1:1) (Fisher #SH20361), 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained in a humidified atmosphere of 95% air and 5% CO2 at a temperature of 37°C. The full growth media was changed every 2-5 days by trypsinizing with 0.25% trypsin/0.1% EDTA. For experiments, cells were plated into 96-well plate at equal concentrations with cell density of 100,000 cells/mL in full growth media DMEM/F12 (1:1)(Fisher #SH20361), with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin for HT22 cells and DMEM/F12 (1:1)(Fisher #SH20361), with 10% FBS, 1% Pen/Strep, $1.4*10^{-4}$ geneticin for CHO cells) at 37◦C, 5% CO_2, and grow to 90% confluency (20–22 h). Cells were serum-starved overnight prior to the treatments; DMEM/F12 was exchanged for treatment media, including different test compounds concentrations (1, 5, 10 µM), co-incubated with Aβ42 (5 µM) for 24 h at 37 ºC. Each well contained 100 µL of treatment media. The 95 µL DMEM/F12, and 5 µL Aβ42 (5 µM final concentration) were added to the Aβ control wells. For wells containing treatment, 94, 90, 80 µL DMEM/F-12 and 5 µL Aβ42 with various concentrations of test compounds (1, 5, 10 µM) in quadruplicates (n = 4) were considered. DMSO and ethanol were used as vehicle control of the compound with the same concentration. After 24 hours of incubation at 37 ºC, the media changed to 10% MTT solution with DMEM/F12 (serum-

free). The cells were placed in the culture incubator for 3-4 hours to allow mitochondrial enzyme deactivation in dead cells. PBS was used as a solvent of MTT reagent ml (thiazolyl blue tetrazolium bromide 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)(Sigma Aldrich) to 5 mg/mL solution. Cells were lysed, and crystals dissolved in solubilization buffer (IPA, 1% HCl (12M), 10% Triton X-100 in propane-2-ol) (Thermo, Fisher, Markham, Ontario). A Molecular Dynamics™ plate reader was used to read plates at 570 nm and 690 nm. The cells treated with different concentrations of test compounds in the absence of Aβ42 were used as a positive control. The results are calculated as the percent cell viability compared to controls. The mentioned procedure and protocols were adapted from Robinson et al. [57].

3.2 Aβ preparation

Amyloid-beta, 1 mg of Aβ42 was dissolved in ultrapure, HFIP (manufactured by rPeptide), to 1 mg/ml in an anhydrous environment (desiccator), incubated for 30 minutes. Aliquots of Aβ in HFIP (100 µL) are prepared in microcentrifuge tubes and placed in the desiccator overnight to produce a thin film of monomeric Aβ. After 24 hours, microcentrifuge tubes were placed at -20ºC and stored with desiccant. Immediately before use, monomeric or oligomeric Aβ was prepared. For oligomeric stock, Aβ film was dissolved in DMSO to 5 mM, vortex for 30 seconds, and sonicated for 10 minutes. Afterward, it was diluted in ice-cold supplemented media to the 100 µM stock and incubated in the fridge overnight at 4 ºC. Immediately before treatment was diluted to the working concentrations in fresh media at 37 ºC . The mentioned protocol was adapted from Robinson & Stine et al. [57, 84].

3.1.3 Statistics

Parametric statistical analyses in GraphPad Prism were used to assess the data. A one-way ANOVA (which assumes that our data are taken from a population with a normal distribution) with Dunnett's multiple comparisons was performed to establish significance between the combination groups ($\alpha = 0.05$). Each concentration was tested in quadruplicates for n = 4 repeats. Also, the unpaired t-test was used to assess changes between every two groups: 1. DMSO as Aβ vehicle and Aβ (to show Aβ toxicity) and 2. endocannabinoids with Aβ and Aβ with endocannabinoids vehicle (to show the protection effect). The graphs of t-test are not shown in the thesis, as more than two groups were compared.

Additionally, a one-way ANOVA with Bonferroni correction was applied to show Aβ toxicity (DMSO group as Aβ vehicle, Aβ, and control group). Given that cell viability data were normalized and graphically present as a fold change relative to the controls, the control data became the baseline, in other words, equal to 1. So, the respective cumulative fold changes statistically were analyzed. All error bars represent the standard error of the mean.

3.3 Western Blot

Western blots were performed to detect the CB1 receptors in CHO cells. After 2 passages, cells were scraped, sheared using 26 gauge needles, and 43 centrifuged at 14,000 × g for 20 min at 4°C. Then they were washed by phosphate-buffered saline (PBS) and lysed in chilled lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 30 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1% Triton X-100; and 1% Halt Protease and Phosphatase Inhibitor (Thermo, Fisher, Markham, Ontario) for a BCA protein assay (Thermo) to measure the total protein before Western Blot. Samples were heated in

3x loading buffer (240 mM Tris-HCl at pH 6.8, 6% w/v SDS, 30% v/v glycerol, 0.02% w/v bromophenol blue, 50 mM DTT, and 5% v/v β-mercaptoethanol) for 15 min at 75°C and 5-20 µg total protein was loaded into polyacrylamide gel wells. Proteins were separated by SDS-PAGE using electrophoresis buffer (25 mM Tris base, 190 mM glycine, 3.5 mM 44 sodium dodecyl sulfate), followed by transfer of proteins to a nitrocellulose membrane by electroblotting with transfer buffer (25 mM Tris base, 190 mM glycine, 20% v/v methanol). Membranes were then blocked with 5% non-fat milk in Tris-buffered saline (20 mM Tris base, 150 mM NaCl, pH 7.6) plus 0.1% Tween (TBS-T) for 1 hour at room temperature or overnight at 4°C, followed by incubation with primary antibody added to blocking buffer for 1 h at room temperature, or overnight at 4°C. Membranes were washed three times with TBS-T and then incubated with a secondary antibody conjugated to horseradish peroxidase (HRP) in the blocking buffer for 1 hour at room temperature. Membranes were washed three additional times with TBS-T. Luminata substrate was used to visualize proteins on the Invitrogen iBright 1500F imaging station, and related imaging software performed densitometric analyses of images. After imaging, membranes were probed with the primary antibody against β-actin and human CB1 receptor (Cayman; 1:200 (rabbit). Anti-mouse (1:10000) and anti-rabbit (1:5000) horseradish peroxidase (HRP) enzyme-conjugated IgG secondary antibodies were used.

3.4 Thioflavin-T (ThT) based Aβ Aggregation Kinetic Studies

The inhibition and/or modulation activity of the selected endocannabinoids and AA was determined using Aβ42 fibrils based on the ThT- fluorescence assay. Thioflavin T (ThT) is a benzothiazole dye used as the most frequent technique to monitor the amyloid aggregation formation. The general chemical structure of ThT contains benzylamine and benzothiazole ring

systems linked by a carbon-carbon single bond (C-C). This single bond allows for free rotation of the molecule in the solution. Therefore, the binding of ThT to Aβ42 oligomers and fibrils prevents the C-C rotation and thereby causes a significant shift in the fluorescence (excitation = 440 nm and emission = 490 nm) (Figure 6). There are three crucial developmental phases during the amyloid aggregation process in fluorescence assay: the lag phase, growth phase, and a steady phase that Aβ tends to follow during its fibrillization. The lag phase represents the monomers of Aβ begin to grow and proliferate to fibrils form detected by ThT when bonded to fibrils. The growth phase consists of oligomers and protofibrils. The plateau phase represents the formation of Aβ fibrils (Figure 7). The assay was performed in Costar, black, clear-bottom 384-well plates. Data were collected every 5 minutes using a BioTek synergy H1 microplate reader, with continuous shaking at 730 cycles per minute (cpm) for 30 seconds, with the temperature maintained at 37 ºC for 24 h. The readings were recorded as fluorescence intensity units, obtained by measuring ThT excitation and emission at 440 nm and 490 nm, respectively. All compounds for concentrations of 1, 5, 10 µM were prepared fresh and diluted in disodium hydrogen phosphate heptahydrate buffer ($Na_2HPO_4.7H_2O$ in UPW, adjusted to pH 7.4). The Aβ42 hexafluoro-2-propanol (HFIP) (AnaSpec, CA, USA) samples were dissolved in 10% NH_4OH and further sonicated at RT for 5 min to ensure homogeneity. Then the peptide solution was diluted to 50 µM in phosphate buffer ($Na_2HPO_4.7H_2O$) and placed on an ice bath for plating. A 15 µM ThT stock solution was prepared fresh using 50 mM glycine and sodium hydroxide (NaOH) buffer (adjusted to pH 7.4) and was protected from light. Methylene blue (MB) and resveratrol were used as known Aβ42 aggregation inhibitors. Plates were sealed with a ThermoSeal film (Sigma Aldrich) before placing them to the plate reader. The results of this assay represent an average value of triplicate reading. Each well accommodated 80 µl of the sample, and therefore, the plating sequence was

per the following: ThT background, Aβ control, endocannabinoids screening, and compounds control, as shown in Table 2**.** The inhibition percentage data were obtained by calculating the RFU difference between Aβ control and endocannabinoids & AA with the tested concentrations at 24 h time point (Table 5). The mentioned procedure and protocols adapted from Tin & Mohamed et al. [85]

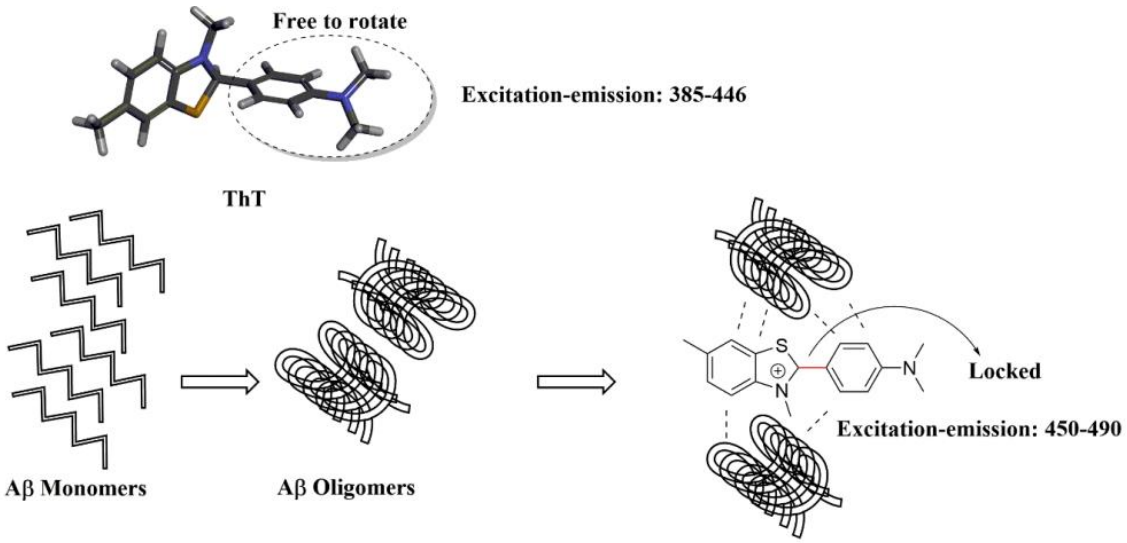


Figure 6. Principle of ThT assay and ThT-Aβ oligomers interaction in Aβ kinetic assay [86]

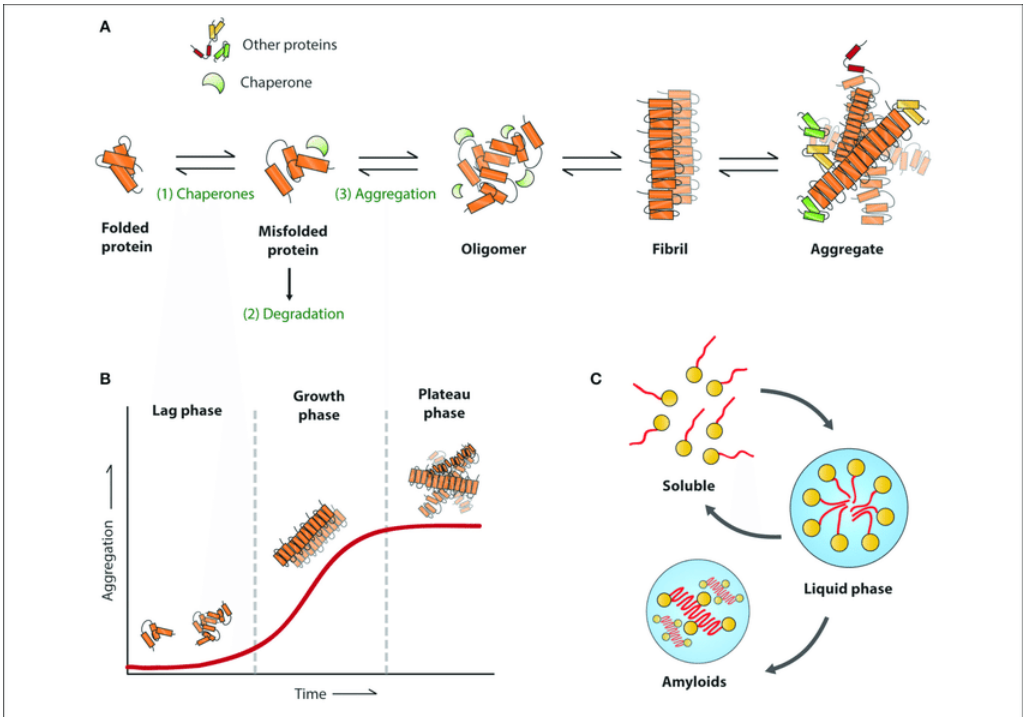


Figure 7. Schematic view of (ThT) based Aβ aggregation kinetic, which represents Aβ toxicity and its correlation with Aβ aggregation process [87].

ThT Background	Aβ42 Control	Compound Screening	Compound Control
44 µl ThT	44 µl ThT	44 µl ThT	44 µl ThT
35 µl Buffer	27 µl Buffer	20 µl Buffer	28 µl Buffer
1 µl DMSO	1 µl DMSO	8 µl Compound	8 µl Compound
-	8 µl Aβ	8 µl Aβ	-

Table 2. Reagents and assay protocol for the ThT-based Aβ aggregation

3.5 Transmission electron microscopy (TEM) studies

TEM experiment was used as a method to observe features such as the structure and morphology of Aβ42 peptides aggregation in the presence and absence of endocannabinoids and AA. The Philips CM 10 transmission electron microscope at 60 kV (Department of Biology, University of Waterloo) was used to scan the grids, and a 14-megapixel AMT camera obtained the micrographs. The samples were obtained directly from the 384 well plates within 1 hour after 24 hours of ThT kinetic aggregation assay. Interference was evaluated by comparing the test image with the compound background. TEM grids were prepared by adding approximately 20 µl of the sample using a Pasture pipette on to the formvar-coated copper grid (400 mesh) and allowed to air-dry overnight. The grids were washed with 2-3 droplets of UPW to remove any precipitated buffer salts and bolted out using a filter paper, and further air-dried for 30 mins. 20 µL of 2% phosphotungstic acid (PTA) was used for staining the samples, which were dried immediately by small pieces of filter paper and kept to dry overnight before imaging. The mentioned procedure and protocols adapted from Tin & Mohamed et al. [85]

Chapter 4: Results

4.1 MTT Test

4.1.1 HT22 Cell Viability

CB1 expression of HT22 cells was investigated by immunocytochemistry and Western blot in many studies previously [59, 67, 88]. Also, CB1 antagonist (AM251 at 5 µM) was used to determine if it could reverse the protection that some of the endocannabinoids have shown. The endocannabinoids were evaluated alone to see whether they exhibited any toxicity. We found that these compounds are non-toxic in the concentrations used in our experiments (Figure 8 A-B, Figure 9 C-F). Results are shown for the following groups: Endocannabinoid-treated groups (at 1, 5, 10 µM) and ethanol-treated groups with the same concentrations as the vehicle. There were no significant differences in cell viability between these groups and their vehicle (Figures 8 and 9 A-F).

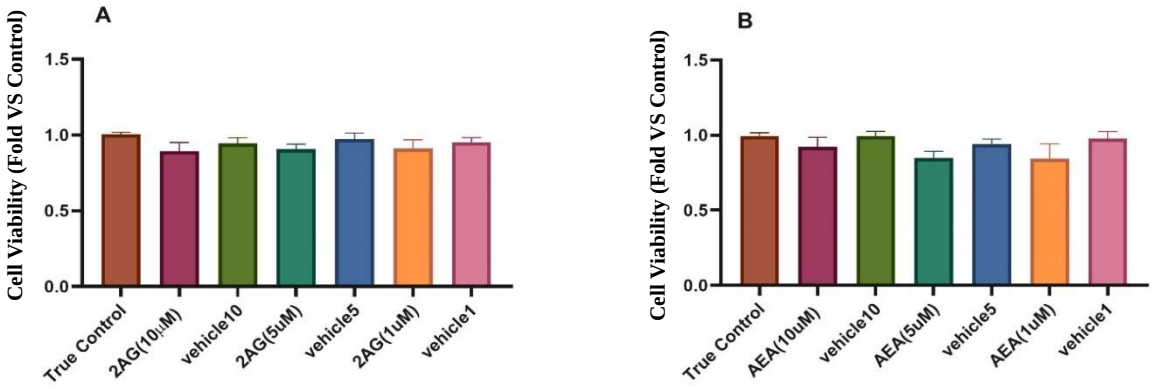


Figure 8. Effect of Endocannabinoids, 2-AG and AEA (1,5,10 µM) and their vehicles on HT22 Cells. There were no significant differences in cell viability between these groups and their vehicle. Endocannabinoids and their vehicle are non toxic at tested concentrations. The results are shown as the average ± standard error of the mean (SEM). A one-way ANOVA performed to establish significance between groups ($\alpha = 0.05$). The data is representative of 4 independent experiments. Each column is representative of 4 replicates.

