

**17-beta estradiol in mitochondrial function and neuroprotection in central
and peripheral nervous systems: Mechanisms and Implications**

by

Pranav Mishra

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Department of Pharmacology and Therapeutics
Max Rady College of Medicine, Rady Faculty of Health Sciences
University of Manitoba
Winnipeg, Canada

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ABSTRACT

Neurodegenerative diseases, such as Alzheimer's disease (AD) and peripheral neuropathies, are characterized by progressive neuronal damage and dysfunction. Estradiol (E2), a potent estrogen, has been shown to exert neuroprotective effects in both the central nervous system (CNS) and peripheral nervous system (PNS). This thesis investigated the mechanisms underlying E2's neurotrophic and neuroprotective actions in dorsal root ganglion (DRG) neurons and cortical neurons, focusing on mitochondrial function, axonal regeneration, neuroinflammation, and neuronal survival.

E2 enhanced mitochondrial respiration and promoted neurite outgrowth in cultured DRG neurons from the PNS through activation of AMP-activated protein kinase (AMPK) and its downstream effectors, including peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) and activating transcription factor 3 (ATF3), which are proteins involved in mitochondrial biogenesis and axonal regeneration, respectively. I identified Ca²⁺/calmodulin-dependent protein kinase kinase beta (CaMKK β) pathway as a key upstream regulator of AMPK activation in DRG neurons. In primary cortical neurons of the CNS, E2 countered amyloid-beta (A β)-induced toxicity by improving mitochondrial function and suppressing pro-inflammatory NF- κ B signaling, underscoring its potential in mitigating AD-related neuronal loss. Notably, pharmacological inhibition of AMPK using Compound C (CC) abolished E2's protective effects, confirming AMPK's central role in mediating E2's actions in cortical neurons.

Collectively, these findings highlight E2's neurotrophic and neuroprotective effects in both peripheral and central neurons, with AMPK emerging as a critical mediator. Additionally, they underscore the therapeutic potential of targeting mitochondrial health and inflammatory pathways,

alongside AMPK, in neurodegenerative disorders. Future research directions include *in vivo* validation, investigation of sex-specific responses, and development of safer estrogen-based alternatives, such as selective estrogen receptor modulators (SERMs) to fully harness estradiol's multifaceted neuroprotective roles in both CNS and PNS disorders.

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DEDICATION

I dedicate this thesis to my lifelong heroes - my family.

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ABBREVIATIONS

°C	Degree Celsius
3xTg	Triple-transgenic
5xFAD	Five familial AD mutations
AD	Alzheimer's Disease
Akt	Protein Kinase B
ALS	Amyotrophic Lateral Sclerosis
AMPK	AMP-activated Protein Kinase
ANOVA	Analysis of Variance
APP	Amyloid Precursor Protein
ArKO	Aromatase knockout
ATF3	Activating Transcription Factor 3
ATP	Adenosine-5'-Triphosphate
A β	Amyloid-beta
BDNF	Brain-Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
Ca ²⁺	Calcium Ion
CaMKK β	Calcium/Calmodulin-dependent Protein Kinase Kinase beta
CBS	Cystathione β -synthase
CC	Compound C (AMPK inhibitor)
CIDP	Chronic Inflammatory Demyelinating Polyneuropathy
CNS	Central Nervous System

COX III	Cytochrome c oxidase III
CREB	CAMP Response Element Binding protein
CTD	C terminus domain
CVD	Cardiovascular Disease
Cy3	Cyanine3 (fluorescent dye)
DAPI	4',6-diamidino-2-phenylindole (nuclear stain)
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPN	Diarylpropionitrile (ER β -selective agonist)
DRG	Dorsal Root Ganglion
E2	17 β -estradiol
ECL	Enhanced Chemi Luminescence
EDTA	Ethylene-Diamine-Tetra-Acetic acid
ER	Estrogen Receptor
ERE	Estrogen response element
ERK	Extracellular Signal-Regulated Kinase
ER α	Estrogen Receptor alpha
ER β	Estrogen Receptor beta
ETC	Electron Transport Chain
FAD	Familial Alzheimer's disease
FBS	Fetal Bovine Serum
FCCP	Carbonyl Cyanide-p-Trifluoro Methoxyphenylhydrazone

GPER	G protein-coupled Estrogen Receptor
HD	Huntington's Disease
HEPES	4-(2-Hydroxy-Ethyl)-1-Piperazine-Ethane Sulfonic acid
hr	Hour
HRP	Horse Radish Peroxidase
IL	Interleukin
ICV	Intracerebroventricular
IMM	Inner Mitochondrial Membrane
I κ B α	Inhibitor of kappa B alpha
JAK/STAT	Janus kinase/signal transducer and activator of transcription
LKB1	Liver Kinase B1
MAPK	Mitogen-Activated Protein Kinase
MS	Multiple Sclerosis
MnSOD	Manganese Superoxide Dismutase
min	minute
ml	Milli Liter
mM	Milli Molar
mtDNA	Mitochondrial DNA
NAFLD	Non-alcoholic fatty liver disease
NDUFB8	NADH:Ubiquinone Oxidoreductase Subunit B8
NF- κ B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
Ngb	Neuroglobin
NGF	Nerve growth factor

NLRP3	Nucleotide-binding domain leucine rich containing family pyrin domain containing-3
nM	Nanomolar
NRF1	Nuclear Respiratory Factor 1
NRF2	Nuclear Respiratory Factor 2
NT-2	Neurotrophin-3
OCR	Oxygen Consumption Rate
OPA1	Optic atrophy 1
OVX	Ovariectomized
OxPhos	Oxidative Phosphorylation
PBS	Phosphate Buffered Saline
PD	Parkinson's Disease
PFA	Paraformaldehyde
PGC-1 α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha
PI3K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
pmol	picomole
PNS	Peripheral Nervous System
PPT	Propyl pyrazole triol (ER α -selective agonist)
RAGs	Regeneration-Associated Genes
RIPA	Radioimmunoprecipitation Assay (buffer)
ROS	Reactive Oxygen Species

SD	Sprague-Dawley (rat strain)
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SERMs	Selective estrogen receptor modulators
SIRT1	Sirtuin 1
SOD2	Superoxide dismutase 2
STO-609	CaMKK β inhibitor
T2D	Type 2 Diabetes
T2D	Type 2 diabetes
TAK1	Transforming Growth Factor- β Activated Kinase 1
TFAM	Mitochondrial Transcription Factor A
Tg CRND8	Transgenic CRND8
TIM	Translocase of Inner Membrane
TNF	Tumor Necrosis Factor
TOM	Translocase of the Outer Membrane
TrkB	Tropomyosin receptor kinase B
μ g	Microgram
μ l	Microliter
μ M	Micro Molar

CHAPTER 1

INTRODUCTION

1.1 Neurodegenerative diseases

Neurodegenerative diseases refer to a group of disorders characterized by the progressive degeneration and/or death of neurons in the central and peripheral nervous systems. These conditions primarily affect the elderly population (Fratiglioni and Qiu, 2009) and have become increasingly prevalent in recent decades, largely due to the rising life expectancy and aging population worldwide, posing a significant challenge to global health while also costing billions every year to the healthcare system (Nandi *et al.*, 2024; Wong, 2020). According to the WHO's (World Health Organization) report, currently over 3 billion people worldwide live with some neurological condition (Steinmetz *et al.*, 2024), which is now the leading cause of ill health and disability globally. Some examples of neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and peripheral neuropathy. These disorders can cause a wide range of symptoms, from memory loss and cognitive decline (Jahn, 2013) to motor dysfunction (Xin *et al.*, 2023) behavioral changes, and sensory loss severely impacting the quality of life of affected individuals. The progressive nature of these conditions often leads to increased dependence on others, placing emotional, financial, and physical burdens on family members and healthcare systems. The etiology of neurodegenerative diseases is complex and multifaceted, involving a combination of genetic factors, environmental influences, and lifestyle choices (Bertram and Tanzi, 2005; Cannon and Greenamyre, 2011; Muddapu *et al.*, 2020). Additionally, cellular and molecular changes (Batoool *et al.*, 2019; Lin *et al.*, 2018; Yang *et al.*, 2023) play a crucial role in the pathogenesis of these disorders, often resulting in changes

in neuronal metabolism, mitochondrial dysfunction, oxidative stress, inflammation, and abnormal protein accumulation, ultimately leading to synaptic loss and neuronal death (Chi *et al.*, 2018).

1.1.1 Neurodegenerative diseases of the central nervous system (CNS)

Neurodegenerative diseases of the central nervous system (CNS) encompass a range of disorders characterized by progressive loss of structure and/or function of neurons in the brain and spinal cord (Shang *et al.*, 2022). These conditions lead to a gradual decline in cognitive abilities, motor function, and overall quality of life. For instance, AD is characterized by memory loss and cognitive decline, which are associated with the accumulation of amyloid plaques and tau tangles in the brain (Weller and Budson, 2018). PD presents with motor symptoms such as tremors, rigidity, and bradykinesia, resulting from the loss of dopaminergic neurons in the substantia nigra (Beitz, 2014). HD is a genetic disorder causing progressive brain damage and loss of motor control (Walker, 2007), and ALS, affects motor neurons in the brain and spinal cord, leading to muscle weakness and paralysis (Hardiman *et al.*, 2017). CNS neurons differ from the peripheral nervous system neurons in several key aspects, including their limited regenerative capacity, the composition of their myelin sheaths, and their structural support systems (Cooke *et al.*, 2022; Gordon, 2016; Stadelmann *et al.*, 2019). CNS neurons have a restricted ability to regenerate compared to PNS neurons, making damage to the CNS often irreversible (Huebner and Strittmatter, 2009). These aforementioned diseases highlight the vulnerability of CNS neurons and the devastating consequences of their degeneration on overall human health and function.

1.1.2 Neurodegenerative diseases of the peripheral nervous system (PNS)

The peripheral nervous system (PNS) consists of nerves branching out from the brain and spinal cord, forming a communication network between the CNS and other body parts. Neurodegenerative diseases of the PNS include disorders characterized by progressive damage to nerves outside these regions (brain and spinal cord). These conditions, collectively known as peripheral neuropathies, can affect sensory, motor, and autonomic nerve fibers, leading to diverse symptoms and functional impairments (Hanewinkel *et al.*, 2016). Peripheral neuropathies can be acquired or inherited (Burns *et al.*, 2006), with causes ranging from metabolic disorders such as diabetes to toxic exposures, infections, and genetic mutations (Martyn and Hughes, 1997). Common symptoms include numbness, tingling, burning sensations, weakness, and autonomic dysfunction. Unlike CNS neurons, PNS neurons have a greater capacity for regeneration, though this ability is often limited in chronic conditions. Diabetic neuropathy is the most prevalent form, affecting millions worldwide and causing significant morbidity (Feldman *et al.*, 2019). Other notable peripheral neuropathies include chemotherapy-induced peripheral neuropathy, Charcot-Marie-Tooth disease, and Guillain-Barré syndrome. The impact of these disorders is profound, affecting quality of life and daily functioning.

1.1.3 Causes of neurodegenerative diseases

The causes of neurodegenerative diseases are complex and multifaceted, involving a combination of genetic, cellular, molecular, environmental, and lifestyle factors (Armstrong, 2020). At the genetic level, certain inherited mutations or polymorphisms increase susceptibility to specific neurodegenerative disorders. For example, the apolipoprotein E4

(APOE4) gene variant is the strongest known genetic risk factor for late-onset, sporadic AD (Raulin *et al.*, 2022). Most cases of AD arise sporadically and reflect a complex interplay between genetic predisposition and various non-genetic risk factors. At the cellular and molecular levels, a common hallmark of many neurodegenerative diseases is the misfolding, aggregation, and accumulation of specific proteins into abnormal, toxic species (Agorogiannis *et al.*, 2004). These protein aggregates, such as amyloid beta (A β) plaques in AD or alpha-synuclein in PD, can lead to neuronal dysfunction and death. Cellular mechanisms such as increased oxidative stress, mitochondrial dysfunction, and inflammation also contribute to the progression of these diseases. Environmental factors also play a significant role in the development of neurodegenerative disorders. For example, long-term exposure to toxins, such as pesticides, herbicides, and heavy metals, has been linked to an increased risk of conditions such as PD (Cannon and Greenamyre, 2011). Additionally, lifestyle factors including diet, physical activity, smoking, and alcohol consumption can influence the risk and progression of neurodegenerative diseases including peripheral neuropathy (Popa-Wagner *et al.*, 2020). Other potential contributors include head trauma, infections, and vascular risk factors such as hypertension and diabetes. Amongst all these, aging remains the most significant risk factor for many neurodegenerative diseases, with risk increasing dramatically later in life (Hou *et al.*, 2019).

1.2 Alzheimer's disease (AD)

Alzheimer's was first recognized in 1906 by a clinical psychiatrist and neuroanatomist, Dr. Alois Alzheimer, who reported it as a disease process of the cerebral cortex at a meeting of German Psychiatrists in Tübingen (Hippius and Neundörfer, 2003). He found plaques and

neurofibrillary tangles (NFTs) in the brain histology of a 50-year-old patient. Today, AD is recognized as a progressive neurodegenerative disorder of the CNS that primarily affects the elderly population. It is the most common form of dementia, characterized by a gradual decline in cognitive function, memory loss, and changes in behavior (Weller and Budson, 2018). According to an article published in *The Lancet* (Emma *et al.*, 2022) more than 57 million people were living with dementia worldwide in 2019, with AD accounting for 70% of those cases and this number is projected to cross 152 million by 2050 if no breakthroughs in treatment or prevention are discovered.

AD can be classified into two main types: familial AD and sporadic AD (Duara *et al.*, 1993). Of the two, familial AD also known as early-onset AD is rarer and accounts for less than 5% of all cases (Chakrabarti *et al.*, 2015; Mendez, 2019). It is caused by specific genetic mutations in three genes [presenilin 1 (PSEN1), presenilin 2 (PSEN2) and amyloid precursor protein (APP)] that are inherited in an autosomal dominant pattern (Bertram and Tanzi, 2004). Sporadic AD, on the other hand, is the more common form, and accounts for over 95% of cases (Chakrabarti *et al.*, 2015). It is thought to result from a complex interaction of genetic and environmental factors, with age being the most significant risk factor. Table 1.1 presents some of the key characteristics of Familial and Sporadic Alzheimer's Disease. AD disproportionately affects women, with nearly two-thirds of Alzheimer's patients being female (Hebert *et al.*, 2001). This sex specific disparity might be attributed to women's longer life expectancy, as age is the greatest risk factor for developing Alzheimer's, however, this by itself does not fully explain the disparity. For example, decline in the levels of the sex hormone estrogen post menopause is also a major contributing factor for higher occurrence of AD in older women (Paganini-Hill and Henderson, 1994).

Feature	Familial Alzheimer's (Early Onset)	Sporadic Alzheimer's (Late Onset)
Age of onset (typical)	Before 65 years	After 65 years
Genetic Involvement	Strong genetic component	Weak or no genetic component
Key Genes Involved	APP, PSEN1, PSEN2	APOE4 (Risk factor)
Inheritance Pattern	Autosomal dominant	No clear inheritance pattern
Prevalence	Rare (<5% of cases)	Common (>95% of cases)
Family History	Strong family history	May or may not have family history

Table 1.1: Comparison of Familial and Sporadic Alzheimer's Disease based on key characteristics. APP: Amyloid Precursor Protein; PSEN1/2: Presenilin 1/2; APOE4: Apolipoprotein E4.

The progression of AD often begins with mild cognitive impairment (MCI), a transitional stage between normal aging and mild AD. MCI is characterized by noticeable memory or cognitive problems that are more pronounced than typical age-related changes (Anderson, 2019), but do not significantly interfere with day-to-day events. As the condition advances to other stages of AD, symptoms begin with subtle short-term memory loss and progress to more severe cognitive impairments, such as difficulties with problem-solving or having trouble in performing everyday tasks with ease. This progression is marked by a gradual decline in cognitive function, with memory lapses becoming more pronounced. As the disease advances

further, patients experience further behavioral changes including neuropsychiatric symptoms such as social withdrawal and agitation (Lyketsos *et al.*, 2011). Persons with AD also start forgetting their personal history and even fail to identify loved ones. The transition from MCI to AD is often subtle, with symptoms emerging gradually and becoming more severe over time, however, much variability is seen behaviorally. Pathologically, AD has two major hallmarks. It is characterized by the accumulation of extracellular A β plaques and intracellular NFTs inside the brain (Gallardo and Holtzman, 2019). These pathological changes lead to synaptic dysfunction (including synaptic pruning), neuronal loss, and brain atrophy, which particularly affects regions crucial for memory and cognitive functions, such as the cortex and hippocampus. In recent years, increased neuroinflammation has emerged as a third major hallmark of AD pathology (Amelimojarad *et al.*, 2024). This chronic inflammatory response inside the brain contributes significantly to disease progression, neuronal damage, and cell death. Importantly, A β accumulation can trigger and exacerbate this inflammatory process, creating a self-perpetuating cycle of neurodegeneration (Cai *et al.*, 2014). The focus of this thesis, particularly in Chapter 3, will be on AD pathology, with special emphasis on A β and neuroinflammation. In the next set of subsections, the major pathological aspects of AD will be discussed, to provide a comprehensive understanding of the complex interplay between A β accumulation and inflammatory responses, and their combined impact on AD progression and severity.

Neurodegeneration

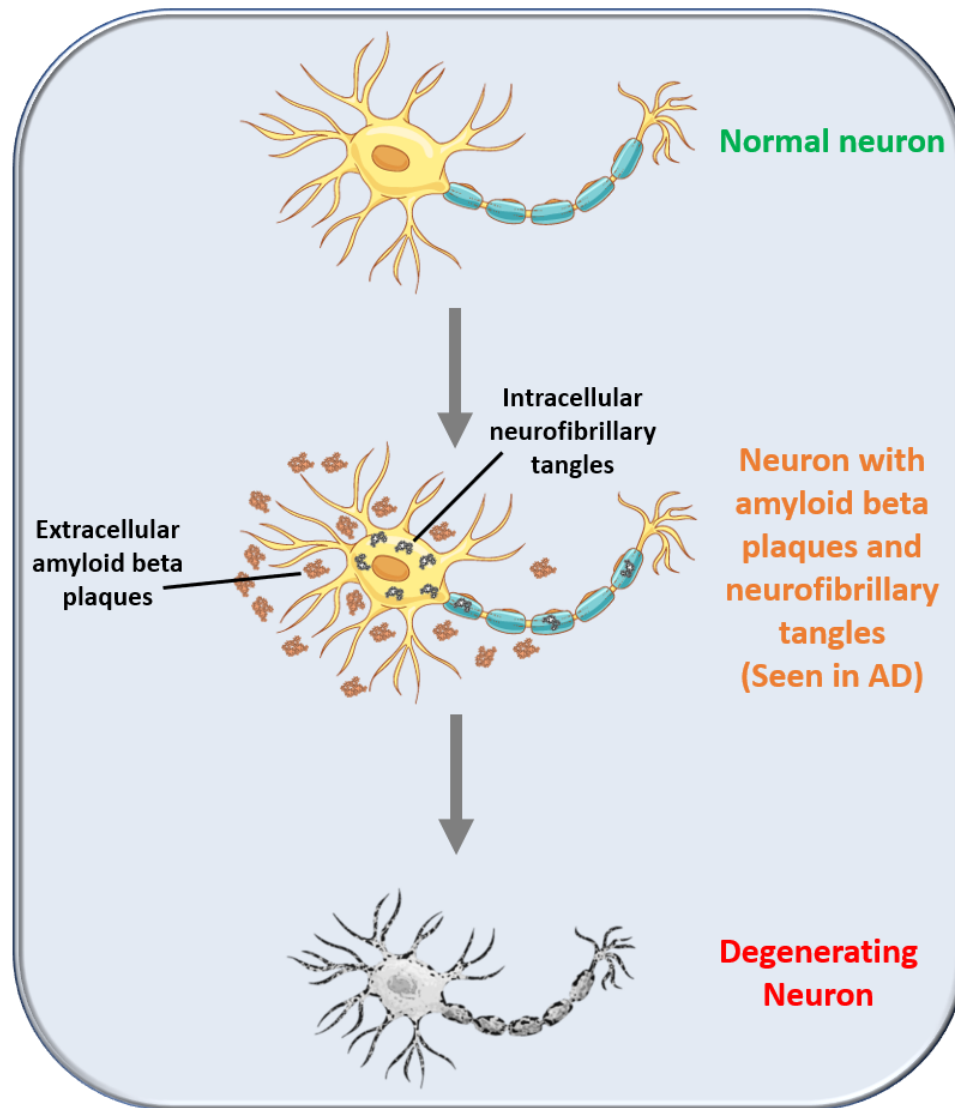


Figure 1.1. Progressive neurodegeneration in Alzheimer's disease. The first image depicts a normal, healthy neuron with intact structure. The second image illustrates a neuron in case of Alzheimer's disease, showing the presence of amyloid beta plaques and neurofibrillary tangles, which are pathological hallmarks of the disease. The third image represents a degenerating neuron, highlighting neuronal shrinkage, dendritic loss, and structural disintegration associated with disease progression. This figure is original and prepared by myself.

1.2.1 Pathology

1.2.1.1 Amyloid beta (A β) in Alzheimer's disease

Amyloid beta (A β) is a peptide derived from the amyloid precursor protein (APP) and plays a central role in the pathogenesis of AD. Under normal physiological conditions, A β plays a role in various cellular processes, including synaptic plasticity and neuronal survival (Parihar and Brewer, 2010). However, A β accumulation in the brain is a major hallmark of AD pathology. The generation of this A β peptide occurs through the sequential cleavage of APP by enzymes; β -secretase (BACE1) followed by γ -secretase. This process, called the amyloidogenic pathway (Hampel *et al.*, 2021), produces A β peptides of varying lengths, primarily A β 40 and A β 42 which are 40 and 42 amino acids long, respectively. Alternatively, APP can be processed via the non-amyloidogenic pathway, where α -secretase cleaves APP, resulting in the release of soluble APP α (sAPP α) and generation of a membrane-bound fragment called C-terminal fragment α (CTF α) (C83). CTF α is further cleaved by γ -secretase, producing a non-toxic peptide called p3 and the APP intracellular domain (AICD). This pathway prevents the formation of toxic A β peptides and is the dominant pathway under normal physiological conditions (Kojro and Fahrenholz, 2005). While cleavage of APP is a part of the normal cellular process, improper or excessive cleavage can lead to an overproduction of A β , particularly the more aggregation-prone A β 42 form. Under typical conditions, the brain maintains a balance between A β production and clearance. However, in AD, this balance is disrupted, leading to the accumulation of A β in the extracellular spaces. This extracellular A β accumulation impairs communication between neurons, disrupts synaptic function, and triggers inflammatory responses inside the brain.

There are different forms of A β that exhibit varying degrees of toxicity (Chen *et al.*, 2017). Monomeric A β is generally considered non-toxic, but as these monomers aggregate, they form oligomers, fibrils, and eventually, the characteristic amyloid plaques found in AD brains capable of disrupting synaptic function. The presence of these A β oligomers and aggregates exacerbates neuroinflammation by activating cellular pro-inflammatory pathways such as nuclear factor kappa B (NF- κ B) (Kaltschmidt *et al.*, 1997; Snow and Albeni, 2016). Once activated, these pathways increase the release of pro-inflammatory cytokines and other inflammatory mediators, contributing to a chronic state of inflammation in the AD brain. This inflammatory environment in turn promotes further production of A β and impairs its clearance, creating a vicious cycle of neurodegeneration. Recent research has shown that while amyloid plaques are neurotoxic and remain a hallmark of AD pathology, it is the A β oligomers (A β Os) that display a much stronger correlation with the initiation and progression of AD (Lublin and Gandy, 2010; Viola and Klein, 2015). These oligomers are considered highly neurotoxic, mediating synaptic dysfunction, mitochondrial damage, and neuronal loss often preceding detectable plaque formation. Thus, A β Os are believed to play a central and causative role in AD pathogenesis, shifting the focus of therapeutic strategies toward targeting these early oligomeric assemblies.

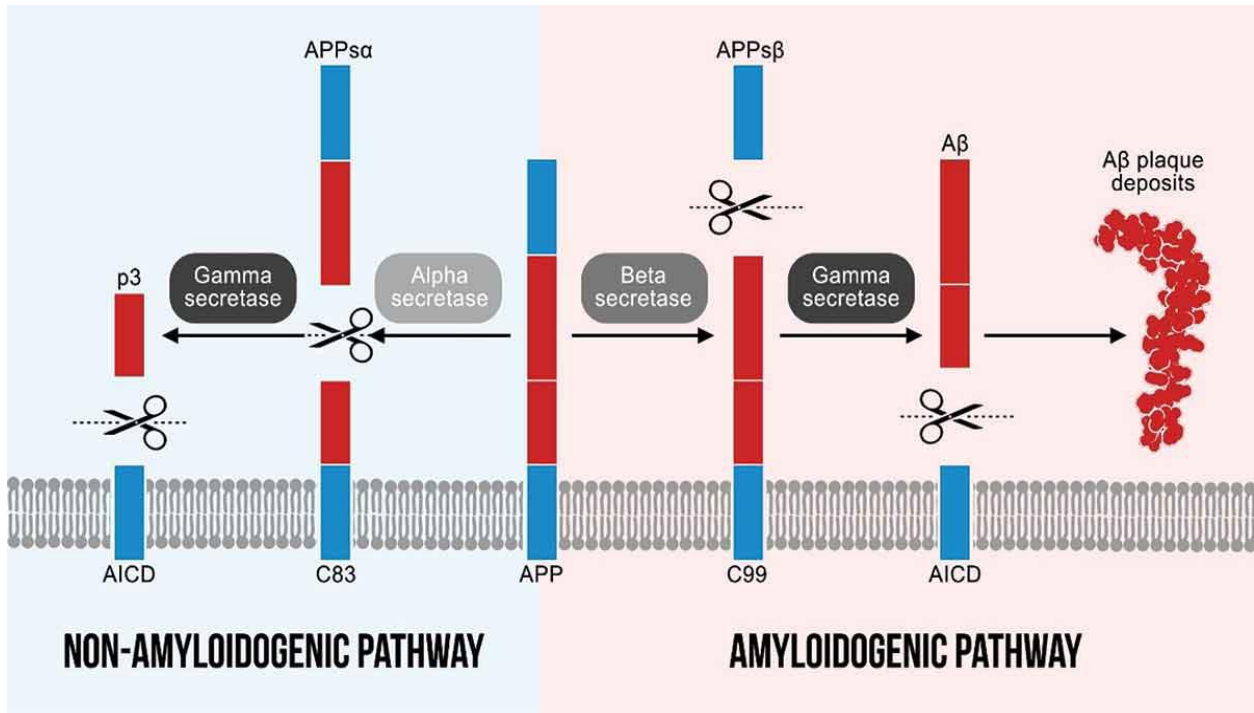


Figure 1.2. Non-amyloidogenic and amyloidogenic pathways of APP processing. The red regions of APP signify the potentially harmful A β portion which is cleaved in the non-amyloidogenic pathway. This figure is reprinted from Biotechnology and Genetic Engineering Reviews article ‘Current viral-mediated gene transfer research for treatment of Alzheimer’s disease’ (Sasmita, 2019) with permission of Informa UK Limited, trading as Taylor & Francis Group.

1.2.1.2 Neurofibrillary tangles (NFTs) in Alzheimer’s disease

Neurofibrillary tangles (NFTs) are intracellular aggregates of hyperphosphorylated tau protein and represent the second major hallmark of AD pathology. Tau is a microtubule-associated protein that normally plays a vital role in stabilizing microtubules while also supporting axonal transport within neurons (Barbier *et al.*, 2019). However, in AD, tau undergoes abnormal hyperphosphorylation due to an imbalance in kinase and phosphatase activities, leading to its detachment from microtubules and subsequent aggregation into

insoluble fibrillary structures known as NFTs (Brion, 1998; Mandelkow and Mandelkow, 1998). As NFTs accumulate within neurons, they disrupt cellular scaffolds and impair various intracellular processes including, axonal transport, synaptic function, and overall neuronal health (Terwel *et al.*, 2002). This disruption leads to neuronal dysfunction and ultimately cell death. Interestingly, NFT formation typically appears after the initial deposition of A β in AD pathology (Gulisano *et al.*, 2018). This temporal relationship suggests that A β accumulation triggers or exacerbates tau pathology. Hence, targeting A β accumulation in the early stages of AD might help prevent or slow down the subsequent formation of NFTs.

1.2.1.3 Neuroinflammation in Alzheimer's disease

Neuroinflammation in the CNS is a critical component of neurodegenerative diseases, including AD, PD, HD, MS, and ALS (Al-Badri and Castorina, 2018; Jia *et al.*, 2022; Liu and Wang, 2017). In these conditions, neuroinflammation is not merely a consequence but a key driver of neurodegeneration, characterized by the activation of immune cells that release pro-inflammatory mediators (Zhang *et al.*, 2023). While inflammation is a natural defense mechanism, sustained neuroinflammation disrupts cellular functions and exacerbates neuronal injury, leading to progressive neurodegeneration as seen in AD (Heneka *et al.*, 2015; Kiraly *et al.*, 2023).

In AD, neuroinflammation plays a pivotal role in disease progression (Long *et al.*, 2025). It is triggered by endogenous factors such as A β Os and NFTs, which activate pro-inflammatory pathways like NF- κ B, leading to chronic inflammation (Bhaskar *et al.*, 2022; Kaltschmidt *et al.*, 2022; Verma *et al.*, 2023). Microglia, the resident immune cells of the CNS, play a central role in AD-related neuroinflammation. Astrocytes are also actively involved in this

neuroinflammatory response, contributing to oxidative stress and the release of pro-inflammatory mediators in AD (González-Reyes *et al.*, 2017). In response to A β accumulation, microglia become activated. Initially, they adopt an anti-inflammatory phenotype, attempting to clear A β deposits but, as the disease progresses, microglia shift towards a pro-inflammatory phenotype (Luo and Chen, 2012). Activated microglia upregulate pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α , as well as reactive oxygen species (ROS), that contribute to synaptic dysfunction and neuronal damage (Lull and Block, 2010). Importantly, A β activates microglia through various receptors, including toll-like receptors (TLRs) and the receptor for advanced glycation end products (RAGE) (Li *et al.*, 2025). This activation triggers pro-inflammatory pathways including NF- κ B, leading to the production of inflammatory mediators and further microglial recruitment, creating a self-perpetuating cycle of inflammation and neurodegeneration.

Recent evidence suggests that neuroinflammation may occur early in AD, even before the appearance of classical pathological hallmarks (Calsolaro and Edison, 2016). Genetic factors, such as the apolipoprotein E4 (APOE4) allele, increase the risk for AD onset and also enhance neuroinflammatory responses (Egensperger *et al.*, 1998; Michaelson, 2014). Notably, the interplay between neuroinflammation and mitochondrial dysfunction is particularly critical, as each condition exacerbates the other, contributing to neuronal death (Wilkins and Swerdlow, 2016). Furthermore, sustained NF- κ B activation as seen in AD, is detrimental. Persistent activation of NF- κ B, a transcription factor that regulates genes involved in inflammation and cell survival (Liu *et al.*, 2017), leads to chronic neuroinflammation and oxidative stress (Kaltschmidt *et al.*, 2022; Shih *et al.*, 2015). NF- κ B subunits can also translocate into the mitochondria, and disrupt mitochondrial function leading to cellular damage (Albensi, 2019).

1.2.1.4 Mitochondrial dysfunction in Alzheimer's disease

1.2.1.4.1 Mitochondria in cellular functions and neurodegeneration

The CNS has high energy requirements, which demands the presence of thousands of mitochondria in each neuron (Misgeld and Schwarz, 2017). Mitochondria (singular; mitochondrion), often referred to as the powerhouse of the cell, are membrane bound cell organelles that play a crucial role in cellular function and survival by generating ATP, the primary energy currency of cells (Javadov and Kuznetsov, 2013). These organelles produce energy through oxidative phosphorylation (OXPHOS), which involves a series of redox reactions occurring within the electron transport chain (ETC) located on the inner mitochondrial membrane (IMM) (Senior, 1988). The resulting proton gradient is harnessed by Complex V, or ATP synthase, to synthesize ATP (Maloney, 1982). In addition to production of ATP, mitochondria drive numerous cellular functions, including protein synthesis, maintenance of membrane potential, and synaptic plasticity (Nicholls, 2004; Sayehmiri *et al.*, 2024). They are also integral to calcium homeostasis and the regulation of overall cell health (Vandecasteele *et al.*, 2001). Given their critical role in cell survival, mitochondrial dysfunction has been implicated as a root cause in various neurodegenerative diseases (Ferryhough, 2015; Van Acker *et al.*, 2025; Wang *et al.*, 2014). Mitochondrial dysfunction results in energy deficiency, which is a key characteristic of many CNS diseases (Johri and Beal, 2012) and plays a role in the pathogenesis of several neurodegenerative disorders, including but not limited to AD, PD, and ALS (Moon and Paek, 2015; Zhao *et al.*, 2022; Zhu *et al.*, 2004).

1.2.1.4.2 Impaired Mitochondrial Function in Alzheimer's Disease

Mitochondrial dysfunction plays a crucial role in the pathogenesis of Alzheimer's disease (AD), significantly contributing to neuronal degeneration (Ashleigh *et al.*, 2023). Mitochondrial bioenergetic deficits become evident even before the appearance of A β and NFTs, suggesting that mitochondrial dysfunction could be an early driver of AD pathology (Hauptmann *et al.*, 2009; Sharma *et al.*, 2021; Yao *et al.*, 2009). Dysfunctional mitochondria fail to produce sufficient ATP, leading to synaptic dysfunction, neuronal loss, and cognitive decline, all characteristic of AD. In 2004, Swerdlow *et al.*, proposed the mitochondrial cascade hypothesis for AD which states that mitochondrial dysfunction contributes to onset of AD (Swerdlow *et al.*, 2010). Additionally, mitochondrial ETC complexes, particularly complex I (NADH ubiquinone oxidoreductase), complex IV (cytochrome oxidase (COX)), and complex V (ATPase) exhibit reduced activity in AD brain mitochondria, leading to decreased ATP production (Johri, 2021). A large number of studies have also reported metabolic defects and reduced rate of brain metabolism in AD (Blass, 2001; Mosconi *et al.*, 2008; Ogawa *et al.*, 1996).

1.2.1.4.3 Mitochondrial dysfunction and neuroinflammation

Neuroinflammation in AD can also damage the mitochondria, impairing their structure and function (Di Filippo *et al.*, 2010). Interestingly, the transcription factor NF- κ B has been localized inside the mitochondria, specifically in the mitochondrial intermembrane space and the inner matrix (Bottero *et al.*, 2001; Cogswell *et al.*, 2003), where it can regulate mitochondrial genes, potentially influencing mitochondrial function (Albensi, 2019). Impaired mitochondria release mitochondrial DNA (mtDNA) and other damage-associated molecular

patterns (DAMPs) that contribute towards the production of excessive amounts of ROS, causing oxidative damage (Dela Cruz and Kang, 2018; Lin *et al.*, 2022). DAMPs further trigger inflammation, creating a vicious cycle where increased neuroinflammation, in turn, exacerbates mitochondrial dysfunction, and leads to neuronal damage.

1.2.1.4.4 Mitochondrial dysfunction and upstream energy regulators

Mitochondrial dysfunction in AD extends beyond the organelles themselves, affecting upstream regulators of mitochondrial function such as AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α), which are involved in cellular energy balance. Production of new mitochondria is regulated, in part, by PGC-1 α which is a transcriptional coactivator (Austin and St-Pierre, 2012), and AMPK can interact with PGC-1 α , to regulate mitochondrial function (Garcia and Shaw, 2017). Downregulated AMPK signaling can lead to impaired mitochondrial biogenesis and contribute to mitochondrial dysfunction (Herzig and Shaw, 2018), further exacerbating the decline in energy levels in neurons (Gureev *et al.*, 2019; Johri and Beal, 2012). Hence, improving mitochondrial function holds great promise as a potential therapeutic strategy to slow the progression of AD. Targeting mitochondrial dysfunction, potentially in combination with approaches addressing neuroinflammatory processes, could offer more effective treatment options.

1.3 Neuropathy

Neuropathy refers to a group of disorders characterized by damage or dysfunction of the nervous system, particularly affecting the peripheral nerves that are part of the PNS. These conditions often result in a wide range of symptoms, including pain, numbness, tingling, and even weakness in various parts of the body (Bril *et al.*, 2018; Marchettini *et al.*, 2006). Neuropathy can develop due to numerous factors such as diabetes, infections, exposure to toxins, vitamin deficiencies and genetic disorders (Staff and Windebank, 2014). While neuropathy can technically involve any component of the nervous system, it generally refers to peripheral neuropathy, which is discussed in detail in the next section of this chapter. The most common form of neuropathy includes diabetic neuropathy (Feldman *et al.*, 2019), which is caused by high blood sugar and lipid levels and results in nerve fiber damage in limbs. Other notable neuropathies include chemotherapy-induced peripheral neuropathy, Guillain-Barré syndrome, and Charcot-Marie-Tooth disease, all of which significantly impact patients' quality of life and their functional independence.

1.3.1 Peripheral Neuropathy

Diabetic neuropathy is the most prevalent form of peripheral neuropathy (Feldman *et al.*, 2019), affecting millions of individuals worldwide and causing significant morbidity. The impact of these disorders can be profound, affecting quality of life and daily functioning. Peripheral neuropathy occurs when the PNS nerves (nerves outside the brain and spinal cord) get damaged. Over 100 different types of peripheral neuropathies exist affecting more than 300 million people worldwide (Feldman *et al.*, 2019; Singh and Bhatti, 2023). This condition can impact sensory, motor, and autonomic nerve fibers, leading to a range of symptoms and

functional impairments. Peripheral neuropathies can be either acquired or inherited (Burns *et al.*, 2006), with causes ranging from metabolic disorders such as diabetes to toxic exposures, infections, and genetic mutations (Staff and Windebank, 2014). Common symptoms include numbness, tingling, burning sensations, weakness, allodynia, hyperalgesia, and autonomic dysfunction (Husain, 2024; Low *et al.*, 2003; Watson and Dyck, 2015). One major difference in neurons of the PNS when compared to CNS is that, unlike CNS neurons, peripheral neurons have a greater capacity for regeneration (Pleasure *et al.*, 1999).

