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DIRECTOR Prof. KATIA VARANI

# PEMFs as a Neuroprotective Strategy: Restoration of Mitochondrial function and CREB/BDNF Pathways in an *in vitro* Model of Alzheimer's Disease

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# ABBREVIATIONS LIST

**AChEI:** Acetylcholinesterase Inhibitors

**AD**: Alzheimer's Disease

**ADLs**: Activities of Daily Living

**AICD**: APP Intracellular Domain

**Akt**: Protein Kinase B

**ANOVA**: Analysis of Variance

**APOE**: Apolipoprotein E

**APP**: Amyloid Precursor Protein

**ARIA:** Amyloid Related Imaging Abnormalities

**ASOs**: Antisense Oligonucleotides

**AT(N)**: Amyloid, Tau, and Neurodegeneration

**Aβ**: β-Amyloid

**BACE:** β-site APP-Cleaving Enzyme

**BBB**: Blood–Brain Barrier

**Bcl-2**: B-cell lymphoma 2

**BDNF**: Brain-Derived Neurotrophic Factor

**BPSD:** Behavioral and Phychological Symptoms of Dementia

**BSA**: Bovine Serum Albumin

**CAA**: Cerebral Amyloid Angiopathy

**cAMP**: Cyclic Adenosine Monophosphate

**CAT**: Catalase

**CDR:** Clinical Dementia Rating

**CP**: Click Peptide

**CREB**: cAMP-Response Element Binding

**CSF:** Cerebrospinal Fluid

**CT:** Computed Tomography

**DCFH-DA**: 2',7'-Dichloro-Dihydrofluorescein

**DCF:** Diclofenac

**DMEM**: Dulbecco's Modified Eagle Medium

**ELF-MFs:** Extremely Low Frequency Magnetic Fields

**EMA**: European Medicines Agency

**EOAD**: Early-onset AD

**EOFAD**: Early-onset familial AD

**ERK1/ERK2**: Extracellular Regulated Kinase 1/2

**FBS**: Fetal Bovine Serum

**FDA**: Food and Drug Administration

**FDG-PET:** Fluorodeoxyglucose Positron Emission Tomografy

**GFAP:** Glial Fibrillary Acidic Protein

**GPx:** Glutathion Peroxidase

**H-LIPEF:** High-Frequency Low-Intensity Pulsed Electric Fields

**$H_2$DCFDA**: 2′,7′-Dichlorodihydrofluorescein Diacetate

**$H_2O_2$**: Hydrogen Peroxide

**HS**: Horse serum

**IL-1β:** Interleukin-1β

**IL-6:** Interleukin-6

**JC-1**: Tetraethyl-Benzimidazolyl-Carbocyanine Iodide

**LPS**: Lipopolysaccharide

**LTP**: Long-Term Potentiation

**MABs**: Monoclonal Antibodies

**MAMs**: Mitochondria-Associated ER Membranes

**MAPK**: Mitogen-Activated Protein Kinase

**MCI**: Mild Cognitive Impairment

**MMP**: Mitochondrial Membrane Potential

**MMSE**: Mini-Mental State Examination

**MoCA:** Montreal Cognitive Assessment

**MRI:** Magnetic Resonance Imaging

**MTS**: [3-(4,5-Dimethylthiazole-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)- 2H-Tetrazolium

**NfL**: Neurofilament Light chain

**NFTs**: Neurofibrillary Tangles

**NGF**: Nerve Growth Factor

**NMDA**: N-Methyl-D-Aspartate

**NSAID**: Non-Steroidal Anti-Inflammatory Drug

**p38 MAPK**: p38 Mitogen-Activated Protein Kinase

**PBS**: Phosphate Buffered Saline

**PDE4**: Phosphodiesterase-4

**PEMFs**: Pulsed Electromagnetic Fields

**PET:** Positron Emission Tomography

**pGlu3- Aβ:** pyroglutamate-modified form of Aβ

**PI3K**: Phosphatidylinositol 3-kinase

**PKA:** Protein Kinase A

**PROTACs:** Proteolysis-Targeting Chimeras

**PSEN1**: Presenilin 1

**PSEN2**: Presenilin 2

**ROS**: Reactive Oxygen Species

**RPMI 1640**: Roswell Park Memorial Institute 1640

**RT**: Room Temperature

**sAPPα**: Soluble APPα

**sAPPβ**: Soluble APPβ

**SDI**: Socio-Demographic Index

**SEM**: Standard Error of Mean

**SOD:** Superoxide Dismutase

**SP:** Senile Plaque

**TBI**: Traumatic Brain Injury

**TNF- α:** Tumor Necrosis Factor- α

**TREM 2:** Triggering Receptor Expressed on Myeloid Cells 2

**TrkB**: Tropomyosin receptor kinase B

**VGCCs:** Voltage-gated calcium channels

**WHO**: World Health Organization

**Δψm**: Mitochondrial Membrane Potential

# ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive impairment and neuronal loss driven by β-amyloid (Aβ) accumulation, oxidative stress, neuroinflammation, and mitochondrial dysfunction. Dysregulation of signalling pathways such as ERK1/2 and the cAMP/CREB/BDNF axis further contributes to impaired neuronal survival and plasticity. Given the limited efficacy and high cost of current therapeutic approaches, non-pharmacological strategies capable of targeting multiple pathological mechanisms are of increasing interest. Pulsed electromagnetic fields (PEMFs) have recently emerged as a potential neuroprotective tool, with evidence suggesting beneficial effects on mitochondrial integrity, oxidative balance, and neurotrophic signalling.

This thesis investigated the neuroprotective potential of PEMFs in an in vitro model of AD using NGF-differentiated PC12 cells exposed to hydrogen peroxide ($H_2O_2$) or a β-amyloid analogue (CP). Cells were treated with PEMFs (1.5 mT, 75 Hz, 1.3 ms) for 24 hours. Experimental endpoints included cell viability, reactive oxygen species (ROS) production, catalase activity, mitochondrial membrane potential, apoptotic markers, and activation of ERK1/2, cAMP, CREB, and BDNF pathways. Pharmacological inhibitors were employed to clarify the contribution of specific signalling cascades.

PEMF exposure significantly mitigated $H_2O_2$- and Aβ-induced cytotoxicity, restoring mitochondrial function, reducing ROS accumulation, and preventing apoptosis. At the molecular level, PEMFs attenuated ERK1/2 phosphorylation while enhancing cAMP production, CREB activation, and BDNF expression. Comparative analyses with diclofenac indicated that PEMFs exert broader, multi-target effects relative to conventional anti-inflammatory treatments.

Overall, the findings demonstrate that PEMFs modulate key stress-response systems involved in oxidative and amyloidogenic injury, supporting their potential as a non-pharmacological strategy for promoting neuronal survival. The results provide mechanistic insights into PEMF-induced neuroprotection and offer a foundation for future preclinical and clinical investigations in the context of AD.

# INTRODUCTION

## *Alzheimer's Disease*

### *An Overview*

*Alzheimer's disease (AD)* is a progressive neurodegenerative disorder and the leading cause of dementia worldwide (Minter et al., 2016). First described by the German psychiatrist Alois Alzheimer in 1906, the disease was initially identified through the case of Auguste Deter, a 51-year-old woman who exhibited short-term memory loss, phobic behaviors, and spatiotemporal disorientation. Postmortem examination revealed cortical atrophy and abnormal brain deposits, later recognized as *Senile Plaques (SPs)* and *Neurofibrillary Tangles (NFTs)* (Maurer et al., 1997; Strassnig & Ganguli, 2005). These pathological hallmarks are central to AD pathogenesis. Clinically, AD is characterized by a gradual decline in cognitive and behavioral functions, including memory, comprehension, reasoning, language, attention, and judgment (Kumar et al., 2024). Early symptoms typically involve episodic memory loss and language difficulties, progressing to impairments in thinking, attention, and behavior such as apathy, aggression, and depression (Braak et al., 2011; Bateman et al., 2012). As AD progresses, the ongoing degeneration of neurons extends to additional brain regions, resulting in a growing dependence on caregivers for daily activities. In the advanced stages, the impairment of areas responsible for essential bodily functions leads to complete loss of autonomy and, ultimately, to a fatal outcome (Ganguli et al., 2005; Tom et al., 2015).

Among all forms of dementia, AD is the most prevalent, responsible for over half of cases in the global population aged 65 and older (Karantzoulis & Galvin, 2011; Kumar et al., 2024).

AD is considered a multifactorial disorder, with advanced age being the most significant risk factor; its incidence doubles approximately every five years after age 65, resulting in late-onset AD (Qiu et al., 2009; Kumar et al., 2024). Although rare, early-onset AD can occur before age 65, accounting for about 5% of cases and often presenting with atypical symptoms that complicate diagnosis and accelerate disease progression (Mendez, 2017). To better understand the complexity of AD and the rationale behind emerging therapeutic strategies, it is essential to explore its epidemiological trends, underlying pathophysiological mechanisms, and clinical manifestations.

## *Epidemiology*

Over a century after its first description, AD has become one of the most prevalent forms of dementia, representing an urgent global health challenge, with the number of affected individuals projected to more than double by 2050 (Figure1) (Nichols et al., 2022; Alzheimer's Association, 2023). The incidence rate of AD increases almost exponentially with advancing age, although it remains unclear whether this trend continues indefinitely or plateaus in the oldest age groups (Qiu et al., 2009). Epidemiological data indicate that AD is more common in women than in men, a difference not fully explained by the greater longevity of females, as the pathological processes underlying AD begin decades before clinical symptoms emerge (Scheyer et al., 2018; Zhu et al., 2021). Several factors, including differences in brain structure and genetic background, are thought to contribute to this increased susceptibility in women (Laws et al., 2018; Zhu et al., 2021). Recent projections estimate that by 2050, the global population affected by AD could reach 132 million, with the steepest increases expected in low- and middle-income countries where healthcare systems may already be under strain (Zhang XX et al., 2021).

Globally, AD is currently the seventh leading cause of death (WHO). In 2019, the economic cost of dementia exceeded $1.3 trillion, and is projected to reach $2.8 trillion by 2030, including direct medical costs, social care, and informal caregiving (Alzheimer Disease International, 2025). Although some predictive models suggest a slight decline in age-standardized incidence and mortality rates, the absolute number of cases will continue to grow due to demographic aging, especially in countries with a high *Socio-Demographic Index (SDI)* (Liu et al., 2025). Regions with higher SDI values tend to show greater burdens of AD, emphasizing the need for targeted public health strategies (Zhang N et al., 2025).

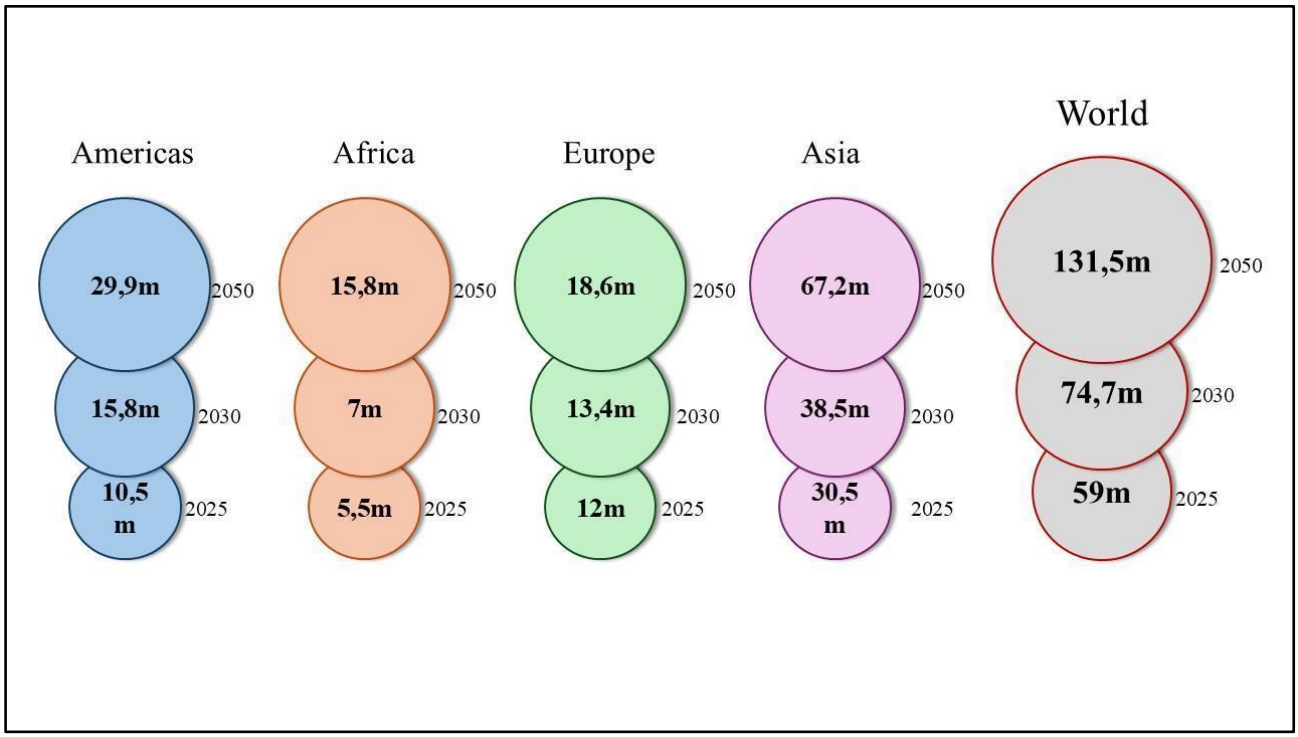


***Figure 1. Global projections of AD incidence.*** *By mid-century, the total number of individuals affected by AD is expected to reach 132 million, with the most significant increase occurring in low- and middle-income countries. (World Health Organization Dementia, 2022).*

## *Risk Factors Associated with AD*

The pathogenesis of AD involves a multifactorial process, and despite significant scientific advances, its precise etiology remains elusive due to the involvement of heterogeneous and interacting risk factors (Figure 2) (Silva et al., 2019; Safiri et al., 2024).

Age remains the most significant risk factor for AD, with prevalence estimates rising from approximately 5–10% among individuals aged 65 and older to nearly 50% in those aged 85 and above (Alzheimer's Association, 2025). Aging is accompanied by physiological changes, such as glucose hypometabolism, cholesterol dysregulation, mitochondrial dysfunction, depression, and cognitive decline, that may obscure early diagnosis (Silva et al., 2019).

AD manifests in two major forms: late-onset, typically affecting individuals over 65, and early-onset, occurring before 65 and accounting for approximately 4–6% of cases. While *Early-Onset AD (EOAD)* is often associated with familial inheritance, the two terms are not synonymous. *Early-onset familial AD (EOFAD)* refers specifically to cases caused by autosomal dominant mutations in genes such as *Amyloid Precursor Protein (APP), Presenilin 1 (PSEN1), and Presenilin 2 (PSEN2)* represents a subset of EOAD (Armstrong, 2019). However, not all EOAD cases are genetically inherited; some

arise sporadically without a clear familial pattern. Although most AD cases are not directly caused by genetic mutations, familial history remains a significant risk factor, with approximately 70% of cases showing genetic influence (Silva et al., 2019). Recent genomic studies have identified over 30 new genes associated with AD susceptibility, further highlighting its genetic complexity (Alzheimer's Association, 2024).

Beyond genetic predisposition, modifiable lifestyle factors such as cognitive engagement, healthy diet, education, and social interaction are protective, while midlife obesity, hypertension, diabetes, smoking, excessive alcohol use, and hearing loss increase risk (Costa-Laparra et al., 2023). Early- and late-onset AD also differ in clinical and biological features, including symptom profiles, neuropathology, and neuroimaging patterns, suggesting distinct underlying mechanisms. Among known genetic mutations, PSEN1 (14q24.3) is the most frequently observed, accounting for approximately 80% of early-onset autosomal dominant cases, followed by APP (21q21) mutations (~15%) and PSEN2 (1q31–q42) mutations (~5%) (Calero et al., 2015). These mutations often disrupt the balance between Aβ isoforms, particularly increasing the $A\beta_{42}/A\beta_{40}$ ratio, thereby accelerating amyloid deposition and neurodegeneration (Bekris et al., 2010). In light of this complex interaction between genetic, environmental and behavioural factors, it is essential to investigate both the main genes involved in the familial form of AD and the modifiable risk factors that can influence the onset and progression of the disease.

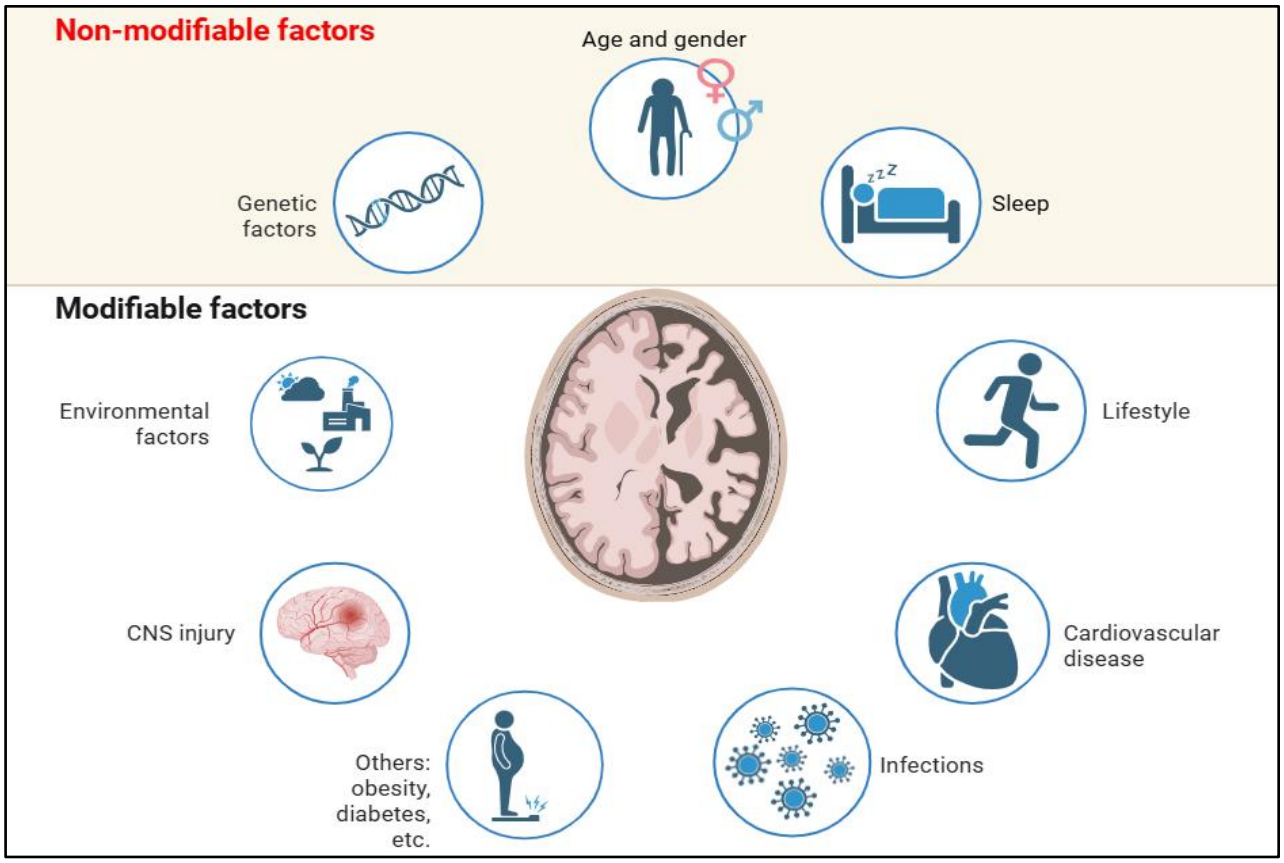


***Figure 2. Overview of risk factors for AD***. *Risk factors are categorized into non-modifiable and modifiable.This distinction underscores the potential for preventive strategies targeting modifiable elements (Armstrong, 2019).*

- **Apolipoprotein E (apoE)** is a glycoprotein involved in lipid metabolism, encoded by the APOE gene located on chromosome 19. This gene exists in three major allelic forms, ε2, ε3, and ε4, which give rise to the protein isoforms apoE2, apoE3, and apoE4. These variants are distributed differently across the population and have distinct implications for AD risk. The ε4 allele is the most significant genetic risk factor for AD. In contrast, the ε2 allele appears to offer a protective effect against the disease. Beyond its role in lipid transport, apoE is essential for maintaining brain homeostasis. It facilitates receptor-mediated endocytosis of lipoprotein particles, including cholesterol, which is crucial for myelin formation and neuronal function (Zhao J et al., 2023). The APOE gene influences several key pathways in the brain, such as synaptic plasticity, glucose metabolism, and membrane trafficking (Liampas et al., 2024). Given that neuronal membranes are rich in cholesterol and phospholipids, apoE-containing lipoproteins play a central role in synaptogenesis and membrane dynamics. Importantly, the ε4 isoform has been linked to increased Aβ deposition and cerebral amyloid angiopathy

(CAA), contributing to AD pathology (Tachibana et al., 2019). Disruption of apoE's physiological functions may also exacerbate other neurodegenerative conditions, including synucleinopathies, highlighting its broader relevance in brain health (Fernandez-Calle et al., 2022).

- **APP** is one of the hallmark features of AD is the accumulation of fibrillar aggregates of Aβ peptides in the brain and cerebral vasculature. These peptides, typically 39 to 43 amino acids in length, are generated through sequential proteolytic cleavage of the APP, a transmembrane glycoprotein encoded by a gene on chromosome 21 (Zheng et al., 2006). APP is highly expressed in neurons and has a molecular weight ranging from 100 to 140 kDa, consisting of 695 amino acids. Structurally, APP includes a large extracellular N-terminal domain (ectodomain, ~90–100 kDa) and a smaller intracellular C-terminal domain (~10 kDa). Three major isoforms of APP have been identified, APP695, APP751, and APP770, arising from alternative splicing and post-translational modifications. APP695 is predominantly expressed in neuronal tissues, while APP751 and APP770 are more commonly found in glial and peripheral cells (Müller et al., 2017). The processing of APP and the resulting imbalance in Aβ isoforms, particularly the increased ratio of Aβ42 to Aβ40, are central to the amyloidogenic cascade that determines AD pathology (Therriault et al., 2024).

- **PSEN1 and PSEN2** are the most common causes of EOFAD. Presenilin acts as the catalytic subunit of γ-secretase. This intramembrane protease cleaves APP to generate Aβ peptides of different lengths, including the aggregation-prone $A\beta_{1-42}$, which is responsible for plaque formation in the brain (Armstrong, 2019). PSEN1 mutations increase $A\beta_{1-42}$ production and disrupt the $A\beta_{42}/A\beta_{40}$ ratio, supporting both the amyloid hypothesis and the presenilin hypothesis of EOFAD pathogenesis (Yang et al., 2023). Beyond APP metabolism, presenilins influence Notch signaling, calcium homeostasis, and gene regulation via *cAMP Response Element-Binding protein (CREB)* (Zhang S et al., 2013; Sun et al., 2016). PSEN2 mutations are rarer but similarly elevate γ-secretase activity and $A\beta_{1-42}$ levels, and have also been linked to other neurodegenerative disorders such as Parkinson's disease-associated dementia and dementia with Lewy bodies (Cai et al., 2015; Kabir et al., 2020).

In addition to genetic and age-related vulnerabilities, several modifiable lifestyle and metabolic factors significantly influence AD onset and progression. One of the most striking epidemiological observations is the sex disparity in AD prevalence, with nearly two-thirds of patients being women. This difference has been partly attributed to the decline in ovarian hormones during menopause. In

particular, estradiol has been shown to modulate Aβ accumulation and cognitive decline, suggesting that hormonal status significantly influences disease progression. These findings underscore the importance of incorporating sex-specific models and considering hormonal fluctuations in AD research (German-Castelan et al., 2024). In parallel, genetic and epigenetic factors contribute to individual susceptibility. While genetic variants such as APOE ε4 are well-established risk factors, recent studies have highlighted the role of epigenetic modifications in regulating AD-related genes. These mechanisms may interact with environmental exposures and lifestyle factors, further shaping disease risk (Lashley et al., 2018; Long et al., 2019).

Among environmental contributors, pollution has emerged as a significant concern. Post-mortem analyses of individuals living in highly polluted areas have revealed hallmark features of AD, including amyloid plaques and phosphorylated Tau (Bateman et al., 2012; Alzheimer's Association, 2019). Moreover, exposure to heavy metals such as arsenic, aluminum, zinc, mercury, manganese, cadmium, and magnesium may disrupt the metabolism of APP and APOE, thereby promoting neurodegeneration (Nisa et al., 2021).

Closely related to environmental exposure is the role of nutrition, which has a profound impact on brain health. Nutritional deficiencies, particularly in calcium, magnesium, and vitamins E, A, D, K, B12, and folate, are common in AD patients and can impair cognitive function. B vitamins, in particular, are essential for DNA synthesis and methylation, and their deficiency may exacerbate epigenetic dysregulation and cognitive decline (Caraci et al., 2018; Scheyer et al., 2018).

These metabolic imbalances often coexist with cardiovascular diseases, such as atrial fibrillation, heart failure, and hypertension, which reduce cerebral perfusion and lead to hypoxia and neuronal damage. Such conditions are known to increase APP expression and Aβ accumulation. Additionally, disruption of the *Blood–Brain Barrier (BBB)* impairs Aβ clearance and facilitates Tau hyperphosphorylation, contributing to the formation of neurofibrillary tangles (Armstrong, 2019; Silva et al., 2019).

Further compounding these effects are metabolic disorders like obesity and type 2 diabetes, which are associated with insulin resistance, oxidative stress, and chronic neuroinflammation. Diabetes, in particular, enhances β- and γ-secretase activity and promotes Tau hyperphosphorylation, accelerating AD pathology. These observations have led to the conceptualization of AD as "type 3 diabetes", emphasizing the central role of impaired glucose metabolism in neurodegeneration (Breijyeh & Karaman, 2020; Alemany et al., 2021; Kciuk et al., 2024).

Another important risk factor is *Traumatic Brain Injury (TBI)*, which has been linked to long-term neurodegenerative changes. TBI can compromise the integrity of the BBB, facilitating the accumulation of neurotoxic proteins such as Aβ and phosphorylated Tau, and triggering neuroinflammation and synaptic dysfunction (Sun et al., 2025).