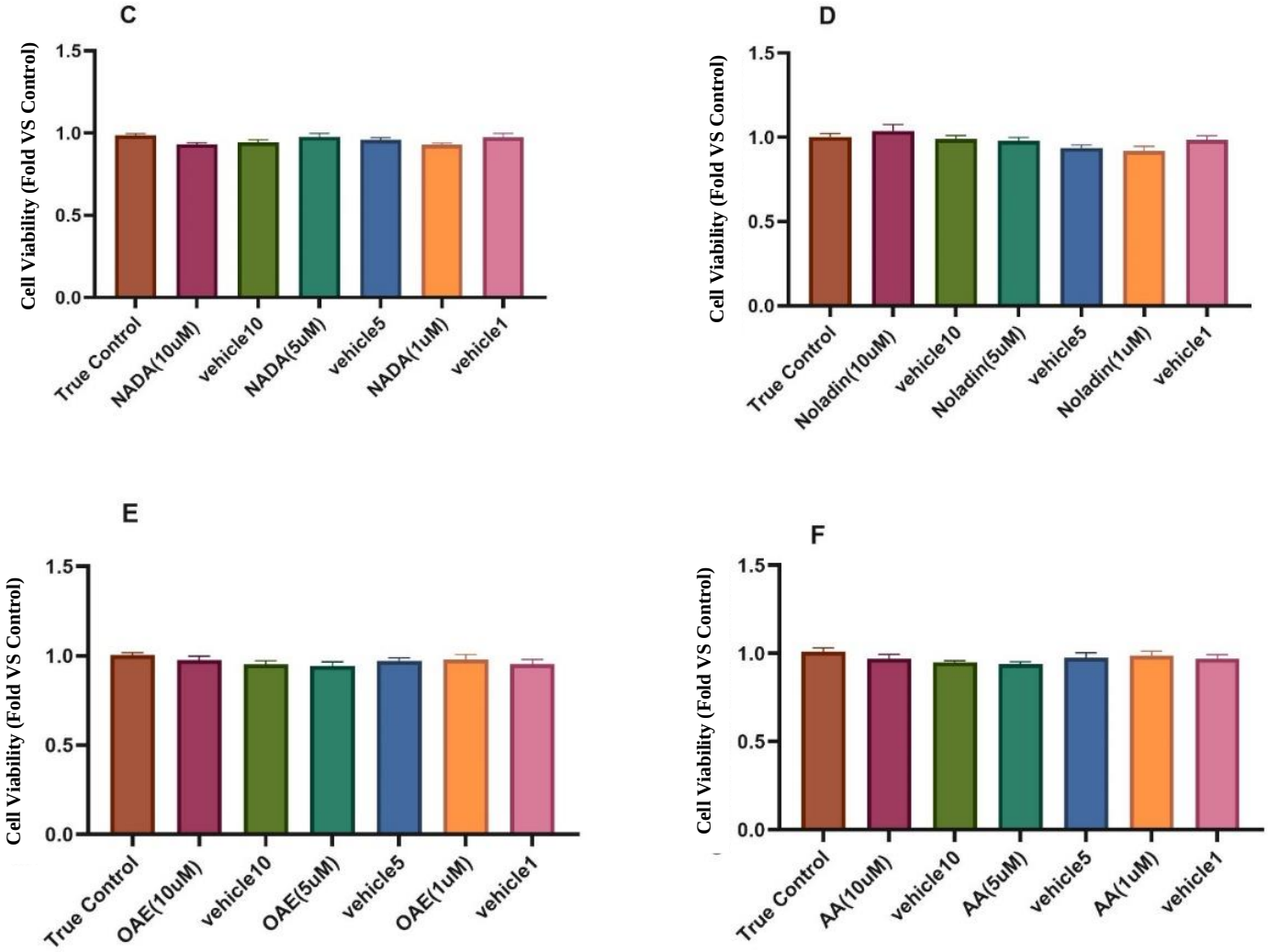


Figure 9. Effect of Endocannabinoids, NADA, noladin, OAE and AA at (1, 5, 10 μM) and their vehicle on HT22 Cells. There were no significant differences in cell viability between these groups and their vehicles. Endocannabinoids and their vehicles are non-toxic at tested concentrations. The results are shown as the average ± standard error of the mean (SEM). The data is representative of 4 independent experiments. Each column is representative of 4 replicates. A one-way ANOVA was performed to establish significance between groups ($\alpha = 0.05$).

The Aβ42 (5 μM) toxicity and DMSO control data are shown in Figure 10. There were no significant differences in cell viability between the DMSO control group and the "True Control" group, which indicates that it does not affect cell viability. However, a significant difference was observed (P<0.0001, ****) between Aβ42 and the True Control group: this shows that Aβ oligomers at 5 μM induced toxicity to the HT22 cells.

The protective effects of 2-AG and NADA at 1, 5, 10 μM against Aβ42 (5 μM) oligomer-induced toxicity in the HT22 cell were small and not statistically significant (Figure 11 and 14, respectively). However, the same experiment for AEA showed that AEA in 10 μM was able to improve the cell viability of HT22 cells significantly (P<0.05 *) (Figure 12). Also, the CB1 antagonist (AM251) at 5 μM could not reverse the AEA (10 μM) neuroprotective effect in HT22 cells (Figure 13).

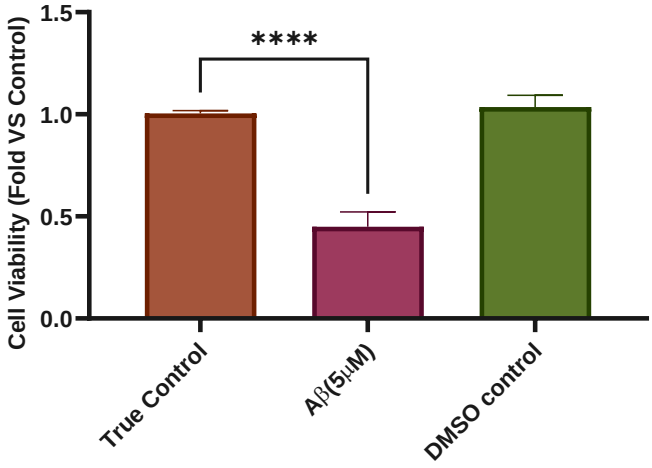


Figure 10. Aβ42 (5 μM) toxicity on HT22 Cells. There are no significant differences in cell viability between the DMSO control group and the True Control group, which indicates that the DMSO as the vehicle does not affect cell viability. However, a significant difference was observed (P<0.0001 ****) between Aβ42 and the True Control group, which shows that Aβ oligomers at 5 μM induced toxicity to the HT22 cells. A one-way ANOVA with the Bonferroni correction was applied to establish significance between groups ($\alpha = 0.05$). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

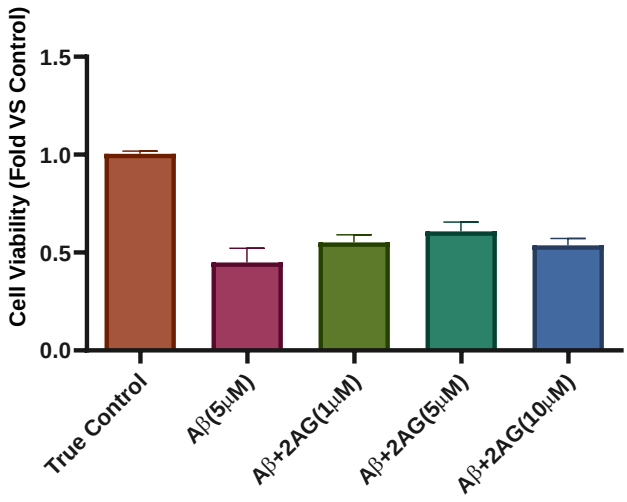


Figure 11. The protective effects of 2-AG at 1, 5, 10 μM aginst Aβ42 (5μM) oligomer-induced toxicity in the HT22 cell were small and not statistically significant. A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

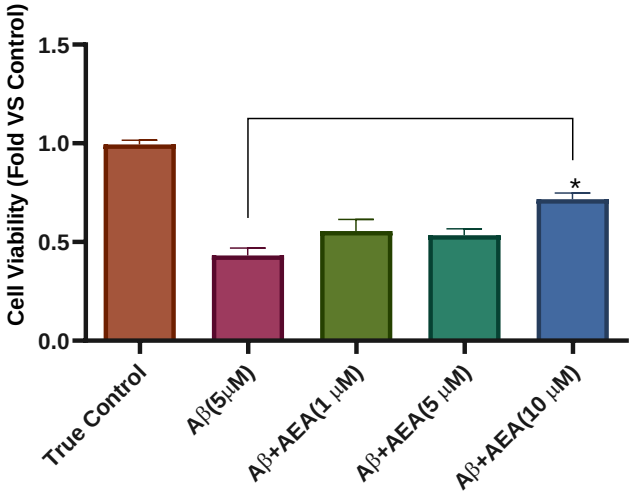


Figure 12. Effect of AEA at 1, 5, 10 μM against Aβ42 (5μM) toxicity on HT22 cells. AEA in 10 μM was able to improve the cell viability significantly (P<0.05 *). A one-way ANOVA with Dunnett's multiple comparisons performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

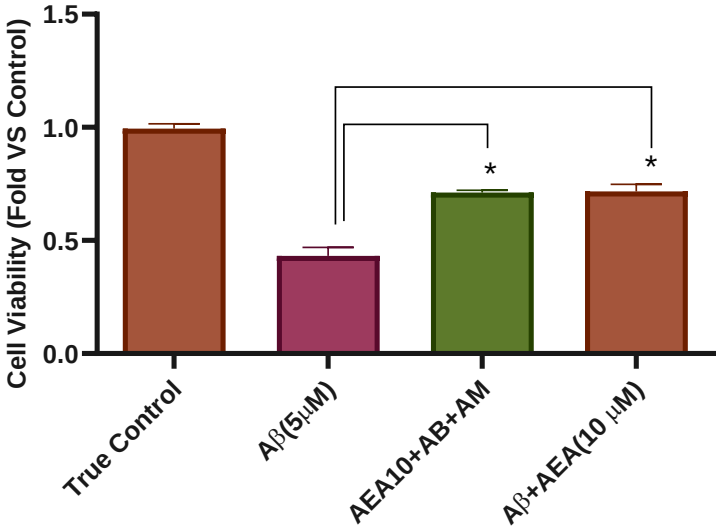


Figure 13. Effect of AEA (10 μM) with AM251 (5μM) against Aβ42 (5 μM) toxicity on HT22 cells. The CB1 antagonist (AM251) could not reverse the AEA(10 μM) neuroprotective effect. A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

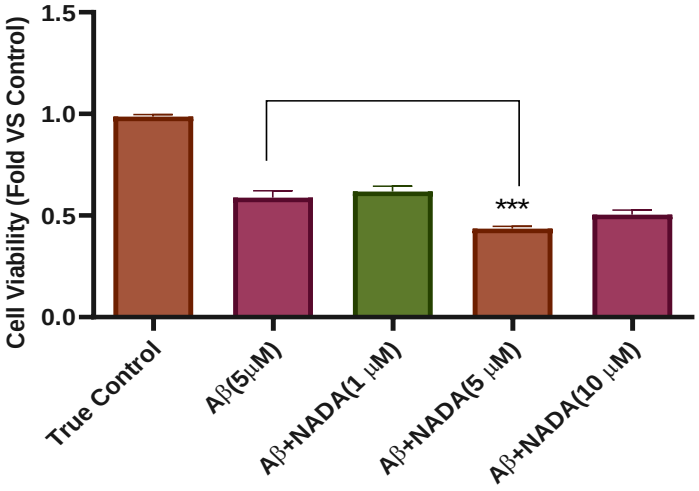


Figure 14. Effect of NADA at 1, 5, 10 μM against Aβ42 (5 μM) toxicity on HT22 cells. The result showed that NADA could not increase the cell viability. A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

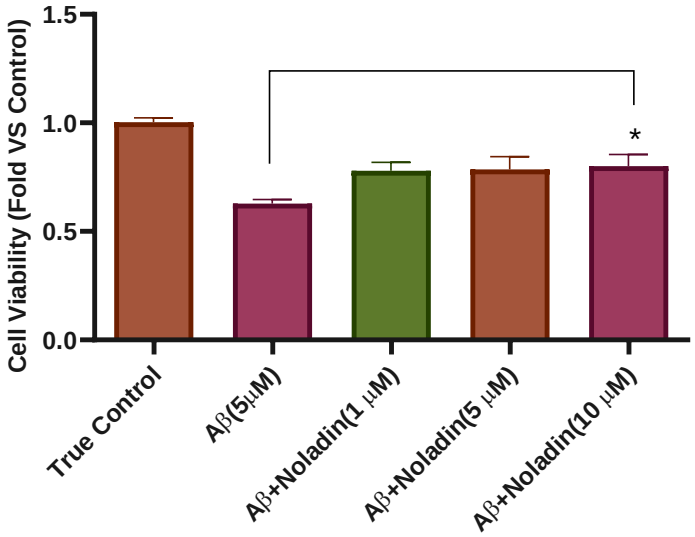


Figure 15. Effect of noaldin at 1, 5, 10 µM against Aβ42 (5 µM) toxicity on HT22 cells. Noladin at 10 µM increased the cell viability significantly (P<0.05 *). A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

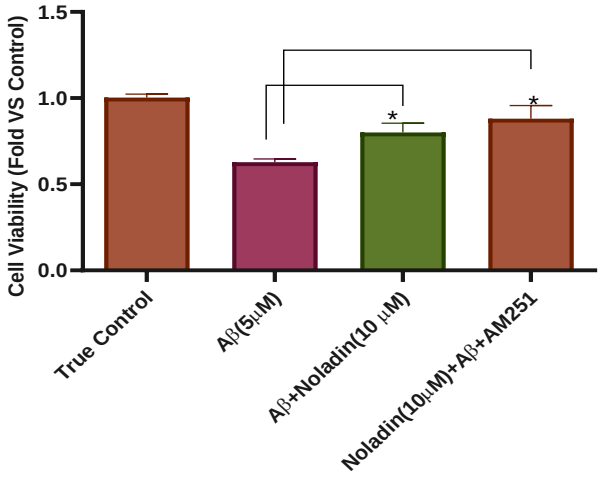


Figure 16. Effect of noaldin (10 µM) with AM251 (5 µM) against Aβ42 (5 µM) toxicity on HT22 Cells. The CB1 antagonist (AM251) could not reverse the noladin (10 µM) neuroprotective effect. A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α= 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

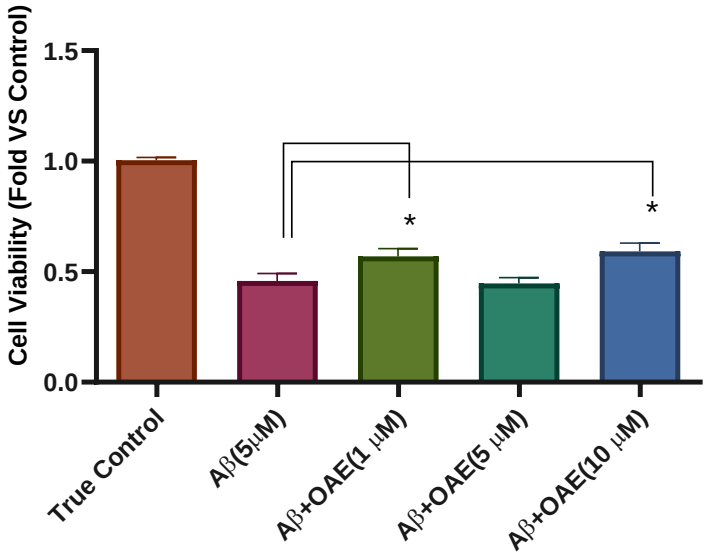


Figure 17. Effect of OAE at 1, 5, 10 μM against Aβ42 (5 μM) toxicity on HT22 cells. OAE at 1, 10 μM increased the cell viability significantly (P<0.05 *). A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