1.3.2 Peripheral neuropathy affecting dorsal root ganglion (DRG) neurons

Peripheral nerve degeneration significantly affects dorsal root ganglion (DRG) neurons (Krames, 2014; Shi *et al.*, 2001), which are crucial components of the PNS and play a vital role in sensory processing. DRGs house the cell bodies of primary sensory neurons and are potential targets for neuronal restoration in peripheral neuropathies (Estivill-Torrús *et al.*, 2024). Situated on either side of the spinal cord, DRGs serve as a critical junction between the PNS and CNS, where they modulate sensory information before it reaches the spinal cord. During peripheral neuropathy, DRGs often become sites of significant cellular and molecular alterations, including neuronal hyperexcitability, changes in ion channel expression, altered pain signaling, and even neuronal death in severe cases (Jang and Garraway, 2024; Zhang and Dougherty, 2014). A major issue observed in DRG neurons in cases of neuropathy is axonal degeneration, which propagates and leads to symptoms of allodynia and hyperalgesia (Jensen and Finnerup, 2014). A key approach in addressing peripheral neuropathy and its effects on DRG neurons is nerve regeneration. PNS neurons possess an intrinsic ability to regenerate their axons. This regenerative capacity is supported by various factors, including the activation of

growth-associated genes, the presence of growth-promoting Schwann cells, and the production of neurotrophic factors (Gordon, 2009; Wei *et al.*, 2024). However, the success of nerve regeneration can be limited by factors such as the severity of the injury or the chronicity of the condition. Understanding the mechanisms of peripheral nerve regeneration, particularly in relation to DRG neurons, can aid in developing effective therapeutic strategies for peripheral neuropathies.

1.3.3 Pathology

While the exact pathology of peripheral neuropathy depends on the underlying disease, previous studies have identified multiple mechanisms that exhibit similar patterns of axonal degeneration and dysfunction of peripheral nerves, including those associated with the DRGs, involving complex processes such as axonal degeneration, demyelination, Wallerian degeneration, and mitochondrial dysfunction (Fernyhough, 2015; Hammi and Yeung, 2025). Axonal degeneration involves the breakdown of axons, often beginning at the distal ends and progressing proximally. This "dying-back" phenomenon is particularly evident in long axons, making the longest nerves in the body most vulnerable (Coleman, 2005). Demyelination, on the other hand, involves damage to the myelin sheath surrounding axons, which can occur independently or in conjunction with axonal degeneration (Sharma *et al.*, 2002). Wallerian degeneration is a process where the distal part of a damaged nerve breaks down, leading to loss of nerve function. It also results in myelin damage, inflammation, and is often associated with neuropathic pain (Üçeyler and Sommer, 2006). In addition to inflammation, mitochondrial dysfunction is another major contributor to the pathogenesis of peripheral neuropathy

(Fernyhough, 2015). Both inflammation and mitochondrial dysfunction are discussed in the next two sub-sections.

1.3.3.1 Mitochondrial dysfunction in neuropathy

Mitochondrial dysfunction plays a crucial role in the pathogenesis of peripheral neuropathies, affecting both motor and sensory neurons in the PNS. Neurons of the PNS are highly dependent on mitochondria for energy production and axonal transport (Baloh, 2008), which are essential for maintaining axonal integrity, supporting transmission, and regulating calcium homeostasis (Lee *et al.*, 2018a; Wang *et al.*, 2021). Key aspects of mitochondrial dysfunction in neuropathy include reduced ATP production, disrupted calcium homeostasis, and altered mitochondrial dynamics (Fernyhough *et al.*, 2010; Sajic, 2014), compromising cellular energy homeostasis and contributing to increased reactive oxygen species (ROS) production, oxidative damage, and axonal degeneration. Mitochondrial defects in neuropathies lead to reduced cellular energy levels, increased oxidative stress, and disrupted calcium buffering, resulting in axonal degeneration, demyelination, and ultimately, neuronal death. Additionally, cellular pathways such as the AMPK pathway are down-regulated, further disrupting metabolic regulation in peripheral nerves. Diabetic neuropathy, one of the most common forms of peripheral neuropathy, affects DRG neurons and exhibits significant mitochondrial involvement (Aghanoori *et al.*, 2017; Fernyhough, 2015). The impaired mitochondrial function is characterized by decreased activity of ETC complexes, reduced mitochondrial membrane potential (MMP), and increased ROS generation (Fernyhough *et al.*, 2010; Zhang *et al.*, 2021). This leads to axonal degeneration, contributing to sensory deficits, neuropathic pain, and allodynia or hyperalgesia (Jensen and Finnerup, 2014). Importantly,

mitochondria play a crucial role in regeneration of axons (Han *et al.*, 2016). Hence, enhancing mitochondrial function could be a key in preventing or reversing the progression of various peripheral neuropathies.

1.3.3.2 Neuroinflammation in neuropathy

Neuroinflammation in the PNS frequently results in nerve dysfunction, neuropathy, or neuropathic pain (Ellis and Bennett, 2013). This inflammatory response arises from conditions such as diabetes, infections, or autoimmune disorders like chronic inflammatory demyelinating polyneuropathy (CIDP), which involves immune cells such as macrophages and T lymphocytes (Bril *et al.*, 2016; Martini and Willison, 2016). Neuroinflammation affects multiple components of the PNS, including DRG neurons, which are critical for sensory transmission. Inflammation in DRG neurons can lead to sensory dysfunction, demyelination, and axonal degeneration, contributing to neuropathic pain and sensory deficits (Cashman and Höke, 2015; Martin *et al.*, 2019). Alongside macrophages, neutrophils, and activated Schwann cells also contribute to the inflammatory milieu around DRG neurons, exacerbating pain signaling and nerve damage (Wei *et al.*, 2019; Yu *et al.*, 2020). The release of pro-inflammatory cytokines and chemokines during inflammation activates nociceptors, causing persistent pain characterized by allodynia or hyperalgesia (Wallace, 2007; Zhang and An, 2007). Additionally, neuroinflammation can disrupt cellular energy metabolism by impairing pathways like AMPK, a key regulator of cellular energy homeostasis. Inflammatory mediators can inhibit AMPK activation (Sag *et al.*, 2008), reducing its neuroprotective effects and contributing to mitochondrial dysfunction. This dysregulation exacerbates nerve damage and also impedes the repair or regeneration processes.

1.4 Estrogen

Estrogen is a steroid hormone primarily known as a female sex hormone (Hamilton *et al.*, 2017). However, it exerts a wide range of functions in both males and females. In females, estrogen regulates the menstrual cycle and is responsible for the development of primary and secondary sexual characteristics (Richards and Hawley, 2011). In males, estrogens play a crucial role in sperm maturation and erectile function (Schulster *et al.*, 2016). Beyond its role in the development of secondary sexual characters and reproduction, estrogen influences the brain, bones, cardiovascular system, metabolism, and immune system (Hara *et al.*, 2015; Stice *et al.*, 2009; Väänänen and Härkönen, 1996; Xiang *et al.*, 2021). Additionally, estrogen plays a significant role in the development and function of the nervous system (McEwen *et al.*, 2012), where it regulates neuronal growth, synaptic plasticity, and neuroinflammatory responses, contributing to overall neural health (Brinton, 2009). Estrogen acts via estrogen receptors (ERs) that are widely expressed across both the CNS and PNS (Patrone *et al.*, 1999; Warfvinge *et al.*, 2020). Its presence in these regions suggests a potential role in regulation of neural functions, which could have implications for neurodegenerative diseases. In fact, several studies have found estrogen to have significant neuroprotective properties (Garcia-Segura *et al.*, 2001), which can help mitigate neurodegenerative disorders such as AD.

1.4.1 Forms of estrogen

There are three main types of endogenous estrogens: estrone (E1), estradiol (E2), and estriol (E3). Estradiol (E2) is the most potent and prevalent form, primarily produced in the ovaries of premenopausal women, where it plays a crucial role in reproductive functions and overall health (Cui *et al.*, 2013). In premenopausal females, circulating plasma E2 levels range

from approximately 0.1 to 2.2 nM, depending on the phase of the menstrual cycle, which is comparable to the levels in males (approximately 0.1 nM) (Vrtačnik *et al.*, 2014). Estrone (E1) is less potent than E2, primarily produced in extragonadal tissues such as adipose tissue, and can be converted to E2 in peripheral tissues (Siiteri, 1987). Estriol (E3) is predominantly synthesized by the placenta during pregnancy. Another form, estetrol (E4), is also produced during pregnancy by the fetal liver (Holinka *et al.*, 2008). These estrogens exert their effects by binding to ERs, which regulate gene expression and influence various physiological processes.

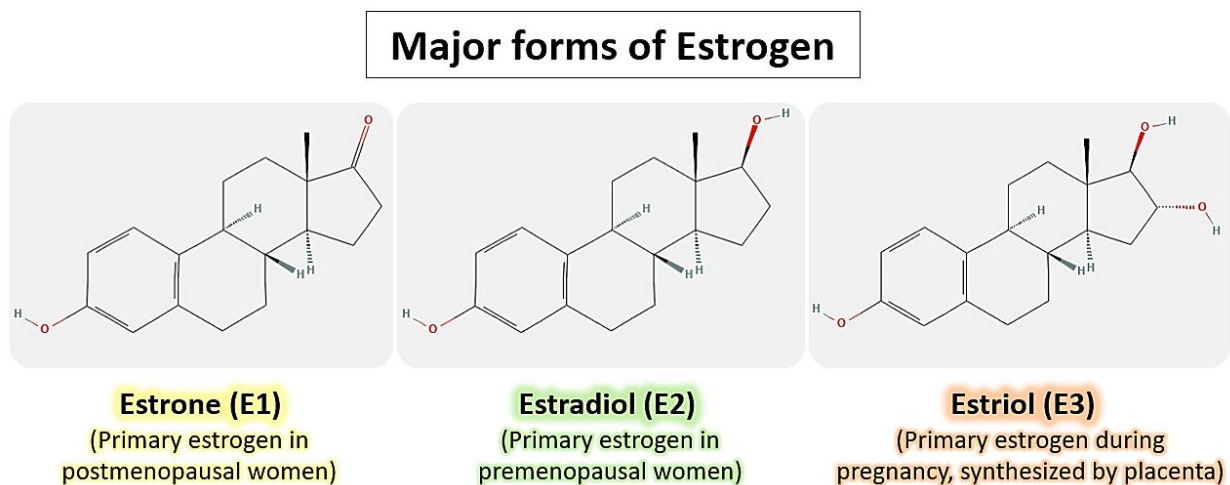


Figure 1.3. The three major forms of estrogen and their roles throughout different life stages. Estradiol (E2): The most potent and active form of estrogen, predominantly present before menopause. **Estrone (E1):** The primary form of estrogen after menopause, with lower potency compared to E2. **Estriol (E3):** Primarily produced during pregnancy by the placenta, with relatively weaker estrogenic effects compared to E2. Chemical structures were obtained from the open-source database PubChem. This figure is original and prepared by myself.

1.4.2 Estrogen synthesis

Estrogen synthesis primarily occurs in the ovaries of premenopausal females and in the testes of males, with additional production in peripheral tissues such as the brain and adipose tissue in both sexes (Cui *et al.*, 2013). Estrogen synthesis begins with cholesterol, which undergoes a series of enzymatic reactions involving cytochrome P450 enzymes, notably aromatase, to convert androgens such as testosterone and androstenedione into estrogens (detailed illustration in Figure 1.4). Estradiol (E2) is the most predominant form of estrogen in premenopausal women, while estrone (E1) becomes the primary estrogen after menopause. Estrogen levels decline with aging in both males and females, leading to symptoms such as decreased sex drive and bone loss. Furthermore, research has linked this decline in estrogen levels with an increased risk of AD (Cholerton *et al.*, 2002), suggesting a potential protective role of estrogen in neurodegenerative processes.

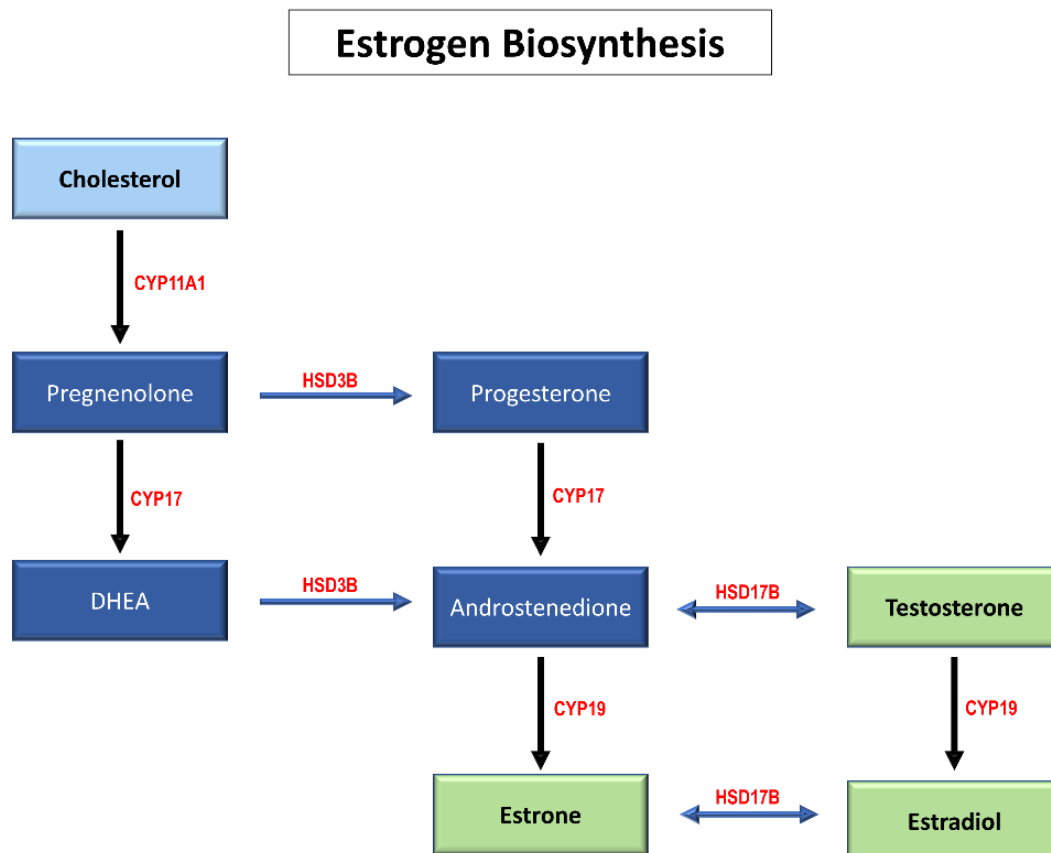


Figure 1.4. Schematic representation of estrogen biosynthesis. Cholesterol serves as the precursor, undergoing enzymatic conversions to form key steroid intermediates such as pregnenolone, progesterone, dehydroepiandrosterone (DHEA), and androstenedione. The final conversion to estrogens; estrone (E1) and estradiol (E2) occurs through the action of enzymes, including aromatase (CYP19) and 17 β -hydroxysteroid dehydrogenase (HSD17B). Enzymes are labeled in red, with arrows indicating the biochemical pathways. This figure is original and prepared by myself.

1.4.3 Estrogen receptors

Estrogen exerts its effects on tissues and organs primarily through ERs, which are members of the nuclear receptor superfamily. The discovery of ERs began with the identification of ER α by Jensen in 1958 (Jensen, 1962), followed by ER β by Kuiper and colleagues in 1996 (Kuiper *et al.*, 1996). Both ER α and ER β function as ligand-activated transcription factors, sharing a similar five-domain structure that includes the N-terminal A/B domain, DNA binding domain (DBD), a hinge region, a ligand binding domain (LBD), and a C-terminal F region (illustrated in Figure 1.5). ER α and ER β differ in their N-terminal regions, with ER β being shorter and having lower sequence homology to ER α (Kumar *et al.*, 2011). ER α , with 595 amino acids, is slightly larger than ER β , which contains 530 amino acids. Both receptors can form homo- and heterodimers, facilitating transcriptional activity. The DBD of ERs binds to estrogen response elements (EREs) in DNA, allowing them to regulate gene expression. Additionally, a more recently identified receptor, G protein-coupled estrogen receptor 1 (GPER1), acts as a transmembrane ER, differing from the nuclear ERs (Revankar *et al.*, 2005). GPER1 localizes in the plasma membrane and plays a role in rapid nongenomic estrogen signaling (described in the next section) (Pupo *et al.*, 2016). Both ER α and ER β are evolutionarily conserved (Callard *et al.*, 2011) and widely distributed in the CNS and PNS, where they play crucial roles

in modulating neural functions. The evolutionary conservation of ERs across species underscores their importance in maintaining physiological homeostasis.

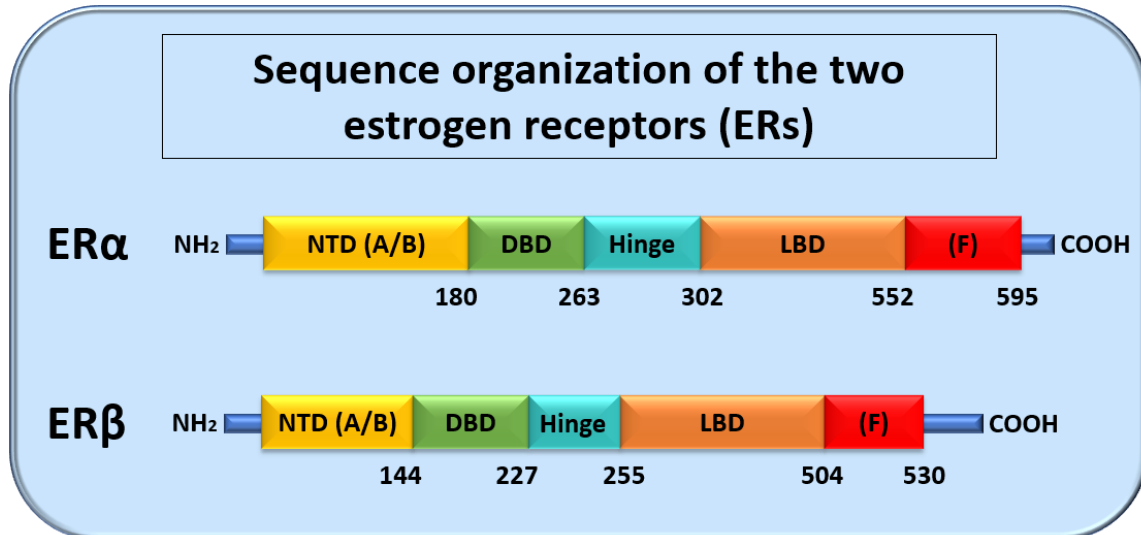


Figure 1.5. Sequence Organization of Estrogen Receptor ER α and ER β . The sequence organization of the two isoforms of estrogen receptors, ER α and ER β , is shown. Different functional domains are highlighted in distinct colors: NTD (A/B) - Amino-terminal domain (yellow), DBD - DNA binding domain (green), Hinge region (blue), LBD - Ligand binding domain (orange), and F region (red). The amino acid sequence positions corresponding to each domain are indicated. This figure is original and prepared by myself.

1.4.4 Estrogen mechanism of action

Estrogen exerts its effects through genomic or non-genomic pathways, depending on the subcellular location of ERs in the cytoplasm or nucleus (Azuma and Inoue, 2012). Genomic signaling primarily involves ER α and ER β . As a steroid hormone, estrogen can easily cross the cell membrane and bind to ERs, which then translocate into the nucleus. There, they bind to EREs on DNA or interact with other transcription factors, such as NF- κ B or activator protein 1 (AP-1), to modulate gene expression (Björnström and Sjöberg, 2004; Xing *et al.*, 2012). This classical pathway allows for direct regulation of transcription by recruiting co-factors that either activate or suppress gene expression. Non-genomic signaling, on the other hand, involves ERs located in the cytoplasm or on the cell membrane, where they do not interact directly with DNA. Instead, ligand binding triggers intracellular signaling cascades that activate protein kinases, which in turn activate transcription factors to alter gene expression. This mode of signaling is characteristic of GPER1-mediated estrogen signaling (Pupo *et al.*, 2016). Through these distinct mechanisms, estrogen can modulate key cellular pathways such as AMPK and NF- κ B, thereby regulating energy metabolism and inflammation inside a cell (Yang and Wang, 2015).

1.4.5 Estradiol in neuroprotection

Estrogen is a multifaceted hormone that regulates various tissues and functions beyond its classical role in reproduction. Research has established its neurotrophic and neuroprotective actions, influencing memory and cognition (Bortz *et al.*, 2022; Hara *et al.*, 2015). Traditionally viewed as a reproductive hormone, estrogen has significant non-reproductive effects on the brain and spinal cord, where it supports neuronal survival and promotes synaptic plasticity.

Extensive studies in rodent models and humans have shown that estrogen can delay the onset and slow the decline of cognitive function associated with neurodegenerative diseases such as Alzheimer's or Parkinson's by preventing neuronal loss (Ali *et al.*, 2023; Saunders-Pullman *et al.*, 1999). Notably, estradiol or 17 beta-estradiol (E2), the most potent form of estrogen, is particularly neuroprotective (Wise *et al.*, 2001). E2 upregulates neuroprotective pathways and downregulates pro-inflammatory pathways, improving functional outcomes in conditions such as spinal cord injury (Shvetcov *et al.*, 2023). In traumatic brain injury (TBI), E2 imparts neuroprotection by increasing cerebral blood flow, promoting angiogenesis, and regulating neurotransmitter levels (Brotfain *et al.*, 2016). Additionally, E2 facilitates rapid phosphorylation of transcription factors such as cAMP-responsive element-binding protein (CREB) (Abrahám *et al.*, 2003), initiating gene transcription and regulating neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF), which support neuronal growth and repair (Azcoitia *et al.*, 2019; Kövesdi *et al.*, 2020). E2 also mitigates oxidative stress by modulating antioxidant enzyme activity (Cagnacci *et al.*, 2023). Estrogen replacement has been linked to improved verbal memory, and studies in TBI models have demonstrated that E2 can reduce neurological severity and improve spatial memory acquisition (Lu *et al.*, 2018). Given the focus of this thesis on Alzheimer's, the potential role of E2 as a neuroprotective factor in AD is discussed further in the next section.

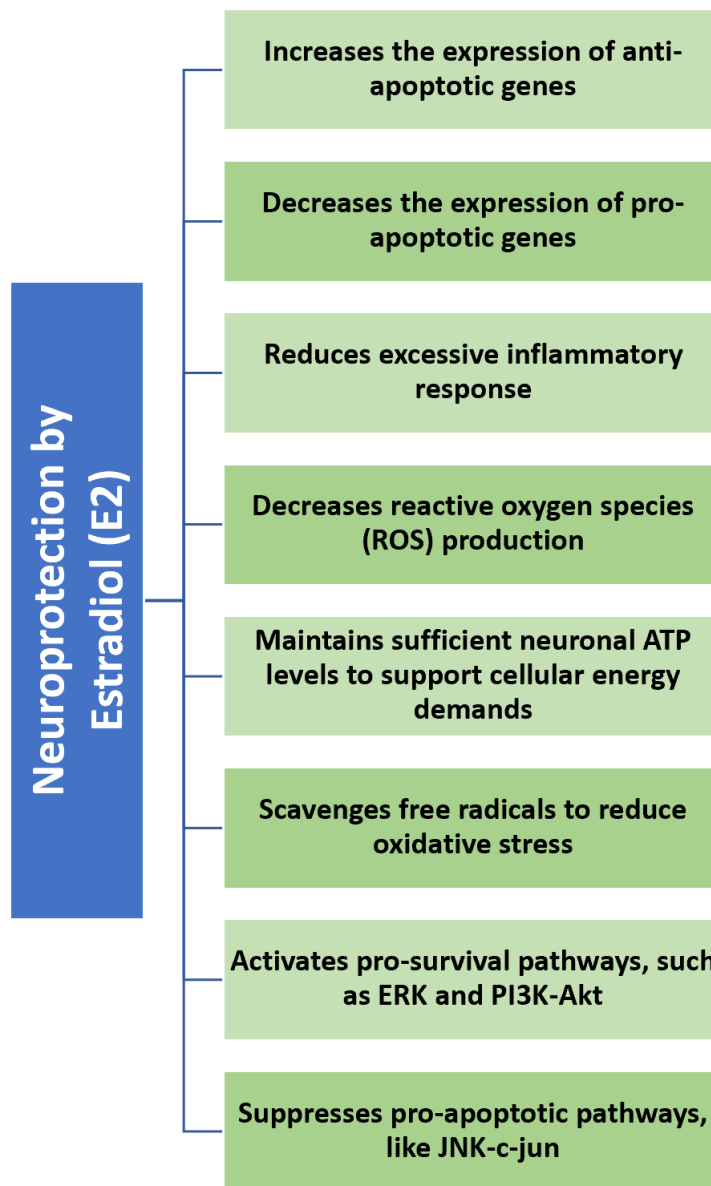


Figure 1.6. Mechanisms of estradiol neuroprotection. This figure summarizes major mechanisms through which estradiol (E2) protects neurons. This figure is reproduced with modifications and permission from the Elsevier article ‘Estrogen neuroprotection and the critical period hypothesis’ (Scott *et al.*, 2012). This figure prepared by myself.

1.4.6 Estradiol and Alzheimer's disease

AD is a neurodegenerative disorder with a higher incidence in women than in age-matched men, partly attributed to the decline in estrogen levels post-menopause (Paganini-Hill and Henderson, 1994). Estradiol (E2), the most prevalent form of estrogen before menopause, rapidly declines with aging and menopause, which is linked to an increased risk of AD onset (Henderson, 1997). Research using AD mouse models and cell cultures has demonstrated that estrogen, particularly the E2 form, exerts neuroprotective effects against AD (Garcia-Segura *et al.*, 2001). Estradiol imparts neuroprotection through multiple mechanisms, including regulation of mitochondrial proteins, reduction in oxidative stress, and by decreasing inflammation (Son *et al.*, 2020). It also modifies and increases the clearance of A β Os, a hallmark of AD pathology (Li *et al.*, 2000). Estradiol provides neuroprotection against A β mediated toxicity (Nilsen *et al.*, 2006) and also improves cognitive functions such as verbal learning and working memory, which are typically impaired in AD (Sherwin, 1998). A study by Zhao *et al.* demonstrated that estrogen deficiency leads to mitochondrial damage prior to the emergence of cognitive deficits, highlighting the importance of estrogen in maintaining mitochondrial health, which is implicated in AD (Albensi, 2020; Zhao *et al.*, 2021). Work with ER antagonists and inhibitors further supports the notion that ER activation is crucial and implicated in neurodegenerative disorders (Pike, 1999). However, clinical trials involving estradiol treatment have shown mixed results, necessitating further research to understand how estradiol-mediated neuroprotection can be effectively translated into therapeutic strategies for AD (Mishra *et al.*, 2023).

1.4.7 Estradiol and mitochondrial function

Estradiol plays a significant role in regulating mitochondrial function. Both estrogen receptors, ER α and ER β , act as transcription factors that bind to genomic and mitochondrial DNA (mtDNA), influencing gene transcription (Klinge, 2017). Additionally, E2 interacts with plasma membrane-associated signaling proteins such as GPER1 to activate intracellular cascades that alter mitochondrial morphology and function. E2 regulates mitochondrial biogenesis by stimulating nuclear respiratory factor-1 (NRF-1), which interacts with the transcriptional coactivator PGC-1 α to regulate nuclear-encoded mitochondrial genes (Chen *et al.*, 2009). NRF-1 also promotes the transcription of mitochondrial transcription factor A (TFAM), which upregulates mtDNA-encoded genes (Scarpulla, 2008; Scarpulla *et al.*, 2012). E2 enhances mitochondrial bioenergetics by increasing baseline oxygen consumption rates (OCR) and ATP production (Klinge, 2020; Wang *et al.*, 2001). Additionally, E2 regulates mitochondrial dynamics as it induces mitochondrial fusion and increases the expression of OXPHOS complex proteins (Klinge, 2017). Furthermore, E2 can elevate AMPK levels, which is upstream of PGC-1 α and affects energy metabolism and mitochondrial biogenesis (Halling and Pilegaard, 2020; Yang and Wang, 2015).

1.4.8 Estradiol and neuroinflammation

Estradiol plays a significant role in modulating neuroinflammation, a critical component of various neurodegenerative disorders (Zhang *et al.*, 2023). Being a steroid hormone, estradiol can cross the blood-brain barrier, influencing neuronal activity and exerting anti-inflammatory effects. These properties of E2 are increasingly recognized, with research suggesting it can decrease neuroinflammation and protect against neurodegeneration (Giraud *et al.*, 2010). Estradiol can downregulate inflammatory gene expression, modulate microglial activity,

reduce cytokine levels, and decrease oxidative stress in the brain (Saeed *et al.*, 2021; Thakkar *et al.*, 2018; Wang *et al.*, 2024a). Estradiol can mitigate lipopolysaccharide (LPS)-induced neuroinflammation in animal models by lowering brain levels of pro-inflammatory cytokines such as IL-1 β and IL-6 (Yilmaz *et al.*, 2019). Additionally, E2 can regulate and suppress the NF- κ B-mediated inflammatory pathway, which is pivotal in neuroinflammatory responses (Murphy *et al.*, 2010). Moreover, estradiol can suppress the pro-inflammatory M1 phenotype of microglia while promoting the anti-inflammatory M2 phenotype (Thakkar *et al.*, 2018), further highlighting its neuroprotective potential in mitigating neuroinflammatory processes.

1.4.9 Selective Estrogen Receptor Modulators

Selective Estrogen Receptor Modulators (SERMs) also known as synthetic estrogen receptor agonists/antagonists (ERAAAs), are a class of compounds that act as ER agonists or antagonists in a tissue-specific manner (Komm and Mirkin, 2014). While estradiol provides significant benefits in maintaining bone density, cardiovascular health, and cognitive function, its use is often limited by adverse effects such as an increased risk of breast cancer, endometrial hyperplasia, and thromboembolism (Cano and Hermenegildo, 2000; Martino *et al.*, 2004). SERMs were developed to provide targeted estrogenic effects without these broad systemic impacts of traditional estrogen therapy. SERMs differ from natural estrogens by selectively activating or inhibiting estrogen receptors in specific tissues, allowing for beneficial effects while minimizing risks such as breast cancer. The need for SERMs arises from the desire to capture estrogen's protective benefits while avoiding its adverse effects, with future potential in therapies that selectively activate specific estrogen receptor subtypes for targeted treatments. Some examples of successful SERMs include raloxifene and lasofoxifene, both of which

reduce breast cancer risk while promoting bone health in postmenopausal women (McClung *et al.*, 2006; Peterson *et al.*, 2011).

1.5 Pathways regulating energy metabolism and inflammation

Energy metabolism and inflammation are intricately linked processes that play critical roles in maintaining physiological homeostasis while responding to pathological conditions. Inflammation, a natural defense mechanism of the body, can regulate energy metabolism by influencing fuel mobilization and expenditure. For example, pro-inflammatory cytokines can induce lipolysis in adipose tissue, releasing fatty acids into the bloodstream for energy production (Mallick *et al.*, 2024). Conversely, chronic inflammation can disrupt energy balance (Le Thuc *et al.*, 2017), contributing to multiple disorders involving metabolism and mitochondrial function.

This thesis has focused mechanistically on pathways involved in energy metabolism and inflammation, with a particular emphasis on AMPK and NF- κ B pathways. AMPK is a key regulator of energy homeostasis, capable of inhibiting NF- κ B-mediated inflammatory responses (Salminen *et al.*, 2011). The NF- κ B pathway, which is central to immune responses, can also influence energy metabolism by modulating the expression of genes involved in inflammation and metabolism (Capece *et al.*, 2022). The following sections will delve into the detailed mechanisms of these pathways, exploring how they interact to regulate energy metabolism and inflammation, and their implications for neurodegenerative diseases.

1.5.1 AMPK pathway

AMPK is a crucial cellular energy sensor that plays a pivotal role in maintaining energy homeostasis (Hardie *et al.*, 2012). Structurally, AMPK is a heterotrimeric complex consisting of a catalytic α subunit and regulatory β and γ subunits, with multiple isoforms for each subunit, allowing for diverse functions under different physiological conditions (Yan *et al.*, 2018). The detailed structure of AMPK is explained in the next section. AMPK is activated in response to low ATP levels inside a cell, which can occur due to various stresses such as low glucose levels (Garcia and Shaw, 2017). This activation is primarily achieved through the binding of AMP or ADP to the γ subunit, making AMPK an attractive substrate for phosphorylation by upstream kinases including liver kinase B1 (LKB1) or calcium/calmodulin-dependent protein kinase kinase β (CaMKK β). Both kinases phosphorylate AMPK through distinct mechanisms. LKB1 primarily regulates AMPK in an energy-dependent manner (Woods *et al.*, 2003). When cellular energy levels decline, resulting in a high AMP:ATP ratio (reflects a decrease in energy supply), AMP or ADP binds to the γ -subunit of AMPK, inducing a conformational change. This allows LKB1 to phosphorylate AMPK at Thr172 on its α -subunit (Kottakis and Bardeesy, 2012; Shaw *et al.*, 2004). In contrast, CaMKK β activates AMPK in response to increased intracellular calcium levels, independent of the cell's energy status. Elevated calcium binds to calmodulin leading to the formation of a calcium-calmodulin complex which then activates CaMKK β . Activated CaMKK β phosphorylates AMPK at Thr172 (Hawley *et al.*, 2005). Once activated, AMPK regulates energy metabolism by promoting ATP-producing pathways such as fatty acid oxidation and glycolysis, while inhibiting ATP-consuming processes that include gluconeogenesis and lipid synthesis (Ke *et al.*, 2018). A list of catabolic and anabolic processes regulated by AMPK are mentioned in Figure. 1.7. AMPK also influences mitochondrial

biogenesis and function through its downstream targets, including PGC-1 α , which is crucial for mitochondrial gene expression and biogenesis (Canto and Auwerx, 2009; Herzig and Shaw, 2018). Additionally, AMPK can modulate inflammatory responses by regulating transcription factors such as ATF3, which plays a role in stress responses and inflammation (Ku and Cheng, 2020). Through its broad influence on cellular processes such as metabolism and mitochondrial function, AMPK serves as a key therapeutic target for multiple disorders including metabolic diseases and neurodegenerative conditions (Hardie, 2008; Mihaylova and Shaw, 2011). Further details on AMPK's interaction with PGC-1 α and ATF3 will be discussed in subsequent sections.

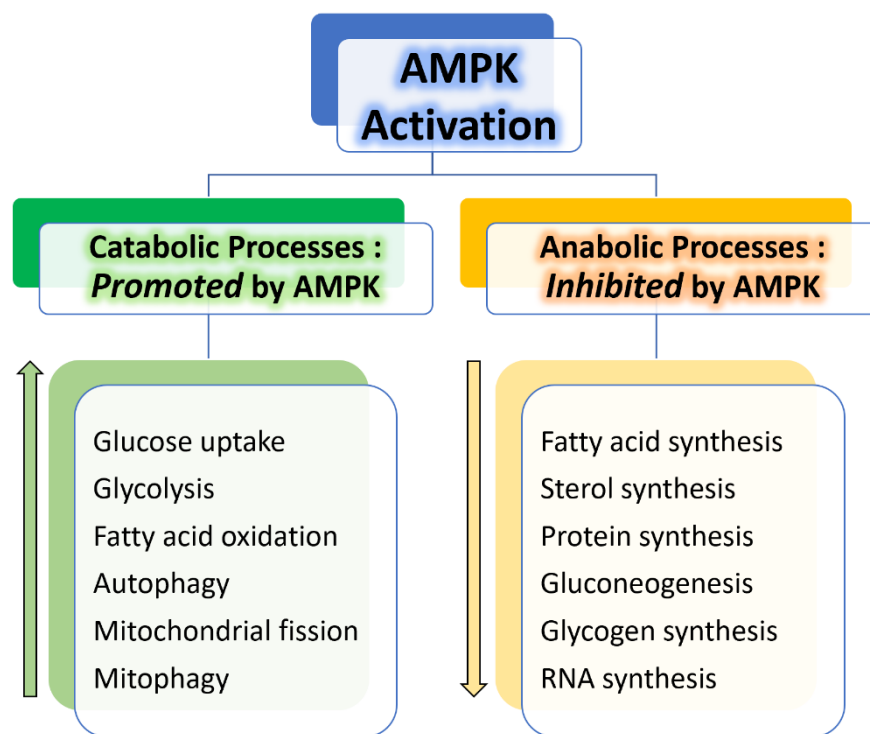


Figure 1.7. Summary of catabolic and anabolic processes regulated by AMPK activation. AMPK (AMP-activated protein kinase) serves as a key energy sensor, promoting catabolic pathways to generate ATP while inhibiting anabolic pathways that consume energy. The figure highlights specific processes affected by AMPK activation. This figure is original and prepared by myself.

1.5.1.1 AMPK structure

AMPK is a heterotrimer consisting of a catalytic α subunit ($\alpha 1$ or $\alpha 2$), regulatory β ($\beta 1$ or $\beta 2$) subunit, and γ subunits ($\gamma 1$, $\gamma 2$, or $\gamma 3$) (Ross *et al.*, 2016). The γ subunit contains four cystathione β -synthase (CBS) domains that bind AMP, ADP, and ATP, enabling AMPK to sense changes in the ATP/AMP ratio and adjust its activity accordingly (Yan *et al.*, 2018). Under conditions of low ATP and high AMP, AMP competitively binds to the CBS domain, enhancing AMPK's basal activity and facilitating the phosphorylation of the α subunit at Thr172 by energy-sensing upstream kinases like LKB1 (Hawley *et al.*, 2005; Woods *et al.*, 2003). AMPK can also be phosphorylated at Thr172 and activated by CaMKK β when intracellular calcium levels rise, independent of AMP levels. This phosphorylation significantly increases AMPK activity, triggering a cascade of downstream effects that promote energy-restoring catabolic pathways such as glucose uptake, glycolysis, fatty acid oxidation, autophagy, mitochondrial fission, and mitophagy, while suppressing energy-consuming anabolic processes including fatty acid synthesis, sterol synthesis, protein synthesis, gluconeogenesis, glycogen synthesis, and RNA synthesis (Grahame Hardie, 2014; Hardie, 2011).