Finally, oxidative stress and mitochondrial dysfunction represent core pathological features of AD. Oxidative stress arises when *Reactive Oxygen Species (ROS)* overwhelm the cell's antioxidant defenses, leading to damage of lipids, proteins, and DNA. This not only compromises cellular integrity but also promotes the accumulation of Aβ and neurofibrillary tangles, creating a vicious cycle that accelerates neuronal death. Mitochondrial dysfunction further exacerbates this process by increasing ROS production. Importantly, elevated oxidative stress can be detected even in the early, asymptomatic stages of AD, although its precise role remains under investigation (Swerdlow et al., 2012; Cioffi et al., 2019; Dhapola et al., 2024; Nasb et al., 2024).

Taken together, these interconnected factors illustrate the multifaceted nature of AD and highlight the need for integrative approaches that consider biological sex, genetic predisposition, environmental exposures, and systemic health in both research and clinical practice.

## *Pathophysiology*

From a pathophysiological perspective, AD is driven by a complex interplay of molecular and cellular mechanisms that contribute to neuronal death, brain atrophy, and clinical decline (Zhang J. et al., 2025). This atrophy is particularly pronounced in regions involved in memory and higher cognitive functions, such as the hippocampus and cerebral cortex, and is closely linked to cognitive impairment (Sabuncu et al., 2011; Planche et al., 2022; Zilioli et al., 2025). At the macroscopic level, AD is characterized by widespread cerebral atrophy, which is most pronounced in the medial temporal lobe, including the hippocampus and entorhinal cortex, and progressively extends to parietal and frontal association cortices (Figure 3).

This atrophy results in marked cortical thinning and enlargement of the ventricular system, reflecting substantial neuronal and synaptic loss. *Magnetic Resonance Imaging (MRI)*-based staging studies indicate that hippocampal and amygdalar shrinkage occur early, followed by involvement of the temporal and parietal lobes, and later the frontal regions (Planche et al., 2022). Post-mortem and imaging analyses consistently show that hippocampal subfields such as CA1 and the subiculum are

particularly vulnerable, correlating with memory impairment and cognitive decline (Mueller et al., 2010; Zilioli et al., 2025). These macroscopic changes are considered hallmarks of AD progression and are closely associated with the accumulation of tau pathology and other neurodegenerative processes (Reijner et al., 2025).

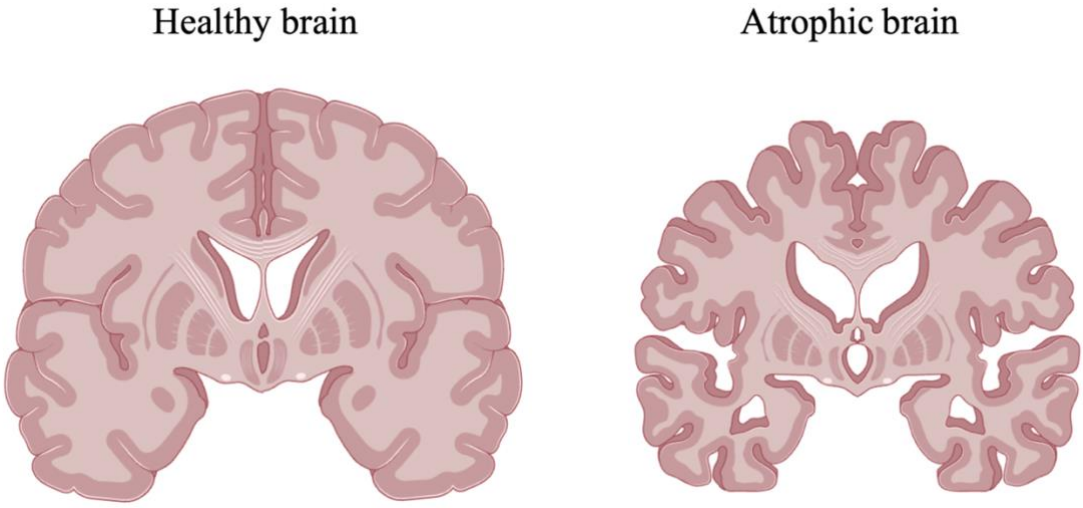


***Figure 3. Comparison between a healthy brain and an atrophic brain***. *The atrophic brain shows significant cortical thinning and enlarged ventricles, typical of neurodegenerative conditions such as AD.*

Over the past few decades, multiple hypotheses have been proposed to explain the interrelated mechanisms contributing to neuronal dysfunction and death. The amyloid cascade hypothesis suggests that the accumulation of Aβ peptides in extracellular plaques triggers a cascade of neurotoxic events, including synaptic dysfunction and neuronal loss (Kepp et al., 2023; Wu T et al., 2022). Complementary to this, the tau hypothesis emphasizes the role of intracellular aggregation of hyperphosphorylated tau proteins that form neurofibrillary tangles, which disrupt microtubule stability and axonal transport (Hong et al., 2025; Kammula et al., 2023). In addition to these classic models, growing evidence highlights the contribution of chronic neuroinflammation, driven by sustained activation of microglia and astrocytes, which exacerbates neuronal damage (Heneka et al., 2025; Han et al., 2025). Furthermore, oxidative stress resulting from mitochondrial dysfunction and the accumulation of reactive oxygen species further accelerates neurodegeneration (Dhapola et al., 2024; Weinstock, 2025). Together, these mechanisms interact in a complex network that underlies the progressive nature of AD. A distinctive neuropathological feature of Alzheimer's disease is the coexistence of extracellular plaques of Aβ protein and intracellular neurofibrillary tangles composed

of hyperphosphorylated tau protein. These lesions often appear in overlapping brain regions, particularly the hippocampus and association cortices, and their combined presence is strongly correlated with synaptic dysfunction and cognitive decline. While Aβ deposition is thought to trigger the pathological cascade, tau pathology shows a closer association with neuronal loss and clinical severity, suggesting a synergistic interaction between these two processes in driving neurodegeneration. (Selkoe and Hardy, 2016; Braak and Del Tredici, 2015; Wang et al., 2025).

## *Amyloid Cascade Hypothesis*

The earliest and most studied pathological events in AD include the abnormal accumulation of Aβ peptides and the formation of NFTs composed of hyperphosphorylated Tau protein. Aβ peptides, generated by the cleavage of APP via β- and γ-secretases, aggregate into extracellular plaques that disrupt synaptic transmission and trigger neuroinflammatory responses, leading to neuronal dysfunction (Karisetty et al., 2020; Iliyasu et al., 2023). In parallel, Tau pathology destabilizes microtubules and impairs intracellular transport, ultimately resulting in neuronal death (Parra-Bravo et al., 2024). The combined effects of Aβ and Tau accumulation drive synaptic loss, neuroinflammation, oxidative stress, and mitochondrial dysfunction, progressively damaging brain circuits involved in memory and cognition (Zhuang et al., 2024). SPs characterized by extracellular Aβ deposition, are neurotoxic and stimulate astrocytes and microglia, causing further synaptic and neuronal damage, particularly in the hippocampus, amygdala, and cortex (Breijyeh & Karaman, 2020; Yu et al., 2024). This accumulation results from an imbalance between Aβ production and clearance, beginning decades before clinical symptoms emerge (Cai et al., 2023).

Given the central role of Aβ in AD pathogenesis, it is essential to understand the molecular pathways underlying its production. APP can be processed through two main pathways: the non-amyloidogenic pathway, which precludes Aβ formation, and the amyloidogenic pathway, which leads to the generation of Aβ peptides. The balance between these pathways is a critical determinant of Aβ accumulation and, consequently, of the risk of plaque formation and neurodegeneration (Selkoe & Hardy, 2016). Under physiological conditions, APP is primarily processed via the non-amyloidogenic pathway at the plasma membrane. In this route, α-secretase cleaves APP within the Aβ domain, thereby preventing Aβ formation and releasing a soluble fragment known as sAPPα, which possesses neuroprotective and trophic properties. The remaining membrane-bound fragment, C83, is subsequently cleaved by γ-secretase, producing the p3 peptide and the *APP Intracellular Domain (AICD)*, which may have signaling functions (Figure 5) (Selkoe, 2001; Dunys et al., 2018).

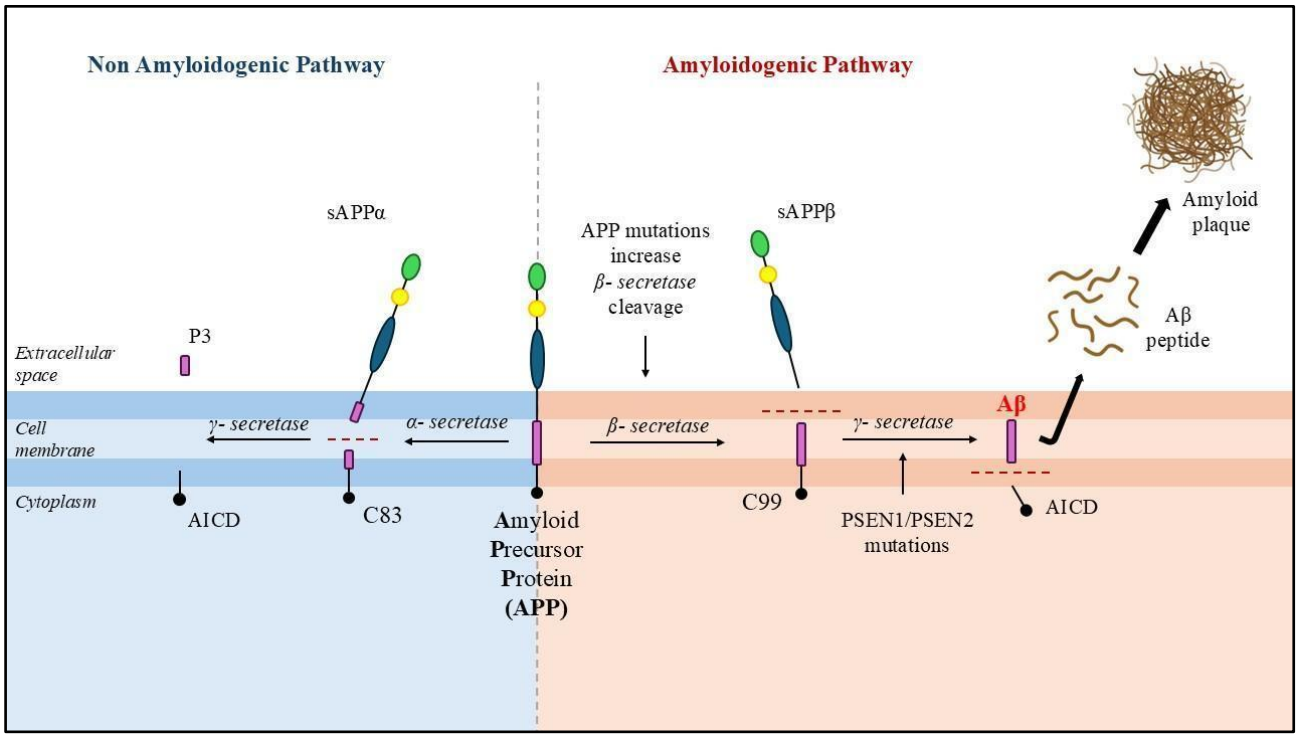


***Figure 5. APP Processing Pathway.*** *Under physiological conditions, APP is cleaved by α-secretase, initiating the non-amyloidogenic pathway. This produces sAPPα, which has neuroprotective effects, and the membrane-bound fragment C83. C83 is then cleaved by γ-secretase to generate the non-toxic peptide p3 and the intracellular domain AICD. In contrast, in Alzheimer's disease, the amyloidogenic pathway predominates: APP is cleaved by β-secretase, producing sAPPβ and C99, which is subsequently processed by γ-secretase to release Aβ peptides and AICD (Orobets & Karamyshev, 2023).*

In contrast, the amyloidogenic pathway predominates under pathological conditions and is initiated within endosomes. Here, β-secretase *(β-site APP-Cleaving Enzyme - BACE1)* cleaves APP to generate sAPPβ and a membrane-bound fragment, C99. This fragment is then processed by γ-secretase, resulting in the release of Aβ peptides, primarily $A\beta_{1\text{-}40}$ and the more aggregation-prone $A\beta_{1\text{-}42}$, as well as the AICD (Dunys et al., 2018). Although the mechanisms driving the shift toward the amyloidogenic pathway in AD remain incompletely understood, its predominance is closely associated with disease progression.

Initially, Aβ is produced as soluble monomers that may play physiological roles in synaptic function. However, when production becomes excessive or dysregulated, these monomers aggregate into oligomers and fibrils, eventually forming senile plaques. Aβ oligomers are particularly neurotoxic, as they interfere with synaptic signaling and contribute to the degeneration of multiple neuronal systems, including cholinergic, serotonergic, noradrenergic, and dopaminergic pathways (Shankar & Walsh, 2009; Chen & Yan, 2010; Crews & Masliah, 2010). During aggregation, Aβ can also generate ROS, especially in the presence of transition metals such as $Fe^{2+}$ and $Cu^{2+}$. These ROS induce lipid

peroxidation and disrupt membrane proteins, including glucose transporters and ATPases, ultimately compromising neuronal homeostasis and increasing vulnerability to apoptosis (Tiwari et al., 2019). Among the Aβ species, $A\beta_{1-40}$ and $A\beta_{1-42}$ are the most prevalent. Although $A\beta_{1-42}$ represents a smaller fraction of total Aβ, it is more hydrophobic and prone to aggregation. Mutations associated with familial AD often increase the $A\beta_{1-42}/A\beta_{1-40}$ ratio, thereby promoting plaque formation and accelerating neurodegeneration (Burdick et al., 1992; Zhang et al., 2011).

## *Tau Protein Hypothesis*

Neurofibrillary tangles, composed of hyperphosphorylated Tau, accumulate within neurons and lead to cytoskeletal degeneration and cell death (Sinsky et al., 2021; Zhang et al., 2023). Recent research also highlights that both Aβ and Tau can activate apoptotic pathways and immune responses, further contributing to neuronal loss (Kumar et al., 2023). These synergistic pathological changes underscore the importance of targeting both Aβ and Tau, as well as their downstream effects, in developing effective therapeutic strategies for AD, although their clinical efficacy remains under investigation (Bloom, 2014; d'Errico & Meyer-Luehmann, 2020). Parallel to Aβ pathology, Tau protein plays a pivotal role in the progression of AD. Under physiological conditions, Tau is a microtubule-associated protein that stabilizes neuronal microtubules and supports axonal transport. However, in AD, Tau undergoes abnormal post-translational modifications which disrupt its binding to microtubules and promote its aggregation into NFTs within neurons (Lin et al., 2025). Hyperphosphorylated Tau loses its normal function and gains toxic properties, contributing to cytoskeletal disintegration, synaptic dysfunction, and mitochondrial impairment (Zhang et al., 2024; Wang et al., 2025). Tau aggregation follows a prion-like propagation mechanism, spreading trans-synaptically across brain regions and correlating more strongly with cognitive decline than Aβ plaque burden (Sonawane et al., 2018; Chaggar et al., 2025). Moreover, Tau pathology is tightly linked to neuroinflammatory responses, with activated microglia and astrocytes exacerbating Tau misfolding and aggregation through inflammatory signaling pathways (Chen & Yu, 2023). This bidirectional relationship between Tau and inflammation amplifies neuronal damage and accelerates disease progression (Figure 6).

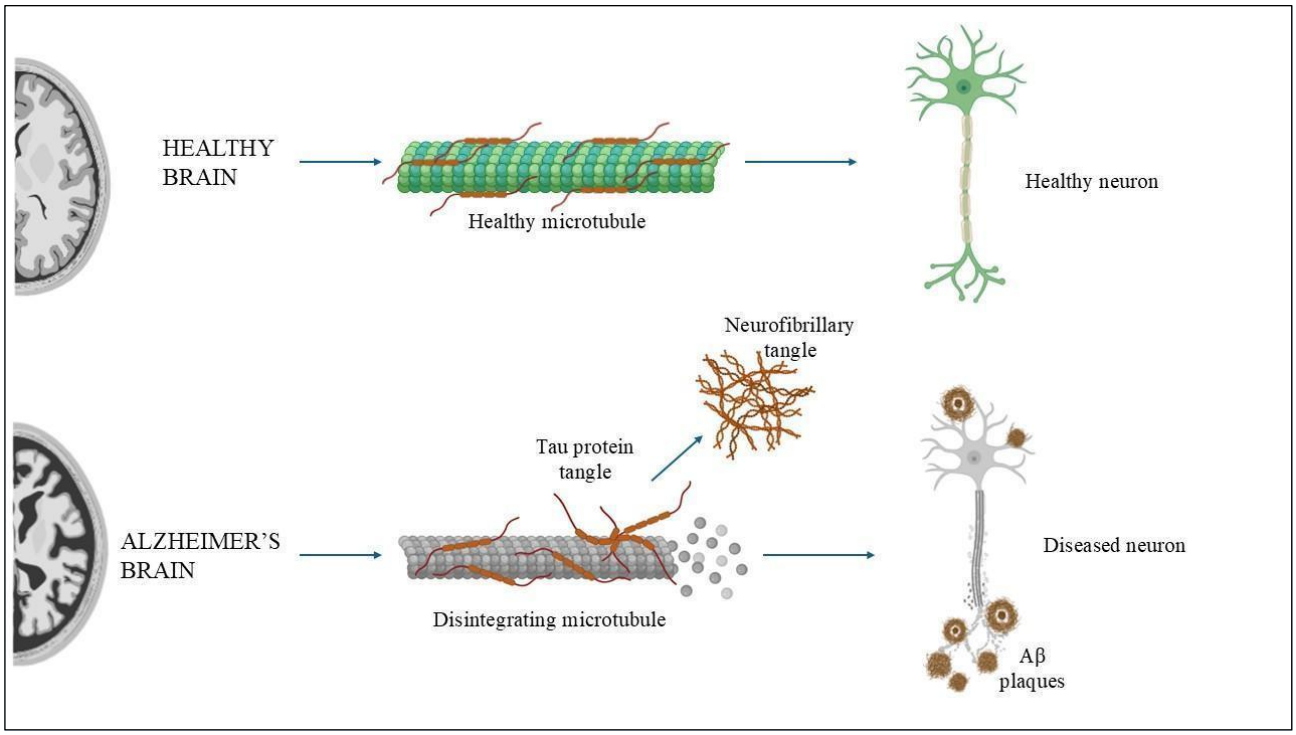


***Figure 6. Microtubule Integrity and Tau Pathology in Alzheimer's Disease (AD).*** *In a healthy brain, microtubules maintain structural integrity and support normal neuronal function. In AD, microtubules disintegrate due to tau protein tangles, leading to neurofibrillary tangles and the formation of amyloid-β (Aβ) plaques, which contribute to neuronal dysfunction and degeneration.*

## *Neuroinflammation Hypothesis*

Beyond being a secondary response, neuroinflammation is increasingly considered a potential primary driver of AD pathogenesis. Activated microglia and astrocytes, the principal glial cells involved in the innate immune response within the central nervous system, play a pivotal role in AD pathogenesis (Figure 7). Evidence from *Positron Emission Tomography (PET)* studies has demonstrated microglial activation in the brains of AD patients even before significant Aβ deposition occurs (Cagnin et al., 2001; van der Flier & Heneka, 2025). Aβ aggregates stimulate the release of pro-inflammatory cytokines such as *Interleukin-1β* (*IL-1β)*, *Interleukin-6 (IL-6)*, and *Tumor Necrosis Factor- α (TNF-α)*, which are consistently found at elevated levels in AD brains (Heneka et al., 2015). Genetic polymorphisms in cytokine genes and acute-phase proteins, including α1-antichymotrypsin, have been associated with increased susceptibility to AD (McGeer & McGeer, 2001; Wang et al., 2002). Furthermore, transcriptomic and genome-wide association studies have identified microglial signaling pathways as critical modulators of disease onset and progression (Wu et al., 2024; Graham et al., 2025). Altered expression of *Triggering Receptor Expressed on Myeloid Cells 2 (TREM2)*, both

centrally and peripherally, has been observed in preclinical stages of AD, suggesting that immune dysregulation may precede neurodegeneration. Astrocytes also contribute to the inflammatory milieu through reactive astrogliosis and by participating in Aβ clearance via autophagic mechanisms (Ferrari-Souza et al., 2025). Neurons, traditionally considered passive targets, have been shown to produce inflammatory mediators in response to toxic stimuli, further amplifying the neuroinflammatory cascade (Choi et al., 2023). Collectively, these findings support the hypothesis that neuroinflammation may act as a "primum movens" in AD, initiating and sustaining pathological processes that culminate in synaptic dysfunction, neuronal loss, and cognitive decline.

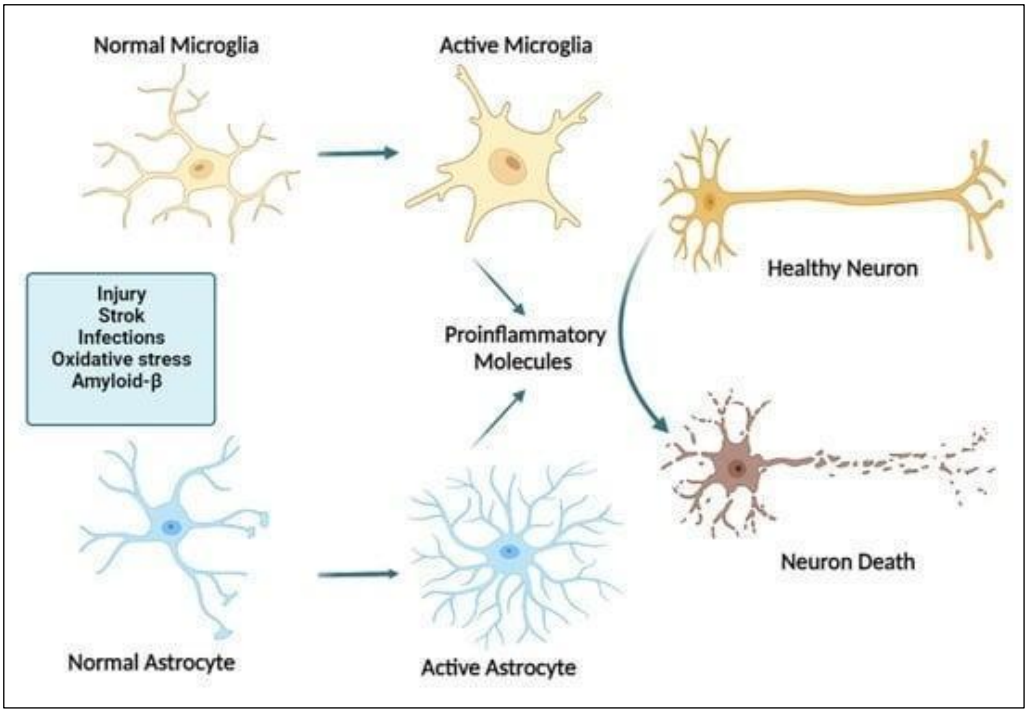


***Figure 7. The role of chronic neuroinflammation in Alzheimer's disease pathogenesis.*** *Activation of microglia and astrocytes in response to injury, oxidative stress, infections, or amyloid-β leads to the release of proinflammatory molecules, which contribute to neuronal damage and death (Al-Ghraiybah et al., 2022).*

## *Oxidative Stress Hypothesis*

Oxidative stress is defined as an imbalance between ROS production and antioxidant defenses, representing a hallmark of many neurodegenerative disorders, including AD (Nasb et al., 2024). ROS such as superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals (•OH), damage cellular components like lipids, proteins, and nucleic acids, thereby compromising neuronal integrity (Huang WJ et al., 2016; Dhapola et al., 2024). In AD, oxidative stress is closely linked to

enhanced deposition of Aβ and NFTs, both of which further amplify ROS generation, creating a perpetuous cycle that accelerates neuronal loss and cognitive decline (Dhapola et al., 2024). Moreover, ROS also modulates several intracellular signaling pathways that are implicated in neurodegeneration (Cioffi et al., 2019). A widely accepted hypothesis suggests that mitochondrial dysfunction, in concert with oxidative stress, plays a central role in AD pathophysiology (Figure 8) (Swerdlow et al., 2012). Mitochondria, essential for ATP production, calcium homeostasis, and apoptosis regulation, are particularly vulnerable to oxidative damage, and their dysfunction is increasingly recognized as a central contributor to AD pathophysiology (Swerdlow et al., 2012; Spina et al., 2025). Evidence indicates that mitochondrial impairment manifests as defective oxidative phosphorylation, reduced ATP synthesis, excessive ROS production, altered dynamics, and impaired mitophagy, leading to accumulation of damaged organelles (Misrani et al., 2021; Farsi, 2023). These abnormalities appear early in AD, even before clinical symptoms, supporting the mitochondrial cascade hypothesis, which posits that mitochondrial dysfunction may initiate disease progression (Spina et al., 2025). Furthermore, structural changes in *Mitochondria-Associated ER Membranes (MAMs)* disrupt calcium signaling and lipid metabolism, promoting Aβ accumulation and tau hyperphosphorylation, while *mitochondrial DNA* (mtDNA) mutations and reduced biogenesis exacerbate energy deficits and oxidative imbalance (Li et al., 2025). This creates a feed-forward loop where oxidative stress and mitochondrial dysfunction perpetuate each other, amplifying neurodegeneration. Although traditional antioxidant therapies have shown limited success, emerging strategies targeting mitochondrial health, including mitochondria-targeted antioxidants (e.g., MitoQ, SS-31), modulators of mitochondrial dynamics, and approaches aimed at enhancing mitophagy, are gaining attention as potential interventions to slow AD progression, although their clinical translation remains challenging (Farsi, 2023; Misrani et al., 2021; Li et al., 2025).

Notably, elevated oxidative stress has been observed even in the preclinical stages of AD. However the relationship between mitochondrial impairment and oxidative imbalance remains under investigation. It is still unclear whether these phenomena are initiating events or downstream consequences of the disease process (Nasb et al., 2024).

