



UNIVERSITY OF MESSINA

DEPARTMENT OF BIOMEDICAL AND DENTAL SCIENCES
AND OF MORPHOLOGICAL AND FUNCTIONAL IMAGES

38th cycle International PhD: Translational and Molecular Medicine and Surgery
S.S.D. BIO/14

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MODULATION OF AUTOPHAGY AND APOPTOSIS PROCESSES IN EXPERIMENTAL MODELS OF NEUROINFLAMMATION

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Academic Year 2025/2026

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INTRODUCTION

Neuroinflammation: Molecular Mechanisms and Implications in Neurodegenerative Diseases

Neuroinflammation is a complex response to injury, trauma, or infection. It's a key part of the brain's immune system, which is biochemically linked to the rest of the body's immune system (Bhardwaj et al., 2025). This inflammatory process can be a double-edged sword; it represents a protective mechanism that helps repair damaged tissue and disrupt pathogens (Bhatia et al., 2022). However, if neuroinflammation becomes chronic or excessive, it can be destructive (Thakur et al., 2023). The over-activation of inflammatory molecules can lead to significant damage to neurons and other brain cells. This prolonged inflammatory state is implicated in various neurodegenerative diseases (Dhapola et al., 2021).

Neuroinflammation causes neurodegeneration, characterised by the loss of physiological neuronal structure and function (Piancone et al., 2021), or neuron death through various mechanisms, including the release of cytokines by activated microglia, reactive astrocytes, and infiltrating peripheral immune cells (Takeuchi et al., 2010).

Neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS), are characterised by a progressive loss of neurons, accompanied by a gradual decline in cognitive and motor functions (Rana et al., 2018). Among these, Alzheimer's is the most common form of dementia, characterised by three main aspects: the extracellular deposition of senile plaques, consisting of β -amyloid ($A\beta$) protein aggregates, the intraneuronal accumulation of hyperphosphorylated and misfolded tau protein, which gives rise to neurofibrillary tangles, and, finally, progressive neuronal death (Bhardwaj et al., 2025). These pathological events not only compromise brain function, but also activate the innate immune response, which, while initially playing a protective role, can also promote the onset of chronic neuroinflammation, thus contributing to the acceleration of neurodegenerative processes (Rana & Musto, 2018).

These events trigger an innate immune response, which, although initially protective, can contribute to the onset of chronic neuroinflammation and accelerate neurodegeneration (Pascual et al., 2021). Although it may represent an attempt by the body to counter threats

and repair injuries, dysregulation of this process can lead to the propagation of neuropathology. It is a feature present in most neurological disorders, where it often contributes to the worsening of neurodegeneration (Heneka et al., 2013).

Brain damage and the accumulation of toxic proteins are recognised as danger signals by the immune system. In the central nervous system (CNS), the main actors in this response are microglia, astrocytes and oligodendrocytes, but neurons themselves can also participate (Xie et al., 2020). Immune activation leads to the release of pro-inflammatory cytokines and chemokines, which, if uncontrolled, fuel a persistent inflammatory state with neurotoxic effects (Kaushal et al., 2015) (Aguzzi et al., 2013).

Microglia constantly monitor the local microenvironment and frequently respond to pathogens or particles with directed processes or via migration to the corresponding site (Wake et al., 2009).

They are implicated in the elimination of apoptotic cells and in the establishment of functional synapses. (Kumar, 2019). Together with microglia, neuroinflammation involves the activation of astrocytes, which are specialised cells of the CNS. Astrocytes can acquire a neurotoxic phenotype, including in response to cytokines secreted by microglia. Leukocytes, including T lymphocytes, monocyte-derived macrophages (MdMs), and B lymphocytes, can infiltrate the CNS, further fuelling neuroinflammation (Pascual et al., 2021).

A significant pathway leading to the development of inflammatory diseases involves the activation of the inflammasome: a multi-protein complex that intensifies inflammatory responses to both pathogenic and endogenous activators (Guo et al., 2015).

The Interplay Between PAMPs, DAMPs, and Toll-Like Receptors

Toll-like receptors (TLRs) are a class of pattern-recognition receptors (PRRs) that play a central role in the activation of the immune system response.

TLRs were the first to be identified and are among the best understood mechanisms of innate immune defence. These receptors are expressed in immune cells (such as macrophages, B lymphocytes, and neutrophils), in non-immune cells (endothelial and epithelial), and, in the CNS, mainly in glial cells, but also in neurons (Piancone et al., 2021).

When TLRs are activated, they trigger a cascade of intracellular signals that aim to protect the body and repair damaged tissues. However, their hyperactivation can alter the immune balance, promoting a state of chronic inflammation that contributes to the progression of neurodegenerative diseases (McKernan, 2020). Furthermore, under pathological conditions, TLRs can even involve the peripheral immune system, aggravating neuronal damage (Takeuchi & Akira, 2010). TLR activation occurs through the recognition of two main categories of signals, PAMPs (Pathogen-Associated Molecular Patterns) and DAMPs (Damage-Associated Molecular Patterns), acting as molecular sensors, they trigger a complex net of molecular pathways (Kawasaki T. & Kawai, T, 2014)

PAMPs are molecules characteristic of pathogenic microorganisms, such as bacterial lipopolysaccharides or viral RNA (Su et al., 2016). DAMPs are endogenous signals released by damaged or stressed cells, such as misfolded proteins like A β and tau protein, extracellular ATP, and DNA fragments (Landreth & Reed-Geaghan, 2009).

In neurodegenerative disorders, DAMPs resulting from neuronal damage and the accumulation of toxic proteins activate TLRs, thereby contributing to the amplification of the inflammatory process and the progression of neuronal degeneration (Kinra et al., 2022). Microglia are often the first to respond, recognising DAMPs and PAMPs (Pascual et al., 2021), in the neurobiological model, microglia are considered the macrophages of the CNS, functioning as sentinel innate immune cells (Kumar, 2019).

Cellular Mechanisms Underlying NLRP3 Inflammasome Activation

PRRs can be classified into four different families: TLRs, C-type lectin receptors (CLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Heneka MT, 2013). The most extensively studied of these sensors is the NOD-like receptor of the pyrin family containing domain 3 (NLRP3) (Sharma & Kanneganti, 2016).

Research has focused on elucidating the mechanism of NLRP3 inflammasome activation and its regulation, since there are a number of sensors capable of inducing its formation (Hafner-Bratkovič & Pelegrín, 2018). The process is initiated by a priming signal induced by the stimulation of pattern-recognition receptors (PRRs). The PRRs then activate nuclear factor κ B (NF- κ B), which transcribes NLRP3 and IL-1 β . (Bauernfeind et al., 2009).

The following event is the oligomerisation with the apoptosis-associated adaptor protein speck-like containing a CARD (ASC), which leads to an activation platform, resulting in the recruitment of pro-caspase-1 (Lu & Wu, 2015). After the inflammasome has been formed, pro-caspase-1 is cleaved, leading to its activation. In turn, this results in the cleavage of pro-IL-1 β into its active form by caspase-1 (Muñoz-Planillo et al., 2013).

IL-1 β is essential in neuroinflammation; it has been shown to increase in neurodegenerative diseases, such as AD, where it upregulates amyloid precursor protein (APP) gene expression, Tau hyperphosphorylation and memory impairment (Beyer et al., 2020).

A strong connection exists between inflammasomes and autophagy, which is a key process through which damaged proteins and organelles are recycled, and intracellular pathogens are destroyed (Kuballa et al., 2012). Autophagy dysfunction has been demonstrated to be a contributing factor to the development of inflammatory diseases, such as inflammatory bowel disease, characterised by hyperinflammation and excessive activation of inflammasomes (Levine et al., 2011).

In addition, it has been demonstrated that innate immune responses instigate autophagy in response to infectious threats (Sun Q., 2017). Furthermore, inflammatory signals have been shown to upregulate autophagy, thereby suppressing excessive responses and protecting the host (Biasizzo & Kopitar-Jerala, 2020).

Autophagy is regulated by a wide panel of proteins, produced from autophagy-related genes (Atg), including LC3, which is frequently used as a marker of autophagosome production (Klionsky et al., 2016).

Autophagosome formation is initiated by the activation of the ULK kinase complex (comprising ULK1 or ULK2, ATG13, FIP200 and ATG101), which targets the class III PI3K complex (comprising Beclin1, VPS15, VPS34 and ATG14) (Kaur et al., 2015).

LC3 is necessary for expanding the autophagic membranes, and the resulting autophagosome that fuses with a lysosome results in the degradation of the enclosed material (Harris et al., 2017). As an important homeostatic mechanism for the degradation and recycling of cytosolic components, autophagy enables the generation of amino acids during starvation and the elimination of damaged and dysfunctional organelles (Biasizzo & Kopitar-Jerala, 2020).

The interplay between neuroinflammation and apoptosis.

Programmed cell death (PCD) plays a crucial role in the pathogenesis of the CNS (Nelson et al., 2018). AD and neuroinflammation, innate immune cells, such as microglia and astrocytes, can trigger programmed cell death through multiple pathways, including apoptosis, which is central to the pathogenesis of AD (Sharma & Kanneganti, 2016)(Nelson et al.,2012).

The resulting cell death may aim to eliminate A β and Tau deposits. In contrast, inflammatory cell death can also be harmful, as it releases proinflammatory cytokines and cellular contents that stimulate severe neuroinflammation (Tahara et al., 2006).

Apoptosis is the main form of PCD employed to maintain homeostasis in the CNS. It is initiated by microenvironmental stimuli that activate initiator caspases (caspase-8 or -9), which then activate effector caspases (caspase-3, -6 and -7) (Galluzzi et al., 2018). In AD, A β and Tau deposits can lead to a dysregulation of apoptotic components, causing the activation of the pro-apoptotic protein Bax and the downregulation of the anti-apoptotic protein Bcl-2 (de Vasconcelos N. M., 2016).

Apoptosis is triggered either by the extrinsic or intrinsic pathway. Extracellular stimuli activate extrinsic apoptosis through the formation of a death-inducing signalling complex (DISC), which involves adaptor proteins (including TRADD and FADD) and pro-caspase-8 (Kischkel et al., 1995). Effector caspases (caspase-3, -6 and -7) are then activated by effector caspases (caspase-8) to drive further substrate cleavage and the execution of apoptotic cell death (Slee et al., 2001).

Intrinsic apoptosis reacts to intracellular changes in equilibrium and involves the activation of Bcl-2 family members, followed by the release of mitochondrial proteins such as cytochrome c. Cytochrome c binds to APAF-1 and activates caspase-9, which then activates downstream effectors, caspases 3, 6, and 7 (Callens et al., 2021).

Exploring the Molecular Basis of Alzheimer's Disease

AD is a neurodegenerative disorder and the most common cause of dementia, affecting around 44 million people worldwide (Kamatham et al., 2024). The progression of the disease varies, and symptoms, which include progressive memory loss and cognitive decline, can develop over decades (Rajesh & Kanneganti, 2022).

At a molecular level, AD is characterised primarily by two abnormalities: β -amyloid plaques and neurofibrillary tangles of tau protein (a type II microtubule-associated protein). The deposition of β -amyloid plaques is caused by the cleavage of amyloid precursor protein (APP) by β -secretase, followed by cleavage by γ -secretase, which generates neurotoxic amyloid β (A β) peptides (Chen et al., 2017).

A β is the main component that aggregates and oligomerises into insoluble structures. These plaques disrupt cell communication, cause microglial activation and inflammation, and ultimately lead to neuronal death (Chasseigneaux & Allinquant, 2012).

Tau protein is expressed in neurons, astrocytes and oligodendrocytes and normally binds to microtubules to stabilise them. However, post-translational abnormalities can cause hyperphosphorylated tau, which dissociates from microtubules and aggregates intracellularly into neurofibrillary tangles (NFTs) (Alonso et al., 2018).

A β deposition, in turn, can exacerbate tau-mediated pathogenesis, contributing to cognitive decline and dementia (Wesseling et al., 2020).

Several forms of programmed cell death play a significant role in neurodegeneration and can be triggered by the assembly of multiprotein complexes, such as the NLRP3 inflammasome (Rajesh & Kanneganti, 2022).

Microglial activation is essential for clearing cellular debris and misfolded proteins (M1 phenotype), but chronic activation can be detrimental (Rea et al., 2018). The activation of astrocytes and microglia releases pro-inflammatory cytokines, including IL-1 β , TNF- α and IL-6, which can trigger tau hyperphosphorylation. Elevated levels of these cytokines have been found in the serum and brain of AD patients. Caspase-1 activation in AD leads to increased IL-1 β release (Beyer et al., 2020).

Cytokines and chemokines, primarily produced by microglia and astrocytes, drive neuroinflammation (Heneka et al., 2015).

They are involved in several processes, including chemoattraction, neuronal injury, and cellular response to A β deposits (Zhang et al., 2020) and influence the development and

progression of AD (Beyer et al., 2020). Several disease conditions can release cytokines into the brain, which have been associated with dementia and loss of neurocognitive function (Dunn et al., 2005). Recent findings have also identified a mechanistic link between cytokine storms, particularly synergism between TNF- α and IFN- γ , and PCD 31, defining cytokine storms as a life-threatening condition caused by excessive production of cytokines mediated by inflammatory cell death (Karki & Kanneganti, 2021). The role of TNF- α in AD is well-established, and there are links between cytokine storm and neuroinflammation. This means that the process is likely to make the pathology of AD worse (Vollmar et al., 2010).

Role of Oxidative Stress in the Pathogenesis of Alzheimer's Disease

Oxidative stress is widely recognised as a possible key mechanism in the pathogenesis of AD (Agrawal & Jha, 2020). In fact, in this context, an increase in the production of reactive oxygen species (ROS) or a reduction in cellular antioxidant defences causes oxidative damage to lipids, proteins and nucleic acids (DNA/RNA) in neurons (Buccellato et al., 2021). In complex interactions with classic AD alterations, such as the accumulation of amyloid-beta (A β) and hyperphosphorylated tau, oxidative stress appears to be more than just a secondary effect. Rather, it is a cofactor that can promote protein aggregation, as well as inflammatory and microglial responses (Alkhalifa et al., 2025). Several ROS-mediated modifications that might be relevant for Alzheimer's disease have already been reported, and more are expected to follow (Cho et al., 2009). Studies on post-mortem human brains, including those in the early stages of cognitive decline, have shown increased levels of markers of oxidation, in particular lipid peroxidation, protein carbonylation, nitration and DNA damage, alongside a reduction in antioxidant enzymes (Zafrilla P., 2006). These alterations are correlated with the degree of cognitive impairment (Ansari & Scheff, 2010). Therefore, even though it is unclear whether oxidative stress is a primary cause or a consequence of the neurodegenerative process, there is a strong body of evidence suggesting that it is an essential element in promoting and amplifies the progression of AD (Pohanka, 2014). Even more intriguing, though, is the interplay between ROS and NLRP3 is crucial in the context of neurotoxicity, particularly in neurodegenerative diseases (Ashrafian et al., 2021). Oxidative stress and excessive ROS production are critical pathological

mechanisms driving neurodegeneration. Conversely, neuroinflammation, mediated by NLRP3 inflammasome activation and the release of pro-inflammatory cytokines, is key in exacerbating neuronal damage. A significant increase in ROS production upregulates NLRP3 expression. (Chiang et al., 2025).

The Role of Microglia in Neuroinflammation

In the ageing brains of mice, rats, and primates, microglia are more sensitive to things that cause inflammation, which is similar to what is seen in microglia in brains where neurodegeneration is still happening (Perry & Teeling, 2013).

This phenomenon is termed 'priming'. Ageing may be associated with microglial senescence, which could be a cause of priming. On the transcriptomic level, the expression of endogenous ligands decreases with age, while the expression of factors involved in host defence and neuroprotection increases (Hickman et al., 2013).

It is unknown yet whether age-related microglia priming is due to cells ageing on their own or whether it's because they've been exposed to an old neural environment for a long time.

Physiologically aged and senescence-accelerated mice showed signs of significant microglia priming, marked by elevated levels of cytokines and ROS, along with augmented phagocytic capability (Heneka et al., 2013).

This model demonstrated that environmental factors, such as neuronal ageing, can trigger microglia priming. Gene expression for microglia priming revealed gene correlation network analysis, featuring increased pattern recognition and expression of interferon signalling genes.

A similar set of genes was seen in experiments with mice that have age-related dementia, including APP/PS1 transgenic mice. Systemic inflammation may polarise microglia by the reaction of the immune system in the body (Orre et al., 2013).

Role of microRNAs in neuroinflammation and neurodegenerative disorders

Recent evidence has highlighted the central role of non-coding RNAs, and in particular microRNAs (miRNAs), in the fine regulation of neuroinflammatory responses and in the molecular mechanisms underlying neurodegenerative diseases (Slota & Booth, 2019). MiRNAs are a class of small regulatory RNAs (approximately 22 nucleotides in length) that function by modulating gene expression in a post-transcriptional manner (Chen et al., 2019). This modulation is achieved through the binding of miRNA to target mRNAs, thereby either repressing their translation or inducing their degradation. Within the CNS, they participate in the control of microglial activation, synaptic plasticity, autophagy, oxidative stress responses and apoptotic pathways (O'Brien et al., 2018). Dysregulation of specific miRNAs has been consistently reported in AD, where A β accumulation, mitochondrial dysfunction and chronic inflammation alter the expression of miRNAs that normally play a key role in maintaining inflammatory homeostasis (Gugliandolo et al., 2020).

Numerous studies have demonstrated that miRNAs, including miR-146a, miR-155, miR-34a, and miR-21, have the capacity to modulate pivotal inflammatory pathways, such as NF- κ B, NLRP3, IL-1 β , and TLR signalling. This regulatory process is pivotal in determining the amplitude and duration of the neuroinflammatory response (Shah & Jain, 2024).

Furthermore, it has been demonstrated that miRNA-mediated regulation is implicated in tau phosphorylation, synaptic dysfunction and alterations of autophagy flux, which are features that contribute significantly to the pathogenesis of Alzheimer's disease (Kim, 2022). These findings lend support to the hypothesis that miRNAs function as an ancillary regulatory layer, capable of integrating inflammatory, metabolic, and degenerative signals. This integration offers both mechanistic insights and potential therapeutic targets for complex neurological disorders (Shah & Jain, 2024). The advent of next-generation sequencing (NGS) has made it possible to characterise the miRNA landscape with unprecedented precision. Small RNA-seq is currently one of the most powerful technologies for profiling regulatory small RNAs in a quantitative and unbiased manner (Wang C. A., 2024).