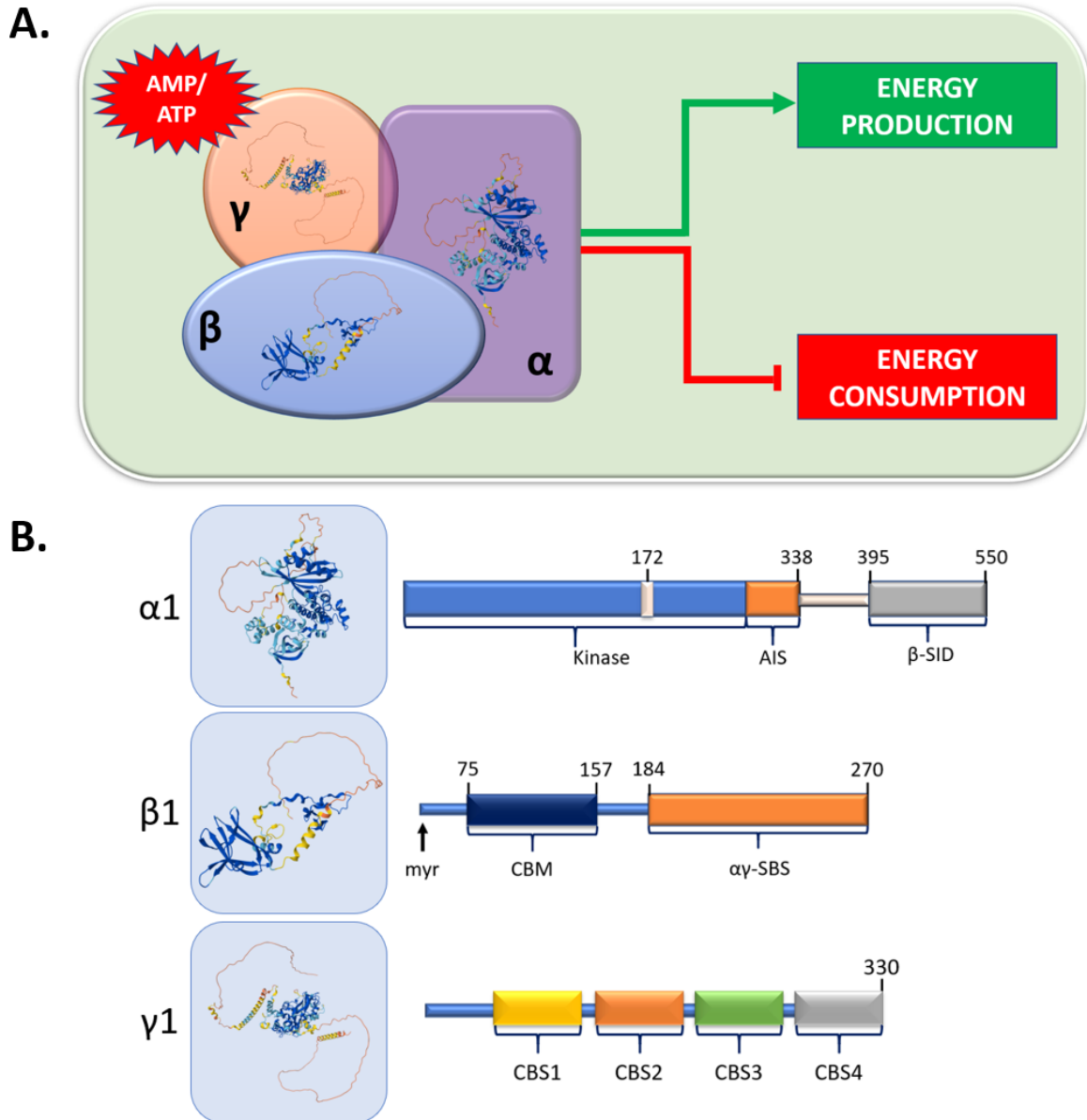


Figure 1.8. AMPK Subunits and domain structure. (A) AMPK Subunits and Function: The AMP-activated protein kinase (AMPK) is a heterotrimeric complex composed of three subunits: α (purple), β (blue), and γ (orange). AMPK acts as a cellular energy sensor, responding to changes in AMP/ATP levels. When activated, it promotes energy production and inhibits energy consumption, thereby maintaining cellular energy homeostasis. **(B)** AMPK Domains and Structure: Domain organization of AMPK subunits, with residue numbering corresponding to human $\alpha 1$, $\beta 1$, and $\gamma 1$ isoforms. The α subunit contains an N-terminal kinase domain, an autoinhibitory sequence (AIS), and a β -subunit interacting domain (β -SID). The β

subunit is N-terminally myristoylated (myr) and includes a carbohydrate-binding module (CBM) and an $\alpha\gamma$ subunit-binding sequence ($\alpha\gamma$ -SBS). The γ subunit features four cystathionine β -synthase (CBS) domains, which pair (CBS1+CBS2 and CBS3+CBS4) to form two Bateman modules, crucial for AMP/ATP binding and regulation. The ribbon diagrams for the α , β , and γ subunits were obtained from the open source AlphaFold Protein Structure Database. Figure (B) is reproduced with modifications and permission from the Elsevier article ‘AMPK functions as an adenylate charge-regulated protein kinase’ (Oakhill *et al.*, 2012). This figure is prepared by myself.

1.5.1.2 AMPK and PGC-1 α

PGC-1 α is recognized as the master regulator of mitochondrial biogenesis (Abu Shelbayeh *et al.*, 2023), enhancing mitochondrial biogenesis and function. There is a significant overlap in the genes transcriptionally regulated by AMPK and those by PGC-1 α , indicating that PGC-1 α is a key mediator of AMPK-induced gene expression (Canto and Auwerx, 2009). Notably, AMPK acts upstream of PGC-1 α , directly phosphorylating it at specific serine (Ser538) and threonine (Thr177) residues. This modification is essential for the role of PGC-1 α in mitochondrial function, enhancing its transcriptional activity and promoting the expression of genes involved in mitochondrial biogenesis, including nuclear respiratory factors 1 and 2 (NRF1 and NRF2), and mitochondrial transcription factor A (TFAM) (Clark and Parikh, 2021; Jäger *et al.*, 2007). Additionally, AMPK-mediated phosphorylation primes PGC-1 α for further activation by Sirtuin1 (SIRT1) through deacetylation, promoting mitochondrial function and energy balance (Canto and Auwerx, 2009). When AMPK is activated in response to low energy states, it enhances PGC-1 α activity, leading to increased mitochondrial biogenesis, oxidative capacity, and mitochondrial antioxidant defenses (Fernandez-Marcos and Auwerx, 2011; Valle *et al.*, 2005), thereby helping to restore cellular homeostasis. PGC-1 α also regulates its own

expression through an autoregulatory feedback loop, further amplifying its effects on mitochondrial function (Handschin *et al.*, 2003). Disruption of this axis, as seen in metabolic disorders, impairs mitochondrial function and contributes to diseases such as neuropathy and neurodegenerative disorders (Panes *et al.*, 2022). Impaired AMPK activity decreases PGC-1 α expression and mitochondrial efficiency (Abu Shelbayeh *et al.*, 2023; Kong *et al.*, 2022), exacerbating metabolic imbalances and disease progression. Understanding this interplay between AMPK and PGC-1 α is crucial for developing therapies to restore energy balance and mitigate metabolic and neurodegenerative diseases.

1.5.1.3 AMPK and ATF3

In addition to mitochondria, several other factors also contribute to nerve regeneration. One key factor is activating transcription factor 3 (ATF3), which is closely linked to AMPK signaling and plays a significant role in promoting nerve regeneration, particularly in response to injury. Genetically, ATF3 is a conserved pro-regenerative transcription factor belonging to the regeneration-associated gene (RAG) cluster (Katz *et al.*, 2022). It is rapidly induced following nerve injury, making it a crucial component of the cellular stress response (Hai *et al.*, 1999; Linda *et al.*, 2011). Notably, ATF3 enhances the intrinsic growth capacity of injured neurons, facilitating axonal sprouting and nerve regeneration (Seijffers *et al.*, 2007). Studies in animal models, such as mice and zebrafish, have demonstrated that ATF3 is essential for axon regrowth - its deletion or knockdown impairs regeneration, while its induction promotes axon sprouting and regrowth (Gey *et al.*, 2016; Wang *et al.*, 2017). Interestingly, AMPK activation regulates ATF3 expression. Research in macrophages shows that AMPK knockdown reduces ATF3 induction, suggesting a direct regulatory link (Kim *et al.*, 2014; Siwakoti *et al.*, 2023).

This AMPK-ATF3 axis is particularly relevant in neuropathic conditions, where enhancing ATF3 expression could improve nerve regeneration. For example, reduced facial nerve regeneration and decreased neurite outgrowth were observed in ATF3 mutant mice, reinforcing ATF3 as a potential therapeutic target for neuropathy and neurodegenerative disorders (Gey *et al.*, 2016; Seijffers *et al.*, 2007).

1.5.1.4 AMPK, PGC-1 α , ATF3 – regulation by estradiol

Estradiol plays a significant role in regulating key metabolic and regenerative pathways (Islamov *et al.*, 2002; Prakapenka and Korol, 2021). Notably, estradiol modulates AMPK activity (Martínez de Morentin *et al.*, 2014). Estradiol can activate AMPK in various cell types, including adipocytes and human endothelial cells, promoting glucose metabolism (Kim *et al.*, 2012). This activation is mediated through ERs, which can interact with or regulate AMPK to have downstream effects on glucose and lipid metabolism (Kim *et al.*, 2012). Furthermore, estradiol modulates hindbrain AMPK, affecting glucose balance, norepinephrine signaling, and hypothalamic metabolic activity (Alenazi *et al.*, 2016; Mahmood *et al.*, 2020). In addition to its effects on AMPK, estradiol influences PGC-1 α , a master regulator of mitochondrial biogenesis. Estradiol enhances PGC-1 α expression through AMPK activation (Jäger *et al.*, 2007; Park *et al.*, 2017), leading to increased mitochondrial function. In addition to its effects on AMPK and PGC-1 α , estradiol also regulates ATF3, a transcription factor involved in stress responses and neuronal regeneration (Hai *et al.*, 1999; Seijffers *et al.*, 2007). While studies indicate that estradiol can upregulate ATF3 expression in human fibroblasts (Liu *et al.*, 2007), direct evidence of this regulation in neurons remains limited. However, since ATF3 promotes nerve regeneration (Linda *et al.*, 2011; Seijffers *et al.*, 2007) and estradiol can upregulate ATF3

in vivo (Liu *et al.*, 2006), it is plausible but somewhat speculative that estradiol could have a similar effect in neurons. Further studies are needed to determine whether estradiol directly affects ATF3 expression in neurons and contributes to neuronal repair and recovery.

Given the focus of Chapter 2 on DRG neurons, estradiol's ability to enhance mitochondrial function through the AMPK and PGC-1 α pathways, as well as its potential to promote neuronal regeneration (Brinton *et al.*, 1997; Gowayed *et al.*, 2024; Pooley *et al.*, 2015; Tian *et al.*, 2023), suggests it may play a beneficial role in improving mitochondrial health and facilitating nerve repair in DRG neurons. This is particularly relevant in neuropathic conditions where impaired mitochondrial function and reduced regenerative capacity contribute to disease progression.

1.5.2 NF- κ B pathway

The NF- κ B signaling pathway is a pivotal regulator of various bodily functions, including immunity, inflammation, synaptic plasticity and memory formation, and cell survival (Albensi, 2012; Liu *et al.*, 2017). It is typically activated in response to injury, infection, inflammation, and other stressful conditions, serving as a transcription factor that induces the expression of pro-inflammatory genes encoding cytokines and chemokines (Lawrence, 2009; Su *et al.*, 2021). NF- κ B also plays a role in inflammasome regulation (Boaru *et al.*, 2015). While inflammation is essential for host defense, deregulated inflammatory responses can cause excessive or prolonged tissue damage, contributing to chronic inflammatory diseases. The importance of NF- κ B is highlighted by studies in knockout mice lacking NF- κ B components, which often suffer from severe immune deficiencies and heightened susceptibility to infections (Pasparakis *et al.*, 2006). Mice lacking NF- κ B subunits p50 or p65 typically die during embryonic development or shortly after birth, and exhibit defects such as impaired epidermal

keratinocyte differentiation, skeletal abnormalities, and alterations in memory (Beg *et al.*, 1995; Li *et al.*, 1999; Oikawa *et al.*, 2012; Takeda *et al.*, 1999). NF- κ B signaling occurs through two main pathways: the canonical pathway, which activates the NF- κ B p50 and p65 subunits via I κ B α degradation, and the non-canonical pathway, which involves the processing of the p100 subunit into p52 by IKK α (Trares *et al.*, 2022). Dysregulation of NF- κ B is implicated in various conditions, including autoimmune and neurodegenerative diseases such as AD, making it a promising target for therapeutic interventions in inflammatory disorders.

1.5.2.1 NF- κ B structure

The NF- κ B family comprises five subunits: p65 (RelA), RelB, c-Rel, p50, and p52. Each of these subunits shares a Rel homology domain (RHD), which facilitates DNA binding, dimerization, and nuclear localization. Among them, RelA, RelB, and c-Rel contain a transactivation domain (TAD), enabling them to directly activate gene expression. In contrast, p50 and p52, derived from p105 and p100, respectively, lack TADs and primarily act as repressors unless paired with TAD-containing subunits. These subunits form homo- or heterodimers to regulate gene transcription, with p65/p50 heterodimer driving the canonical NF- κ B pathway, crucial for inflammation and immunity, while the p52/RelB heterodimer mediates the non-canonical pathway. The structural arrangement of NF- κ B allows it to bind to specific DNA sequences known as κ B sites, thereby regulating the expression of target genes involved in inflammation and immune responses.

NF- κ B Subunits

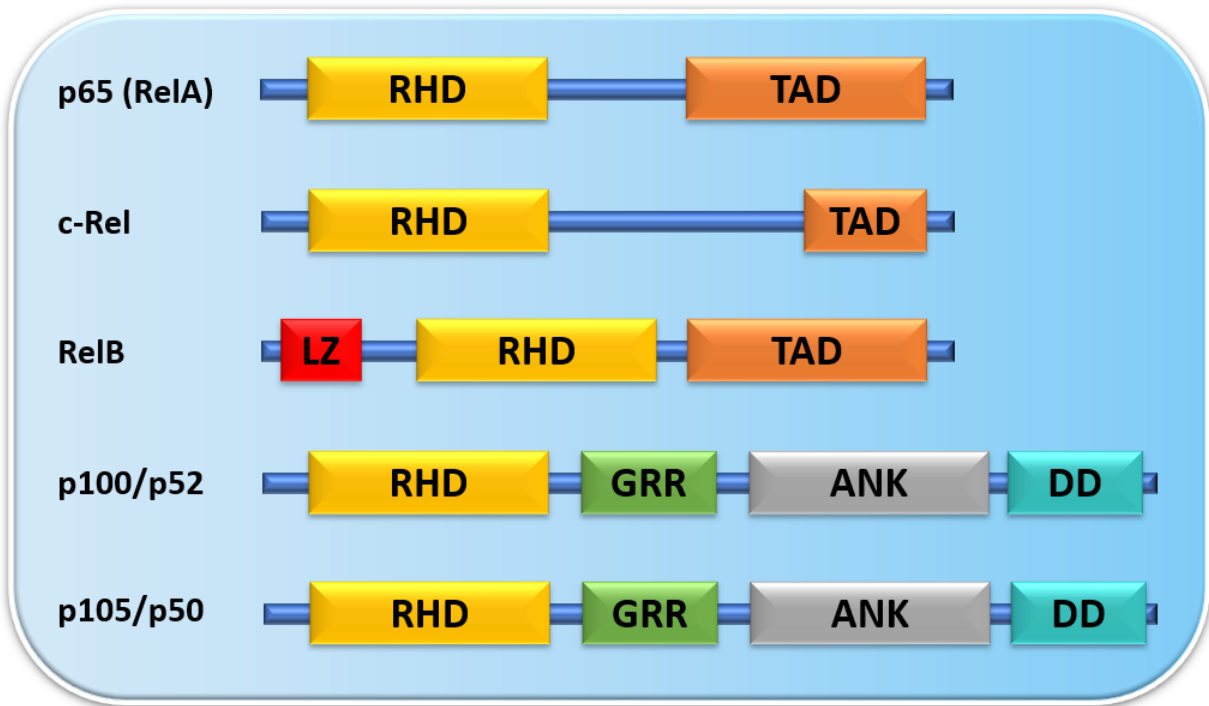


Figure 1.9. NF- κ B family members. All NF- κ B subunits share the Rel Homology Domain (RHD), which is essential for DNA binding, dimerization, and nuclear localization. The diagram illustrates the functional domains of each subunit: TAD (transcription activation domain), LZ (leucine zipper), GRR (glycine-rich region), ANK (ankyrin repeats), and DD (death domain). RelA, RelB, and c-Rel possess a TAD, allowing them to activate gene expression, whereas p50 and p52, generated from p105 and p100, respectively, lack TADs and primarily function as repressors unless dimerized with TAD-containing subunits. This figure is original and prepared by myself.

1.5.2.2 NF- κ B and gene regulation

In its inactive state, NF- κ B consists of homo- or heterodimers bound to the inhibitory protein I κ B α . In neurons, the most common configuration consists of the p50-p65 and I κ B α subunits (Mincheva-Tasheva and Soler, 2013). NF- κ B activation in the canonical pathway involves I κ B kinase (IKK), which phosphorylates and degrades I κ B α , allowing the NF- κ B dimer to translocate to the nucleus and bind to specific DNA sequences, and either induce or suppress gene expression (Snow and Albensi, 2016). Notably, NF- κ B can interact with various transcription factors including AP-1, p53, and CREB to modulate gene expression, influencing immune responses, cell proliferation, and stress responses (Carrà *et al.*, 2020; Fujioka *et al.*, 2004; Snow and Albensi, 2016). NF- κ B regulates multiple genes crucial for inflammation, including pro-inflammatory cytokines and chemokines such as TNF- α , IL-1 β , IL-6, and IL-8, which mediate inflammatory responses (Carrà *et al.*, 2020; Zhou *et al.*, 2001). It also regulates anti-apoptotic genes such as Bcl-2 and Bcl-xL, which protect cells from apoptosis, and pro-apoptotic genes like FasL and Bim, which induce programmed cell death (Catz and Johnson, 2001; Liu *et al.*, 2012). NF- κ B being central to immune responses, also regulates genes involved in antigen presentation (MHC class I & II) and immune cell activation (CD40, CD80, CD86) (Molinero *et al.*, 2004; Srahna *et al.*, 2001). Additionally, NF- κ B modulates enzymes and metabolic genes such as iNOS and COX-2, which are involved in inflammatory responses and prostaglandin synthesis. Furthermore, NF- κ B has also been shown to have many antioxidant targets, with one of the well-known targets being manganese superoxide dismutase (MnSOD), which plays a role in mitigating oxidative stress (Lingappan, 2018; Snow and Albensi, 2016).

Inflammation involves both innate and adaptive immune components (Caamaño and Hunter, 2002). NF- κ B plays a crucial role in regulating innate immune cells such as macrophages and dendritic cells, as well as adaptive immune components, particularly CD4+ T-helper cells (Liu *et al.*, 2017). Deregulated NF- κ B activation can lead to aberrant T-cell activation, contributing to autoimmune and inflammatory responses. Additionally, NF- κ B also regulates inflammasomes, such as NLRP3, which are critical in initiating inflammatory diseases (Caamaño and Hunter, 2002). Its dysregulation is implicated in various conditions, including inflammatory bowel disease (IBD), rheumatoid arthritis, multiple sclerosis, and neurodegenerative diseases like Alzheimer's and Parkinson's (Liu *et al.*, 2017; Park and Hong, 2016; Singh *et al.*, 2024; Sun *et al.*, 2022), where NF- κ B activation is often increased in affected brain regions.

1.5.2.3 NF- κ B and mitochondrial dysfunction

NF- κ B and mitochondrial function are intricately linked, where one can influence the other. Mitochondria produce ROS, which can activate NF- κ B, leading to the transcription of genes involved in inflammation and immune responses (Morgan and Liu, 2011). Conversely, NF- κ B can regulate mitochondrial function by modulating genes involved in mitochondrial biogenesis and metabolism (Cherry and Piantadosi, 2015). Notably, excessive ROS from dysfunctional mitochondria can chronically activate NF- κ B, resulting in sustained inflammation and cell damage, which is often associated with neurodegenerative disorders. Interestingly, NF- κ B subunits have been found within mitochondria, with studies indicating the presence of I κ B α and NF- κ B p65 subunits in the mitochondrial intermembrane space (Albensi, 2019; Bottero *et al.*, 2001; Zamora *et al.*, 2004). Additionally, electron microscopy has revealed the localization

of NF- κ B subunits p50 and p65 within the mitochondrial matrix (Cogswell *et al.*, 2003). This mitochondrial localization suggests that NF- κ B may play a role in regulating mitochondrial-specific gene expression. While the mechanism of NF- κ B translocation into the mitochondria is not fully understood, hypoxia is believed to trigger its entry inside mitochondria via translocase of the outer mitochondrial membrane (TOM40) and the matrix chaperone mortalin (Ivanova and Perkins, 2019). Furthermore, NF- κ B influences mitochondrial dynamics by affecting proteins involved in mitochondrial fission and fusion, such as optic atrophy 1 protein (OPA1), which is crucial for mitochondrial inner membrane fusion and cristae remodeling (Garcia *et al.*, 2018; Olichon *et al.*, 2006). The absence of NF- κ B IKK α has been associated with reduced OPA1 expression and altered mitochondrial morphology, suggesting that NF- κ B may upregulate OPA1 to maintain mitochondrial structure and function (Laforge *et al.*, 2016). However, NF- κ B mediates A β induced mitochondrial dysfunction, affecting the expression of mitochondrial respiratory chain components such as cytochrome c oxidase III (COX III) (Shi *et al.*, 2014). Shi and colleagues in 2014 demonstrated that intramitochondrial NF- κ B signaling plays a role in A β -induced mitochondrial dysfunction. Using HT22 mouse hippocampal neuronal cells, they showed that A β exposure reduced mitochondrial function and COX III activity, and this effect was associated with NF- κ B activation, as inhibiting NF- κ B prevented A β -induced mitochondrial impairment. Notably, NF- κ B DNA binding activity within mitochondria is low under basal conditions but increases upon TNF α stimulation (Cogswell *et al.*, 2003). Moreover, the p65 subunit of NF- κ B has been found to bind to the D-loop region of mtDNA, where it negatively regulates oxidative phosphorylation by repressing the expression of mitochondrial genes coding for COX III and cytochrome b (Johnson *et al.*, 2011; Kracht *et al.*, 2020). The interplay between NF- κ B and mitochondria is critical for maintaining cellular

homeostasis, and understanding this relationship is essential for developing therapeutic strategies to mitigate mitochondrial dysfunction.

1.5.2.4 NF- κ B regulation by estradiol

Estradiol plays a significant role in regulating NF- κ B activity, influencing inflammation, immune responses, and cell survival (Dai *et al.*, 2009). It can modulate NF- κ B through multiple mechanisms, both directly and indirectly. Directly, estradiol can interfere with NF- κ B binding to DNA, while indirectly, it can promote the expression of inhibitory proteins like I κ B α , which keeps NF- κ B inactive in the cytoplasm (Evans *et al.*, 2001; Xing *et al.*, 2012). For instance, estradiol suppresses NF- κ B activation by regulating microRNAs, enhancing I κ B α levels to prevent NF- κ B nuclear translocation, and blocking p65 binding to inflammatory gene promoters (Murphy *et al.*, 2010; Xing *et al.*, 2012). This anti-inflammatory effect is beneficial in autoimmune and neuroinflammatory disorders, including AD, where estradiol can help suppress excessive inflammation (Mishra *et al.*, 2023). Estradiol's influence on mitochondria also contributes to its anti-inflammatory effects. By maintaining mitochondrial function and reducing ROS production (Razmara *et al.*, 2007), estradiol can suppress excessive NF- κ B-driven inflammation. Additionally, the decline in estradiol levels after menopause can lead to increased NF- κ B activity, contributing to chronic inflammation observed in neurodegenerative diseases, which may explain why women are at higher risk for AD post-menopause (Paganini-Hill and Henderson, 1994).

1.5.3 Cross talk between NF- κ B pathway and AMPK pathway

The NF- κ B and AMPK pathways exhibit significant cross-talk, reciprocally influencing activities in regulating inflammation and energy metabolism. AMPK can inhibit NF- κ B signaling indirectly through its downstream targets, such as SIRT1, PGC-1 α , and Forkhead box O (FoxO), which collectively repress inflammatory responses mediated by NF- κ B (Salminen *et al.*, 2011). Furthermore, activation of AMPK signaling downregulates NF- κ B function, by suppressing pro-inflammatory responses via upregulation of CREB activity (Cui *et al.*, 2023). This cross-talk is beneficial in reducing chronic inflammation associated with metabolic disorders and aging. Conversely, NF- κ B can indirectly modulate AMPK activity by activating inflammation, which in turn inhibits AMPK. However, direct interactions between these molecules are less defined. Importantly, the interplay between these pathways is crucial for maintaining cellular homeostasis, by balancing energy metabolism and inflammatory responses. Understanding this cross-talk is essential for developing therapeutic strategies to mitigate inflammatory and metabolic disorders.

RATIONALE, HYPOTHESES, AND AIMS

Rationale

Neurodegenerative diseases affecting both the peripheral and central nervous systems are characterized by progressive neuronal loss, chronic inflammation, and mitochondrial dysfunction, leading to profound neurological deficits (Klemmensen *et al.*, 2024; Wilson *et al.*, 2023). Estradiol (E2), the primary form of estrogen, exhibits neuroprotective, anti-inflammatory, and mitochondrial-enhancing properties in various cellular contexts, including neurons (Garcia-Segura *et al.*, 2001; Klinge, 2020; Son *et al.*, 2020). Notably, AMPK, a key regulator of cellular energy homeostasis, plays a significant role in modulating energy metabolism and inflammation in neurodegenerative diseases (Domise and Vingtdoux, 2016). Despite these insights, the precise mechanisms by which E2 confers neuroprotection, particularly its interaction with AMPK and other pathways, remain incompletely understood. Given the wide expression of estrogen receptors, ER α and ER β , across both nervous systems (Papka and Mowa, 2003; Sato *et al.*, 2023), this project aimed to investigate the neuroprotective mechanisms of E2 using well-established neuronal models: dorsal root ganglion (DRG) sensory neurons for the PNS (Project 1) and primary cortical neurons for the CNS (Project 2).

Rationale for Project 1: Work with DRG Neurons (PNS)

DRG neurons, located along the dorsal aspect of the spinal cord, relay sensory signals from the periphery to the CNS and are implicated in various peripheral neuropathies (Kajander *et al.*, 1992; Shiers *et al.*, 2024). They express both ER α and ER β (Patrone *et al.*, 1999), making them a suitable model for investigating the neuroprotective and neurotrophic effects of E2. Previous studies have shown that estrogen supports DRG neuronal survival and regeneration (Chakrabarty *et al.*, 2008; Patrone *et al.*, 1999), but the molecular pathways, especially those involving energy metabolism

and neurite outgrowth, remain poorly defined. As DRG neurons are susceptible to metabolic stress and degeneration, exploring E2's action on mitochondrial function and axonal sprouting is critical. A major energy-sensing pathway in DRG neurons includes AMPK (Kong *et al.*, 2020), which regulates ATF3 and PGC-1 α proteins involved in neuronal regeneration and mitochondrial biogenesis, respectively (Clark and Parikh, 2021; Katz *et al.*, 2022). Hence, we aimed to explore the mechanism by which E2 acts on DRG neurons to regulate energy metabolism and axonal sprouting.

Hypothesis: E2 treatment, resulting in ER activation increases energy metabolism and mitochondrial function in DRG neurons, leading to enhanced axonal sprouting.

Aims:

1. Determine the effects of E2 on AMPK activity and PGC-1 α levels.
2. Assess the effects of E2 on mitochondrial respiration and ATP production.
3. Investigate the effects of E2 on the transcription factor ATF3 and assess neurite outgrowth as a measure of axonal sprouting.
4. Examine the mechanism by which E2 exerts its effects on DRG neurons.

Rationale for Project 2: Work with Cortical Neurons (CNS)

Alzheimer's disease (AD) is the most common form of dementia, with increasing age being the greatest known risk factor (Hou *et al.*, 2019). The loss of estrogen, particularly in post-menopausal women, contributes to AD development (Paganini-Hill and Henderson, 1994). Estradiol (E2) has major neuroprotective effects across the CNS (Garcia-Segura *et al.*, 2001), and its declining levels can trigger inflammation and mitochondrial dysfunction, contributing to memory loss and cognitive decline (Long *et al.*, 2012; Zhang *et al.*, 2024). E2 also mediates A β clearance (Li *et al.*, 2000), a major pathological hallmark of AD. A β can trigger inflammation that contributes to neurodegeneration through the activation of the pro-inflammatory NF- κ B pathway (Granic *et al.*,

2009; Kinney *et al.*, 2018). Additionally, A β and inflammation can lead to deficits in brain energy metabolism, resulting in mitochondrial dysfunction and worsening AD symptoms (Wang *et al.*, 2020b). ERs are widely present in the brain, and E2 exhibits neuroprotection through anti-inflammatory properties by inhibiting NF- κ B activity and mitochondrial mechanisms (Simpkins and Dykens, 2008; Xing *et al.*, 2012). Hence, we aimed to investigate the neuroprotective potential of E2 in preventing mitochondrial dysfunction and inflammatory responses that contribute to the progression of AD.

Hypothesis: Estradiol treatment decreases A β -driven, NF- κ B pro-inflammatory signaling, thereby enhancing mitochondrial function and increasing neuronal survival.

Aims:

1. Assess the effects of E2 and A β on NF- κ B-mediated inflammatory signaling and mitochondrial function.
2. Investigate the neuroprotective effects of E2 in mitigating A β -induced inflammation, mitochondrial dysfunction, and neuronal cell death.
3. Determine the role of AMPK signaling in mediating the protective effects of E2 against A β neurotoxicity.

Transition statement one

Following the rationale for investigating the neuroprotective effects of estradiol across both the peripheral and central nervous systems, I first focused on exploring its role in enhancing neurite outgrowth in DRG sensory neurons. Next chapter (Chapter 2) addresses the PNS component of my research and fulfills the aims outlined in Project 1. My manuscript, titled "*Estradiol activates the CaMKK β /AMPK pathway to enhance neurite outgrowth in cultured adult sensory neurons*" provides a detailed account of this investigation. I used DRG neurons obtained from SD rats and employed an axotomy model to simulate injury, mimicking conditions found in peripheral neuropathies. Treatment with E2 allowed me to assess its impact on mitochondrial function and axonal regeneration, evaluating its potential utility in treating PNS diseases. Additionally, I utilized pharmacological inhibitors to elucidate the mechanisms underlying E2's effects, particularly its interaction with the AMPK pathway. This study provides critical insights into how E2 promotes neuronal recovery and regeneration in the PNS, setting the stage for understanding its broader neuroprotective roles across different neuronal populations.

CHAPTER 2

Estradiol activates the CaMKK β /AMPK pathway to enhance neurite outgrowth in cultured adult sensory neurons

Pranav Mishra ^{a, b}, Benedict C. Albensi ^{a, b, c, 1} and Paul Fernyhough ^{a, b, *}

¹Division of Neurodegenerative Disorders, St. Boniface Hospital Albrechtsen Research Centre, University of Manitoba, Winnipeg, Canada. ²Department of Pharmacology and Therapeutics, Max Rady College of Medicine, Rady Faculty of Health Sciences, University of Manitoba, Winnipeg, Canada. ³Department of Pharmaceutical Sciences, Barry & Judy Silverman College of Pharmacy, Nova Southeastern University, Fort Lauderdale, FL, USA.

¹Co-senior author

*Corresponding author:

Dr. Paul Fernyhough, BSc, PhD

Professor, Department of Pharmacology & Therapeutics,
Rady Faculty of Health Sciences, University of Manitoba.

Director, Division of Neurodegenerative Disorders,
St. Boniface Hospital Albrechtsen Research Centre,
Winnipeg, Manitoba, Canada.

E-mail: pfernyhough@sbrc.ca

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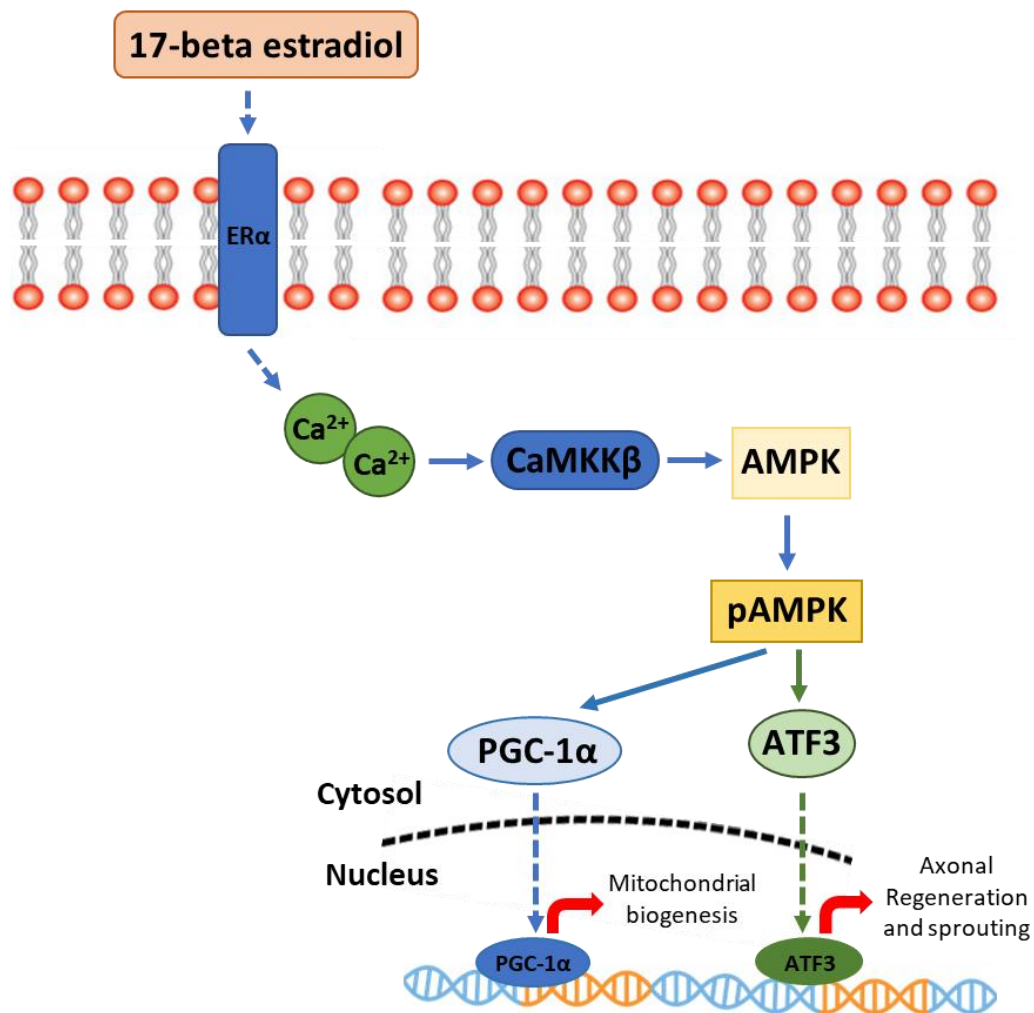
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2.1 Abstract

Adult rat dorsal root ganglion (DRG) sensory neurons express estrogen receptors (ERs) α and β . Estrogen regulates multiple aspects of the nervous system including development, survival, and axonal outgrowth of DRG neurons. While previous studies have established estrogen's neuroprotective role in these neurons, the specific ER subtypes and downstream signaling pathways mediating these effects remain poorly defined. The objective of our study was to investigate the effects of 17 beta-estradiol (E2) on mitochondrial function and axonal regeneration of cultured DRG neurons and explore the pathways by which E2 acts. We observed that E2 treatment upregulated the levels of phosphorylated AMP-activated protein kinase (AMPK). E2 also increased the levels of peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) and activating transcription factor 3 (ATF3), which are proteins involved in mitochondrial biogenesis and axonal regeneration. The Seahorse assay showed that E2 elevated basal respiration in cultured DRG neurons. Additionally, E2 treatment for 24h significantly increased total neurite outgrowth of DRG neurons. Pharmacological inhibition of AMPK using Compound C inhibited E2-mediated increases in ATF3 expression and neurite outgrowth. The Ca^{2+} /calmodulin-dependent protein kinase kinase β (CaMKK β) inhibitor STO-609 blocked E2-mediated AMPK activation. Furthermore, we assessed whether these effects were mediated by ER α or ER β by using the ER α selective agonist propyl pyrazole triol (PPT) and ER β selective agonist diarylpropionitrile (DPN). PPT upregulated phosphorylated AMPK levels and increased total neurite outgrowth, whereas DPN was ineffective. The results demonstrate that E2 acts through ER α to promote neurite outgrowth via a pathway involving activation of CaMKK β /AMPK in adult DRG neurons. Our findings identify ER α -mediated AMPK activation as a therapeutic target for enhancing neuronal regeneration and mitochondrial function in neurodegenerative disorders.

Graphical Abstract



Proposed mechanism for E2-mediated AMPK activation and downstream effects in adult rat DRG neurons. Estradiol (E2) acts via ERα to increase intracellular calcium, leading to CaMKKβ activation. This results in phosphorylation and activation of AMPK (pAMPK). Activated AMPK (pAMPK) then enhances expression of PGC-1α and ATF3, promoting mitochondrial biogenesis and axonal regeneration, respectively.

Highlights

- Estradiol (E2) increases AMPK activation, PGC-1 α and ATF3 expression in DRG neurons
- E2 activates CaMKK β /AMPK pathway to promote neurite outgrowth in adult DRG neurons
- ER α mediates E2-induced enhancement of mitochondrial function and neurite outgrowth
- Findings suggest potential for ER α -targeted therapies in peripheral nerve disease

2.2 Introduction

Estrogen, a steroid hormone renowned for its critical functions in the reproductive system, also plays an essential role within the nervous system, and contributes to neural health. It has three major forms: estrone, estradiol, and estriol. Of the three, estradiol, or 17 beta-estradiol (E2) is the most potent (Wise *et al.*, 2009) and predominant form (E2 is also referred to as estradiol or estrogen from this point onwards). The biological effects of E2 are mediated through two pathways: genomic and non-genomic. In the genomic pathway, E2 acts primarily via two nuclear estrogen receptors (ERs), ER α and ER β , which function as ligand-activated transcription factors to regulate gene expression. This pathway involves direct binding of E2-ER complexes to estrogen response elements (EREs) in DNA, leading to modulation of target gene transcription (Heldring *et al.*, 2007). In contrast, the non-genomic pathway involves rapid signaling events initiated by membrane-associated ERs, which include a subpopulation of the classical ERs, ER α and ER β (Björnström and Sjöberg, 2005; Norfleet *et al.*, 1999), as well as the G protein-coupled estrogen receptor (GPER) (Mishra *et al.*, 2023; Tirado-Garibay *et al.*, 2023). These receptors activate various intracellular signaling cascades, such as mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, and calcium signaling pathways, which can rapidly modulate cellular functions without directly altering gene expression; although they can ultimately lead to changes in gene transcription through the activation of other transcription factors. ER α and ER β are expressed in dorsal root ganglion (DRG) sensory neurons during early postnatal development and in culture (Patrone *et al.*, 1999), suggesting that they are a target of E2. Studies have demonstrated a role of E2 in promoting survival (Patrone *et al.*, 1999) and regeneration (Chakrabarty *et al.*, 2008) of DRG neurons. Moreover, it can regulate the expression of

neurotrophin receptors in DRG neurons suggesting a role in modulating neuronal sensitivity to neurotrophins like nerve growth factor (NGF) (Sohrabji *et al.*, 1994; Toran-Allerand, 1996).