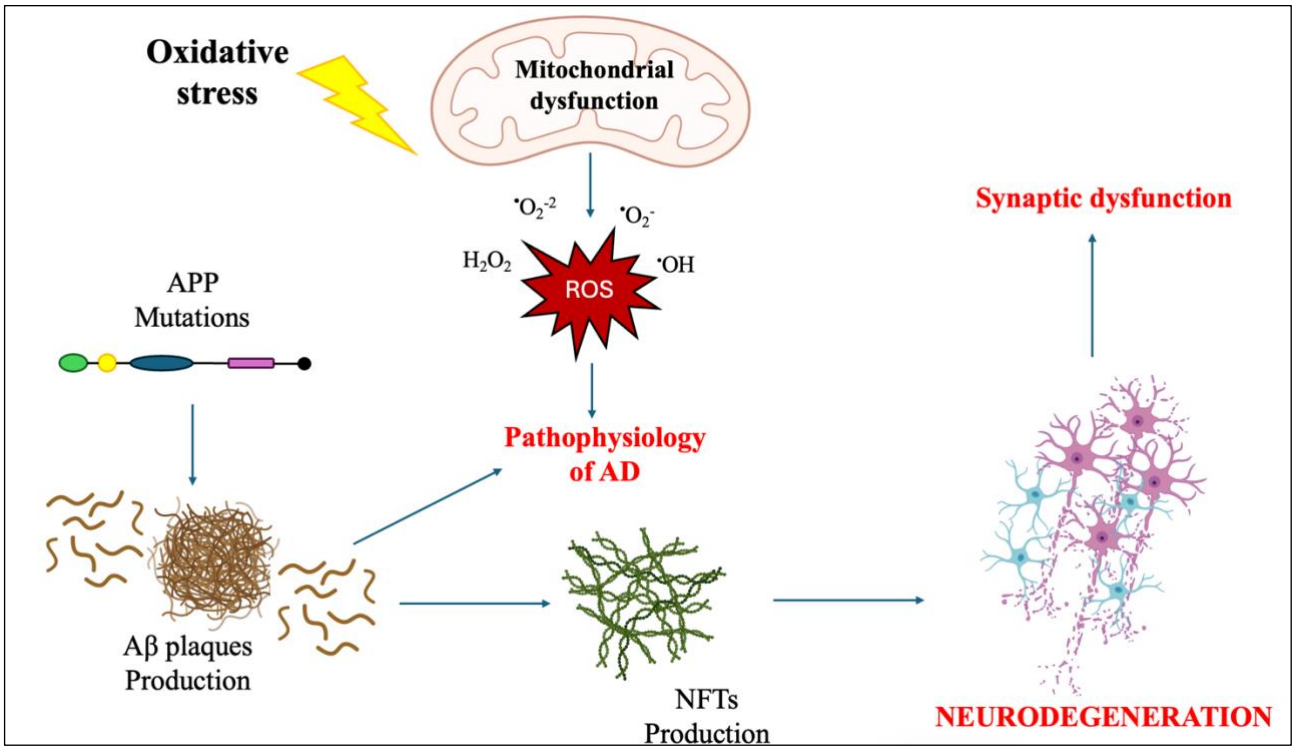


***Figure 8***. *Oxidative stress and mitochondrial dysfunction promote the generation of reactive oxygen species (ROS), which drive the pathophysiology of Alzheimer's disease. These processes contribute to amyloid-β (Aβ) plaque formation, neurofibrillary tangle (NFT) accumulation, synaptic dysfunction, and ultimately neurodegeneration.*

## *ERK/CREB/BDNF Pathway*

Beyond the well-established accumulation of Aβ and Tau proteins, AD is characterized by profound impairments in molecular pathways that regulate neuronal survival, synaptic plasticity, and cognitive function (Misrani et al., 2021). One of the most affected systems is the neurotrophic signaling cascade involving *Brain-Derived Neurotrophic Factor (BDNF)* and the transcription factor CREB, both of which are essential for memory formation and synaptic integrity (Azman et al., 2022; Alqahtani et al., 2025). In AD, Aβ oligomers and Tau pathology interfere with CREB phosphorylation, leading to reduced BDNF expression, impaired *Long-Term Potentiation (LTP)*, and progressive cognitive deficits (Misrnai et al., 2021; Gao et al., 2022). Post-mortem studies of AD brains consistently show decreased levels of phosphorylated CREB and BDNF, particularly in the hippocampus and cortex (Amidfar et al., 2020). These molecular alterations are closely linked to oxidative stress and mitochondrial dysfunction, which play a central role in the neurodegenerative process. Damaged mitochondria produce excessive amounts of ROS, which amplify Aβ and Tau

pathology, stimulate inflammatory responses, and trigger programmed cell death. These events collectively destabilize neuronal networks and contribute to cognitive impairment (Amidfar et al., 2020). Furthermore, oxidative stress impairs CREB-dependent transcription of neuroprotective genes, while dysregulated activation of the *Extracellular signal-regulated kinase (ERK)* signaling pathway has been implicated in both neuroinflammation and Tau hyperphosphorylation (Khezri et al., 2023). Importantly, ERK acts as an upstream regulator of CREB, and its aberrant activation in AD disrupts synaptic signaling and promotes neurodegeneration (Khezri et al., 2023). Elevated ERK activity has been observed in the hippocampus and glial cells of AD patients, where it contributes to inflammatory responses and neuronal damage (Khezri et al., 2023). This suggests that ERK/CREB/BDNF signaling dysfunction forms a critical axis in the progression of AD (Wu SY et al., 2020). Additionally, cAMP signaling plays a pivotal role in CREB activation and synaptic plasticity. Reduced cAMP levels have been reported in AD brains, impairing *Protein Kinase A (PKA)*-mediated phosphorylation of CREB and downstream transcription of neuroprotective genes (Pugazhenthi et al., 2011; Shen et al., 2022). Activation of the ERK/CREB pathway increases BDNF production in cortical neurons (Alqahtani et al., 2025), and BDNF protects hippocampal neurons from glutamate toxicity through transient activation of ERK (Almeida et al., 2005). The cAMP response is a pivotal transcription factor that regulates genes involved in synaptic plasticity, neuronal survival, and memory consolidation. Activation of CREB through phosphorylation triggers the transcription of neuroprotective genes, among which BDNF is one of the most critical. BDNF supports neuronal differentiation, dendritic growth, and synaptogenesis, thereby maintaining the structural and functional integrity of neural circuits essential for learning and memory (Amidfar et al., 2020; Gao et al., 2022). Dysregulation of this signaling axis has profound consequences: reduced CREB activity and diminished BDNF expression are consistently observed in the early stages of AD, contributing to synaptic dysfunction and cognitive decline (Sharma & Singh, 2020; Alqahtani et al., 2025). Amyloid-β oligomers and hyperphosphorylated tau interfere with CREB activation, either by disrupting upstream cAMP/PKA signaling or by promoting kinase imbalances that prevent CREB phosphorylation (Tropea et al., 2022). This inhibition reduces BDNF transcription and weakens neurotrophic support. Moreover, oxidative stress and neuroinflammation exacerbate this dysfunction, as reactive oxygen species and pro-inflammatory cytokines further suppress CREB activity and impair TrkB receptor signaling, amplifying synaptic loss and neuronal vulnerability (Wu A et al., 2004; Karvandi et al., 2023). Restoring CREB-BDNF signaling represents a promising therapeutic strategy for AD. Several approaches are under investigation, including pharmacological agents that enhance cAMP/PKA signaling, TrkB agonists, and epigenetic modulators to boost BDNF transcription (Amidfar et al., 2020; Sharma & Singh, 2020). Lifestyle interventions such as physical

exercise have also been shown to increase CREB phosphorylation and BDNF levels, improving synaptic plasticity and memory performance (Jaberi & Fahnestock, 2023). Additionally, natural compounds (e.g., flavonoids, ginsenosides) and gene therapy techniques targeting this pathway are emerging as innovative options to counteract neurodegeneration (Gupta et al., 2025; Alqahtani et al., 2025). Therapeutic interventions aimed at restoring CREB-BDNF signaling, such as *Phosphodiesterase-4 (PDE4)* inhibitors like rolipram, physical activity, and enriched environments, have shown beneficial effects in preclinical models, improving memory and reducing synaptic loss (Adlard et al., 2005; Lazarov et al., 2005; Sheng et al., 2022). These findings highlight the potential of targeting neurotrophic and intracellular signaling pathways to counteract neurodegenerative processes in AD.

## *Clinical features*

AD typically begins with a subtle onset and gradually intensifies over the course of several years. The rate of progression and the specific symptoms exhibited can vary significantly between individuals. Early manifestations are predominantly cognitive, with impairments most commonly affecting the ability to form new memories (Kumar et al., 2023). As the disease progresses, deficits extend beyond memory to encompass other cognitive domains, including language, executive function, visuospatial abilities, and attention.

The cognitive and behavioral progression of AD is typically categorized into three main stages: mild (early), moderate (middle), and severe (late).

- In the mild stage, patients often present with subtle memory lapses, particularly affecting recent events and the ability to learn new information. Additional signs include disorientation, difficulty in planning or organizing tasks, and mild changes in mood or personality, such as anxiety or irritability (Kumar et al., 2023). At this stage, individuals may still maintain independence in most daily activities, although compensatory strategies become necessary.

- During the moderate stage, cognitive deficits become more pronounced, extending beyond memory to language, executive function, visuospatial abilities, and attention. Patients frequently experience word-finding difficulties (anomia), impaired judgment, and reduced problem-solving skills. *Behavioral and psychological symptoms of dementia (BPSD)* often emerge, including apathy, depression, irritability, agitation, and, in some cases, psychotic

features such as delusions and hallucinations (Kales et al., 2015; Cerejeira et al., 2012). Functional decline necessitates assistance with daily activities and supervision to ensure safety.

- In the severe stage, profound cognitive deterioration occurs. Patients lose the ability to communicate coherently, recognize loved ones, and perform basic self-care. Behavioral symptoms such as aggression, apathy, and psychosis may persist, while neurological signs including muscle rigidity and gait disturbances often appear. At this point, individuals are completely dependent on caregivers, and quality of life is significantly compromised (Mayo Clinic, 2025).

These stages are not strictly linear and can vary in duration and intensity among individuals. Clinical tools such as the *Clinical Dementia Rating (CDR)* and *Mini-Mental State Examination (MMSE)* are widely used to assess disease severity and monitor progression (Morris, 1993; Folstein et al., 1975). A crucial phase in the clinical continuum is the transition from *Mild Cognitive Impairment (MCI)* to AD (Jack et al., 2018; Wang Y. et al., 2024). MCI is defined by measurable cognitive decline that does not yet interfere significantly with daily functioning. However, individuals with amnestic MCI are at high risk of progressing to AD. This evolution is marked by worsening episodic memory and the emergence of deficits in executive function, language, and visuospatial skills. Behavioral changes such as apathy and anxiety may also appear. Neurobiologically, this phase is associated with increasing β-amyloid deposition, tau pathology, and hippocampal atrophy, detectable through cerebrospinal fluid biomarkers and neuroimaging techniques. Multimodal approaches combining clinical, imaging, and genetic data (e.g., APOE ε4 status) enhance the prediction of MCI-to-AD conversion (Da et al., 2013; Mieling et al., 2025).

## *Diagnosis*

AD is a progressive neurodegenerative disorder characterized by a sequence of distinct clinical stages, each characterized by specific cognitive, functional, and behavioral symptoms (Khan et al., 2025). Historically, diagnosis relied on clinical symptoms and exclusion of other causes of cognitive decline. However, the advent of *in vivo* biomarkers and disease-modifying therapies has shifted the paradigm toward a biological definition of AD, emphasizing early detection and staging based on neuropathological processes rather than clinical presentation (Dubois et al., 2023; Jack et

al., 2024). This approach recognizes AD as a continuum that begins decades before symptom onset, enabling earlier intervention and more accurate prognostication.

Despite these advances, there is no single definitive test for AD. Diagnosis remains complex and requires a multidisciplinary approach involving neurologists, psychiatrists, geriatricians and neuropsychologists, as well as input from family members to compensate for the patient's limited ability to accurately report symptoms (Newhouse et al., 2020). A differential diagnosis is made that excludes other conditions that mimic dementia, such as vascular cognitive impairment, depression, or metabolic disorders (Pimentel, 2009). It is important to note that definitive confirmation of AD pathology is still based on post-mortem examination, which reveals the presence of amyloid plaques and neurofibrillary tangles (Thal & Braak, 2005).

In addition to these traditional methods, the *Amyloid, Tau, and Neurodegeneration* - AT(N) biomarker framework has become central to modern diagnosis. Amyloid or *Cerebrospinal Fluid (CSF)* $A\beta_{42}/A\beta_{40}$ ratio indicate Amyloid pathology (A), Tau PET or CSF/plasma p-tau reflect tau pathology (T), and MRI or *Fluorodeoxyglucose Positron Emission Tomografy (FDG-PET)* assess Neurodegeneration (N) (Jack et al., 2018; Jack et al., 2024). Blood-based biomarkers such as plasma p-tau217, *Glial Fibrillary Acidic Protein (GFAP),* and N*eurofilament light (NfL)* are emerging as cost-effective alternatives for early detection and staging (Eratne et al., 2025; Dark et al., 2024).

This integrated approach improves diagnostic accuracy, supports personalized treatment strategies, and facilitates enrollment in clinical trials targeting early disease stages. However, challenges remain, including cost, accessibility, and ethical considerations regarding disclosure of preclinical AD status (Erickson et al., 2021; Alpinar-Sencan & Schicktanz, 2020).
Building on these advances, clinical practice integrates traditional assessments with biomarker-based frameworks to ensure a comprehensive evaluation. This structured workflow typically includes:

**1. Medical History (Anamnesis):** a comprehensive medical history is the fundament of AD diagnosis. Clinicians collect detailed information on symptom onset, progression, lifestyle factors, medications, comorbidities, and family history to identify risk factors and exclude alternative causes of cognitive decline. Genetic predisposition (e.g., APOE ε4 allele), cardiovascular risk factors, and psychosocial elements are considered during this phase (Safiri et al., 2024; Silva et al., 2019). Structured interviews with patients and informants help document functional changes and behavioral symptoms (Alzheimer's Association, 2025).

**2. Physical Examination:** Physical assessment focuses on general health and sensory functions, as impairments in vision or hearing can exacerbate cognitive symptoms and mimic dementia. Recent evidence links sensory deficits to increased dementia risk (Zumbrunn et al., 2025; Davoudi et al., 2025). Evaluating cardiovascular health and systemic conditions is essential to rule out reversible contributors.

**3. Neurological Examination:** A standardized neurological exam assesses reflexes, motor coordination, cranial nerve function, and gait, identifying abnormalities that may indicate neurodegenerative processes or alternative diagnoses. Findings such as abnormal deep tendon reflexes, tremor, and gait disturbances are common in autosomal dominant AD and correlate with faster cognitive decline (Vöglein et al., 2023). This step remains a base of diagnosis, even before advanced imaging.

**4. Cognitive and Neuropsychological Testing:** Cognitive screening tools such as the MMSE and *Montreal Cognitive Assessment (MoCA)* are widely used to evaluate memory, attention, language, and executive function. MMSE scores below 24 typically indicate cognitive impairment, and longitudinal decline of ~3 points per year is common in AD (Folstein et al., 1975; McLaughlin et al., 2024). In addition to basic screening, more advanced tools are available that offer greater accuracy in monitoring disease progression and predicting the transition from MCI to dementia (Wang et al., 2023; Tsoi et al., 2015).

**5. Laboratory Tests:** Laboratory investigations aim to exclude reversible causes of cognitive decline, such as thyroid dysfunction, vitamin B12 deficiency, and metabolic disorders. Blood-based biomarkers are increasingly used to support diagnosis, including plasma phosphorylated tau (p-tau217), NfL, and GFAP, which demonstrate high diagnostic accuracy for AD pathology (Dark et al., 2024; Palmqvist et al., 2024).

**6. Neuroimaging:** in cases suspected of AD, based on clinical evaluation and cognitive testing, neuroimaging techniques are employed to support diagnosis and exclude alternative causes of cognitive decline. In particular, structural imaging techniques such as MRI and *Computed Tomography (CT)*, play a fundamental role in the diagnostic process by excluding alternative causes of cognitive decline, including brain tumors, strokes, and hydrocephalus (Harper et al., 2014). These modalities also enable visualization of characteristic patterns of hippocampal and medial temporal lobe atrophy, which are considered hallmark features of AD and correlate strongly with disease progression (de Flores et al., 2015; Zhao W et al., 2019). MRI provides high-resolution images that allow detection of early structural changes, while CT is primarily used to rule out acute lesions or

vascular abnormalities (Zhao G. et al., 2024). Beyond structural assessment, functional imaging offers complementary insights by evaluating brain metabolism and pathological protein deposition. FDG-PET measures cerebral glucose metabolism and typically reveals hypometabolism in temporoparietal regions in AD patients (Ortner et al., 2019; Bouter et al., 2025). Additionally, amyloid PET and tau PET scans enable direct visualization of amyloid-β plaques and tau neurofibrillary tangles, providing a robust basis for biological staging and differential diagnosis (Høilund-Carlsen et al., 2023; Patterson et al., 2010). These imaging modalities are increasingly integrated into clinical and research protocols to confirm AD pathology and distinguish it from other forms of dementia (Dang et al., 2023). Recent advances emphasize multimodal diagnostic strategies, combining neuroimaging with blood-based biomarkers such as plasma p-tau217 and NfL. This approach improves diagnostic accuracy, reduces invasiveness, and lowers costs compared to traditional CSF or PET-only methods (Palmqvist et al., 2024; Yim et al., 2025). Multimodal frameworks are particularly promising for early detection in preclinical stages and for stratifying patients in clinical trials, where precise staging is critical for evaluating disease-modifying therapies (Tripathi et al., 2025; World Alzheimer Report, 2025). However, despite these technological advances, the only definitive confirmation of AD still requires *post-mortem* neuropathological examination of the brain. This involves microscopic identification of hallmark lesions like SPs and NFTs (Thal et al., 2025; DeTure et al., 2019; Shakir & Dugger, 2022). Autopsy studies not only validate clinical and biomarker-based diagnoses but also provide critical insights into co-pathologies such as Lewy bodies and TDP-43 inclusions, which cannot be fully assessed *in vivo* (Thal et al., 2025; Gibson et al., 2025).

Understanding the stages is essential for early diagnosis and targeted therapeutic management. AD progresses through distinct stages that reflect both molecular pathology and clinical symptoms. According to the revised criteria proposed by Jack et al. (2024), biological staging is anchored in core biomarkers detected via PET imaging or fluid assays.

The biological progression of AD can be categorized into distinct stages based on the distribution and severity of amyloid and tau pathology:

- **Stage A** represents the earliest detectable biological change, characterized by amyloid positivity in the brain while tau pathology remains absent. This stage reflects initial amyloid deposition, which typically begins years before clinical symptoms emerge, and is considered a critical window for preventive interventions.

- **Stage B** marks the onset of tau pathology, which remains confined to medial temporal regions such as the entorhinal cortex and hippocampus. These areas are essential for memory processing, and their involvement explains the subtle cognitive changes often observed during this phase.

- **Stage C** is defined by a moderate spread of tau pathology into neocortical regions. At this point, neurodegeneration accelerates, and cognitive impairment becomes more pronounced, often progressing from MCI toward early dementia.

- **Stage D** represents the most advanced biological stage, characterized by widespread tau deposition across associative cortices. This extensive pathology correlates with severe neurodegeneration and global cognitive decline, ultimately leading to loss of functional independence.

These stages illustrate the sequential and overlapping nature of amyloid and tau accumulation, emphasizing that AD is a *continuum* rather than a sudden onset condition (Jack et al., 2024). Understanding these biological phases is crucial for developing stage-specific diagnostic tools and therapeutic strategies aimed at slowing or halting disease progression. This staging correlates with disease severity but does not always align with clinical impairment due to factors such as cognitive reserve and co-pathologies (Pichet Binette et al., 2025).

Instead, clinical staging is defined as symptom-based stages divided into six categories as shown in Table 1.

| Stages | |
|---|---|
| Stage 0<br>Asymptomatic, deterministic gene | No evidence of clinical change. Biomarkers in normal range. |
| Stage 1<br>Asymptomatic, biomarker evidence only | Performance within expected range on objective cognitive tests.<br><br>No evidence of recent cognitive decline or new symptoms. |
| Stage 2<br>Transitional decline: mild detectable change, but minimal impact on daily function | Normal performance within expected range on objective cognitive tests. |

| | |
|---|---|
| | Decline from previous level of cognitive or neurobehavioral function that represents a change from individual baseline within the past 1 to 3 years, and has been persistent for at least 6 months.<br><br>May be documented by evidence of subtle decline on longitudinal cognitive testing, which may involve memory or other cognitive domains but performance still within normal range.<br><br>May be documented through a subjective report of cognitive decline.<br><br>May be documented with recent-onset change in mood, anxiety, motivation not explained by life events.<br><br>Remains fully independent with no or minimal functional impact on activities of daily living (ADLs) |
| Stage 3<br>Cognitive impairment with early functional impact | Performance in the impaired/abnormal range on objective cognitive tests.<br><br>Evidence of decline from baseline, documented by the individual's report or by an observer's (e.g., study partner) report or by change on longitudinal cognitive testing or neurobehavioral assessments.<br><br>Performs daily life activities independently but cognitive difficulty may result in detectable functional impact on complex ADLs (i.e., may take more time or be less efficient but still can |

| | |
|---|---|
| | complete, either self-reported or corroborated by an observer). |
| Stage 4<br>Dementia with mild functional impairment | Progressive cognitive and mild functional impairment on instrumental ADLs, with independence in basic ADLs. |
| Stage 5<br>Dementia with moderate functional impairment | Progressive cognitive and moderate functional impairment on basic ADLs requiring assistance. |
| Stage 6<br>Dementia with severe functional impairment | Progressive cognitive and functional impairment, and complete dependence for basic ADLs. |

***Table 1. Clinical staging of individuals along the AD continuum, from asymptomatic to severe dementia****. This staging framework illustrates the progressive nature of AD, starting from preclinical phases characterized by biomarker positivity without symptoms, through mild cognitive impairment, and ultimately to severe dementia. It provides a standardized approach for diagnosis, patient stratification, and clinical trial design, enabling early identification and targeted interventions across different disease stages (Jack et al., 2024).*

## *Current Treatments and Limitations*

Despite extensive research efforts, AD remains a neurodegenerative disorder without a definitive pharmacological cure. Existing treatments primarily aim to alleviate symptoms and delay progression through the early and intermediate stages, which are characterized by milder cognitive and behavioral impairments. However, no drug has yet demonstrated the ability to halt or reverse the underlying neurodegenerative processes (Salomone et al., 2012; Briggs et al., 2016). Over the past decade, considerable attention has been devoted to developing disease-modifying therapies that target the pathophysiological mechanisms of AD, such as Tau protein aggregation, Aβ peptide accumulation, neuroinflammation, and oxidative stress (Kisby et al., 2019; Se Thoe et al., 2021). Nevertheless, the complexity of AD has posed significant challenges, and no effective disease-modifying agent has emerged to date (Frisoni et al., 2022).

Currently, only four drugs have been approved by both the *Food and Drug Administration (FDA) and European Medicines Agency (EMA)* for AD treatment: Donepezil, Rivastigmine, Galantamine, and Memantine (Barthold et al., 2020). The first three are *Acetylcholinesterase Inhibitors (AChEIs)*, which enhance cholinergic transmission by preventing the breakdown of acetylcholine, essential for

memory, learning, and attention (Ferreira-Viera et al., 2016; Zhang XX et al., 2021). These drugs are primarily used in mild-to-moderate AD and offer modest improvements in cognitive and behavioral symptoms. However, therapeutic response varies, with approximately one-third of patients showing no benefit and another third experiencing side effects (Birks & Harvey, 2003; Briggs et al., 2016). While Memantine, an *N-Methyl-D-Aspartate (NMDA)* receptor antagonist, is indicated for moderate-to-severe AD. It modulates excessive glutamatergic activity, which can lead to excitotoxicity and neuronal damage due to abnormal calcium influx (Tang et al., 2023; Folch et al., 2017). Although memantine does not alter the disease course, it may help stabilize symptoms and improve quality of life (Folch et al., 2017). Combining AChEIs with NMDA receptor antagonists has shown promise in enhancing therapeutic outcomes due to their complementary mechanisms of action (Farrimond et al., 2012; Matsunaga et al., 2015). While these treatments remain symptomatic, they represent the best available pharmacological options pending the development of more effective, disease-modifying strategies.

- **AChEI**: Acetylcholine is a major neurotransmitter in the brain, with activity throughout the cortex, basal ganglia, basal forebrain, and cholinergic synapses, which are critical for memory, learning, and attention (Zhang XX et al., 2021). Therefore, the cholinergic system plays a central role in the quest to find a cure for AD: the cholinergic hypothesis focuses precisely on the progressive loss of limbic and neocortical cholinergic innervation (Ferreira-Viera et al., 2016). AChEIs are able to increase the acetylcholine available to synapses by blocking the enzyme that degrades it (Pozzi et al., 2022). These drugs improve cognitive function and some behavioural manifestations of AD in mild cases (Moreta et al., 2021), so the benefit of administering these compounds has been shown to be mild (Marucci et al., 2021). Despite the limited activity of AchEIs, pending more effective approaches, these drugs still represent a therapeutic resource for the treatment of AD.

- **NMDA Receptor Inhibitors**: The NMDA receptor is a ligand for glutamate, the primary excitatory neurotransmitter in the human brain (Jewett & Thapa, 2022). This receptor plays a fundamental role in synaptic transmission and synaptic plasticity and is thought to underlie learning and memory processes (Baez et al., 2018). These receptors have a high permeability to $Ca^{2+}$ ion, and several lines of evidence suggest that neuronal toxicity is mostly mediated by excessive $Ca^{2+}$ entry via the NMDA receptor (Choi, 1988). Pathological $Ca^{2+}$ signaling leads to a gradual loss of synaptic function and neuronal death (Wang & Reddy, 2017). This therefore justifies the use of Memantine, a non-competitive NMDA receptor antagonist, for the symptomatic treatment of AD (Tampi & Van Dyck, 2007).

The combined administration of donepezil, and memantine, has been shown to provide enhanced symptomatic relief in patients with moderate to severe AD, compared to monotherapy in terms of cognitive and functional outcomes (Tariot et al., 2004; Grossberg et al., 2013; Kim et al., 2024).