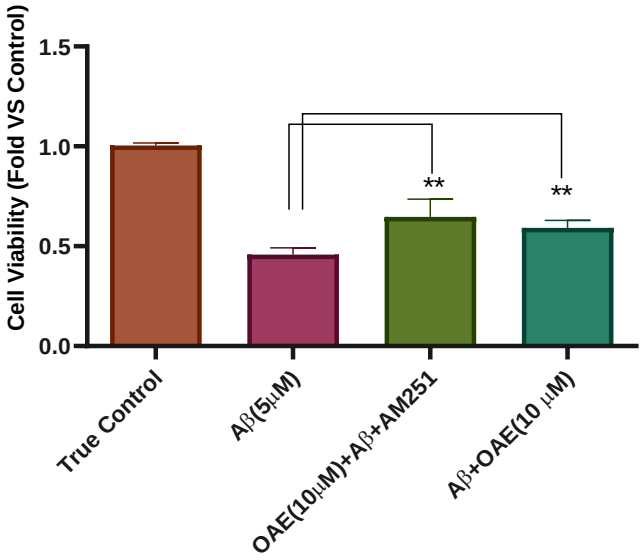


Figure 18. Effect of OAE (10 μM) with AM251 (5 μM) against Aβ42(5 μM) toxicity on HT22 cells. The CB1 antagonist (AM251) could not reverse the OAE (10 μM) neuroprotective effect. A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

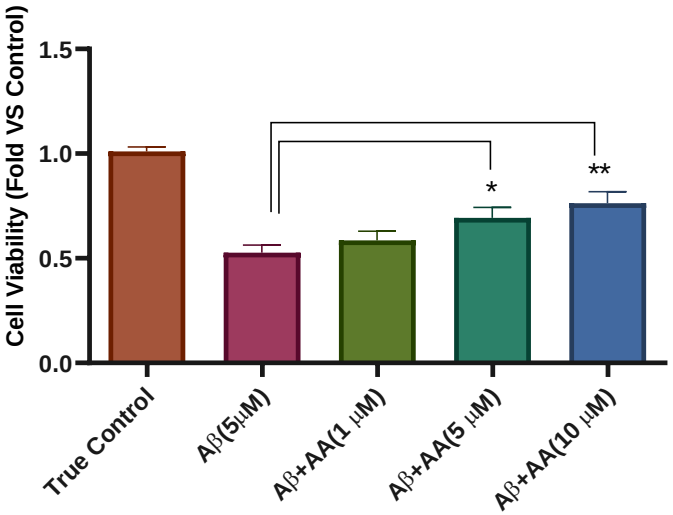


Figure 19. Effect of AA at 1, 5, 10 μM against Aβ42 (5μM) toxicity on HT22 cells. AA at 1, 10 μM increased the cell viability significantly (P<0.05 *). A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

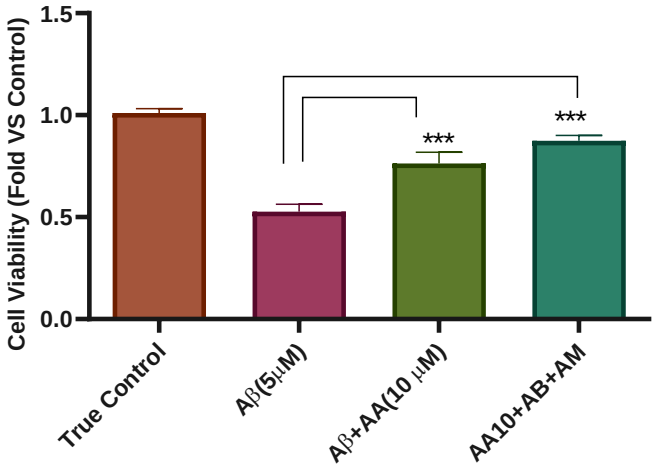


Figure 20. Effect of AA (10 μM) with AM251 (5 μM) against Aβ42 (5 μM) toxicity on HT22 cells. The CB1 antagonist (AM251) could not reverse the AA (10 μM) neuroprotective effect. A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

Moreover, noladin increased the cell viability significantly (P<0.05 *) in HT22 cells at the highest concentration (Figure 15). However, AM251 as a CB1 antagonist could reverse this protection in HT22 cells (Figure 16). Also, OAE was able to inhibit Aβ42 aggregation significantly at (1, 10 μM) in HT22 cells (P<0.05 *) (Figure 17), and AM251 could not reverse this protection (Figure 18).

AA, as a common metabolite and also precursor of endocannabinoids, showed a significant protective effect in HT22 cells at 5, 10 μM (P<0.05, *, P<0.01, ** respectively) (Figure 19), although AM251 could not reverse this effect) (Figure 20).

Endocannabinoid / **Concentrations**	**Significant neuroprotective effect against Aβ42 (5 μM) on cell viability of HT22 cells**			**Reversed by CB1 antagonist (AM251 at 5 μM)**
	1 μM	**5 μM**	**10 μM**	
2-AG	Not Significant	Not Significant	Not Significant	Not Significant
AEA	Not Significant	Not Significant	**Significant**	Not Significant
NADA	Not Significant	Not Significant	Not Significant	Not Significant
Noladin	Not Significant	Not Significant	**Significant**	Not Significant
OAE	**Significant**	Not Significant	**Significant**	Not Significant
AA	Not Significant	Significant	**Significant**	Not Significant

Table 3. Summary of endocannabinoids'effect on cell viability of HT22 cells. A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05).

4.2 CHO Cells Expressing CB1 Receptor

As we hypothesized that CB1 receptors might be involved in the endocannabinoids-induced protection against Aβ42 toxicity, CHO cells transfected with human CB1 receptors were used to determine the endocannabinoids' effect on Aβ42 treated cells. Western blot analysis of cell lysates detected the presence of a major CB1 immunoreactive bond using Fisher BioReagents™ EZ-Run™ Prestained Rec Protein Ladder, Fisher BioReagents (Catalog No.BP3603500), which was close to the expected molecular mass of the CB1 receptor (approximately 43 kDa) (Figure 21).

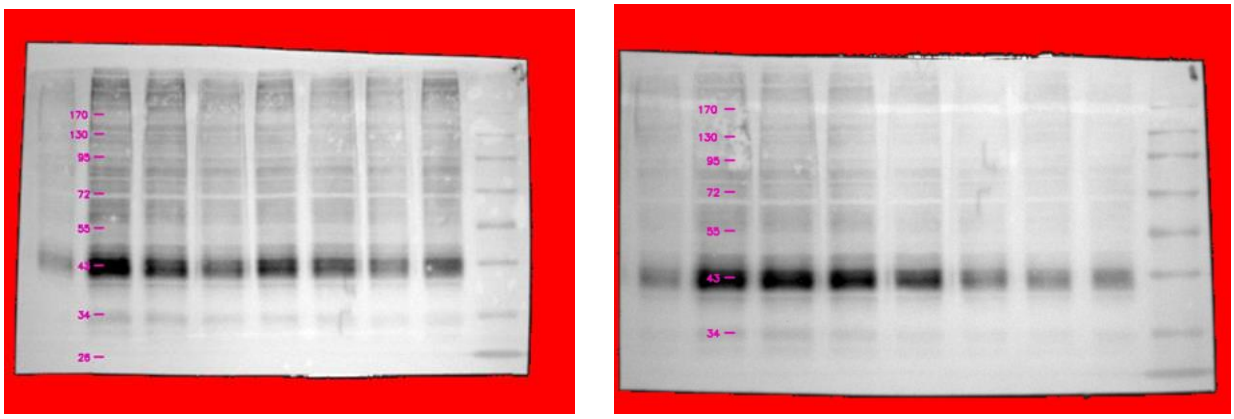


Figure 21. CB1-CHO cells were lysed and evaluated by Western blots as described in the methods, detected the presence of CB1r bond, which was close to the expected molecular mass of the CB1 receptor (approximately 43 kDa) (n = 2).

4.3 CB1-CHO Cell Viability

We observed that CB1 was successfully expressed in CHO cells, and these cells were used to determine the effects of endocannabinoids' in Aβ42 treated cells to detect their potential interaction with human CB1 receptors. The results are shown as the average ± standard error of the mean (SEM). The data is representative of 4 independent experiments. Each column is representative of 4 replicates.

All of the endocannabinoids were evaluated alone to see whether they exhibited any toxicity. We found that these compounds are non-toxic at the concentration used in these experiments (Figure 23 A-F). Results are shown for the following groups: Endocannabinoid-treated groups (1, 5, 10 µM) and ethanol-treated groups with the same concentrations as a vehicle. There were no significant differences in cell viability between these groups and their vehicle (Figure 23 A-F).

The cell viability data for Aβ42 (5 µM) treated and DMSO control is shown in Figure 22. There were no significant differences in cell viability between the DMSO control group and the control group, which indicates that it does not affect cell viability. However, the significant difference ($P<0.0001$ ****) between Aβ42 and the control group shows that Aβ oligomers at 5 µM induce toxicity to the CB1-CHO cell.

Also, the protective effects of 2-AG, AEA, NADA at 1, 5, 10 µM against Aβ42 (5µM) oligomer-induced toxicity on the CB1-CHO cell were not insignificant (Figure 24 A-C). A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups ($\alpha = 0.05$). Each column is representative of 4 replicates, and $n = 4$ independent experiments were performed.

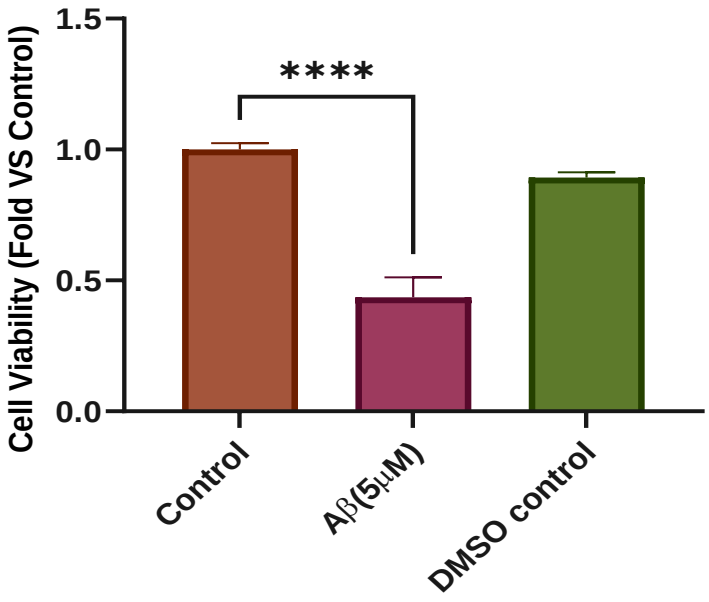


Figure 22. Aβ42 (5 μM) toxicity on CB1-CHO cells. Aβ42 (5 μM) toxicity on HT22 Cells. There are no significant differences in cell viability between the DMSO control group and the True Control group, which indicates that the DMSO as the vehicle does not affect cell viability. However, a significant difference was observed (P<0.0001****) between Aβ42 and the True Control group, which shows that Aβ oligomers at 5 μM induced toxicity to the HT22 cells. A one-way ANOVA with Bonferroni correction was applied to establish significance between groups ($\alpha = 0.05$). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

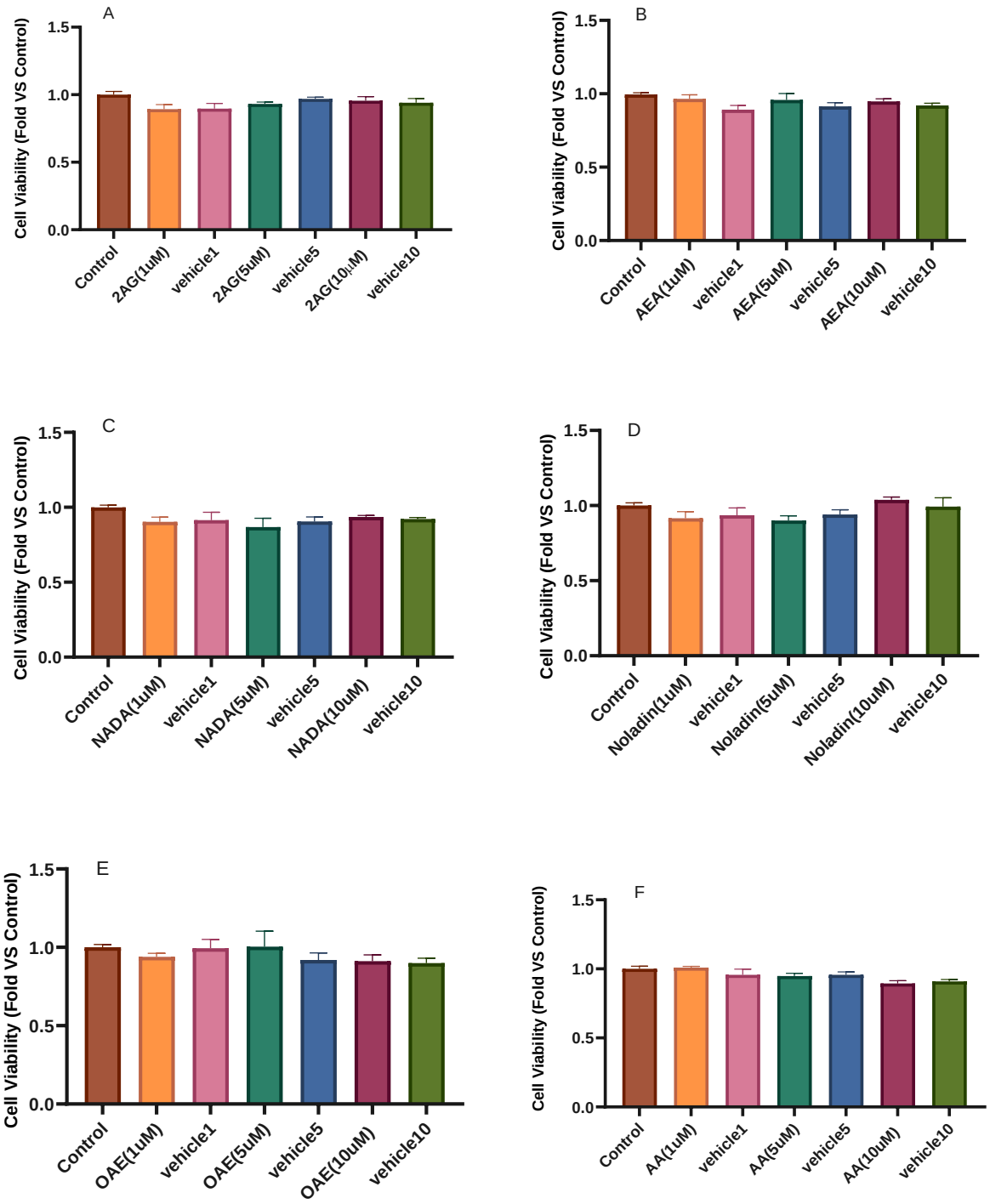


Figure 23. Effect of endocannabinoids (1, 5, 10 μM) and their vehicle on CB1-CHO Cells. There were no significant differences in cell viability between these groups and their vehicles. Endocannabinoids and their vehicles are non-toxic at tested concentrations. The results are shown as the average ± standard error of the mean (SEM). The data is representative of 4 independent experiments. Each column is representative of 4 replicates. A one-way ANOVA was performed to establish significance between groups ($\alpha = 0.05$).