E2 also upregulates the expression of proteins critical for mitochondrial biogenesis in rat skeletal muscles, namely peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α), a transcriptional coactivator also termed the master regulator of mitochondrial biogenesis (Capllonch-Amer *et al.*, 2014). AMP-activated protein kinase (AMPK), a master regulator of energy metabolism can interact with PGC-1 α to regulate mitochondrial function (Jäger *et al.*, 2007). Also, AMPK's activation is regulated by one of its three upstream kinases; liver kinase B1 (LKB1) (Woods *et al.*, 2003), the transforming growth factor- β activated protein kinase-1 (TAK1) (Momcilovic *et al.*, 2006), or the Ca²⁺/calmodulin-dependent protein kinase kinase β (CaMKK β) (Hawley *et al.*, 2005). CaMKK β activates AMPK in response to an increase in intracellular Ca²⁺ levels, which then modulates mitochondrial function and biogenesis. The axonal outgrowth of DRG neurons, requires high amounts of ATP that is produced by the mitochondria (Bernstein and Bamberg, 2003). Additionally, the neuronal expression of activating transcription factor 3 (ATF3) is crucial for nerve regeneration (Gey *et al.*, 2016) and gets rapidly upregulated following neuronal injury or stress (Hunt *et al.*, 2012). ATF3 promotes neuronal regeneration through modulation of gene expression, leading to the activation of regeneration-associated genes (RAGs) (Gey *et al.*, 2016). E2 can activate AMPK (Yang and Wang, 2015) and promote PGC-1 α activity, and can also influence the expression of ATF3 (Liu *et al.*, 2007). These interactions indicate that E2 plays an important role in maintaining cellular energy homeostasis and mediating the cellular response to injury or stress.

In the present study, we aimed to elucidate the mechanism by which E2 promotes axonal sprouting and/or regeneration in adult DRG neurons. We hypothesized that E2 acts through

specific ERs to activate the AMPK pathway, leading to increased mitochondrial function and axonal regeneration. To test this hypothesis, we utilized cultured DRG neurons from adult Sprague-Dawley (SD) rats and employed a combination of pharmacological interventions and specific ER agonists. We investigated the effects of E2 on AMPK phosphorylation, PGC-1 α and ATF3 levels, mitochondrial function, and neurite outgrowth. Furthermore, we explored the potential involvement of CaMKK β in E2-mediated AMPK activation and the contributions of ER α and ER β in mediating these effects. This comprehensive approach allowed us to dissect the signaling pathways underlying E2's neurotrophic effects in promoting neuronal regeneration of adult rat DRG neurons.

2.3 Materials and Methods

2.3.1 Adult DRG neuron culture

DRG neurons from 2-month-old adult male and female SD rats (weight 200-250 grams) were dissected, isolated and dissociated using methods previously described (Calcutt *et al.*, 2017). All animal procedures followed the guidelines of University of Manitoba's Animal Care Committee under the rules of Canadian Committee on Animal Care (CCAC). DRG neurons were seeded onto plates coated with 0.5mg/ml poly-d-l-ornithine hydrobromide followed by 2µg/ml laminin at a density of 20,000 cells/well (5,952 cells/cm²) for 12-well plates or 10,000 cells/well (5,482 cells/cm²) for 24-well plates. Neurons were cultured for 24 hours in Hams F12 media containing 10 mM D-glucose (318015CL, Wisent Inc, Saint-Jean-Baptiste, Quebec, Canada) supplemented with 1 µM L-glutamine (SH300340, Cytiva, Marlborough, Massachusetts, USA), 1X antibiotic antimycotic solution (15240062, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and modified Bottenstein's N2 additives (30 nM sodium selenite, 0.1 mg/ml transferrin, 100 mM putrescine, 20 nM progesterone, 0.1 mg/ml BSA; all additives were from MilliporeSigma Canada Ltd, Oakville, Ontario, Canada). No neurotrophins or insulin was added to any of the DRG neuron cultures. Treatments were added at specific time points to maintain a consistent total culture period of 24 hours for all experiments. For 24-hour treatments, agents were added at the time of seeding. For shorter 1-hour treatments, agents were added 23 hours after seeding. We used the following drugs and pharmacological inhibitors: 10 nM 17 beta-estradiol (E2) (E2758, MilliporeSigma Canada Ltd, Oakville, Ontario, Canada), 3 µM Compound C, to inhibit AMPK (171261, MilliporeSigma Canada Ltd, Oakville, Ontario, Canada), 1 µM STO-609, to inhibit CAMKKβ (1551, Bio-Techne Canada, Toronto, ON, Canada), 10 nM ERα selective agonist propyl pyrazole triol (PPT) (1426, Bio-Techne Canada, Toronto, ON, Canada), and 10 nM ERβ selective agonist

diarylpropionitrile (DPN) (1494, Bio-Techne Canada, Toronto, ON, Canada). It should be noted that while the DRG cultures were cultured under defined conditions leading to removal of vast majority of Schwann cells and fibroblasts, there are remaining satellite cells that are found closely associated with neurons. The cytoplasmic volume of these non-neuronal cells is insignificant compared with neuronal cytoplasmic volume within the culture.

2.3.2 Quantitative Western blotting

Cell lysates were harvested 24 hours after seeding the DRG neuronal cultures and homogenized in ice-cold RIPA buffer containing 25 mM Tris pH 8, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, and protease and phosphatase inhibitor cocktail (78440, Thermo Fisher Scientific, Waltham, Massachusetts, USA). This time point was selected based on pilot experiments to ensure sufficient protein expression while maintaining neuronal viability. Next, a protein assay was performed using a detergent compatible (DC) colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA) and, proteins were resolved and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred on to a nitrocellulose membrane (66485, Cytiva, Marlborough, Massachusetts, USA) using Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA) and immunoblotted with specific antibodies against phosphorylated AMPK (pAMPK) Thr172 (1:1000, Cell Signaling, Danvers, Massachusetts, USA), total AMPK (T-AMPK, 1:5000, Abcam, Waltham, Massachusetts, USA), total OxPhos (1:1000, Thermo Fisher Scientific, Waltham, Massachusetts, USA; antibody cocktail containing multiple OxPhos antibodies against complex I to V), total ERK (T-ERK, 1:1000, Santa Cruz Biotechnology Inc. Dallas, Texas, USA), PGC-1 α (1:1000, Thermo Fisher Scientific, Waltham, Massachusetts, USA), ATF3 (1:500, Santa Cruz Biotechnology Inc. Dallas, Texas, USA), ER α (1:1000, Abcam, Waltham, Massachusetts, USA), and ER β (1:1000, Thermo Fisher

Scientific, Waltham, Massachusetts, USA). Total protein was captured using chemiluminescent imaging of the blot following gel activation (TGX Stain-Free FastCast Acrylamide Solutions, Bio-Rad, Hercules, CA, USA) in addition to T-ERK for target protein normalization. Secondary antibodies used were HRP-conjugated goat anti-rabbit IgG (H + L) or goat anti-mouse IgG (H + L) (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA). Blots were incubated in SignalFire ECL Reagent (Cell Signaling, Danvers, Massachusetts, USA) or Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) and imaged using a ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA) (Table 2.1).

Primary Antibody	Dilution	Supplier
Anti-pAMPK Thr172	1:1000	Cell Signaling, Danvers, Massachusetts, USA
Anti-Total AMPK	1:5000	Abcam, Waltham, Massachusetts, USA
Total OxPhos	1:1000	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Anti-Total ERK	1:1000	Santa Cruz Biotechnology Inc., Dallas, Texas, USA
Anti-PGC-1 α	1:1000	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Anti-ATF3	1:500	Santa Cruz Biotechnology Inc., Dallas, Texas, USA
Anti-ER α	1:1000	Abcam, Waltham, Massachusetts, USA
Anti-ER β	1:1000	Thermo Fisher Scientific, Waltham, Massachusetts, USA

Table 2.1: Primary Antibodies used for Western blotting

2.3.3 Measurement of mitochondrial respiration

We used the Seahorse XF24 Analyzer (Agilent Technologies Inc, Santa Clara, California, USA) to measure the levels of basal mitochondrial oxygen consumption rate (OCR), the maximal respiration, and the spare respiratory capacity. DRG neurons were isolated and cultured on Seahorse XF24 plates at a density of 12,000 cells/well (37,500 cells/cm²). Neurons were cultured for a total of 24 hours, with E2 treatments added either 3 hours or 6 hours before running the experiment. The culture media was changed 1 hour prior to the assay to an unbuffered DMEM (Dulbecco's modified Eagle's medium, pH 7.4) media supplemented with 1 mM sodium pyruvate and 10 mM D-glucose. Three drug cocktails containing mitochondrial complex inhibitors; oligomycin (1 μ M), carbonyl cyanide p-trifluoro methoxyphenylhydrazone (FCCP) (1 μ M), and rotenone/antimycin A (1 μ M) were injected sequentially through ports of the Seahorse FluxPak cartridges (Agilent Technologies Inc, Santa Clara, California, USA). Oligomycin is an irreversible inhibitor of ATP synthase while FCCP acts as an uncoupler, and rotenone and antimycin A inhibit Complex I and III respectively, of the electron transport chain (ETC). All these chemicals were from MilliporeSigma, Canada, Oakville, Ontario, Canada. Following OCR measurements, the cells lysates were harvested individually from each well in RIPA buffer and the DC protein assay was performed. OCR measurements were normalized to the amount of protein in each well and reported as pmole/min/mg protein.

2.3.4 Immunostaining for ERs and ATF3

DRG neurons were cultured on glass coverslips (NC0615200, Thermo Fisher Scientific, Waltham, Massachusetts, USA) coated with poly-d-l-ornithine hydrobromide (0.5 mg/ml in ddH₂O) (MilliporeSigma Canada, Oakville, Ontario, Canada) and laminin (2 μ g/ml in F12 media) at a

density of 10,000 cells/well (5,482 cells/cm²) for 24 hours prior to immunostaining. Cultured neurons were fixed using 4% paraformaldehyde (PFA) (J61899, Thermo Fisher Scientific, Waltham, Massachusetts, USA) in phosphate-buffered saline (PBS) for 15 minutes. Coverslips were washed with PBS (3 x 10 minutes) and permeabilized using 0.3% Triton X-100 (X100, MilliporeSigma Canada Ltd, Oakville, Ontario, Canada) for 5 minutes. After washing, coverslips were incubated in blocking buffer (5% BSA in PBS) for 1 hour, followed by overnight incubation with primary antibodies at 4°C. For ER staining, coverslips were incubated with β -tubulin III primary antibody (mouse anti- β -tubulin III antibody, T8578, 1:1000, MilliporeSigma Canada Ltd, Oakville, Ontario, Canada) overnight. The next day, after three PBS washes, coverslips were incubated with Cyanine3 (Cy3) conjugated anti-mouse secondary antibody (1:500, Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) for 1 hour at room temperature. Coverslips were then washed in PBS, blocked again for 1 hour, and incubated with ER α (rabbit anti-estrogen receptor alpha antibody, ab32063, 1:200, Abcam, Waltham, Massachusetts, USA) or ER β (rabbit anti-estrogen receptor beta antibody, PA1-313, 1:200, Thermo Fisher Scientific, Waltham, Massachusetts, USA) primary antibodies at 4°C overnight. On day 3, coverslips were washed and stained with secondary antibody Alexa Fluor 488 (anti-rabbit, A-11008, 1:500, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 1 hour at room temperature. For ATF3 staining, coverslips were incubated with ATF3 primary antibody (mouse anti-ATF3 antibody, sc-81189, 1:100, Santa Cruz Biotechnology Inc., Dallas, Texas, USA) overnight at 4°C. The next day, after three PBS washes, coverslips were incubated with secondary antibody Alexa Fluor 488 (anti-mouse, A-11001, 1:500, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 1 hour at room temperature. After final washes, coverslips were incubated in 1:3000 DAPI in PBS for 5 minutes, then washed again with PBS (3 x 10 minutes) and mounted in PermaFluor

aqueous mounting medium (TA030FM, Eprelia, Kalamazoo, Michigan, USA). Appropriate negative controls (incubated with secondary antibody only) were included for each group to ensure specificity of staining. ER α + β -tubulin III, ER β + β -tubulin III, and ATF3 staining were assessed independently and visualized using an IX83-Olympus fluorescence microscope.

2.3.5 Measurement of neurite outgrowth and Sholl analysis

DRG neurons were cultured on glass coverslips coated with poly-d-l-ornithine hydrobromide (0.5 mg/ml in ddH₂O) followed by laminin (2 μ g/ml in F12 media). Neurons were seeded at a density of 10,000 cells/well (5,482 cells/cm²) and cultured for 24 hours. E2 treatments (1 nM and 10 nM) were added at the time of seeding. After 24 hours, neurons were fixed using 4% paraformaldehyde (PFA) in PBS for 15 minutes, permeabilized with 0.3% Triton X-100 for 5 minutes and immunostained. Cells were incubated with 5% BSA in PBS for 1 hour followed by overnight incubation in β -tubulin III primary antibody (1:1000, T8578, MilliporeSigma Canada Ltd, Oakville, Ontario, Canada) at 4°C. Next day, after three PBS washes, DRG neurons were incubated in Cy3 conjugated secondary antibody (1:500, Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) for 1 hour at room temperature, and coverslips were mounted on glass slides using PermaFluor aqueous mounting medium. For neurite outgrowth analysis, DRG neurons were imaged using a Carl Zeiss Axioscope-2 fluorescence microscope equipped with an AxioCam. Random images were captured, and total neurite outgrowth was quantified by measuring the mean pixel area of captured images using ImageJ software (Version 1.48v). Total pixel area was normalized to the number of cell bodies to determine total neurite outgrowth per neuron. To measure the length of the longest neurite, DRG neurons were first categorized into three groups based on cell diameter: small (<25 μ m), medium (25-35 μ m), and large (>35 μ m) neurons. Using the straight-line tool in ImageJ software, we measured the distance from the center of the soma to

the furthest neurite tip for each neuron. The lengths were then summed and normalized to the cell count within each size category. To quantify the morphological complexity of DRG neurons, we employed Sholl analysis. Random images of DRG neurons with their soma were captured and analyzed using FIJI (Version 1.53f51) software. For each neuron, the image was imported into FIJI to remove the background and enhance the visibility of fine neuronal structures that allowed the reconstruction of a neurite tree, including all its branches. After processing, the images were subjected to Sholl analysis. The measurement was set from the center of the soma and Sholl analysis plugin was run with the parameters: Start radius = 10 μm , Step size = 10 μm , End radius = 500 μm . This enabled neurite tracing using a grid of concentric rings, equivalent to 10 μm spacing. The number of intersections with these circles was used to estimate the maximum radius/length of the neuron, as well as to quantify the branching complexity of neuritic processes at various distances from the soma.

2.3.6 Statistical analysis

Data is expressed as mean $\pm$ standard error of the mean (SEM) and analyzed using two-tailed t-test or one-way ANOVA followed by Dunnett's post hoc tests, as indicated. For comparisons between multiple treatment groups, we used one-way ANOVA followed Tukey's post hoc test. GraphPad Prism (version 8.0) software was used to perform these statistical analyses. A *P* value of < 0.05 was considered statistically significant in all the experiments.

2.4 Results

Please note that, with the exception of some figures in the supplementary data, all experiments were conducted using DRG neurons derived from adult male SD rats.

2.4.1 E2 treatment increases AMPK phosphorylation and elevates PGC-1 α and ATF3 levels

Treatment of cultured adult rat DRG neurons with E2 at doses of 0.1 nM, 1 nM, and 10 nM for 1 hour led to a significant, increase in AMPK phosphorylation (Fig. 2.1A) at Thr172 (primary site for AMPK activation) relative to total AMPK levels. The highest increase was observed at 10 nM. A time course study revealed that 10 nM E2 elevated pAMPK levels. This increase was observed as early as 15 minutes and continued progressively at 30 min and 60 minutes (Fig. 2.1B), up to 24 hours (Supplementary Fig. 2.1) indicating a sustained phosphorylation and activation of the AMPK pathway following E2 treatment. DRG cultures derived from male vs female adult rats were also treated with E2 and the AMPK response was compared. Supplementary figure 2.1 shows that pAMPK was elevated by 10 nM E2 over a 24-hour period in male and female cultures, with female cultures revealing a larger fold increase in pAMPK levels. Next, we hypothesized that E2 mediated AMPK phosphorylation and activation could enhance PGC-1 α and ATF3 levels, which are key proteins involved in regulation of mitochondrial function (Halling and Pilegaard, 2020) and nerve regeneration (Linda *et al.*, 2011), respectively. In Fig. 2.1C, following 10 nM E2 treatment, PGC-1 α levels, normalized to total ERK, were significantly elevated at 30 minutes and continued to the 60-minute time point. In Fig. 2.1D, following 10 nM E2 treatment for 15 min, 30 min, and 60 minutes, we observed an increase in ATF3, normalized to total ERK.

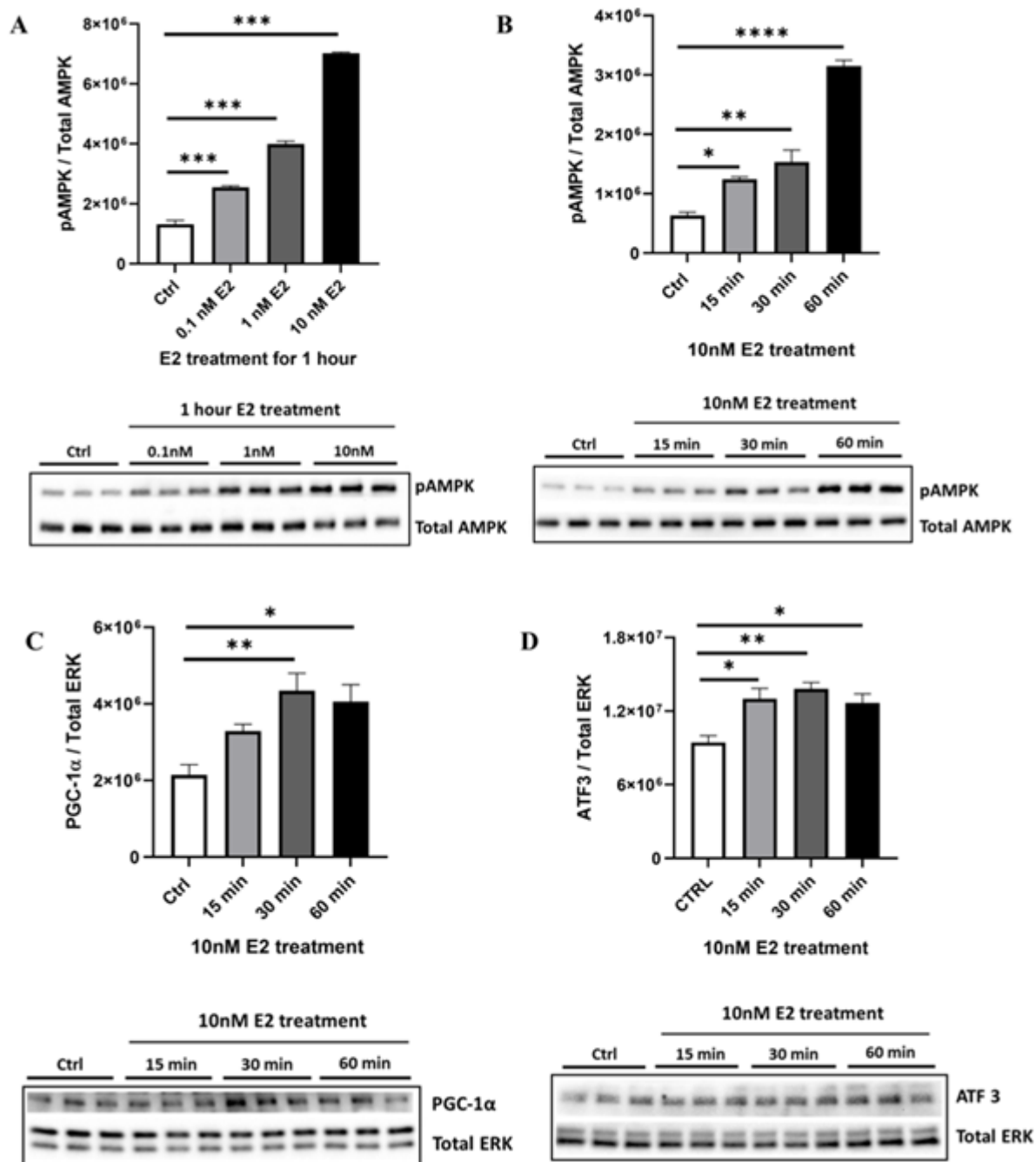


Figure 2.1. E2 treatment increases AMPK phosphorylation in a time dependent manner and enhances levels of PGC-1 α and ATF3. (A) DRG neurons derived from adult control rats were treated with/without 0.1 nM, 1 nM or 10 nM E2 for 1 h. E2 was dissolved in DMSO (vehicle), where the final DMSO concentration was < 0.1% in the cultures. Cell lysates were subject to

Western blotting. Levels of pAMPK are presented relative to Total AMPK. **(Fig B-D)** DRG neurons derived from adult control rats were treated with/without 10 nM E2 for 15 min, 30 min, and 60 min. Cell lysates were subject to Western blotting. In **(B)**, Levels of pAMPK are presented relative to Total AMPK. In **(C)** levels of PGC-1 α and **(D)** levels of ATF3 are presented relative to Total ERK. Data are expressed as mean $\pm$ SEM, n = 3 replicate cultures, *p < 0.05 or **p < 0.01 or ***p < 0.001 or ****p < 0.0001, analyzed by one-way ANOVA with Dunnett's *post hoc* test.

2.4.2 E2 enhances respiratory chain protein expression and mitochondrial function in DRG neurons

E2 treatment significantly elevated mitochondrial respiratory chain Complex I-NDUFB8 protein levels in DRG neurons at a 6-hour time point (Fig. 2.2A). In addition, mitochondrial basal respiration was also enhanced with E2 treatment (Fig. 2.2C), as measured using OCR (Fig. 2.2B) via the Seahorse XF24 Analyzer. The amount of ATP production, as assessed via Seahorse analysis, was also increased, although it did not reach statistical significance ($P = 0.057$) (Fig. 2.2D).

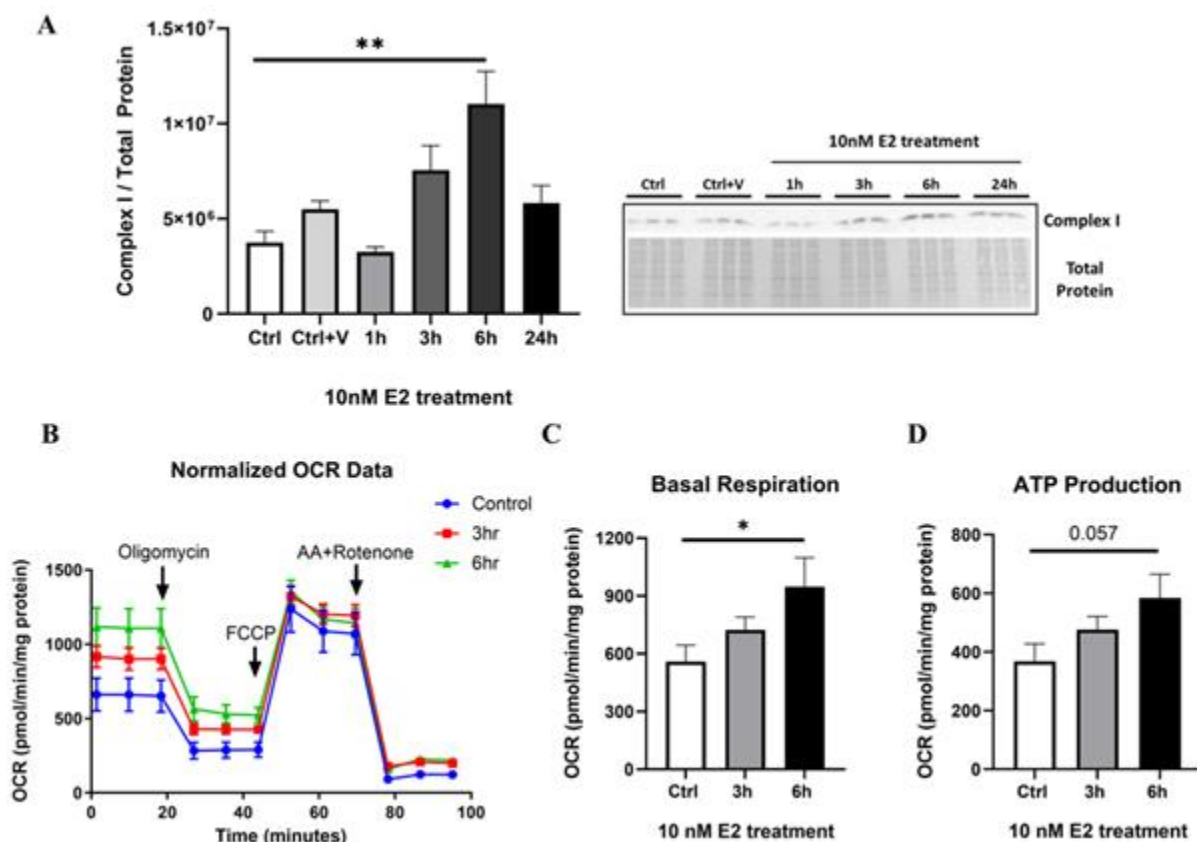


Figure 2.2. E2 treatment increases the expression of ETC complex I protein levels and basal respiration levels in DRG neurons. (A) DRG neurons derived from adult control rats were treated

with/without 10 nM E2 for 1 h, 3 h, 6 h, 24 h. Cell lysates were subject to Western blotting. Levels of mitochondrial electron transport chain complex I-NDUFB8 levels are presented relative to Total Protein. The Ctrl+V group refers to the control group that was treated only with vehicle (DMSO) for 24 h. Data is expressed as mean $\pm$ SEM, n = 3 replicate cultures, **p < 0.01, analyzed by one-way ANOVA with Dunnett's *post-hoc* test. **(Fig B-D)** DRG neurons derived from adult control rats were treated with/without 10 nM E2 for 3 h and 6 h. Mitochondrial respiration was measured using Seahorse XF24 Analyzer. Data was normalized to protein concentration units per well prior to statistical analysis. Data is expressed as mean $\pm$ SEM, n = 4-7 replicate cultures (Fig B-D), *p < 0.05 analyzed by one-way ANOVA with Dunnett's *post hoc* test.

2.4.3 E2 increases neurite outgrowth, longest neurite and branching in DRG neurons

E2 treatment stimulated neurite outgrowth in cultured DRG neurons (Fig. 2.3A). In Fig. 2.3B, DRG neurons treated with 10 nM E2 for 24 hours exhibited a significant increase in total neurite outgrowth compared to untreated controls. In a comparison of male vs female DRG cultures the maximal level of neurite outgrowth induced by 10 nM E2 for 24 hours was similar, although female-derived DRG cultures exhibited higher sensitivity to E2 (Supplementary figure 2.2). Further analysis of neuronal morphology using the Sholl analysis method, revealed that E2 treatment increased neuronal length and branching complexity. Sholl analysis revealed that 10 nM E2 treatment for 24 hours significantly increased the longest neurite length (Fig. 2.3C) and enhanced neurite branching at 50 μ m distance from soma, as evidenced by an increased number of intersections (Fig. 2.3D).

2.4.4 AMPK inhibition suppresses E2-mediated AMPK activation and ATF3 upregulation

Previously, we saw that E2 increases AMPK phosphorylation and upregulates ATF3 levels. To investigate a potential link between these findings, we inhibited AMPK using Compound C (CC), a direct inhibitor of AMPK's metabolic actions (Razmara *et al.*, 2008; Shait Mohammed *et al.*, 2021). Cultured DRG neurons were pretreated with 3 μ M CC for 1 hour prior to treatment with 10 nM E2 for 1 hour. Western blotting revealed that E2-mediated upregulation of ATF3 is dependent on AMPK phosphorylation. In Fig. 2.4A, pretreatment with CC attenuated the E2-mediated increase in pAMPK levels. Similarly, CC pretreatment suppressed E2-induced upregulation of ATF3 (Fig. 2.4B), supporting AMPK acts upstream of ATF3 in adult rat DRG neurons.

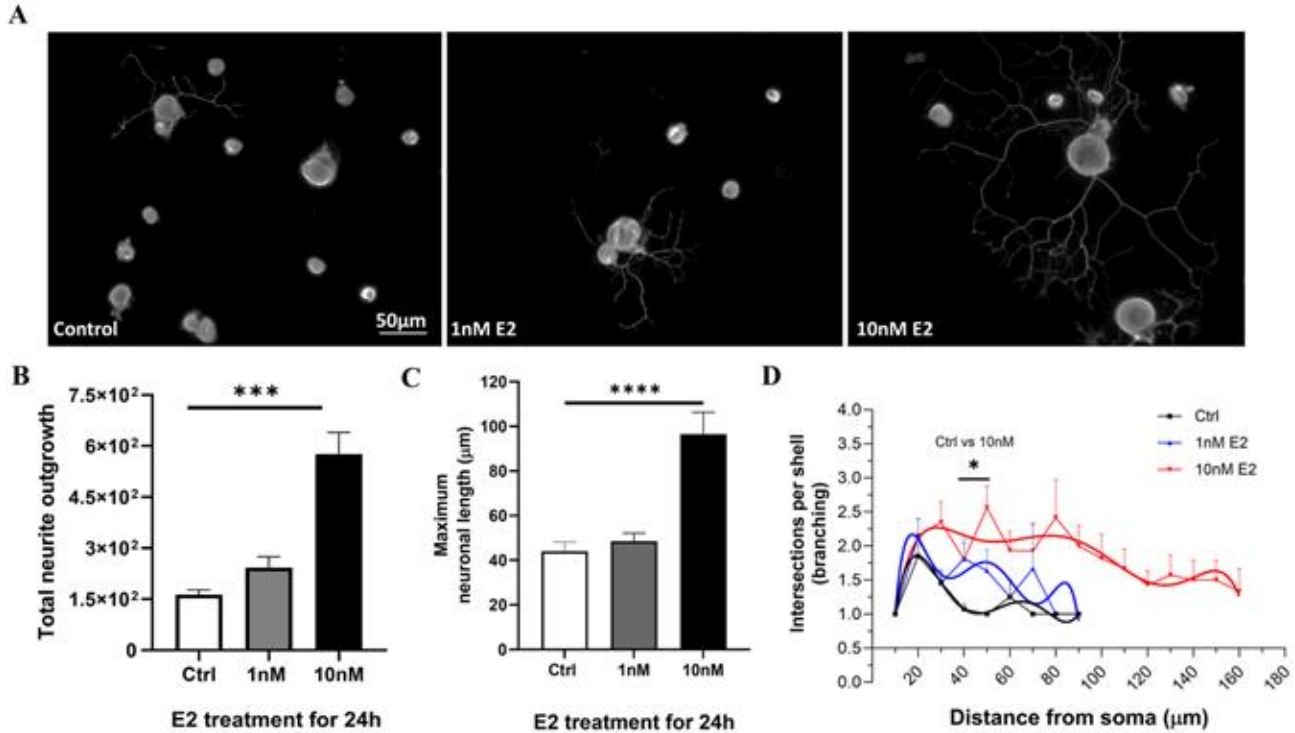


Figure 2.3 E2 treatment increases neurite outgrowth, longest neurite length and branching of cultured DRG neurons. (A) DRG neurons derived from adult control rats were cultured with/without 1 nM and 10 nM E2 for 24 h. Neurons were fixed and immunostained using β -tubulin III antibody and subjected to total neurite outgrowth and Sholl analysis. (B) Total neurite outgrowth data is presented relative to neuron number. Data is expressed as mean $\pm$ SEM, $n = 4$ replicate cultures, *** $p < 0.001$, analyzed by one-way ANOVA with Dunnett's *post hoc* test. (C) Neurite length was measured from the center of the soma. Data is expressed as mean $\pm$ SEM, $n = 20$, **** $p < 0.0001$, analyzed using one-way ANOVA with Dunnett's *post hoc* test. (D) Number of intersections of neuronal processes with concentric circles at 10 μ m intervals from the soma were quantified to measure branching complexity. Data were analyzed using multiple unpaired t -tests and expressed as mean $\pm$ SEM, $n = 20$, * $p < 0.05$.

2.4.5 AMPK activation is required for E2-mediated increase in neurite outgrowth in DRG neurons

To determine the role of AMPK activation in E2-mediated neurite outgrowth, DRG neurons were cultured and pretreated with 3 μ M CC for 1 hour prior to treatment with 10 nM E2 for 24 hours (Fig. 2.4C). Total neurite outgrowth analysis revealed that CC pretreatment abolished the increase in outgrowth that was observed in response to E2 treatment (Fig. 2.4D).

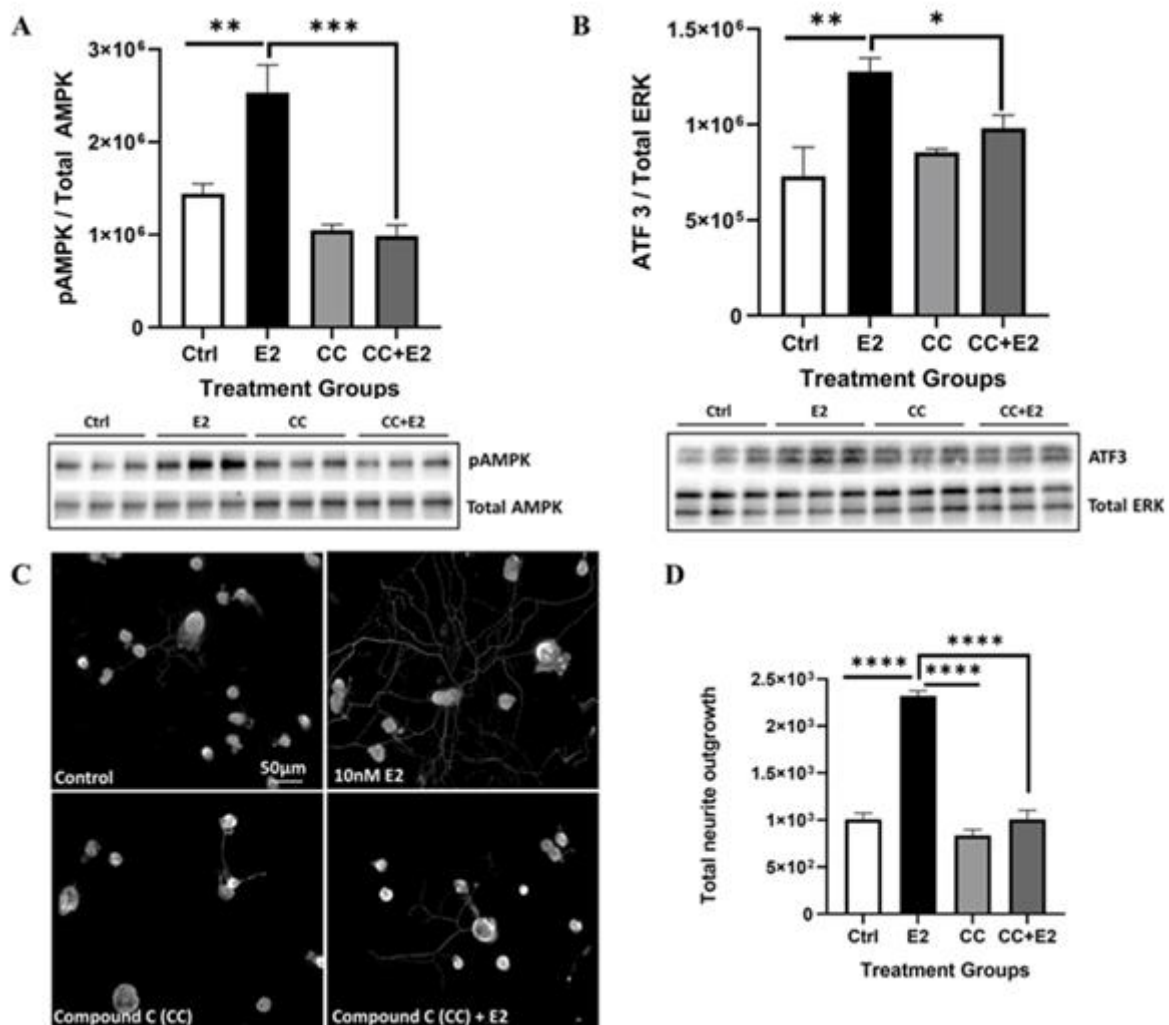


Figure 2.4. Blocking AMPK, inhibits E2-mediated AMPK activation and elevation of ATF3 and also suppresses E2-mediated neurite outgrowth. DRG neurons derived from adult control

rats were pretreated for 1 h with 3 μ M AMPK inhibitor CC followed by treatment with/without 10 nM E2 for 1 h. Cell lysates were subject to Western blotting. **(A)** Levels of pAMPK are presented relative to Total AMPK, with all groups analyzed using one-way ANOVA with Tukey's *post-hoc* test. **(B)** Levels of ATF3 are presented relative to Total ERK. The Ctrl and E2 groups were analyzed using one-way ANOVA with Dunnett's *post-hoc* test, with the Ctrl group as the control. The E2 and CC+E2 groups were then analyzed using one-way ANOVA with Dunnett's *post hoc* test, with the E2 group as the control. Data is expressed as mean $\pm$ SEM, n = 3 replicate cultures, * $p < 0.05$ or ** $p < 0.01$ or *** $p < 0.001$. **(C)** DRG neuronal cultures were pretreated for 1 h with 3 μ M AMPK inhibitor CC followed by treatment with/without 10 nM E2 for 24 h. **(D)** Total neurite outgrowth data is presented relative to neuron number. Data is expressed as mean $\pm$ SEM, n = 6 replicate cultures, **** $p < 0.0001$, analyzed by one-way ANOVA with Tukey's *post hoc* test.