The analytical workflow typically involves adapter trimming, quality filtering, alignment to reference genomes, identification of known and novel miRNAs, and differential expression analysis (Cheng et al., 2013). This approach allows the investigation of subtle transcriptional rewiring induced by inflammatory stimuli or pharmacological treatments and is particularly suited to neurodegenerative models where regulatory mechanisms operate at multiple levels (Schipper HM, 2007). Bioinformatic tools such as hierarchical clustering, principal component analysis, and functional enrichment (GO/KEGG) enable the integration of miRNA data with biological pathways, revealing networks implicated in inflammasome activation, oxidative stress responses, apoptosis, and autophagy (Love et al., 2014). Recent studies applying small RNA-seq to AD models have identified miRNA signatures associated with early A β toxicity, microglial priming and synaptic impairment, reinforcing the value of this technology for dissecting complex molecular phenotypes (Yuen et al., 2021). Through comprehensive miRNA profiling, it becomes possible to define regulatory patterns underlying neuroinflammatory responses and evaluate how neuroprotective compounds, such as cannabinoids, modulate the post-transcriptional landscape (Wang et al., 2011).

Structural and Functional Roles of Drebrin

Drebrin (DBN) plays a crucial role in synaptic plasticity and cytoskeletal remodelling. It is also closely linked to ageing and cellular senescence, particularly in the brain (Shirao et al., 2017).

DBN is an actin-binding protein highly expressed in the CNS, particularly in mature neurons, where it plays a crucial role in modelling and stabilising dendritic spines (Harigaya et al., 1996).

Its binding to actin filaments (F-actin) induces structural modifications resulting in significant functional effects on cytoskeletal dynamics. In particular, DBN modifies the helical pitch of actin filaments (Mizui, 2014).

The association of DBN induces an increase in helical twist, altering the conformation of the filament and its interaction with other actin-regulating proteins. These structural modifications give DBN-decorated F-actin unique physical and dynamic characteristics compared to undecorated filaments (Peitsch et al., 2006).

Notably, DBN slows down treadmilling by reducing the rate of monomer exchange at the filament poles (Wilson et al., 2010). Treadmilling is a continuous process in which actin monomers (G-actin) are continually added to the plus end of an actin filament and removed from the minus end, maintaining a stable filament length while allowing subunits to move through the filament (Koskinen et al., 2012).

Furthermore, the presence of DBN leads to reduced depolymerisation by decreasing the rate of monomer loss from the pointed end, probably due to interference with the access of depolymerising proteins such as cofilin (Wilson C. A., 2010). The result is greater structural and thermal stability for decorated filaments, which are more resistant to fragmentation and less sensitive to temperature changes (Shirao et al., 2017).

In a neuronal context, these effects lead to the stabilisation of dendritic spine structure and the modulation of synaptic plasticity, as DBN helps to maintain the balance between the stable (F-actin) and dynamic (G-actin) forms of actin (Wilson et al., 2010).

DBN disappears from dendritic spines in the brains of AD patients (Harigaya et al., 1996), and its decline particularly affects neuronal developmental processes, leading to pathological conditions where actin regulation is compromised, such as neurodegenerative diseases.

Cannabinoid system implications in neuroinflammatory and oxidative stress molecular patterns

Cannabinoids are of great interest, particularly concerning neurological and psychiatric diseases involving neuroinflammation, neurodegeneration and pain (Duncan, 2022).

The cannabinoid system is complex and includes endogenous, phytocannabinoids, and synthetic cannabinoids, along with their target receptors and enzymes (Vecchiarelli et al., 2022).

Dysregulation of this system has been linked to neuroinflammatory, neurodegenerative and algal processes. The role of the cannabinoid system in the nervous system is an interesting aspect for further investigation on non-neuronal cells present in the CNS. For instance, microglia cells are particularly relevant for the pathogenesis, maintenance or attenuation of neurological disease (Kelly et al., 2020).

The primary endocannabinoid, N-arachidonylethanolamine (anandamide or AEA), is a fundamental component of the system (Duncan et al., 2024). AEA is metabolised by the

enzyme fatty acid amide hydrolase (FAAH), which also breaks down related N-acyl ethanolamine molecules, such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) (Cabral et al., 2008). Reduced FAAH activity has been shown to lead to decreased levels of IL-1 α and TNF- α , suggesting that FAAH or the compounds it metabolises may be involved in their basal regulation at the central level (Duncan et al., 2022). Conversely, AEA is regarded as a possible modulator of inflammatory responses. It is synthesised by the enzyme N-acyl ethanolamide-specific phospholipase D (NAPE-PLD). The increase in neurite growth promoted by AEA may be protective, generating AEA that leads to the observed increase (Berger et al., 2004).

Cannabidiol (CBD), a non-psychoactive cannabinoid derived from *Cannabis sativa*, has been examined for its impact on lifespan and brain health, emphasising advantageous anti-ageing characteristics (Cabral et al., 2008). Studies conducted using in vitro and in vivo models (particularly the nematode worm *C. elegans*) have demonstrated that CBD can extend lifespan and mitigate the effects of age-related physiological decline (Wang et al., 2022).

Autophagy is the main molecular mechanism through which CBD exerts its anti-ageing and neuroprotective effects. It is an evolutionarily conserved catabolic process that eliminates defective cellular molecules and damaged intracellular components via lysosome-mediated degradation (Aman et al., 2021).

Impaired autophagy is associated with accelerated ageing, whereas stimulating it can have powerful anti-ageing effects (Madeo et al., 2010). CBD treatment has been shown to trigger neuronal autophagy in both in vitro (SH-SY5Y neuronal cells and primary mouse hippocampal neurons) and in vivo (*C. elegans*) models. The earliest response to CBD treatment was shown by the nerve-ring neurons of *C. elegans* compared to other tissues (Madeo et al., 2010).

CBD counteracts the decrease in autophagic activity typically observed in nerve-ring neurons, knocking down autophagy genes (*bec-1*, *vps-34* and *sqst-1*) via RNA interference (RNAi), proving that all three are necessary for the enhancement of longevity by CBD (Morselli et al., 2010).

Neuroprotection: CBD prevents the development of abnormal features (like beading and blebbing) in tactile receptor neurons (TRNs) as we age. Treatment with CBD may help maintain neuronal structure (Wang et al., 2022).

CBD was found to enhance neurite outgrowth and increase dendritic spine density in SH-SY5Y neuronal cells and hippocampal neurons. These effects were counteracted by inhibiting SIRT1, validating that SIRT1 plays a pivotal role in promoting CBD-facilitated neurite growth (Morselli, 2010). As well as CBD also Cannabigerol (CBG) is also well-known for its neuroprotective and anti-inflammatory properties (Gowran et al., 2011).

A protective effect is exerted by CBG in the in vitro neuroinflammation model proposed by Gugliandolo et al., with a reduction in neuronal death, inflammation and oxidative stress being key outcomes. By restoring levels of the transcription factor Nrf2, CBG could represent a key regulator for redox homeostasis in neuronal cells, protecting them from cellular damage induced by oxidative stress (Bryk & Starowicz, 2021).

The neuroprotective and differentiating actions of cannabinoids are not merely the result of direct intracellular modulation but also involve the activation of specific signalling pathways associated with the endocannabinoid system (Elmazoglu et al., 2020),

In this context, the cannabinoid receptors CB1 and CB2 act as key molecular mediators translating cannabinoid exposure into cellular responses related to neuronal differentiation, survival, and adaptation to stress (Vecchiarelli et al., 2022).

The levels of cannabinoid receptors (CB1 and CB2) increased in neural precursor cells exposed to oxidative stress. The CB1 receptor is involved in promoting the differentiation and maturation of neural stem cells, while the CB2 receptor is a key focus of cannabinoid research (Compagnucci et al., 2013).

Activation of CB2 is generally considered to have anti-inflammatory properties and to be involved in cytokine signalling (Olianas et al., 2021).

However, this effect can vary depending on ligand-specific signalling biases. CB2 plays a role in schizophrenia, a condition associated with inflammatory risk factors and microglia-mediated mechanisms (Atwood et al., 2012). In hippocampal microglia, CB2 receptors are implicated in memory-related behaviours (Young et al., 2021).

Both CBD and CBG appear to modulate the activity of the endocannabinoid system through multifaceted mechanisms that may contribute to neuroprotection. This complex regulatory network is involved in maintaining neuronal homeostasis and balancing neuroinflammation (Howlett et al., 2017).

BDNF in Alzheimer's Disease, A β -Driven Neuroinflammation, and Its Interaction with the Endocannabinoid System

The Brain-Derived Neurotrophic Factor (BDNF) plays a central role in neuronal survival, synaptic plasticity, and cognitive function (Numakawa et al., 2010). In AD, a consistent reduction in BDNF expression and TrkB signalling has been reported, although it remains debated whether this decrease represents an early driver of synaptic vulnerability or a downstream consequence of chronic neuroinflammation and A β toxicity (Soman et al., 2025). B-amyloid accumulation suppresses BDNF transcription and impairs long-term potentiation, thereby exacerbating dendritic spine loss and hippocampal circuit dysfunction (Peng et al., 2005). Pro-inflammatory cytokines such as IL-1 β and TNF- α downregulate BDNF pathways, establishing positive feedback in which neuroinflammation reduces neurotrophic support, and diminished BDNF signalling in turn facilitates inflammatory propagation and synaptic degeneration (Toader et al., 2025). Growing evidence indicates a functional crosstalk between BDNF and the endocannabinoid system (ECS) (Khaspekov et al., 2004). Neuronal CB1 and CB2 receptors activation enhances BDNF expression via ERK/CREB pathways, promoting resilience against A β -mediated stress and restoring a microenvironment favourable to BDNF-dependent neuroprotection (Yeh et al., 2017). Conversely, BDNF can regulate endocannabinoid tone by modulating the synthesis and release of anandamide and 2-AG (Burstein, 2018). This bidirectional interaction suggests that ECS modulation may indirectly rescue impaired neurotrophic signalling in AD (Yeh et al., 2017).

AIM OF THE STUDY

Given the growing evidence of the pivotal mechanisms underlying neuronal dysfunction and death in AD as being oxidative imbalance and chronic neuroinflammation, this experiment aimed to better characterise molecular mechanisms involved in neuroinflammation, in particular, those associated with inflammasome activation and autophagy, and to define a pattern of ncRNA associated with neuroinflammation

In this context, we wanted to elucidate the anti-neurodegenerative properties of cannabinoids CBD and CBG.

Autophagy is a process whose role in neurodegeneration is still being debated, as it can contribute to either neuronal survival or cell death, depending on the context.

By providing a deeper understanding of the molecular mechanisms, it would be interesting to speculate how cannabinoids may exert therapeutic or preventive effects against Alzheimer's disease-related neurotoxicity.

A more comprehensive insight into how cell death pathways and innate immune sensors regulate neuroinflammation and A β /Tau clearance will be crucial for identifying innovative molecular targets, paving the way for cannabinoid-based strategies aimed at mitigating the progression of Alzheimer's disease.

METHODS

Cell cultures

Human neuroblastoma SH-SY5Y cells and human microglial HMC3 cells, both purchased from ATCC (Manassas, VA, USA), were used to reproduce key pathological features associated with Alzheimer's disease (AD). SH-SY5Y cells were maintained in a 1:1 mixture of Eagle's Minimum Essential Medium and F12 medium (EMEM/F12), whereas HMC3 cells were cultured in Eagle's Minimum Essential Medium (EMEM; Sigma-Aldrich, St. Louis, MO, USA). Both media were supplemented with 10% fetal bovine serum (FBS; ATCC, Manassas, VA, USA) and 1% penicillin–streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cultures were kept at 37 °C in a humidified incubator with 5% CO₂, and the medium was refreshed every 48 hours.

Cell treatments

To induce AD-like stress conditions, cells were exposed to oligomeric β -amyloid_{1–42}. β -amyloid_{1–42} was provided by GenScript (Piscataway, New Jersey, United States). The peptide was prepared by diluting β -amyloid_{1–42} in the respective culture media and allowing it to oligomerize under agitation (100 rpm) for 1 hour at 37 °C. SH-SY5Y and HMC3 cells were subsequently treated with different concentrations of β -amyloid_{1–42} oligomers, respectively 10 μ M and 20 μ M. All experiments were performed using cells between passages 3 and 8 to ensure stable phenotypic characteristics. To obtain β -amyloid oligomers, it was diluted in culture medium and stirred for 1 hour (100 rpm) at 37°C. Both cell lines were triggered with β -amyloid_{1–42} oligomers for 72h, adding fresh medium containing the stimulus, and on the third day, after 48h, β -amyloid_{1–42} was administered concomitantly with cannabidiol (CBD) (Bio-Techne, Tocris, Minneapolis, MN, USA) and cannabigerol (CBG) (Bio-Techne, Tocris, Minneapolis, MN, USA). β -amyloid_{1–42}, CBD and CBG were added to the medium, alone or in combination at different doses. Doses and timing for β -amyloid_{1–42} stimulation were chosen based on data from quantitative PCR analysis for inflammatory and apoptotic targets (**Figure 3 and 4**). CBD and CBG doses were selected based on MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] data and *NRF2* dose-response curve (**Figure 2**).

MTT assay

The MTT assay was used to assess cell viability and determine the cytotoxicity of CBD and CBG. The MTT assay was used to assess cell viability and determine the cytotoxicity of CBD, CBG. SH-SY5Y cells were seeded at a density of 6×10^4 cells, while hMC3 cells were seeded at a density of 4×10^4 cells per well in a 96-well plate. Upon reaching confluence, the cells were treated with CBD and CBG at concentrations of 1, 2.5, 5, 10, and 20 μ M for 24 hours.

Then 5 mg/mL of tetrazolium dye MTT (Sigma Aldrich, St. Louis, MO, USA) was thawed in sterile PBS, and 20 μ L were added into each well, two hours before the end of the treatments. Twenty-four hours after the start of the treatment, the formazan crystals were solubilised using 200 μ L of dimethyl sulfoxide (DMSO). Cytotoxicity was determined using a VICTOR Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA) at wavelengths of 540 and 620 nm. Data are expressed as a percentage of the viability of the control cells.

RNA extraction and Quantitative RT-PCR

Both cellular lines were treated with different concentrations of β -amyloid₁₋₄₂ at different time-points to determine the optimal conditions for stimulation by quantifying the expression of the main pro-inflammatory cytokines. After treatment, extraction of total RNA was performed from SH-SY5Y and hMC3 cells with PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) and quantified with a spectrophotometer (NanoDrop Lite; Thermo Fisher Scientific). Total RNA (1 μ g) was reverse transcribed in the cDNA using SuperScript VILO Master Mix (Thermo Fisher, Waltham, Massachusetts, USA).

The obtained cDNA (1 μ L) was added to the PowerUp SYBR Green Master Mix Master Mix (AppliedBiosystem, ThermoFisher, Waltham, Massachusetts, USA) to measure the mRNA level of target. QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, CA, USA) was used to perform qPCR and the GAPDH gene was used as the housekeeping gene. The qPCR reaction was examined by using QuantStudio 6 Flex (Thermo Fisher Scientific, Monza, MB, Italy), and the changes in mRNA expression were measured using the $2^{-\Delta\Delta CT}$ method, using GAPDH as a housekeeping gene and the control group as a calibrator. The primers for targets and reference genes are listed in Table 1.

Evaluation of intracellular ROS Production

The anti-oxidant effect of natural compounds was evaluated in a model of neuroinflammation triggered by β -amyloid 1-42 (GenScript, Hong Kong, China) using a probe called CM-H₂DCFDA. This probe can detect the production of intracellular ROS in both cell lines. Cells were stimulated with β -amyloid, SH-SY5Y cells at 10 μ M, while hMC3 at 20 μ M, for 72h and treated with CBD, CBG, or a combination of the two for 24 hours. Fluorescein diacetate (FDA) is an esterase substrate that can be used to determine enzymatic activity (the hydrolysis of the substrate and formation of the fluorescent product) and cell membrane integrity.

The increase in fluorescence can be detected with a fluorescence microscope. This is due to the oxidation of the probe. Excitation sources and filters appropriate for fluorescein (FITC) are used.

Finally, the cells were incubated with 5 μ L of the CM-H₂DCFDA probe (Thermo Fisher, Carlsbad, CA, USA) for 15 min 37 °C with 5% CO₂. Following the cells were washed with sterile PBS, the cells were observed under a fluorescent microscope, and the fluorescent intensity was quantified using ImageJ 1.53e software for Windows (Softonic, Barcelona, Spain).

Western blot analysis

Cells treated were recovered using RIPA buffer (25 mM Tris/HCl, pH 7.4; 1.0 mM EGTA; 1.0 mM EDTA) with NP40 (1%), phenyl methylsulfonyl fluoride (PMSF, 0.5%), aprotinin, leupeptin and pepstatin (10 μ g/mL each) and centrifuged at 15,000 rpm for 15 min at 4 °C to obtain the supernatant from each sample.

The total amount of protein was evaluated in cell supernatants using the Bradford method. Then, 30 μ g of protein was fractionated by electrophoresis on a 10% SDS polyacrylamide gel and transferred to PVDF membranes (Bio-Rad, California, USA) using a specific transfer buffer. After washing in a TBS-0.1% Tween buffer solution and incubating in a 5% non-fat dry milk solution, the membranes were incubated with a primary antibody specific for: Caspase-3 (Cell Signaling, 9662S, Danvers, MA, USA), LC3B (Abcam, ab192890, Cambridge, UK), P62 (Abcam, ab109012, Cambridge, UK), NLRP3 (Cell Signaling, 15101S, Danvers, MA, USA), β -actin (Cell Signaling, 4967S, Danvers, MA,

USA). Stock solution was diluted in TBS-0.1% Tween as required by data sheet and incubated overnight, at 4 °C.

The following day, the membranes were washed three times with TBS-0.15% Tween buffer. A secondary peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (GeneTex, Irvine, CA, USA) was then used for 1 hour at room temperature (RT) to bind to and detect the target proteins, according to the antibody used.

After three further washes with TBS-0.15% Tween buffer, the membranes were analysed using a chemiluminescence system (LumiGlo reserve; Seracare, Milford, MA, USA). An image analysis system (C-DiGit, Li-Cor, Lincoln, NE, USA) was used to obtain and quantify the images via scanning densitometry. The results were expressed as relative integrated intensity, with β -actin (Cell Signalling Technology, Danvers, MA, USA) used as a loading control.

Immunofluorescence

SH-SY5Y cells were seeded at a density of 2×10^4 cell/well in 8-well chamber slides. Cells were triggered with β -amyloid1-42 (10 μ M). After 48h, β -amyloid1-42 (10 μ M) was administered concomitantly with CBD 10 μ M and/or CBG 10 μ M, alone and in combination (CBD 10 μ M CBG 7,5 μ M) for 24h, the end of the treatments, cells were fixed with 4% of paraformaldehyde (PFA) in 0.2 M phosphate buffer (pH 7.4) for 10 min at RT; cells were washed 3 times for 10 min with sterile phosphate-buffered saline (PBS). β -amyloid1-42 Cells were, also, incubated with 0.3% triton X-100 in PBS for 10 min to induce permeabilization of the membranes and with 1% bovine serum albumin (BSA) in PBS, for 1 h. at RT, in order to block non-specific binding sites. At the end of incubation, cells were incubated with a rabbit polyclonal anti-DBN antibody (1:100 dilution) (Abcam, ab60933, Cambridge, UK) overnight at 4 °C. The day after, cells were rinsed with PBS and a FITC conjugated IgG anti-rabbit antibody was added (GeneTex, Irvine, CA, USA) for 1 h. at RT, in order to detect the primary antibody. Nuclei were stained with DAPI diluted to 1:1000 in PBS (Thermo Fisher Scientific, Carlsbad, CA, USA) for 10 min RT. At the end of this process, cells were rinsed in PBS, the coverslips were mounted on slides and fluorescence was monitored using a fluorescent microscope.