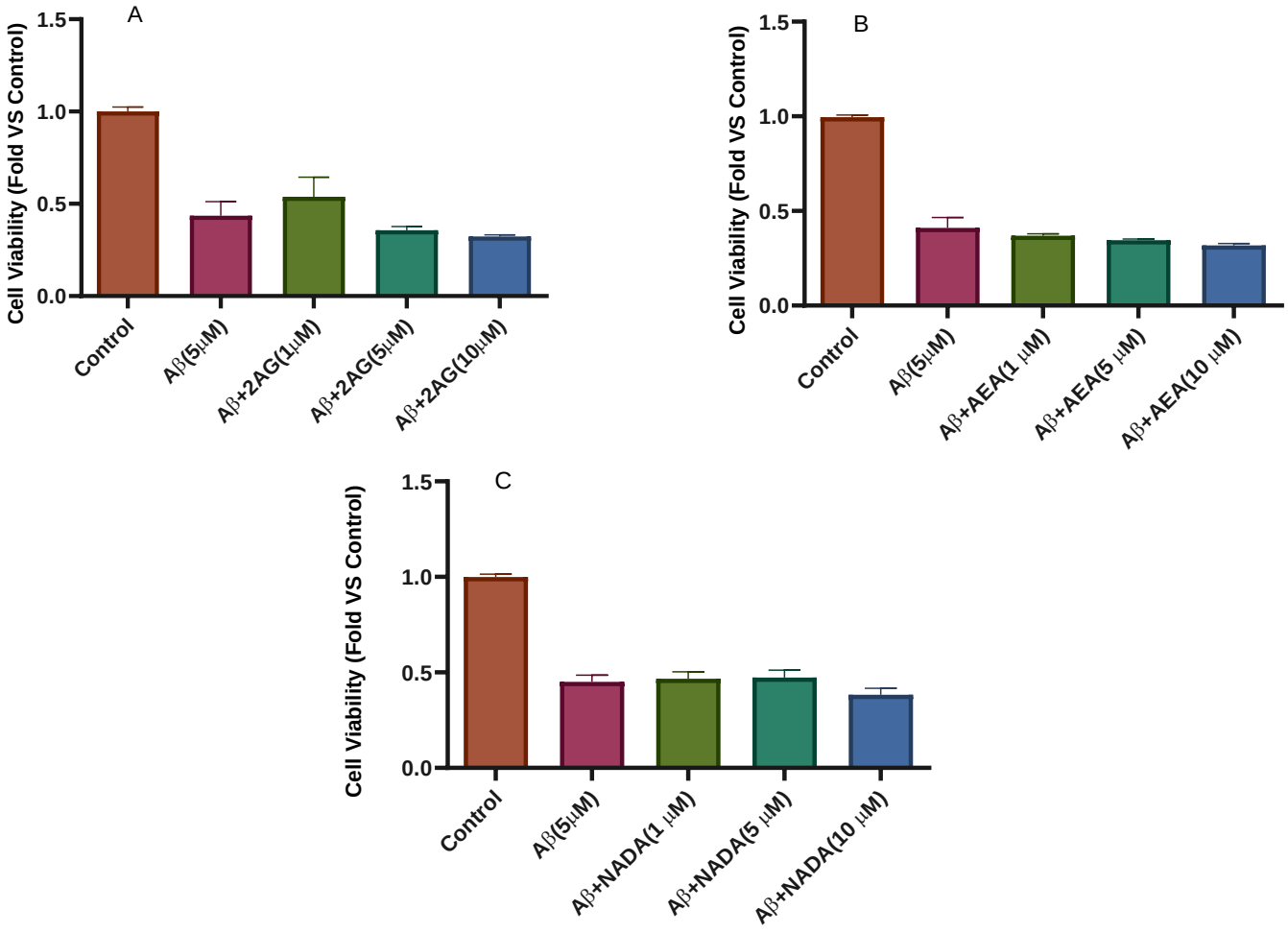


Figure 24. Effect of endocannabinoids against Aβ42 toxicity on CB1-CHO cells. A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups ($\alpha = 0.05$). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

A: The protective effects of 2-AG at 1, 5, 10 μM against Aβ42 (5 μM) oligomer-induced toxicity the was not statistically significant.

B: The protective effects of AEA at 1, 5, 10 μM against Aβ42 (5 μM) oligomer-induced toxicity the was not statistically significan.

C: The protective effects of NADA at 1, 5, 10 μM against Aβ42 (5 μM) oligomer-induced toxicity the was not statistically significant.

On the other hand, noladin increased the cell viability significantly (P<0.05 *) in CB1-CHO cells at the highest concentration (Figure 25), and AM251 as a CB1 antagonist could reverse this protection in CB1-CHO cells (P<0.05 *) (Figure 26). Also, OAE at all the concentrations tested (1, 5, 10 µM) increased the cell viability significantly in CHO cells (P<0.05 *) and AM251 reversed this protection in CB1-CHO cells (P<0.05 *) (Figure 27, 28). Lastly, AA at all tested concentrations (1, 5, 10 µM) improved the cell viability of CB1-CHO cells treated with Aβ42 (5µM), and AM251 was able to reverse this protection (Figures 29 and 30).

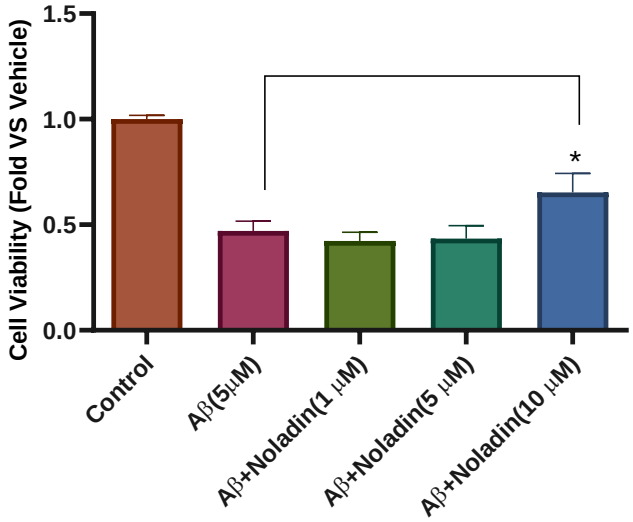


Figure 25. Effect of noladin ether at 1, 5, 10 µM against Aβ42 (5 µM) toxicity on CB1-CHO cells. Noladin at 10 µM increased the cell viability significantly (P<0.05*). A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α=0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

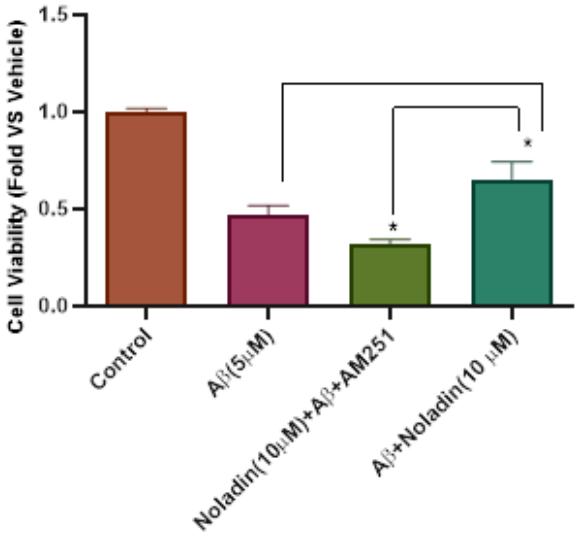


Figure 26. Effect of noladin (10 μM) with AM251 (5 μM) against Aβ42 (5 μM) toxicity on CB1-CHO. The CB1 antagonist (AM251) reversed the noladin neuroprotective effect significantly (P<0.05*). A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

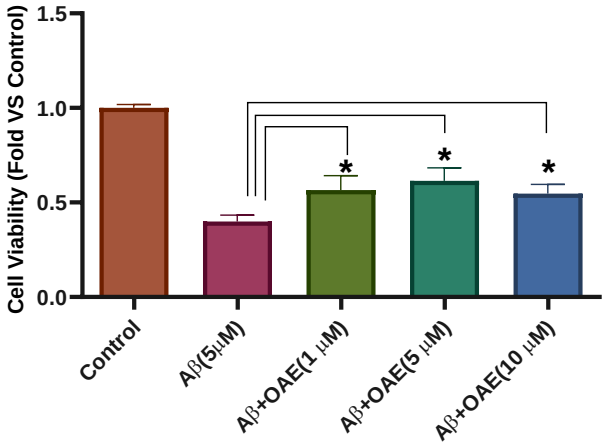


Figure 27. Effect of OAE at 1, 5, 10 μM against Aβ42 (5 μM) toxicity on CB1-CHO cells. OAE at all tested concentrations increased the cell viability significantly (P<0.05*). A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

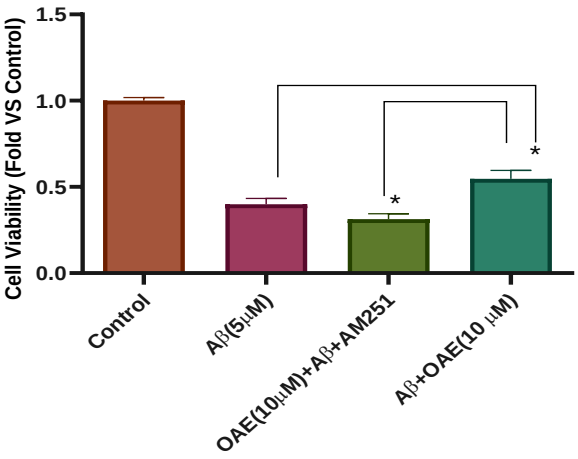


Figure 29. Effect of OAE (10 μM) with AM251 (5 μM) against Aβ42 (5 μM) toxicity on CB1-CHO. The CB1 antagonist (AM251) reversed the OAE neuroprotective effect significantly (P<0.05*). A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

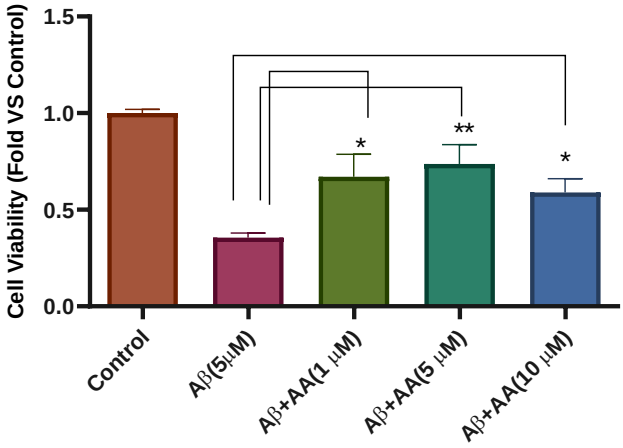


Figure 28. Effect of AA at 1, 5, 10 μM against Aβ42 (5 μM) toxicity on CB1-CHO cells. AA at all tested concentrations increased the cell viability significantly (P<0.05* for 1,10 μM and P<0.05** or 5 μM). A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

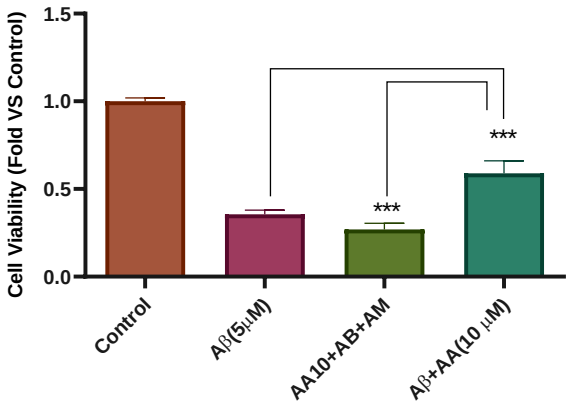


Figure 30. Effect of AA (10 μM) with AM251 (5 μM) against Aβ42 (5 μM) toxicity on CB1-CHO. The CB1 antagonist (AM251) reversed the AA neuroprotective effect significantly (P<0.05***). A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α = 0.05). Each column is representative of 4 replicates. The data is representative of 4 independent experiments.

Endocannabinoids / **Concentrations**	**Significant neuroprotective effect against Aβ42 (5μM) on cell viability of CB1-CHO cells**			**Reversed by CB1 antagonist (AM251 at 5 μM)**
	1 μM	**5 μM**	**10 μM**	
2-AG	Not Significant	Not Significant	Not Significant	Not Significant
AEA	Not Significant	Not Significant	Not Significant	Not Significant
NADA	Not Significant	Not Significant	Not Significant	Not Significant
Noladin	Not Significant	Not Significant	**Significant**	**Significant**
OAE	**Significant**	**Significant**	**Significant**	**Significant**
AA	**Significant**	**Significant**	**Significant**	**Significant**

Table 4. Summary of endocannabinoids'effect on cell viability of CB1-CHO cells. A one-way ANOVA with Dunnett's multiple comparisons was performed to establish significance between groups (α=0.05).

4.4 ThT based Aβ Aggregation Kinetic assay

The aggregation kinetic assay results of 2-AG showed 22.7%, 3.5%, 12.7% inhibition at 1, 5, 10 µM, respectively (Figure 31). The same experiment for AEA with the same concentrations showed that this compound was able to exhibit 29.% and 14.05% inhibition against Aβ42 aggregation at 1,5 µM, respectively at 24 h time point, with better inhibition at higher concentrations (10 µM = 93.3% inhibition) (Figure 32). The aggregation kinetic studies of NADA showed that it was a weak inhibitor of Aβ42 aggregation (8.4% to maximum 37.% inhibition) (Figure 33). However, noladin showed maximum inhibition of 72.9% at the highest concentration tested (10 µM), with 19% and 14.6 % inhibition at 1,5 µM, respectively, at 24 h time point (Figure 34). The OAE inhibition on Aβ42 aggregation ranged from 11% to 25.% inhibition (Figure 35). The aggregation kinetic study for AA showed that it can directly interact with Aβ42 aggregates and reduce fibrillogenesis (25%, 86, 94% inhibition respectively at 1, 5, 10 µM) at 24 h time point (Figure 36). ThT fluorescence spectroscopy monitored aggregation kinetics assay at excitation = 440 nm and emission = 490.

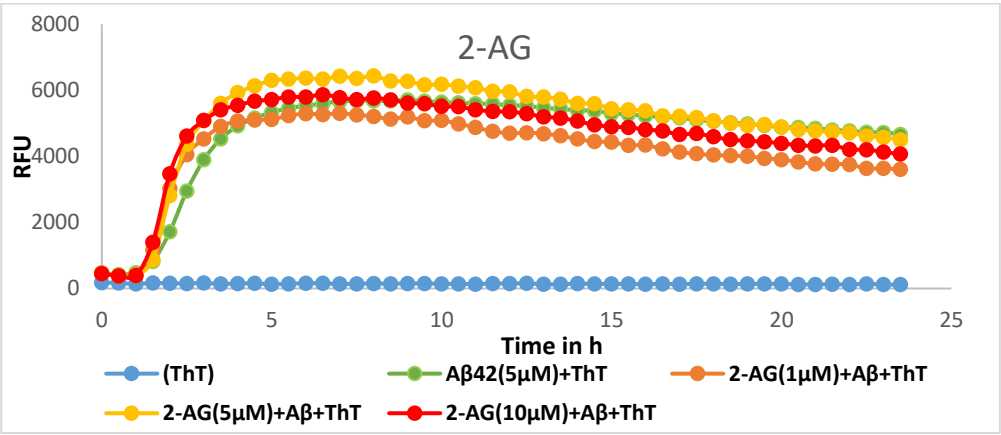


Figure 31. ThT-monitored 24 h kinetics of Aβ42 (5 µM) in the presence of 1, 5, 10 µM of 2-AG that showed 22.7%, 3.5%, 12.7% inhibition respectively at 24 h time point. Aggregation kinetics were monitored by ThT-fluorescence spectroscopy (excitation = 440 nm, emission = 490 nm) at pH 7.4, 37 °C in phosphate buffer.

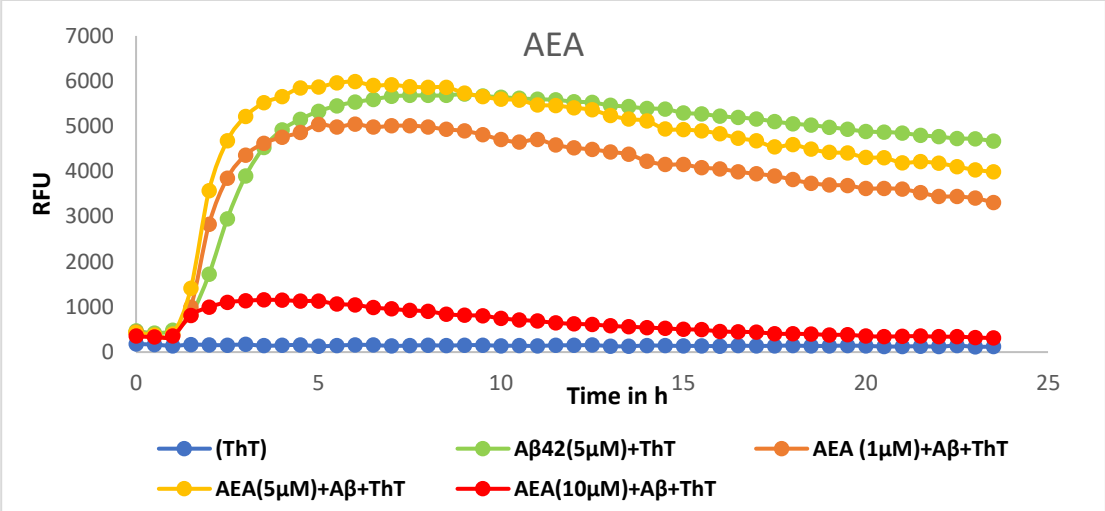


Figure 32. ThT-monitored 24h kinetics of Aβ42 (5 μM) in the presence of 1, 5, 10 μM of AEA that showed 29% , 14.05%, and 93% inhibition respectively at 24 h time point. Aggregation kinetics were monitored by ThT-fluorescence spectroscopy (excitation = 440 nm, emission = 490 nm) at pH 7.4, 37 °C in phosphate buffer.