At the same time, therapeutic strategies have increasingly shifted toward disease modification, particularly through the development of *monoclonal antibodies (MABs)* that selectively target aggregated forms of Aβ, intending to reduce plaque burden and interrupt the neurodegenerative cascade associated with AD (Tolar et al., 2020; Van Dyck et al., 2023; Kim et al., 2024). This approach represents a significant advancement in AD treatment, moving beyond symptomatic management. The rationale for these therapies is rooted in the amyloid hypothesis, which posits that aberrant processing and accumulation of Aβ peptides are central to AD pathogenesis, a theory that has guided the majority of clinical trials in recent years (Glenner & Wong, 1984; Hardy & Allsop, 1991; Liu et al., 2019). These biologics are designed to bind aggregated Aβ species, facilitating their clearance and potentially altering the course of the disease (Tolar et al., 2020; Van Dyck et al., 2023; Kim et al., 2024). Among the most extensively studied agents are:

- **Aducanumab** (Aduhelm™) received accelerated approval from the FDA in 2021 based on its ability to reduce Aβ plaque load, but was rejected by the EMA. Its approval was highly controversial because the pivotal trials produced inconsistent results. In particular, one trial showed a modest slowing of cognitive decline in a high-dose subgroup, while the other failed to demonstrate benefit. Moreover, safety concerns such as *Amyloid-Related Imaging Abnormalities (ARIA),* combined with limited insurance coverage and high cost, further undermined its clinical adoption. Ultimately, Biogen discontinued Aducanumab in 2024, citing lack of clear efficacy, unresolved safety issues, and inability to secure partners for post-marketing studies (Whitehouse et al., 2022; Knopman et al., 2021; Biogen, 2024).

- **Lecanemab** (Leqembi™), approved in 2023, demonstrated a 27% slowing of cognitive decline in the CLARITY-AD trial, along with reductions in cerebrospinal fluid biomarkers such as pTau181 and markers of glial activation (Van Dyck et al., 2023). However, in July 2024 the EMA rejected marketing authorization, stating that the modest clinical benefit did not outweigh the risks, particularly the high incidence of ARIA. As a result, Lecanemab remains available in the U.S., Japan, and other regions, but is not approved in Europe (EMA, 2024).

- **Donanemab** (Kisunla™) is a humanized IgG1 monoclonal antibody that selectively targets a *pyroglutamate-modified form of Aβ (pGlu3-Aβ),* which is highly aggregation-prone and prevalent in plaques. By binding this, Donanemab facilitates plaque clearance and slows tau accumulation. In the phase 3 TRAILBLAZER-ALZ 2 trial, Donanemab significantly slowed cognitive and functional decline by approximately 32% on the Integrated Alzheimer's Disease Rating Scale over 76 weeks compared to placebo. Amyloid clearance was often achieved within 12–18 months, allowing for treatment discontinuation in some patients. However, ARIA events occurred in 26–30% of treated individuals, requiring monitoring. Donanemab received full FDA approval in 2024 for early symptomatic AD (Sims et al., 2023; Rashad et al., 2023).

Considering the limitations of anti-Aβ monoclonal antibodies, attention has shifted to alternative therapeutic targets, particularly neuroinflammation, now recognised as an early and crucial event in the pathogenesis of AD. Genomic association studies have linked the risk of AD to immune genes such as TREM2 and CD33, highlighting the role of microglia in the onset of the disease. Microglial activation initially plays a protective role by promoting the removal of amyloid plaques; however, its persistence induces a continuous release of pro-inflammatory cytokines (e.g., IL-1β, TNF-α), oxidative stress, and synaptic dysfunction, creating a vicious cycle that accelerates tau pathology and neuronal loss (Lista et al., 2024; Han et al., 2025; Erdag & Haskologlu, 2025). Therapeutic strategies under investigation include molecules that modulate microglial signalling (e.g. TREM2 agonists), NLRP3 inflammasome inhibitors, and approaches targeting systemic inflammation and gut-brain axis interactions. Immunomodulatory therapies aimed at restoring microglial homeostasis represent a promising avenue for modifying disease progression (Ameli-Mojarad et al., 2024; Ebrahimi et al., 2025).

At the same time, therapies targeting tau protein are increasing, given the strong correlation between tau pathology and cognitive decline. Current approaches include GSK-3β and CDK5 kinase inhibitors, tau-specific phosphatase activators (e.g., PP2A), *antisense oligonucleotides (ASOs)* such as BIIB080, monoclonal antibodies (e.g., gosuranemab, semorinemab), and emerging technologies such as *Proteolysis-Targeting Chimeras (PROTACs),* designed to promote tau degradation (Congdon et al., 2023; Schreiner et al., 2025; Harris et al., 2025). ASOs have shown encouraging results in early-stage trials, reducing tau levels in cerebrospinal fluid and demonstrating favourable safety profiles, while immunotherapies against extracellular tau are in advanced clinical stages (Congdon et al., 2023). Despite progress, the clinical efficacy of tau-centric interventions has yet to be confirmed,

but their potential to modify the disease positions them as a key element in future therapeutic paradigms for AD.

In conclusion, the management of AD will require a multimodal approach that integrates anti-amyloid therapies, anti-tau strategies and interventions targeting neuroinflammation, with the aim of slowing progression and improving patients' quality of life.

## *Pulsed Electromagnetic Fields*

Despite the availability of FDA-approved therapies, including cholinesterase inhibitors, NMDA receptor antagonists, and recently anti-amyloid monoclonal antibodies, these treatments offer only modest symptomatic relief or limited disease-modifying effects, and their long-term efficacy remains uncertain (Van Dyck et al., 2023; Wu W et al., 2023; Huang et al., 2023). Moreover, anti-amyloid antibodies require amyloid confirmation and MRI monitoring due to risks of ARIA, raising concerns about safety, cost, and accessibility (Jin & Noble, 2024). Consequently, there is growing interest in alternative and complementary therapeutic strategies that can target multiple pathogenic mechanisms beyond amyloid clearance.

Among these, *Pulsed Electromagnetic Fields (PEMFs)* have gained attention as non-invasive biophysical stimuli capable of modulating cellular activity. PEMFs represent a specific class of electromagnetic fields generated by pulsed signals with peak magnetic flux densities in the millitesla (mT) range. These fields are characterized by rapid variations in magnetic field intensity ($d\Phi/dt$) occurring on a millisecond timescale, with a well-defined waveform and amplitude parameters (Capone et al., 2009). PEMFs are produced by coils through circulating electric currents, which induce secondary time-varying electric fields within exposed tissues (Capone et al., 2022). Unlike direct electrical stimulation, PEMFs do not require electrodes on the skin or invasive implantation, making them a non-invasive approach for targeting injured or pathological tissues without interfering with nerve or muscle function (Hug & Röösli, 2012).

PEMFs influence key molecular targets, particularly ion channels and purinergic receptors, which play essential roles in cellular homeostasis and neuroprotection. *Voltage-gated calcium channels (VGCCs)* are highly responsive to PEMF exposure, facilitating transient calcium influx which serve as a pivotal second messenger in numerous signaling cascades. This calcium entry triggers downstream signaling pathways involved in mitochondrial metabolism, regulation of apoptosis, and modulation of inflammatory responses (Wang et al., 2024; Song et al., 2024). Calcium signaling

under PEMF stimulation has been linked to enhanced mitochondrial biogenesis and improved ATP production, which are essential for neuronal survival under stress conditions.

While VGCC activation appears central to PEMF-mediated effects, evidence suggests that other voltage-sensitive channels may also contribute, such as sodium and potassium channels, may also contribute to changes in membrane excitability and synaptic transmission (Funk, 2018; Peng et al., 2021). These findings highlight the complexity of PEMF interactions with excitable membranes and underscore the need for further mechanistic studies to delineate the full spectrum of ion channel involvement.

Concurrently, PEMFs also modulate purinergic signaling by upregulating $A_{2A}$ and $A_3$ receptor expression. These G protein-coupled receptors play critical roles in regulating anti-inflammatory and cytoprotective pathways (Vincenzi et al., 2013). Activation of adenosine receptors under PEMF exposure has been associated with suppression of pro-inflammatory cytokine release, inhibition of microglial activation, and prevention of programmed cell death (; Capone et al., 2022). Mechanistically, $A_2A$ receptor stimulation enhances intracellular cAMP levels, activating protein kinase A (PKA) and downstream transcription factors such as CREB, which promote neurotrophic factor expression and synaptic plasticity). $A_3$ receptor activation, on the other hand, is linked to anti-apoptotic signaling and mitochondrial protection (Vincenzi et al., 2013). This receptor-driven mechanism provides a plausible explanation for the observed neuroprotective effects of PEMFs in models of neurodegeneration, including AD and Parkinson's disease.

PEMFs also modulate key intracellular signaling cascades implicated in neuronal survival and plasticity. Experimental evidence suggests that PEMFs activate PI3K/Akt, MAPK/ERK, and CREB pathways, which are essential for promoting cell survival, differentiation, and synaptic remodeling (Capone et al., 2022; Wang et al., 2024). These pathways converge on transcriptional programs that enhance neurotrophic factor expression (e.g. BDNF), mitochondrial function, and antioxidant defenses, thereby mitigating oxidative damage and apoptosis.

They have been shown to reduce oxidative stress, enhance mitochondrial function, and promote synaptic plasticity (Funk et al., 2021; Merighi et al., 2025). In neuronal models, PEMFs activate pathways such as PI3K/Akt, CREB, and MAPK/ERK, which are critical for neuronal differentiation, survival, and repair (Urnukhsaikhan et al., 2016; Merighi et al., 2025). Experimental evidence from both *in vitro* and *in vivo* studies supports the anti-inflammatory, anti-apoptotic, and neuroprotective properties of PEMFs in models of neurodegeneration (Capone et al., 2022; Vincenzi et al., 2017).

Specifically, PEMFs have been shown to protect neurons from oxidative damage by reducing ROS production, increasing antioxidant enzyme activity, and activating protective signaling pathways such as p38 MAPK and CREB (Merighi et al., 2024; Gessi et al., 2019). They also promote the expression of neurotrophic and anti-apoptotic proteins, including BDNF and Bcl-2, which are essential for neuronal regeneration and survival (Lin et al., 2023; Gessi et al., 2019).

Furthermore, PEMFs have been shown to significantly enhance neurite outgrowth and elevate intracellular cAMP levels, both of which are critical mediators of synaptic remodeling and neuronal connectivity. In MN9D dopaminergic neurons, PEMF exposure promotes neurite elongation and branching, processes essential for restoring dopaminergic circuitry in neurodegenerative conditions (Lekhraj et al., 2014).

Beyond structural remodeling, emerging evidence suggests that PEMFs exert systemic effects on gene regulation. Studies indicate that PEMFs can modulate gene expression in *peripheral blood mononuclear cells (PBMCs)*, influencing immune responses and inflammatory pathways. This modulation appears to involve epigenetic mechanisms, including alterations in microRNA (miRNA) activity, which play a pivotal role in post-transcriptional regulation of genes associated with neuroinflammation and synaptic function (Capelli et al., 2017).

For example, PEMF-induced changes in miRNA profiles may downregulate pro-inflammatory mediators while upregulating neuroprotective factors, thereby contributing to the rebalancing of dysregulated signaling pathways observed in AD (Capelli et al., 2017).

Epigenetic modulation by electromagnetic fields, including ELF-MF and H-LIPEF, is an emerging area of research. Evidence suggests that these fields can influence gene expression through mechanisms such as DNA methylation, histone acetylation, and miRNA regulation, which are critical for neuronal survival and inflammatory control (Consales et al., 2018; Falone et al., 2016; Urnukhsaikhan et al., 2016). Although direct chromatin remodeling under PEMF exposure remains to be fully elucidated, studies on related electromagnetic modalities support the hypothesis that these fields may restore cellular homeostasis by modulating epigenetic and transcriptional programs (Giorgi & Del Re, 2021). This multi-level regulatory capacity underscores the potential of electromagnetic stimulation not only as a physical stimulus for neuronal repair but also as a systemic modulator of molecular networks implicated in neurodegeneration.

Recent studies using SH-SY5Y and N9 cells exposed to oxidative insults such as *hydrogen peroxide ($H_2O_2$)* and an $A\beta_{1-42}$ analog (O-acyl isopeptide, CP) have demonstrated that PEMFs significantly

reduce ROS levels, improve mitochondrial function, and decrease cell death, providing compelling evidence of their neuroprotective potential (Merighi et al., 2024; Merighi et al., 2025). Clinically, pilot trials such as the study *Effects of PEMF Treatment on Patients With Mild to Moderate Dementia* are investigating pulsed electromagnetic field therapy in AD, aiming to assess safety, cognitive outcomes, and disease progression (ClinicalTrials.gov, 2025; Herrick Medical LLC, 2025). Separately, preliminary evidence from earlier research suggests improvements in cognitive performance and functional connectivity, supporting PEMFs as a promising adjunctive therapy (Arendash, 2016).

Although research on the effects of PEMFs is still in its early stages and further studies are required, current evidence suggests that brain stimulation with PEMFs can provide tangible clinical benefits for patients with AD (Capelli et al., 2017).

Currently, PEMF therapy is recognized by the FDA as a non-invasive, safe, and effective modality within magnetic therapy for managing various health conditions, including bone fractures, osteoporosis, joint and muscle pain, osteoarthritis, rheumatoid arthritis, algodystrophy, articular cartilage disorders, and fibromyalgia (Waldorff et al., 2017).

# AIM OF THE THESIS

The aim of this thesis was to investigate the cellular and molecular mechanisms underlying the neuroprotective effects of PEMFs in the context of AD. Specifically, the study focused on evaluating key parameters such as cell viability, oxidative stress, mitochondrial membrane potential, and the modulation of intracellular signaling pathways including ERK1/2 and the cAMP/CREB/BDNF axis. To achieve this, *Nerve Grow Factor (NGF)*-differentiated PC12 cells were employed as an *in vitro* model of AD, subjected to oxidative and amyloidogenic insults induced by hydrogen peroxide and amyloid-β analogs. This approach allowed for a comprehensive assessment of PEMFs as a non-invasive therapeutic strategy aimed at counteracting neuronal damage and promoting cellular resilience.

Notably, this study has been published by the journal *International Journal of Molecular Sciences (IJMS)*:

*"PEMFs Restore Mitochondrial and CREB/BDNF Signaling in Oxidatively Stressed PC12 Cells Targeting Neurodegeneration"*

# MATERIALS AND METHODS

## *PEMFs Exposure System*

PC12 cells were exposed to PEMFs generated by a pair of rectangular coils (14 × 23 cm), made of 1,400 turns of copper wire placed opposite each other. The complete exposure system has already been described in detail (Cadossi et al., 1992; Varani et al., 2012).

The cells were placed between coils so that the plane of the coils was perpendicular to the culture plates. The coils were powered by the PEMF generator system (IGEA, Carpi, Italy). The selection of PEMF parameters (pulse duration 1.3 ms, frequency 75 Hz, magnetic field intensity 1.5 ± 0.2 mT) was based on previous studies using the same exposure system in neuronal and glial models (Massot et al., 2000; Varani et al., 2012; Barbault et al., 2009; De Mattei et al., 2009; Vincenzi et al., 2013). In particular, both 1.5 mT and 3.0 mT PEMFs, applied with identical pulse characteristics, induced consistent biological effects (Varani et al., 2012). The 1.5 mT intensity was selected in the present study as it represents the lowest effective dose capable of eliciting neuroprotective responses, ensuring both biological efficacy and safety. The peak intensity of the magnetic field and the peak intensity of the induced electric voltage were detected in air between two coils from one side to the other, at the level of the culture plates. The peak values measured between two coils in air had a maximum variation of 1% in the whole area in which the culture plates were placed. The peak intensity of the magnetic field was detected using the Hall probe (HTD61-0608-05-T, 8 F.W. Bell, Sypris Solutions, Louisville, KY) of a gaussmeter (DG500, Laboratorio Elettrofisico, Milan, Italy) with a reading sensitivity of 0.2%. The corresponding peak amplitude of the induced electric voltage was 2.0 ± 0.5 mV. It was detected using a standard coil probe (50 turns, 0.5 cm internal diameter of the coil probe, 0.2 mm copper diameter), and the temporal pattern of the signal was displayed using a digital oscilloscope (Le Croy, Chestnut Ridge, NY). The shape of the induced electric voltage and its impulse length were kept constant (Figure 9).

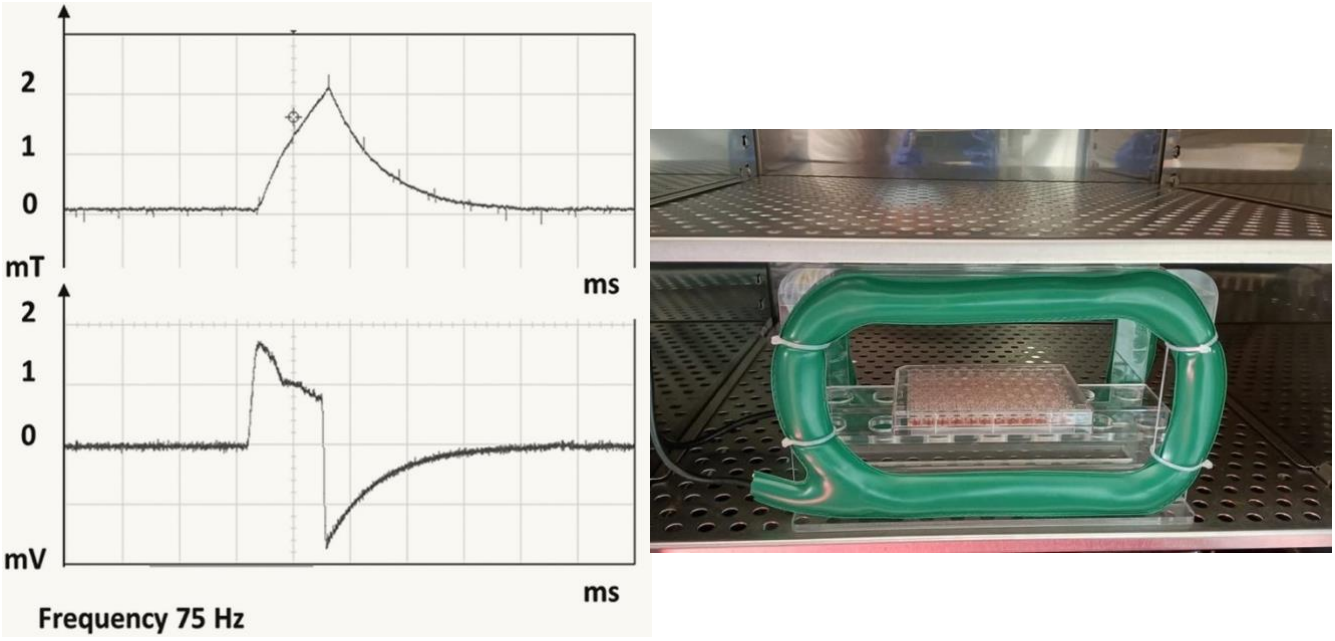


***Figure 9. signal waveform of IGEA PEMFs therapy.*** *The peak intensity of the magnetic field is 1.5 ± 0.2 mT, detected using the Hall probe (HTD61-0608-05-T; F.W. Bell, Sypris Solutions, Louisville, KY) of a gasometer (DG500; Laboratorio Elettrofisico, Milan, Italy) with a reading sensitivity of 0,2%. The corresponding peak amplitude of the induced electric voltage is 0.2 ± 0.5 mV, detected using a standard coil probe (50 turns, 0.5 cm internal diameter of the coil probe, 0.2 mm copper diameter) and the temporal pattern of the signal is displayed using a digital oscilloscope (Capone et al., 2022).*

## *Preparation of 26-O-Acyl Isopeptide (CP) Stock*

The 26-O-Acyl Isopeptide or Click Peptide (CP) of $A\beta_{1-42}$, possessing an ester bond at the Gly(25)-Ser(26) sequence, is a water-soluble and non-aggregative precursor molecule and is capable of producing monomer $A\beta_{1-42}$. In addition, the CP is promptly converted to $A\beta_{1-42}$ (t1/2 10 s) at pH 7.4, via an O-to-N intramolecular acyl migration reaction (Figure 10).

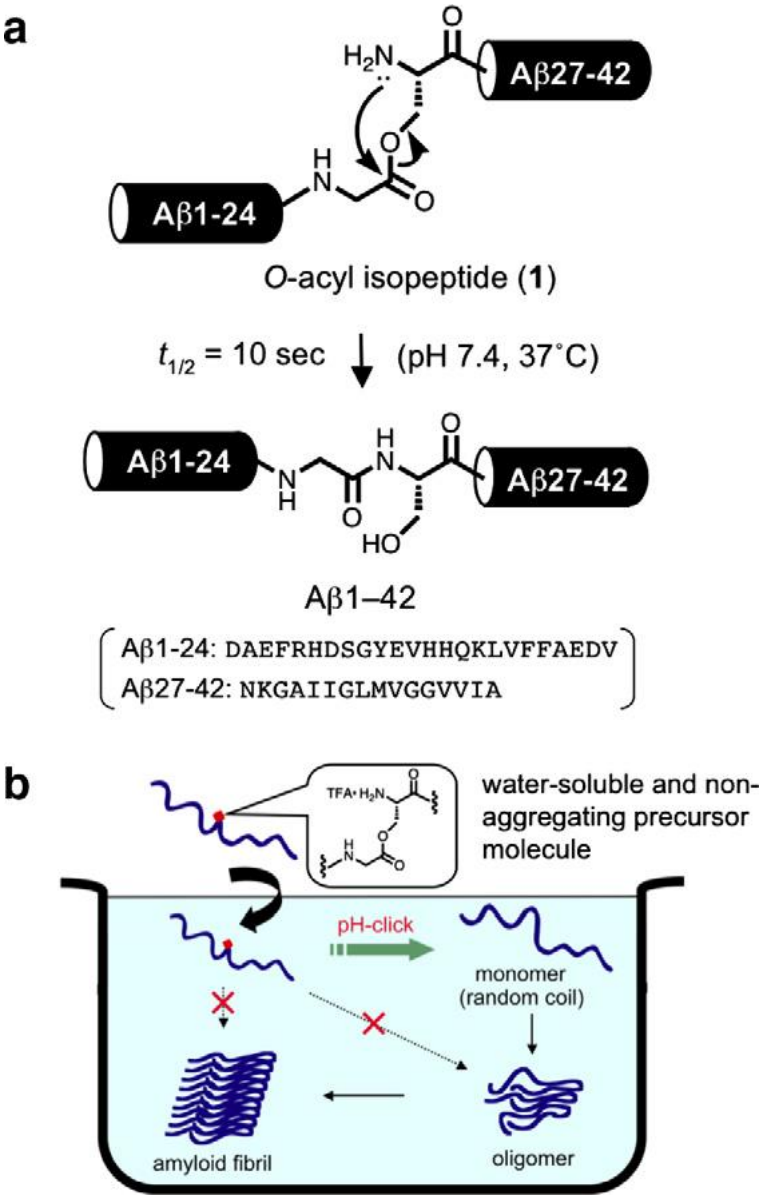


***Figure 10. Intramolecular acyl migration scheme to obtain Aβ$_{1-42}$ in monomeric form***. *The CP of Aβ$_{1-42}$ features an ester bond between the Gly(25) and Ser(26) residues. This precursor is soluble in water and does not aggregate, and it can yield monomeric Aβ$_{1-42}$. At a pH of 7.4, it rapidly transforms into Aβ$_{1-42}$, with a half-life of 10 seconds, via an intramolecular acyl migration reaction from O to N (Kawashima et al., 2014).*

For resuspension, dried CP was brought to *Room Temperature (RT)* for 30 min and 500 µL of 20 mM NaOH was added to 1 mg of the CP. Following this, the CP was diluted into *Phosphate Buffered Saline (PBS)* at pH 7.4 to a final concentration of 100 µM (Roberts et al., 2020). Ultimately, 100 µM CP was aggregated by incubating for 24 h at 4 °C (Ruotolo et al., 2020).

## *Cell Culture*

PC12 cells originate from a pheochromocytoma of the rat adrenal medulla and have an embryonic lineage traced back to the neural crest (Green et al., 1976). This cell line includes a combination of neuroblastic and eosinophilic cells. Due to their embryological background and the presence of neuroblastic features, PC12 cells have the capacity to differentiate into neuron-like cells, although they are not classified as mature neurons (Westerink et al., 2007). The term "neuron-like" refers to their ability to exhibit neuronal characteristics, such as neurotransmitter release via vesicular mechanisms (Howard et al., 1986). Upon exposure to NGF, PC12 cells cease proliferation and undergo terminal differentiation, acquiring features such as neurite outgrowth and the ability to synthesize and secrete catecholamines like dopamine and acetylcholine. These properties make PC12 cells a widely used and valuable in vitro model in neurobiological and neuropharmacological research, particularly for studying neuronal differentiation, neurotransmitter release, neurosecretion, and neurotoxicity (Oprea et al., 2022).

PC12 cells were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 1% (v/v) penicillin/streptomycin, 10% (v/v) horse serum (HS), and 5% (v/v) fetal bovine serum (FBS), under standard conditions at 37°C in a humidified incubator with 5% $CO_2$. To induce differentiation into a neuron-like phenotype, cells were cultured in high-glucose DMEM containing the same supplements (2 mM L-glutamine, 1% penicillin/streptomycin, 10% HS, and 5% FBS) along with 100 ng/mL NGF. This differentiation medium was applied for 3 days. All experiments in this study were conducted using NGF-differentiated PC12 cells. Before treatments, the differentiation medium was replaced with a fresh, serum-free version of the same medium to ensure optimal experimental conditions (Wiatrak et al., 2020).

To simulate cellular stress, two compounds were employed: (i) $H_2O_2$ at concentrations of 200 or 1000 μM, representing an oxidative agent known to induce neuronal toxicity, and (ii) CP at 20 μM, used to trigger both toxicity and inflammatory responses. Treatments were performed with or without exposure to PEMFs. Exposure times were selected based on preliminary dose-response experiments, which identified 24 hours as optimal for inducing cellular damage with 1 mM $H_2O_2$ and 20 μM CP. Time points were further tailored to the dynamics of the biological processes under investigation: 90 minutes for acute responses such as mitochondrial membrane potential depolarization (assessed via JC-1 assay) and chromatin changes (Hoechst 33342 staining); 20 minutes for rapid signaling events like ERK1/2 and CREB phosphorylation; 1 hour for cAMP quantification, and 4 hours for evaluating catalase enzymatic activity.