Samples were observed using a Zeiss LSM 510 confocal microscope, which was equipped with an Argon laser (458 nm and 488 nm) and two HeNe lasers (543 nm and 633 nm).

All images were digitally scanned at a resolution of 8 bits into an array of 2048×2048 pixels. Optical sections of the fluorescent specimens were obtained at 488 nm with an average scanning speed of 62 s and up to eight repetitions. The optimal resolution was achieved by setting the pinhole, and contrast and brightness were established by examining the brightest pixels and selecting settings that allowed clear visualisation of structural details while maintaining pixel intensities close to 200. These parameters were used for all observations. Digital images were subsequently cropped, and figure montages were prepared using Adobe Photoshop 7.0.

Bioinformatic Methods

1. Raw Data Processing and Quality Control

The bioinformatic workflow began with the conversion of Illumina BCL files into FASTQ format using the instrument's standard base-calling pipeline. The quality of the raw reads was then assessed, and all sequences underwent preprocessing with fastp, an integrated solution for high-throughput FASTQ quality control. During this step, adapter-contaminated reads, sequences containing more than 10% undetermined bases, and reads with over half of their nucleotides exhibiting a Phred score below five were removed. Standard quality indicators, including Q20, Q30 and GC content, were calculated for each sample. Only reads meeting the required thresholds were retained as “clean reads” for downstream analyses, ensuring that subsequent steps were not influenced by technical artefacts or low-quality sequencing output.

2. Alignment to the Human Genome

Clean reads between 18 and 40 nucleotides in length—corresponding to the expected size distribution of miRNAs and other small RNAs—were aligned to the human reference genome using Bowtie. This aligner is particularly well suited for small RNA-seq because of its ability to map short sequences with high sensitivity through a zero-mismatch seed strategy.

Mapping statistics were generated to evaluate the proportion of uniquely mapped and multi-mapped reads, as well as the distribution of reads across exonic, intronic, intergenic and repetitive regions.

3. Annotation of Known miRNAs

Annotated miRNAs were identified by comparing mapped reads with curated entries in the miRBase database. This was achieved through miRDeep2, which integrates structural and positional information, reconstructing precursor hairpin motifs and verifying the compatibility of read stacks with canonical Drosha and Dicer cleavage patterns. This approach allowed confident identification and quantification of all known human miRNAs across the sequencing dataset.

4. Removal of Non-miRNA Small RNA Species

To avoid contamination by other classes of small RNAs, a hierarchical annotation strategy was applied. Reads were sequentially matched against databases of rRNA, tRNA, snRNA, snoRNA, repetitive elements and protein-coding transcripts. Each read was assigned to the most likely origin based on biologically informed priority rules. This ensured that only bona fide miRNAs and candidate novel miRNA sequences were retained for further quantification and differential expression analysis.

The procedure effectively minimized false discovery due to cross-mapping or misannotation of structurally similar small RNA species.

5. Novel miRNA Prediction

The identification of previously unannotated miRNAs was carried out with miREvo and miRDeep2, which evaluate secondary structure characteristics, free energy folding profiles, read-stack patterns and Dicer cleavage signatures. Only candidates that met stringent structural and thermodynamic criteria were considered valid predictions. These novel miRNA candidates were incorporated into the expression matrix together with known miRNAs, thereby expanding the regulatory landscape assessed in the study.

6. Quantification and Normalization of miRNA Expression

Expression levels for each miRNA, both known and novel, were calculated using the Transcripts Per Million (TPM) normalization method. TPM accounts for differences in sequencing depth among samples and enables direct comparison of miRNA abundance across experimental conditions. TPM-based normalization is widely adopted in small RNA sequencing studies due to its robustness and suitability for datasets with heterogeneous total read counts.

7. Differential Expression Analysis

Differential expression analysis was performed using DESeq2 for all comparisons involving biological replicates. DESeq2 models count data through a negative binomial generalized linear model, estimating dispersion parameters and computing log₂ fold changes and Wald test statistics. Multiple testing correction was applied using the Benjamini–Hochberg false discovery rate (FDR), providing adjusted p-values to assess statistical significance at the transcriptome level. In comparisons lacking replicate structure, normalization and dispersion estimation relied on the Trimmed Mean of M-values (TMM) method implemented in edgeR, which provides a reliable framework for normalization in small RNA-seq datasets where replicate constraints limit variance modelling. This dual strategy ensured statistical rigor across all conditions.

8. Prediction of miRNA Target Genes

To determine the potential regulatory impact of differentially expressed miRNAs, mRNA targets were predicted using the complementary algorithms miRanda and RNAhybrid. miRanda evaluates sequence complementarity and thermodynamic feasibility of miRNA–mRNA interactions, whereas RNAhybrid identifies minimum free energy hybridization sites within 3′ untranslated regions. Target prediction was restricted to the intersection of the two algorithms, thereby increasing the confidence of inferred regulatory interactions and reducing false positives.

9. Functional Enrichment and Pathway Analysis

Functional interpretation of miRNA-mediated regulatory effects was achieved through enrichment analysis of the predicted target genes. Using the clusterProfiler framework, both Gene Ontology (GO) terms and KEGG pathways were evaluated for significance based on the adjusted p-values obtained from define it as False Discovery Rate (FDR) correction. This analysis provided a comprehensive overview of biological processes and molecular pathways modulated by the differentially expressed miRNAs, highlighting key mechanisms implicated in apoptosis, mitochondrial dysfunction, oxidative stress, calcium signaling and inflammatory responses. These enriched pathways were subsequently integrated with experimental findings to contextualize the regulatory networks underlying A β -induced cytotoxicity and cannabinoid-mediated neuroprotection.

Statistical analysis

All results are expressed as means and standard deviation ($\pm$ SD). For different groups analysis, t-test and two-way ANOVA with Tukey's post-test for intergroup comparisons. A p-value < 0.05 was considered significant. Graphs were realised using GraphPad Prism10.

The KEGG enrichment analysis is not based on experimentally measured gene expression, but on the predicted targets of differentially expressed miRNAs. Up- or down-regulated miRNAs were mapped to their potential mRNA targets using established prediction databases (TargetScan, miRanda, miRTarBase), and the resulting target list was used to identify overrepresented pathways. Therefore, the enrichment reflects biological processes that are potentially regulated by the altered miRNAs, rather than direct changes in mRNA expression.

The p-value represents the statistical significance of a single test, while the adjusted p-value (FDR), obtained by correction for multiple comparisons according to Benjamini-Hochberg, controls the false positive rate in RNA-seq analyses. Although several miRNAs showed low nominal p-values, none exceeded the significance threshold after correction ($p_{adj} < 0.05$), suggesting that the observed changes represent biological trends to be interpreted with caution.

RESULTS

CBD and CBG, alone, do not significantly affect cell viability

Before testing anti-inflammatory mechanisms, MTT assays were conducted to determine the nontoxic concentrations of the compounds (**Figure 1**). Neither CBD nor CBG alone significantly affected the viability of hMC3 (**Panel A**) and SH-SY5Y (**Panel B**) cells at concentrations of up to 10 μ M. Whereas higher doses of both CBD and CBG, 15 and 20 μ M, resulted in a significant ($p < 0.0001$) decrease in viability, suggesting potential cytotoxicity at these levels. The trend was comparable across the two cell lines.

Combined treatments with nontoxic concentrations of CBD and CBG did not significantly reduce viability in either model. Across all tested combinations, cell viability remained close to control values, with only modest variations that did not suggest additive or synergistic toxicity.

Dose-Response curves of NRF2 transcript expression

To determine the most appropriate concentrations for subsequent experiments, we first evaluated the dose-dependent modulation of NRF2 transcript levels following exposure to CBD or CBG (**Figure 2**). In hMC3 cells (**Figure 2, Panel A**), the induction of *NRF2* by CBG was significantly higher compared to that of CBD. Across all concentrations tested, CBG reached its peak induction (17.7 ± 0.5 fold-change) at 7.5 μ M and it was maintained at 10.0 μ M ($p < 0.0005$). A similar pattern was observed in SH-SY5Y cells (**Figure 2, Panel B**), where CBG elicited a significant ($p < 0.005$) induction already at 5 μ M, reaching the highest expression values at 7.5–10 μ M ($p < 0.0005$). CBD displayed a more moderate, gradual increase, reaching its peak at the concentration of 10 μ M in both hMC3 ($p < 0.005$) and SH-SY5Y ($p < 0.0005$) cell lines.

Based on these results, CBD at a concentration of 10 μ M and CBG at a concentration of 5 μ M and 7.5 μ M (in SH-SY5Y) and 10 μ M (in hMC3) were selected for subsequent protective co-treatment experiments.

Evaluation of chronic stimulation with β -amyloid₁₋₄₂

To assess the response of microglia to chronic stimulation by β -amyloid, hMC3 cells were exposed to 20 μ M of β -amyloid₁₋₄₂ for 24, 48 and 72 hours. RT-qPCR analysis of gene expression revealed time-dependent changes in markers related to inflammasome activation and apoptosis.

As shown in **Figure 3, Panel A**, in hMC3 exposure to β -amyloid resulted a progressive and significant increase in both *NLRP3* and *IL-1 β* expression observed over time. *NLRP3* transcript was significantly upregulated after 48h ($p < 0.005$) reaching the highest levels of expression after 72 hours of β -amyloid exposure ($p < 0.0005$), indicating sustained inflammasome activation. *IL-1 β* showed a similar trend, with levels significantly increased from 48 hours onwards ($p < 0.005$), reaching its maximum at 72 hours ($p < 0.0005$), compared to the control group.

Exposure to β -amyloid in SH-SY5Y resulted in a significant increase in *NLRP3* and *IL-1 β* expression after 72 hours ($p < 0.0005$) compared with the other timepoints, indicating long-term activation of inflammasome-related signalling (**Figure 3, Panel B**).

These results suggest that the inflammasome pathway is activated and becomes progressively more active following exposure to β -amyloid, consistent with the development of a chronic inflammatory phenotype in microglial and neuroblastoma cells. In addition, the expression of mitochondrial apoptosis-related genes was evaluated (*BAX*, *BCL2*, *CASP3* and *CASP9*) in both cell lines, to determine whether chronic β -amyloid exposure triggers apoptotic signalling **Figure 4**.

In hMC3 cell (**Figure 4, Panel A**), a significant upregulation of *CASP3*, and *CASP9* was observed, after 48 hours onwards ($p < 0.05$ and $p < 0.005$) with a further increase at 72 hours ($p < 0.0005$) for both targets, suggesting the activation of the intrinsic apoptotic pathway. *BCL2* expression showed a significant increase ($p < 0.0005$) at 48 hours followed by a marked reduction at 72 hours, suggesting an early compensatory mechanism that declines over time. Conversely, *BAX* significant upregulation was observed after 72 hours ($p < 0.0005$).

In SH-SY5Y, a significant upregulation of *BAX*, *CASP3* and *CASP9* was observed from 48 hours onwards, with further increases at 72 hours ($p < 0.0005$), indicating the activation of pro-apoptotic signalling pathways (**Figure 5**). In contrast, *BCL2* expression showed a significant induction at 24 hours ($p < 0.0005$) and decreased at successive time

points, suggesting that early compensatory survival responses progressively diminish with prolonged A β ₁₋₄₂ exposure.

Cannabinoid Modulation of NLRP3 and IL-1 β transcripts in β -Amyloid-triggered cells

A β ₁₋₄₂ exposure (20 μ M in hMC3; 10 μ M in SH-SY5Y) significantly upregulated the mRNA expression of both *NLRP3* and *IL-1 β* compared to the untreated control, confirming the activation of an inflammatory response in both microglial and neuronal cells (**Figure 6**). In hMC3 cells (**Figure 6, Panel A**) single treatments with CBD (10 μ M) or CBG (10 μ M) significantly reduced A β -induced expression of *NLRP3* ($p < 0.0005$ for CBD 10 μ M and $p < 0.05$ for CBG 10 μ M).

CBD (10 μ M) and CBG (10 μ M) significantly reduce *IL-1 β* levels ($p < 0.0005$ for CBD 10 μ M and $p < 0.0001$ for CBG 10 μ M) compared to cells treated with A β ₁₋₄₂ alone. The combined treatment with CBD (10 μ M) + CBG (7.5 μ M) resulted in the strongest decrease in both transcripts, indicating a more effective attenuation of the inflammatory response than either compound alone. A similar trend emerged in SH-SY5Y cells (**Figure 7, Panel B**). A β ₁₋₄₂ induced a significant increase in both inflammatory markers ($p < 0.0005$ for *NLRP3* and $p < 0.0001$ for *IL-1 β*).

Consistent with the hMC3 results, co-treatment with **CBD** (10 μ M) + **CBG** (5 μ M) produced the most significant reduction in *NLRP3* and *IL-1 β* mRNA expression, supporting a higher anti-inflammatory effect when the two cannabinoids are administered together ($p < 0.0001$ for *NLRP3* and $p < 0.005$ for *IL-1 β*).

Cannabinoid modulation of Intrinsic Apoptotic Signalling

To evaluate the anti-apoptotic efficacy of the cannabinoids, the expression of the mitochondrial apoptosis-related genes, pro-apoptotic and anti-apoptotic, and their ratio were analysed (**Figure 7**).

The ratio BAX/BCL2 serves as a critical indicator of the cell's commitment to the intrinsic apoptotic pathway.

In hMC3 cells (**Figure 7, Panel A**), exposure to A β ₁₋₄₂ (20 μ M) significantly upregulated BAX expression ($p < 0.005$) compared to the control. Treatment with CBD 10 μ M

significantly reduced the A β ₁₋₄₂ (20 μ M) induced transcription ($p < 0.005$). Conversely, the association of CBD 10 μ M + CBG 7.5 μ M significantly increased expression of BCL2 compared to the inflammatory condition A β ₁₋₄₂-induced ($p < 0.005$). Crucially, stimulation led to a robust 3-fold increase in the BAX/BCL2 ratio ($p < 0.0001$) compared to the CTRL, which was significantly reduced by CBD 10 μ M alone ($p < 0.0001$), CBG 10 μ M alone ($p < 0.0001$), and most notably by the co-treatment ($p < 0.0001$).

A stronger apoptotic imbalance was observed in SH-SY5Y cells (**Figure 8, Panel B**), where A β ₁₋₄₂ (10 μ M) induced a strong and significant increase in BAX transcription ($p < 0.0001$) and a tendency toward reduced BCL2. All treatments CBD (10 μ M), CBG (7.5 μ M), and the combination CBD (10 μ M) + CBG (5 μ M), significantly attenuated the A β -induced upregulation of BAX ($p < 0.0001$).

Furthermore, the and co-treatment resulted in the strongest induction of expression of BCL2 ($p < 0.0001$). The BAX/BCL2 ratio was significantly exacerbated in SH-SY5Y A β ₁₋₄₂ treated compared to the control condition ($p < 0.0001$), but all cannabinoid treatments significantly reversed this pro-apoptotic shift and with the co-treatment showing near complete restoration to control levels ($p < 0.0001$). These results indicate that both cannabinoids effectively block the initiation of the mitochondrial apoptotic pathway, with the combined treatment exerting the strongest overall protective effect.

Cannabinoid-Mediated Downregulation of Caspase-3 and Caspase-9 Transcripts

To further elucidate the mechanisms by which cannabinoids mitigate A β ₁₋₄₂-induced apoptosis, gene expression of Caspase-9 (*CASP9*), the initiator of the intrinsic mitochondrial pathway, and Caspase-3 (*CASP3*), the primary executioner of apoptosis was analysed. In hMC3 microglial cells (**Figure 8, Panel A**), exposure to A β ₁₋₄₂ triggered a significant upregulation of both caspases compared to control levels ($p < 0.0001$), confirming the activation of the apoptotic cascade. Treatment with CBD (10 μ M) and CBG (10 μ M) significantly reduced this response (respectively $p < 0.0005$, $p < 0.0001$). Specifically, for *CASP3*, the combined treatment (CBD 10 μ M + CBG 7.5 μ M) proved to be the most effective intervention, reducing mRNA levels significantly more than either cannabinoid alone.

Regarding *CASP9*, while single treatments with CBD (10 μ M) showed a significant efficacy ($p < 0.0001$), the co-treatment gave the best results in term of reduction of the transcript levels compared to the A β_{1-42} -stimulated condition.

A similar pattern was observed in SH-SY5Y neuroblastoma cells (**Figure 8, Panel B**). A β_{1-42} stimulation induced a significant increase in *CASP3* and *CASP9* transcripts ($p < 0.0001$). Both CBD (10 μ M) and CBG (7.5 μ M) administered independently, were able to significantly reduce the expression of the executioner *CASP3* ($p < 0.0005$). However, the strongest modulation was again achieved with the CBD (10 μ M) + CBG (5 μ M) co-treatment, which induced a significant downregulation of both the initiator (*CASP9*) and executioner (*CASP3*) caspases ($p < 0.0001$ vs. A β), effectively counteracting the pro-apoptotic signal engaged by the amyloid insult.

Western Blot Analysis

Western Blot analysis confirmed that in hMC3 β -amyloid $_{1-42}$ (20 μ M) stimulation caused a significant increase ($p < 0.0001$) in the expression of NLRP3, key component of the inflammasome (**Figure 9, Panel A**). Treatment with CBD (10 μ M) and CBG (10 μ M) alone significantly reduced NLRP3 expression compared to the A β_{1-42} -treated cells, even if the best results came from CBD (10 μ M) + CBG (7.5 μ M) combination ($p < 0.0001$).

β -amyloid $_{1-42}$ (20 μ M) stimulation also resulted in a significant ($p < 0.0005$) up-regulation of Caspase-3, central executioner of apoptosis (programmed cell death). Consistent with the other findings, both CBD (10 μ M) and the combined CBD (10 μ M) + CBG (7.5 μ M) treatment significantly ($p < 0.0005$) suppressed the β -amyloid $_{1-42}$ -induced Caspase-3 expression. These findings suggest a neuroprotective, anti-inflammatory and anti-apoptotic mechanism.