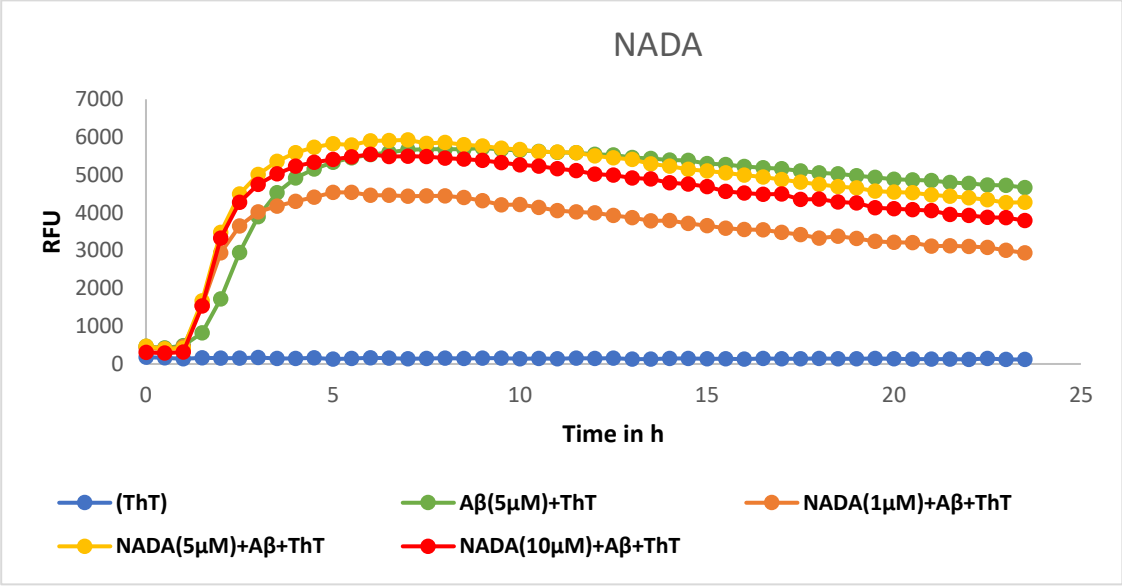


Figure 33. ThT- monitored 24h kinetics of Aβ42 (5 μM) in the presence of 1, 5, 10 μM of NADA showed 37%, 9% and 19% inhibition respectively at 24 h time point. Aggregation kinetic were monitored by ThT-fluorescence spectroscopy (excitation = 440 nm, emission = 490 nm) at pH 7.4, 37 °C in phosphate buffer.

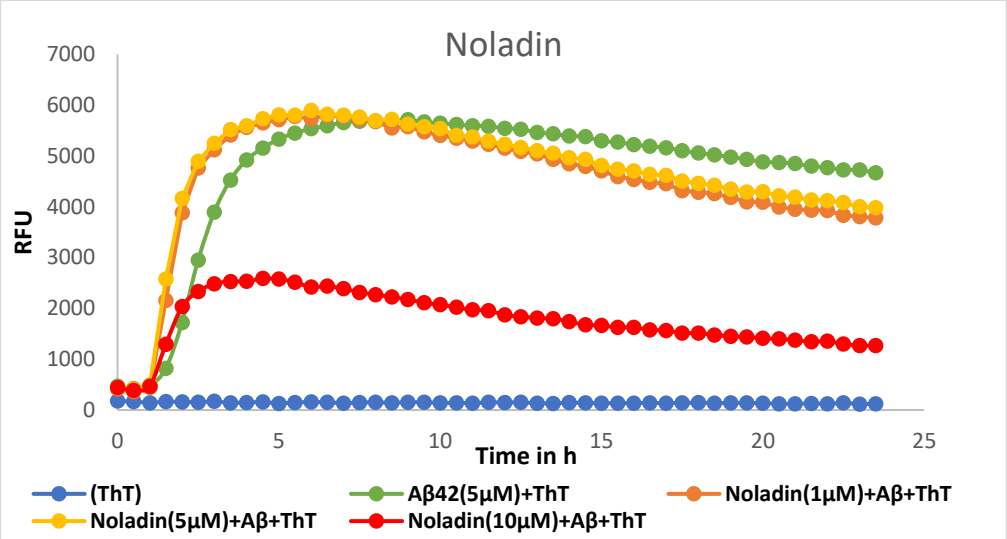


Figure 34. shows ThT-monitored 24h kinetics of Aβ42 (5 μM) in the presence of 1, 5, 10 μM of Noladin that showed 19% and 15 % and 73% inhibition rectively at 24 h time point. Aggregation kinetics were monitored by ThT-fluorescence spectroscopy (excitation = 440 nm, emission = 490nm) at pH 7.4, 37 °C in phosphate buffer.

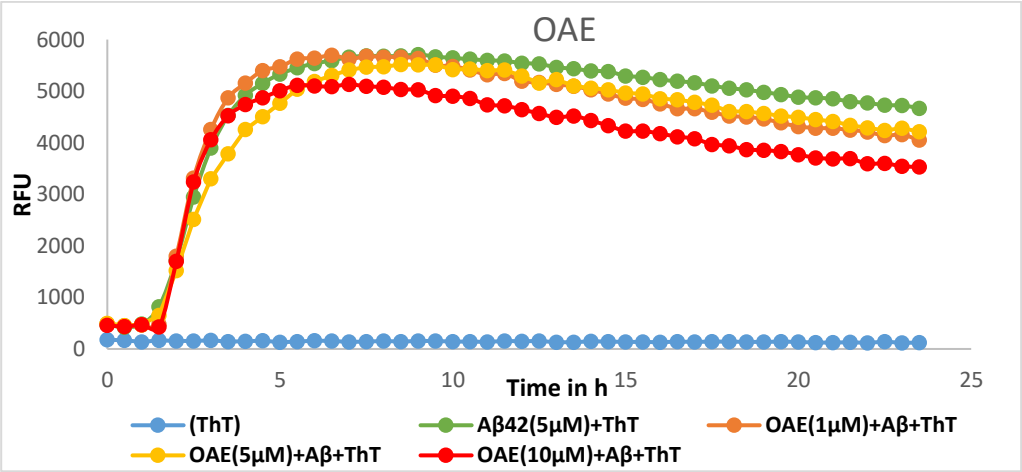


Figure 35. shows ThT-monitored 24h kinetics of Aβ42 (5 μM) in the presence of 1, 5, 10 μM of OAE that showed 15%, 11% to 25 % inhibition respectively at 24 h time point. Aggregation kinetics were monitored by ThT-fluorescence spectroscopy (excitation = 440 nm, emission = 490nm) at pH 7.4.0, 37 °C in phosphate buffer.

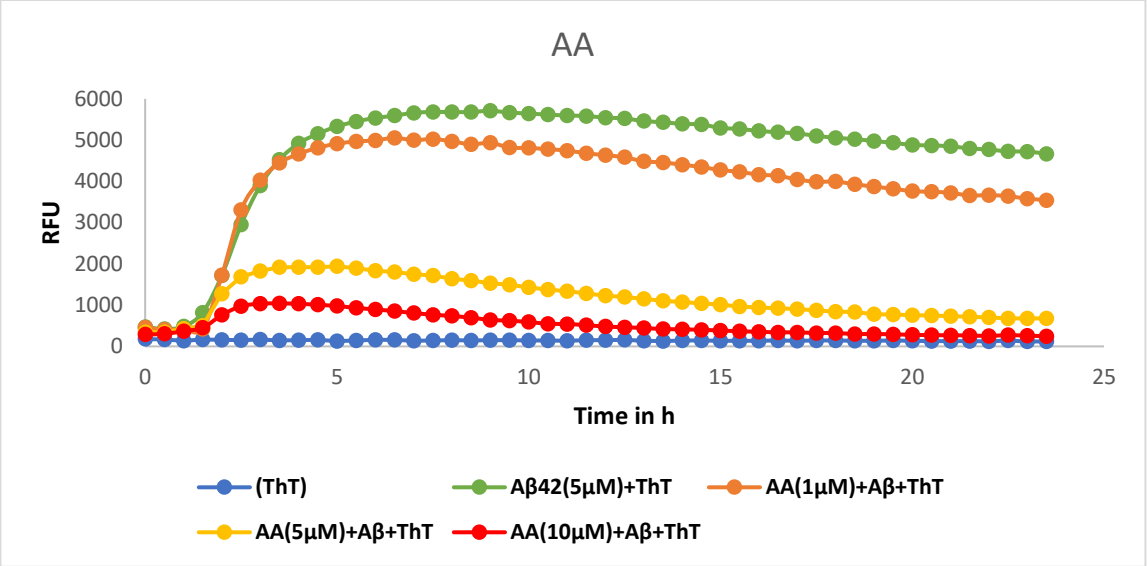


Figure 36. shows ThT-monitored 24h kinetics of Aβ42 (5 μM) in the presence of 1, 5, 10 μM of AA that showed 25%, 86% to 94.5% inhibition respectively at 24 h time point. Aggregation kinetics were monitored by ThT-fluorescence spectroscopy (excitation = 440 nm, emission = 490 nm) at pH 7.4, 37 °C in phosphate buffer.

Compounds	2-AG	AEA	NADA	NOLADIN	OAE	AA
Inhibition percentage (1 μM)	22.8	29	37	19	14.7	25.3
Inhibition percentage (5 μM)	3.5	14.5	8.3	14.6	11	86
Inhibition percentage (10 μM)	12.8	93.3	18.8	72.9	25.1	94.5

Table 5. shows the inhibition percentage of ThT-monitored 24 h aggregation kinetics of Aβ42 (5 μM) in the presence of 1, 5, 10 μM of endocannabinoids at pH 7.4, 37 °C in phosphate buffer at 24 h time point. Aggregation kinetics were monitored by ThT fluorescence spectroscopy (excitation = 440 nm, emission = 490 nm).

4.3 Transmission electron microscopy (TEM) data

The morphology of Aβ42 was investigated in the presence and absence of the Endocannabinoids at their highest concentration (10 µM). Figure 37 is the image of Aβ42 alone at 5 µM. Scale 100 nm.

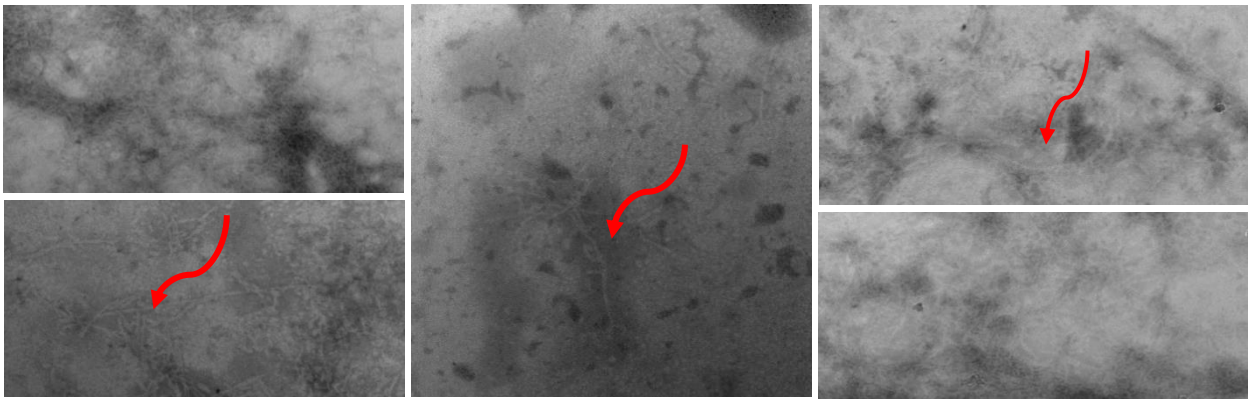


Figure 37. Morphology of aggregate species of Aβ42 (5 µM) alone after 24 h incubation at 37 °C. Scale 100 nm.

The TEM assessment of Aβ42 morphology in the presence of 2-AG at 10 µM did not inhibit aggregation, which correlates with ThT-based inhibition assay result at the same concentration (12.8%) (Figure 38). At the same tested concentrations, AEA was able to reduce the formation of Aβ42 aggregates (Figure 39).

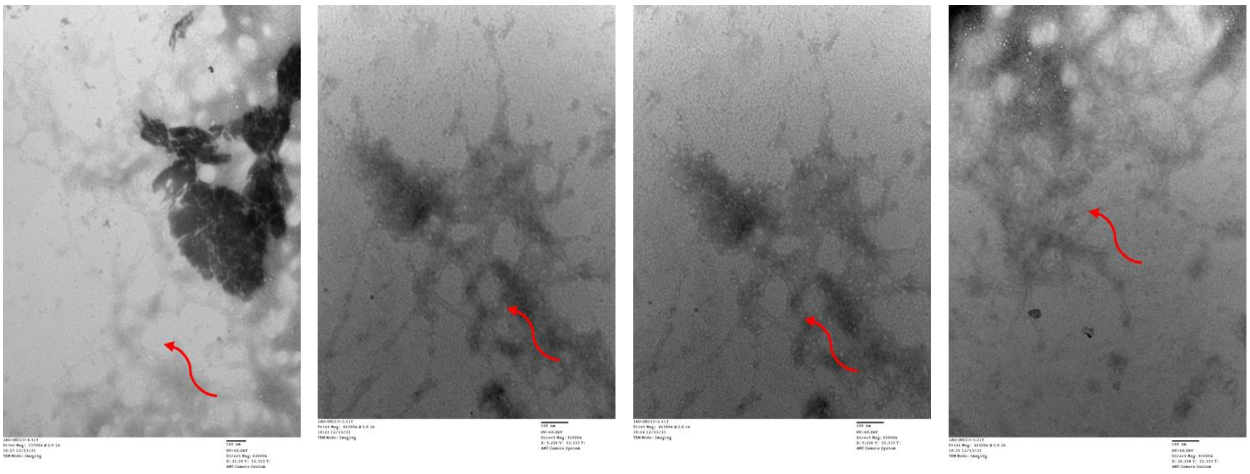


Figure 38. Morphology Aβ42 in the presence of 2AG (10 µM) after 24 h incubation at 37 °C, which did not inhibit aggregation. Scale 100 nm.

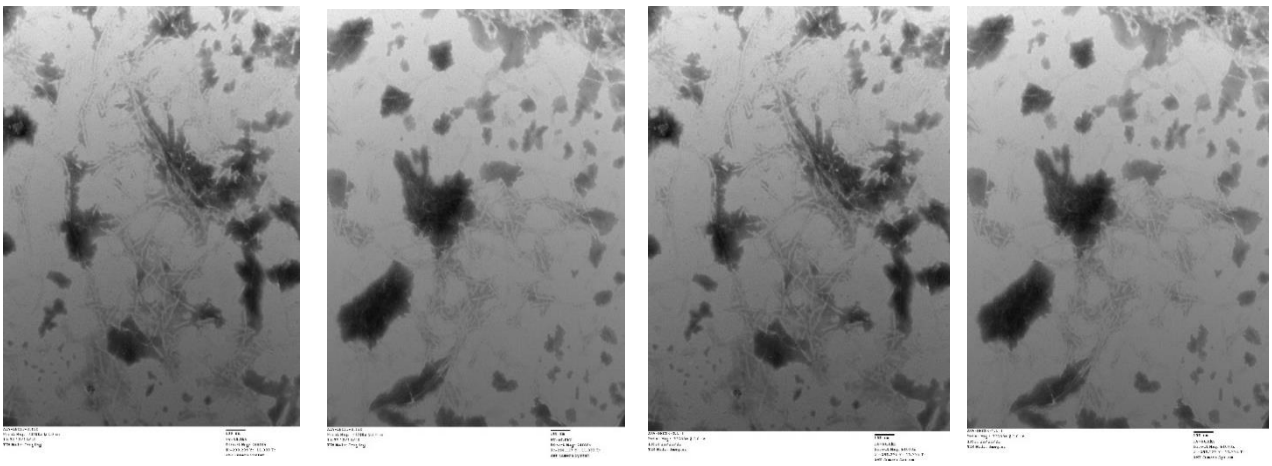


Figure 39. Morphology Aβ42 in the presence of AEA (10 µM) after 24 h incubation at 37 °C, shows inhibition of aggregation. Scale 100 nm.

Moreover, the TEM images of noladin at 10 μM showed the presence of smaller aggregates compared to the Aβ42 control fibril. However, the aggregate size and distribution do not seem similar to the significant fibril formation of the Aβ42 control (Figure 40). The TEM image of OAE (at 10 μM) did not show any significant inhibition of Aβ42 aggregation (Figure 41).