2.4.6 E2 activates AMPK to enhance neurite outgrowth via the CaMKK β pathway

To confirm a role for CaMKK β in AMPK activation in adult DRG neurons, we employed the CaMKK selective inhibitor STO-609 (Razmara *et al.*, 2008; Tokumitsu *et al.*, 2002). DRG neurons were pretreated with 1 μ M STO-609 for 1 hour, followed by treatment with 10 nM E2 for 1 hour. Western blotting revealed that, STO-609 pretreatment substantially attenuated E2-induced AMPK phosphorylation (Fig. 2.5A). Additionally, as shown in Fig. 2.5B, E2-induced upregulation of PGC-1 α was also suppressed by STO-609 pretreatment. To further investigate the effects of CaMKK β blockade on neurite outgrowth, DRG neurons were pretreated with 1 μ M STO-609 for 1 hour, followed by 10 nM E2 for 24 hours (Fig. 2.5C). We observed that STO-609 pretreatment inhibited the E2-mediated increase in neurite outgrowth (Fig. 2.5D). These findings confirm that

AMPK activation is essential for E2-mediated neurite outgrowth (Fig. 2.4D and 2.5D) and corroborates that E2 activates AMPK via the CaMKK β pathway in DRG neurons (Fig. 2.5A).

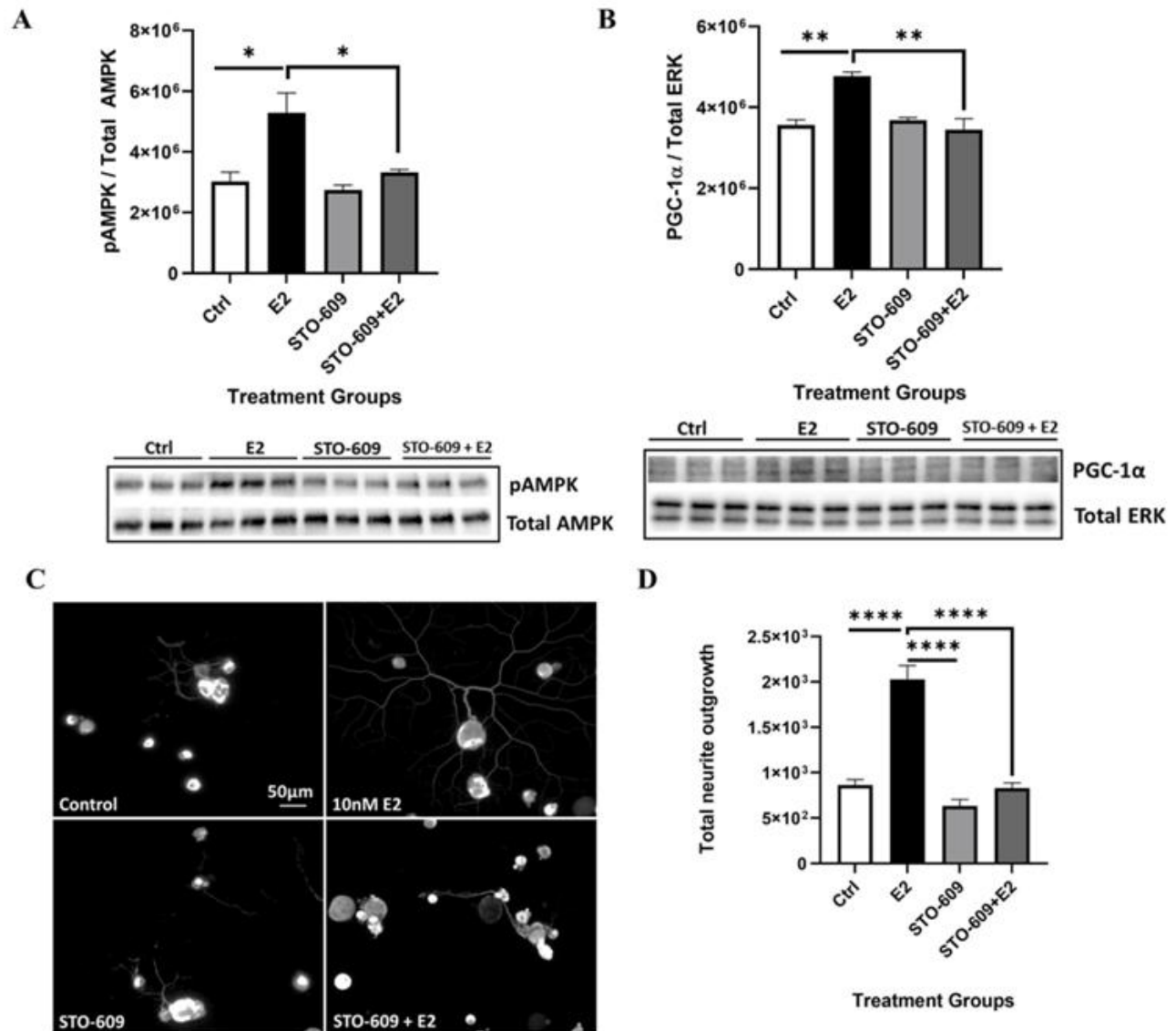


Figure 2.5. Blocking CaMKK inhibits E2-mediated AMPK activation and elevation of PGC-1 α levels, as well as E2-induced neurite outgrowth. DRG neurons derived from adult control rats were cultured, pretreated for 1 h with 1 μ M CaMKK inhibitor STO-609 followed by treatment with/without 10 nM E2 for 1 h. Cell lysates were subject to Western blotting. **(A)** Levels of pAMPK are presented relative to Total AMPK. **(B)** Levels of PGC-1 α are presented relative to

Total ERK. Data is expressed as mean $\pm$ SEM, n = 3 replicate cultures, *p < 0.05 or **p < 0.01, analyzed by one-way ANOVA with Tukey's *post hoc* test. (C) DRG neuronal cultures were pretreated for 1 h with 1 μ M CaMKK inhibitor STO-609 followed by treatment with/without 10 nM E2 for 24 h. (D) Total neurite outgrowth data is presented relative to neuron number. Data is expressed as mean $\pm$ SEM, n = 6 replicate cultures, ****p < 0.0001, analyzed by one-way ANOVA with Tukey's *post hoc* test.

2.4.7 ER α and ER β are expressed in DRG neurons

To elucidate the roles of ER subtypes in mediating the effects of E2 in adult DRG neurons, we first sought to confirm their expression in our experimental model. As shown in Fig. 2.6A and 2.6B, immunostaining revealed that both ER subtypes, ER α (Fig. 2.6A) and ER β (Fig. 2.6B) were localized in DRG neurons. Notably, almost all DRG neurons in our cultures were positive for both ER α and ER β . Western blots in Fig. 2.6C and 2.6D, show distinct bands corresponding to ER α (Fig. 2.6C) and ER β (Fig. 2.6D).

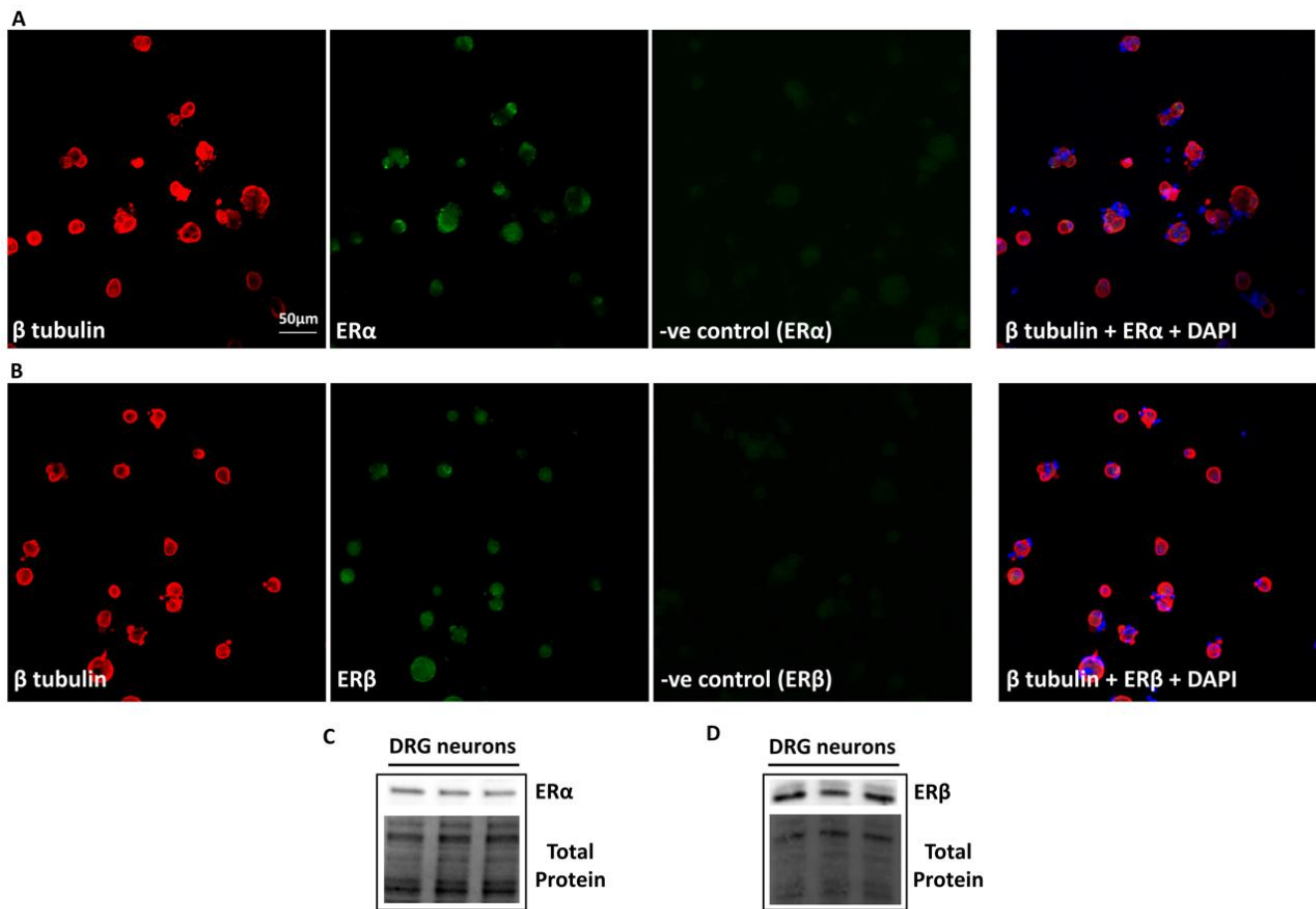


Figure 2.6. Expression of ERα and ERβ in adult rat DRG neurons. DRG neurons derived from adult rats were analyzed for ER expression using immunostaining and Western blotting. **(A)** Immunostaining of ERα in DRG neurons. Cells were labeled with β tubulin III antibody (red, to mark DRG neurons), ERα antibody (green, to mark ERα), and DAPI (blue, to mark nuclei). **(B)** Immunostaining of ERβ in DRG neurons. Cells were labeled as in (A), but with ERβ antibody (to mark ERβ) instead of ERα. Bar = 50 μm. Western blot analysis of ERα **(C)** and ERβ **(D)** protein expression in DRG neuronal cultures. Representative blots from three replicate cultures for ERα and ERβ are shown in (C) and (D) respectively.

2.4.8 E2 acts via ER α to mediate AMPK activation, augment mitochondrial respiration and induce neurite outgrowth

To elucidate if these actions of E2 are mediated by a specific ER subtype, we treated the DRG neuronal cultures with an ER α -selective agonist propylpyrazoletriol (PPT) or an ER β -selective agonist diarylpropionitrile (DPN) for 1 hour. Treatment with ER α agonist PPT resulted in an increase in AMPK phosphorylation (Fig. 2.7A). In contrast, treatment with DPN had no effect on pAMPK levels (Fig. 2.7B), suggesting that E2 actions on AMPK phosphorylation are primarily mediated through ER α in adult rat DRG neurons. Following this, to investigate which ER subtype mediates neurite outgrowth, we treated cultured DRG neurons with different doses of PPT and DPN (Fig. 2.7C). Treatment with 10 nM PPT for 24 hours significantly increased total neurite outgrowth compared to the control group (Fig. 2.7D). Meanwhile, DPN treatment had no significant effect on neurite outgrowth. To determine which DRG neurons exhibited the maximum outgrowth, we categorized the neurons based on cell diameter: small (<25 μ m), medium (25-35 μ m), and large (>35 μ m). We measured the longest neurite in control and 24-hour 10 nM PPT-treated neurons, as 10 nM PPT induced maximal outgrowth. Analysis of the longest neurite for each cell size category revealed that medium and large-sized neurons showed the most pronounced increases, with average maximum lengths increasing by 54% and 73%, respectively (Fig. 2.7E). To further investigate the downstream effects of ER α activation on ATF3 nuclear localization we performed immunocytochemistry (Fig. 2.8). DRG neurons treated with 10 nM PPT for 24 hours showed increased nuclear ATF3 expression (Fig. 2.8A and 2.8B). Quantification of nuclear ATF3-positive neurons revealed size-dependent differences where, almost all small sized DRG neurons in both control and PPT-treated groups expressed nuclear ATF3. However, in the control group, only 55% of medium and 26% of large sized neurons showed nuclear ATF3 expression. 24 hour

10 nM PPT treatment increased nuclear ATF3 localization to 79% for medium and 81% for large neurons (Fig. 2.8C). Treatment with 10 nM PPT for 24 hours also led to augmentation in multiple parameters of mitochondrial function, as measured by the Seahorse XF24 Analyzer (Fig. 2.9A). We observed significant increases in basal respiration, maximal respiration, and spare respiratory capacity (Fig. 2.9B-D). Relative ATP production also increased, although it did not reach statistical significance ($p=0.06$) (Fig. 2.9E).

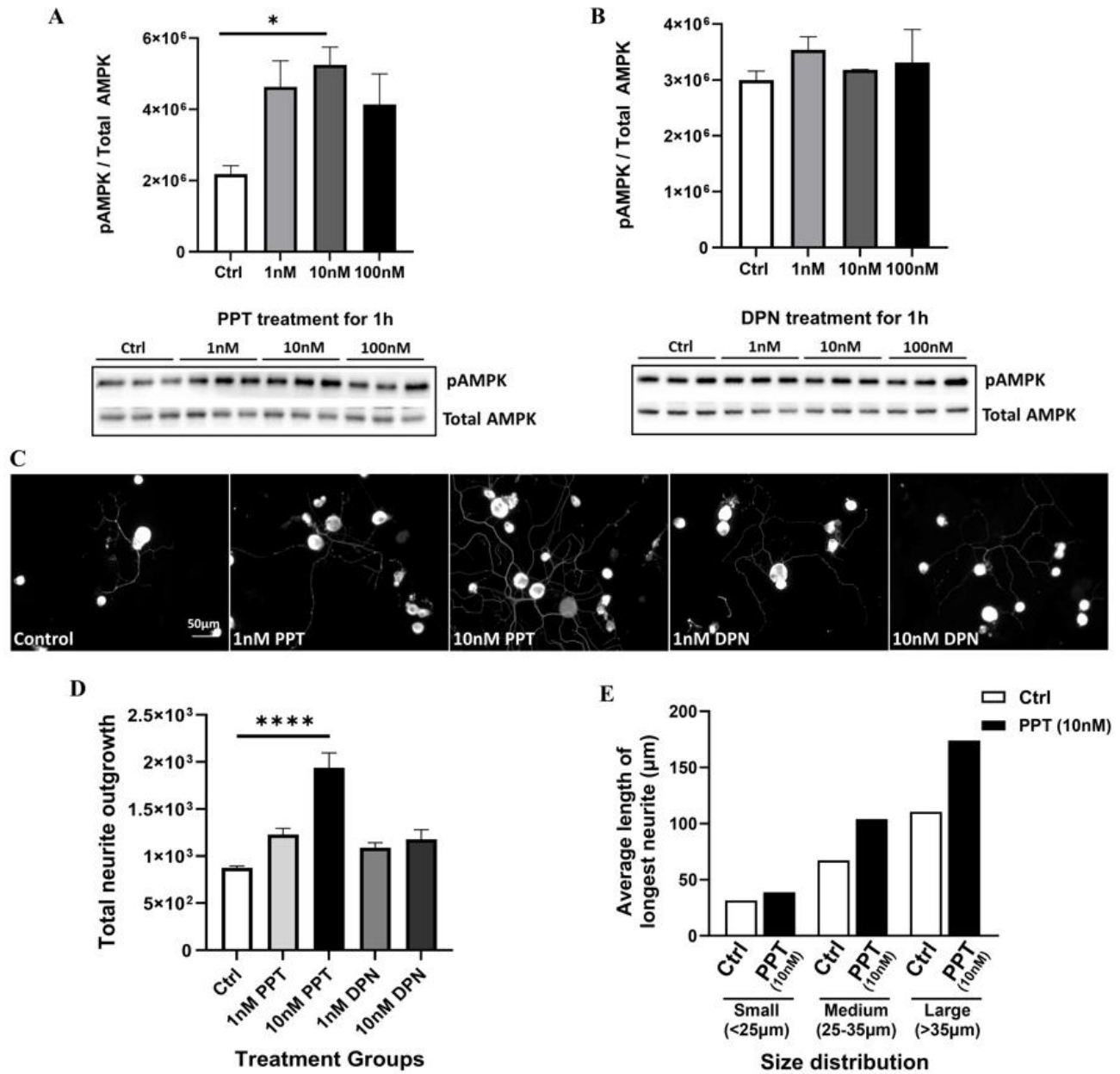


Figure 2.7. ER α agonist propylpyrazoletriol increases AMPK phosphorylation and mediates neurite outgrowth in cultured DRG neurons. DRG neurons derived from adult control rats were treated with/without 1 nM, 10 nM or 100 nM propylpyrazoletriol (PPT) (**A**) or diarylpropionitrile (DPN) (**B**) for 1 h. Cell lysates were subject to Western blotting. Levels of pAMPK are presented relative to Total AMPK in PPT (**A**) and DPN (**B**) treated cells respectively. Data are expressed as mean $\pm$ SEM, n = 3 replicate cultures, *p < 0.05, analyzed by one-way ANOVA with Dunnett's

post-hoc test. **(C)** DRG neuronal cultures were treated with/without 1 nM and 10 nM PPT or DPN for 24 h. Neurons were fixed and immunostained using β -tubulin III antibody and analyzed for neurite outgrowth. **(D)** Total neurite outgrowth data is presented relative to neuron number. Data is expressed as mean $\pm$ SEM, n = 4 replicate cultures, ****p < 0.0001, analyzed by one-way ANOVA with Tukey's *post hoc* test. **(E)** Data represents average length of the longest neurite in control vs 10 nM PPT treated DRG neurons of sizes: small (<25 μ m), medium (25-35 μ m), and large (>35 μ m). A minimum of 90 DRG neurons were measured in each group, with at least 30 neurons in each small, medium or large size categories.

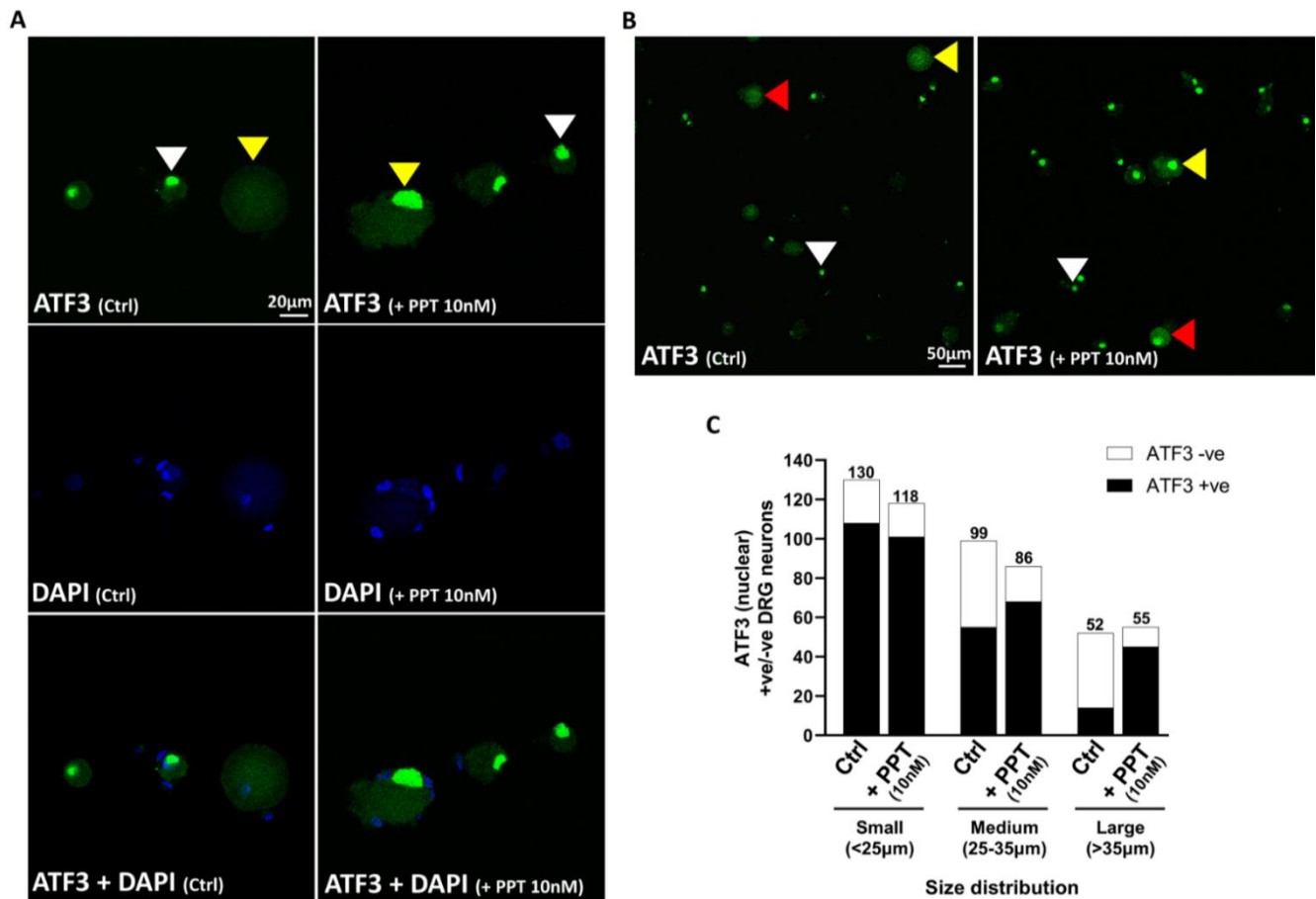
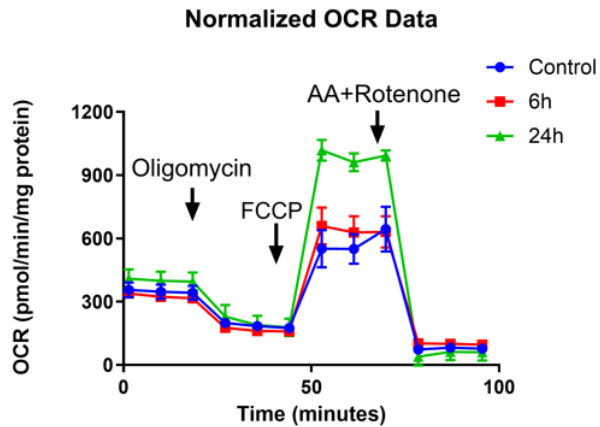
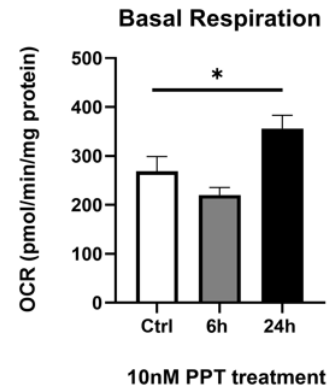


Figure 2.8. ER α activation induces nuclear ATF3 expression in adult rat DRG neurons. DRG neurons from adult rats were treated with/without 10 nM propylpyrazoletriol (PPT) for 24 h, then fixed and immunostained for ATF3 expression. Cells were labeled with ATF3 antibody (green) and DAPI (blue, to mark nuclei). (A) Representative high-magnification images showing ATF3 localization in control vs. 10 nM PPT treated DRG neurons. Scale bar = 20 μ m. (B) Low-magnification images showing immunostaining of ATF3 in control vs 10 nM PPT treated DRG neurons. Scale bar = 50 μ m. Yellow arrows indicate: large DRG neurons; red arrows indicate: medium DRG neurons; white arrows indicate: small DRG neurons. (C) Quantification of nuclear ATF3 +ve and -ve DRG neurons by size category: small (<25 μ m), medium (25-35 μ m), and large (>35 μ m). Numbers above bars indicate total cells analyzed for nuclear ATF3 levels in each size category. At least 255 cells were analyzed in each control/PPT treated group.

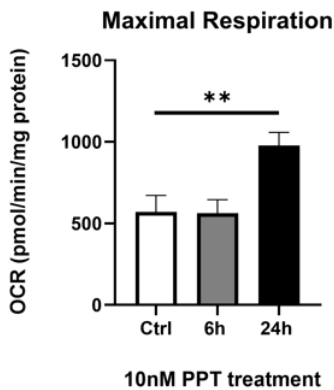
A



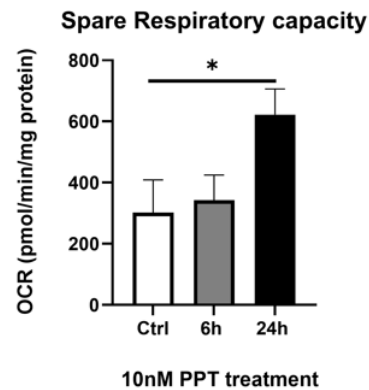
B



C



D



E

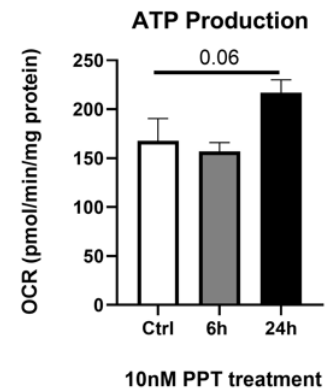


Figure 2.9. ER α agonist PPT increases mitochondrial function in DRG neurons. DRG neurons derived from adult control rats were treated with/without 10 nM PPT for 6 h and 24 h. Mitochondrial respiration was measured using Seahorse XF24 Analyzer. Data were normalized to protein concentration units per well prior to statistical analysis. Data is expressed as mean $\pm$ SEM, $n = 6-7$ replicate cultures, * $p < 0.05$, ** $p < 0.01$, analyzed by one-way ANOVA with Dunnett's *post hoc* test.

2.5 Discussion

In this study, we investigated the effects of E2 on cultured adult rat DRG sensory neurons, focusing on AMPK signaling, mitochondrial function, and neurite outgrowth. While prior work established E2's neurotrophic effects in neonatal DRG neurons (Chakrabarty *et al.*, 2008) our findings reveal novel mechanisms in adult DRG sensory neurons, linking the CaMKK β /AMPK signaling axis to mitochondrial function and axonal regeneration. We demonstrated that E2 activates AMPK in a time-dependent manner (Fig. 2.1A and 2.1B), leading to upregulation of downstream targets PGC-1 α (Fig. 2.1C) and ATF3 (Fig. 2.1D). This AMPK activation occurred via the CaMKK β pathway (Fig. 2.5A). E2 treatment also enhanced mitochondrial function (Fig. 2.2) and promoted axonal regeneration (Fig. 2.3B). Crucially, we established that AMPK activation is essential for E2-mediated axonal regeneration and ATF3 upregulation (Fig. 2.4). Also, for the first time we showed that E2 acts primarily via ER α to mediate AMPK activation (Fig. 2.7A) driving both mitochondrial upregulation (Fig. 2.9) and neurite outgrowth (Fig. 2.7D) in adult DRG neurons, distinguishing our work from studies in non-neuronal systems (Yang and Wang, 2015) or developmental models (Patrone *et al.*, 1999).

E2 can exert its effects via genomic or non-genomic pathways and activates AMPK in various cell types (D'Eon *et al.*, 2008; Lipovka *et al.*, 2015; Yang and Wang, 2015). The rapid activation of AMPK by E2 observed in our study, occurring as early as 15 minutes (Fig. 2.1B), suggests a non-genomic mechanism of action and extends upon previous reports of E2's non-genomic effects (Banks *et al.*, 2024). This quick response indicates that E2 plays a role in rapid energy balance regulation in adult rat DRG neurons that aligns with its calcium-mobilizing effects observed in hippocampal neurons (Wu *et al.*, 2011), but diverges from LKB1-dominated pathways reported in murine embryonic fibroblasts (MEFs) (Shaw *et al.*, 2004). We provide the first evidence in adult

DRG neurons that E2-induced AMPK activation requires CaMKK β , as evidenced by blockade of E2-induced AMPK phosphorylation when DRG neurons were pretreated with the CaMKK inhibitor STO-609 (Fig. 2.5A). This is consistent with previous studies showing that E2 activates AMPK via the CaMKK β pathway in human endothelial cells (Yang and Wang, 2015), and CaMKK can activate AMPK in response to increases in intracellular Ca²⁺ levels (Gormand *et al.*, 2011; Hawley *et al.*, 2005; Hurley *et al.*, 2005).

The intracellular calcium signal required for this AMPK activation in DRG neurons could originate from either intracellular stores, such as the endoplasmic reticulum (ER), or extracellular sources. Previous work has established that E2 can regulate calcium levels in various cell types, including hippocampal neurons (Wu *et al.*, 2011), aortic endothelial cells (Goetz *et al.*, 1999), and cardiomyocytes (Mahmoodzadeh and Dworatzek, 2019). In 2010, Thor and colleagues demonstrated that E2, acting via ER α , regulated intracellular calcium homeostasis in human endothelial cells (Thor *et al.*, 2010). Furthermore, a study by Dopp *et al.*, directly implicated E2 in calcium release from the ER, demonstrating that E2 increased intracellular calcium in RUCA-I cells, an effect that was blocked by an ER antagonist (Dopp *et al.*, 1999).

ATF3 has emerged as a highly conserved pro-regenerative transcription factor in the vertebrate nervous system (Renthal *et al.*, 2020), and is implicated in various physiological processes, including lipid metabolism, glucose metabolism, apoptosis, and inflammation, as well as in diseases such as cancer (Hu *et al.*, 2023; Ku and Cheng, 2020; Liu *et al.*, 2024). ATF3 also contributes to nerve regeneration, as ATF3 mutant mice exhibit reduced axon regeneration (Gey *et al.*, 2016). Our study demonstrates a rapid increase in both AMPK activation (Fig. 2.1B) and ATF3 expression (Fig. 2.1D) in DRG neurons as early as 15 minutes following E2 treatment. This observation aligns with previous findings, such as those by Liu and colleagues, who reported that

ATF3 is estrogen-responsive in vivo (Liu *et al.*, 2006) and E2 can upregulate ATF3 expression in human foreskin fibroblasts (Liu *et al.*, 2007). In murine macrophages, AMPK knockdown diminished the effects of metformin on ATF3 induction (Kim *et al.*, 2014). Our results validate this relationship in DRG neurons, as we observed that blocking AMPK inhibited E2-mediated elevation of ATF3 (Fig. 2.4B). The rapid increase in ATF3 levels (within 15 minutes) observed in our study raises questions about the underlying mechanisms, but also hints towards a novel post-transcriptional regulation in DRG neurons, differing from the canonical transcriptional regulation reported in fibroblasts (Liu *et al.*, 2007). While de novo transcription of ATF3 mRNA seems unlikely (but not impossible) within this short timeframe, other possibilities exist. One potential mechanism is ATF3 mRNA stabilization. Previous studies have shown that certain stimuli, such as anisomycin (an antibiotic retrieved from the bacteria *Streptomyces griseolus*), can both activate the ATF3 promoter and stabilize ATF3 mRNA (Liang *et al.*, 1996). This dual action could explain the rapid increase in ATF3 levels that we observed.

AMPK activation has been consistently linked to increased PGC-1 α expression through multiple mechanisms. Firstly, AMPK directly phosphorylates PGC-1 α at Thr177 and Ser538, which enhances its transcriptional activity (Jäger *et al.*, 2007; Qian *et al.*, 2024). This phosphorylation is crucial for PGC-1 α -dependent induction of its own promoter, creating a positive feedback loop (Handschin *et al.*, 2003). Additionally, AMPK indirectly regulates PGC 1 α through its effects on another key metabolic sensor SIRT1, where AMPK increases intracellular NAD⁺ levels, which in turn activates SIRT1, leading to deacetylation and activation of PGC-1 α (Canto and Auwerx, 2009; Qian *et al.*, 2024). Other pathways involved in PGC-1 α regulation include p38 MAPK, which can phosphorylate and activate PGC-1 α in muscle cells (Akimoto *et al.*, 2005), and Akt (Protein kinase B) which can inhibit PGC-1 α activity through direct phosphorylation at Ser570 (Li *et al.*, 2007).

Our observation that CaMKK inhibitor STO-609, blocked both E2-mediated AMPK activation (Fig. 2.5A) and PGC-1 α elevation (Fig. 2.5B) establishes a unique CaMKK β /AMPK/PGC-1 α signaling axis in adult DRG neurons and supports the critical role of the AMPK in regulating PGC-1 α levels. This finding aligns with the established CaMKK-AMPK-PGC-1 α signaling cascade, where cyanidin 3-glucoside increased PGC-1 α expression by elevating intracellular Ca²⁺ levels and activating the CaMKK-AMPK pathway in skeletal muscle (Matsukawa *et al.*, 2017).

PGC-1 α can interact with nuclear respiratory factors NRF1 and NRF2 and mitochondrial transcription factor A (TFAM) to modulate mitochondrial biogenesis and energy metabolism (Chen *et al.*, 2022) leading to increased expression of respiratory chain subunits. In adult sensory neurons antimuscarinic drugs and the negative allosteric modulator, muscarinic toxin 7 (MT7), activate PGC-1 α downstream from the CaMKK β /AMPK pathway to drive mitochondrial function and neurite outgrowth (Saleh *et al.*, 2020). Interestingly, our study observed an increase only in Complex I subunit levels of the respiratory chain following E2 treatment (Fig. 2.2A), without significant changes in other complexes. This selective upregulation of Complex I is intriguing and warrants further discussion. Complex I, which is a major entry point for electrons, is also the largest complex of the respiratory chain (Zhao *et al.*, 2019), and a major site of ROS production (Kowaltowski *et al.*, 2009). Therefore, its upregulation significantly impacts overall mitochondrial function. Additionally, Capllonch and colleagues demonstrated that E2 enhances mitochondrial biogenesis and reduces oxidative stress in skeletal muscles (Capllonch-Amer *et al.*, 2014). This effect may be mediated through PGC-1 α , which protects cells from oxidative stress by regulating antioxidant genes, including superoxide dismutase and catalase, thus preventing oxidative injury (Rius-Pérez *et al.*, 2020). Similarly, work performed by Tian and his colleagues showed that E2 upregulated PGC-1 α , leading to improved mitochondrial function (Tian *et al.*, 2023).

Several possibilities could explain the selective increase in Complex I observed in our study. Temporal differences in complex assembly might play a role, as Complex I, being the largest and most intricate, could be the first to show measurable increases in response to E2-mediated PGC-1 α activation. Furthermore, the upregulation of Complex I could directly contribute to the observed increase in basal respiration (Fig. 2.2C). Additionally, the organization of respiratory chain complexes into supercomplexes is also significant. Lopez-Fabuel et al. demonstrated that assembly of Complex I into supercomplexes determines differential mitochondrial respiration levels in neurons and astrocytes, with neurons showing Complex I mostly embedded in supercomplexes, resulting in high mitochondrial respiration (Lopez-Fabuel *et al.*, 2016). Hence, the selective upregulation of Complex I by E2 in the DRG neurons could potentially reflect a shift towards a more neuron-like supercomplex organization, to optimize energy production. This reorganization could explain the observed increase in Complex I and basal respiration without a corresponding increase in other ETC complexes.

E2 targets and exerts its effects through both ER α and ER β , which can lead to complex cellular responses. In our investigation, we initially observed that E2 elevated basal respiration in DRG neurons (Fig. 2.2C). To elucidate the specific mechanisms, we first confirmed the presence of both ERs in these neurons through immunostaining and Western blotting (Fig. 2.6A-D). Our results demonstrated that both ER subtypes are widely expressed in DRG neurons, with almost all neurons in our cultures showing immunoreactivity for ER α and ER β . Subsequent experiments with selective ER agonist revealed that only ER α activation increased AMPK levels (Fig. 2.7A), prompting us to perform Seahorse analysis with the ER α agonist PPT. Interestingly, PPT treatment demonstrated a more pronounced effect on mitochondrial OCR compared to E2, significantly increasing basal respiration, maximal respiration, and spare respiratory capacity (Fig. 2.9B-D).

These findings are in line with the work of Bauzá-Thorbrügge *et al.*, who demonstrated ER α -mediated enhancement of mitochondrial function in adipocytes through a PKA-dependent mechanism (Bauza-Thorbrugge *et al.*, 2019). The discrepancy between E2 and PPT effects is difficult to interpret, given that E2 activates ER α , ER β and GPER. One possibility is that ER β and GPER might exert inhibitory effects on mitochondrial function or ER α signaling, offsetting the positive effects of ER α activation. This hypothesis is supported by several studies. Manente *et al.*, demonstrated that ER β activation impaired mitochondrial oxidative metabolism in mesothelioma cells, compromising mitochondrial activity and reducing ATP production (Manente *et al.*, 2013). Additionally, GPER activation inhibited ER α -dependent uterine growth by suppressing ERK1/2 and ER α phosphorylation signals in response to E2 (Gao *et al.*, 2011). This complex interplay between ER α , ER β , and GPER may explain why E2, which activates all three receptors, showed less pronounced effects on mitochondrial function compared to the ER α agonist PPT in our study.