Treatment with β -amyloid $_{1-42}$ (20 μ M) significantly increased the LC3B II/LC3B I ratio ($p < 0.0001$) compared to the control (CTRL), suggesting an induction of autophagy or an accumulation of autophagosomes (**Figure 10**). Treatment with CBD 10 μ M or the combination of CBD (10 μ M) + CBG (7.5 μ M) significantly reduced this ratio to levels comparable to the control ($p < 0.0001$), indicating a potential restoration of autophagic flux. Similarly, Beclin expression significantly ($p < 0.0001$) increases in cells triggered with A β_{1-42} (20 μ M). The treatment with CBD (10 μ M) and CBD (10 μ M) alone significantly reduced expression of Beclin ($p < 0.005$), the association CBD (10 μ M) +

CBG (7.5 μ M) combination significantly restored Beclin levels towards the control baseline ($p < 0.0001$ vs $A\beta_{1-42}$ 20 μ M).

Consistently, in SH-SY5Y cells exposed to β -amyloid₁₋₄₂ (10 μ M), expression of NLRP3 and Caspase-3 was significantly augmented ($p < 0.0001$). CBD (10 μ M) and CBG (7.5 μ M) alone or in combination (CBD 10 μ M + CBG 5 μ M) significantly reduced $A\beta_{1-42}$ -triggered neuroinflammation. $A\beta_{1-42}$ treatment significantly increased autophagy markers expression ($p < 0.0001$) compared to the control condition. LC3B II/I ratio showed modulation consistent with cannabinoid-mediated regulation of autophagic flux, in particular CBG (7.5 μ M) and in association with CBD (10 μ M) ($p < 0.0001$).

The trend of Beclin expression showed a moderate increase with CBD (10 μ M) and a significant reduction with the association of CBD (10 μ M) + CBG (5 μ M), compared to cells triggered with $A\beta_{1-42}$ (**Figure 10, Panel B**).

ROS detection

To assess whether cannabinoids counteract the oxidative imbalance triggered by β -amyloid, intracellular ROS levels were examined in hMC3 microglial cells and SH-SY5Y neuroblastoma cells using the CM-H₂DCFDA fluorescence probe (**Figure 11, Panels A and B**). In hMC3 cells, $A\beta_{1-42}$ exposure (20 μ M) resulted in a significant increase in green fluorescence intensity ($p < 0.0001$), indicating elevated ROS production compared with untreated controls. Treatment with CBD (10 μ M), CBG (10 μ M) significantly reduced ROS-associated fluorescence ($p < 0.005$), their combination (CBD 10 μ M + CBG 7.5 μ M), exhibited the most pronounced attenuation ($p < 0.0001$) referred to $A\beta_{1-42}$ treated only cells. All images were acquired under identical exposure and gain settings and are representative of three independent experiments.

A comparable pattern was observed in SH-SY5Y cells, where β -amyloid₁₋₄₂ (10 μ M) induced a strong oxidative impairment.

CBD (10 μ M) significantly reduced ROS levels compared with $A\beta_{1-42}$ stimulation alone ($p < 0.005$), while CBG (7.5 μ M) produced an even stronger decrease ($p < 0.0001$).

The combined treatment (CBD 10 μ M + CBG 5 μ M) led to the most significant attenuation of ROS accumulation among all conditions. Representative fluorescence images, acquired under identical exposure settings, reinforce these findings and visually confirm the cannabinoid-mediated reduction of β -amyloid-induced oxidative stress.

Cannabinoid-mediated modulation of BDNF expression in SH-SY5Y cells exposed to β -amyloid₁₋₄₂

To evaluate the neuroprotective and trophic potential of the treatments, the expression of the brain-derived neurotrophic factor (BDNF) gene was investigated using RT-qPCR. As shown in **Figure 12**, exposure of SH-SY5Y treatment with cannabinoids resulted in significant BDNF expression compared to the amyloid stress condition (**Figure 12**). Notably, a single administration of 10 μ M CBD and 7.5 μ M CBG resulted in a significant increase in transcript levels ($p < 0.001$ and $p < 0.05$, respectively, versus β -amyloid). The most significant effect was observed with co-treatment (CBD 10 μ M + CBG 5 μ M). The combination of the two compounds increased BDNF expression by over 20-fold compared to the control group ($2^{-\Delta\Delta C_t} > 20$). Statistical analysis confirmed that the efficacy of the combined formulation was significantly higher than that of the group treated with CBD and CBG alone ($p < 0.0001$), suggesting synergistic action between the two cannabinoids in stimulating the synthesis of neurotrophic factors.

CBD and CBG restored DBN Expression and Cellular Morphology Following β -amyloid₁₋₄₂ exposure

Immunofluorescence microscopy was employed to evaluate the protective effects of CBD) and CBG against β -amyloid₁₋₄₂ induced toxicity in SH-SY5Y neuroblastoma cells. The analysis focused on the expression and distribution of DBN, a β -actin-binding protein crucial for maintaining the integrity and structure of dendritic spines (**Figure 13**). DBN was visualised using an indirect immunofluorescence technique (green signal via FITC-conjugated secondary antibody), and cell nuclei were counterstained with DAPI (blue). Untreated SH-SY5Y cells exhibited a normal baseline cellular morphology. This was characterised by a homogeneous, continuous, and intense DBN signal (green), reflecting the physiological organisation of the β -actin cytoskeleton. Nuclei (blue) were normal and uniformly distributed, showing no signs of cellular stress.

Exposure to β -amyloid₁₋₄₂ (10 μ M) induced severe morphological damage. This was primarily evidenced by a significant reduction in DBN expression signal intensity ($p < 0.0001$). Morphologically, the green signal appeared fragmented and sparse. Furthermore,

the nuclei (blue) were more sparsely distributed, reflecting β -amyloid₁₋₄₂ mediated toxicity.

Treatment with CBD 10 μ M partially restored DBN expression relative to the β -amyloid₁₋₄₂-treated samples. The fluorescence signal (green) appeared more continuous and denser. The nuclear distribution (blue) was also closer to the control pattern, suggesting a protective effect against cellular damage.

CBG 7.5 μ M treatment resulted in a significant enhancement of DBN expression ($p < 0.0001$) compared to the β -amyloid₁₋₄₂ condition. The significant recovery of the DBN signal assessed the capacity of CBG to counteract A β ₁₋₄₂ induced loss of this key protein. Co-treatment with CBD (10 μ M) and CBG (5 μ M) yielded the strongest recovery of DBN expression. The green signal became dense and continuous, closely approximating the healthy pattern observed in control cells. Concurrently, the nuclei were uniformly distributed, indicating the most pronounced amelioration of A β ₁₋₄₂-induced morphological damage via a synergistic action of the two cannabinoids.

Bioinformatic Differential Expression Analysis

Differential expression analysis ($p < 0.05$) revealed that CBD (10 μ M), CBG (10 μ M) and their combination CBD(10 μ M) + CBG (7,5 μ M), in hMC3, significantly modulated the expression of several miRNAs dysregulated by A β ₁₋₄₂. Volcano plots (**Figure 14**) show differential miRNA expression analysis revealed distinct transcriptional responses to A β ₁₋₄₂ exposure and cannabinoid treatments in both hMC3 and SH-SY5Y models.

In A β -treated microglia (**Panel A**), 42 miRNAs were significantly modulated ($p < 0.05$), with 22 upregulated and 20 downregulated relative to untreated controls, indicating a balanced yet substantial shift in miRNA expression following A β exposure.

Treatment with CBD (10 μ M) (**Panel B**) produced 58 significant changes, with a predominance of downregulated miRNAs (28 down vs 30 up), suggesting that CBD exerts a broad regulatory influence capable of reversing multiple A β -induced alterations. Similarly, CBG (10 μ M) treatment (**Panel C**) resulted in 29 differentially expressed miRNAs (21 up, 8 down), indicating a more selective transcriptional response compared with CBD. The combined CBD (10 μ M) + CBG (10 μ M) treatment (**Panel D**) induced the largest modulation, with 109 miRNAs significantly affected (59 up, 50 down). This

broader transcriptional reprogramming suggests additive or synergistic effects between the two cannabinoids in reshaping microglial regulatory networks.

Exposure of SH-SY5Y cells to A β _{1–42} (*Panel E*) led to 55 differentially expressed miRNAs (23 up, 32 down), revealing a strong imbalance characterised mainly by miRNA suppression. Treatment with CBD (10 μ M) (*Panel F*) produced a strong transcriptional response, with 192 miRNAs significantly modulated (132 up, 60 down), indicating that CBD robustly counteracts A β -driven miRNA alterations and activates additional regulatory pathways. In contrast, CBG (5 μ M) treatment (*Panel G*) generated a more moderate profile, with 71 significant changes (50 up, 21 down), reflecting a milder but still notable impact on neuronal miRNA expression. The combined CBD (10 μ M) + CBG (5 μ M) treatment (**Figure H**) resulted in 70 modulated miRNAs (57 up, 13 down), again showing a marked predominance of upregulated miRNAs, consistent with a restorative or compensatory response counteracting A β toxicity. Across both cell types, A β _{1–42} induced a characteristic but cell-type-specific miRNA dysregulation, whereas cannabinoid treatments restored or reshaped these profiles to varying degrees. Notably, the combination treatment produced the most extensive regulatory shift in microglia, while CBD alone exerted the strongest effect in neurons. The predominance of upregulated miRNAs under cannabinoid treatment suggests activation of compensatory post-transcriptional programs associated with survival, stress mitigation and inflammatory resolution.

In **Tables 2** and **3**, miRNAs of biological interest were identified by comparing those dysregulated following A β _{1–42} exposure with those subsequently restored by cannabinoid treatments. These miRNAs were therefore selected for focused analysis, as they represent key regulatory nodes potentially involved in effects of A β _{1–42}

and the protective mechanisms activated by cannabinoids. CBD downregulated miR-592, miR-4787-3p, miR-548b-5p and miR-380-5p, all of which were strongly induced by A β _{1–42}. Conversely, miR-335-3p, miR-21-3p, miR-539-3p, miR-656-3p and miR-7974 were upregulated. CBG (10 μ M) showed a partially overlapping profile, affecting miR-592, miR-335-3p and miR-7974. The combined CBD CBG (10 μ M) + CBG (7,5 μ M) treatment (MIX) exhibited the broadest range of modulation, regulating miR-1246, miR-1260b, miR-378c, miR-4685-3p and miR-543 in addition to those targeted by the individual cannabinoids. Globally, cannabinoids induced a coordinated shift in miRNA

expression, involving regulators of inflammatory signalling, oxidative stress, and apoptosis, suggesting that CBD, CBG, and their combination remodel the ncRNA landscape disrupted by A β_{1-42} exposure.

Also, in SHSY-5Y treated with CBD (10 μ M), CBG (7,5 μ M) and their combination CBD (10 μ M) + CBG (5 μ M) significantly modulated several miRNAs dysregulated by A β_{1-42} in SH-SY5Y cells (**Table 3**). CBD (10 μ M) upregulated miR-103a-2-5p, miR-135a-5p, miR-19b-3p, miR-301a-3p, miR-4521, miR-500a-5p, miR-502-5p, and let-7f-1-3p, while downregulating novel_64. CBG (7,5 μ M) produced a largely overlapping regulatory profile, enhancing miR-103a-2-5p, miR-135a-5p, miR-19b-3p, miR-301a-3p, miR-4521, miR-500a-5p and miR-362-5p, and additionally modulating miR-7974. The combined treatment CBD(10 μ M) + CBG (5 μ M) resulted in positive modulation of the same main miRNAs, miR-103a-2-5p, miR-135a-5p, miR-19b-3p, miR-301a-3p, miR-4521 and miR-500a-5p. Overall, cannabinoid treatments produced coherent and partially overlapping miRNA signatures across the three conditions, affecting miRNAs associated with inflammatory signalling, apoptosis and oxidative stress regulation in SH-SY5Y neurons.

KEGG Enrichment Analysis

KEGG enrichment analysis of miRNA-predicted targets revealed that exposure of hMC3 microglia to A β_{1-42} induced a broad activation of apoptotic signalling pathways (Figure 16). Several components of the extrinsic apoptotic cascade, including Fas, TRAIL-R, FADD and TRADD, were predicted to be upregulated, indicating enhanced death receptor-mediated signalling. At the mitochondrial level, pro-apoptotic mediators such as Bid, Bak, Bim, NOXA and PUMA were enriched among upregulated targets, while anti-apoptotic regulators, including Bcl-2 and Bcl-xL tended to be downregulated. Pathway nodes linked to endoplasmic reticulum stress, PERK, ATF4 and CHOP, also showed positive enrichment, supporting the activation of unfolded protein response-associated apoptosis. Caspase-related modules (CASP3, CASP6, CASP7), together with mitochondrial destabilisation components such as cytochrome c and Apaf-1, further contributed to a transcriptional landscape consistent with enhanced apoptotic priming in A β -treated microglia.

In contrast, hMC3 cells treated with CBD (10 μ M) + CBG (10 μ M) showed a marked attenuation of this A β -driven apoptotic signature (Figure 17). Key pro-apoptotic genes,

including FAS, TNFRSF1A, CASP8, CASP3, BAK1 and BAX, were predicted to be downregulated, while anti-apoptotic or pro-survival factors such as BCL2, BCL2L1, MCL1 and BIRC3 were upregulated. The cannabinoid combination additionally reduced the expression of genes involved in mitochondrial outer membrane permeabilisation (MOMP) and DNA fragmentation, indicating a coordinated suppression of both the extrinsic and intrinsic apoptotic pathways and a shift toward a more cytoprotective transcriptional state.

A comparable pattern was observed in SH-SY5Y neurons. Exposure to A β ₁₋₄₂ (10 μ M) resulted in a robust activation of the KEGG apoptosis pathway (Figure 18), with upregulation of pro-apoptotic factors in both pathway branches. Genes such as BAX, BAK1, BID, BAD, CASP9, CASP3, FAS, TNFRSF1A, TRADD and FADD were consistently increased, while anti-apoptotic regulators (BCL2, BIRC2, BIRC3, MCL1) were downregulated. This pattern reflects the well-established mitochondrial dysfunction and caspase cascade activation elicited by A β toxicity.

Conversely, treatment of SH-SY5Y cells with CBD (10 μ M) + CBG (5 μ M) substantially reversed this profile (Figure 19). Pro-apoptotic components such as FAS, TNFRSF1A, TRADD, FADD, CASP8, CASP9, CASP3, BAX and BAK1 were downregulated, while BCL2, BCL2L1, MCL1, BIRC2 and BIRC3 were increased. The cannabinoid combination also reduced the predicted activation of MOMP, further supporting a suppression of mitochondrial apoptosis. Overall, the enrichment patterns demonstrate that CBD + CBG counteract A β -induced apoptotic signalling in both microglial and neuronal models, promoting a transcriptional environment that favours cell survival and mitochondrial protection.

DISCUSSION

The present study revealed that cannabidiol (CBD) and cannabigerol (CBG) have significant anti-inflammatory, anti-apoptotic, antioxidant and neurotrophic properties in both microglial (hMC3) and neuronal (SH-SY5Y) models of β -amyloid₁₋₄₂-induced neurotoxicity. The results consistently demonstrate that prolonged exposure to β -amyloid oligomers ($A\beta_{1-42}$) in both hMC3 microglial cells and the SH-SY5Y neuronal model reproduces many of the key pathological alterations that characterise Alzheimer's disease. This multifactorial response is consistent with canonical models of chronic microglial activation and neuronal damage, involving progressive activation of the NLRP3 inflammasome, increased IL-1 β levels, ROS production, impaired mitochondrial apoptosis, and autophagic dysfunction.

This chronic neuroinflammatory condition aligns with contemporary literature describing the interactions between microglia, oxidative stress, and neurodegeneration (Heneka et al., 2013). Neuroinflammation is a complex physiological response which, if prolonged or excessive, becomes destructive and exacerbates neurodegenerative processes.

However, it is important to recognise that, although the concentrations of $A\beta_{1-42}$ used are standard in the field and in line with current scientific literature, they probably induce a stronger and more synchronous inflammatory response than occurs physiologically.

The findings support the model that neuroinflammation is a self-amplifying process involving inflammation, oxidative stress and mitochondrial dysfunction (Cho et al., 2009). The increase in ROS observed in both cell lines following $A\beta_{1-42}$ exposure aligns with extensive evidence indicating that ROS act as upstream activators of the NLRP3 inflammasome, thereby promoting IL-1 β maturation and perpetuating a self-reinforcing cycle of neuroinflammation and cellular damage. This relationship is well supported by consolidated research showing that $A\beta$ -induced mitochondrial dysfunction leads to the release of mtROS and oxidised mtDNA, both of which serve as potent stimuli for NLRP3 activation (Vodovotz et al., 1996). Consistent with this framework, the proposed *in vitro* model reproduces these dynamics *in vitro*: in hMC3 cells, oxidative stress precedes or accompanies the upregulation of NLRP3 and the activation of Caspase-3, suggesting a functional interdependence between mitochondrial injury, inflammasome activation, and the progression of $A\beta$ -mediated neurotoxicity (Chiang et al., 2025).

A β oligomers act as DAMPs (damage-associated molecular patterns), activating pattern recognition receptors (PRRs) and triggering the innate immune response of microglia. Chronic exposure to A β ₁₋₄₂ resulted in a progressive and significant increase in NLRP3 and IL-1 β expression in both cell lines, indicating prolonged inflammasome activation and a chronic inflammatory phenotype. The activation of the NLRP3 inflammasome is a crucial event in the pro-inflammatory response of microglia, leading to the maturation and release of IL-1 β . Increased IL-1 β levels are well documented in AD and are associated with tau protein hyperphosphorylation, altered synaptic plasticity, and progressive cognitive decline (Alonso et al., 2018).

Transcriptional analyses and Western blots revealed strong induction of caspase-3, the main caspase executing the intrinsic apoptosis pathway, following stimulation with A β ₁₋₄₂ in the cell models studied. Another interesting finding relates to the results concerning LC3B and Beclin. A β ₁₋₄₂ induces an increase in the LC3B II/I ratio and Beclin levels, which suggests either an accumulation of autophagosomes or a blockage in the autophagic flux. Inflammasome hyperactivation leads to autophagy impairment, resulting in amplification of NLRP3 signalling (Muñoz-Planillo et al., 2013).

The findings support the hypothesis that cannabinoids can mitigate multiple mechanisms of neurodegeneration that are relevant to Alzheimer's pathology. However, several mechanistic aspects require cautious interpretation, as *in vitro* responses cannot be linearly translated to *in vivo* systems.

Both CBD and CBG have demonstrated anti-inflammatory, anti-apoptotic, antioxidant and neurotrophic properties in models stimulated by A β .

Suppression of NLRP3 and IL-1 β transcription, which has been confirmed at the protein level for NLRP3, suggests interference with the events that control upstream inflammasome activation.

One possible mechanism is the stabilisation of mitochondrial function and reduction of ROS, given the strong correlation between oxidative stress and NLRP3 activation. However, the current data do not analyse the temporal sequence of these events directly, so alternative interpretations remain possible.