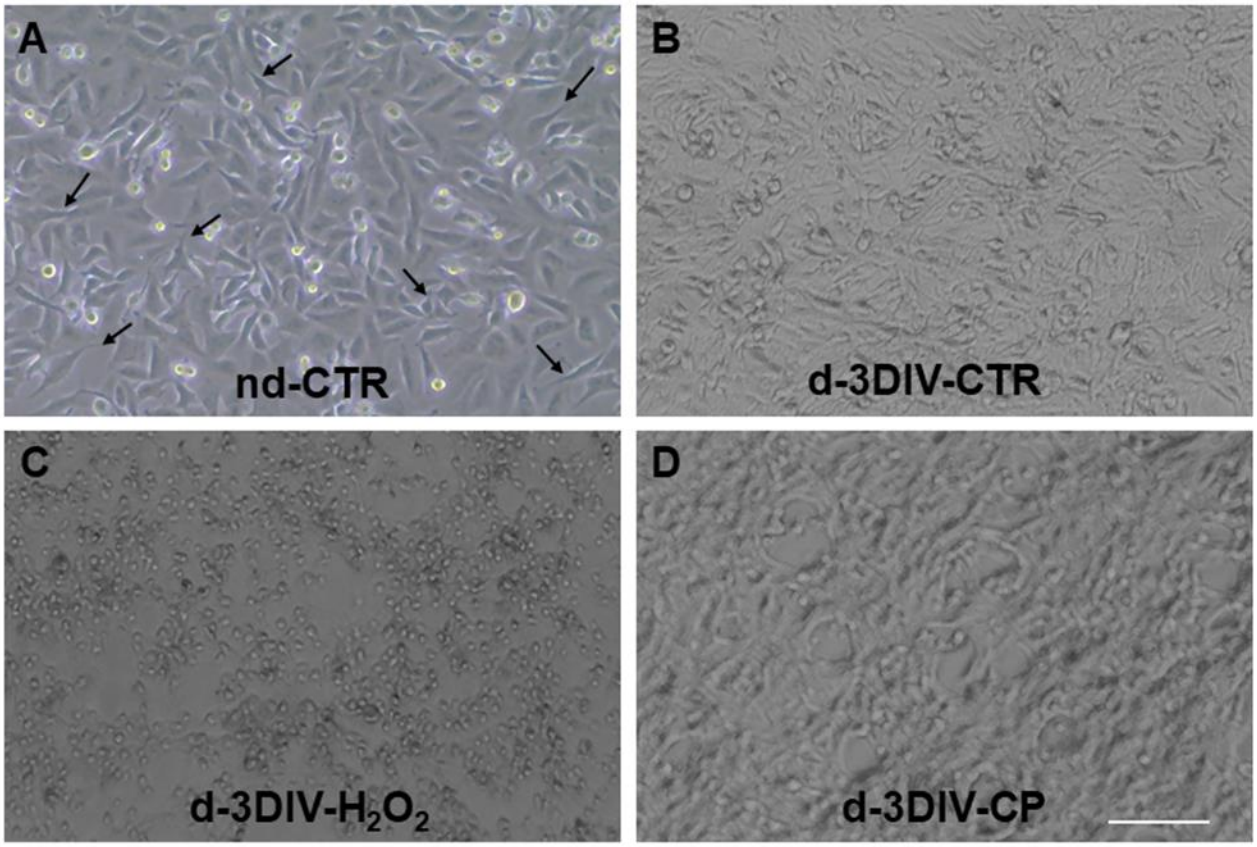


***Figure 11. Representative images of PC12 morphology under different experimental conditions.*** *A. Non-differentiated cells (nd-CTR). B. Differentiated cells (d-3 days in vitro (DIV-CTR) with NGF-containing medium. C. Differentiated cells treated with 1 mM H2O2 for 24h. D. Differentiated cells treated with 20 μM CP for 24h. Arrows indicate an irregularly polyhedral cell shape. Scale bar 100 μm.*

## *Pharmacological inhibitors*

To investigate the involvement of specific signaling pathways, selective pharmacological inhibitors were employed. Inhibitor concentrations were determined through preliminary dose–response and toxicity optimization experiments performed in the experimental model to ensure effective pathway inhibition while avoiding nonspecific cytotoxicity (data not shown), as commonly practiced in *in vitro* pharmacological studies.

The caspase-3 inhibitor was used at 1 μM, a concentration selected based on preliminary dose–response experiments and on previous evidence supporting the neuroprotective role of caspase-3 inhibition in neuronal models (Vaudry et al., 2000). The p38 MAPK inhibitor SB202190, the ERK1/2 inhibitor U0126, the JNK1/2 inhibitor SP600125, and the Akt/PKB inhibitor SH-5 were each applied at 1 μM, in line with previously reported concentrations and experimental conditions in similar

neuronal cell studies (Favata et al., 1998; Gessi et al., 2019). Inhibition of the NF-κB signaling pathway was achieved using BAY 11-7082, as described by Cao et al. (2021).

Pharmacological inhibition of the NLRP3 inflammasome was performed using MCC950 at a concentration of 1 μM, in line with prior in vitro and in vivo studies in central nervous system–related models, including neuroinflammatory and neurodegenerative contexts (Li et al., 2023). This concentration was selected to ensure robust suppression of NLRP3 activity without inducing cytotoxic effects. Finally, the CREB transcriptional inhibitor 666-15 was used at a concentration of 10 μM. This concentration falls within the micromolar range previously shown to effectively suppress CREB-dependent gene transcription in in vitro cellular systems, including transcriptional reporter assays and cancer cell lines (Xie et al. 2015). All concentrations are within the working ranges reported in manufacturers' technical datasheets and are supported by published biochemical potency data ($IC_{50}$/Ki).

## *MTS*

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2h-tetrazolium (MTS) assay was performed to determine cell viability following the manufacturer's instructions. In brief, $3.1x10^4$ cells/$cm^2$ were seeded in 96 multiwell plates (Nunc™ MicroWell™ 96-Well Microplate, Thermo Fisher Scientific) and cultured for 3 days in differentiation medium. The day of the assay, the medium was removed, and cell treatments were performed in serum-free differentiation medium. For the PEMFs-exposed group of cells, the culture plate was positioned inside the PEMFs exposure system for 4 h before treatments, then for 24 h during the compound's treatment time. At the end of the incubation period, 20 μL of MTS solution was added to each well, incubated for 1h, and the optical density was read using the EnSight multimode plate reader (Perkin Elmer, Milan, Italy) at 490 nm.

## *Caspase-3 and Chromatin Condensation (Immunocytochemistry)*

PC12 cells were seeded onto chamber slides pre-coated with Poly-L-Lysine at a density of $3.6 \times 10^5$ cells/$cm^2$ and cultured for three days in NGF-supplemented medium. On the day of the assay, cells were exposed to serum-free medium containing either 1 mM $H_2O_2$ or 20 μM CP for 90 minutes, followed by fixation with 4% paraformaldehyde for 20 minutes at RT.

To block nonspecific binding and permeabilize the cells, a solution of 0.3% Triton X-100 and 2% bovine serum albumin (BSA) in PBS was applied for 1 hour at RT. Subsequently, cells were

incubated overnight at 4°C with a rabbit primary antibody targeting cleaved caspase-3 (Asp175) at a dilution of 1:250. After washing, cells were incubated for 1 hour at RT with an Alexa Fluor™ 488-conjugated goat anti-rabbit secondary antibody (1:500).

For nuclear staining, cells were treated with Hoechst 33342 (1 μg/mL) for 20 minutes at RT and rinsed with PBS. Slides were then mounted using an antifade solution containing 1,4-phenylenediamine in PBS:glycerol (2.5%, 1:3). To assess nonspecific binding of the secondary antibody, control staining was performed without the primary antibody, and no fluorescence signal was detected (data not shown).

Fluorescence images were acquired using a Nikon Eclipse 5i microscope. Quantitative analysis of cleaved caspase-3 and Hoechst 33342 signals was conducted using NIS Elements v5.11 software, evaluating at least five distinct fields per sample well. Total cell counts and the number of nuclei exhibiting chromatin alterations were determined based on Hoechst staining.

## *$H_2$DCFDA Assay*

ROS production in PC12 cells was assessed using the $H_2$DCFDA assay. To measure intracellular ROS levels, the *2',7'-Dichlorofluorescein Diacetate ($H_2$DCFDA)* Assay Kit was employed. Once internalized by the cells, the cell-permeable fluorogenic dye $H_2$DCFDA undergoes deacetylation by intracellular esterases, forming the non-fluorescent intermediate $H_2$DCF. In the presence of ROS, $H_2$DCF is rapidly oxidized to the highly fluorescent compound *2',7'-Dichlorofluorescein (DCF)*, with fluorescence intensity directly correlating to ROS levels.

Specifically, PC12 (10.000 cells/well) were seeded into black 96-well plates and allowed to adhere overnight. The following day, the culture medium was removed and replaced with 100 μL of 20 μM $H_2$DCFDA solution. Plates were incubated in the dark at 37°C for 1 hour, followed by a wash with 1× Assay Buffer. Treatments were then applied in serum-free medium.

Fluorescence was measured using the Ensight multimodal plate reader (Perkin Elmer) with excitation at 485 nm and emission at 538 nm. For experiments involving PEMFs, the culture plates were placed within the PEMF exposure system during the treatment period.

## *Catalase Activity Assay*

CAT activity of PC12 cells was quantitatively measured using a catalase colorimetric kit. Cells were seeded onto a 6-well plate at a density of $5.3\times10^5$ cells/cm$^2$ and cultured for 3 days in differentiation conditions.

The Catalase Colorimetric Activity Kit was used to measure the activity of the antioxidant enzyme catalase. In particular, PC12 differentiated with NGF cells were seeded into a 6-well plate (1.000.000 cells/well) in 1 ml of complete medium and incubated overnight for attachment. Subsequently, the cells were treated. At the end of the treatment, after preparing the cell lysates and diluting the samples in Assay Buffer 1X, they were added to the wells of a transparent half-area plate. Subsequently, 25 µL of $H_2O_2$ Reagent was added to each well, and the plate was incubated at RT for 30 min. After incubation, 25 µL of Substrate was added, followed by the addition of another 25 µL of Horseradish Peroxidase Solution 1X. Then, the plate was incubated for another 15 min at RT. During this incubation, Horseradish Peroxidase reacts with the Substrate in the presence of $H_2O_2$ to convert the colourless Substrate to a pink product. Increasing catalase levels in the samples causes a decrease in the $H_2O_2$ concentration and a reduction in the pink product. Finally, the colored product was read with the Ensight multimodal plate reader (Perkin Elmer) at a wavelength of 560 nm.

## *JC-1 assay*

*Mitochondrial membrane potential (Δψm)* was evaluated using the JC-1 Mitochondrial Membrane Potential Assay Kit, which utilizes the cationic dye *Tetraethyl-Benzimidazolyl-Carbocyanine Iodide (JC-1)*. This dye selectively accumulates in mitochondria, where its fluorescence properties vary depending on membrane potential. At low Δψm, JC-1 remains in its monomeric form, emitting green fluorescence (530 nm). At high Δψm, the dye forms aggregates that emit red to orange fluorescence (590 nm), allowing for a ratiometric assessment of mitochondrial polarization.

For the assay, PC12 (20.000 cells/well) were seeded into black 96-well plates and incubated overnight to allow cell attachment. The following day, the medium was replaced with 100 µL of 20 µM JC-1 solution, and the plate was incubated in the dark at 37°C for 30 minutes. After incubation, cells were washed twice with 1× Dilution Buffer. Treatments were applied during the second wash step using the same buffer. Fluorescence was measured using the Ensight multimodal plate reader (Perkin Elmer).

## *Microscopy Imaging of JC-1 MMP*

MMP was studied using a fluorescence microscope. For this purpose, cells were seeded in 8 well-chamber slides (Millicell EZ SLIDE 8-well glass slide, Merck, Italy) at $3.6x10^5$ cells/$cm^2$, and cultured for 3 days in NGF differentiation conditions. The assay was performed following the protocol described above. The day of the assay, after the treatment with 1 mM $H_2O_2$ or 20 µM CP for

90 min, cells were analyzed under a fluorescence microscope with TRITC and FITC filters. Red and green fluorescence images were acquired using the Nikon Eclipse 5i fluorescence microscope equipped with a cooled charge-coupled device (CCD) camera (Nikon DS-Qi1). The images were analyzed using the software NIS Elements v 5.11.

## *ERK1/2 Assay*

Total and phospho ERK1/2 protein levels were determined by the InstantOne™ ELISA kit. The protein levels were measured according to the manufacturer's instructions. Cells were seeded in 24-multiwell plates at a density of $1\times10^5$ cells/cm$^2$ and cultured for 3 days in differentiation medium. On the day of the assay the medium was replaced with serum-free differentiation medium and properly treated with either 1 mM $H_2O_2$ or 20 μM CP for 20 minutes. Cell lysates were prepared using the kit's proprietary lysis buffer. Briefly, samples were incubated for 1 hour at room temperature with a cocktail of capture and detection antibodies specific for total or phospho-ERK1/2. After washing, wells were incubated with the detection reagent for 20 minutes at RT, followed by the addition of stop solution. Absorbance was measured at 450 nm using the EnSight multimode plate reader (Perkin Elmer, Milan, Italy).

## *BDNF Assay*

Extracellular BDNF levels were quantified in the supernatant of PC12 cells using a BDNF ELISA assay kit, following the manufacturer's instructions. Cells were seeded into 24-well plates at a density of $1.6 \times 10^5$ cells/cm² and cultured for three days in differentiation medium. On the day of treatment, the medium was replaced with a serum-free differentiation medium, and cells were exposed to either 200 μM $H_2O_2$ or 20 μM CP for 24 hours.

For the assay, 100 μL of each sample or standard was added to the ELISA plate wells and incubated at 37°C for 1 hour and 20 minutes. After three washes with 1× wash buffer, 100 μL of biotinylated BDNF antibody solution (1:100 dilution in antibody diluent) was added to each well, followed by a 50-minute incubation at 37°C. Wells were then washed again (3×), and 100 μL of streptavidin-HRP solution (1:100 dilution in HRP diluent) was added, with a further 50-minute incubation at 37°C.

After five additional washes, 90 μL of TMB substrate solution was added to each well and incubated in the dark at 37°C for 20 minutes. The reaction was stopped by adding 50 μL of stop solution, and the plate was gently shaken for 1 minute to ensure mixing. Absorbance was immediately measured at 450 nm using the EnSight multimode plate reader (Perkin Elmer, Milan, Italy).

## *Total/Phospho-CREB Assay*

Phosphorylated and total CREB protein levels were measured using the AlphaLISA™ SureFire® Ultra™ assay, following the manufacturer's instructions. PC12 cells were seeded into 24-well plates at a density of $1 \times 10^5$ cells/cm² and cultured for three days in differentiation medium. On the day of the assay, the medium was replaced with serum-free differentiation medium, and cells were treated with either 1 mM $H_2O_2$ or 20 μM CP for 20 minutes.

After treatment, the medium was removed, and cell lysates were prepared using the lysis buffer provided in the kit. The assay was conducted in a ½ area OptiPlate™ 96-well plate (Perkin Elmer, Milan, Italy), protected from direct light. Briefly, 30 μL of each sample was incubated with an acceptor mix containing reaction buffer 1, reaction buffer 2, activation buffer, and acceptor beads for 1 hour at room temperature. Following this, a donor mix composed of donor beads and diluent was added, and samples were incubated for an additional hour at RT.

Luminescence was measured using the EnSight multimode plate reader (Perkin Elmer, Milan, Italy). The intensity of the emitted signal is directly proportional to the concentration of the target protein in the sample.

## *cAMP Quantification*

Intracellular cAMP levels were measured using the ALPHAscreen cAMP assay kit, following the manufacturer's protocol. ALPHAscreen (*Amplified Luminescent Proximity Homogeneous Assay*) is a bead-based technology used to quantify cAMP levels in cell lysates. The assay relies on the interaction between donor and acceptor beads brought into proximity by a biotinylated cAMP tracer and streptavidin-coated beads. Upon laser excitation, donor beads generate singlet oxygen, which induces a luminescent signal in nearby acceptor beads. The signal intensity is inversely proportional to the amount of endogenous cAMP: higher cellular cAMP competes with the tracer, reducing bead proximity and thus the luminescent signal. This competitive binding principle allows accurate quantification of intracellular cAMP concentrations.

PC12 cells were cultured for three days in differentiation medium. On the day of the assay, cells were resuspended in a stimulation buffer composed of HBSS, 5 mM HEPES, 0.5 mM $MgCl_2$, 100 μM RO, and 0.05% BSA (pH 7.4). After counting, a cell suspension was prepared at a concentration of $10^7$ cells/mL and mixed 1:1 with 10× acceptor beads.

Reactions were carried out in 96-well half-area microplates, with each well containing $2.5 \times 10^4$ cells in 5× acceptor beads. Where indicated, cells were treated with 1 mM $H_2O_2$ or 20 μM CP for 1 hour

at 37°C. Subsequently, 1 μM forskolin was added, and samples were incubated for an additional 30 minutes at 37°C.

To initiate detection, a mix containing 41.7 nM biotin-cAMP tracer and 33.3 μg/mL streptavidin-coated donor beads, diluted in 1× immunoassay buffer, was added to each well. Plates were incubated for 1 hour at 37°C in the dark. A standard curve was prepared and processed in parallel with the samples. Fluorescence was measured using the EnSight multimode plate reader (Perkin Elmer, Milan, Italy).

## *Data Analysis and Statistics*

All data in the figures and text are presented as mean ± standard error of mean (SEM) of at least three independent experiments performed in duplicate. Data sets were examined by one-way analysis of variance (ANOVA) and Sidak's multiple comparison test. P-value ($p < 0.05$) was considered a standard for statistically significant differences among the groups. Data analysis and statistical analysis were performed using GraphPad Prism 8.0.1 software.

# RESULTS

## *PEMFs' Effects on Viability in $H_2O_2$- and CP-injured PC12 cells*

Neuronal cell viability is a fundamental parameter for evaluating cellular integrity and functional resilience under conditions of stress. To investigate the cytotoxic effects of oxidative ($H_2O_2$) and amyloidogenic (CP) insults, as well as the potential neuroprotective role of PEMFs, NGF-differentiated PC12 cells were exposed to increasing concentrations of $H_2O_2$ (500 μM and 1 mM) or CP (10, 20 and 50 μM) for 24, 48, and 72 hours. Cell viability was quantified using the MTS assay. As illustrated in Figure 12, both $H_2O_2$ and CP induced a statistically significant, concentration- and time-dependent reduction in cell viability.

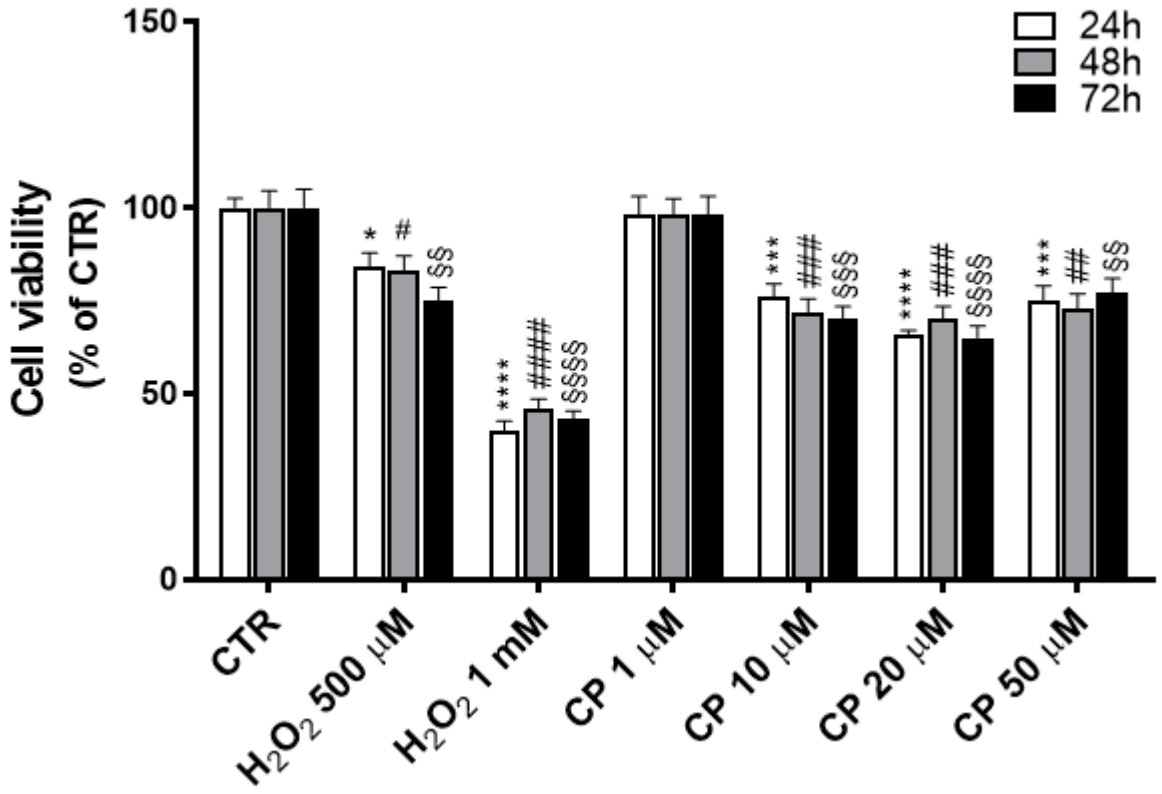


***Figure 12. Effects of $H_2O_2$ and CP treatments in PC12 cells.*** *Cells were treated with different concentrations of $H_2O_2$ and CP for 24, 48 and 72h. Results are presented as mean ± SEM values of at least three independent experiments performed in duplicate ($^{*}p<0.05$, $^{***}p<0.001$, $^{****}p<0.0001$, vs control (CTR) for 24h; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$, vs CTR for 48h; $^{§§}p<0.01$, $^{§§§}p<0.001$, $^{§§§§}p<0.0001$, vs CTR for 72h). Statistical analysis has been performed by one-way analysis of variance (ANOVA) and Sidak's multiple comparison test. CP, Click Peptide; $H_2O_2$: Hydrogen peroxide.*

Based on these preliminary findings, the conditions selected for subsequent experiments were 1 mM $H_2O_2$ and 20 μM CP for 24 hours, which consistently resulted in a pronounced decrease in viability (40 ± 3 and 66 ± 1 % compared to the control, respectively, $^{****}p<0.0001$). Notably, co-treatment with PEMFs for 24 hours significantly attenuated the cytotoxic effects.

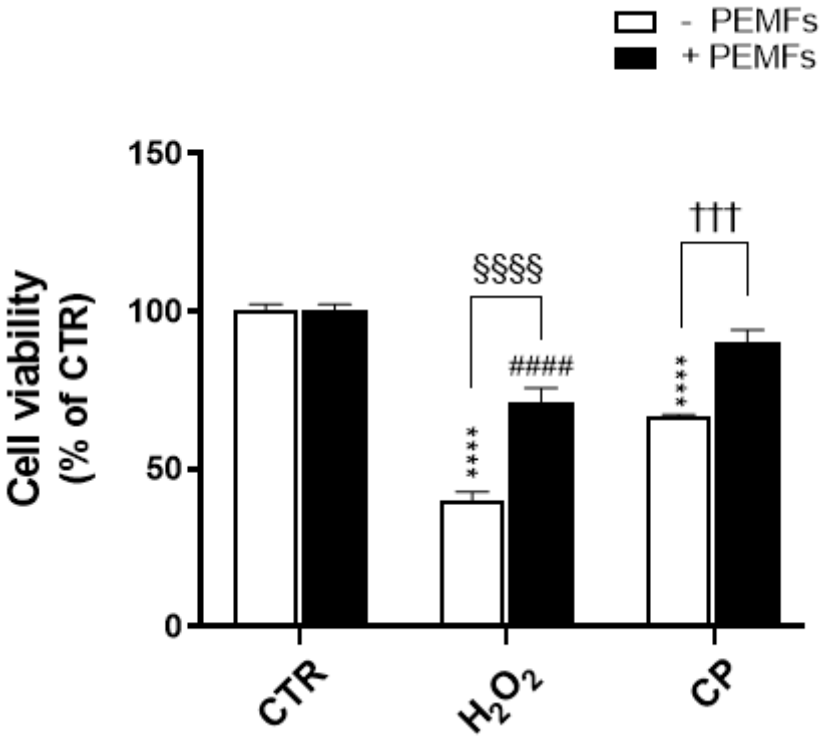


***Figure 13. PEMFs' effects on cell viability in PC12 cells*** *Cell viability was studied in 1 mM $H_2O_2$- and 20 μM CP-injured cells for 24h with and without PEMFs. Results are presented as mean ± SEM values of three or more experiments performed in duplicate ($^{****}p<0.0001$ vs CTR without PEMFs; $^{####}p<0.0001$ vs CTR with PEMFs; $^{§§§§}p<0.0001$, $^{†††}p<0.001$). Statistical analysis has been performed by one-way analysis of variance (ANOVA) and Sidak's multiple comparison test. CP: Click Peptide; $H_2O_2$: Hydrogen peroxide; PEMFs: Pulsed Electromagnetic Fields.*

As shown in Figure 13, PEMF exposure increased cell viability to 71 ± 5% in the $H_2O_2$-treated group ($^{§§§§}p<0.0001$) and to 90 ± 4% in the CP-treated group ($^{†††}p<0.001$), indicating a robust protective effect.

These results demonstrate that PEMFs can partially counteract the deleterious effects of both oxidative and amyloidogenic stressors in PC12 cells. The observed enhancement in cell viability supports the hypothesis of a neuroprotective action of PEMFs, thereby justifying further investigation into the underlying molecular mechanisms, including modulation of oxidative stress pathways and inhibition of apoptosis.

To elucidate the mechanisms underlying the reduction in cell viability observed following exposure to oxidative and amyloidogenic insults, we first examined the role of oxidative stress. PC12 cells were pre-treated with 500 μM Trolox, a water-soluble analogue of vitamin E with antioxidant properties, for 30 minutes before the application of injury stimuli. As shown in Figure 14, Trolox significantly mitigated cell death induced by both 1 mM $H_2O_2$ (73 ± 5% vs. 40 ± 3%, $^{§§§§}p<0.0001$) and 20 μM CP (79 ± 1% vs. 66 ± 1%, $^{†}p<0.05$), supporting the hypothesis that oxidative stress is a major contributor to the cytotoxic effects of these treatments.