E2 significantly increased neurite outgrowth in adult DRG neuronal cultures (Fig. 2.3B). This finding conforms to previous studies demonstrating E2's neurotrophic effects across various neuronal populations, including neocortical, hypothalamic and neuroblastoma cells (Brinton *et al.*, 1997; Cisternas *et al.*, 2020; Takahashi *et al.*, 2011). Our ER subtype analysis resolved questions about E2's receptor selectivity in DRG neuronal regeneration. We showed that only ER α activation elevated neurite outgrowth, while ER β activation did not (Fig. 2.7D). This ER α -specific effect is consistent with prior findings showing that E2 enhances neurite outgrowth in PC12 cells transfected with ER α (Gollapudi and Oblinger, 2001). Furthermore, our observation aligns with the work of Chakrabarty *et al.*, who reported that the ER α agonist induced neurite outgrowth in cultured neonatal DRG neurons, whereas the ER β agonist was ineffective (Takahashi *et al.*, 2011). The mechanisms underlying this ER α -specific effect may be multifaceted. Our findings that ER α

activation enhanced mitochondrial function (Fig. 2.9) offers one potential explanation. Additionally, ER α activation induces differential neuronal phenotypes, specifically increasing both the length and number of neurites in neuroblastoma cells (Patrone *et al.*, 2000). This suggests that ER α may preferentially activate pathways that enhance both mitochondrial function and growth cone dynamics, leading to more robust neurite outgrowth.

Interestingly, the medium and large neurons that showed increased nuclear ATF3 expression in response to the ER α agonist PPT (Fig. 2.8C) also exhibited the most pronounced neurite length (Fig. 2.7E), suggesting a potential link between ATF3 activation and neurite extension in response to ER α activation. In addition, the response in this sub-class of medium to large diameter sensory neurons does correspond with expression of trkB and elevated synthesis of endogenous BDNF upon axotomy (Michael *et al.*, 1997; Michael *et al.*, 1999; Usoskin *et al.*, 2015; Wright and Snider, 1995). Specifically, trkB expressing sensory neurons are myelinated low-threshold mechanoreceptors (LTMRs) of the A δ and A β sub-type (Usoskin *et al.*, 2015). Previous studies have highlighted the critical role of endogenous BDNF in regulating adult sensory neuron survival following axotomy (Acheson *et al.*, 1995) and facilitating their transition from a homeostatic to a regenerating state after peripheral nerve injury (Geremia *et al.*, 2010). Inhibition of endogenous BDNF in lesioned rat DRG neurons resulted in decreased expression of regeneration-associated genes and significantly impaired their intrinsic capacity to extend neurites (Geremia *et al.*, 2010). Furthermore, the work of Toran-Allerand *et al.* identified an estrogen response element within the BDNF gene (Sohrabji *et al.*, 1995). Thus, it could be speculated that activation of ER α receptor may mediate elevated neurite outgrowth (and possibly survival) via up-regulation of endogenous BDNF expression, synthesis and release.

While our study focused on male rats and general female comparisons, it's crucial to consider how responses might differ in female rats during specific estrous cycle phases, particularly proestrus. During proestrus, estradiol levels peak (Butcher *et al.*, 1974), and this could potentially amplify the effects we observed. Previous studies have shown that proestrus is associated with enhanced neuroplasticity and increased neuronal excitability. For instance, E2 significantly increased the firing rate of gonadotropin-releasing hormone (GnRH) neurons during proestrus (Farkas *et al.*, 2018), and cell proliferation in the dentate gyrus was also elevated during this phase (Tanapat *et al.*, 1999). In the context of DRG neurons, this could potentially lead to even greater AMPK activation and neurite outgrowth during proestrus stage compared to non-cycling or ovariectomized females. These heightened responses could result in more pronounced effects on mitochondrial function and axonal regeneration. Future studies could examine DRG neuron responses across all estrous cycle phases to fully elucidate the impact of fluctuating estrogen levels on AMPK signaling and neurite outgrowth in sensory neurons. Our current study significantly extends the therapeutic paradigm for E2's signaling in neurodegenerative diseases. The fact that E2 activates the CaMKK β /AMPK pathway to promote neurite outgrowth has significant clinical potential, particularly in the context of peripheral nerve injuries and neurodegenerative disorders such as sensory neuropathies. E2 has long been recognized for its neuroprotective and neurotrophic properties, but our study provides crucial insights into the specific mechanisms and receptors involved in these processes in adult DRG neurons. The identification of ER α as the primary mediator of E2's neurotrophic effects, coupled with its role in enhancing mitochondrial function and driving AMPK-dependent neurite outgrowth (Fig. 2.7D), suggests that AMPK activation could mimic E2's benefits in sensory neuropathies while avoiding the hormonal side effects of non-selective estrogen therapies. While our study identifies the CaMKK β /AMPK/ER α axis as

critical for E2-mediated neurite outgrowth in cultured adult DRG neurons (in vitro), translating these findings to in vivo models could involve several approaches. Localized ER α agonist delivery (intrathecal or nerve-targeted) in peripheral nerve injury models could be used to assess axonal regeneration and functional recovery. Additionally, ovariectomy followed by ER α agonist administration in rodent models of diabetic neuropathy could be employed to investigate its therapeutic effects on sensory deficits and promotion of nerve regeneration. These in vivo experiments could validate our in vitro findings and aid in paving the way for targeted therapies in peripheral neuropathies and nerve injuries.

Credit authorship contribution statement

Pranav Mishra: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Benedict C. Albensi:** Writing – review & editing, Visualization, Validation, Funding acquisition. **Paul Fernyhough:** Writing – review & editing, Validation, Supervision, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

P.M. and B.C.A. declare that they have no competing interests. P.F. is a cofounder and shareholder in a small biotech company, WinSanTor Inc, that is developing antimuscarinic drugs for treatment of peripheral neuropathy.

Data availability

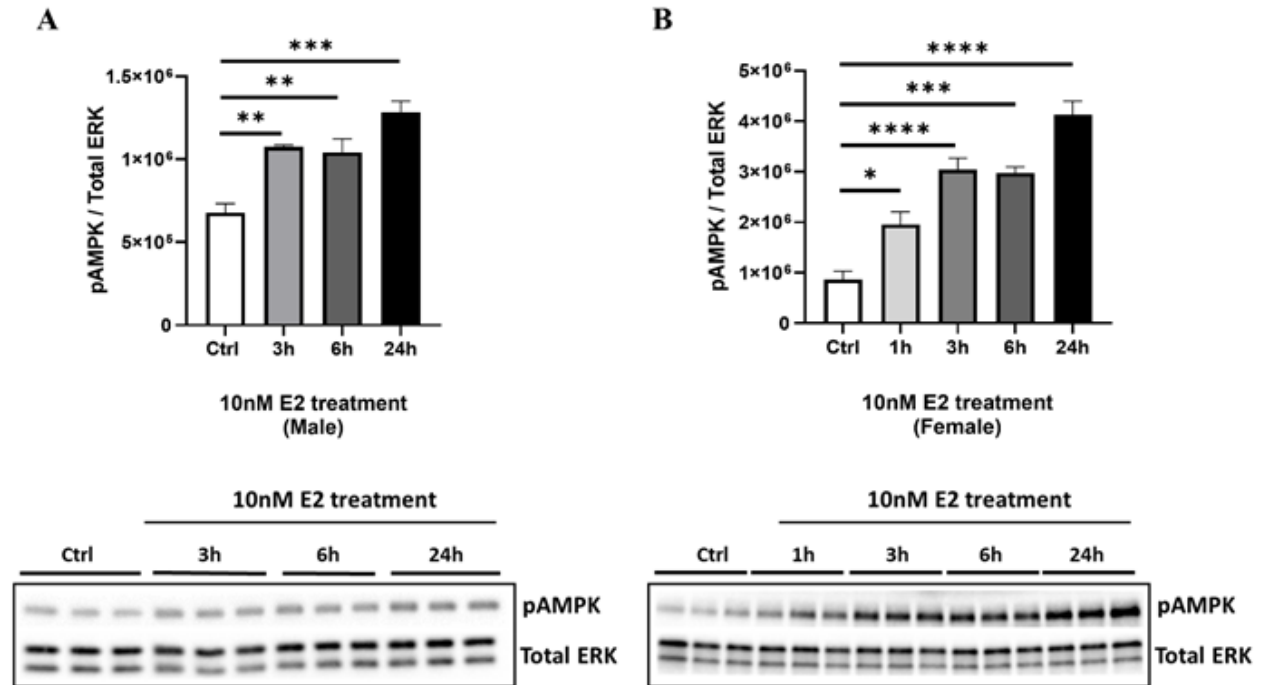
Data will be made available on request.

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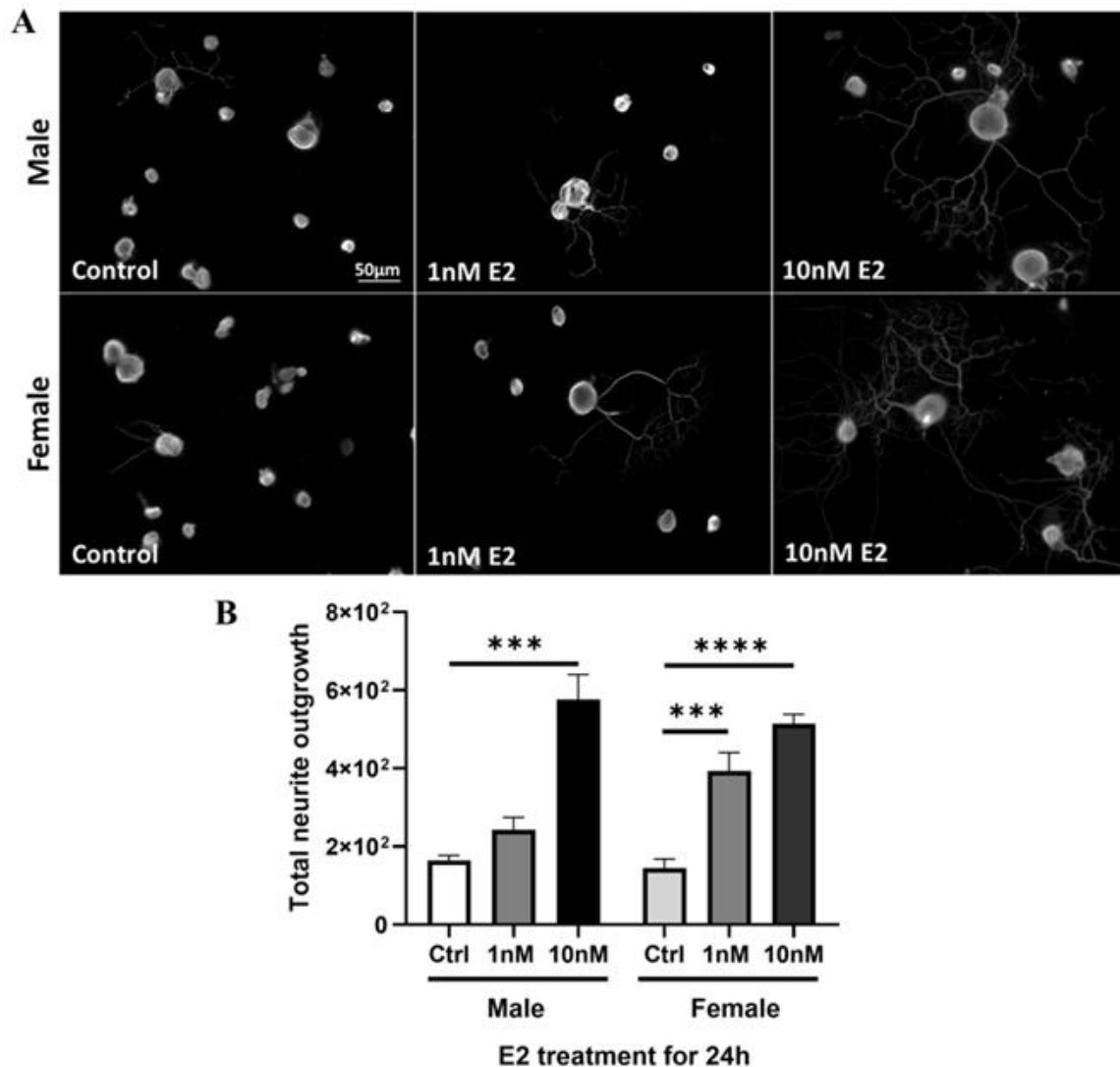
2.6 Supplementary data

Supplementary Figure S2.1.



Supplementary figure S2.1. E2 treatment increases AMPK phosphorylation in a time dependent manner in male and female cultured DRG neurons. DRG neurons derived from adult control male and female rats were treated with/without 10 nM E2 for up to 24 h. Cell lysates were subject to Western blotting. Levels of pAMPK are presented relative to Total ERK for male (**Fig A**) and female (**Fig B**) samples. Data are expressed as mean ± SEM, n = 3 replicate cultures, *p < 0.05 or **p < 0.01 or ***p < 0.001 or ****p < 0.0001, analyzed by one-way ANOVA with Dunnett's post-hoc test.

Supplementary Figure S2.2.



Supplementary figure S2.2. E2 treatment increases neurite outgrowth in male and female cultured DRG neurons. (A) DRG neurons derived from adult control male and female rats were cultured with/without 1 nM and 10 nM E2 for 24 h. Neurons were fixed and immunostained using β -tubulin III antibody. Total neurite outgrowth data is presented relative to neuron number. Data is expressed as mean $\pm$ SEM, n = 4 replicate cultures, ***p < 0.001 or ****p < 0.0001, analyzed by one-way ANOVA with Dunnett's post-hoc test.

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Transition statement two

Having explored the neuroprotective effects of estradiol in the PNS through its impact on DRG sensory neurons, I shifted the focus to the CNS. The next section presents my manuscript titled *"Estradiol prevents amyloid-beta-induced mitochondrial dysfunction and neurotoxicity in Alzheimer's disease via AMPK-dependent suppression of NF- κ B signaling."* This chapter (Chapter 3) addresses the CNS component of my research and fulfills the aims outlined in Project 2. In this study, I utilized primary cortical neurons obtained from mouse embryos to model AD pathology by treating them with A β . I examined the effects of A β on mitochondrial function, inflammation, and cell survival, simulating the neurodegenerative conditions characteristic of AD. Subsequently, I applied E2 pretreatment to assess its neuroprotective effects against A β -induced toxicity, evaluating its potential utility in treating CNS diseases. To elucidate the mechanisms underlying E2's actions, I employed pharmacological inhibitors, focusing on its interaction with the AMPK and NF- κ B pathways. This investigation provides crucial insights into how E2 protects against A β -induced neurotoxicity, further illuminating its neuroprotective roles across different neuronal populations.

CHAPTER 3

Estradiol prevents amyloid beta-induced mitochondrial dysfunction and neurotoxicity in Alzheimer's disease via AMPK-dependent suppression of NF- κ B signaling

Pranav Mishra^{1,2}, Ehsan K. Esfahani¹, Paul Fernyhough^{*,1,2}, and Benedict C. Albensi^{**,1,2,3}

¹Division of Neurodegenerative & Neurodevelopmental Disorders, St. Boniface Hospital Albrechtsen Research Centre, University of Manitoba, Winnipeg, Canada. ²Department of Pharmacology and Therapeutics, Max Rady College of Medicine, Rady Faculty of Health Sciences, University of Manitoba, Winnipeg, Canada. ³Department of Pharmaceutical Sciences, Barry & Judy Silverman College of Pharmacy, Nova Southeastern University, Fort Lauderdale, FL, USA.

*Co-senior author

**Corresponding author:

Dr. Benedict C. Albensi, Ph.D., BCMAS, CRQM

Professor and Chair,

Department of Pharmaceutical Sciences,

Barry & Judy Silverman College of Pharmacy,

Nova Southeastern University, Fort Lauderdale, FL, USA.

Division of Neurodegenerative & Neurodevelopmental Disorders,

St. Boniface Hospital Albrechtsen Research Centre,

Winnipeg, Manitoba, Canada.

Department of Pharmacology & Therapeutics, Max Rady College of Medicine,

University of Manitoba, Winnipeg, Canada.

E-mail: balbensi@nova.edu

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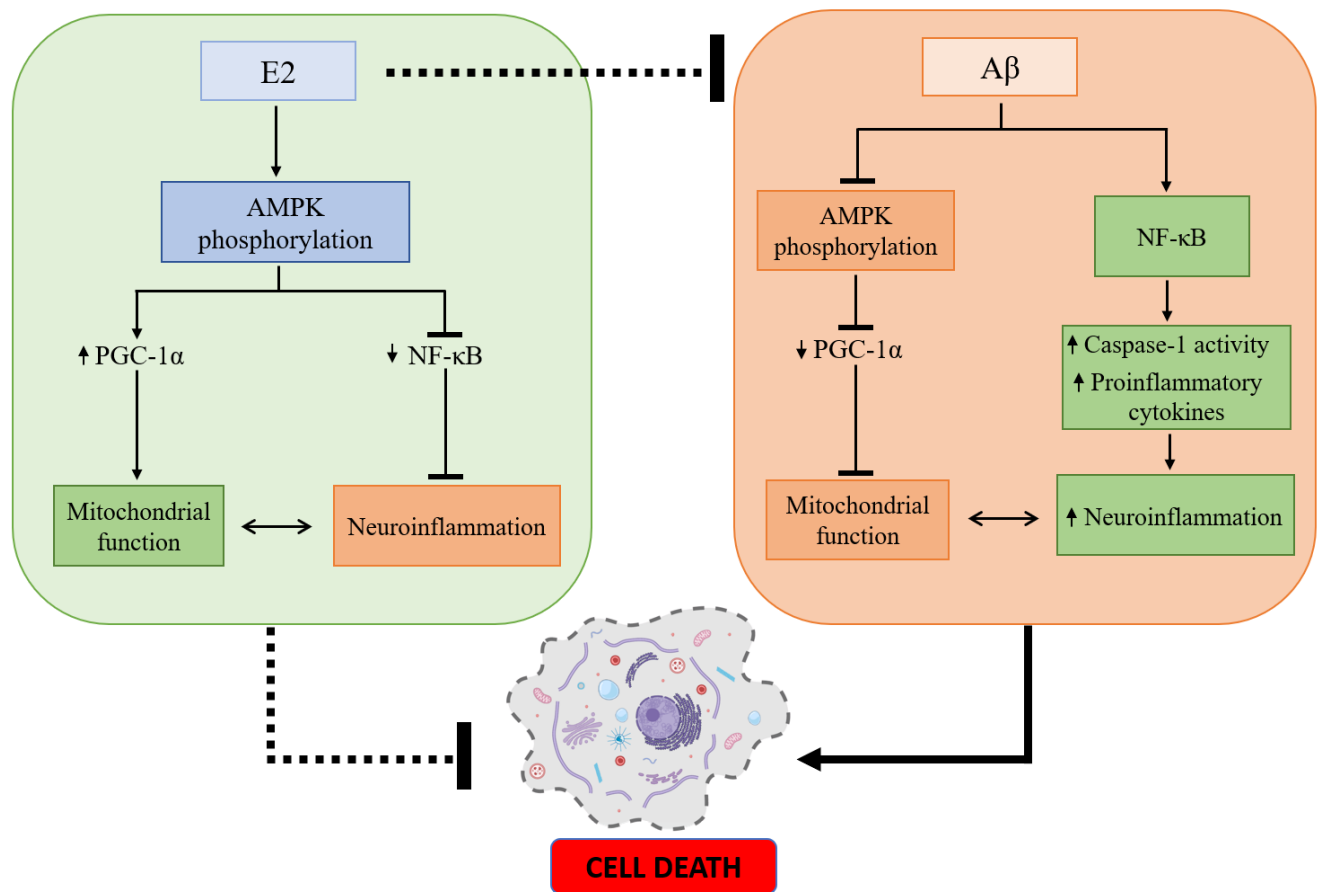
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3.1 Abstract

Alzheimer's disease (AD), the most common form of dementia, is a progressive neurodegenerative disorder characterized by memory loss and cognitive decline. In addition to its two major pathological hallmarks, extracellular amyloid beta (A β) plaques and intracellular neuro-fibrillary tangles (NFTs), recent evidence highlights the critical roles of mitochondrial dysfunction and neuroinflammation in disease progression. A β impairs mitochondrial function, which, in part, can subsequently trigger inflammatory cascades, creating a vicious cycle of neuronal damage. Estrogen receptors (ERs) are widely expressed throughout the brain, and the sex hormone 17 β -estradiol (E2), exerts neuroprotection through both anti-inflammatory and mitochondrial mechanisms. While E2 exhibits neuroprotective properties, its mechanisms against A β toxicity remain incompletely understood. In this study, we investigated the neuroprotective effects of E2 against A β -induced mitochondrial dysfunction and neuroinflammation in primary cortical neurons, with a particular focus on the role of AMP-activated protein kinase (AMPK). We found that E2 treatment significantly increased phosphorylated AMPK and upregulated the expression of mitochondrial biogenesis regulator peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α), leading to improved mitochondrial respiration. In contrast, A β suppressed AMPK and PGC-1 α signaling, impaired mitochondrial function, activated pro-inflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), and reduced neuronal viability. E2 pretreatment also rescued A β -induced mitochondrial dysfunction, suppressed NF- κ B activation, and importantly, prevented the decline in neuronal viability. However, pharmacological inhibition of AMPK using Compound C (CC) abolished these protective effects, resulting in mitochondrial collapse, elevated inflammation, and cell death, highlighting AMPK's critical role in mediating E2's actions. Interestingly, while NF- κ B inhibition

using BAY 11-7082 partially restored mitochondrial respiration, it failed to prevent A β -induced cytotoxicity, suggesting that E2's full neuroprotective effects rely on broader AMPK-dependent mechanisms beyond NF- κ B suppression alone. Together, these findings establish AMPK as a key mediator of E2's protective effects against A β -driven mitochondrial dysfunction and neuroinflammation, providing new insights into estrogen-based therapeutic strategies for AD.

Graphical abstract



E2 prevents Aβ-induced neuronal death through AMPK pathway activation. E2 promotes AMPK phosphorylation, upregulating PGC-1α, enhancing mitochondrial function, and suppressing NF-κB-mediated neuroinflammation. In contrast, Aβ inhibits AMPK, downregulates PGC-1α, impairs mitochondrial function, and activates NF-κB signaling, leading to neuroinflammation and cell death. E2 counteracts Aβ toxicity primarily by restoring AMPK signaling.

Highlights

- Estradiol (E2) rescues A β -induced mitochondrial dysfunction.
- E2 suppresses A β -induced NF- κ B-mediated neuroinflammation.
- AMPK inhibition abolishes E2's neuroprotective effects against A β -mediated toxicity.
- AMPK and estrogen pathways are promising therapeutic targets for Alzheimer's disease (AD).

3.2 Introduction

Alzheimer's disease (AD), the most prevalent neurodegenerative disorder, is marked by progressive synaptic loss, neuronal death, and cognitive impairments (Jahn, 2013; Santiago and Potashkin, 2021). These changes lead to deficits in memory and behavior, ultimately interfering with daily functioning and quality of life (Howe, 2008). In 2019, over 57 million people worldwide were affected by dementia, with AD accounting for approximately 70% of cases. This number is projected to rise to 152 million by 2050, posing a growing global public health crisis (Emma *et al.*, 2022). AD is characterized by two pathological hallmarks: extracellular amyloid-beta ($A\beta$) plaques and intracellular neurofibrillary tangles (NFTs) (Gallardo and Holtzman, 2019). While the disease affects various brain regions, damage typically begins in the cortex and hippocampus that are areas critical for higher cognitive functions including memory formation (Braak *et al.*, 2006; Frisoni *et al.*, 1999). In addition to $A\beta$ and NFTs, recent research has identified mitochondrial dysfunction and chronic neuroinflammation as key contributors to AD progression (Amelimojarad *et al.*, 2024; Ashleigh *et al.*, 2023; Zhu *et al.*, 2004). These processes are interlinked, as mitochondrial impairment can initiate and/or exacerbate inflammatory signaling, and sustained neuroinflammation can in turn further disrupt mitochondrial function (Di Filippo *et al.*, 2010).

Some investigators contend that $A\beta$ accumulation is a central pathological feature of AD and also contributes directly to mitochondrial dysfunction by disrupting oxidative phosphorylation (OXPHOS) and reducing adenosine triphosphate (ATP) production, ultimately causing synaptic failure and neuronal loss (Chen and Yan, 2007; Reddy and Beal, 2008; Wang *et al.*, 2020). In parallel, $A\beta$ also activates inflammatory signaling cascades within neurons (Salminen *et al.*, 2008), and involves the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway (Valerio *et al.*, 2006). NF- κ B, a key regulator of pro-inflammatory gene expression, is aberrantly

activated in early AD, with elevated p65 levels observed in neurons and astrocytes near amyloid plaques (Boissière *et al.*, 1997; Kaltschmidt *et al.*, 2022). Under normal conditions, NF- κ B remains sequestered in the cytoplasm by its inhibitor, I κ B α . Upon triggering by pathological stimuli such as A β , I κ B α becomes phosphorylated and undergoes proteasomal degradation, allowing the release and nuclear translocation of NF- κ B (Snow and Albeni, 2016). Once in the nucleus, NF- κ B binds to specific DNA sequences and promotes the transcription of pro-inflammatory mediators, driving neuroinflammation and contributing to disease progression. Interestingly, NF- κ B also influences mitochondrial function by regulating genes involved in mitochondrial biogenesis and metabolism (Cherry and Piantadosi, 2015). Beyond its nuclear role, NF- κ B subunits have been detected within mitochondria, suggesting a direct role in mitochondrial gene regulation (Albeni, 2019; Bottero *et al.*, 2001; Cogswell *et al.*, 2003; Zamora *et al.*, 2004). Dysfunctional mitochondria release mitochondrial DNA (mtDNA) and other damage-associated molecular patterns (DAMPs), which trigger inflammatory responses (Roh and Sohn, 2018). This creates a vicious cycle where neuroinflammation and mitochondrial dysfunction reinforce each other, forming a self-perpetuating cycle of neurodegeneration (Van Horssen *et al.*, 2019).

Estrogen, a steroid hormone primarily known for its role in reproduction, also exerts significant effects on the central nervous system (CNS) (McEwen *et al.*, 2012). Among the three main endogenous estrogens, 17 β -estradiol (E2) is the most potent form (Wise *et al.*, 2009). Estrogen receptors (ERs) are widely distributed across brain regions, where they regulate neuronal growth, synaptic plasticity, and neuroinflammatory responses, thereby supporting neural health and cognitive function (Brinton, 2009; Warfvinge *et al.*, 2020). The widespread presence of ERs highlights the potential regulatory role of estrogen in maintaining brain health. E2 possesses significant neuroprotective properties (Garcia-Segura *et al.*, 2001) and promotes neuronal survival

by upregulating neurotrophic factors, enhancing mitochondrial function, and attenuating oxidative stress (Azcoitia *et al.*, 2019; Klinge, 2017; Kövesdi *et al.*, 2020; Son *et al.*, 2020). E2 also regulates cellular energy homeostasis through the activation of AMP-activated protein kinase (AMPK) and its downstream target peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α), both of which are essential for mitochondrial biogenesis (Halling and Pilegaard, 2020; Yang and Wang, 2015). Additionally, E2 exerts anti-inflammatory effects by suppressing NF- κ B mediated gene expression and modulating microglial activity, thereby limiting neuroinflammation (Murphy *et al.*, 2010; Saeed *et al.*, 2021). Collectively, these multifaceted actions highlight the critical role of E2 in maintaining neuronal integrity in the context of neurodegenerative disorders.

AMPK plays a dual role in maintaining mitochondrial integrity and regulating inflammatory responses. By activating PGC-1 α , AMPK promotes mitochondrial biogenesis and enhances OXPHOS, which are essential for neuronal energy homeostasis (Canto and Auwerx, 2009; Herzig and Shaw, 2018). Additionally, AMPK exerts anti-inflammatory effects across various cell types, including human aortic smooth muscle cells (HASMCs) and macrophages (Cui *et al.*, 2023; He *et al.*, 2015; Ku and Cheng, 2020). Notably, E2 has been identified as an upstream activator of AMPK, thereby promoting metabolic efficiency and mitochondrial resilience (Kim *et al.*, 2012; Martínez de Morentin *et al.*, 2014). In this study, we investigated whether E2 protects neurons from A β toxicity and explored the involvement of AMPK signaling and NF- κ B mediated inflammation in this process. Using primary cortical neurons exposed to A β , we assessed mitochondrial respiration, ATP production, inflammation, and neuronal viability. To delineate the contribution of each pathway, we employed pharmacological inhibitors of AMPK and NF- κ B. This approach enabled us to dissect the distinct and overlapping roles of AMPK and NF- κ B signaling in mediating E2's protective effects against A β toxicity. Overall, our findings suggest that E2 protects neurons from

A β -induced mitochondrial dysfunction and inflammation through an AMPK-dependent mechanism. This not only advances our understanding of E2's neuroprotective pathways, but also identifies AMPK as a potential therapeutic target in AD. Targeting AMPK activation or modulating estrogen signaling may therefore offer a promising strategy for mitigating neurodegeneration in affected individuals.

3.3 Materials and Methods

3.3.1 Cell culture

Primary neuron cultures were prepared from the cortices of embryonic day 18.5 (E18.5) C57BL/6N mice, using methods previously described (Hunt *et al.*, 2010). All animal procedures were conducted in accordance with the guidelines set by the University of Manitoba's Animal Care Committee and the Canadian Committee on Animal Care (CCAC). Cells were seeded onto poly-D-lysine-coated cell culture plates at specific densities: 500,000 cells per well for 12 well plates and 50,000 cells per well for 96-well plates. The culture medium used was Neurobasal-A Medium, with no D-glucose, no sodium pyruvate (21103049, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and supplemented with 3mM D-glucose (G7528, MilliporeSigma Canada Ltd, Oakville, Ontario, Canada), 0.22mM sodium pyruvate (11360070, Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1x B-27 (A3582801, Thermo Fisher Scientific, Waltham, Massachusetts, USA), 100µg/ml penicillin-streptomycin (SV30010, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and 5mM HEPES (SH3023701, Thermo Fisher Scientific, Waltham, Massachusetts, USA) (NB media). Five percent heat inactivated charcoal-stripped fetal bovine serum (FBS) and 1x Glutamax (35050079, Thermo Fisher Scientific, Waltham, Massachusetts, USA) were added during initial seeding. FBS was removed from the cultures the following day. To prevent glial cell proliferation, cytosine arabinofuranoside (2µM) (C1768, MilliporeSigma Canada Ltd, Oakville, Ontario, Canada) was added to the cultures on day 2. The culture media was replaced on day 3 with fresh NB media supplemented with 1x Glutamax. Cultures were maintained in a humidified incubator at 37°C with 5% CO₂. The media was partially changed every 48 hours and cultures were used for experiments on day 7. For pharmacological interventions, the following drugs and inhibitors were used: 10nM 17 beta-estradiol (E2) (E2758,

MilliporeSigma Canada Ltd, Oakville, Ontario, Canada), 10 μ M A β (A-1163-2, rPeptide, Watkinsville, GA, USA), 5 μ M compound C (CC) (171261, MilliporeSigma Canada Ltd, Oakville, Ontario, Canada) to inhibit AMPK, and 3 μ M BAY 11-7082 (196871, MilliporeSigma Canada Ltd, Oakville, Ontario, Canada) was used to inhibit phosphorylation of I κ B α (thus blocking NF- κ B activation).

3.3.2 Amyloid beta preparation

Monomeric A β ₁₋₄₂ peptide was obtained from rPeptide (A-1163, rPeptide, Watkinsville, GA, USA; sequence: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA), expressed recombinantly in *E. coli* and purified to >97% by mass spectrometry to ensure high purity and consistency. The peptide was supplied as a clear dry film, pretreated with hexafluoroisopropanol (HFIP), which disrupts pre-existing aggregates and yields a highly monomeric starting material. The lyophilized peptide film was resuspended in dimethyl sulfoxide (DMSO) (276855, MilliporeSigma Canada Ltd, Oakville, Ontario, Canada) to a final concentration of 10mM and sonicated in a water bath for 10 minutes to ensure complete dissolution. The resulting stock solution was aliquoted and stored at -80 °C. For oligomer formation, the 10mM stock was diluted to 10 μ M in cell culture media and incubated at 4°C for 24 hours, following a published protocol with minor modifications (Dahlgren *et al.*, 2002). This protocol is widely used to generate A β oligomers, which are considered the most neurotoxic species relevant to AD pathology. The A β oligomer solution was used to treat cells, ensuring that the final DMSO concentration was less than 0.1%.

3.3.3 Quantitative Western blotting

To analyze protein expression levels, cell lysates were harvested and homogenized in ice-cold RIPA buffer (ab156034, Abcam, Waltham, Massachusetts, USA) containing 1% nonylphenol, 0.8% sodium chloride, 0.6% Tris (pH 7.5), 0.5% sodium deoxycholate, 0.1% sodium pyrophosphate decahydrate, 0.11% sodium lauryl sulfate, 0.03% EGTA, 0.03% EDTA, 0.03% disodium dihydroxypropyl phosphate, 0.01% sodium orthovanadate, and a protease and phosphatase inhibitor cocktail (78440, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Nuclear proteins were extracted using the nuclear extraction kit (78833, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. A detergent-compatible (DC) colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA) was used to determine protein concentrations. Proteins were resolved and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (1620112, Bio-Rad, Hercules, CA, USA) using the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). Membranes were immunoblotted with specific primary antibodies overnight at 4°C. We also used the following primary antibodies: phosphorylated AMPK (pAMPK) Thr172 (1:1000, Cell Signaling, Danvers, MA, USA), Total AMPK (T-AMPK) (1:5000, Abcam, Waltham, MA, USA), Total OxPhos (1:1000, Thermo Fisher Scientific, Waltham, MA, USA; antibody cocktail against complexes I to V), β -actin (1:1000, Cell Signaling, Danvers, MA, USA), PGC-1 α (1:1000, MilliporeSigma Canada Ltd, Oakville, ON, Canada), Phospho-IK β (1:1000, Cell Signaling, Danvers, MA, USA), NF- κ B p65 (1:1000, Santa Cruz Biotechnology Inc., Dallas, TX, USA), and Lamin A/C (1:1000, Santa Cruz Biotechnology Inc., Dallas, TX, USA). After primary antibody incubation, membranes were incubated with HRP-conjugated secondary antibodies; goat anti-rabbit IgG (H + L) or goat

anti-mouse IgG (H + L) at 1:5000 dilution (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hour at room temperature. Total protein was visualized using chemiluminescent imaging of the blot following gel activation (TGX Stain-Free FastCast Acrylamide Solutions, Bio-Rad, Hercules, CA, USA) in addition to β -actin for target protein normalization. Blots were developed using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) or SignalFire ECL Reagent (Cell Signaling, Danvers, Massachusetts, USA) and imaged using a ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA).

3.3.4 Seahorse assay to measure mitochondrial respiration

Mitochondrial respiration in primary embryonic cortical neurons was assessed using the Seahorse XF24 Analyzer (Agilent Technologies Inc, Santa Clara, California, USA). Neurons were cultured on poly-D-lysine-coated Seahorse XF24 plates at a density of 50,000 cells per well for a total of 7 days before treatments were added and the experiment was conducted. One hour prior to the assay, the culture medium was replaced with unbuffered DMEM (Dulbecco's modified Eagle's medium, pH 7.4) supplemented with 0.22mM sodium pyruvate and 3mM D-glucose. The Seahorse assay measured basal mitochondrial oxygen consumption rate (OCR), maximal respiration, and spare respiratory capacity. Three drug cocktails containing mitochondrial complex inhibitors were sequentially injected through ports of the Seahorse FluxPak cartridges: oligomycin (1 μ M), carbonyl cyanide p-trifluoro methoxyphenylhydrazone (FCCP) (2 μ M), and rotenone/antimycin A (1 μ M). Oligomycin irreversibly inhibits ATP synthase, FCCP acts as an uncoupler, and rotenone and antimycin A inhibit Complex I and III of the electron transport chain (ETC), respectively. All four drugs were obtained from MilliporeSigma, Canada, Oakville, Ontario, Canada. Following OCR measurements, cell lysates were harvested individually from each well in RIPA buffer, and

a DC protein assay was performed. OCR measurements were normalized to the amount of protein in each well and reported as pmole/min/mg protein.

3.3.5 MTT assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (ab211091, Abcam, Waltham, MA, USA) according to the manufacturer's protocol. This colorimetric assay relies on the reduction of MTT to formazan by metabolically active cells, providing a quantitative measure of cell viability based on metabolic activity. Primary cortical neurons were seeded onto poly-D-lysine-coated 96 well plates and cultured under conditions previously described. Cells were pretreated with or without 10nM E2 for 48 hours, followed by treatment with 10 μ M A β for an additional 48 hours. After treatment, the media was discarded, and 50 μ L of serum-free media along with 50 μ L of MTT reagent was added to each well. The plate was incubated at 37°C for 3 hours. Following incubation, 150 μ L of MTT solvent was added to each well. The plate was wrapped in foil to protect from light and shaken for 30 minutes to ensure complete solubilization of the formazan crystals. Absorbance was measured at 590 nm using a BioTek Synergy Neo2 (Agilent Technologies Inc., Santa Clara, CA, USA) plate reader to determine cell viability.

3.3.6 LDH assay

Cytotoxicity was evaluated using the LDH Cytotoxicity Assay Kit (C20300, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. This colorimetric assay relies on the conversion of lactate to pyruvate by lactate dehydrogenase (LDH), which is released into the culture medium upon cell membrane damage, providing a quantitative measure of cytotoxicity based on the extent of cellular damage. Primary cortical neurons were seeded on to

96 well plates and treated as previously described. After treatment, 50 μ L of media from each well was transferred to a new 96 well plate, and 50 μ L of freshly prepared reaction mixture was added. The plate was gently mixed while avoiding bubble formation and incubated at room temperature for 30 minutes in the dark. Following incubation, 50 μ L of stop solution was added to each well and mixed by gentle tapping. Absorbance was measured at 490nm and 680nm using the BioTek Synergy Neo2 microplate reader to determine LDH activity, which correlates with the level of cellular damage.