CBD and CBG were found to significantly attenuate A β -induced inflammatory signalling, with the combined treatment consistently outperforming the individual compounds. The suppression of *NLRP3* and *IL-1 β* transcription, which was confirmed at

the protein level for NLRP3, suggests interference with the events that control the assembly of the inflammasome.

In line with this, a reduction in ROS was observed in both hMC3 and SH-SY5Y cells, confirming the correlation between oxidative stress and *NLRP3* activation. Nevertheless, the current data do not analyse the temporal sequence of these events directly, and alternative interpretations remain possible. CBD and CBG acted by restoring the *BAX/BCL2* balance, inhibiting the transcription of both initiator (*CASP9*) and executor (*CASP3*) caspases, and reducing Caspase-3 protein levels. These convergent results suggest the intrinsic apoptotic pathway is blocked. Cannabinoid treatments restored LC3B II/I ratios and normalised Beclin levels, suggesting an improvement in autophagic clearance or relief from autophagy blockade. It is plausible, and methodologically correct to emphasise, that the anti-apoptotic effects are downstream of the reduction in oxidative stress and inflammasome, rather than independent. The research results indicate that cannabidiol (CBD) and cannabigerol (CBG) have significant multi-target neuroprotective effects against A β 1-42-induced cytotoxicity. In parallel, the expression of Brain-Derived Neurotrophic Factor (*BDNF*), a key element for neuronal survival, synaptic plasticity and memory, which is typically reduced under conditions of A β stress and neuroinflammation, was also significantly increased by cannabinoids in the SH-SY5Y model. The CBD + CBG combination produced an increase of more than 20 times higher than the control. Exposure to A β 1-42 resulted in significant structural damage, as evidenced by a reduction in DBN protein levels in SH-SY5Y neuronal cells. Since DBN is essential for stabilising dendritic spines (the loss of which is an early event in Alzheimer's disease pathogenesis), its decline indicates severe damage to the actin cytoskeleton and structural plasticity. CBD and CBG, particularly when used together, significantly restored DBN expression and neuronal morphology. The recovery of DBN and cytoskeletal morphology provides strong functional evidence that cannabinoid treatment helps to preserve neuronal structure under amyloid stress.

Across all results, the combination of CBD and CBG showed the strongest protective effects consistently, suggesting additive or synergistic interactions. Overall, these findings suggest that CBD and CBG have a multi-target neuroprotective effect, which is consistent with the idea that complex neurodegenerative conditions could benefit more from pleiotropic modulators than from single-target drugs.

However, it is necessary to recognise the inherent limitations of immortalised cell lines, supraphysiological A β concentrations and static exposure conditions before extrapolating therapeutic implications. Despite these limitations, the simultaneous action of CBD and CBG on trophism, synaptic structure and inflammatory pathways support their potential application as pleiotropic modulators in the treatment of complex neurodegenerative conditions.

Differential Expression Analysis identified a set of miRNA of biological interest, by comparing those dysregulated following A β_{1-42} exposure with those subsequently restored by cannabinoid treatments. Several miRNAs showed a characteristic pattern in which their expression was significantly altered in response to A β -induced inflammation and then reverted, partially or fully, toward control levels after treatment with CBD, CBG, or their combination.

The miRNAs modulated by cannabinoids converge on several pathways central to neuroinflammation, oxidative stress and apoptotic control. Many belong to regulatory networks involving p53, PI3K/AKT, TGF- β /Wnt, FOXO3 and caspase-8.

miR-380-5p, a direct negative regulator of p53 (Swarbrick et al., 2010), was reduced by CBD and MIX, suggesting restoration of p53-mediated apoptotic surveillance suppressed by A β_{1-42} . miR-335-3p, miR-592 and miR-1260b target components of TGF- β /Wnt, FOXO3 and caspase-8, respectively, and therefore influence apoptotic and stress-response pathways (Han et al., 2024). The consistent downregulation of miR-592 by all treatments is particularly relevant, as this miRNA represses FOXO3, a key transcription factor controlling antioxidant defences and inflammation; its overexpression has been associated with exacerbated oxidative stress (Fu et al., 2022).

Cannabinoid-induced reduction of miR-592 is therefore consistent with the observed decrease in ROS and inflammatory mediators. Similarly, miR-21-3p, which promotes NF- κ B signalling and PI3K/AKT activation through suppression of PTEN (Sheedy, 2015), was significantly reduced by CBD and MIX, counteracting an important pro-inflammatory axis engaged by A β_{1-42} . Members of the miR-19 family and miR-543, all converging on PTEN and PI3K/AKT (Rivas et al., 2021; Scheper et al., 2023), were also reshaped by cannabinoid exposure, supporting a global attenuation of PI3K/AKT-driven inflammatory signalling.

The pro-apoptotic and antioxidant effects of cannabinoids are further supported by changes in miR-380-5p, which participates in redox-regulatory circuits (Wang et al., 2021), and by upregulation of miR-335-3p, known to suppress PI3K/AKT survival signalling (Bano et al., 2025).

Among inflammation-associated miRNAs, miR-539-3p, an inhibitor of LRP6 and Wnt signalling (Tripathi et al., 2022), was strongly upregulated by CBD and MIX, potentially counteracting Wnt hyperactivation linked to neuroinflammatory states. Additionally, MIX increased miR-4685-3p, which may affect MMP9 activity (Ma et al., 2024), suggesting cannabinoid-mediated modulation of matrix remodelling and extracellular inflammatory processes. Altogether, these results indicate that cannabinoids exert their neuroprotective effects not through a single pathway but by reconfiguring a network of miRNAs that collectively regulate p53-dependent apoptosis, FOXO3-driven oxidative stress responses, and PI3K/AKT- and Wnt-mediated inflammatory signalling. This multilayered mechanism aligns with the broad biochemical and morphological protective effects observed in A β ₁₋₄₂-treated cells.

The miRNA modulation observed in SH-SY5Y cells exposed to cannabinoids reveals a regulatory landscape consistent with attenuation of A β ₁₋₄₂-induced neurotoxicity.

Several of the miRNAs upregulated by CBD and CBG, including miR-103a-2-5p, miR-135a-5p, miR-19b-3p, miR-301a-3p, miR-4521 and miR-500a-5p, are involved in neuronal stress adaptation pathways or inflammatory control (Mingardi et al., 2023) (Feng et al., 2019). For instance, miR-19 family members regulate PTEN and thereby modulate PI3K/AKT signalling, a pathway disrupted early in Alzheimer's disease and strongly linked to synaptic failure and neuronal vulnerability (Li et al., 2019).

Their modulation by cannabinoids suggests a potential rebalancing of this central survival pathway. Moreover, miR-135a-5p and miR-301a-3p have been implicated in apoptotic regulation and cytoskeletal homeostasis, processes particularly sensitive to A β -induced toxicity (Mingardi et al., 2023).

A β exposure triggers p53 activation, BAX translocation and caspase-dependent neuronal apoptosis (Zhang et al., 2021), and modulation of p53-related miRNAs, including miRNAs that intersect with p53 or FOXO3 networks, may reflect a compensatory mechanism aimed at restricting excessive programmed cell death.

Exposure to A β ₁₋₄₂ elicited a remarkably similar pro-apoptotic profile across both microglial (hMC3) and neuronal (SH-SY5Y) models. In both cell types, the peptide initiated the extrinsic death-receptor pathway by activating FAS and TNFRSF1A and then propagating through TRADD and FADD to activate caspase-8.

This was accompanied by parallel amplification of the intrinsic mitochondrial apoptotic process, as indicated by the activation of BID, BAX and BAK1, the release of cytochrome c, and the subsequent activation of caspase-9. Meanwhile, key anti-apoptotic regulators, including BCL2, BCL2L1, MCL1 and members of the BIRC family, were consistently suppressed, further tipping the cellular balance towards apoptosis. In microglia, this response also extended to ER stress-associated apoptosis, reinforcing the view that A β induces a multidimensional apoptotic process that destabilises both the innate immune system and neuronal compartments.

Treatment with CBD + CBG reversed these pathological signatures in both cell types. The cannabinoid combination was associated with a reversal of predicted apoptotic signalling and executioner caspases, restored mitochondrial stability by upregulating BCL2 family survival factors and decreased markers associated with MOMP and downstream DNA fragmentation. This coordinated suppression of extrinsic and intrinsic apoptosis suggests that cannabinoids act at multiple hierarchical levels of the apoptotic cascade rather than targeting a single node.

Overall, integrating results from both models reveals that A β ₁₋₄₂ triggers a comprehensive apoptotic response in microglia and neurons, while CBD and CBG together elicit a unifying protective effect, reestablishing survival signals and counteracting molecular pathways associated with apoptosis. This dual-cell-type protection highlights the potential of cannabinoid combinations as multi-target modulators capable of mitigating key degenerative processes relevant to Alzheimer's disease.

Despite the biological coherence of the observed miRNA patterns, some important limitations must be acknowledged. First, although several miRNAs reached nominal statistical significance, they did not withstand correction for multiple testing using False Discovery Rate (FDR) adjustment. This statistical constraint indicates that the present findings should be interpreted as exploratory and hypothesis-generating rather than definitive. Independent validation in larger datasets will be required to confirm the robustness of these associations.

Furthermore, the miRNA–target interactions identified in this study rely upon bioinformatic predictive algorithms and established literature, rather than direct functional verification. While the enrichment of predicted targets within the p53, PI3K/AKT, FOXO3, and Wnt signalling pathways aligns with current biological paradigms, these regulatory axes remain to be experimentally demonstrated. Future investigations utilizing luciferase reporter assays or gain- and loss-of-function experiments are requisite to empirically confirm these putative interactions.

CONCLUSION

This study demonstrates that cannabidiol (CBD) and cannabigerol (CBG) reduce several aspects of amyloid-beta ($A\beta_{1-42}$) induced toxicity in both neuronal and microglial cell models. These effects include the reduction of neuroinflammation, oxidative stress, mitochondrial dysfunction, apoptosis, altered autophagy and cytoskeletal damage. Cannabinoids do not simply act as anti-inflammatories; their response profile involves multiple functional levels, which is consistent with current interpretations of the pathophysiology of Alzheimer's disease as discussed in the reference literature. While each compound exerted a measurable protective effect, their co-administration produced the most pronounced results in terms of molecular, biochemical and morphological parameters.

Overall, the data suggest that CBD and CBG provide multidimensional protection in the AD cell model and that a combination of the two is more effective than individual treatments, indicating additive or potentially synergistic interactions. However, in the absence of dedicated quantitative analyses, the nature of this interaction cannot be formally defined.

While these results outline a simultaneous mitigation of oxidative, inflammatory, apoptotic, and autophagic processes, they require cautious interpretation as the responses observed *in vitro* are not directly translatable to *in vivo* or clinical systems. Future studies should clarify the mechanisms underlying the interaction between CBD and CBG, explore the temporal dynamics of molecular events modulated by treatments and verify their reproducibility in primary cells and animal models of AD.

Despite these limitations, the obtained evidence reinforces the rationale for investigating cannabinoids as potential modulators of the early neuroinflammatory and neurodegenerative processes associated with amyloid pathology.

GENE NAME	SEQUENCE
GAPDH HUMAN	Primer F GAG TCA ACG GAT TTG GIC GT Primer R TTG ATT TTG GAG GGA TCT CG
NRF2 HUMAN	Primer F AGA CTC CAA GGC GGT TGA A Primer R TGG GCA CTT GGA GTT GAA CT
IL-1 β HUMAN	Primer F GGG CCT CAA GGA AAA GAA TC Primer R TTC TGC TTG AGAGGT GCT GA
TNF-a HUMAN	Primer F TCCTTC AGA CAC CCT CAA CC Primer R AGG CCC CAG TTT GAA TTC TT
IL-6 HUMAN	Primer F TTT TCT GCC AGT GCC TCT TT Primer R TAC CCC CAG GAG AAG ATT CC
CASPASE-3	Primer F GCA GAG TCA AAG GCT GGA AC Primer R GAG TCA ACC AAG TTC GCA CA
CASPASE-9	Primer F GAT GCT GTC CCC TAT CAG GA Primer R GGG ACT GCA GGT CTT CAG AG

Table 1. Primers used for quantitative PCR.

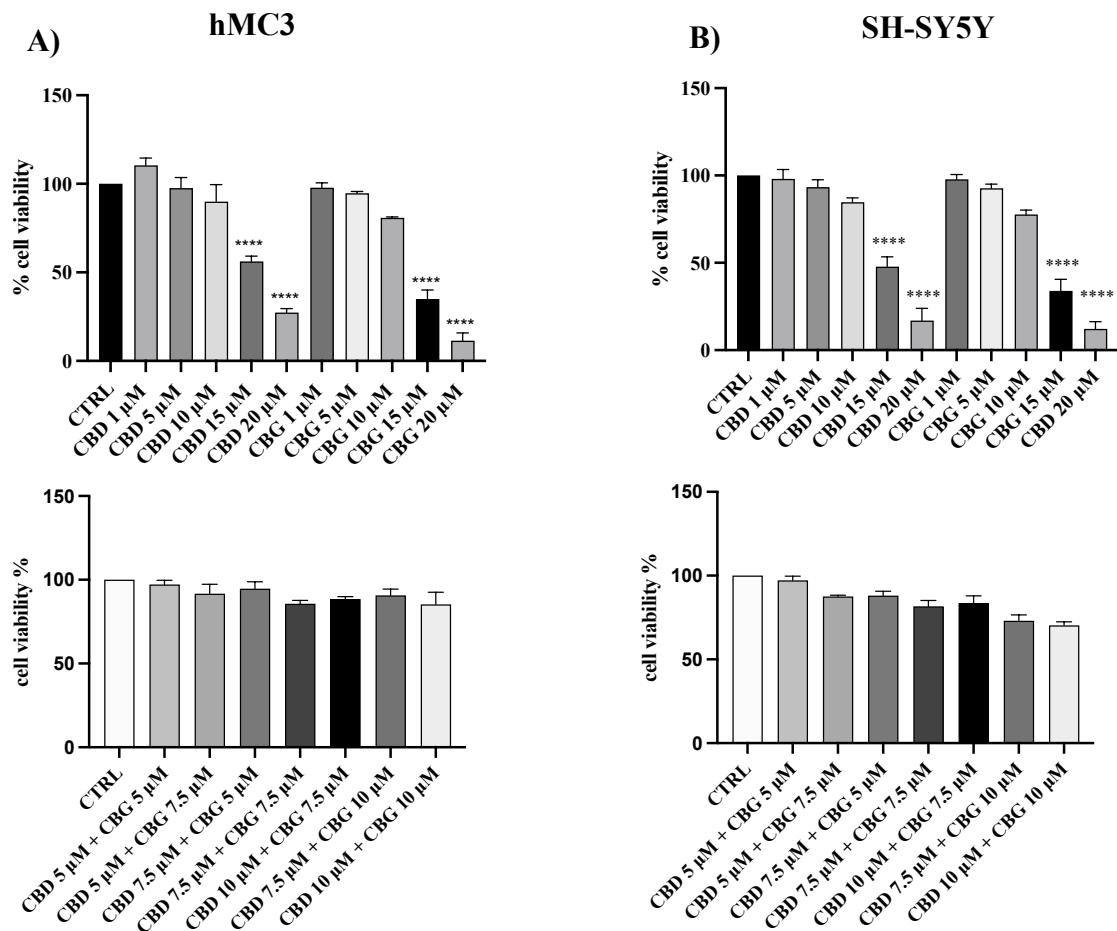


Figure 1. MTT assay following 24 h of incubation with CBD (1-20 μ M), CBG (1-20 μ M) alone and in combination in both hMC3 (A) and SH-SY5Y (B).

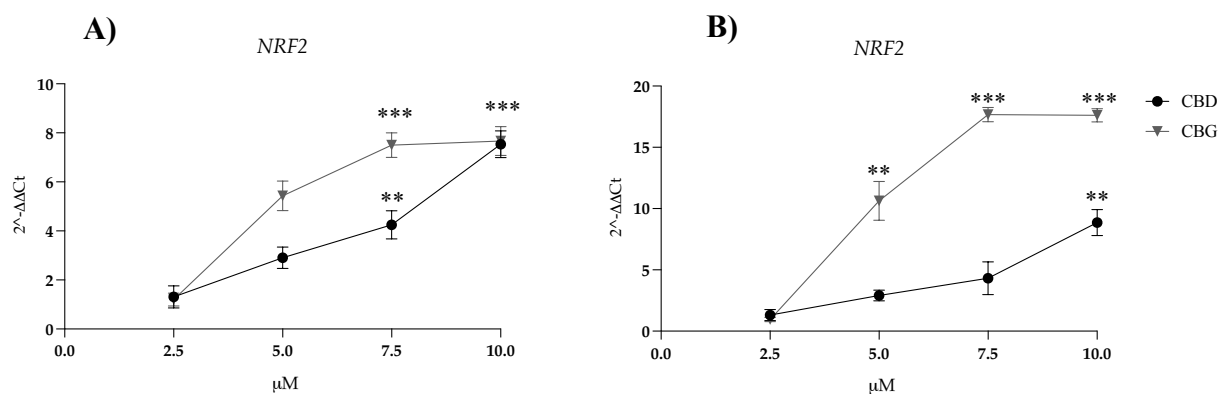


Figure 2. Dose-response curves of NRF2 transcript expression following treatment with increasing concentrations of CBD or CBG.

NRF2 mRNA levels were quantified by RT-qPCR and expressed as $2^{-\Delta\Delta C_t}$ relative to untreated controls in hMC3 (A) and SH-SY5Y (B).