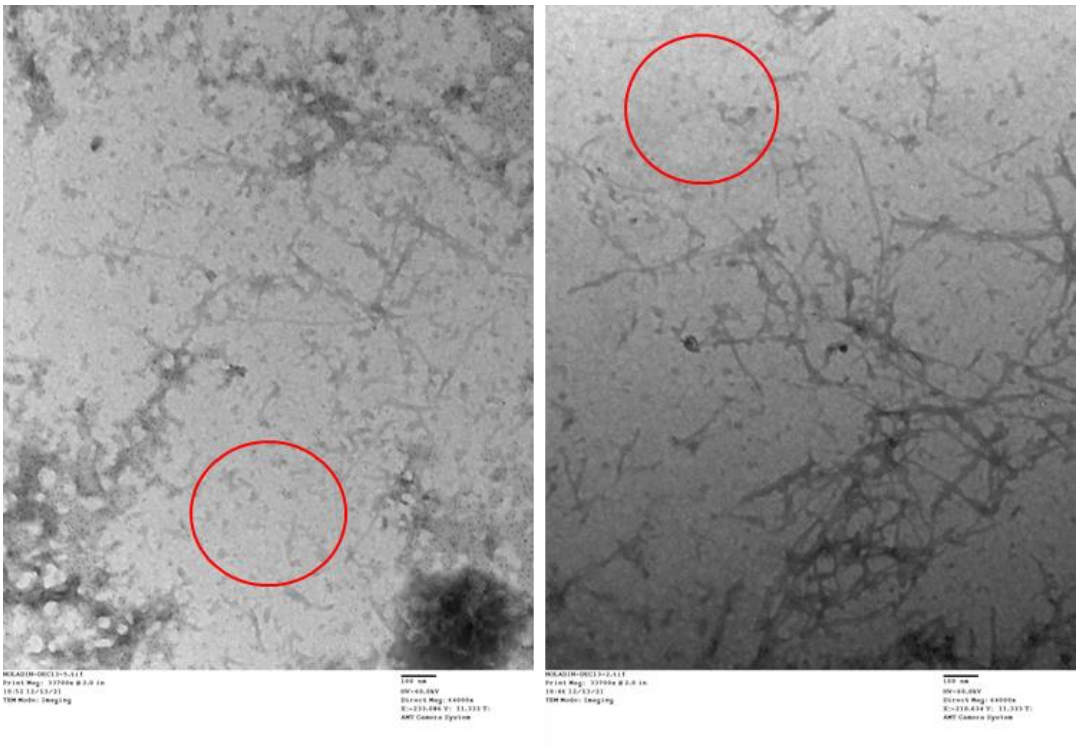


Figure 40. Morphology Aβ42 in the presence of noladin (10 μM) after 24 h incubation at 37 °C. The image does not show similar aggregation morphology compared to Aβ42, which indicates the inhibition of Aβ42 aggregation . Scale 100 nm.

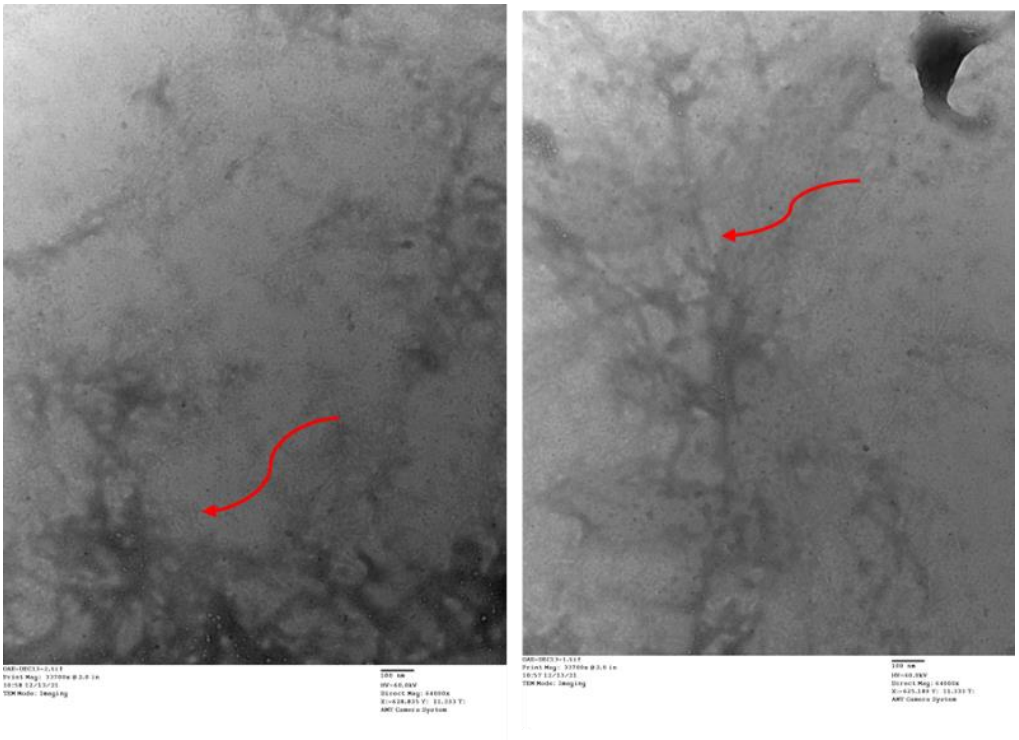


Figure 41. Morphology Aβ42 in the presence of OAE (10 µM) after 24 h incubation at 37 °C. The image showes similar aggregation morphology compared to Aβ42, which indicates that OAE did not inhibit the Aβ aggregation. Scale 100 nm.

Chapter 5: Discussion

This study aimed to explore the endocannabinoids and their mechanism in a comparative manner against Aβ oligomerization and toxicity by *in vitro* studies. The aim was achieved through testing various endocannabinoids and their main metabolite, AA, at various concentrations on HT22 cells to determine their neuroprotective effects, noting that none of the compounds exhibited any direct effects on cell viability at the range of tested concentrations.

To examine the role of the CB1 receptor, we used the CB1 receptor antagonist, AM251, to determine whether it could reverse the neuroprotection induced by endocannabinoids. Our cells studies on HT22 showed that some, but not all of the endocannabinoids, including AEA, noladin, OAE and AA, were able to exhibit neuroprotective effects against Aβ-induced toxicity at 10 µM. However, AM251, a CB1 receptor antagonist, could not reverse this neuroprotection. Since Aβ toxicity is associated with oxidative stress, the beneficial properties of these agents (AEA, noladin, OAE, AA) might result from reducing oxidative damage directly; however, other receptor-mediated and receptor-independent mechanisms should be considered as well [10].

According to previous studies, different toxic mechanisms have been identified as Aβ oligomerization neurotoxicities, such as oxidative and nitrosative stress, NMDA receptor overactivation leading to excitotoxicity, elevated intracellular Ca^{2+} in neuronal cells, protein S-nitrosylation after NOS activation and NO production [89]. A recent study has shown that endocannabinoids, such as AEA, is able to inhibit oxidative stress via receptor-independent pathways [90]. Oxidative stress is responsible for neuronal damage through lipid peroxidation, protein oxidation as well as DNA damage, and inflammation [90]. Direct neuroprotective effects of endocannabinoids may also result from activating anti-apoptotic pathways as a response to

oxidative stress [31, 33]. AEA, noladin, and OAE have been reported to show anti-inflammatory and neuroprotective effects as well as their specific antioxidant capacity that could be involved in the endocannabinoids' neuroprotective properties. Also, it has been demonstrated that the exposure of HT22 hippocampal cells to the Aβ peptide induces apoptotic cell death [90]. Oxidative and nitrosative stress, ROS and RNS production, as well as antioxidant enzyme activities alternation, inflammatory endpoints, and mitochondrial membrane impairment, were all prevented in a differential manner by endocannabinoids [10].

Additional conditions, including neurotransmission signalling disruption and endosomal-lysosomal pathway impairment, play a role in the dysfunctional properties of neurons due to amyloid aggregation [91]. Endocannabinoid ligands may affect various physiological and pathological changes intra/extra-cellularly without binding to a receptor. For instance, AEA directly alters the voltage-gated ion channels or vanilloid receptors at physiologically relevant concentrations [31, 33]. According to previous studies, the protective mechanism of AEA and noladin against Aβ toxicity primarily involves the activation of a MAP kinase cascade as well as induction of survival/proliferative events, antioxidant and anti-inflammatory effects [10, 33]. Additional study on HT22 cells, using endocannabinoids against Aβ42 neurotoxicity, reported the upregulation of PKC expression in HT22 cell membrane, which indicated the PKC role in neuroprotection properties of endocannabinoids CHECK REF [78].

Since AM251, as a CB1 antagonist, could not reverse the effects of any endocannabinoids, which have shown neuroprotective ability against Aβ-induced toxicity, our result suggests that the protective effects of AEA, noladin, OAE, and AA on the HT22 cells might be unrelated to the CB1 receptor pathway and may partially involve previously-mentioned mechanisms. The failure of the CB1 antagonist to reverse the endocannabinoids activity in HT22 cells in our study is

consistent with previous evidence reported on some endocannabinoids, such as AEA activity on neuronal cells occurred independently of the CB1 pathway [90, 92, 93].

In addition to the reported evidence that CB1R-enriched neurons were significantly reduced in areas of microglial activation, the reduction of CB1 receptor expression in human AD may account for our result [69]. Therefore, our results suggest that CB1 receptor-independent effects of endocannabinoids on HT22, including G protein-coupled receptors modulation, transient receptor potential (TRP) receptors regulation, metabolic conversion of anandamide via COX-2 into biologically active component, and lipoxygenase mediators as well as neuroprotective activity at PPARa receptors, highlight the role of endocannabinoids via non-cannabinoid receptor-mediated actions in the present study similar to previously reported evidence [94-96].

Furthermore, animal studies of AD reported by MAGL inhibition (a degrading enzyme of endocannabinoids), the production and accumulation of Aβ significantly suppressed via reducing the expression of amyloid precursor protein cleaving enzyme 1 (BACE1). Through MAGL inhibition, microglia and astrocytes are also inhibited, which prevents neuroinflammation and neurodegeneration. Also, MAGL inhibition leading to BACE1 suppression results in Aβ reduction, enhanced long-term potentiation, synaptic plasticity, spatial learning, and memory by maintaining hippocampal synaptic structure and function [97]. The non-selective CB receptor endocannabinoid agonists such as WIN 55,212-2 and JWH133 diminish neuroinflammation, reduce Aβ levels, and improve cognitive performance by binding competitively to the peripheral anionic site of acetylcholine esterase (AChE) and prevents AChE-induced Aβ-peptide aggregation on the animal model of AD.

Moreover, *in vito* studies on CB1 agonists using different human CNS cell lines, such as MC65 cells and PC12 neuronal cells, showed attenuation of Aβ accumulation that induced

proteotoxicity, which triggers the inflammatory response. These studies indicate that CB receptor agonists were able to decrease reactive oxygen species production, lipid peroxidation, caspase-3-mediated apoptosis, the elevation of the intracellular calcium concentration, and mitochondrial activity through CB1 independent pathways, which highlight the non-CB receptor properties of endocannabinoids [98-100].

In line with these studies, another recent study of lipid rafts has demonstrated that endocannabinoids can affect the stability of several lipid rafts, which may impact intracellular processes. Since endocannabinoids are lipophilic molecules, these non-CB-mediated actions could occur independently of cannabinoid receptors but require membrane cholesterol.

The protection afforded by endocannabinoids seems to be through a ROS-dependent mechanism independently from the cannabinoid receptors CB1 [31, 32, 101]. These findings suggest that signalling pathways other than those associated with CB1 seem to be responsible for the beneficial effects of endocannabinoids, especially in neural cells [102].

Our next step was looking for endocannabinoids' impact on CHO cells expressing the human CB1 receptors, as well as using CB1 antagonist as a means to evaluate the impact of the pharmacological role of the CB1 receptor as an endocannabinoid mechanism to prevent Aβ induced toxicity. AM251 was able to inhibit the protective effects of some, but not all, of the endocannabinoids in CB1-CHO cells. AM251, as a CB1 antagonist, reversed the endocannabinoids' effects, including noladin, OAE, and AA, which increased the cell viability against the Aβ toxicity. This matches several studies that reported CB1 receptor-dependent neuroprotective actions of endocannabinoids in the extraneuronal cells, which may account for the result of our study on CB1-CHO cells [103-105].

Additionally, there is strong evidence that both CB1 and CB2 receptors activated in glia and microglia provide neuroprotection via suppressing elevated ERK/p38MAPK phosphorylation and COX-2 expression induced by Aβ42 [90, 106]. Endocannabinoids such as AEA and noladin (at nanomolar concentrations) on NT-2 cells have been demonstrated the inhibition of Aβ peptide-induced neurotoxicity via CB1 receptor and mitogen-activated protein kinase-dependent mechanisms that reversed by CB1 antagonist [33]. Two MAP kinases, ERK1/2 and p38MAPK, have been found to act as critical mediators of neuronal survival and death [66]. Since the Aβ induces apoptotic cell death via TNFR1 receptor activation, the apoptotic response is triggered by p38MAPK activation and balanced by the cytokine-induced activation of ERK1/2 all through the CB1 receptor activation [66, 107].

Moreover, the activity of CB1R was found to be upregulated in the anterior thalamus in animal studies of AD [108]. In addition to the G-protein coupling and CB1 protein expression, which are significantly reduced in AD patients, it has been found that senile plaques expressing CB1 are associated with microglial activation markers [69]. However, the nitration of CB1 and CB2 proteins was correlated with Aβ42 levels and senile plaque score as molecular markers of AD, along with the enhanced CB2 levels in the AD brain [69, 109].

Studies have demonstrated that the activation of CB1Rs or inhibition of endocannabinoid-degrading enzymes (FAAH, MAGL, and alpha/beta-hydrolase domain-containing) could facilitate Aβ clearance across the blood brain barrier by increasing the expression of the low-density lipoprotein receptor-related protein 1 (LRP1), which function is Aβ transporter from the brain to blood [110].

A previous transitional study showed that CB1 activation leads to PPARc signalling up-regulation that improves neuroinflammation, neurodegeneration, and spatial memory impairment

induced by Aβ peptide [111]. CB1 location within the brain may account for activating the protective signalling pathways of endocannabinoids through CB1, which involves MAP kinase activation that prevents the toxicity and neurodegeneration caused by Aβ [112]. An experimental study using the AD rat model, injected with Aβ to induce neurotoxicity, reported a neuroprotective effect of CB1 activation via voltage-gated Ca^{2+} channel inhibitory effect and suppression of Ca^{2+} activated-K^{+} channel in hippocampus CA1 pyramidal neurons. Based on the reported results, the CB1 protected neurons from electrophysiological changes caused by the Aβ injection. Therefore, the CB1 agonist has exhibited neuroprotection through CB1 activation [65]. In line with the results of this study, similar *in vivo* and *in vitro* studies reported the neuroprotective effects of these lipid mediators such as noladin and OAE via their selectivity at CB1, which result in preventing tau hyperphosphorylation [33].

On the other hand, the results of this study on the endocannabinoids, which did not show a protective effect on the CB1-CHO such as 2-AG, AEA, and NADA, support the reported evidence indicating a lack of direct cannabinoid receptor activity associated with these molecules. No neuroprotection on pretreated cells reported in a similar study using CB1 receptor-selective agonists such as ACEA [90].

In our cell studies on HT22 and CB1-CHO cells, using CB1 antagonist, the CB1 receptor seems to play a key role. The different expression levels of CB1 receptor in these two cell lines, as well as the different sources of these receptor (CHO cells express human CB1, while HT22 express mouse CB1 recptor), could be considered with respect to the different results from these two cell lines. The mechanisms responsible for the endocannabinoids' properties remain unclear; however, they are often considered beneficial [32]. We suggest further investigations on assessing endocannabinoids' role and pathway precisely through the animal models lacking CB receptors as

well as the ligand-binding assays to reveal the potential effect of endocannabinoids dependently or independently of CB1 receptors.

Additionally, as a next step, we investigated potential structural interactions between these endocannabinoids and Aβ aggregation as a potential mechanism of neuroprotection. The results of this study from the ThT fluorescence assay suggest that the structural interaction of endocannabinoids such as AEA, noladin, and AA with Aβ amyloid had an inhibitory effect on Aβ fibril formation. In the previous study, these endocannabinoids also showed direct interaction with the Aβ peptide[33]. However, OAE that showed significant inhibition of Aβ aggregation in cell studies was not a potent inhibitor in the *in vitro* ThT fluorescence assay. The microscopic evidence of amyloid fibril formation of the endocannabinoids was not clear and significant to state the neuroprotective properties of these compounds. It is important to note that this finding highlights potential limitations of ThT assay as a fibril inhibition indicator because they may have interfered with the spectroscopic properties of ThT [113]. However, endocannabinoids have been tested for their competitive interference with ThT binding to fibrils. For instance, polyphenol compounds have quenched the fluorescence of ThT, leading to false positive results for fibril inhibition [90]. Additionally, endocannabinoids as lipophilic groups may interfere with ThT micelle formation in aqueous environments, affecting the dye's affinity for amyloid and its fluorescent properties [114].

While endocannabinoid ligands variably altered the morphology of Aβ fibrils and aggregates, no strong evidence or clear correlation has been demonstrated between effects on Aβ morphology and neuroprotective actions of endocannabinoids, which explains our result in TEM images that did not show significant changes in Aβ morphology [115]. However, it is still not clear whether some of the endocannabinoids, at μmolar concentrations, are able to protect neurons from beta-amyloid-induced neurodegeneration, which is in line with the result of this study on 2-AG and

NADA that failed to increase cell viability in cell studies and did not prevent how β-amyloid aggregation [106].