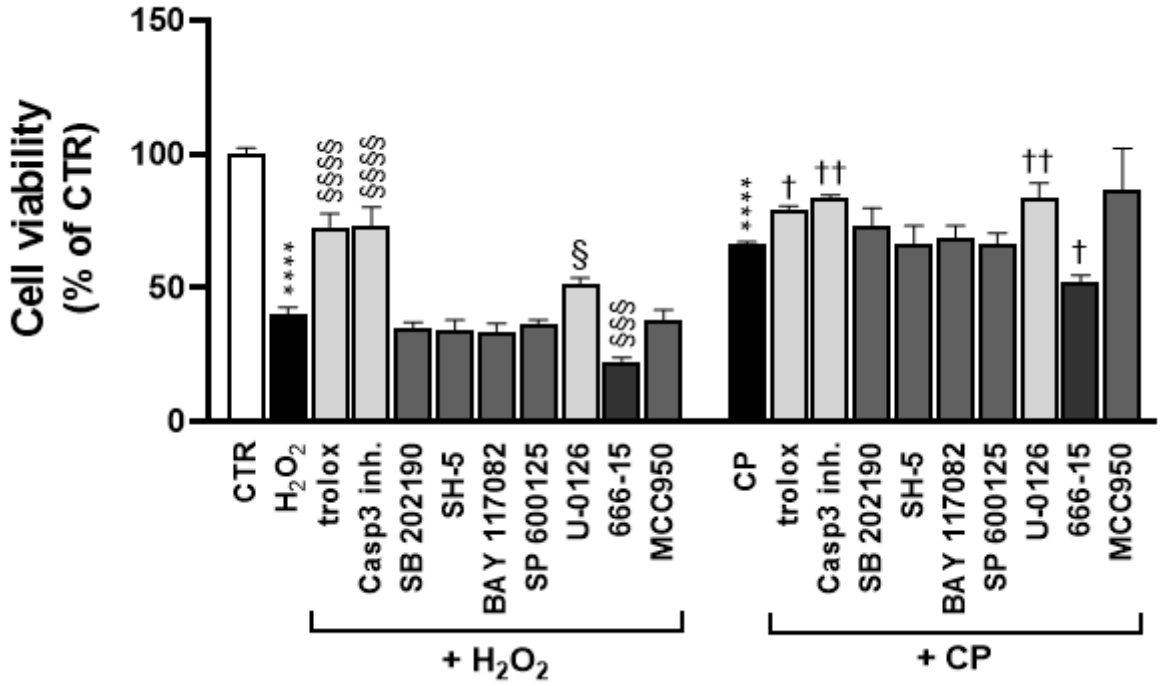


***Figure 14. Trolox effects on cell viability in PC12 cells.*** *Effects of cell pre-treatment for 30 min with 500 μM trolox**,** 1 μM caspase-3 inhibitor, SB 202190 (p38), SH-5 (Akt), BAY 117082 (NF-kB), SP 600125 (JNK1/2), U-0126 (ERK 1/2), MCC950 (NLRP3) and 10 μM 666-15 (CREB) on the viability of PC12 cells injured with 1 mM $H_2O_2$ or 20 μM CP. Results are presented as mean ± SEM values of at least three independent experiments performed in duplicate ($^{****}p<0.0001$ vs CTR; $^{§§§§}p<0.0001$, $^{§§§}p<0.001$, $^{§}p<0.05$ vs $H_2O_2$-treated cells; $^{††}p<0.01$, $^{†}p<0.05$ vs CP-treated cells). Statistical analysis has been performed by one-way analysis of variance (ANOVA) and Sidak's multiple comparison test. CP: Click Peptide; $H_2O_2$: Hydrogen peroxide; PEMFs: Pulsed Electromagnetic Fields.*

Subsequently, we investigated the involvement of programmed cell death, focusing on the caspase-3-dependent apoptotic pathway, which plays a central role in the execution phase of apoptosis. Cells were pre-incubated with a caspase-3 inhibitor (1 μM, 30 min) before exposure to $H_2O_2$ or CP.

The inhibitor significantly increased cell viability in both treatment groups: $H_2O_2$-treated cells (73 ± 7% vs. 40 ± 3%, $^{§§§§}p<0.0001$) and CP-treated cells (83 ± 1% vs. 66 ± 1%, $^{††}p<0.01$) (Figure 14), indicating that caspase-3 activation contributes to the observed cell death.

To further explore the molecular mechanisms underlying the protective effects of PEMFs, we performed a screening of pharmacological inhibitors targeting key signaling molecules and transcription factors involved in pro-survival and pro-apoptotic pathways. Using the MTS assay, we tested the following inhibitors: SB 202190 (p38 MAPK inhibitor), SH-5 (Akt inhibitor), BAY 117082 (NF-κB inhibitor), SP 600125 (JNK inhibitor), U-0126 (MEK/ERK1/2 inhibitor), MCC950 (NLRP3 inflammasome inhibitor), and 666-15 (CREB inhibitor). Cells were pre-treated with each compound (1 μM, except for 666-15 at 10 μM) for 30 minutes before exposure to $H_2O_2$ or CP for 24 hours. Notably, none of the inhibitors alone exhibited cytotoxic effects (data not shown).

Among the tested compounds, U-0126 and 666-15 were found to significantly modulate cell viability in response to both $H_2O_2$ and CP. Specifically, U-0126 partially rescued cells from $H_2O_2$-induced death (52 ± 2% vs. 40 ± 3%, $^{§}p<0.05$) and CP-induced death (83 ± 6% and 66 ± 1%, $^{††}p<0.01$), suggesting a detrimental role of ERK1/2 signaling in this context. Conversely, 666-15 exacerbated cell death induced by both $H_2O_2$ (22 ± 2% and 40 ± 3%, $^{§§§}p<0.001$) and CP (52 ± 2% and 66 ± 1%, $^{†}p<0.05$), indicating a protective role for CREB signaling in maintaining neuronal cell viability under stress conditions.

These findings provide mechanistic insights into the cellular pathways involved in PC12 cell response to oxidative and amyloidogenic stress, and highlight potential targets for enhancing neuroprotection, including the modulation of oxidative stress, apoptosis, and CREB-mediated transcriptional activity.

## *PEMFs' Effects on Cleaved Caspase-3 Expression and Chromatin Condensation in $H_2O_2$- and CP-injured PC12 cells*

Subsequently, to investigate the potential of PEMFs to counteract cell death induced by $H_2O_2$ and CP, and considering the involvement of caspase-3 in apoptotic pathways, we examined the expression of cleaved caspase-3 by immunocytochemistry. Exposure of PC12 cells to $H_2O_2$ and CP for 90 minutes resulted in a significant upregulation of cleaved caspase-3 expression, reaching 235 ± 10% and 230 ± 15% compared to control levels (100 ± 4%, $^{****}p<0.0001$), indicating activation of apoptosis in response to both oxidative and amyloidogenic stress.

Importantly, continuous exposure to PEMFs for 90 minutes, applied immediately following the $H_2O_2$ or CP treatments, significantly reduced cleaved caspase-3 levels to 184 ± 6 % in the $H_2O_2$ group ($^{§§§}p<0.001$) and 155 ± 9% in the CP group ($^{††††}p<0.0001$), suggesting a partial protective effect of PEMFs against apoptosis.

Representative images of cells double-stained for cleaved caspase-3 and Hoechst 33342 are presented in Figure 15. Quantitative analysis of cleaved caspase-3 fluorescence intensity, normalized to the total number of cells and expressed as a percentage relative to the control group, is shown in the accompanying graph (Figure 16).

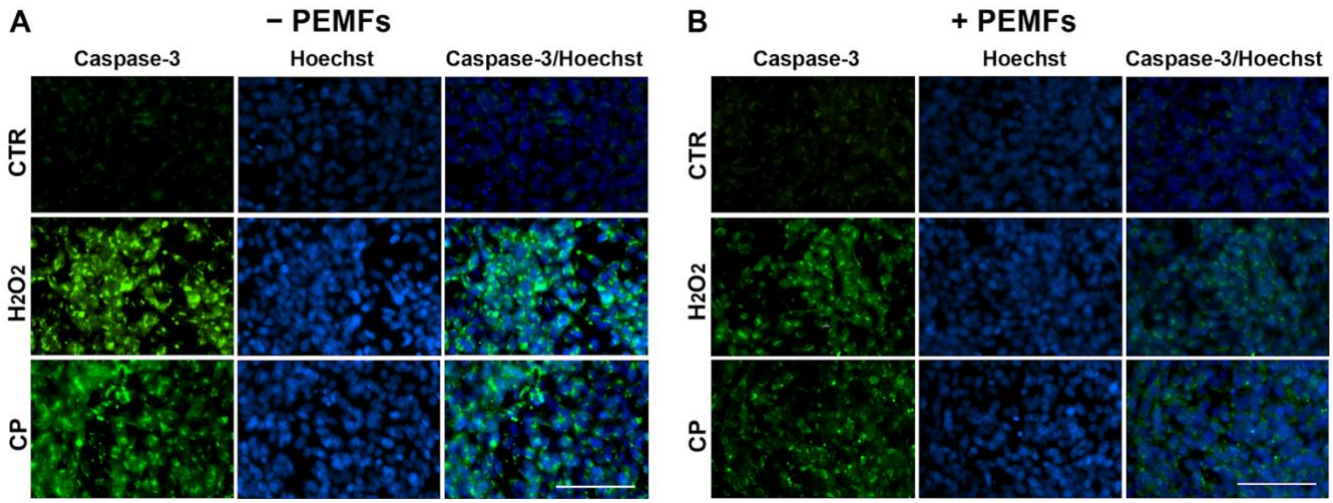


***Figure 15. Representative images of cleaved caspase-3*** *positive PC12 cells (green) in control (CTR) and with 1 mM $H_2O_2$ or 20 µM CP treatment for 90 min, without (**A**) and with (**B**) PEMFs. Hoechst 33342 nuclear staining (blue) and a merge of cleaved caspase-3/Hoechst 33342 nuclear staining have been included. Scale bar 100 µm.*

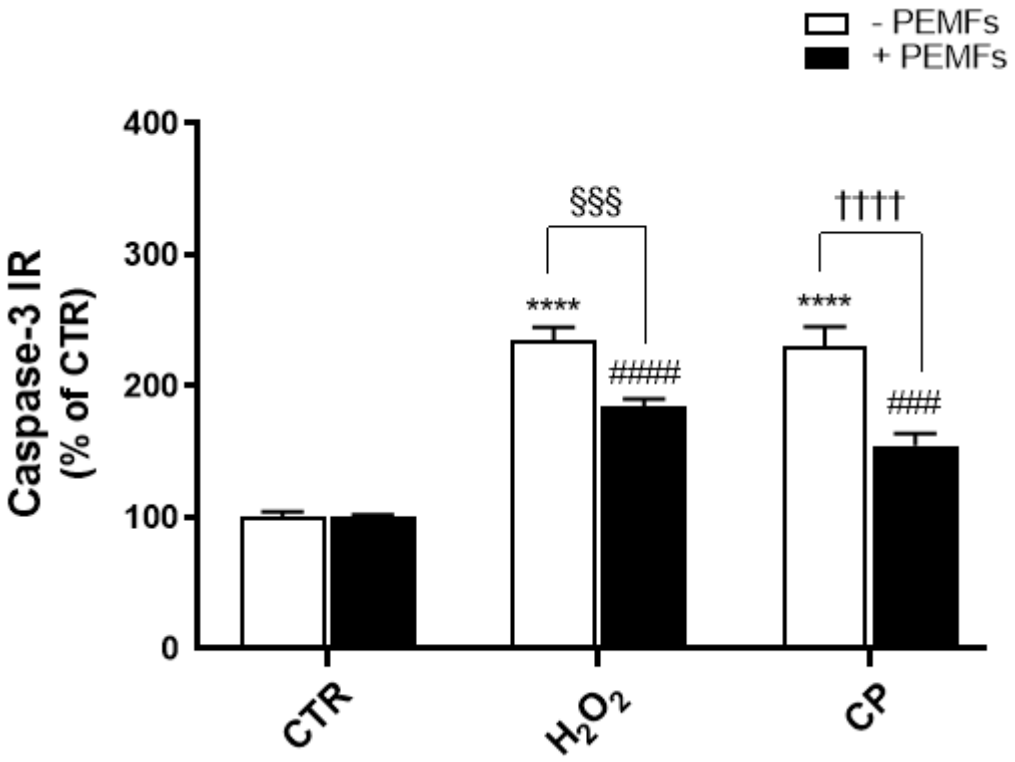


***Figure 16. PEMFs' effects on cleaved caspase-3 in PC12 cells***. *Analysis of cleaved caspase-3 immunoreactivity (IR) normalized to the number of cells. Results are presented as mean ± SEM values of at least three independent experiments performed in duplicate* ($^{****}p<0.0001$ *vs CTR without PEMFs;* $^{\#\#\#\#}p<0.0001$ *and* $^{\#\#\#}p<0.001$ *vs CTR with PEMFs, respectively;* $^{§§§}p<0.001$; $^{††††}p<0.0001$). *CP: Click Peptide;* $H_2O_2$*: Hydrogen peroxide; PEMFs: Pulsed Electromagnetic Fields.*

To investigate the pro-survival effects of PEMFs on PC12 neuronal-like cells, we employed Hoechst 33342 staining, a widely used method for assessing nuclear morphology and detecting apoptotic changes. This DNA-binding fluorescent dye enables the visualization of nuclear condensation and fragmentation by producing bright, compact signals in apoptotic nuclei, in contrast to the uniform staining observed in healthy cells. Additionally, Hoechst 33342 allows for the identification of replicating cells and the estimation of DNA content, making it a versatile tool for cell cycle and viability studies.

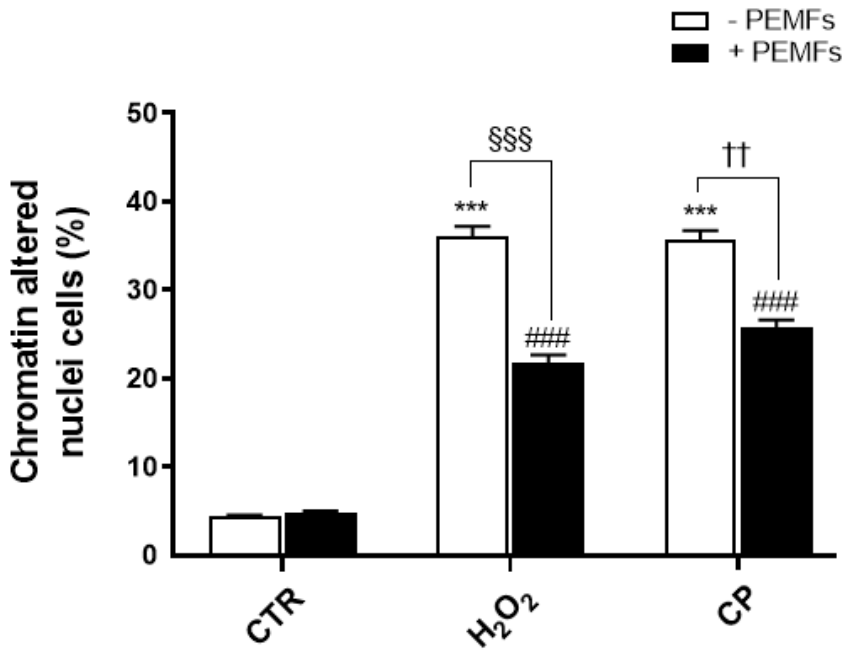


***Figure 17. Analysis of Hoechst 33342 chromatin-altered nuclei cells.*** *Data are presented as mean ± SEM values ($^{***}p<0.001$ vs CTR without PEMFs; $^{\#\#\#}p<0.001$ vs CTR with PEMFs; $^{§§§}p<0.001$; $^{††}p<0.01$). Statistical analysis has been performed by one-way ANOVA and Sidak's multiple comparison test. CP: Click Peptide; $H_2O_2$: Hydrogen peroxide; PEMFs: Pulsed Electromagnetic Fields.*

In our experimental model, PC12 cells treated with $H_2O_2$ or CP for 90 minutes exhibited a significant increase in the percentage of cells showing chromatin-altered nuclei (36 ± 2% and 36 ± 1%, respectively) compared to control cells (4 ± 1%, $^{***}p<0.001$), indicating activation of apoptotic pathways.

Importantly, PEMF exposure significantly reduced the proportion of cells with condensed chromatin to 22 ± 1% in the $H_2O_2$ group ($^{\#\#\#}p<0.001$) and 26 ± 1% in the CP group ($^{§§§}p<0.001$; $^{††}p<0.01$), suggesting a protective effect at the nuclear level (Figure 17). No significant differences were observed between PEMF-exposed and non-exposed control groups (4 ± 1% vs. 5 ±1%), confirming that PEMFs do not alter nuclear morphology under basal conditions.

To further support these findings, we quantified the mean fluorescence intensity of Hoechst 33342-stained nuclei across all experimental conditions. The results closely mirrored the percentage of chromatin-condensed nuclei (Figure 18), reinforcing the association between increased Hoechst fluorescence and apoptotic nuclear changes.

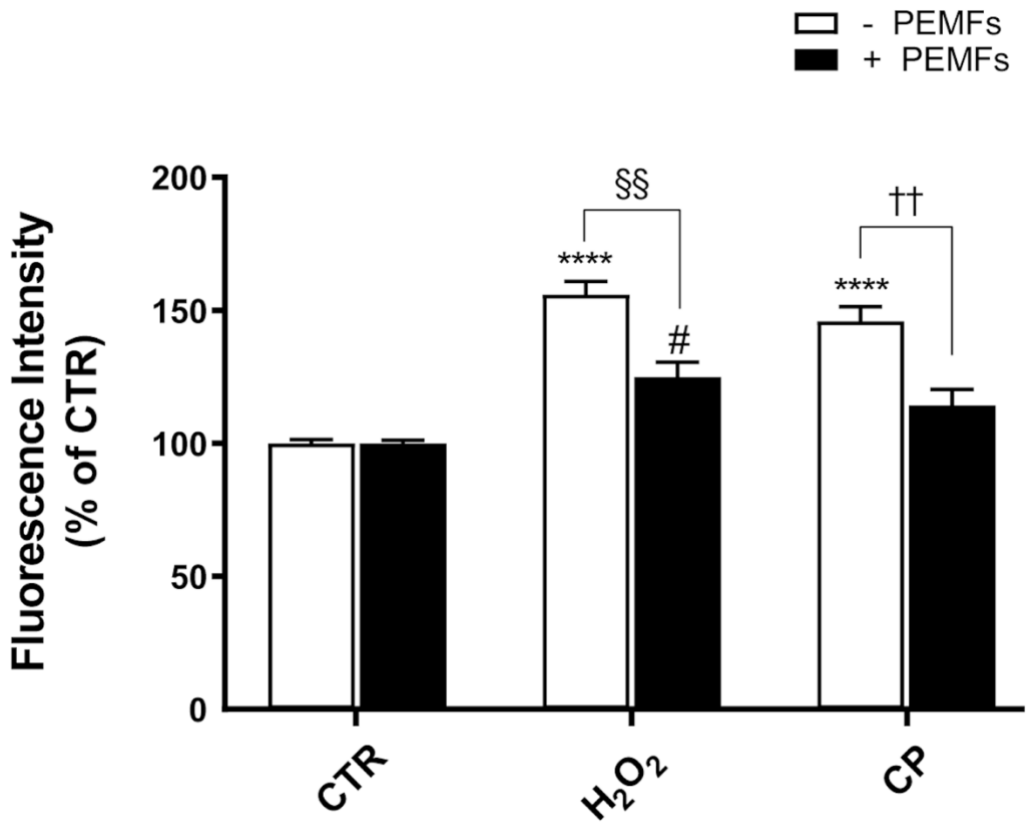


***Figure 18. Hoechst 33342 cell staining mean intensity.*** *The mean fluorescent intensity of Hoechst 33342 staining has been measured and analysed. Results, expressed as percentage of the control group, are presented as mean ± SEM values and the statistical analysis has been performed by student's t test ($^{***}p<0.001$ and $^{**}p<0.001$ vs CTR without PEMFs; $^{\#}p<0.05$ and $^{\$}p<0.05$ vs CTR with PEMF; $^{\S\S}p<0.01$; $^{\dagger\dagger}p<0.01$ ). CP: Click Peptide; $H_2O_2$: Hydrogen peroxide; PEMFs: Pulsed Electromagnetic Fields.*

## *PEMFs Effects on Oxidative Stress and Catalase Activity in $H_2O_2$- and CP-injured PC12 cells*

The use of $H_2$DCFDA to measure ROS is supported by its established role as a sensitive probe for intracellular redox changes. As ROS are key mediators of oxidative damage, their quantification is essential for assessing cellular stress responses.

The fluorogenic dye $H_2$DCFDA is commonly used to assess intracellular ROS activity. The resulting fluorescence intensity directly correlates with ROS levels in the cytosol, which are known to contribute to DNA damage and the oxidation of proteins and lipids, ultimately leading to oxidative stress. We evaluated ROS production 24 hours after treatment with 1 mM $H_2O_2$ and 20 μM CP. As shown in Figure 19, both $H_2O_2$ and CP significantly increased fluorescence intensity compared to untreated control cells (176 ± 13% and 129 ± 7% vs. 100 ± 6%, $^{****}p<0.0001$ and $^{*}p<0.05$,

respectively), confirming that these toxic agents induce oxidative stress in this cellular model. Notably, when cells were exposed to PEMFs for 24 hours immediately following treatment with $H_2O_2$ and CP, ROS levels were markedly reduced (125 ± 13% and 91 ± 11%, $^{§§}p<0.01$ and $^{†}p<0.05$, respectively), as illustrated in Figure 19.

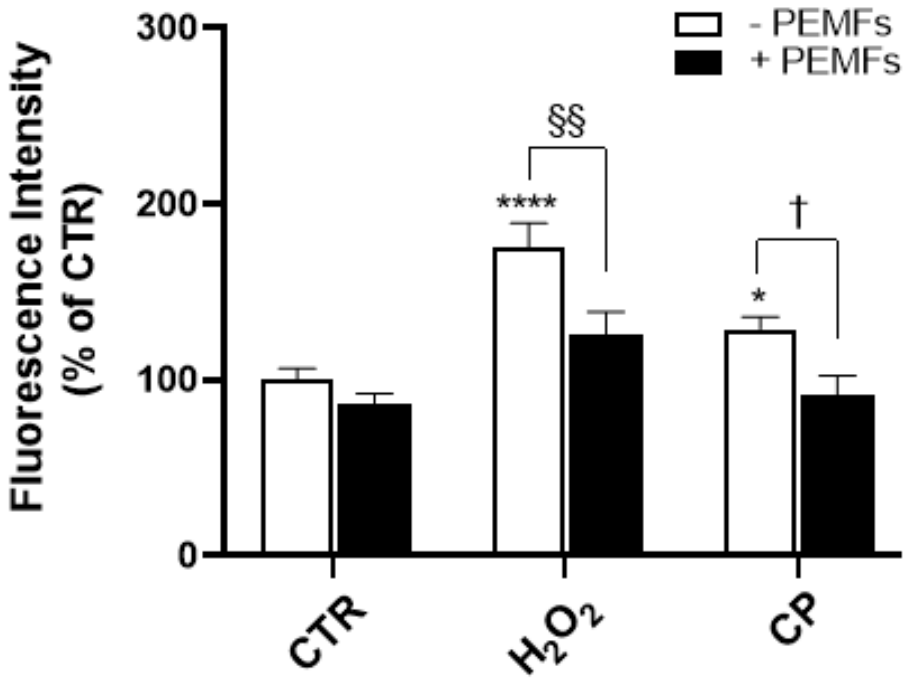


***Figure 19. PEMFs' effects on oxidative stress in $H_2O_2$- and CP-injured PC12 cells.*** *ROS levels in PC12 cells treated with 1 mM $H_2O_2$ or 20 μM CP for 24h through the $H_2$DCFDA test. Results are presented as mean ± SEM values of at least three independent experiments performed in duplicate ($^{****}p<0.0001$ and $^{*}p<0.05$ vs control (CTR) without PEMFs, respectively; $^{§§}p<0.01$ vs $H_2O_2$-injured cells; $^{†}p<0.05$ vs CP-injured cells). Statistical analysis has been performed by one-way ANOVA and Sidak's multiple comparison test. CP: Click Peptide; $H_2$DCFDA, 2',7'-Dichlorodihydrofluorescein Diacetate; $H_2O_2$: Hydrogen peroxide; PEMFs: Pulsed Electromagnetic Fields; ROS, reactive oxygen species.*

Based on these findings, we further explored whether PEMF exposure could influence the activity of endogenous antioxidant defense mechanisms, specifically focusing on catalase (CAT), an enzyme responsible for converting $H_2O_2$ into oxygen and water. PC12 cells were treated with 200 μM $H_2O_2$ and 20 μM CP for 4 hours, with or without concurrent PEMF exposure, and CAT activity was subsequently measured. As illustrated in Figure 20, treatment with $H_2O_2$ and CP led to a reduction in CAT activity compared to the control group (82 ± 6% and 85 ± 3% vs. 100 ± 3%, $^{*}p<0.05$).

Remarkably, PEMF exposure restored CAT activity to levels comparable to the untreated control following both treatments (100 ± 5% and 100 ±4%, $^{\S}p<0.05$ and $^{\dagger}p<0.05$, respectively) (Figure 20). These results support the hypothesis that PEMFs exert a protective antioxidant effect, not only by limiting ROS accumulation but also by maintaining CAT enzyme functionality.