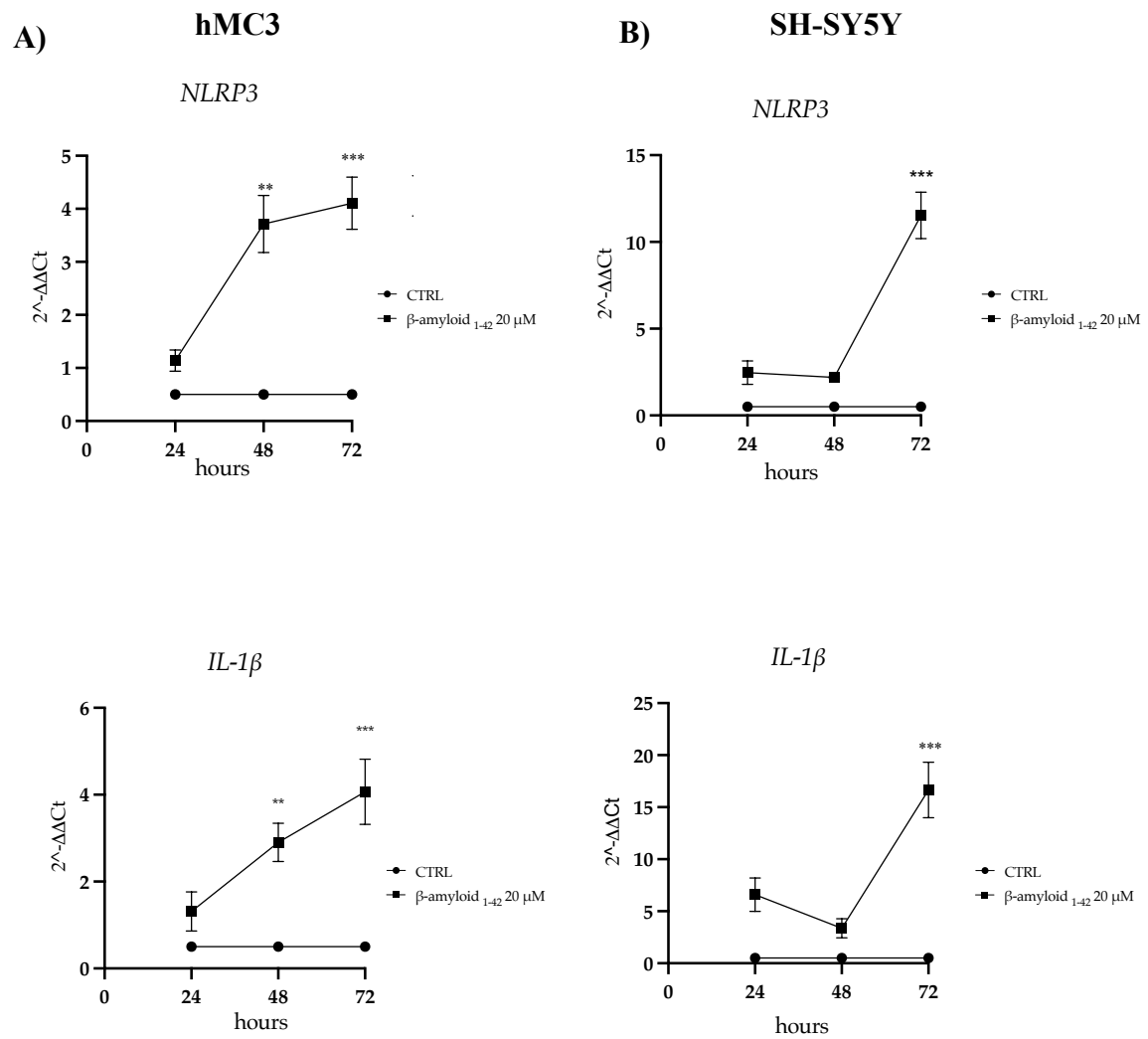


Figure 3. Chronic β -amyloid exposure induces a time-dependent upregulation of inflammasome components.

Gene expression analysis of NLRP3 and IL-1 β in hMC3 (A) and SH-SY5Y (B) exposed to β -amyloid₁₋₄₂ (20 μ M) for 24, 48, and 72 hours. Data are expressed as $2^{-\Delta\Delta C_t}$ relative to untreated control cells (CTRL). Values are presented as mean $\pm$ SEM from three independent experiments.

hMC3

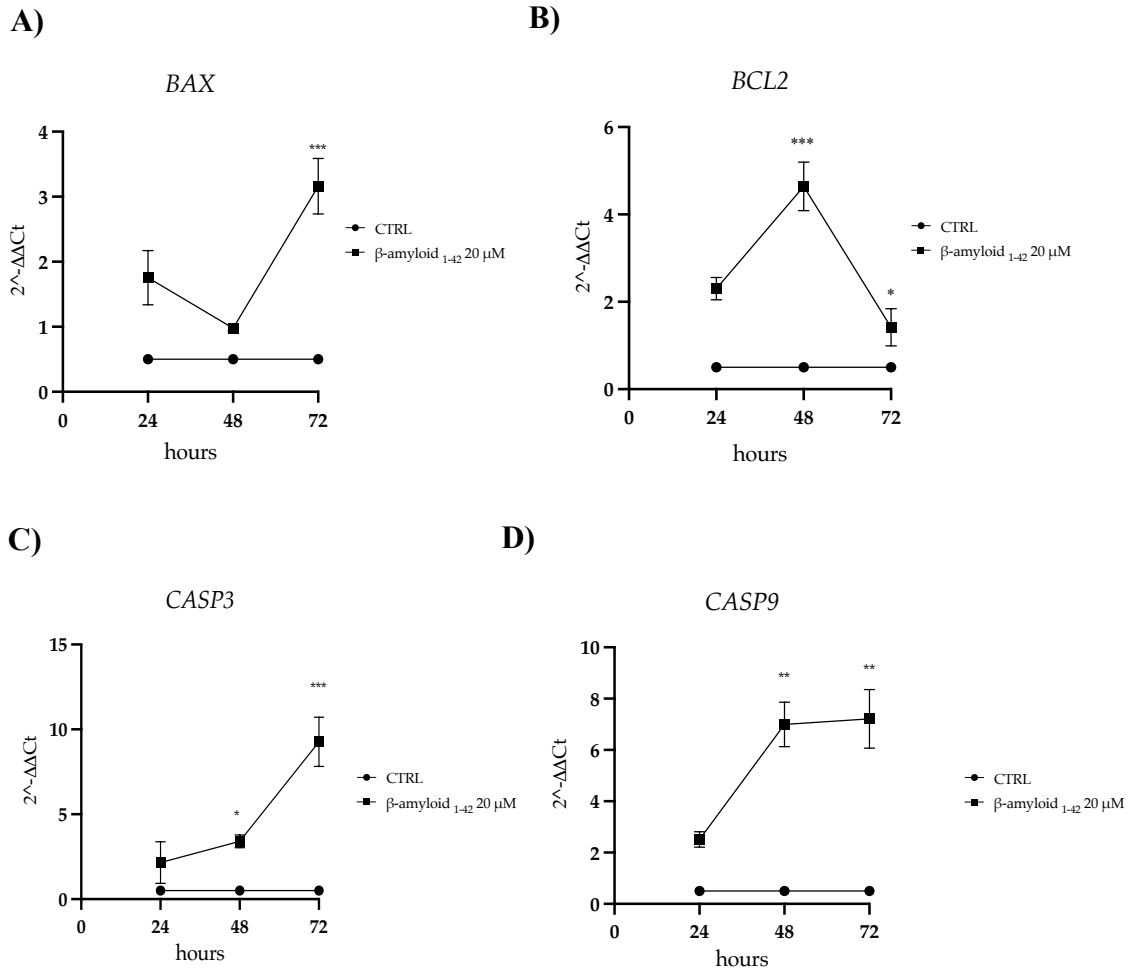


Figure 4. β -amyloid triggers a time-dependent modulation of apoptotic gene expression in hMC3 microglial cells.

Expression levels of pro-apoptotic **A) BAX**, **C) CASP3**, **D) CASP9** and anti-apoptotic **C) BCL2** genes in hMC3 cells exposed to β -amyloid₁₋₄₂ (20 μ M) for 24, 48, and 72 hours. Data are expressed as $2^{-\Delta\Delta C_t}$ relative to untreated control cells (CTRL). Values are presented as mean $\pm$ SEM from three independent experiments.

SH-SY5Y

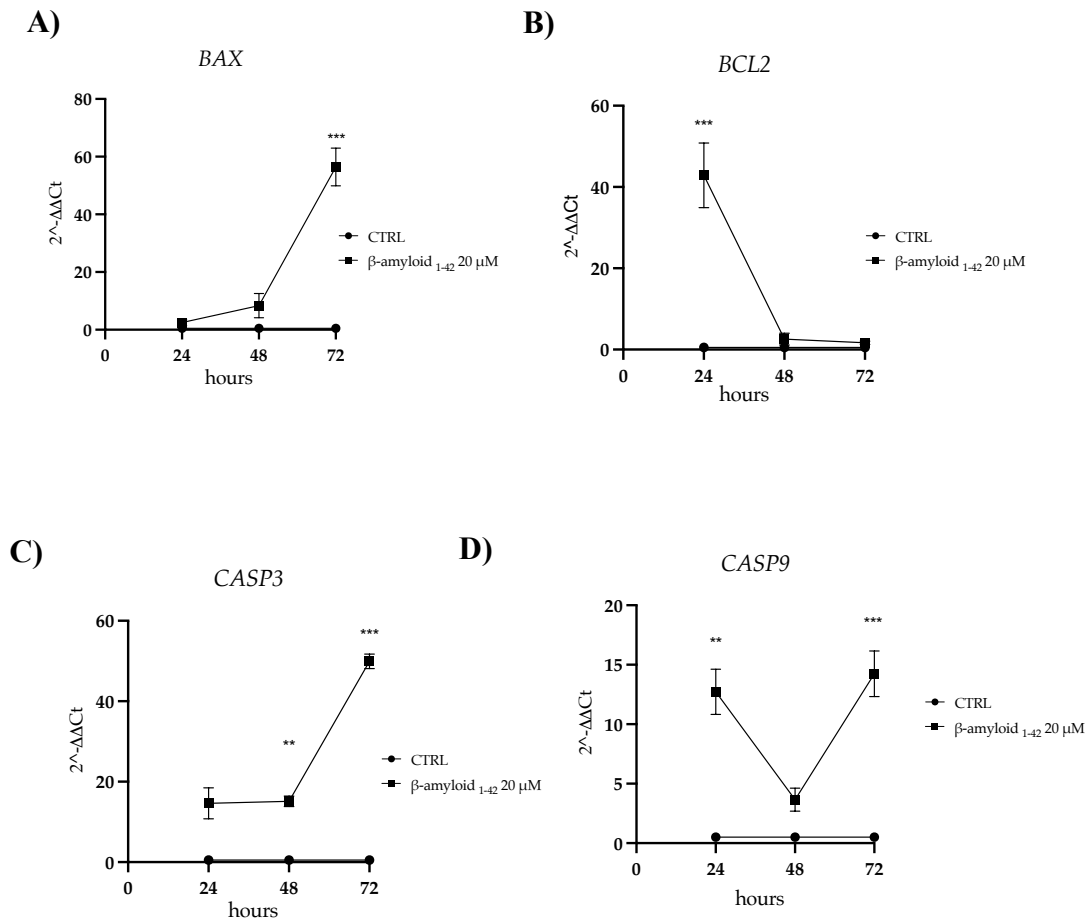


Figure 5. β -amyloid triggers a time-dependent modulation of apoptotic gene expression in SH-SY5Y cells.

Expression levels of pro-apoptotic **A) BAX**, **C) CASP3**, **D) CASP9** and anti-apoptotic **C) BCL2** genes in hMC3 cells exposed to β -amyloid₁₋₄₂ (20 μ M) for 24, 48, and 72 hours. Data are expressed as $2^{-\Delta\Delta Ct}$ relative to untreated control cells (CTRL). Values are presented as mean $\pm$ SEM from three independent experiments.

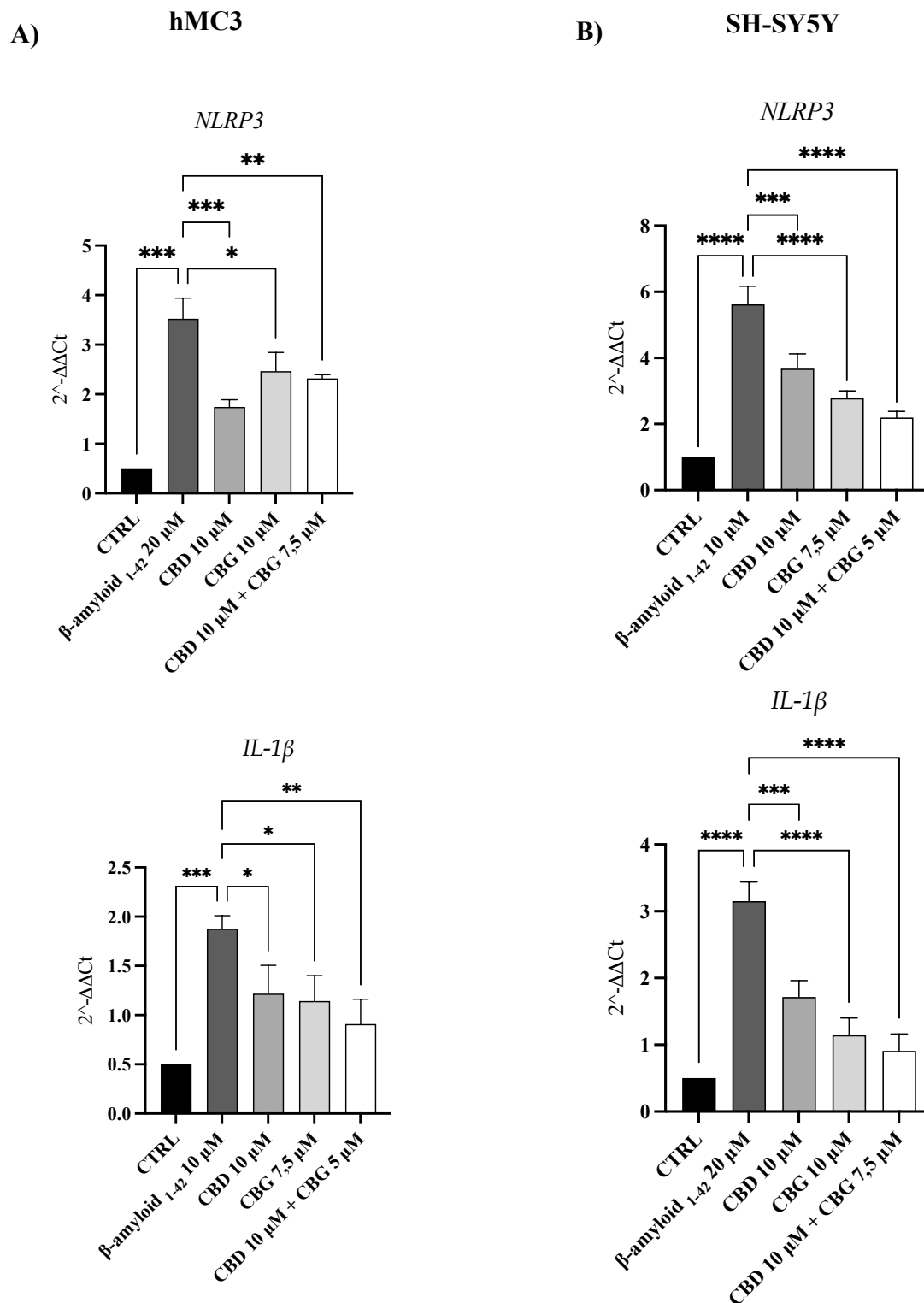


Figure 6. Gene expression analysis of inflammasome-related targets in cells exposed to β -amyloid $_{1-42}$ and treated with cannabinoids.

The graph reports the mRNA expression levels of *NLRP3* and *IL-1 β* in hMC3 (A) and SH-SY5Y (B). Data are expressed as $2^{-\Delta\Delta C_t}$ relative to untreated control cells (CTRL). Values are presented as mean $\pm$ SEM from three independent experiments.

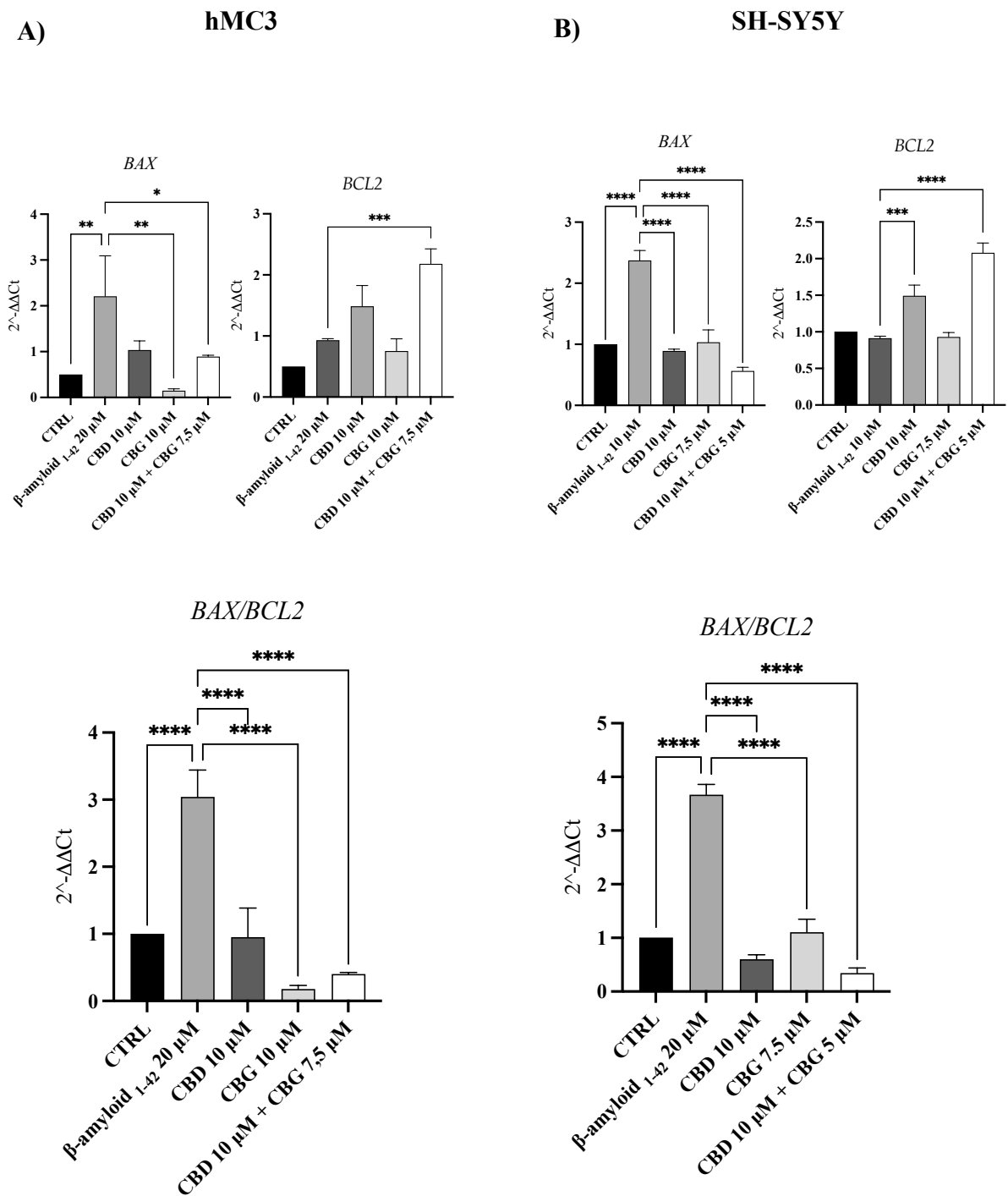


Figure 7. Gene expression analysis of BAX and BCL-2 in cells exposed to β -amyloid₁₋₄₂ and treated with cannabinoids.