3.3.7 NF- κ B p65 activation assay

NF- κ B activation was assessed using the NF- κ B p65 Transcription Factor Assay Kit (ab133112, Abcam, Waltham, MA, USA), an enzyme-linked immunosorbent assay (ELISA) based method that quantifies the binding of the NF- κ B p65 subunit to a specific double-stranded DNA (dsDNA) sequence containing the NF- κ B response element immobilized on a 96-well plate. Nuclear extracts were prepared from control and treated primary cortical neurons using the Nuclear Extraction Kit (78833, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Extracts were added to the wells and incubated overnight at 4°C to allow specific binding of NF- κ B p65 to the immobilized DNA. After washing, a primary antibody specific to NF- κ B p65 (provided in the kit) was added and incubated for 1 hour at room temperature on an orbital shaker. Following additional washes, an HRP-conjugated secondary antibody was added and incubated under the same conditions. After final washes, 100 μ L of developing solution was added to each well and incubated for 30 minutes at room temperature with shaking. The reaction was stopped with 100 μ L of stop solution, and absorbance was measured at 450nm using a microplate reader to quantify NF- κ B p65 activation. Data normalization was performed using total protein concentrations of the nuclear extracts, measured by a DC protein assay.

3.3.8 Measurement of Caspase-1 activity

Caspase-1 is activated downstream of the NLRP3 inflammasome and plays a central role in the maturation of pro-inflammatory cytokines such as IL-1 β . Caspase-1 activity, a hallmark of inflammasome activation, was quantified using the Caspase-Glo-1 Inflammasome Assay Kit (G9951, Promega, Madison, WI, USA), according to the manufacturer's protocol. Neurons were seeded on 96-well plates and treated as previously described. Following treatment, the plates were removed from the incubator and equilibrated to room temperature for 10 minutes. Next, Caspase-Glo-1 Reagent or Caspase-Glo-1 YVAD-CHO Reagent was added to each well. The Caspase-Glo-1 Reagent contains MG-132 to inhibit proteasome-related nonspecific activity, while the Caspase-Glo-1 YVAD-CHO Reagent includes both MG-132 and the selective caspase-1 inhibitor Ac-YVAD-CHO to confirm the caspase-1 specificity of the observed luminescent signal. Plates were gently mixed on a plate shaker at 450 rpm for 5 minutes and then incubated at room temperature for 1 hour, protected from light by wrapping in foil. Luminescence was measured using a microplate reader, and signal intensity was used to assess caspase-1 activity.

3.3.9 ATP measurement

Cellular ATP levels were quantified using the luminescent ATP Determination Kit (A22066, Thermo Fisher Scientific, Waltham, MA, USA), which utilizes firefly luciferase to catalyze the reaction of D-luciferin with ATP, producing light. The luminescence generated is directly proportional to ATP concentration, enabling sensitive detection of cellular energy levels. Cells were cultured and treated as described. After treatment, cultures were washed with ice-cold PBS and lysed to release intracellular ATP. The lysates were centrifuged at 14,000 rpm for 10 minutes, and the supernatants were collected for ATP quantification according to the manufacturer's guidelines. Appropriate background controls were included to subtract background luminescence.

Samples were read at 560nm using a microplate reader. ATP levels were normalized to total protein levels in each sample.

3.3.10 ELISA

Cytokine levels in cell culture supernatants were measured using commercially available ELISA kits for mouse IL-10 (ab100697), TNF α (ab208348), IL-6 (ab222503) (all from Abcam, Waltham, MA, USA), IL-18 (BMS618-3, Thermo Fisher Scientific, Waltham, MA, USA) and IL-1 β (BMS6002, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturers' instructions. Interleukin concentrations were calculated based on standard curves generated for each respective cytokine and reported as fold change relative to the untreated control group.

3.3.11 Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SEM) and analyzed using one-way ANOVA followed by Dunnett's or Tukey's *post hoc* tests, as indicated. For comparisons between multiple treatment groups, one-way ANOVA followed by Tukey's *post hoc* test was used. Statistical analyses were performed using GraphPad Prism (version 8.0) software. A P value of less than 0.05 (< 0.05) was considered statistically significant in all experiments.

3.4 Results

3.4.1 E2 activates AMPK in a time dependent manner and enhances PGC-1 α levels

To assess whether E2 modulates energy metabolism in neurons, we examined its effect on AMPK activation. Treatment of primary cortical neurons with E2 at doses of 0.1 nM, 1 nM, and 10 nM for 1 hour induced a significant, increase in AMPK phosphorylation (pAMPK) at Thr172 relative to total AMPK (Fig. 3.1A). Maximal activation was observed at 10 nM E2, which increased pAMPK levels by approximately 4-fold compared to untreated controls. A time-course study revealed that 10 nM E2 rapidly elevated pAMPK within 30 minutes (Fig. 3.1B). Sustained AMPK activation persisted for up to 24 hours (Fig. 3.1C), indicating long-term modulation of energy metabolism pathways. Given that AMPK is a key upstream regulator of PGC-1 α (Jäger *et al.*, 2007), we assessed PGC-1 α protein expression in response to E2. Acute exposure to 10 nM E2 for 30–60 minutes significantly increased PGC-1 α levels (normalized to β -actin; Fig. 3.1D). This elevation was maintained over prolonged treatments, with PGC-1 α levels remaining elevated at 6 and 24 hours (Fig. 3.1E).

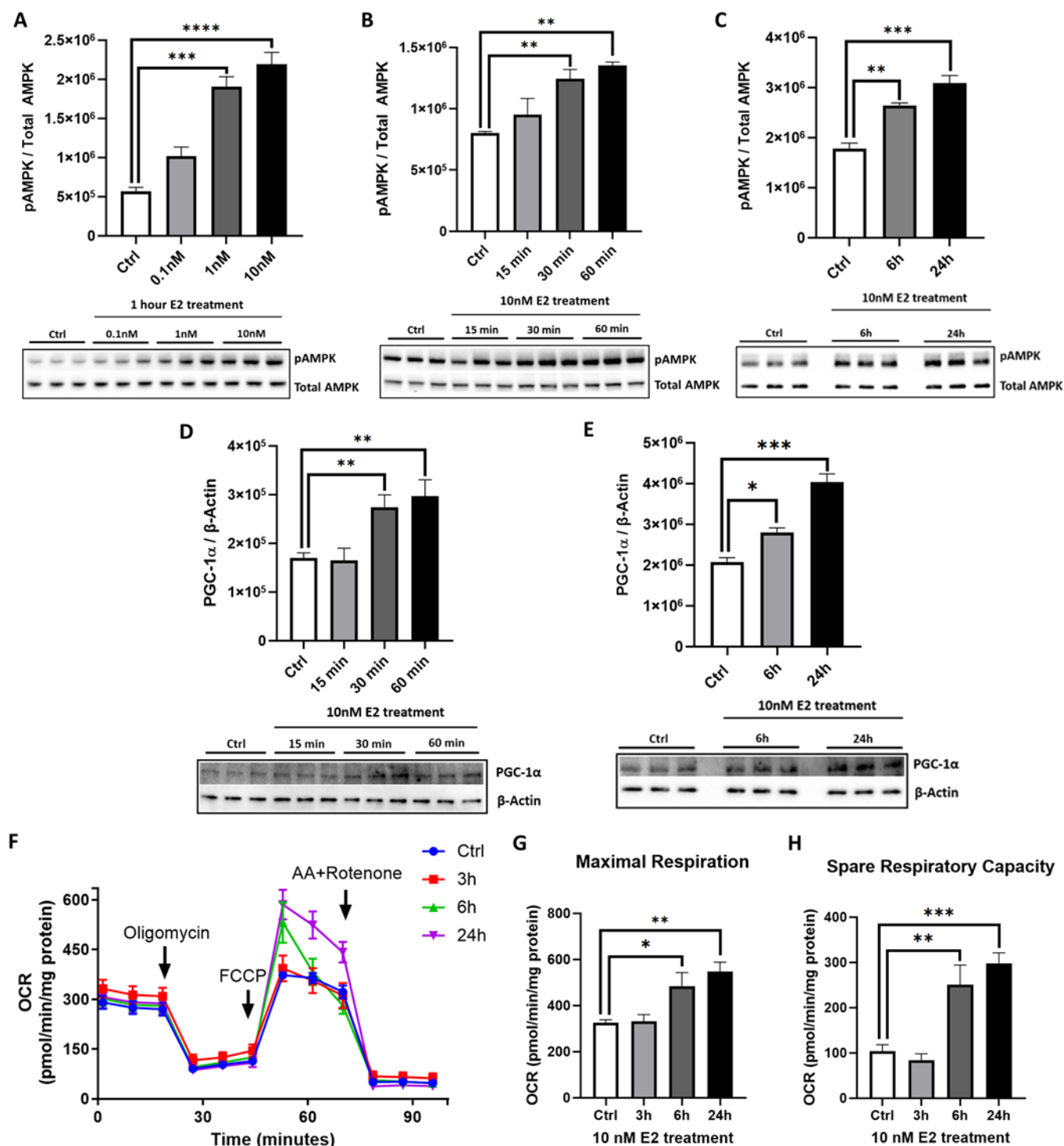


Figure 3.1. E2 activates AMPK, increases PGC-1 α levels, enhances mitochondrial function in primary cortical neurons. Primary cortical neurons were treated with or without E2 at various doses (0.1–10 nM) and for different durations (15 min to 24 h) as indicated. E2 was dissolved in

DMSO (vehicle, final concentration <0.1%). Cell lysates were analyzed by Western blotting for (A–C) pAMPK (normalized to total AMPK), and (D–E) PGC-1 α (normalized to β -actin). (F–H) Mitochondrial oxygen consumption rate (OCR), maximal respiration, and spare respiratory capacity were measured via Seahorse XF24 Analyzer after 3h, 6h, and 24h E2 treatment and OCR (O₂ in pmol/min) was normalized to protein concentration per well. Data are expressed as mean $\pm$ SEM, with n=3 replicate cultures for Western blot analyses (A–E) and n=5 replicate cultures for Seahorse assays (F–H). Statistical significance was determined by one-way ANOVA with Dunnett's *post hoc* test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.4.2 E2 enhances mitochondrial respiration and suppresses NF- κ B subunit expression

To assess functional mitochondrial outcomes, we measured OCR in live cortical neurons using the Seahorse XF24 Analyzer. Treatment with 10 nM E2 for 6 and 24 hours significantly increased both maximal respiration and spare respiratory capacity (Fig. 3.1F–G), indicating enhanced mitochondrial efficiency and metabolic flexibility. In addition to its effects on energy metabolism, E2 also modulated inflammatory signaling pathways. Notably, E2 suppressed NF- κ B activation, as evidenced by a time-dependent reduction in phosphorylated I κ B α (pI κ B α), a marker of NF- κ B pathway activation (Fig. 3.2). Acute E2 treatment for up to 60 minutes significantly reduced pI κ B α levels (Fig. 3.2A), with sustained suppression observed at 6 and 24 hours (Fig. 3.2B). Together, these findings suggest that E2 enhances mitochondrial function while also attenuating initial NF- κ B-driven neuroinflammatory signaling.

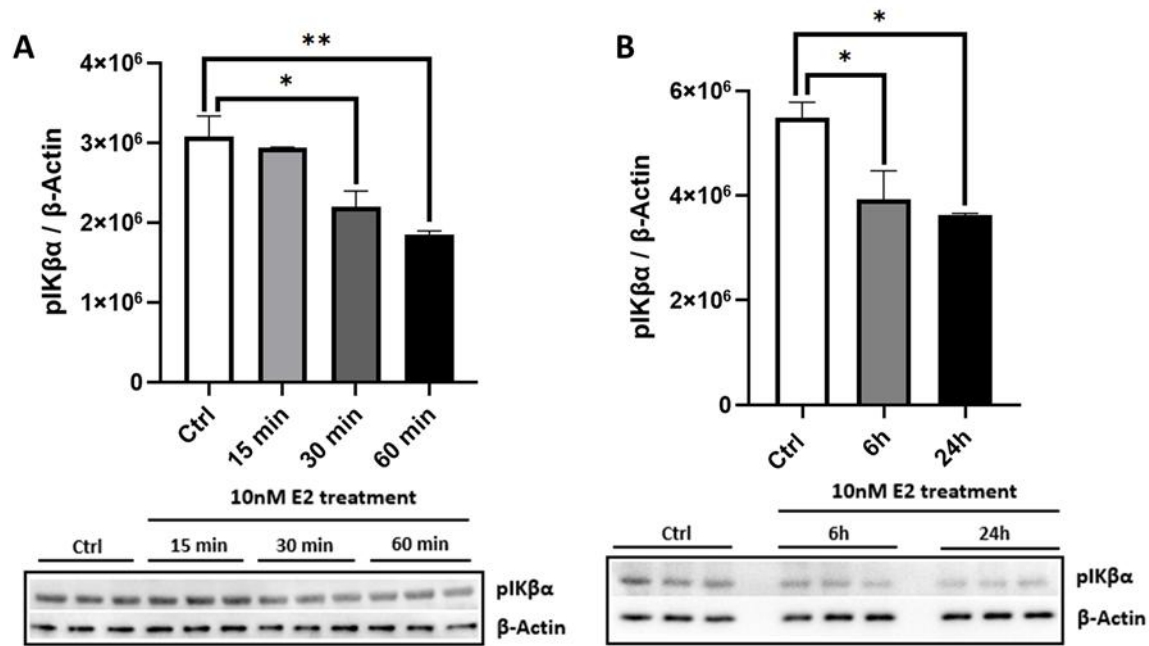


Figure 3.2. E2 suppresses NF-κB signaling in primary cortical neurons. Primary cortical neurons were treated with 10 nM E2 for various durations (15 min to 24 h). Cell lysates were analyzed by Western blotting for **(A)** acute (15–60 min) and **(B)** prolonged (6–24 h) changes in phosphorylated IκBα (pIKβα) levels, normalized to β-actin. Data are expressed as mean ± SEM, with n=3 replicate cultures. Statistical significance was determined by one-way ANOVA with Dunnett’s *post hoc* test; *p<0.05, **p<0.01.

3.4.3 A β suppresses AMPK activation, reduces PGC-1 α levels, and impairs mitochondrial function

To determine the pathological impact of A β on neuronal energy regulation, we examined AMPK signaling in primary cortical neurons. Exposure to A β induced a time-dependent suppression of AMPK phosphorylation in primary cortical neurons, with significant reductions observed at 3, 6, and 24 hours (Fig. 3.3A). Additionally, A β decreased PGC-1 α levels by nearly 50% at 24 hours (Fig. 3.3B). Functional assessment of mitochondrial respiration using the Seahorse XF24 Analyzer revealed that A β impaired the OCR, significantly reducing basal respiration, maximal respiration, and ATP production (Fig. 3.3C–F), consistent with mitochondrial dysfunction (Reddy and Beal, 2008).

3.4.4 A β activates NF- κ B and reduces cell viability

A β triggered NF- κ B pathway activation, as demonstrated by a time-dependent reduction in pI κ B α levels (which normally facilitates subsequent NF- κ B nuclear translocation; Fig. 3.3G). This proximal pro-inflammatory response coincided with a significant reduction in neuronal viability. A β exposure for 48 hours reduced cell viability to about 60% of control levels (Fig. 3.3H).

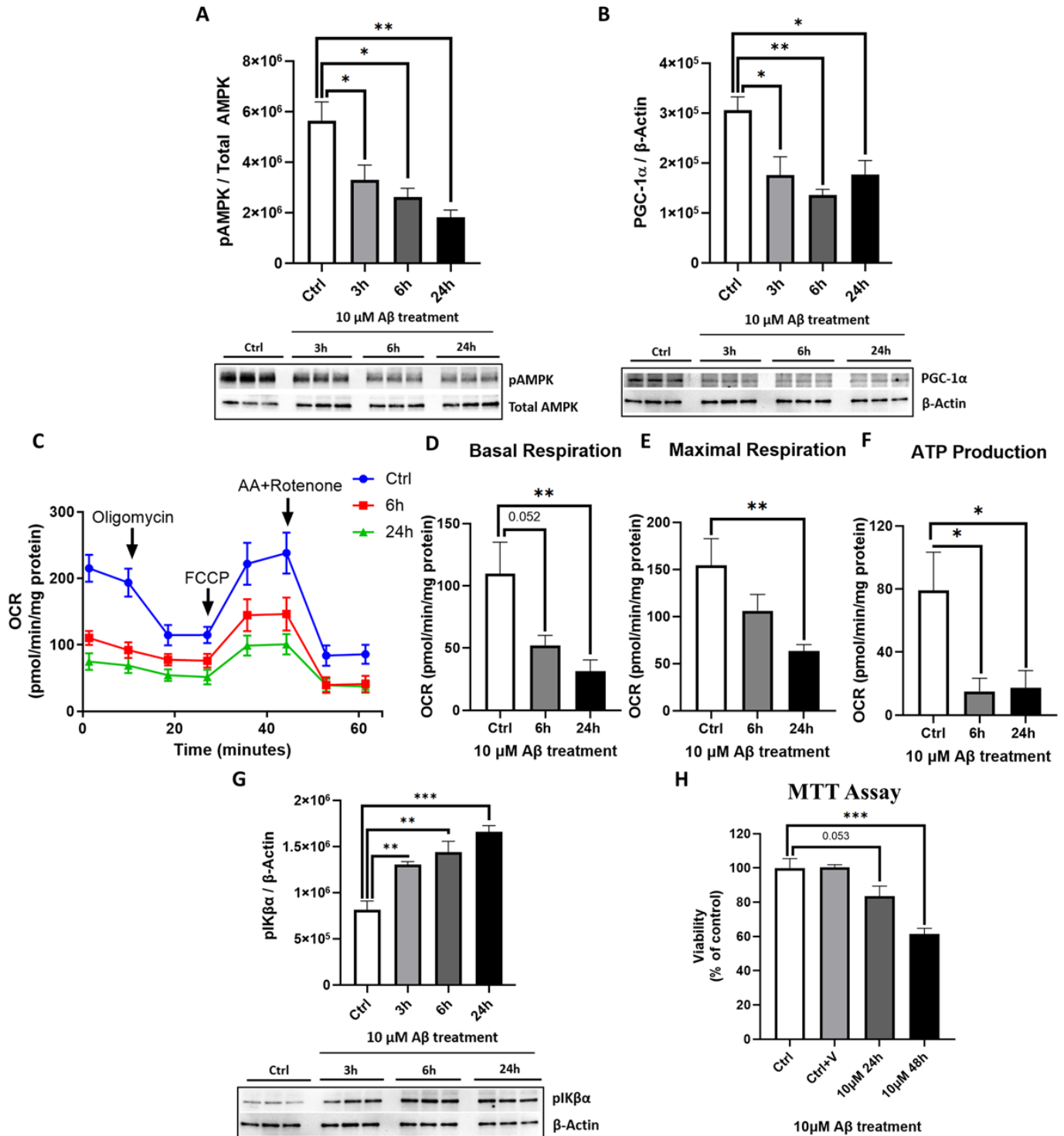


Figure 3.3. Aβ suppresses AMPK activation, decreases PGC-1α levels, impairs mitochondrial function, activates NF-κB, and reduces cell viability in primary cortical neurons. Primary cortical neurons were treated with 10 μM Aβ for 3, 6, or 24 hours as indicated.

Cell lysates were analyzed by Western blotting for **(A)** pAMPK (normalized to total AMPK), **(B)** PGC-1 α (normalized to β -actin), and **(G)** pI κ B α (normalized to β -actin). **(C–F)** OCR, including basal respiration, maximal respiration, and ATP production, was measured using the Seahorse XF24 Analyzer after 6 and 24 hours of A β treatment (OCR was normalized to protein concentration per well). **(H)** Cell viability was assessed by MTT assay after 24 or 48 hours of A β treatment, with data shown as a percentage of the control group. Data are expressed as mean $\pm$ SEM, with n=3 replicate cultures for Western blot analyses (A,B,G), n=6–7 replicate cultures for Seahorse assays (C–F), and n=4 replicate cultures for MTT assay (H). Statistical significance was determined by one-way ANOVA with Dunnett's *post hoc* test; *p<0.05, **p<0.01, ***p<0.001.

3.4.5 E2 pretreatment rescues A β -induced mitochondrial dysfunction, restores AMPK and PGC-1 α signaling, and preserves mitochondrial electron transport chain integrity

To investigate whether E2 pretreatment could counteract A β toxicity, primary cortical neurons were pretreated with 10 nM E2 for 48 hours prior to 24-hour A β exposure. A β significantly reduced AMPK phosphorylation, while E2 pretreatment maintained pAMPK levels (Fig. 3.4A). Similarly, A β suppressed PGC-1 α expression, but E2 pretreatment prevented the suppression of PGC-1 α levels (Fig. 3.4B), indicating protection of pathways involved in cellular energy homeostasis and mitochondrial biogenesis. Functional assessment of mitochondrial respiration in live cells via the Seahorse XF24 Analyzer revealed that A β markedly impaired mitochondrial respiration, reducing basal respiration, maximal respiration, and spare respiratory capacity (Fig. 3.4C–G). E2 pretreatment showed a trend toward reversing these impairments and improving all OCR parameters, although the changes did not reach statistical significance. Consistent with these findings, A β significantly reduced cellular ATP levels by approximately 50%, which were rescued by E2 pretreatment (Fig. 3.4H). To further investigate the impact on mitochondrial structure and function, we analyzed the protein levels of ETC complex subunits. A β exposure to primary cortical neurons significantly decreased mitochondrial ETC complex subunit levels, including proteins of Complex I (NDUFB8), Complex II (SDHB), Complex IV (MTCO1), and Complex V (ATP5A) (Fig. 3.5A–E). E2 pretreatment prevented this decline, restoring all four ETC complex subunit levels.

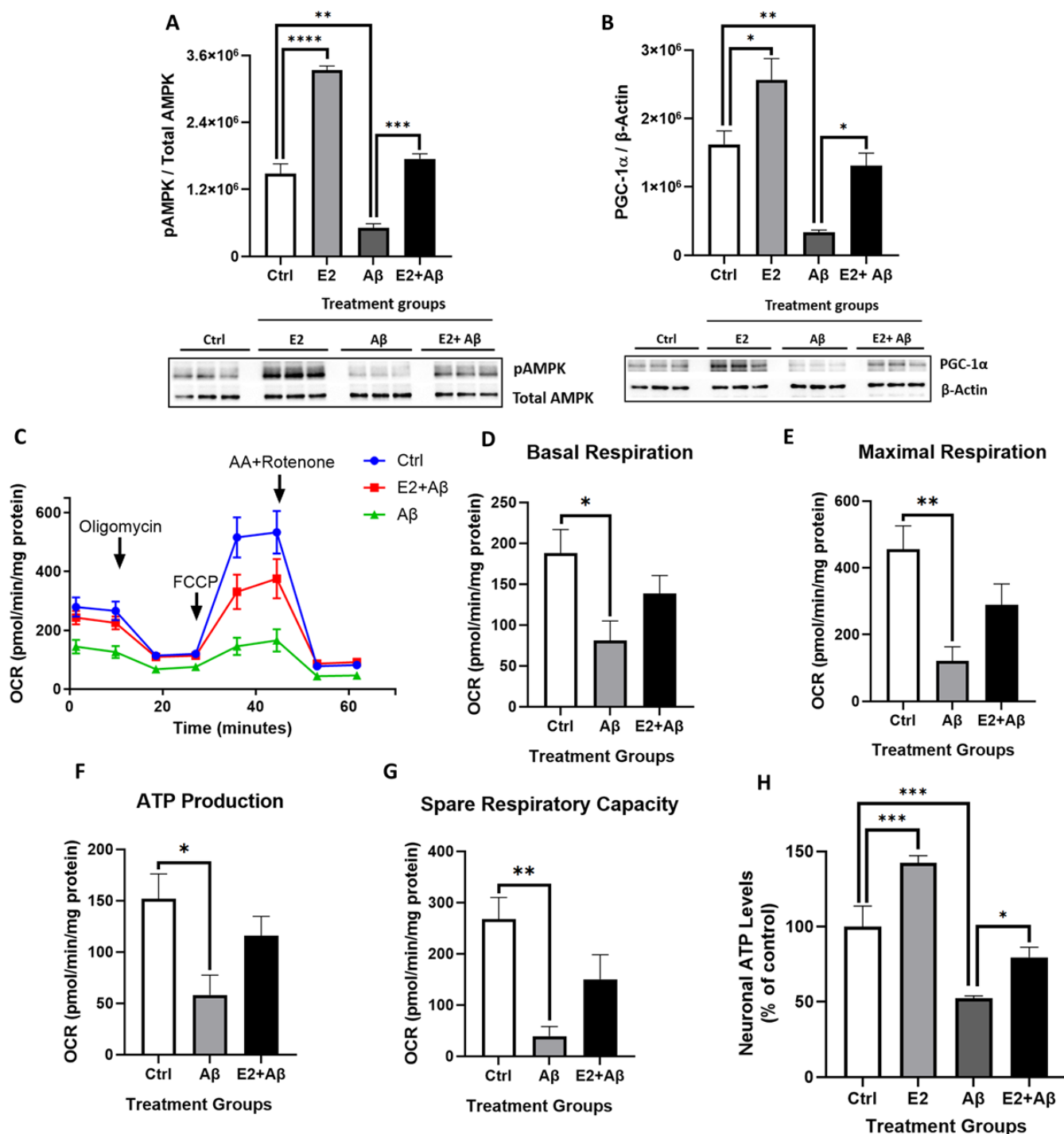


Figure 3.4. E2 pretreatment rescues Aβ-induced mitochondrial dysfunction, restores AMPK and PGC-1α levels, and enhances mitochondrial bioenergetics. Primary cortical neurons were pretreated with 10 nM E2 for 48 hours before exposure to 10 μM Aβ for 24 hours. **(A)** pAMPK levels (normalized to total AMPK) and **(B)** PGC-1α levels (normalized to β-actin) were analyzed

by Western blotting. **(C–G)** OCR including basal respiration, maximal respiration, ATP production, and spare respiratory capacity was measured using the Seahorse XF24 Analyzer and normalized to protein concentration per well. **(H)** Cellular ATP levels (normalized to total protein) were measured using a luminescent ATP determination kit. Data are expressed as mean $\pm$ SEM, with n=3 replicate cultures for Western blot analyses (A–B) and ATP assay (H), and n=6–7 replicate cultures for Seahorse assay (C–G). Statistical significance was determined by one-way ANOVA with Tukey's *post hoc* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

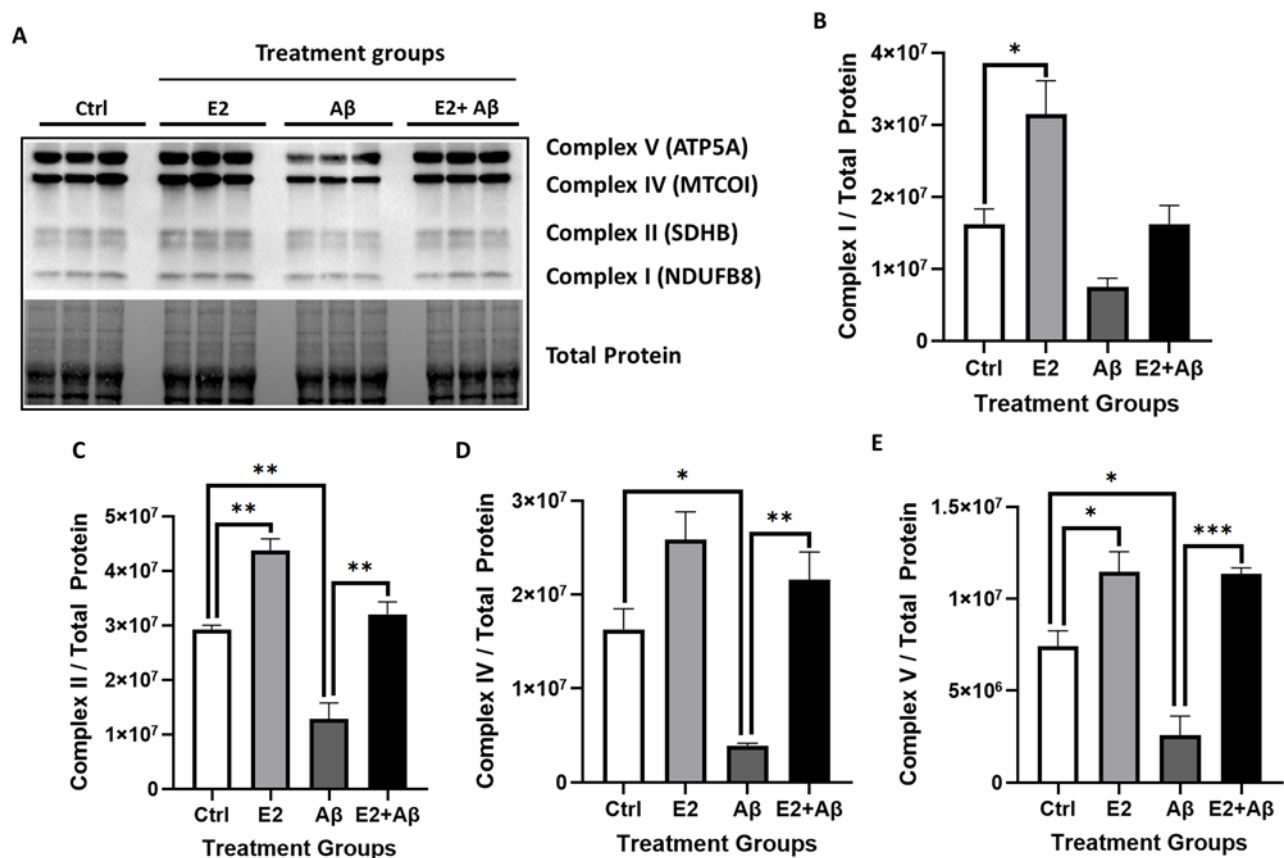


Figure 3.5. E2 pretreatment restores Aβ-induced loss of mitochondrial electron transport chain (ETC) complexes. Primary cortical neurons were pretreated with 10 nM E2 for 48 hours before exposure to 10 μM Aβ for 24 hours. Mitochondrial ETC complex levels were assessed by Western blotting: (A-E) Complex I (NDUFB8), Complex II (SDHB), Complex IV (MTCO1), and Complex V (ATP5A) (normalized to total protein). Data are expressed as mean ± SEM, with n=3 replicate cultures. Statistical significance was determined by one-way ANOVA with Tukey's *post hoc* test; *p<0.05, **p<0.01, ***p<0.001.

3.4.6 E2 pretreatment suppresses A β -induced neuroinflammation by inhibiting NF- κ B and inflammasome activity

To further investigate E2's neuroprotective mechanisms, we examined its effects on A β -induced neuroinflammatory signaling. A β significantly triggered neuroinflammatory responses in primary cortical neurons, as shown by increased phosphorylation of I κ B α (Fig. 3.6A). A β significantly enhanced nuclear translocation of the NF- κ B p65 subunit (Fig. 3.6B) and its DNA binding activity (Fig. 3.6C), indicating sustained activation of the NF- κ B pathway. While E2 alone significantly reduced phospho-I κ B α levels, its effect on nuclear p65 levels showed a trend toward reduction but did not reach statistical significance ($p = 0.06$) (Fig. 3.6A–B). E2 pretreatment significantly mitigated A β effects by reducing I κ B α phosphorylation, suppressing nuclear translocation of p65, and inhibiting NF- κ B p65 DNA-binding activity compared to A β -treated neurons. Given that NF- κ B activation can promote inflammasome assembly and caspase-1 activation (Liu *et al.*, 2017), we next assessed caspase-1 activity. A β significantly increased caspase-1 activity (Fig. 6D), a hallmark of NLRP3 inflammasome activation (Blevins *et al.*, 2022). This increase was abolished by the selective caspase-1 inhibitor Ac-YVAD-CHO, confirming assay specificity. Importantly, E2 pretreatment significantly reduced A β -induced caspase-1 activity, indicating suppression of inflammasome signaling. Consistent with this, A β elevated the levels of pro-inflammatory cytokines including IL-1 β , IL-6, TNF α , and IL-18, while reducing the anti-inflammatory cytokine IL-10 (Fig. 3.6E). E2 pretreatment prevented the A β -induced increase in pro-inflammatory cytokines and restored levels of the anti-inflammatory cytokine IL-10 to those seen in control neurons. Collectively, these findings demonstrate that E2 attenuates A β -induced neuroinflammation by suppressing NF- κ B activation, inhibiting inflammasome activity, and restoring a balanced cytokine profile.

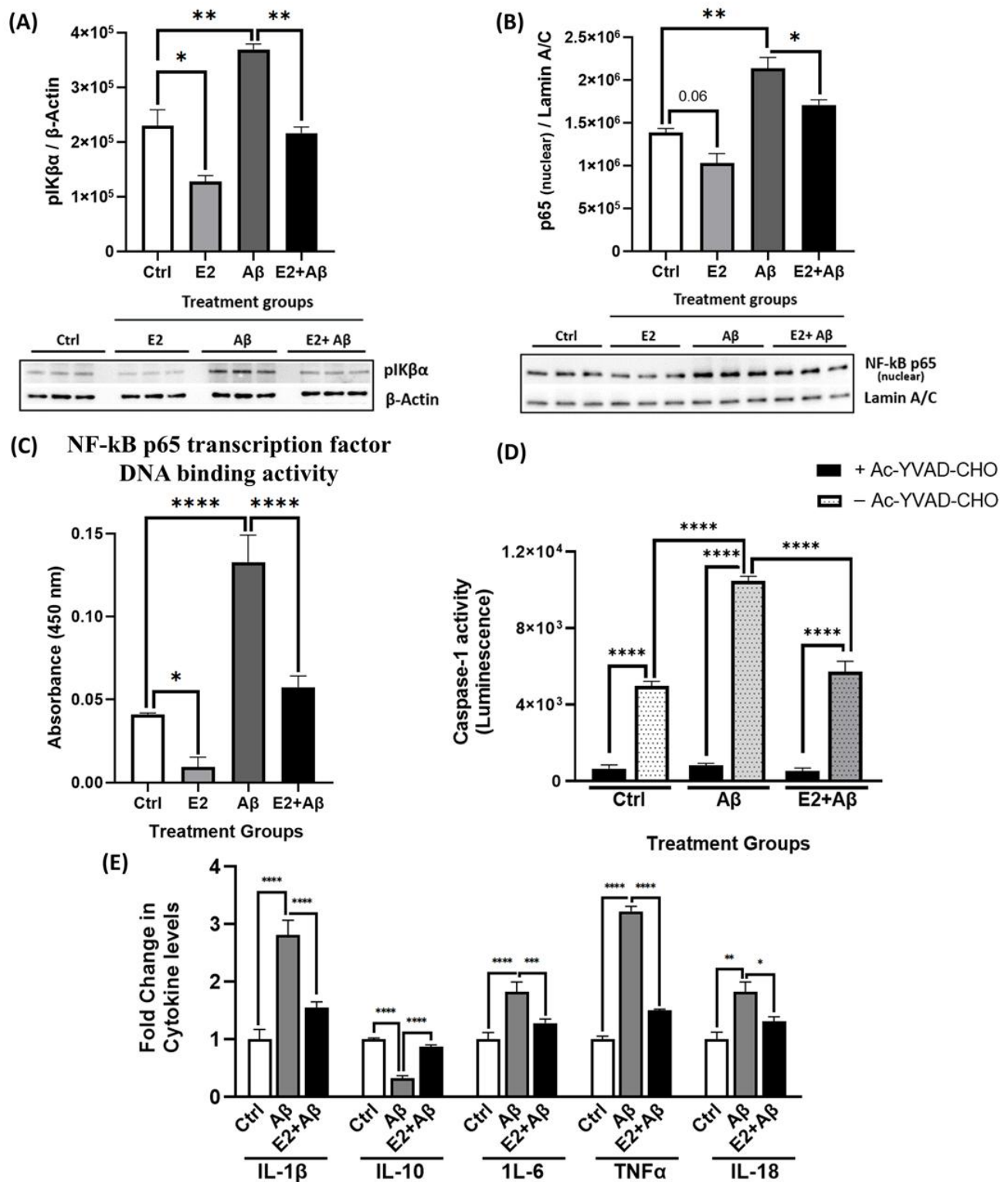


Figure 3.6. E2 pretreatment suppresses Aβ-induced NF-κB activation, inflammasome activity, and pro-inflammatory cytokine production in primary cortical neurons. Primary cortical neurons were pretreated with 10 nM E2 for 48 hours before exposure to 10 μM Aβ for 24

hours. **(A)** pIkB α levels (normalized to β -actin) and **(B)** nuclear NF- κ B p65 levels (normalized to Lamin A/C) were determined by Western blotting. **(C)** NF- κ B activation was assessed by measuring the DNA binding activity of the NF- κ B p65 subunit to a specific dsDNA sequence containing the NF- κ B response element, with data normalized to total protein concentration in nuclear extracts. **(D)** Caspase-1 activity, a measure of inflammasome activation, was determined by luminescence assay; the selective caspase-1 inhibitor Ac-YVAD-CHO was used to confirm specificity. **(E)** Pro-inflammatory (IL-1 β , IL-6, TNF α , IL-18) and anti-inflammatory (IL-10) cytokine levels in cell culture supernatants were measured by ELISA and reported as fold change relative to the control group. Data are expressed as mean $\pm$ SEM, with n=3 replicate cultures for Western blot and NF- κ B DNA binding assays (A–C) and n=4 replicate cultures for caspase-1 activity and ELISA (D–E). Statistical significance was determined by one-way ANOVA with Tukey's *post hoc* test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.4.7 E2 pretreatment rescues A β -induced cytotoxicity and preserves neuronal viability during prolonged exposure

To evaluate the longer-term neuroprotective potential of E2, primary cortical neurons were pretreated with 10 nM E2 for 48 hours before exposure to 10 μ M A β for 48 hours. A β significantly reduced neuronal viability, as measured by the MTT assay (Fig. 3.7A), and increased cytotoxicity, as indicated by elevated LDH release (Fig. 3.7B). E2 pretreatment maintained neuronal viability and significantly reduced LDH release compared to A β -treated neurons. Notably, E2 alone had no significant impact on viability or cytotoxicity, confirming its non-toxic effects under the experimental conditions.