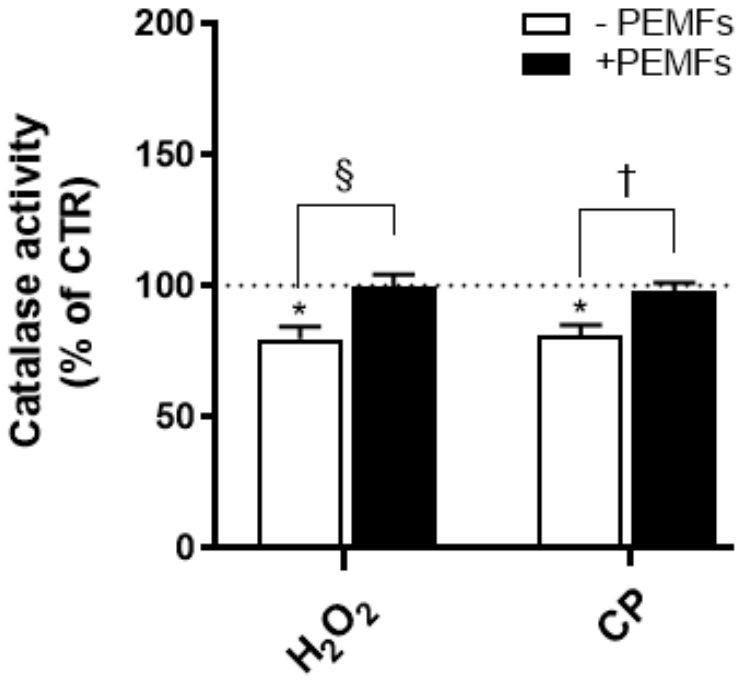


***Figure 20. PEMFs' effects catalase enzyme activity in $H_2O_2$- and CP-injured PC12 cells.*** *Catalase activity measured after cell treatment with 200 μM $H_2O_2$ or 20 μM CP for 4h. Results are presented as mean ± SEM values of at least three independent experiments performed in duplicate (*$p<0.05$ vs CTR without PEMFs; $^{\S}p<0.05$ vs $H_2O_2$-treated cells; $^{\dagger}p<0.05$ vs CP-treated cells. Statistical analysis has been performed by one-way ANOVA and Sidak's multiple comparison test. CP: Click Peptide; CAT: Catalase; $H_2O_2$: Hydrogen peroxide; PEMFs: Pulsed Electromagnetic Fields.*

## *PEMFs' Effects Mitochondrial Membrane Potential in $H_2O_2$- and CP-injured PC12 cells*

To assess mitochondrial membrane potential (MMP), we employed JC-1, a cationic carbocyanine dye that accumulates in mitochondria in a potential-dependent manner. In healthy control cells, JC-1 forms red fluorescent aggregates (dimers) within the mitochondria. In contrast, in damaged cells with reduced MMP, JC-1 accumulates in lower amounts, remaining in its green fluorescent monomeric form.

PC12 cells were loaded with JC-1 and exposed to injury stimuli, 1 mM $H_2O_2$ or 20 µM CP for 90 minutes, with or without PEMF exposure. MMP was evaluated using two complementary approaches: live-cell fluorescence microscopy and a fluorescence plate reader. For the live microscopy imaging, cells were cultured in 8-well chamber slides and analyzed immediately after treatment. Representative images are shown in Figure 21, where red dimers and green monomers are visible in control and injured cells, respectively. As can be observed, both stimuli, $H_2O_2$ and CP, induced a huge increase of green monomers (Figure 21 A), indicating MMP depolarization, whereas in the PEMF-exposed group, the stimuli effects were lower and dimers/monomers colocalization can be observed (Figure 21 B).

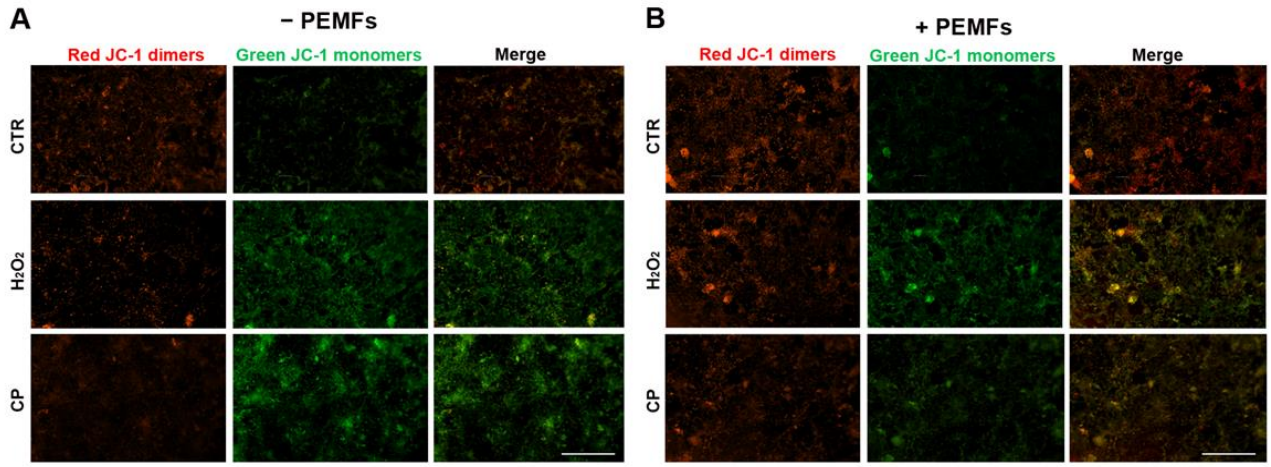


***Figure 21. PEMFs' effects on MMP depolarization in PC12 cells.*** *Representative images of JC-1 staining in 1 mM $H_2O_2$- or 20 µM CP-treated cells for 90 min, without PEMFs (A) and with PEMFs (B). Scale bar 100 µm. CP: Click Peptide; JC-1, Tetraethyl-Benzimidazolyl-Carbocyanine Iodide; $H_2O_2$: Hydrogen peroxide; MMP: Mitochondrial membrane potential; PEMFs: Pulsed Electromagnetic Fields.*

Results obtained from the analysis of the mean fluorescent intensity expressed as a red/green ratio percentage of the control group (relative ratio) have been included in the graph of Figure 23. Treatment with 1 mM $H_2O_2$ and 20 µM CP significantly reduce the red/green ratio % (14 ± 1% and 22 ± 1% versus 100 ± 3%, $^{****}p<0.0001$), while PEMFs exposure in part reverted this effect (33 ± 4% and 57 ± 5% versus 100 ± 9%, $^{###}p<0.001$ and $^{##}p<0.01$, respectively; $^{§}p<0.05$ and $^{††}p<0.01$). Higher magnification images further illustrate JC-1 staining patterns, with red aggregates in control cells and green monomers in $H_2O_2$-treated cells (Figure 22).

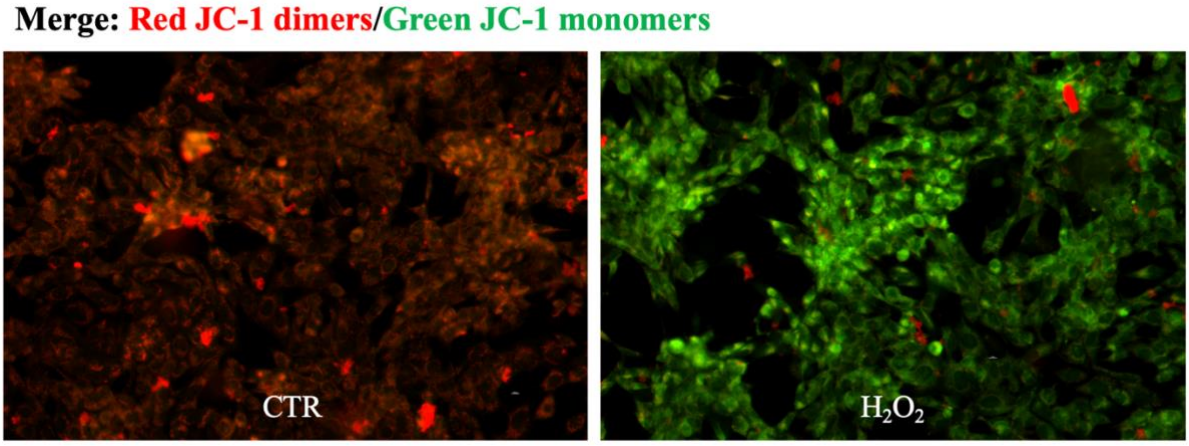


***Figure 22. PEMFs' effects on MMP depolarization in PC12 cells.*** *Representative high-magnification images of JC-1 staining cells under CTR and after treatment with 1 mM $H_2O_2$.*

For fluorescence-based measurements, cells were seeded in black 96-well plates to minimize background signal and enhance detection sensitivity. At the end of treatments, fluorescence was measured using a multimode plate reader. Fluorescence intensity values, corresponding to JC-1 aggregate and monomer species, were analyzed and expressed as the aggregate/monomer ratio relative to control. These results confirmed the trend observed from live cell imaging. Specifically, treatment with 1 mM $H_2O_2$ and 20 µM CP induced a significant MMP depolarization (15 ± 5% and 21 ± 1%, respectively, vs. 100 ± 10% in control, $^{****}p<0.0001$), which was partially reversed by PEMFs (56 ± 11% and 42 ±2%, respectively, vs. 100 ± 10% in control, $^{##}p<0.01$, $^{#####}p<0.0001$, $^{§}p<0.05$, $^{††}p<0.01$), further confirming the protective effect of PEMFs exposure on mitochondrial function (Figure 23).

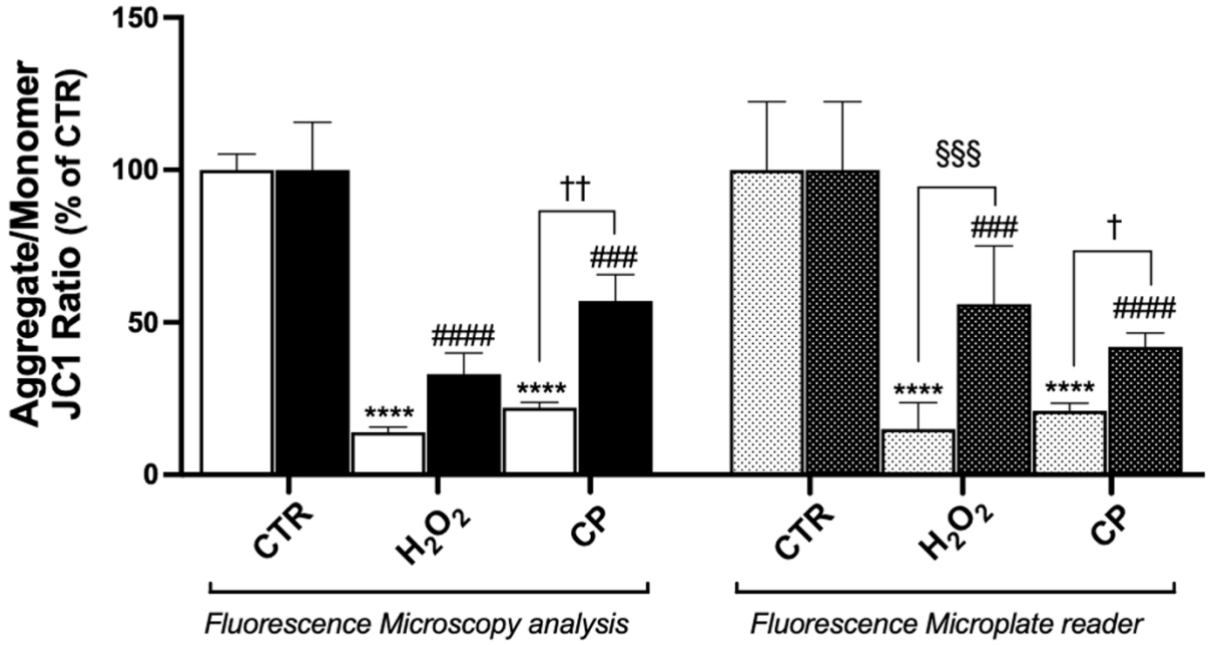


***Figure 23. PEMFs' effects on MMP depolarization in PC12 cells.*** *The graph includes the results of fluorescence microscope images analysis and fluorescence microplate reader analysis, expressed as the red/green ratio percentage of control groups (CTR). These results are presented as mean ± SEM values of at least three independent experiments performed in duplicate ($^{****}p<0.0001$ vs CTR without PEMFs; $^{\#\#\#\#\#}p<0.0001$, $^{\#\#\#}p<0.001$, $^{\#\#}p<0.01$, vs CTR with PEMFs; $^{\S}p<0.05$ vs $H_2O_2$-treated cells; $^{\dagger\dagger}p<0.01$ vs CP-treated cells). Statistical analysis has been performed by one-way ANOVA and Sidak's multiple comparison test. CP: Click Peptide; $H_2O_2$: Hydrogen peroxide; MMP: Mitochondrial membrane potential; PEMFs: Pulsed Electromagnetic Fields.*

## *PEMFs' Effects on Inhibition of ERK1/2 Phosphorylation in $H_2O_2$- and CP-injured PC12 cells*

Given the observed protective effects of the MEK/ERK1/2 inhibitor U0126 against $H_2O_2$- and CP-induced cytotoxicity, we further examined the activation status of the ERK1/2 signaling pathway under these conditions. ERK is a key signaling cascade within the mitogen-activated protein kinase (MAPK) family and is widely recognized for its role in promoting cell survival (Khezri et al., 2023). Once phosphorylated, activated ERK proteins translocate into the nucleus, where they phosphorylate specific transcription factors, thereby influencing the expression of genes involved in cell survival (Almeida et al., 2005). Specifically, we analyzed the expression levels of both total and phosphorylated ERK1/2 in injured cells, with and without PEMF exposure. As shown in Figure 24, treatment with 1 mM $H_2O_2$ and 20 μM CP for 20 minutes significantly increased the p-/T-ERK1/2

ratio (178 ± 11% and 139 ± 10% vs. 100 ± 13%, $^{***}p<0.001$ and $^{*}p<0.05$, respectively), indicating activation of the ERK1/2 pathway in response to oxidative stress.

Interestingly, PEMF exposure markedly reduced the p-/T-ERK1/2 ratio in both treatment groups (123 ± 9% and 95 ± 2%, $^{§§}p<0.01$ and $^{†}p<0.05$, respectively), bringing the values closer to those observed in control cells. These findings suggest that PEMFs can mitigate the abnormal activation of the ERK1/2 signaling cascade triggered by $H_2O_2$ and CP, potentially contributing to their overall cytoprotective effects.

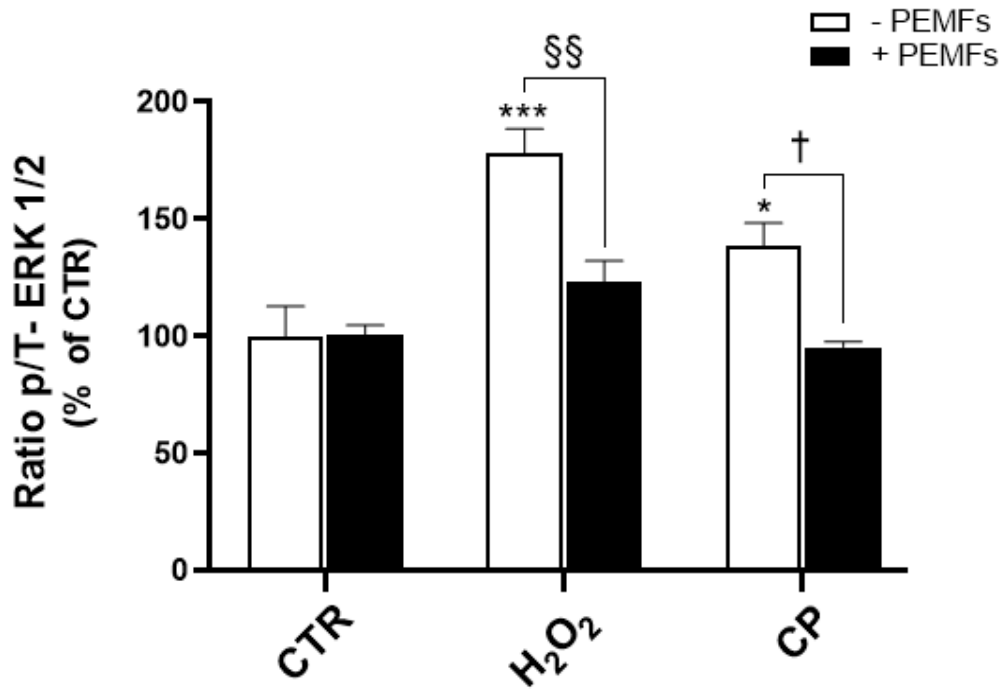


***Figure 24. PEMFs' effects on ERK1/2 in PC12 cells.*** *Quantification of total (T) and phosphorylated (p) forms of ERK1/2 after 1 mM $H_2O_2$ or 20 μM CP treatment of cells for 20 min. Results are presented as mean ± SEM values of at least three independent experiments performed in duplicate ($^{***}p<0.001$ and $^{*}p<0.05$ vs control (CTR) without PEMFs; $^{§§}p<0.01$ vs $H_2O_2$-treated cells; $^{†}p<0.05$ vs CP-treated cells). Statistical analysis has been performed by one-way ANOVA and Sidak's multiple comparison test. CP: Click Peptide; ERK: Extracellular signal-regulated kinase; $H_2O_2$: Hydrogen peroxide; PEMFs: Pulsed Electromagnetic Fields.*

### *PEMFs' Effects on Activation of cAMP/CREB/BDNF Axis in $H_2O_2$- and CP-injured PC12 cells*

To further explore the role of CREB in our cellular injury model and considering that inhibition of CREB with 666-15 exacerbated $H_2O_2$- and CP-induced cell death, we examined the expression levels of both total and phosphorylated CREB. A 20-minute treatment with 1 mM $H_2O_2$ or 20 μM CP significantly reduced the phospho/total-CREB ratio compared to control cells (85 ± 4% and 72 ± 5% vs. 100 ± 2%; $^{*}p<0.05$), indicating a downregulation of CREB activation in response to oxidative and amyloidogenic stress (Figure 25).

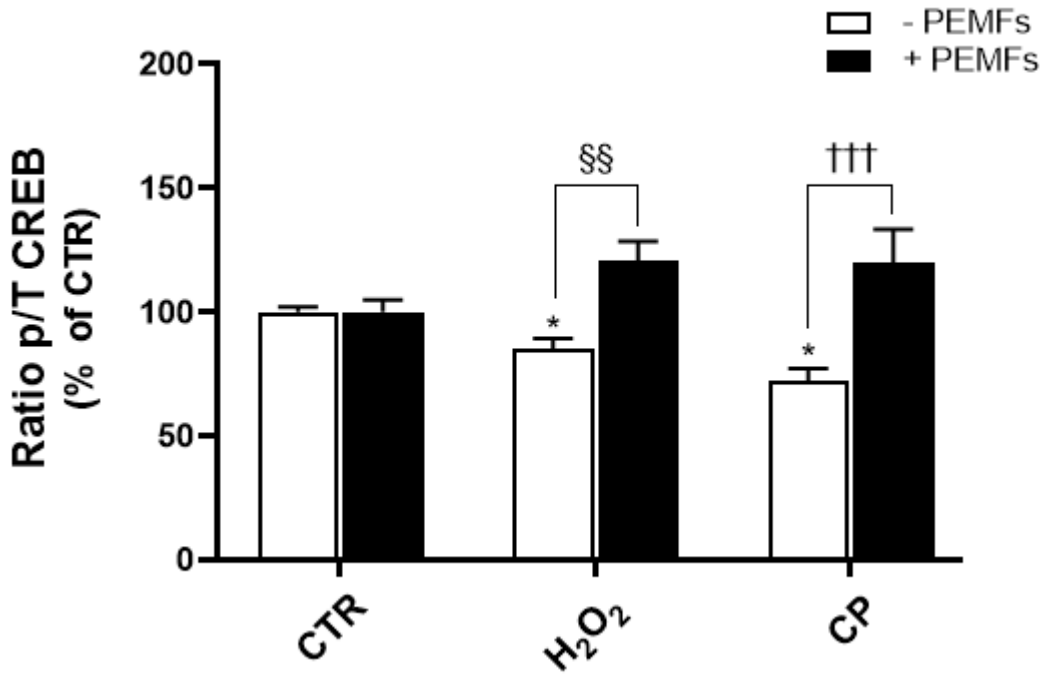


***Figure 25. PEMFs' effects on CREB in PC12 cells.*** *Quantification of total (T) and phosphorylated (p) forms of CREB after 1 mM $H_2O_2$ or 20 μM CP treatment of cells for 20 min. Results are presented as mean ± SEM values of at least four independent experiments performed in duplicate ($^{*}p<0.05$ vs control (CTR) without PEMFs; $^{§§}p<0.01$, vs $H_2O_2$-treated cells; $^{†††}p<0.001$ vs CP-treated cells). Statistical analysis has been performed by one-way ANOVA and Sidak's multiple comparison test. CP: Click Peptide; CREB: cAMP response element-binding protein; $H_2O_2$: Hydrogen peroxide; PEMFs: Pulsed Electromagnetic Fields.*

Interestingly, when cells were exposed to PEMFs immediately following the injury stimuli, a significant increase in the phospho/total-CREB ratio was observed (121 ± 8% and 120 ± 13%, respectively; $^{§§}p<0.01$ and $^{†††}p<0.001$), suggesting that PEMFs may exert a protective effect by promoting CREB activation. Given CREB's involvement in cellular stress responses, we investigated

upstream regulatory mechanisms, focusing on cAMP signaling. As shown in Figure 26, cAMP production was markedly reduced following treatment with $H_2O_2$ and CP (42 ± 9% and 57 ± 4% vs. 100 ± 6%; $^{****}p<0.0001$ and $^{***}p<0.001$, respectively). However, PEMF exposure during treatment partially restored cAMP levels (87 ± 5% and 86 ± 4%, $^{§§§}p<0.001$ and $^{†}p<0.05$, respectively), indicating a potential link between PEMF-induced CREB phosphorylation and cAMP signaling, and supporting the hypothesis that PEMFs act upstream of CREB activation.

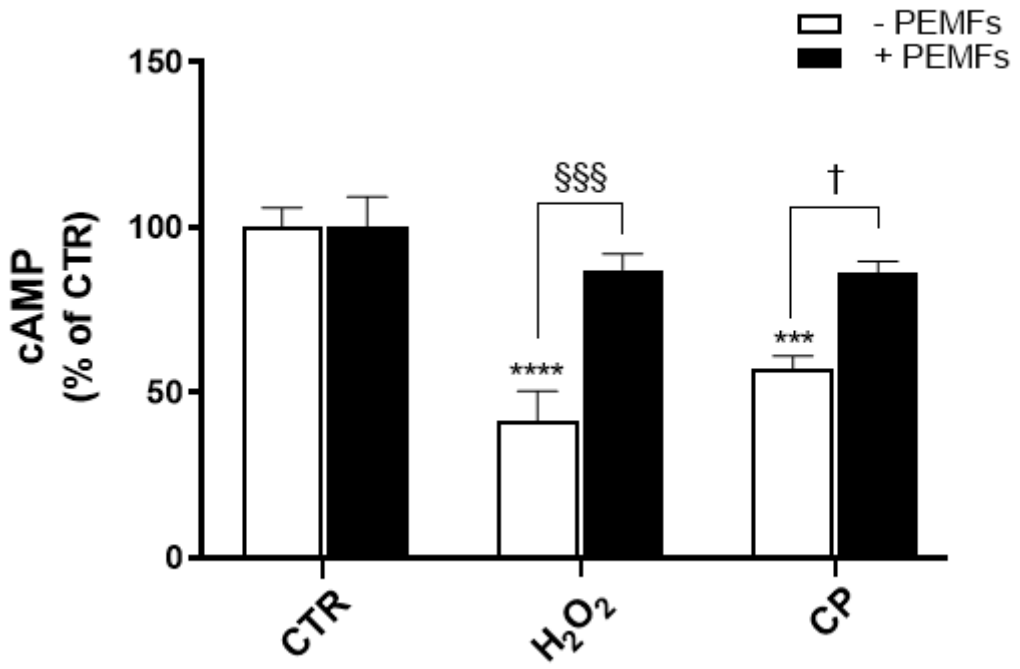


***Figure 26. PEMFs' effects on cAMP in PC12 cells***. *cAMP production after 1 mM $H_2O_2$ and 20 μM CP cell treatment. Results are presented as mean ± SEM values of at least three independent experiments performed in duplicate ($^{****}p<0.0001$ and $^{***}p<0.001$ vs CTR without PEMFs, respectively; $^{§§§}p<0.001$ vs $H_2O_2$-injured cells; $^{†}p<0.05$ vs CP-injured cells). Statistical analysis has been performed by one-way ANOVA and Sidak's multiple comparison test. cAMP: cyclic adenosine monophosphate; CP: Click Peptide; $H_2O_2$: Hydrogen peroxide; PEMFs: Pulsed Electromagnetic Fields.*

To assess the functional consequences of CREB modulation, we measured the release of brain-derived neurotrophic factor (BDNF), a key neurotrophic factor regulated by CREB. Treatment with 200 μM $H_2O_2$ or 20 μM CP for 24 hours significantly reduced extracellular BDNF levels (62 ± 12% and 77 ± 8% vs. 100 ± 5% in control; $^{*}p<0.05$), reflecting impaired neurotrophic support under stress conditions (Figure 27). Importantly, PEMF exposure significantly enhanced BDNF release (104 ±

7% and 113 ± 11%, respectively; $^{\S}p<0.05$ and $^{\dagger}p<0.05$), further supporting the neuroprotective role of PEMFs through CREB activation and its downstream signaling.

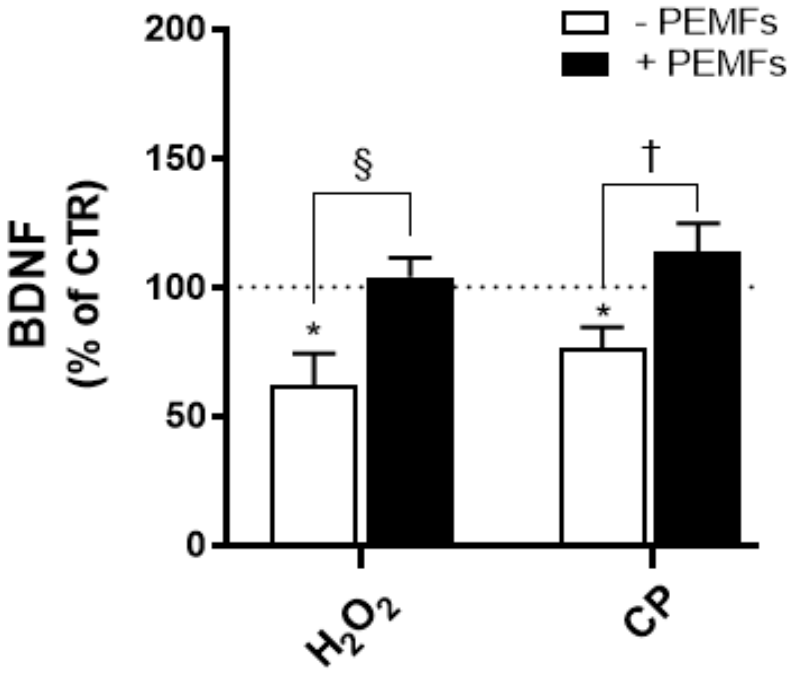


***Figure 27. PEMFs' effects on BDNF in PC12 cells.*** *Quantification of extracellular BDNF levels after 200 μM $H_2O_2$ or 20 μM CP treatment for 24h. Results are presented as mean ± SEM values of at least three independent experiments performed in duplicate ($^{*}p<0.05$ vs CTR without PEMFs; $^{\S}p<0.05$ vs $H_2O_2$-injured cells; $^{\dagger}p<0.05$ vs CP-injured cells). Statistical analysis has been performed by one-way ANOVA and Sidak's multiple comparison test. BDNF: Brain-Derived Neurotrophic Factor. CP: Click Peptide; $H_2O_2$: Hydrogen peroxide; PEMFs: Pulsed Electromagnetic Fields.*

## *Diclofenac Effects in $H_2O_2$- and CP-injured PC12 cells*

We evaluated the effect of diclofenac (DCF), a widely used non-steroidal anti-inflammatory drug (NSAID), in our oxidative and amyloidogenic stress model. Cells were pretreated with DCF at concentrations of 1 μM or 5 μM for 30 minutes before a 24-hour exposure to 1 mM $H_2O_2$ or 20 μM CP. As shown in Figure 28, both DCF concentrations provided partial protection against $H_2O_2$-induced cytotoxicity. Specifically, cell viability increased from 40% in $H_2O_2$-treated cells to 78 ± 12% and 73 ± 11% following pretreatment with 1 μM and 5 μM DCF, respectively ($^{\S\S\S\S}p<0.0001$ for both comparisons). A protective effect was also observed in CP-treated cells, with viability rising from 66 ± 1% (CP alone) to 88 ± 4% with 1 μM DCF ($^{\dagger}p<0.05$) and to 97 ± 4% with 5 μM DCF ($^{\dagger\dagger\dagger}p<0.001$), suggesting a dose-dependent cytoprotective effect.