It should be noted that the interactions and inhibition properties can vary at different test compound concentrations due to changes in the ratios of Aβ vs test compounds. In the cell culture experiments, assessing the CB1 expression before and after each experiment may provide more insights. Using different neural cells overexpressed with CB1 would make a better understanding of these compounds as well. MAGL inhibition as a degrading enzyme of endocannabinoids or using endocannabinoids reuptake inhibitors would also help evaluate the endocannabinoids level and their impact on neuroinflammation-associated Aβ42 accumulation and neurodegeneration.

In summary, the present study provides evidence that some of the endocannabinoids, not all of them, including noladin, OAE and their main metabolite AA, were able to induce protection against Aβ peptide in the cell studies. It should be noted that the CB1 receptor antagonist could not reverse the protection of AEA, noladin, OAE and AA in HT22 cells, which may point to the non-CB receptor-mediated actions of endocannabinoids. Although, the effect of the CB1 receptor antagonist on CB1-CHO cells highlights the involvement of CB1 receptors in the protective properties of endocannabinoids such as noladin and OAE. Also, another possible mechanism for endocannabinoids, such as AEA and NADA, could be through direct interaction with Aβ42 peptides that prevent aggregation and reduce the toxic formation of Aβ oligomers. Further investigation on the structure activity relationship of these molecules, as well as their modulation via CB1 receptor and their activity on non-CB ligand by in vivo and in vitro studies, will reveal the charactrezation of endocannabinoids with the respect to their neuroprotective properties.

Chapter 6: Conclusion and Future Directions

6.1 Conclusion

This thesis has addressed the questions that it sought to explore. This is the first report which investigated the effect of endocannabinoids against the Aβ toxicity. This thesis had three major aims; the foundational goal was to determine the endocannabinoid's effect against Aβ toxicity through *in vitro* study. We achieved this by demonstrating this using cell culture studies. The second aim of this thesis was to explore the mechanism of these molecules using the CB1 receptor over-expressing cell line (CB1-CHO) and their related proteins (CB1 agonist and antagonist). After investigating the endocannabinoids' impact on different cell lines, the next step was to determine whether they interfere with Aβ aggregation.

Overall, it is crucial to control the Aβ neurotoxicity in order to reduce and potentially cure the AD burden. Also, there has been considerable progress in understanding the biological function of the endocannabinoids and other components of the endocannabinoid system in neurodegenerative diseases over the last few years. Moreover, the development of novel therapeutic approaches for neurodegenerative diseases has not only focused on neuroprotective properties but also on alleviating symptoms of such diseases. Therefore, endocannabinoids, through their anti-inflammatory ability and neuroprotective functions, have been recognized as potential therapeutic targets for neurodegenerative disorders.

Since different types of endocannabinoids may be helpful for therapeutic exploitation, several functions are defined by the endocannabinoid system under physiological conditions. Endocannabinoids in the CNS are involved in short-term and long-term synaptic plasticity, including depolarization-induced suppression of excitatory and inhibitory neurotransmission and long-term potentiation and depression [37]. As a result, neuronal circuits in the cortex,

hippocampus, and amygdala contribute to the regulation of cognitive function and emotions, and the reinforcement of substance abuse in the mesolimbic system is also enhanced by the regulation of endocannabinoids ligands [81]. Neuromodulatory actions of endocannabinoids in the sensory and autonomic nervous systems are supported by the beneficial activities of the cannabinoid receptor in neurodegenerative disorders, including multiple sclerosis, Parkinson's disease, and vascular dementia, which have been well documented [33]. As a result of these findings, we can now speculate about additional mechanisms by which endocannabinoid-based strategies may promote Aβ clearance, attenuate synaptic plasticity deficits, and reverse learning and memory deficits in the AD brain.

6.2 Future Directions

Further experimental studies will be necessary to directly address the question of whether endocannabinoids are able to induce neuroprotection effects via CB1 and non-CB1 pathways. Additional *in vitro* experiments should be performed to assess the beneficial effects of neuronal CB1 receptor activation and whether this activation could increase neuronal survival rates and prevent cell toxicity. Moreover, considering the upregulation of CB1 expression in the neuronal and microglial cells through pathological conditions, such as AD, the CB1 receptor could be considered a disease-associated target in AD pathophysiology.

Future directions include further studies to explore the mechanisms by which endocannabinoids induce neuroprotective effects through their SAR and biological activities. The development of more selective compounds targeting cannabinoid receptors to modulate the endocannabinoid system for therapeutic purposes will be promising in the drug discovery pipeline. Although a clear understanding on CB receptors pathways under physiological and pathological

conditions seem necessary to consider the endocannabinoid system for therapeutic approaches.

In light of the latter studies, the results from this thesis suggest further screening on a more extensive set of endocannabinoids and/or modified endocannabinoid compounds as Aβ inhibitors may be required. This thesis sets a benchmark for future *in vitro* and *in vivo* studies of endocannabinoids as amyloid aggregation inhibitors. It should be emphasized that introducing the neuroprotective potential of endocannabinoids as well as determining the optimal therapeutic concentrations for neurodegenerative disorders, such as Alzheimer's disease, should be investigated as the next step in the drug development research.

References

[1] N. Maroof, "'No title," *The role of endocannabinoids in Alzheimer's disease*.

[2] M. Maccarrone, "'CB2 receptors in reproduction," *Br.J.Pharmacol.*, vol. 153, no. 2, pp. 189-198.

[3] J. Noonan, "'No title," *Endocannabinoids and Neuroprotection in an in Vitro Model of Alzheimer's Disease*.

[4] G. Talarico, A. Trebbastoni, G. Bruno and C. de Lena, "'Modulation of the cannabinoid system: a new perspective for the treatment of the Alzheimer's disease," *Current neuropharmacology*, vol. 17, no. 2, pp. 176-183.

[5] D. Rapaka, V.R. Bitra, S.R. Challa and P.C. Adiukwu, "'Potentiation of microglial endocannabinoid signaling alleviates neuroinflammation in Alzheimer's disease," *Neuropeptides*, vol. 90, pp. 102196.

[6] K.L. Wright, M. Duncan and K.A. Sharkey, "'Cannabinoid CB2 receptors in the gastrointestinal tract: a regulatory system in states of inflammation," *Br.J.Pharmacol.*, vol. 153, no. 2, pp. 263-270.

[7] O. Ofek, M. Karsak, N. Leclerc, M. Fogel, B. Frenkel, K. Wright, J. Tam, M. Attar-Namdar, V. Kram and E. Shohami, "'Peripheral cannabinoid receptor, CB2, regulates bone mass," *Proceedings of the National Academy of Sciences*, vol. 103, no. 3, pp. 696-701.

[8] B.K. Atwood and K. Mackie, "'CB2: a cannabinoid receptor with an identity crisis," *Br.J.Pharmacol.*, vol. 160, no. 3, pp. 467-479.

[9] I. Katona and T.F. Freund, "'Multiple functions of endocannabinoid signaling in the brain," *Annu.Rev.Neurosci.*, vol. 35, pp. 529-558.

[10] Z. Elmazoglu, E. Rangel-López, O.N. Medina-Campos, J. Pedraza-Chaverri, I. Túnez, M. Aschner, A. Santamaría and Ç Karasu, "'Cannabinoid-profiled agents improve cell survival via reduction of oxidative stress and inflammation, and Nrf2 activation in a toxic model combining hyperglycemia Aβ1-42 peptide in rat hippocampal neurons," *Neurochem.Int.*, vol. 140, pp. 104817.

[11] M. Oz, "'Receptor-independent actions of cannabinoids on cell membranes: focus on endocannabinoids," *Pharmacology & therapeutics (Oxford)*, vol. 111, no. 1, Jul, pp. 114-144.

[12] P.E. Castillo, T.J. Younts, A.E. Chávez and Y. Hashimotodani, "'Endocannabinoid signaling and synaptic function," *Neuron*, vol. 76, no. 1, pp. 70-81.

[13] M. Kano, T. Ohno-Shosaku, Y. Hashimotodani, M. Uchigashima and M. Watanabe, "'Endocannabinoid-mediated control of synaptic transmission," *Physiol.Rev.*, vol. 89, no. 1, Jan, pp. 309-380.

[14] S. Galiègue, S. Mary, J. Marchand, D. Dussossoy, D. Carrière, P. Carayon, M. Bouaboula, D. Shire, G. LE Fur and P. Casellas, "'Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations," *European journal of biochemistry*, vol. 232, no. 1, pp. 54-61.

[15] M. Herkenham, A.B. Lynn, M.D. Little, M.R. Johnson, L.S. Melvin, B.R. De Costa and K.C. Rice, "'Cannabinoid receptor localization in brain," *Proceedings of the national Academy of sciences*, vol. 87, no. 5, pp. 1932-1936.

[16] A. Gorzkiewicz and J. Szemraj, "'Brain endocannabinoid signaling exhibits remarkable complexity," *Brain Res.Bull.*, vol. 142, pp. 33-46.

[17] J. Noonan, R. Tanveer, A. Klompas, A. Gowran, J. McKiernan and V.A. Campbell, "'Endocannabinoids prevent β-amyloid-mediated lysosomal destabilization in cultured neurons," *J.Biol.Chem.*, vol. 285, no. 49, pp. 38543-38554.

[18] A.C. Howlett and J. Shim, "'Cannabinoid receptors and signal transduction,".

[19] S.M. Augustin and D.M. Lovinger, "'Functional relevance of endocannabinoid-dependent synaptic plasticity in the central nervous system," *ACS chemical neuroscience*, vol. 9, no. 9, pp. 2146-2161.

[20] D.J. Araujo, K. Tjoa and K. Saijo, "'The endocannabinoid system as a window into microglial biology and its relationship to autism," *Frontiers in Cellular Neuroscience*, pp. 424.

[21] V.L. Alves, J.L. Gonçalves, J. Aguiar, H.M. Teixeira and J.S. Câmara, "'The synthetic cannabinoids phenomenon: from structure to toxicological properties. A review," *Crit.Rev.Toxicol.*, vol. 50, no. 5, pp. 359-382.

[22] Y. Liu, H. Liu, S. Li, W. Ma, D. Wu, H. Li, A. Xiao, L. Liu, F. Zhu and R. Gan, "'Cannabis sativa bioactive compounds and their extraction, separation, purification, and identification technologies: An updated review," *TrAC Trends in Analytical Chemistry*, vol. 149, pp. 116554.

[23] D. McGolrick and N. Frey, "'Nabilone for Chronic Pain Management: A Review of Clinical Effectiveness and Guidelines–An Update,".

[24] F. Fezza, M. Bari, R. Florio, E. Talamonti, M. Feole and M. Maccarrone, "'Endocannabinoids, related compounds and their metabolic routes," *Molecules*, vol. 19, no. 11, pp. 17078-17106.

[25] N. Maroof, M.C. Pardon and D.A. Kendall, "'Endocannabinoid signalling in Alzheimer's disease," *Biochem.Soc.Trans.*, vol. 41, no. 6, pp. 1583-1587.

[26] G. Morris, K. Walder, S. Kloiber, P. Amminger, M. Berk, C.C. Bortolasci, M. Maes, B.K. Puri and A.F. Carvalho, "'The Endocannabinoidome in Neuropsychiatry: Opportunities and Potential Risks," *Pharmacological Research*, pp. 105729.

[27] S.I. Farah, S. Hilston, N. Tran, N. Zvonok and A. Makriyannis, "'1-, 2-and 3-AG as substrates of the endocannabinoid enzymes and endogenous ligands of the cannabinoid receptor 1," *Biochem.Biophys.Res.Commun.*, vol. 591, pp. 31-36.

[28] E. Dainese, S. Oddi and M. Maccarrone, "'Interaction of endocannabinoid receptors with biological membranes," *Curr.Med.Chem.*, vol. 17, no. 14, pp. 1487-1499.

[29] S.S. Hu and K. Mackie, "'Distribution of the endocannabinoid system in the central nervous system," *Endocannabinoids*, pp. 59-93.

[30] V.D. Marzo, "'Endocannabinoids: synthesis and degradation," *Reviews of Physiology Biochemistry and Pharmacology*, pp. 1-24.

[31] A. Wojtalla, F. Herweck, M. Granzow, S. Klein, J. Trebicka, S. Huss, R. Lerner, B. Lutz, F.A. Schildberg and P.A. Knolle, "'The endocannabinoid N-arachidonoyl dopamine (NADA) selectively induces oxidative stress-mediated cell death in hepatic stellate cells but not in hepatocytes," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 302, no. 8, pp. G873-G887.

[32] K.L. Mustonen, "'No title," *Endocannabinoid System in the Planarian Model*.

[33] N.G. Milton, "'Anandamide and noladin ether prevent neurotoxicity of the human amyloid-β peptide," *Neurosci.Lett.*, vol. 332, no. 2, pp. 127-130.

[34] P. Lunardi, L.W. de Souza, B. dos Santos, B. Popik and L. de Oliveira Alvares, "'Effect of the Endocannabinoid System in Memory Updating and Forgetting," *Neuroscience*, vol. 444, pp. 33-42.

[35] D. Kumar, A. Sharma, R. Taliyan, M.T. Urmera, O. Herrera-Calderon, T. Heinbockel, S. Rahman and R. Goyal, "'Orchestration of the circadian clock and its association with Alzheimer's disease: Role of endocannabinoid signaling," *Ageing Research Reviews*, vol. 73, pp. 101533.

[36] D. Piomelli, "'The molecular logic of endocannabinoid signalling," *Nature Reviews Neuroscience*, vol. 4, no. 11, pp. 873-884.

[37] C. Grimaldi and M. Bifulco, "'Anandamide, an Endogenous Ligand of Cannabinoid Receptors, Inhibits Human Breast Cancer Cell Proliferation Through a Lipid Rafts Mediated Mechanism," *Pharmacologyonline*, vol. 1, pp. 1-45.

[38] T. Bisogno, A. Ligresti and V. Di Marzo, "'The endocannabinoid signalling system: biochemical aspects," *Pharmacology Biochemistry and Behavior*, vol. 81, no. 2, pp. 224-238.

[39] D.K. Giang and B.F. Cravatt, "'Molecular characterization of human and mouse fatty acid amide hydrolases," *Proceedings of the National Academy of Sciences*, vol. 94, no. 6, pp. 2238-2242.

[40] W. Wuli, S. Tsai, T. Chiou and H. Harn, "'Human-Induced Pluripotent Stem Cells and Herbal Small-Molecule Drugs for Treatment of Alzheimer's Disease," *International journal of molecular sciences*, vol. 21, no. 4, pp. 1327.

[41] A. El Shatshat, A.T. Pham and P.P. Rao, "'Interactions of polyunsaturated fatty acids with amyloid peptides Aβ40 and Aβ42," *Arch.Biochem.Biophys.*, vol. 663, pp. 34-43.

[42] Z. Arvanitakis, R.C. Shah and D.A. Bennett, "'Diagnosis and management of dementia," *JAMA*, vol. 322, no. 16, pp. 1589-1599.

[43] M.G. Galety and S. Gupta, "'Artificial Intelligence in the Detection of Alzheimer's Disease,", pp. 136-155.

[44] Alzheimer's Association, "'2018 Alzheimer's disease facts and figures," *Alzheimer's & Dementia*, vol. 14, no. 3, pp. 367-429.

[45] J.Y. Ho and Y. Franco, "'The rising burden of Alzheimer's disease mortality in rural America," *SSM-Population Health*, pp. 101052.

[46] C.D. Keene, T. Montine and L. Kuller, "'Epidemiology, pathology, and pathogenesis of Alzheimer disease,", vol. 2020, no. Nov, Jan 19,.

[47] Z. Breijyeh and R. Karaman, "'Comprehensive review on Alzheimer's disease: Causes and treatment," *Molecules*, vol. 25, no. 24, pp. 5789.

[48] Y. Rolland, G.A. van Kan and B. Vellas, "'Physical activity and Alzheimer's disease: from prevention to therapeutic perspectives," *Journal of the American Medical Directors Association*, vol. 9, no. 6, pp. 390-405.

[49] M.T. Kabir, M. Uddin, M. Begum, S. Thangapandiyan, M. Rahman, L. Aleya, B. Mathew, M. Ahmed, G.E. Barreto and G.M. Ashraf, "'Cholinesterase inhibitors for Alzheimer's disease: multitargeting strategy based on anti-Alzheimer's drugs repositioning," *Curr.Pharm.Des.*, vol. 25, no. 33, pp. 3519-3535.

[50] D. Press and S.S. Buss, "'Treatment of Alzheimer disease,".

[51] US Food and Drug Administration, "'FDA Grants Accelerated Approval for Alzheimer's Drug,", Jun 07,.

[52] G.D. Rabinovici, "'Controversy and progress in Alzheimer's disease—FDA approval of aducanumab," *N.Engl.J.Med.*, vol. 385, no. 9, pp. 771-774.

[53] R.A. Salinas, "'Aducanumab for Alzheimer's disease: expediting approval and delaying science," *BMJ Evidence-Based Medicine*, vol. 26, no. 5, pp. 214-215.

[54] H.E. Marei, A. Althani, J. Suhonen, M.E. El and T. Caceci, "'Recent perspective about the amyloid cascade hypothesis and stem cell-based therapy in the treatment of alzheimer's disease," *Frontiers in Clinical Drug Research-Alzheimer Disorders.BENTHAM SCIENCE PUBLISHERS*.