The graph reports the mRNA expression levels of BAX, BCL2 and related ratio in hMC3 (A) and SH-SY5Y (B). Data are expressed as $2^{-\Delta\Delta C_t}$ relative to untreated control cells (CTRL). Values are presented as mean $\pm$ SEM from three independent experiments.

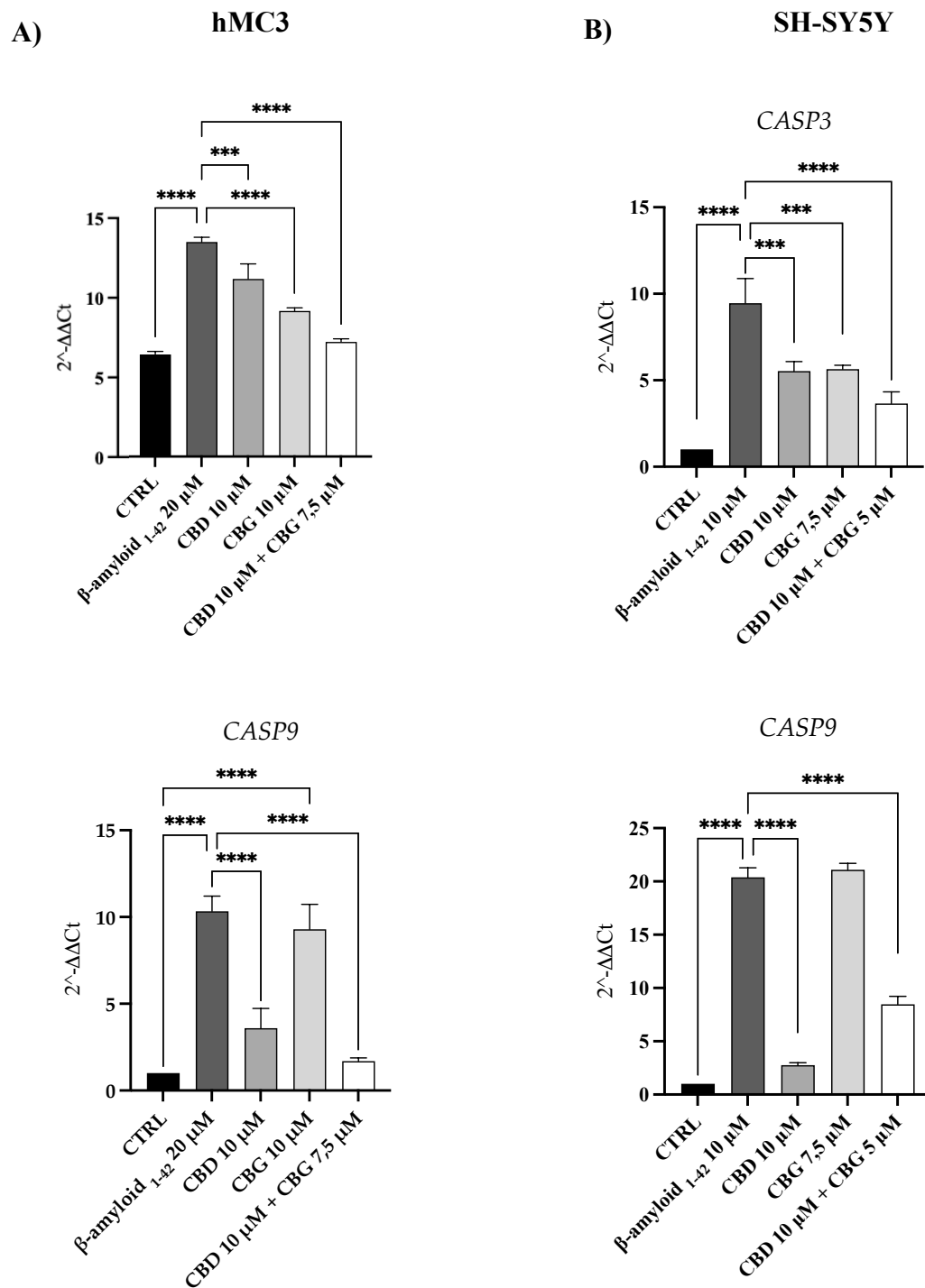


Figure 8. Gene expression analysis of apoptosis-related caspase in cells exposed to β -amyloid and treated with cannabinoids.

The graph shows the relative mRNA expression levels of CASP3 and CASP9 in hMC3 (A) and in SH-SY5Y (B). Data are expressed as $2^{-\Delta\Delta Ct}$ relative to untreated control cells (CTRL). Values are presented as mean $\pm$ SEM from three independent experiments.

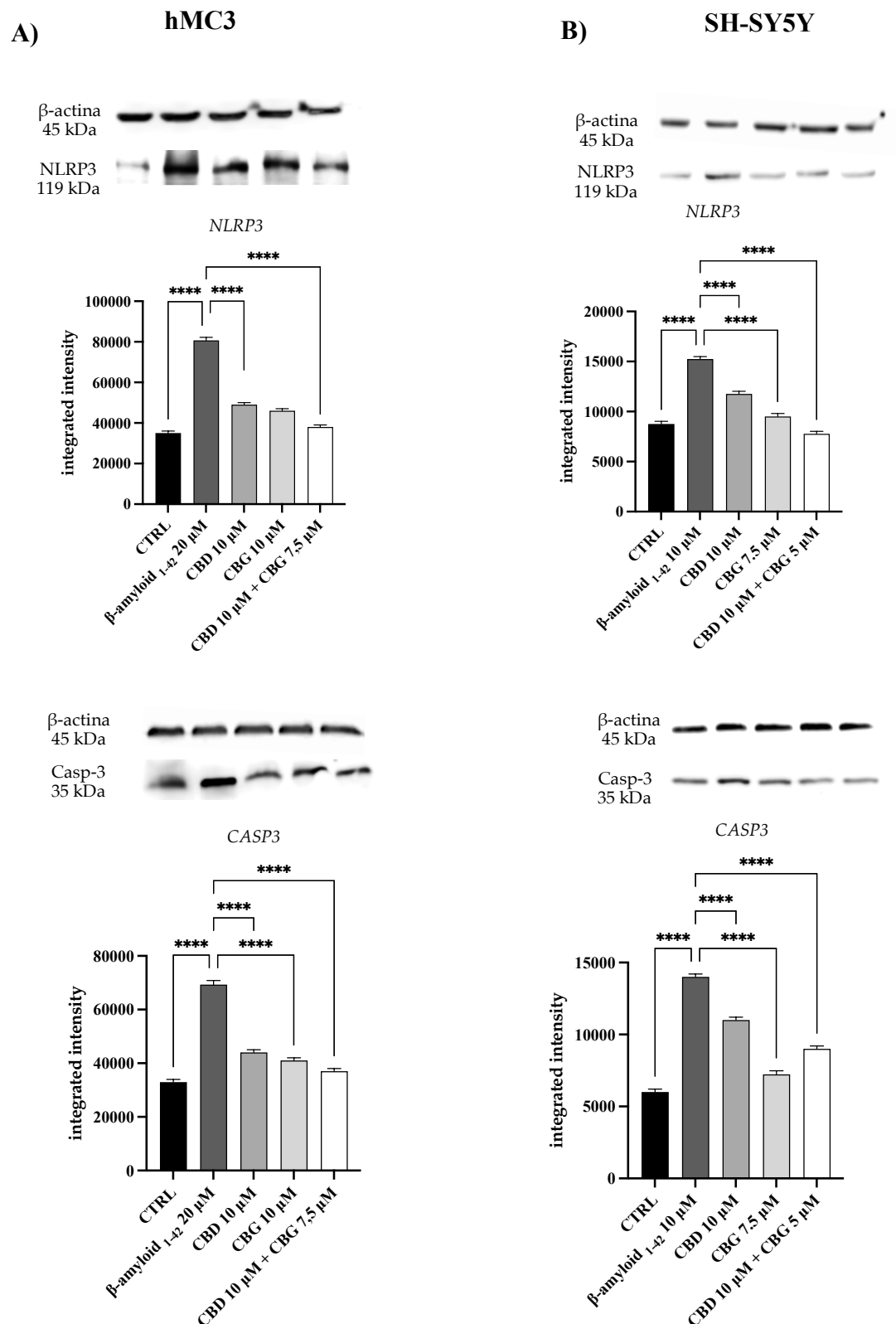


Figure 9, Western Blot Analysis of Autophagy and Inflammasome Markers in hMC3 (A) and SH-SY5Y (B) cell lines triggered with β -amyloid₁₋₄₂ and treated with cannabinoids.

The expression levels of two inflammasome components, NLRP3, Caspase-3 (CASP3), were analysed via Western Blot to evaluate the effects of β -amyloid₁₋₄₂ (20 μ M) and the potential neuroprotective effects of cannabinoids. B-actin was used as a loading control for all panels.

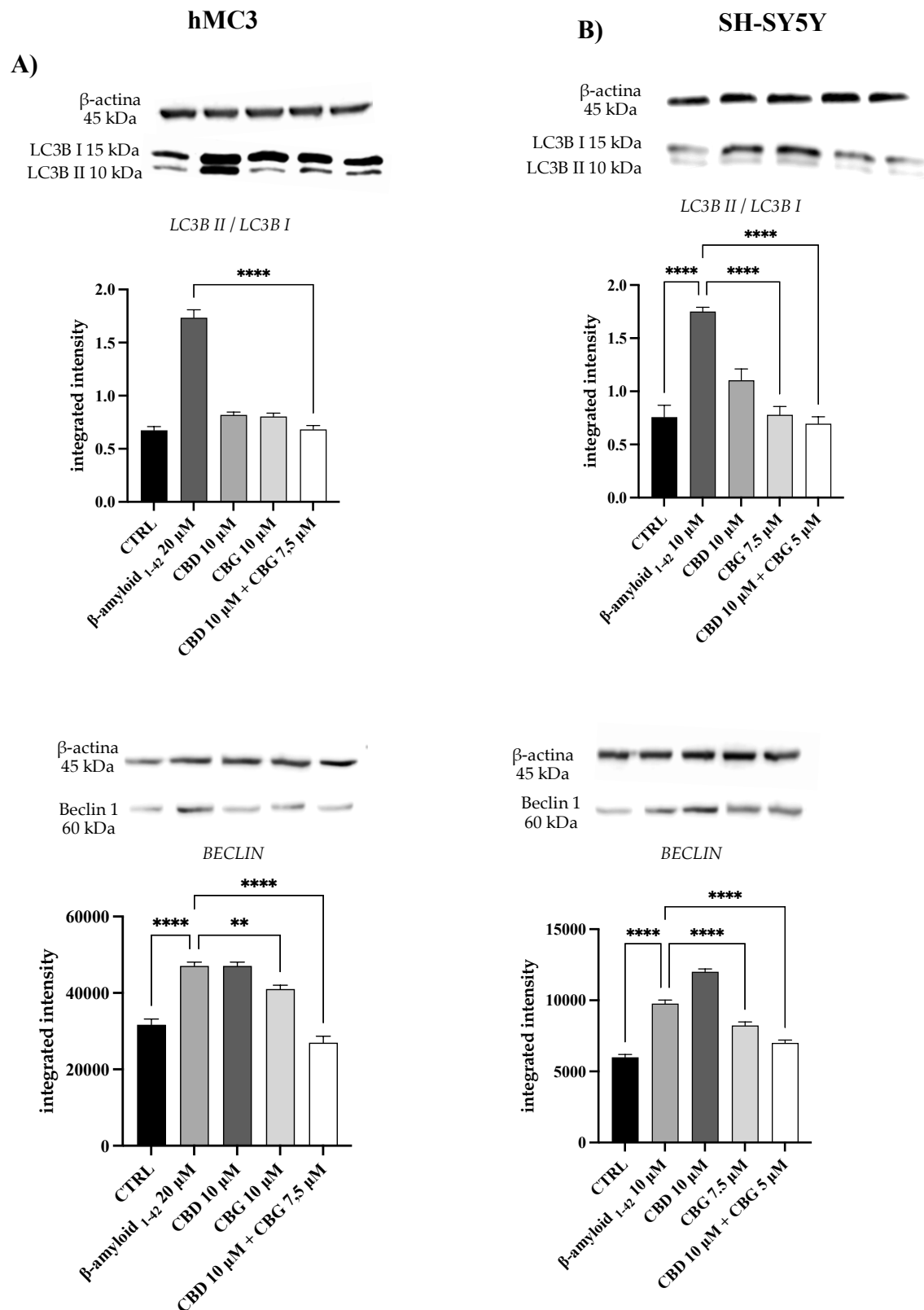


Figure 10. Western Blot Analysis of Autophagy and Inflammasome Markers hMC3 (A) and SH-SY5Y (B) cell lines triggered with β -amyloid₁₋₄₂ and treated with cannabinoids.

The expression levels of two autophagy key targets Beclin, and LC3B I/II were analysed via Western Blot to evaluate the effects of β -amyloid₁₋₄₂ (20 μ M) and the potential neuroprotective effects cannabinoids. B-actin was used as a loading control for all panels.

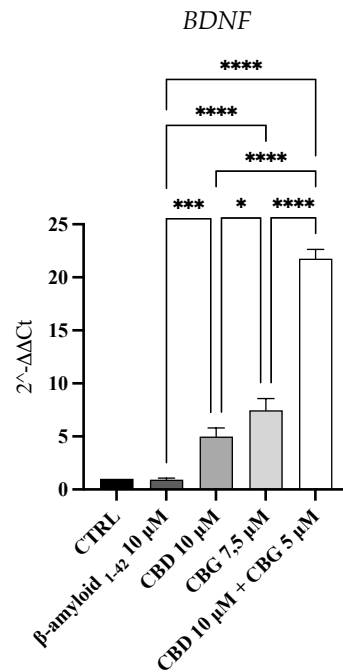


Figure 12. BDNF mRNA expression in SH-SY5Y cells following β -amyloid stimulation and cannabinoids treatments. The graph shows the relative mRNA expression levels of BDNF in SH-SY5Y cells. Data are expressed as $2^{-\Delta\Delta C_t}$ relative to untreated control cells (CTRL). Values are presented as mean $\pm$ SEM from three independent experiments.

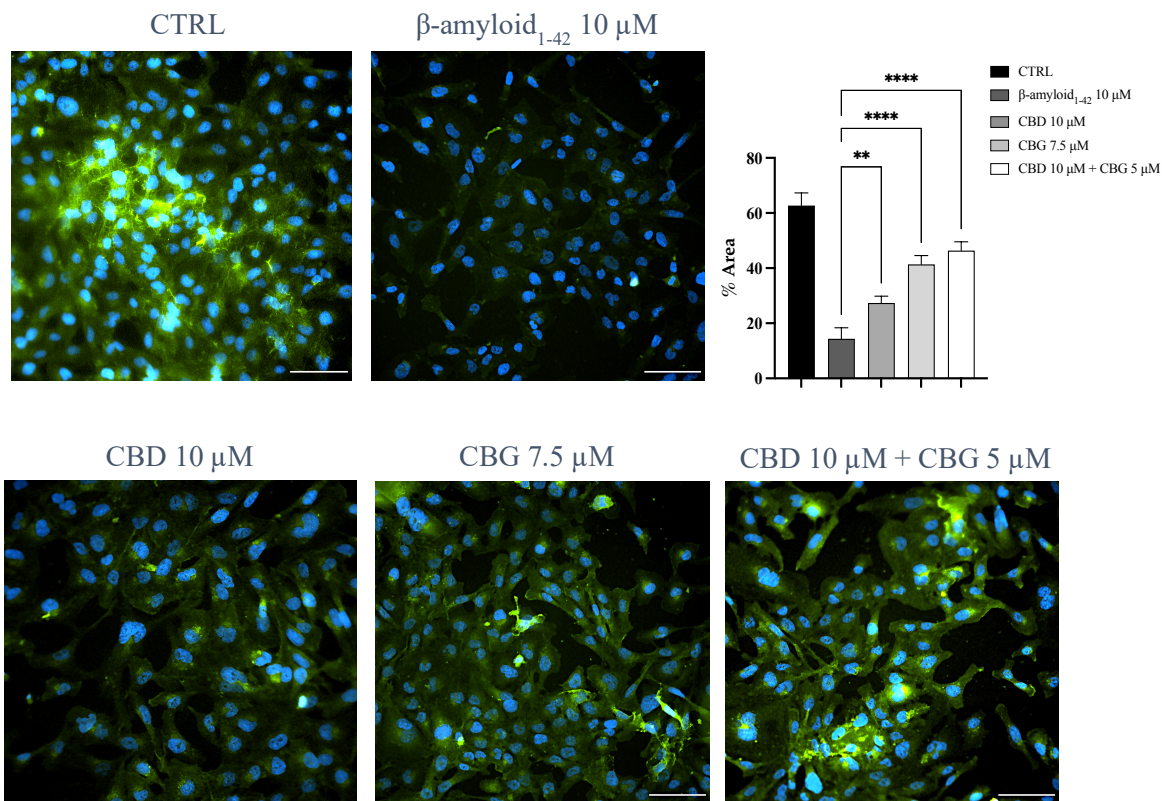


Figure 13. Cannabinoid-mediated protection from β -amyloid induced DBN loss in SH-SY5Y cells.