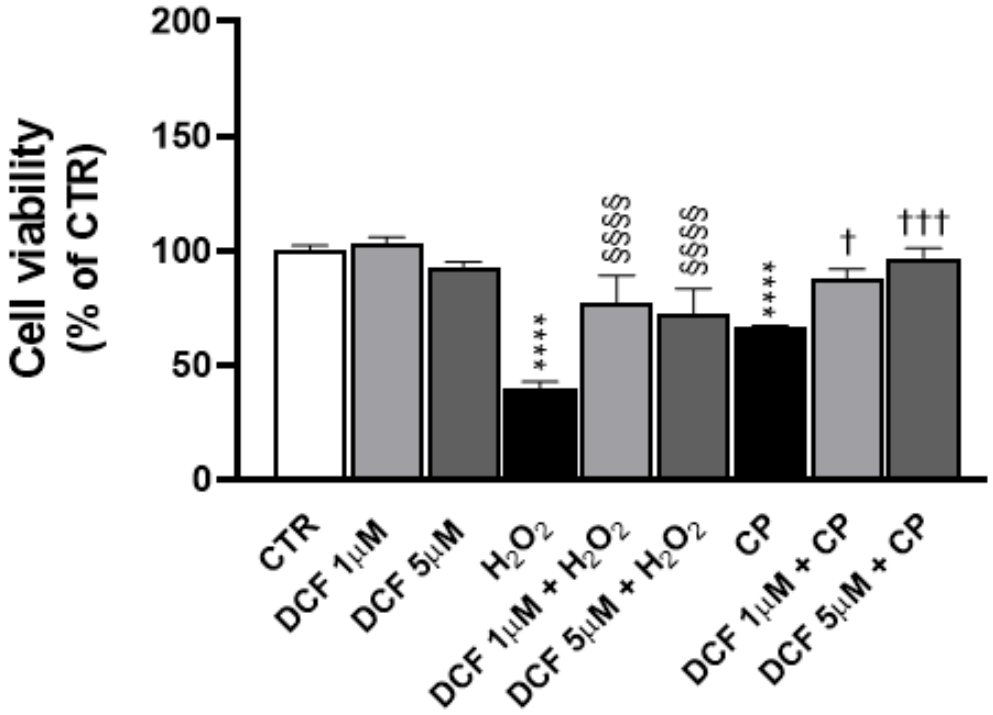


***Figure 28. Diclofenac (DCF) effect in injured PC12 cells.*** *Cell viability was studied in cells pretreated for 30 min with 1 μM or 5 μM DCF and then treated with 1 mM $H_2O_2$ or 20 μM CP for 24h, through MTS assay. Results are presented as mean ± SEM values of at least four independent experiments performed in duplicate ($^{****}p<0.0001$ vs control, CTR; $^{§§§§}p<0.0001$ vs $H_2O_2$-injured cells; $^{†}p<0.05$ and $^{†††}p<0.001$ vs CP-injured cells). Statistical analysis has been performed by one-way ANOVA and Sidak's multiple comparison test. CP: Click Peptide; DCF: Diclofenac; $H_2O_2$: Hydrogen peroxide; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.*

Regarding ROS production, both $H_2O_2$ and CP treatments significantly increased ROS levels compared to control cells (177 ± 16% and 138 ± 5% vs. 100 ± 8%, $^{***}p<0.001$ and $^{*}p<0.05$). Pretreatment with DCF at 1 μM and 5 μM effectively reduced ROS generation in response to $H_2O_2$ (84 ± 18% and 88 ± 7%, $^{§§§}p<0.001$ and $^{§§§§}p<0.0001$, respectively) and CP (95 ± 14% and 76 ± 21%, respectively, $^{†}p<0.05$), as shown in Figure 29, indicating that DCF also exerts antioxidant effects in this model. These findings support the hypothesis that DCF may confer cytoprotection not only through anti-inflammatory pathways but also by mitigating oxidative damage.

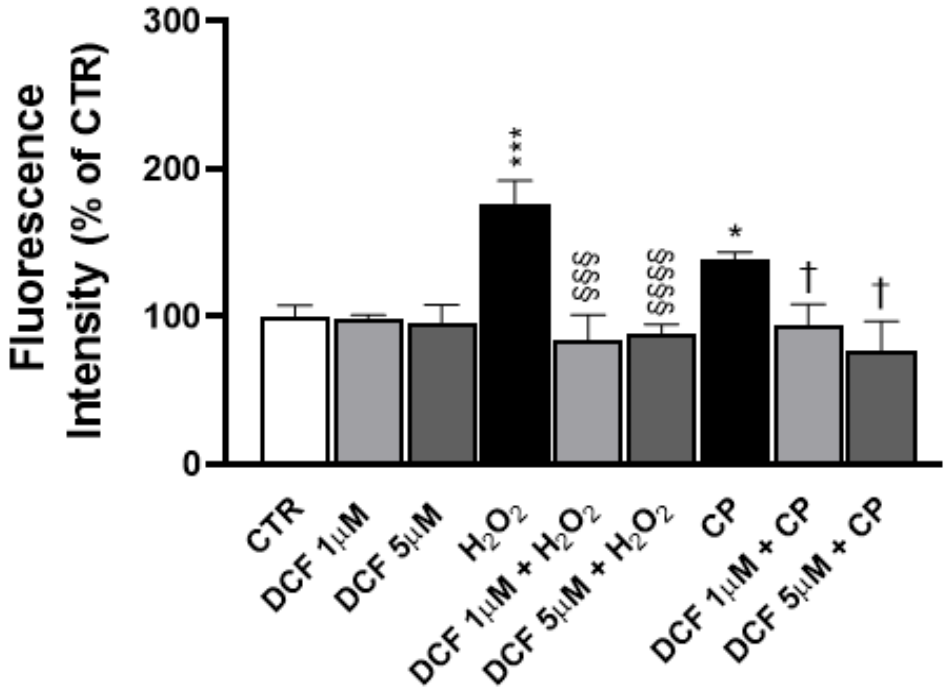


***Figure 29. Diclofenac (DCF) effect in injured PC12 cells.*** *ROS production was studied in cells pretreated for 30 min with 1 μM or 5 μM DCF and then treated with 1 mM $H_2O_2$ or 20 μM CP for 24h, through $H_2$DCFDA assay. The graph shows mean ± SEM values from three independent experiments performed in duplicate ($^{***}p<0.001$ and $^{*}p<0.05$ vs CTR; $^{§§§}p<0.001$ and $^{§§§§}p<0.0001$ vs $H_2O_2$-injured cells; $^{†}p<0.05$ vs CP-injured cells). Statistical analysis has been performed by one-way ANOVA and Sidak's multiple comparison test. CP: Click Peptide; DCF: Diclofenac; $H_2$DCFDA: 2',7'-Dichlorofluorescein Diacetate; $H_2O_2$: Hydrogen peroxide; ROS: Reactive Oxigen Species.*

# DISCUSSION AND CONCLUSIONS

AD is the most common form of dementia and poses a major global health concern. As life expectancy rises, the prevalence of AD is also increasing and is projected to double by 2050. This condition is a progressive neurodegenerative disorder that initially manifests as memory deficits and cognitive deterioration. Although its exact etiology remains unclear, aging, genetic predisposition, and environmental factors are considered key contributors (Suresh et al, 2023).

The disease is characterized by memory loss and cognitive impairment, driven by neuropathological hallmarks such as Aβ plaques, NFTs, astrogliosis, and neuronal degeneration. These alterations are strongly associated with oxidative stress, mitochondrial dysfunction, and regulated cell death pathways (Jurcău et al., 2022; Ekundayo et al., 2024; Wang et al., 2020; Misrani et al., 2021; Goel et al., 2022). ROS, primarily generated by impaired mitochondria, exacerbate Aβ and tau accumulation, trigger neuroinflammation, and promote neuronal death. Such processes disrupt neuronal homeostasis, leading to synaptic failure and cognitive decline.

Several hypotheses have been proposed to explain the mechanisms underlying AD. The amyloid cascade hypothesis suggests that the accumulation of β-amyloid (Aβ) peptides in the brain initiates a series of pathological events, including synaptic dysfunction, neuroinflammation, and neuronal death (Jurcău et al., 2022; Ekundayo et al., 2024). Another key feature is the formation of neurofibrillary NFTs composed of hyperphosphorylated tau protein, which disrupts microtubule stability and axonal transport (Sinsky et al., 2021). These processes are closely linked to oxidative stress and mitochondrial dysfunction, which exacerbate neuronal damage through excessive production of ROS (Wang et al., 2020; Misrani et al., 2021). ROS not only promote Aβ and tau aggregation but also activate inflammatory pathways and regulate cell death, contributing to synaptic failure and cognitive decline.

In addition to these mechanisms, dysregulation of intracellular signaling pathways such as CREB and ERK plays a critical role in AD progression. Oxidative stress induced by Aβ can inhibit CREB-mediated transcription of neuroprotective genes like BDNF, while abnormal ERK activity has been associated with tau phosphorylation and neuroinflammation (Pugazhenthi et al., 2011; Khezri et al., 2023).

Current FDA-approved treatments, including acetylcholinesterase inhibitors, memantine, and anti-amyloid antibodies, primarily offer symptomatic relief or modest disease-modifying effects. However, their long-term efficacy remains limited, and none of these therapies can halt or reverse

disease progression. This therapeutic gap underscores the urgent need for alternative strategies targeting multiple pathological mechanisms. In response, therapeutic development has increasingly shifted toward disease modification, particularly through monoclonal antibodies that selectively target aggregated forms of Aβ, aiming to reduce plaque burden and interrupt the neurodegenerative cascade associated with AD (Tolar et al., 2020; Van Dyck et al., 2023; Kim et al., 2024).

In this context, PEMFs have emerged as a promising non-invasive approach. PEMFs can modulate cellular activity by influencing membrane components such as voltage-gated calcium channels and activating intracellular signaling cascades (Funk et al., 2021). Experimental evidence indicates that PEMFs reduce oxidative stress, improve mitochondrial function, and enhance neuronal survival through pathways like PI3K/Akt, CREB, and MAPK/ERK (Urnukhsaikhan et al., 2016; Capone et al., 2022; Vincenzi et al., 2017). Furthermore, PEMFs promote neurotrophic factor expression (e.g., BDNF), support synaptic plasticity, and exert anti-inflammatory and anti-apoptotic effects in both in vitro and in vivo models of neurodegeneration (Gessi et al., 2019; Merighi et al., 2020; Lin et al., 2023; Merighi et al., 2024).In SH-SY5Y and N9 cells exposed to oxidative stress ($H_2O_2$) and an Aβ1-42 analog (CP), PEMF treatment reduced ROS, improved mitochondrial function, and decreased cell death (Merighi et al., 2024). These results suggest that PEMFs may counteract key pathological processes in AD by restoring redox balance and activating protective signaling pathways. Although the precise molecular mechanisms require further investigation, the evidence supports the potential of PEMFs as an adjunctive therapy for AD.

Therefore this study aimed to investigate the molecular mechanisms at the basis of the protective effects of PEMFs on rat neuron-like PC12 cells insulted with $H_2O_2$ and Aβ peptide. Due to their neuronal characteristics and oxidative stress sensitivity, PC12 cells are widely used as an in vitro model for studying AD-related mechanisms (Xie at al., 2023). $H_2O_2$ is a powerful oxidative stressor producing ROS in PC12 cells, thus exacerbating oxidative damage (Clementi et al., 2019; Wang et al., 2023). Similarly, the Aβ peptide contributes to the formation of senile plaques and induces oxidative stress through mitochondrial dysfunction and other cellular pathways. In particular, in PC12 cells, Aβ exposure leads to elevated ROS production, which in turn promotes cellular damage and apoptosis (Hong et al., 2012; Wang et al., 2023).

The present study offers a comprehensive view of the neuroprotective action of PEMFs, acting via an orchestrated modulation of multiple signaling pathways in NGF-differentiated PC12 cells exposed to oxidative and amyloidogenic insults. The data highlight that PEMFs do not act through a single

molecular target but through a multitarget signaling that stabilizes redox balance, suppresses pro-death agents, and activates neurotrophic pathways.

In particular, this study demonstrates that PEMF exposure significantly reduced neuronal cell death induced by $H_2O_2$ and CP in PC12 cells. As for the mechanistic aspect of their protection, it has been found that the damage caused by these insults was at least in part recovered by a caspase-3 inhibitor and by Trolox, a known antioxidant agent, suggesting that the protective effects of PEMFs may be mediated through the modulation of apoptotic pathways and oxidative stress.

Caspase-3 is a key enzyme in the apoptotic pathway and serves as a critical marker in neurodegenerative diseases such as AD. In our study, the use of a caspase-3 inhibitor protected injured PC12 cells from cell death, suggesting the involvement of this apoptotic protein in the observed damage. To gain deeper insight into this mechanism, we analyzed cleaved caspase-3 protein expression, which was significantly upregulated in cells treated with $H_2O_2$ and CP, confirming its role in the programmed cell death pathways activated in our model. At the nuclear level, PEMFs suppressed caspase-3 activation and reduced chromatin condensation, demonstrating a block of the executioner phase of apoptosis. These findings align with prior *in vitro* and *in vivo* evidence demonstrating decreased neuronal or neuroblastoma cell line death, decreased infarct volume, and enhanced recovery following *Extremely Low Frequency Magnetic Fields (ELF-MFs)* or PEMF therapy in experimental stroke models (Oda et al., 2004; Pirozzoli et al., 2003; Pena-philippides et al., 2014; Grant et al., 1994; Capone et al., 2024). Similar results were found following chronic exposure to 50 Hz ELF-MFs in primary rat cortical neurons that enhanced cell viability and reduced apoptosis without inducing overt oxidative damage. Although ROS levels showed a slight increase, this was effectively counterbalanced by intracellular glutathione, whose levels correlated inversely with apoptosis and directly with ROS, suggesting a dynamic redox adaptation (Di Loreto et al., 2009).

Trolox, a water-soluble analogue of vitamin E, is well known for its potent antioxidant properties. It exerts protective effects on $H_2O_2$-treated PC12 cells through multiple mechanisms, including direct scavenging of ROS, attenuation of oxidative stress, and prevention of cellular damage. Additionally, trolox inhibits lipid peroxidation, thereby preserving membrane integrity, and enhances endogenous antioxidant defenses by upregulating antioxidant enzymes (Horakova et al., 2003). Consistent with previous findings in the AD mouse model, our results show that trolox partially mitigated the cytotoxic effects induced by $H_2O_2$ or CP in PC12 cells, supporting the involvement of oxidative stress in the observed cell death (Tahir et al., 2024). Specifically, exposure to PEMF significantly reduces oxidative stress. This is evidenced by decreased levels of ROS and the restoration of catalase activity,

indicating that the treatment acts upstream in the oxidative cascade. This result was confirmed in SH-SY5Y cells damaged with $H_2O_2$ or CP, where *High-Frequency Low-intensity Pulsed Electric Fields (H-LIPEF)* as well as PEMFs were able to reduce ROS (Merighi et al., 2024; Lin et al., 2023).

While our study focused on CAT activity as a marker of antioxidant defense, other enzymes such as *Superoxide Dismutase (SOD)* and *Glutathione Peroxidase (GPx*) also play crucial roles in cellular redox homeostasis. CAT was selected due to its direct role in detoxifying $H_2O_2$, the primary oxidative agent used in our model. This choice was supported by previous studies demonstrating CAT's sensitivity to oxidative stress in PC12 cells (Zhu et al., 2024; Vincenzi et al., 2020). Nonetheless, future investigations will include a broader panel of antioxidant enzymes to fully elucidate the spectrum of PEMF-induced antioxidant responses.

In PC12 cells, exposure to $H_2O_2$ results in a significant loss of MMP, indicating impaired mitochondrial function (Zhu et al., 2024). This disruption of MMP can trigger the release of cytochrome c from mitochondria to the cytosol, activating caspases and promoting apoptosis (Wang et al., 2025). On the other hand, Aβ in mitochondria specifically can directly induce apoptotic cell death by disrupting mitochondrial function (Yoshioka et al., 2006). We employed the JC-1 assay to evaluate MMP as an indicator of mitochondrial integrity (Falone et al., 2016). Our results demonstrated that treatment with $H_2O_2$ and CP led to a significant reduction in MMP. However, exposure to PEMFs partially mitigated this effect. In the groups subjected to PEMFs during injury treatments, an increased presence of JC-1 aggregates (dimers), which colocalized with monomers, was observed, suggesting a protective effect on mitochondrial function. The preservation of MMP further confirms the centrality of mitochondrial protection in PEMF-mediated effects. These findings align with prior studies showing that PEMFs or H-LIPEF mitigate mitochondrial dysfunction and apoptosis in SH-SY5Y cells exposed to $H_2O_2$ or Aβ (Merighi et al., 2024; Park et al., 2013).

Existing research indicates that ERK1/2 is dysregulated in AD patients (Khezri et l., 2023). In particular, the altered APP expression and thus Aβ production cause activation and phosphorylation of ERK1/2 proteins with potential implications in AD pathology, including Tau phosphorylation and neuroinflammation (Khezri et al., 2023). Our findings confirm that exposure of PC12 cells to the toxic agents $H_2O_2$ and CP results in a marked increase in ERK1/2 phosphorylation, suggesting activation of stress-related signaling pathways. Interestingly, treatment with PEMFs effectively counteracted this effect, reducing ERK1/2 activation as shown by the reduced phosphorylation of ERK1/2. The pharmacological inhibition of MEK with U0126 produced partial protection, reinforcing the contribution of ERK in the cytotoxic response. These data confirm a trend already

observed in SH-SY5Y cells insulted with $H_2O_2$ or Aβ, where stimuli were applied for a short period of time, like 20 min (Merighi et al., 2024). However, it is important to note that the role of ERK signaling in neuronal survival is highly context-dependent and not always pro-apoptotic. Indeed, H-LIPEF applied to SH-SY5Y cells exposed to $H_2O_2$ or Aβ for 24 h resulted in activation of the ERK pathway, which was associated with neuroprotection (Lin et al., 2023; Park et al., 2013). These findings highlight the complex and time-dependent nature of ERK signaling in neuronal cells, suggesting that its modulation by PEMFs may have different outcomes depending on the cellular context and timing of activation.

In addition, evidence from literature reports CREB to be involved in neurodegenerative diseases. Actually, its downregulation is related to the pathology of AD since reduced CREB phosphorylation results in lower transcriptional activity, which in turn affects synaptic plasticity and neuronal loss. Thus, increasing CREB expression has been considered as a potential therapeutic strategy for AD (Cha et al., 2012; Sivandzade et al., 2019). In PC12 cells, the inhibition of CREB has been described to reduce the protective effects of neurotrophic factors, making cells more susceptible to oxidative stress and Aβ-induced damage (Chen et al., 2021). PEMFs reactivate the cAMP/CREB/BDNF axis, a key neurotrophic signaling pathway typically impaired under oxidative or amyloidogenic stress. Our experiments showed that PEMFs restored intracellular cAMP levels, increased CREB phosphorylation, and enhanced BDNF secretion. The CREB inhibitor 666-15 increases cell death induced by $H_2O_2$ and CP, confirming the role of CREB as a downstream effector. Accordingly, in SH-SY5Y cells, application of H-LIPEF activated the ERK/CREB pathway, producing protective prosurvival effects (Lin et al., 2023; Park et al., 2013). Notably, PEMF exposure activated BDNF/TrkB/Akt signaling in ischemic mice, contributing to synaptic recovery and functional improvement (Khakha et al., 2023). Furthermore, the ability of PEMFs to simultaneously enhance cAMP, p-CREB, and BDNF expression while downregulating ERK1/2 activity highlights their potential to provide neuroprotective effects. This dynamic interplay underscores the importance of targeting multiple signaling pathways to restore neuronal homeostasis and support cognitive function.

A visual summary of the main mechanisms involved in the neuroprotective action of PEMFs is provided in Figure 30, which integrates the key findings of this study into a unified mechanistic model. The schematic highlights how PEMFs exert a coordinated multitarget response capable of counteracting both oxidative and amyloidogenic damage. Specifically, PEMF exposure reduces ROS generation and restores catalase activity, converging with a marked inhibition of the apoptotic cascade, as shown by the reduced activation of caspase-3 and the attenuation of nuclear chromatin condensation. In parallel, PEMFs activate prosurvival signaling pathways, including ERK/CREB,

ultimately enhancing BDNF expression and supporting neuronal survival. Overall, this representation underscores the integrated nature of PEMF-mediated protection in PC12 cells and contextualizes the mechanistic findings discussed below.

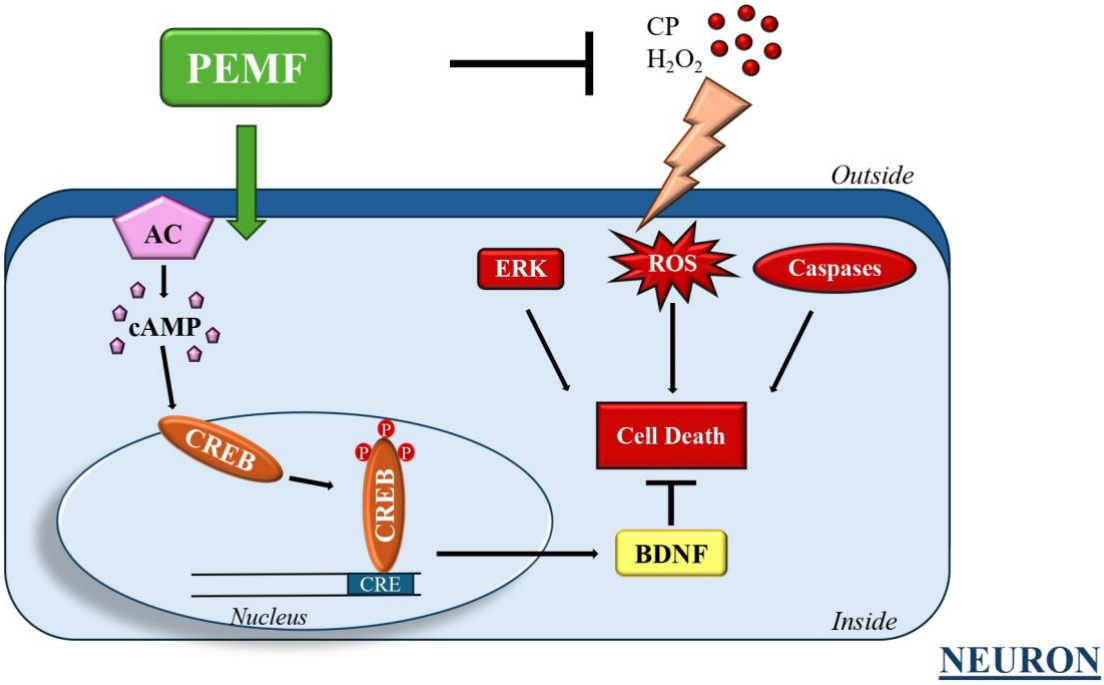


***Figure 30. Schematic representation of the molecular mechanisms underlying the protective effects of PEMFs in NGF-differentiated PC12 cells.*** *Pulsed Electromagnetic Fields (PEMFs) counteract oxidative and amyloidogenic insults ($H_2O_2$ and CP) by reducing intracellular ROS accumulation, restoring catalase activity, and limiting the activation of apoptotic pathways, including caspase-3 cleavage. Concurrently, PEMFs activate prosurvival signaling cascades, particularly ERK/CREB, promoting CREB phosphorylation and BDNF expression, ultimately enhancing neuronal survival and preventing chromatin condensation and apoptosis. The depicted multitarget action highlights the coordinated antioxidant, anti-apoptotic, and neurotrophic mechanisms engaged by PEMFs in sustaining neuronal homeostasis.*

Finally, this study also compared the effects of PEMFs with those of DCF, a widely used NSAIDs. DCF exerts its anti-inflammatory action by inhibiting cyclooxygenase enzymes, thereby reducing the synthesis of pro-inflammatory prostaglandins. This mechanism directly targets the inflammatory cascade, which is a key contributor to neurodegeneration (Amidfar et al., 2020). In contrast, PEMFs act through biophysical modulation of intracellular signaling pathways, including the suppression of ERK1/2 activation and the enhancement of the cAMP/CREB/BDNF axis, as shown in our study. These distinct modes of action suggest that PEMFs and NSAIDs could be used in combination to achieve broader neuroprotection. Future studies should investigate the potential synergistic or

additive effects of such combined approaches, which may offer enhanced efficacy while minimizing the dosage and side effects of pharmacological agents.

Overall, our findings suggest that PEMFs positively modulate the cAMP/CREB/BDNF signaling pathway in PC12 cells subjected to oxidative and chemical injury, thereby enhancing cell survival and promoting neuroprotection

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**Technical datasheet**

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