[55] S. Gandy and S.T. DeKosky, "'Toward the treatment and prevention of Alzheimer's disease: rational strategies and recent progress," *Annu.Rev.Med.*, vol. 64, pp. 367-383.

[56] N. Strohminger and S. Nichols, "'Neurodegeneration and identity," *Psychological Science*, vol. 26, no. 9, pp. 1469-1479.

[57] M. Robinson, J. Lou, B. Mehrazma, A. Rauk, M. Beazely and Z. Leonenko, "'Pseudopeptide Amyloid Aggregation Inhibitors: In Silico, Single Molecule and Cell Viability Studies," *International Journal of Molecular Sciences*, vol. 22, no. 3, pp. 1051.

[58] R. Sangubotla and J. Kim, "'Recent trends in analytical approaches for detecting neurotransmitters in Alzheimer's disease," *TrAC Trends in Analytical Chemistry*, vol. 105, pp. 240-250.

[59] L. Zhang, Z. Zhou, Z. Wang, Y. Du, Z. He, C. Cao and S. Zhou, "'Coffee and caffeine potentiate the antiamyloidogenic activity of melatonin via inhibition of Aβ oligomerization and modulation of the Tau-mediated pathway in N2a/APP cells," *Drug design, development and therapy*, vol. 9, pp. 241.

[60] B.R. Troutwine, L. Hamid, C.R. Lysaker, T.A. Strope and H.M. Wilkins, "'Apolipoprotein E and Alzheimer's disease," *Acta Pharmaceutica Sinica B*.

[61] E. Drolle, F. Hane, B. Lee and Z. Leonenko, "'Atomic force microscopy to study molecular mechanisms of amyloid fibril formation and toxicity in Alzheimer's disease," *Drug Metab.Rev.*, vol. 46, no. 2, pp. 207-223.

[62] B. Lakshmi, M. Sudhakar and K.S. Prakash, "'Protective effect of selenium against aluminum chloride-induced Alzheimer's disease: behavioral and biochemical alterations in rats," *Biol.Trace Elem.Res.*, vol. 165, no. 1, pp. 67-74.

[63] X. Sun, W. Chen and Y. Wang, "'β-Amyloid: the key peptide in the pathogenesis of Alzheimer's disease," *Frontiers in pharmacology*, vol. 6, pp. 221.

[64] B.S. Basavarajappa, M. Shivakumar, V. Joshi and S. Subbanna, "'Endocannabinoid system in neurodegenerative disorders," *J.Neurochem.*, vol. 142, no. 5, pp. 624-648.

[65] R. Cooray, V. Gupta and C. Suphioglu, "'Current aspects of the endocannabinoid system and targeted THC and CBD phytocannabinoids as potential therapeutics for Parkinson's and Alzheimer's diseases: a review," *Mol.Neurobiol.*, vol. 57, no. 11, pp. 4878-4890.

[66] M.C. Olianas, S. Dedoni and P. Onali, "'Cannabinoid CB1 and CB2 receptors differentially regulate TNF-α-induced apoptosis and LPA1-mediated pro-survival signaling in HT22 hippocampal cells," *Life Sci.*, vol. 276, pp. 119407.

[67] M. Wu, J. Jia, C. Lei, L. Ji, X. Chen, H. Sang and L. Xiong, "'Cannabinoid Receptor CB1 Is Involved in Nicotine-Induced Protection Against Aβ1–42 Neurotoxicity in HT22 Cells," *Journal of Molecular Neuroscience*, vol. 55, no. 3, pp. 778-787.

[68] H. Matsushima, S. Shimohama, M. Chachin, T. Taniguchi and J. Kimura, "'Ca2 -Dependent and Ca2 -Independent Protein Kinase C Changes in the Brains of Patients with Alzheimer's Disease," *J.Neurochem.*, vol. 67, no. 1, pp. 317-323.

[69] B.G. Ramírez, C. Blázquez, T.G. del Pulgar, M. Guzmán and M.L. de Ceballos, "'Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation," *Journal of Neuroscience*, vol. 25, no. 8, pp. 1904-1913.

[70] R. Rozenfeld and L.A. Devi, "'Regulation of CB1 cannabinoid receptor trafficking by the adaptor protein AP-3," *The FASEB Journal*, vol. 22, no. 7, pp. 2311-2322.

[71] V. Chiurchiù, L. Scipioni, B. Arosio, D. Mari, S. Oddi and M. Maccarrone, "'Anti-inflammatory effects of fatty acid amide hydrolase inhibition in monocytes/macrophages from alzheimer's disease patients," *Biomolecules*, vol. 11, no. 4, pp. 502.

[72] P. Zogopoulos, I. Vasileiou, E. Patsouris and S. Theocharis, "'The neuroprotective role of endocannabinoids against chemical-induced injury and other adverse effects," *Journal of Applied Toxicology*, vol. 33, no. 4, pp. 246-264.

[73] C.A. Gallelli, S. Calcagnini, A. Romano, J.B. Koczwara, M. De Ceglia, D. Dante, R. Villani, A.M. Giudetti, T. Cassano and S. Gaetani, "'Modulation of the oxidative stress and lipid peroxidation by endocannabinoids and their lipid analogues," *Antioxidants*, vol. 7, no. 7, pp. 93.

[74] R. Mechoulam and E. Shohami, "'Endocannabinoids and traumatic brain injury," *Mol.Neurobiol.*, vol. 36, no. 1, pp. 68-74.

[75] R. Gajardo-Gómez, V.C. Labra, C.J. Maturana, K.F. Shoji, C.A. Santibañez, J.C. Sáez, C. Giaume and J.A. Orellana, "'Cannabinoids prevent the amyloid β-induced activation of astroglial hemichannels: A neuroprotective mechanism," *Glia*, vol. 65, no. 1, pp. 122-137.

[76] M. Van der Stelt, C. Mazzola, G. Esposito, I. Matias, S. Petrosino, D.D. Filippis, V. Micale, L. Steardo, F. Drago and T. Iuvone, "'Endocannabinoids and β-amyloid-induced neurotoxicity in vivo: effect of pharmacological elevation of endocannabinoid levels," *Cellular and Molecular Life Sciences CMLS*, vol. 63, no. 12, pp. 1410-1424.

[77] R. Tanveer, A. Gowran, J. Noonan, S.E. Keating, A.G. Bowie and V.A. Campbell, "'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.*, vol. 287, no. 41, pp. 34709-34721.

[78] A. Stumpf, "'Cannabinoid type 2 receptor-mediated cell type-specific self-inhibition in hippocampal and cortical neurons,", 2019.

[79] M. Maroso, G.G. Szabo, H.K. Kim, A. Alexander, A.D. Bui, S. Lee, B. Lutz and I. Soltesz, "'Cannabinoid control of learning and memory through HCN channels," *Neuron*, vol. 89, no. 5, pp. 1059-1073.

[80] V. Micale, C. Mazzola and F. Drago, "'Endocannabinoids and neurodegenerative diseases," *Pharmacological Research*, vol. 56, no. 5, pp. 382-392.

[81] T. Bisogno and V. Di Marzo, "'The role of the endocannabinoid system in Alzheimer's disease: facts and hypotheses," *Curr.Pharm.Des.*, vol. 14, no. 23, pp. 2299-2305.

[82] C. Benito, E. Núñez, R.M. Tolón, E.J. Carrier, A. Rábano, C.J. Hillard and J. Romero, "'Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains," *Journal of Neuroscience*, vol. 23, no. 35, pp. 11136-11141.

[83] S.L. Palmer, A.D. Khanolkar and A. Makriyannis, "'Natural and synthetic endocannabinoids and their structure-activity relationships," *Curr.Pharm.Des.*, vol. 6, no. 13, pp. 1381-1397.

[84] W.B. Stine, L. Jungbauer, C. Yu and M.J. LaDu, "'Preparing synthetic Aβ in different aggregation states,", pp. 13-32.

[85] G. Tin, T. Mohamed, A. Shakeri, A.T. Pham and P.P. Rao, "'Interactions of selective serotonin reuptake inhibitors with β-amyloid," *ACS chemical neuroscience*, vol. 10, no. 1, pp. 226-234.

[86] A. Shakeri, "'No title," *Design, Synthesis and Biological Evaluation of Novel Adamantane Derivatives as Potential Treatments for Alzheimer's Disease*.

[87] E. Stroo, M. Koopman, E.A. Nollen and A. Mata-Cabana, "'Cellular regulation of amyloid formation in aging and disease," *Frontiers in neuroscience*, vol. 11, pp. 64.

[88] R. Cannarsa, D. Carretta, F. Lattanzio, S. Candeletti and P. Romualdi, "'Δ 9-Tetrahydrocannabinol Decreases NOP Receptor Density and mRNA Levels in Human SH-SY5Y Cells," *Journal of Molecular Neuroscience*, vol. 46, no. 2, pp. 285-292.

[89] M.W. Akhtar, S. Sanz-Blasco, N. Dolatabadi, J. Parker, K. Chon, M.S. Lee, W. Soussou, S.R. McKercher, R. Ambasudhan and T. Nakamura, "'Elevated glucose and oligomeric β-amyloid disrupt synapses via a common pathway of aberrant protein S-nitrosylation," *Nature communications*, vol. 7, no. 1, pp. 1-11.

[90] B.S. Harvey, K.S. Ohlsson, J.L. Mååg, I.F. Musgrave and S.D. Smid, '"Contrasting protective effects of cannabinoids against oxidative stress and amyloid-β evoked neurotoxicity in vitro," *Neurotoxicology,* vol. 33, no. 1, pp. 138-146.

[91] K.E. Marshall, D.M. Vadukul, K. Staras and L.C. Serpell, '"Misfolded amyloid-β-42 impairs the endosomal–lysosomal pathway," *Cellular and molecular life sciences,* vol. 77, no. 23, pp. 5031-5043.

[92] K.P. Sarker and I. Maruyama, '"Anandamide induces cell death independently of cannabinoid receptors or vanilloid receptor 1: possible involvement of lipid rafts," *Cellular and Molecular Life Sciences CMLS,* vol. 60, no. 6, pp. 1200-1208.

[93] M. Maccarrone, T. Lorenzon, M. Bari, G. Melino and A. Finazzi-Agro, '"Anandamide induces apoptosis in human cells via vanilloid receptors: evidence for a protective role of cannabinoid receptors," *J.Biol.Chem.*, vol. 275, no. 41, pp. 31938-31945.

[94] Y. Sun and A. Bennett, '"Cannabinoids: a new group of agonists of PPARs," *PPAR research,* vol. 2007.

[95] C.J. Fowler, '"The contribution of cyclooxygenase-2 to endocannabinoid metabolism and action," *Br.J.Pharmacol.*, vol. 152, no. 5, pp. 594-601.

[96] L. De Petrocellis and V. Di Marzo, '"Non-CB1, non-CB2 receptors for endocannabinoids, plant cannabinoids, and synthetic cannabimimetics: focus on G-protein-coupled receptors and transient receptor potential channels," *Journal of Neuroimmune Pharmacology,* vol. 5, no. 1, pp. 103-121.

[97] R. Chen, J. Zhang, Y. Wu, D. Wang, G. Feng, Y. Tang, Z. Teng and C. Chen, '"Monoacylglycerol lipase is a therapeutic target for Alzheimer's disease," *Cell reports,* vol. 2, no. 5, pp. 1329-1339.

[98] A.M. Martín-Moreno, B. Brera, C. Spuch, E. Carro, L. García-García, M. Delgado, M.A. Pozo, N.G. Innamorato, A. Cuadrado and M.L. de Ceballos, '"Prolonged oral cannabinoid administration prevents neuroinflammation, lowers β-amyloid levels and improves cognitive performance in Tg APP 2576 mice," *Journal of neuroinflammation,* vol. 9, no. 1, pp. 1-15.

[99] A. Currais, O. Quehenberger, A. M Armando, D. Daugherty, P. Maher and D. Schubert, '"Amyloid proteotoxicity initiates an inflammatory response blocked by cannabinoids," *NPJ aging and mechanisms of disease,* vol. 2, no. 1, pp. 1-8.

[100] L.M. Eubanks, C.J. Rogers, A.E. Beuscher IV, G.F. Koob, A.J. Olson, T.J. Dickerson and K.D. Janda, '"A molecular link between the active component of marijuana and Alzheimer's disease pathology," *Molecular pharmaceutics,* vol. 3, no. 6, pp. 773-777.

[101] M.R. Elphick and M. Egertova, "'The neurobiology and evolution of cannabinoid signalling," *Philosophical Transactions of the Royal Society of London.Series B: Biological Sciences*, vol. 356, no. 1407, pp. 381-408.

[102] T. Iuvone, G. Esposito, R. Esposito, R. Santamaria, M. Di Rosa and A.A. Izzo, "'Neuroprotective effect of cannabidiol, a non-psychoactive component from Cannabis sativa, on β-amyloid-induced toxicity in PC12 cells," *J.Neurochem.*, vol. 89, no. 1, pp. 134-141.

[103] F. Facchinetti, E. Del Giudice, S. Furegato, M. Passarotto and A. Leon, "'Cannabinoids ablate release of TNFα in rat microglial cells stimulated with lypopolysaccharide," *Glia*, vol. 41, no. 2, pp. 161-168.

[104] E.J. Carrier, C.S. Kearn, A.J. Barkmeier, N.M. Breese, W. Yang, K. Nithipatikom, S.L. Pfister, W.B. Campbell and C.J. Hillard, "'Cultured rat microglial cells synthesize the endocannabinoid 2-arachidonylglycerol, which increases proliferation via a CB2 receptor-dependent mechanism," *Mol.Pharmacol.*, vol. 65, no. 4, pp. 999-1007.

[105] E. Eljaschewitsch, A. Witting, C. Mawrin, T. Lee, P.M. Schmidt, S. Wolf, H. Hoertnagl, C.S. Raine, R. Schneider-Stock and R. Nitsch, "'The endocannabinoid anandamide protects neurons during CNS inflammation by induction of MKP-1 in microglial cells," *Neuron*, vol. 49, no. 1, pp. 67-79.

[106] X. Chen, J. Zhang and C. Chen, "'Endocannabinoid 2-arachidonoylglycerol protects neurons against β-amyloid insults," *Neuroscience*, vol. 178, pp. 159-168.

[107] M. Obulesu and M.J. Lakshmi, "'Apoptosis in Alzheimer's disease: an understanding of the physiology, pathology and therapeutic avenues," *Neurochem.Res.*, vol. 39, no. 12, pp. 2301-2312.

[108] I. Manuel, L. Lombardero, F.M. LaFerla, L. Giménez-Llort and R. Rodríguez-Puertas, "'Activity of muscarinic, galanin and cannabinoid receptors in the prodromal and advanced stages in the triple transgenic mice model of Alzheimer's disease," *Neuroscience*, vol. 329, pp. 284-293.

[109] M. Solas, P.T. Francis, R. Franco and M.J. Ramirez, "'CB2 receptor and amyloid pathology in frontal cortex of Alzheimer's disease patients," *Neurobiol.Aging*, vol. 34, no. 3, pp. 805-808.

[110] M. Shibata, S. Yamada, S.R. Kumar, M. Calero, J. Bading, B. Frangione, D.M. Holtzman, C.A. Miller, D.K. Strickland and J. Ghiso, "'Clearance of Alzheimer's amyloid-β 1-40 peptide from brain by LDL receptor–related protein-1 at the blood-brain barrier," *J.Clin.Invest.*, vol. 106, no. 12, pp. 1489-1499.

[111] G. Fakhfouri, R. Rahimian, J. Ghia, W.I. Khan and A.R. Dehpour, "'Impact of 5-HT3 receptor antagonists on peripheral and central diseases," *Drug Discov.Today*, vol. 17, no. 13-14, pp. 741-747.

[112] M.J. McFarland, A.C. Porter, F.R. Rakhshan, D.S. Rawat, R.A. Gibbs and E.L. Barker, "'A role for caveolae/lipid rafts in the uptake and recycling of the endogenous cannabinoid anandamide," *J.Biol.Chem.*, vol. 279, no. 40, pp. 41991-41997.

[113] S.A. Hudson, H. Ecroyd, T.W. Kee and J.A. Carver, "'The thioflavin T fluorescence assay for amyloid fibril detection can be biased by the presence of exogenous compounds," *The FEBS journal*, vol. 276, no. 20, pp. 5960-5972.

[114] R. Khurana, C. Coleman, C. Ionescu-Zanetti, S.A. Carter, V. Krishna, R.K. Grover, R. Roy and S. Singh, "'Mechanism of thioflavin T binding to amyloid fibrils," *J.Struct.Biol.*, vol. 151, no. 3, pp. 229-238.

[115] E. Janefjord, J.L. Mååg, B.S. Harvey and S.D. Smid, "'Cannabinoid effects on β amyloid fibril and aggregate formation, neuronal and microglial-activated neurotoxicity in vitro," *Cell.Mol.Neurobiol.*, vol. 34, no. 1, pp. 31-42.