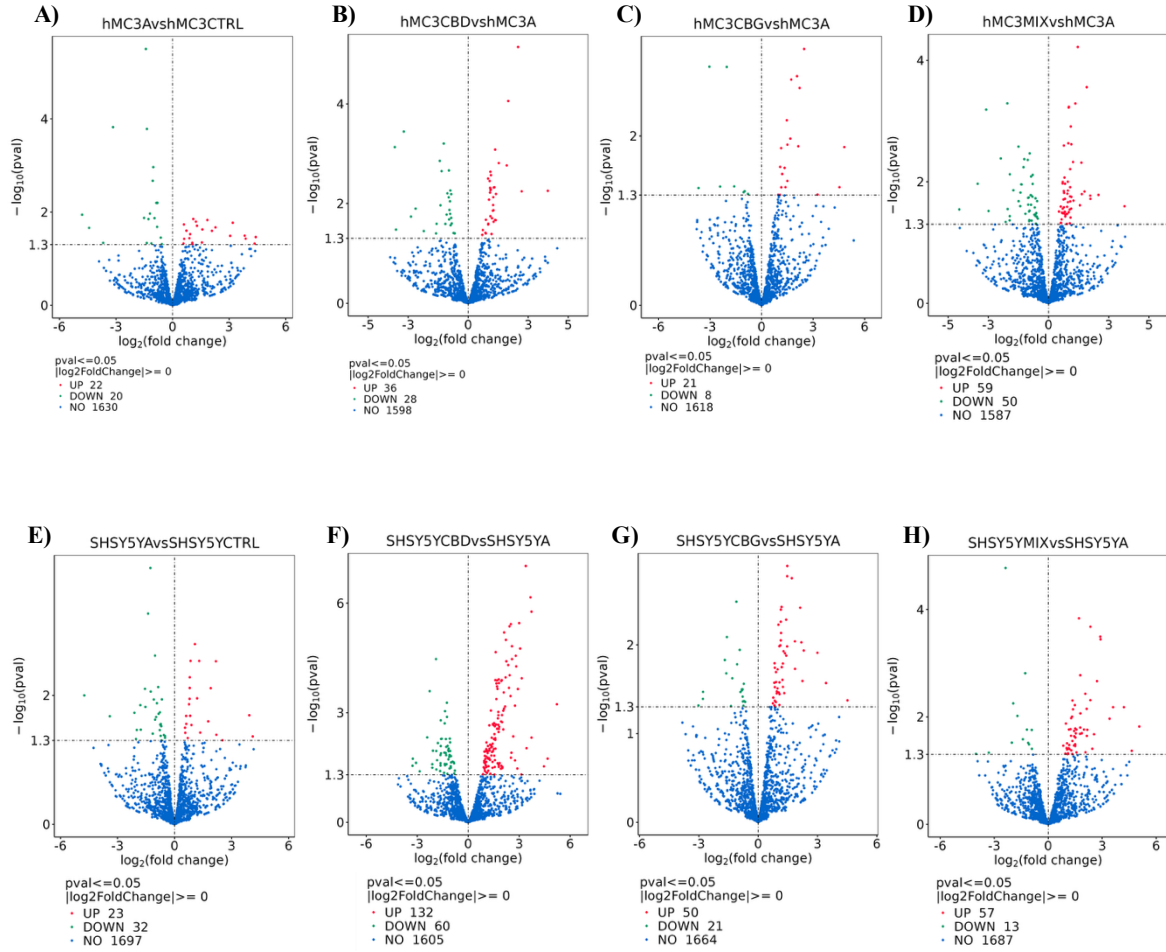


Figure 14. Volcano plots of differential comparisons in HMC3 and SHSY5Y. Each point represents a gene; the x-axis shows $\log_2(\text{fold change})$, while the y-axis represents $-\log_{10}(\text{p-value})$. Red dots indicate significantly up-regulated genes ($p < 0.05$), green dots indicate significantly down-regulated genes, while blue dots represent non-significant genes. The eight panels show, respectively: **A)** hMC3A vs hMC3CTRL (effect of stimulation with $A\beta_{1-42}$), **B)** hMC3CBD vs hMC3A (effect of treatment with CBD), **C)** hMC3CBG vs hMC3A (effect of treatment with CBG), **D)** hMC3MIX vs hMC3A (combined effect of CBD+CBG), **E)** SHSY-5YA vs SHSY-5YCTRL (effect of stimulation with $A\beta_{1-42}$), **F)** SHSY-5YCBD vs SHSY-5YA (effect of treatment with CBD), **G)** SHSY-5YCBG vs SHSY-5YA (effect of treatment with CBG), and **H)** SHSY-5YMIX vs SHSY-5YA (combined effect of CBD+CBG). The number of up-/down-regulated genes in each comparison is shown in the figure.

Treatment	miRNA	log ₂ FoldChange with β - Amyloid	log ₂ FoldChange with β -Amyloid + Treatment
CBD (10 μ M)	hsa-miR-194-3p	1,582928401	-1,578999663
CBD (10 μ M)	hsa-miR-21-3p	-1,200186783	1,529699818
CBD (10 μ M)	hsa-miR-335-3p	-1,413765704	1,073562284
CBD (10 μ M)	hsa-miR-380-5p	-1,125531504	1,376085209
CBD (10 μ M)	hsa-miR-4787-3p	3,056354055	-3,60826676
CBD (10 μ M)	hsa-miR-539-3p	-3,155319241	2,672778785
CBD (10 μ M)	hsa-miR-548b-5p	1,236973973	-1,385341649
CBD (10 μ M)	hsa-miR-592	3,192455745	-3,215375204
CBD (10 μ M)	hsa-miR-656-3p	-1,509926239	1,349674193
CBD (10 μ M)	hsa-miR-7974	-1,021311665	1,091409739
CBG (10 μ M)	hsa-miR-1257	-1,358756804	1,673844317
CBG (10 μ M)g	hsa-miR-335-3p	-1,413765704	1,140727494
CBG (10 μ M)	hsa-miR-592	3,192455745	-3,037465479
CBG (10 μ M)	hsa-miR-7974	-1,021311665	1,730146342
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-1246	1,296362861	-1,400877809
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-1260b	1,098228579	-1,245417387
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-194-3p	1,582928401	-1,927101541
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-21-3p	-1,200186783	1,072814151
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-1246	1,296362861	-1,400877809
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-1260b	1,098228579	-1,245417387
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-21-3p	-1,200186783	1,072814151
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-335-3p	-1,413765704	1,002230763
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-378c	1,047024649	-1,33769429
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-380-5p	-1,125531504	1,060469106
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-4685-3p	2,088655755	-2,037308709
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-539-3p	-3,155319241	2,486327502
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-543	-1,049990076	1,108575346
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-592	3,192455745	-3,530838139
CBD(10 μ M) + CBG (7,5 μ M)	hsa-miR-656-3p	-1,509926239	1,902361246

Table 2. Differential expression of miRNAs following treatment with CBD, CBG, or their combination (mix), relative to β -amyloid and control conditions in A) hMC3.

The column *log₂FoldChange Treatment* reports the differential expression of each miRNA in the indicated treatment condition (CBD, CBG, or mix) relative to A β ₁₋₄₂-stimulated cells. The column *log₂FoldChange β -Amyloid* displays the expression change of the same miRNAs relative to untreated control cells. Positive values indicate upregulation, negative values indicate downregulation. miRNA included showed p value < 0.05.

Treatment	miRNA	log2FoldChange with β -Amyloid + Treatment	log2FoldChange with β -Amyloid
CBD(10 μ M)	hsa-let-7f-1-3p	-4,749428097	4,457809764
CBD(10 μ M)	hsa-miR-103a-2-5p	-1,531182262	3,664879912
CBD(10 μ M)	hsa-miR-135a-5p	-1,149372986	1,657005241
CBD(10 μ M)	hsa-let-7f-1-3p	-4,749428097	4,457809764
CBD(10 μ M)	hsa-miR-103a-2-5p	-1,531182262	3,664879912
CBD(10 μ M)	hsa-miR-19b-3p	-1,984884606	1,80212982
CBD(10 μ M)	hsa-miR-301a-3p	-2,112501185	2,823325355
CBD(10 μ M)	hsa-miR-4521	-1,391567943	1,112146363
CBD(10 μ M)	hsa-miR-500a-5p	-1,023772367	2,092730692
CBD(10 μ M)	hsa-miR-502-5p	-1,554857294	2,17097681
CBD(10 μ M)	novel 64	1,29686787	-1,601415341
CBD(10 μ M)	hsa-miR-4521	-1,391567943	1,112146363
CBG (7,5 μ M)	hsa-miR-103a-2-5p	-1,531182262	2,123213696
CBG (7,5 μ M)	hsa-miR-500a-5p	-1,023772367	2,092730692
CBG (7,5 μ M)	hsa-miR-135a-5p	-1,149372986	1,700669351
CBG (7,5 μ M)	hsa-miR-19b-3p	-1,984884606	2,995067001
CBG (7,5 μ M)	hsa-miR-301a-3p	-2,112501185	2,186220557
CBG (7,5 μ M)	hsa-miR-362-5p	-1,0905642	1,249764251
CBG (7,5 μ M)	hsa-miR-4521	-1,391567943	2,302899239
CBG (7,5 μ M)	hsa-miR-500a-5p	-1,023772367	1,227350387
CBG (7,5 μ M)	hsa-miR-4521	-1,391567943	2,302899239
CBG (7,5 μ M)	hsa-miR-7974	-1,269387688	1,146020303
CBD(10 μ M) + CBG (5 μ M)	hsa-miR-103a-2-5p	-1,531182262	2,894918926
CBD(10 μ M) + CBG (5 μ M)	hsa-miR-135a-5p	-1,149372986	1,567485993
CBD(10 μ M) + CBG (5 μ M)	hsa-miR-19b-3p	-1,984884606	1,79163855
CBD(10 μ M) + CBG (5 μ M)	hsa-miR-301a-3p	-2,112501185	2,341468648
CBD(10 μ M) + CBG (5 μ M)	hsa-miR-4521	-1,391567943	1,438483267
CBD(10 μ M) + CBG (5 μ M)	hsa-miR-500a-5p	-1,023772367	1,501787811

Table 2. Differential expression of miRNAs following treatment with CBD, CBG, or their combination (mix), relative to β -amyloid and control conditions in B) SHSY5Y.

The column *log₂FoldChange Treatment* reports the differential expression of each miRNA in the indicated treatment condition (CBD, CBG, or mix) relative to A β ₁₋₄₂-stimulated cells. The column *log₂FoldChange β -Amyloid* displays the expression change of the same miRNAs relative to untreated control cells. Positive values indicate upregulation, whereas negative values indicate downregulation. This dual comparison highlights treatment-induced miRNA modulation in contrast to β -amyloid-driven dysregulation. miRNAincluded showed p value < 0.05.

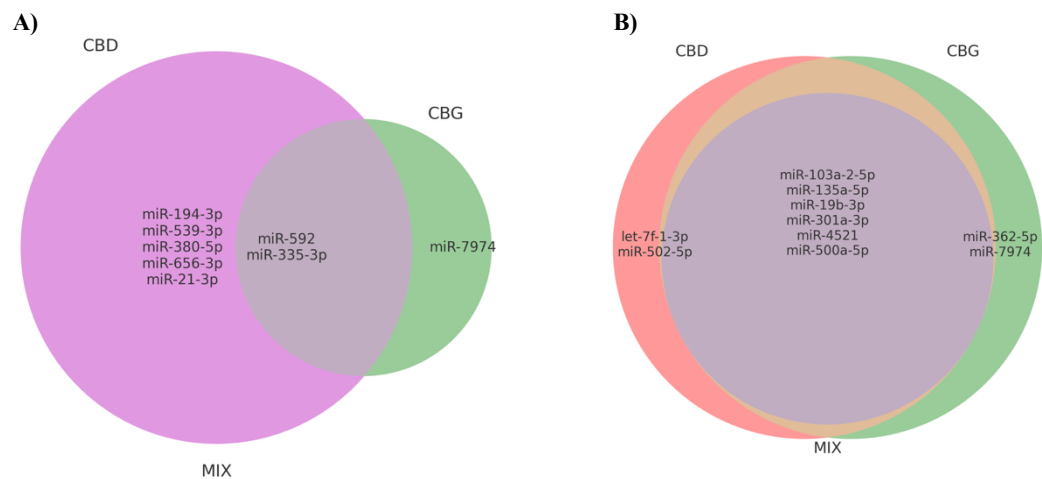


Figure 15. Venn diagram showing the overlap of significantly regulated miRNAs ($p < 0.05$) in hMC3 cells (A) and (B) SHSY-5Y treated with CBD, CBG, or the combined treatment (MIX).

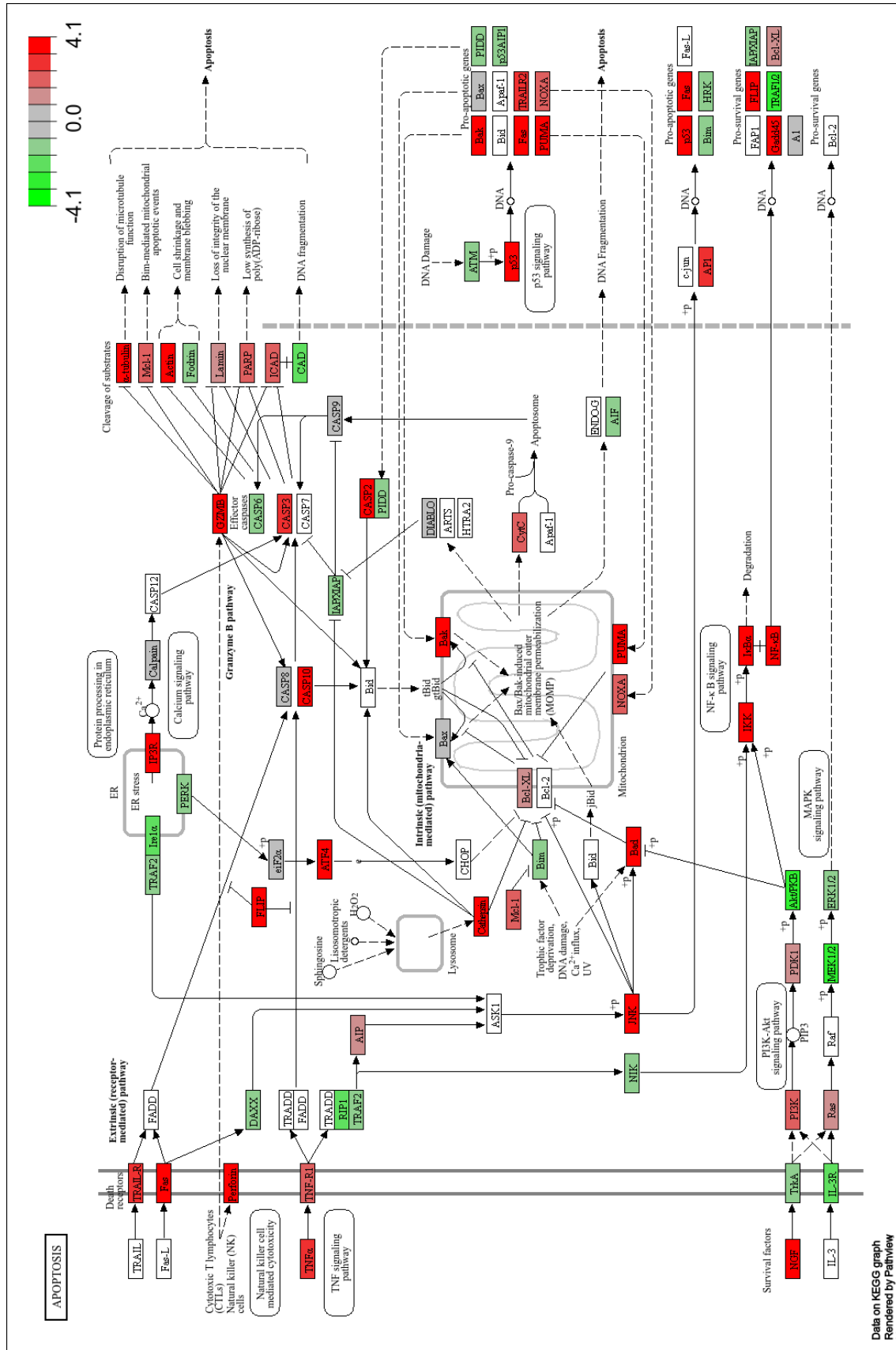


Figure 18. KEGG Apoptosis pathway enrichment in SH-SY5Y cells treated with $A\beta_{1-42}$ (10 μ M) compared to untreated controls.

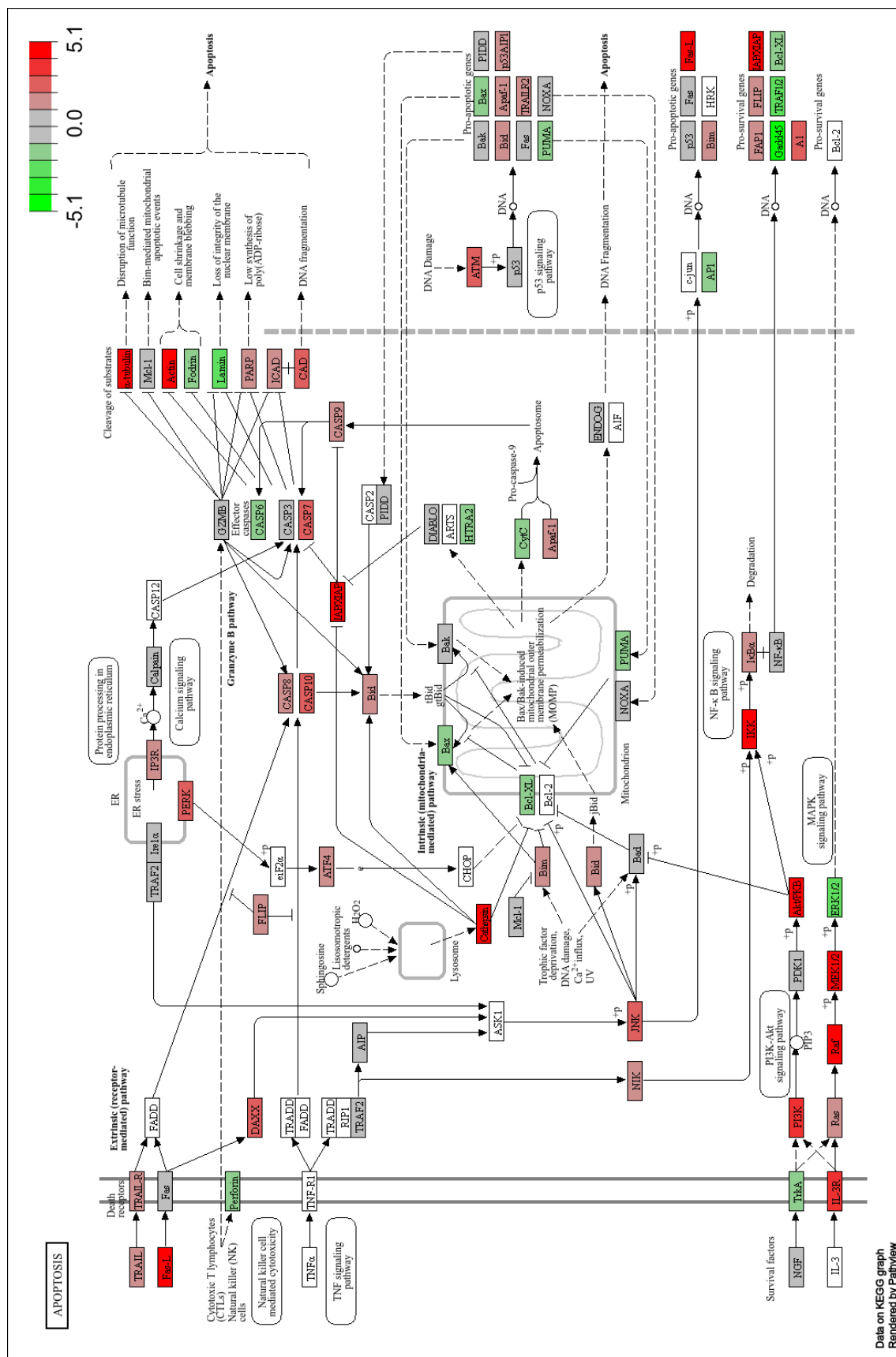


Figure 19. KEGG Apoptosis pathway enrichment in SH-SY5Y cells treated with CBD (10 μ M) + CBG (5 μ M) compared to A β ₁₋₄₂ (10 μ M) conditions.

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