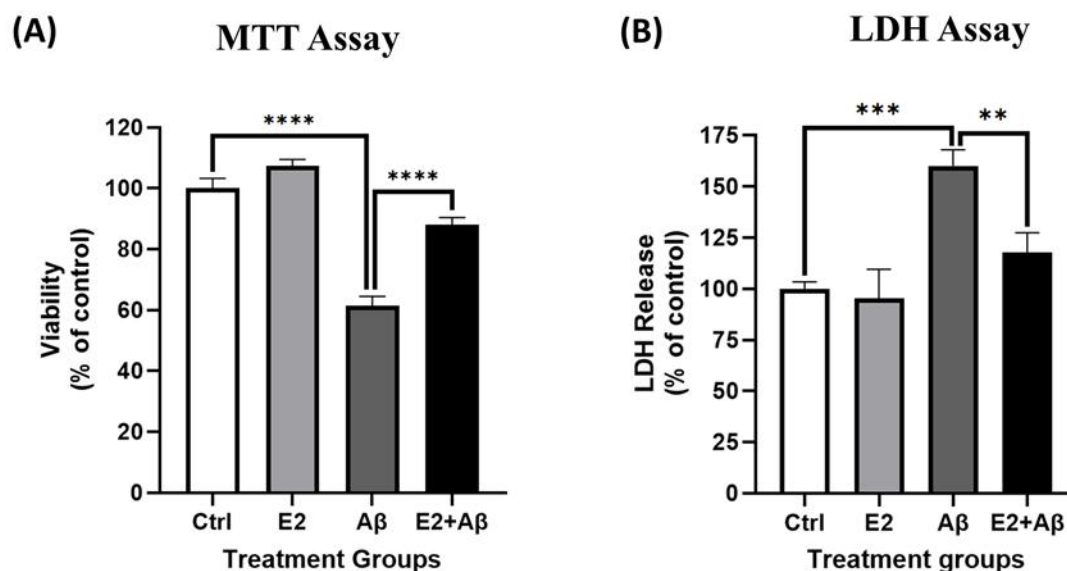


Figure 3.7. E2 pretreatment rescues A β -induced cytotoxicity and improves neuronal viability. Primary cortical neurons were pretreated with 10 nM E2 for 48 hours before exposure to 10 μ M A β for 48 hours. **(A)** Cell viability was assessed using the MTT assay, and **(B)** cytotoxicity was measured using the LDH assay. Data are presented as a percentage of the control group and expressed as mean $\pm$ SEM, with $n = 3-4$ replicate cultures. Statistical significance was determined by one-way ANOVA followed by Tukey's *post hoc* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.4.8 AMPK mediates E2's neuroprotective effects against A β -induced mitochondrial dysfunction and neurotoxicity

To confirm the role of AMPK in E2-mediated neuroprotection, we first optimized the dose of the AMPK inhibitor Compound C (CC). Pretreatment of primary cortical neurons with increasing doses of CC (1–5 μ M) for 1 hour prior to 10 nM E2 exposure revealed that 5 μ M CC completely blocked E2-induced AMPK phosphorylation (Supplementary Fig. 3.1), consistent with previous studies demonstrating the efficacy of this concentration in neuronal systems (Liu *et al.*, 2014). In A β treated neurons, AMPK phosphorylation and PGC-1 α levels were significantly reduced (Fig. 3.8A–B). E2 pretreatment maintained both pAMPK and PGC-1 α levels; however, this rescue was completely abolished by CC, confirming the requirement of AMPK for E2-mediated PGC-1 α upregulation (Fig. 3.8A–B). Functionally, A β significantly impaired mitochondrial respiration as indicated by reductions in basal respiration, maximal respiration, and ATP production, which was rescued by E2 pretreatment (Fig. 3.8C–F). These protective effects of E2 were fully reversed by CC, with OCR parameters reduced to levels comparable to A β treatment alone (Fig. 3.8C–F). Consistent with mitochondrial dysfunction, A β significantly reduced neuronal viability and increased cytotoxicity in our cultures, which was prevented by E2 pretreatment (Fig. 3.8G–H). However, this rescue was abolished when AMPK was inhibited using CC, which completely blocked E2's protective effect, reducing viability to levels observed with A β alone. Collectively, these findings establish that AMPK activation is essential for E2's ability to counteract A β -induced mitochondrial dysfunction and neurotoxicity.

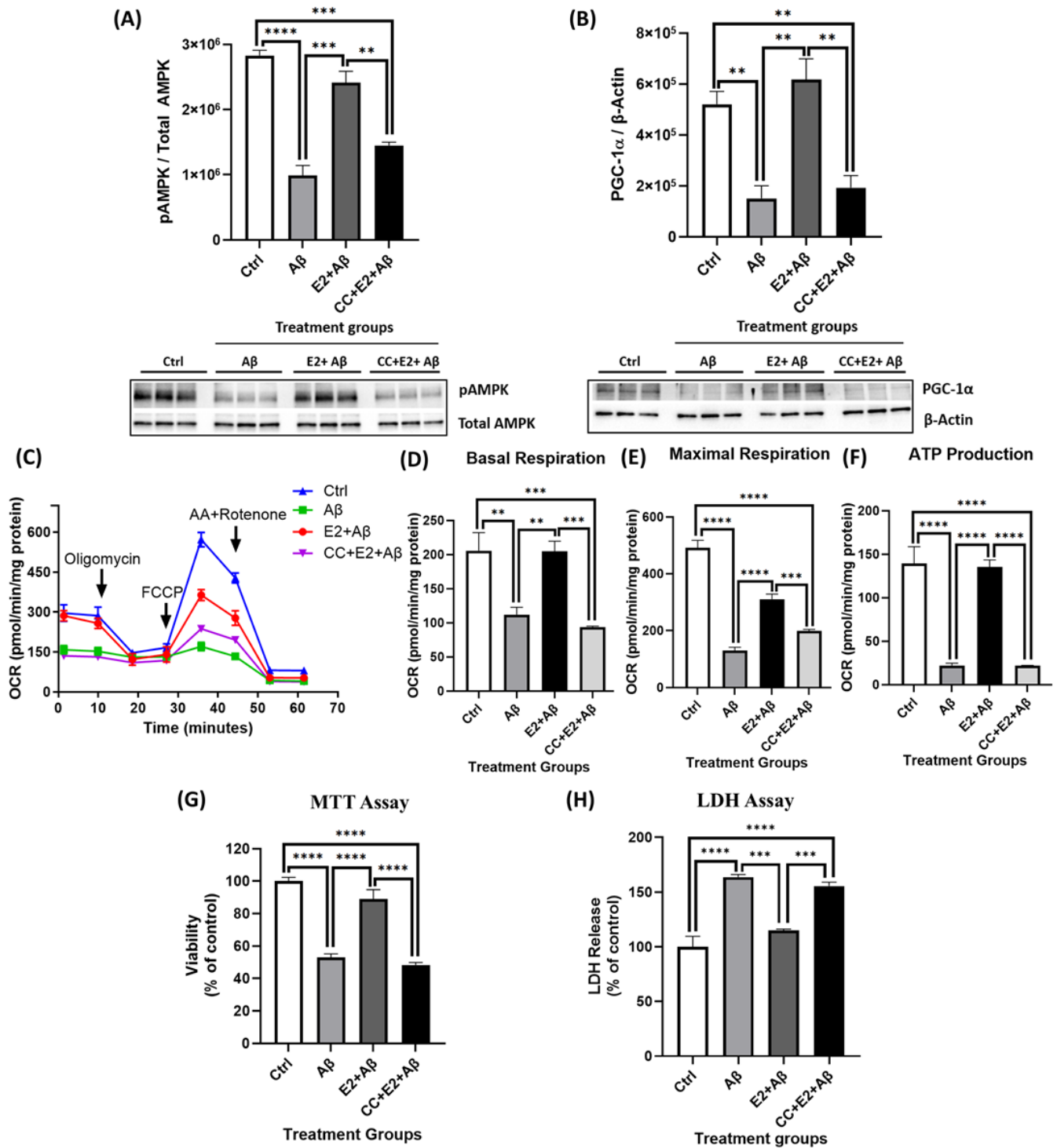


Figure 3.8. Blocking AMPK abolishes E2's protective effects against Aβ-induced mitochondrial dysfunction and neurotoxicity. Primary cortical neurons were treated with 10 μM Aβ alone for 24 hours, pretreated with 10 nM E2 for 48 hours followed by Aβ, or pretreated with

5 μ M Compound C (CC) for 1 hour followed by E2 and A β . **(A)** pAMPK levels (normalized to total AMPK) and **(B)** PGC-1 α levels (normalized to β -actin) were analyzed by Western blotting. **(C–F)** OCR, including basal respiration, maximal respiration, and ATP production was measured via Seahorse XF24 Analyzer and normalized to protein concentration per well. **(G)** Cell viability was assessed by MTT assay, and **(H)** cytotoxicity was measured using the LDH assay, with data expressed as a per-centage of the control group. Data are presented as mean $\pm$ SEM, with n=3 replicate cultures for Western blot analyses (A–B), n=5 replicate cultures for Seahorse assay (C–F), and n=4 replicate cultures for MTT (G) and LDH (H) assays. Statistical significance was determined by one-way ANOVA with Tukey's *post hoc* test; **p<0.01, ***p<0.001, ****p<0.0001.

3.4.9 E2 acts via AMPK to suppress A β -induced NF- κ B subunit expression

We next assessed whether E2's anti-inflammatory effects were AMPK-dependent. A β exposure significantly increased phosphorylated I κ B α levels and promoted the nuclear translocation of NF- κ B p65 subunit, indicating robust activation of the NF- κ B pathway (Fig. 3.9A–B). E2 pretreatment attenuated these effects by reducing both pI κ B α levels and nuclear p65 translocation compared to A β -treated neurons. However, the AMPK inhibitor CC abolished E2's suppressive effect on NF- κ B activation, restoring pI κ B α and nuclear p65 levels to those observed with A β treatment alone (Fig. 3.9A–B). These findings confirm that AMPK activation is essential for E2-mediated suppression of NF- κ B signaling, placing AMPK upstream of NF- κ B in E2's anti-inflammatory mechanism.

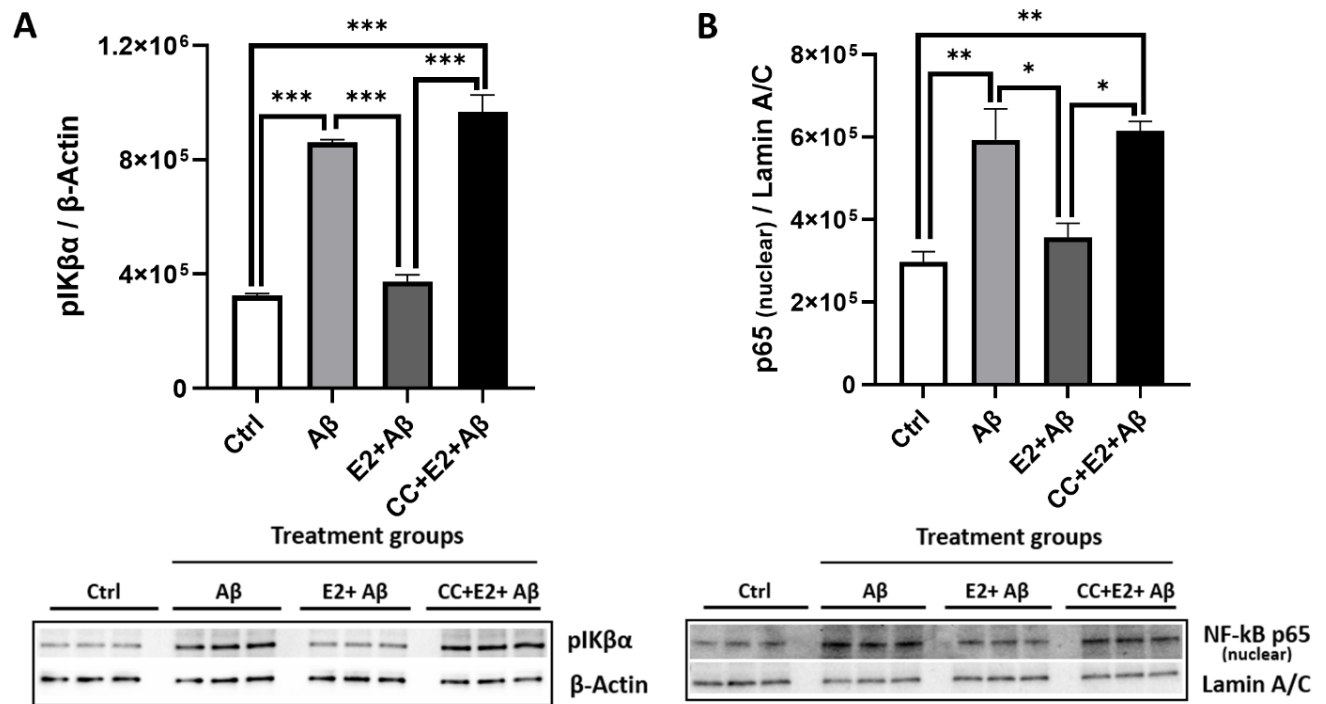


Figure 3.9. AMPK inhibition abolishes E2's suppression of Aβ-induced NF-κB subunit expression and translocation. Primary cortical neurons were treated with 10 μM Aβ alone for 24 hours, pretreated with 10 nM E2 for 48 hours followed by Aβ, or pretreated with 5 μM Compound C (CC) for 1 hour before E2 and Aβ. **(A)** pIκBα levels (normalized to β-actin) and **(B)** nuclear NF-κB p65 levels (normalized to Lamin A/C) were analyzed by Western blotting. Data are presented as mean ± SEM, with n=3 replicate cultures. Statistical significance was determined by one-way ANOVA with Tukey's *post hoc* test; *p<0.05, **p<0.01, ***p<0.001.

3.4.10 NF- κ B inhibition partially rescues mitochondrial dysfunction but fails to prevent A β -induced cytotoxicity

A β exposure significantly impaired mitochondrial respiration, as revealed by significant reductions in basal respiration, maximal respiration, ATP production, and spare respiratory capacity (Fig. 3.10A–E). Consistent with previous findings, E2 pretreatment significantly rescued these parameters. The NF- κ B inhibitor BAY 11-7082, at an optimized dose determined in our culture system (Supplementary Fig. 3.2), also rescued mitochondrial function similarly to E2 pretreatment and restored maximal respiration, ATP production, and spare respiratory capacity (Fig. 3.10C–E), although it showed only a non-significant trend toward improving basal respiration (Fig. 3.10B). In contrast, BAY 11-7082 failed to protect against A β -induced cytotoxicity. A β significantly reduced neuronal viability (Fig. 3.10F) and increased cytotoxicity (Fig. 3.10G) in our cultures (Fig. 3.10F), and while E2 pretreatment prevented this decline, BAY 11-7082 had no protective effect.

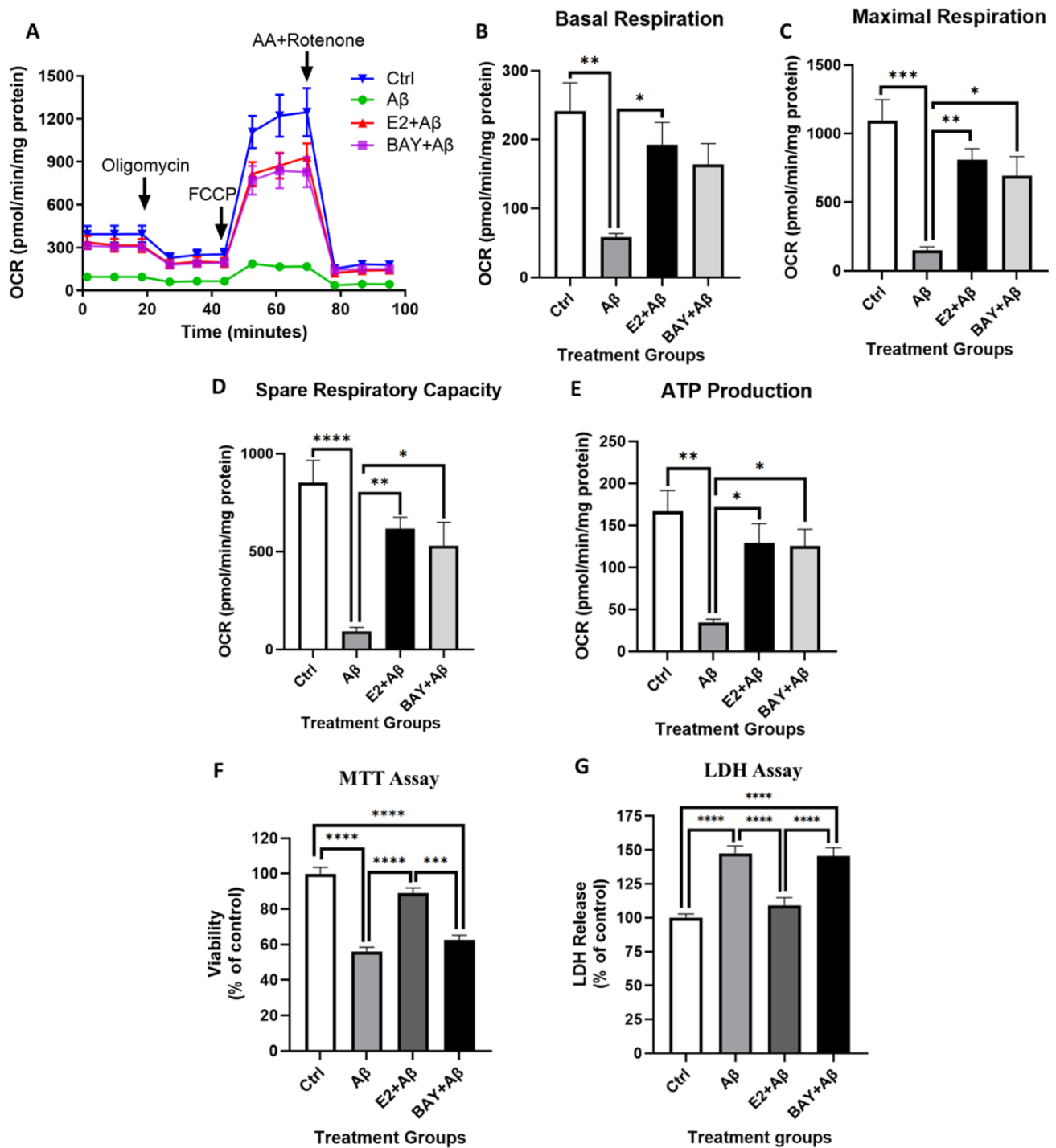


Figure 3.10. NF- κ B inhibition rescues mitochondrial dysfunction but not A β -induced cytotoxicity. Primary cortical neurons were treated with 10 μ M A β alone, pretreated with 10 nM E2 for 48 hours followed by A β , or pretreated with 3 μ M BAY 11-7082 (NF- κ B inhibitor) for 1 hour before A β exposure. **(A-E)** Mitochondrial OCR parameters including basal respiration,

maximal respiration, ATP production, and spare respiratory capacity were measured using the Seahorse XF24 Analyzer after 24 hours of A β exposure. Data were normalized to protein concentration per well and are presented as mean $\pm$ SEM, n = 5 replicate cultures. **(F)** Cell viability was assessed by MTT assay, and **(G)** cytotoxicity was measured using the LDH assay, after 48 hours of A β exposure, with data shown as a percentage of the control group and presented as mean $\pm$ SEM, n = 4 replicate cultures. Statistical significance was determined by one-way ANOVA followed by Tukey's *post hoc* test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.5 Discussion

In this study, we investigated the neuroprotective effects of E2 in primary cortical neurons exposed to A β , with a focus on AMPK signaling and NF- κ B-mediated inflammation. AD is marked by progressive accumulation of A β in the cortex (Frisoni *et al.*, 1999; Reilly *et al.*, 2003). To model this pathological hallmark *in vitro*, we treated cortical neurons derived from mouse embryos with A β oligomers, as established in previous studies (Hughes *et al.*, 2020; Keskin *et al.*, 2023; Meng *et al.*, 2024). Although E2 has been previously shown to confer neuroprotection, our findings offer novel insights into the mechanisms underlying this effect in the context of A β -induced toxicity. We demonstrated that E2 robustly activates AMPK signaling and its downstream effector PGC-1 α (Fig. 3.1A–E), promoting mitochondrial respiration (Fig. 3.1F–H), and enhancing ETC complex protein expression (Fig. 3.5A–E). These results align with previous findings highlighting AMPK's role in supporting mitochondrial function via PGC-1 α (Canto and Auwerx, 2009; Herzig and Shaw, 2018; Saleh *et al.*, 2020).

E2 also reduced NF- κ B activity by attenuating I κ B α phosphorylation (Fig. 3.2A–B), consistent with its established anti-inflammatory properties (Murphy *et al.*, 2010; Vegeto *et al.*, 2008). A β exposure led to a significant reduction in pAMPK and PGC-1 α levels (Fig. 3.3A–B), impaired mitochondrial respiration (Fig. 3.3C–F), and increased NF- κ B activation via enhanced phosphorylation and degradation of its inhibitor I κ B α (Fig. 3.3G), culminating in pronounced neurotoxicity (Fig. 3.3H). E2 pretreatment effectively reversed these pathological changes: it suppressed NF- κ B activation (Fig. 3.6A–C), attenuated caspase-1 activity and proinflammatory cytokine expression (Fig. 3.6D–E), restored AMPK and PGC-1 α levels (Fig. 3.4A–B), rescued mitochondrial deficits (Fig. 3.4C–H), and improved neuronal viability (Fig. 3.7A–B). Crucially, pharmacological inhibition of AMPK abolished E2-mediated mitochondrial and neuroprotective

effects (Fig. 3.8A–G and Fig. 3.9A–B), establishing AMPK as a pivotal mediator of the actions of E2. Interestingly, inhibition of NF- κ B partially rescued mitochondrial respiration, but did not prevent A β -induced neuronal death (Fig. 3.10A–F), suggesting that the effects of E2 involve AMPK-dependent pathways that extend beyond NF- κ B suppression. Together, these results highlight AMPK as a central player in the neuroprotective actions of E2 against A β -induced neurotoxicity.

E2 exerts its neuroprotective effects through both genomic and non-genomic signaling pathways (Brann *et al.*, 2007), mediated by ERs widely expressed in the brain (Foster, 2012). Genomic actions of ER α and ER β control transcription of neuroprotective factors including, but not limited to, brain-derived neurotrophic factor (BDNF), PGC-1 α , and even anti-apoptotic proteins (Schacter *et al.*, 2014; Scharfman and MacLusky, 2006; Zhao *et al.*, 2023). Non-genomic mechanisms, involve rapid activation of membrane bound ERs or GPER, triggering downstream protein kinase cascades including AMPK activation to modulate gene expression (Pupo *et al.*, 2016; Yang and Wang, 2015). All three ERs: ER α , ER β , and GPER contribute to the neuroprotective effects of E2 via both pathways (Elzer *et al.*, 2010; Morissette *et al.*, 2008; Prokai and Simpkins, 2007). For instance, ER α has been implicated in mediating E2's anti-inflammatory effects in the brain (Vegeto *et al.*, 2003) and in providing protection against stroke in both male and female mice (Elzer *et al.*, 2010), while ER β supports learning and memory in postmenopausal models of AD by maintaining mitochondrial homeostasis through NRF1/PGC-1 α signaling (Zhao *et al.*, 2023). GPER and ER α both enhance mitochondrial function (Bauza-Thorbrugge *et al.*, 2019) and studies in ER α and ER β knockout mice further confirm their roles in modulating inflammatory cytokine production (Brown *et al.*, 2010). These findings collectively support the idea that E2's neuroprotective actions are mediated by a complex interplay of receptor subtypes.

Notably, ERs localize in mitochondria (Yager and Chen, 2007), where they may directly regulate mitochondrial function and contribute to the protective effects of estrogen. In our study, it is likely that both pathways mediate the observed benefits of E2. However, the complete loss of E2's protective effects upon AMPK inhibition (Fig. 3.8 and Fig. 3.9) underscores the critical role of membrane-initiated non-genomic signaling in coordinating mitochondrial function and inflammation. This aligns with reports that E2 rapidly activates AMPK through non-genomic mechanisms (Mishra *et al.*, 2025), and with prior reviews emphasizing the role of non-genomic signaling in mediating E2's neuroprotective effects (Fiocchetti *et al.*, 2012).

AMPK regulates PGC-1 α through: direct phosphorylation to enhance its transcriptional activity (Jäger *et al.*, 2007) and indirect activation via Sirtuin 1 (SIRT1) mediated deacetylation (Canto and Auwerx, 2009). This cascade was evident in our study, where E2 enhanced mitochondrial respiration and function (Fig. 3.1). AMPK inhibition abolished the ability of E2 to restore PGC-1 α levels (Fig. 3.8B), indicating that AMPK functions upstream of PGC-1 α in primary cortical neurons and is essential for E2-driven mitochondrial biogenesis. Concurrently, E2 suppressed NF- κ B activation, as evidenced by reduced I κ B α phosphorylation (Fig. 3.2), consistent with its anti-inflammatory effects (Vegeto *et al.*, 2008; Zhang *et al.*, 2014). Together, these findings highlight the ability of E2 to coordinate mitochondrial and inflammatory regulation likely through AMPK-PGC-1 α signaling. A β , a key pathogenic factor in AD, is linked to mitochondrial dysfunction, neuroinflammation, and neuronal death. Its toxicity varies with its aggregation state (Chen *et al.*, 2017). Monomers are generally non-toxic, but oligomers and fibrils disrupt synaptic function and exacerbate neuroinflammation by activating pro-inflammatory pathways like NF- κ B (Kaltschmidt *et al.*, 1997), leading pro-inflammatory cytokine release and chronic inflammation. A β also impairs mitochondrial function, as observed in our study where A β resulted in decreased

pAMPK and PGC-1 α levels, impaired mitochondrial respiration (Fig. 3.3A–F), and enhanced NF- κ B activation (Fig. 3.3G), resulting in neuronal death (Fig. 3.3H). These findings align with previous studies demonstrating A β -induced mitochondrial dysfunction (Chen and Yan, 2007) and cell death (Kadowaki *et al.*, 2005; Reifert *et al.*, 2011), underscoring the pathogenic role of A β in AD.

Consistent with our hypothesis, E2 pretreatment effectively counteracted A β -induced mitochondrial and inflammatory deficits, demonstrating its neuroprotective potential. Specifically, E2 restored pAMPK and PGC-1 α levels (Fig. 3.4A–B), improved mitochondrial respiration (Fig. 3.4C–G), rescued ATP production (Fig. 3.4H), and enhanced ETC complex expression (Fig. 3.5A–E). Mechanistically, E2-mediated activation of AMPK and upregulation of PGC-1 α , likely promote mitochondrial biogenesis through the transcriptional induction of key downstream factors such as nuclear respiratory factors 1 (NRF1), nuclear respiratory factors 2 (NRF2), and mitochondrial transcription factor A (TFAM) (Gureev *et al.*, 2019; Taherzadeh-Fard *et al.*, 2011). Additionally, E2 enhances mitochondrial antioxidant capacity by upregulating superoxide dismutase 2 (SOD2) (Liu *et al.*, 2014) and stabilizes calcium homeostasis, countering A β -induced calcium overload that exacerbates mitochondrial dysfunction (Bhallamudi *et al.*, 2020; Brewer *et al.*, 2006; Nilsen *et al.*, 2006).

In our study, A β exposure increased nuclear translocation of the NF- κ B subunit p65 (Fig. 3.3G) and its DNA-binding activity (Fig. 3.6C), reflecting heightened inflammatory signaling. E2 pretreatment suppressed A β -enhanced NF- κ B responses (Fig. 3.6A–C), supporting prior studies demonstrating E2's inhibition of NF- κ B in macrophages and rat aortic smooth muscle cells (RASMCs) (Murphy *et al.*, 2010; Xing *et al.*, 2012). This suggests rescue of mitochondrial dysfunction by E2 in primary neurons involves NF- κ B inhibition and subsequent attenuation of

inflammatory cascades. A β further triggered caspase-1 activity and elevated pro-inflammatory cytokines (Fig. 3.6D–E), processes associated with NLRP3 inflammasome activation and IL-1 β /IL-18 maturation (Franchi *et al.*, 2009; Galliher-Beckley *et al.*, 2013). E2 mitigated these effects, reducing caspase-1 activity and pro-inflammatory cytokine levels, thereby attenuating neuroinflammatory markers. E2 pretreatment also improved A β -mediated decline in cell viability (Fig. 3.7A–B), likely through its dual actions on mitochondrial function and inflammation. This supports earlier findings showing E2 protects against A β toxicity. For instance, in rat hippocampal neurons, E2 prevented A β -induced apoptosis by upregulating the anti-apoptotic Bcl-2, preventing Bax translocation to mitochondria, and maintaining calcium homeostasis (Nilsen *et al.*, 2006). Similarly, in the SN56 cholinergic cells, E2 prevented cell death induced by A β (Marin *et al.*, 2003). Building on this, our findings suggest that rescuing mitochondrial respiration and ETC complex proteins (Fig. 3.4 and 3.5) would enhance energy metabolism, while the suppressing NF- κ B and caspase-1 (Fig. 3.6) would reduce inflammatory stress, collectively contributing to improved neuronal survival.

Although NF- κ B plays a pivotal role in A β -induced neuroinflammation, it is not the sole contributor. Other pathways including Janus kinase/signal transducer and activator of transcription (JAK/STAT), mitogen-activated protein kinases (MAPK), and NLRP3 inflammasome also mediate A β toxicity (Ageeva *et al.*, 2024; Jain *et al.*, 2021; Jung *et al.*, 2022; Longpré *et al.*, 2006; Rather *et al.*, 2021; Viorel *et al.*, 2024). Importantly, E2 regulates these pathways (Guo *et al.*, 2022; Shi *et al.*, 2018) indicating a broader anti-inflammatory role, beyond NF- κ B suppression. This could explain why E2, unlike NF- κ B inhibition, rescued both mitochondrial function and neuronal cell death. To elucidate NF- κ B's role in A β -induced neuronal damage, we used the selective NF- κ B inhibitor BAY11-7082 (Lang *et al.*, 2022), which blocks I κ B α phosphorylation

and degradation, preventing NF- κ B nuclear translocation (Mori *et al.*, 2002). NF- κ B inhibition partially restored mitochondrial function to levels comparable to E2 treatment (Fig. 3.10A–E). This aligns with previous findings implicating NF- κ B in mitochondrial dysfunction across various models (Zhang *et al.*, 2017). NF- κ B mediates A β -induced mitochondrial impairment by reducing mitochondrial function and cytochrome c oxidase III (COX III) activity (Shi *et al.*, 2014). NF- κ B can translocate into mitochondria and bind mitochondrial DNA (mtDNA), repressing genes such as cytochrome b and COX III (Albensi, 2019; Johnson *et al.*, 2011; Kracht *et al.*, 2020). Despite restoring mitochondrial function, BAY11-7082 failed to prevent A β -induced neuronal cell death (Fig. 3.10F), suggesting that NF- κ B inhibition alone is insufficient for full neuroprotection. This indicates that mitochondrial dysfunction is only one aspect of A β toxicity. Other mechanisms, such as calcium dysregulation (Small *et al.*, 2009), oxidative stress (Butterfield and Boyd-Kimball, 2018), and apoptotic signaling pathways (Fonseca *et al.*, 2013), may also contribute to neuronal death.

The activation of AMPK signaling downregulates NF- κ B activity through downstream targets such as SIRT1 and PGC-1 α , thereby reducing inflammation (Bai *et al.*, 2010; Salminen *et al.*, 2011; Yang *et al.*, 2010). We found that A β exposure lowered levels of pAMPK and PGC-1 α , but E2 pretreatment reversed these effects. However, when AMPK was inhibited with CC prior to E2 treatment, it abolished E2's protective effects preventing the restoration of pAMPK and PGC-1 α (Fig. 3.8A–B), impairing mitochondrial respiration (Fig. 3.8C–F), and reducing neuronal survival (Fig. 3.8G). Similarly, CC blocked E2's suppression of NF- κ B activation (Fig. 3.9A–B). Collectively, these findings position AMPK as a central upstream regulator of PGC-1 α -dependent mitochondrial function and NF- κ B-mediated inflammatory signaling in primary cortical neurons, highlighting its pivotal role in mediating the neuroprotective effects of E2. AMPK is therefore a

likely key mediator of E2's neuroprotective effects against A β -induced toxicity, coordinating mitochondrial function and anti-inflammatory responses.

Despite the valuable insights gained from our study, several limitations must be acknowledged. Our experiments were conducted *in vitro*, which, although enabling detailed mechanistic analysis, lacks the complexity of the intact brain. While animal and cell culture models of A β toxicity have contributed substantially to AD research (Fontana *et al.*, 2020; Xu *et al.*, 2001), they often focus primarily on amyloid accumulation and fail to replicate the full spectrum of human pathology, particularly NFTs. Tau pathology plays a central role in AD and is strongly associated with neuronal loss and cognitive decline (Bejanin *et al.*, 2017; Thal and Tomé, 2022), yet it was not examined in this study. While our results support A β -induced mitochondrial and neuronal dysfunction, the specific mitochondrial sites or mechanisms involved were not directly assessed. Prior studies suggest that A β may enter mitochondria via the TOM/TIM complex (Pagani and Eckert, 2011), but this interaction remains unexplored in our model. Moreover, A β plaque burden in the human brain does not consistently correlate with cognitive impairment (Aizenstein *et al.*, 2008), highlighting the importance of additional factors such as tau in disease progression. Future research should address these gaps using *in vivo* models and tau-related endpoints to provide a more comprehensive understanding of AD pathogenesis.

Clinically, these findings highlight the therapeutic potential of targeting AMPK and estrogen signaling pathways in AD. While clinical trials on estrogen replacement therapy have produced mixed results (Henderson, 2014; Mishra *et al.*, 2023; Mulnard *et al.*, 2000; Wharton *et al.*, 2011), a combinatorial approach integrating AMPK activators with estrogen-based treatments may yield improved outcomes. Importantly, the higher risk of AD in postmenopausal women (Scheyer *et al.*, 2018; Wood Alexander *et al.*, 2025), underscores the relevance of estrogen's

neuroprotective actions in this population. *In vivo* studies using postmenopausal models will be essential to validate these findings in a physiologically relevant context. Such models could also help delineate the interplay between systemic estrogen loss and AMPK signaling in AD pathogenesis. Investigating the optimal timing, formulation, and delivery of estrogen therapies, as well as evaluating how AMPK-targeted strategies might benefit both sexes will be important for developing effective interventions to prevent disease progression.

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Authorship contributions

Pranav Mishra: Writing - original draft, Writing - review and editing, Visualization, Investigation, Methodology, **Ehsan Esfahani:** Visualization, Investigation, Methodology, **Paul Fernyhough:** Writing- review and editing, Visualization, Investigation, Methodology, Supervision, Funding acquisition, and Conceptualization, **Benedict C. Albensi:** Writing - review and editing, Visualization, Investigation, Methodology, Supervision, Funding acquisition, and Conceptualization.

Declaration of interests

P.M, E.E, and B.C.A declare that they have no competing interests. P.F. is a cofounder and shareholder in a small biotech company, WinSanTor Inc, that is developing antimuscarinic drugs for treatment of peripheral neuropathy.

Data availability statement:

Data will be made available upon request to the corresponding author.

Institutional Review Board Statement:

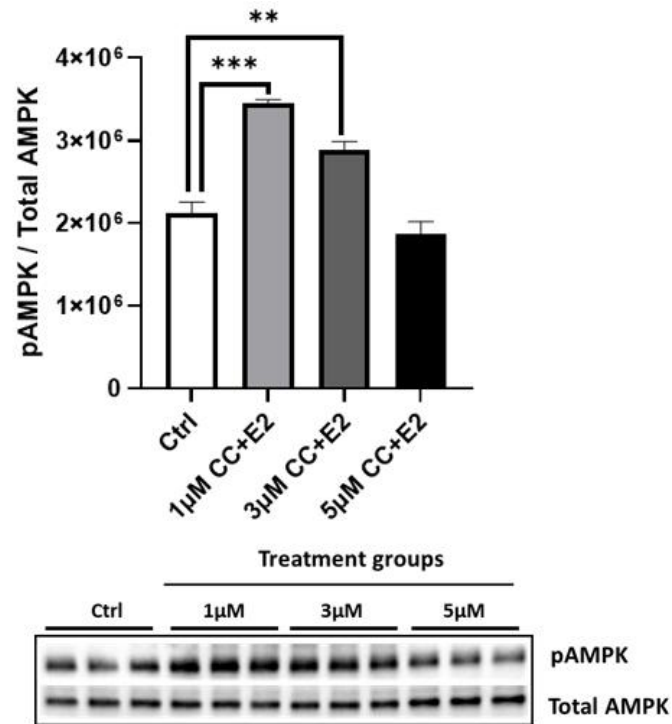
All animal procedures were conducted in accordance with the guidelines set by the University of Manitoba's Animal Care Committee and the Canadian Committee on Animal Care (CCAC).

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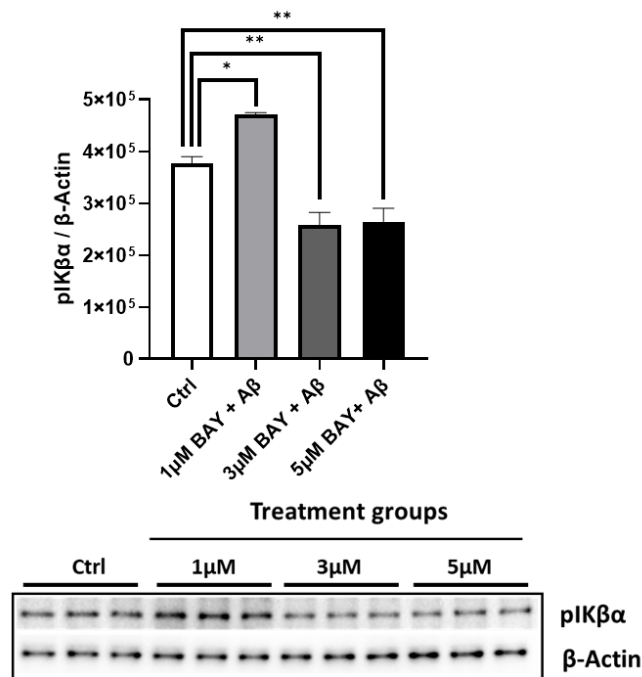
3.6 Supplementary data

Supplementary Figure S3.1.



Supplementary Figure S3.1. Dose optimization of Compound C (CC) for AMPK inhibition in E2-treated cortical neurons. Primary cortical neurons were pretreated with increasing doses of CC (1 μM, 3 μM, 5 μM) for 1 hour, followed by 10 nM E2 for 48 hours. Cell lysates were analyzed by Western blotting for pAMPK levels (normalized to total AMPK). Data are presented as mean ± SEM, with n=3 replicate cultures. Statistical significance was determined by one-way ANOVA followed by Dunnett's *post hoc* test; **p<0.01, ***p<0.001. The 5 μM CC dose was selected for subsequent experiments due to its efficacy in blocking E2-induced AMPK phosphorylation.

Supplementary Figure S3.2.



Supplementary Figure S3.2. Dose optimization of the NF-κB inhibitor BAY 11-7082 in Aβ-treated cortical neurons. Primary cortical neurons were pretreated with increasing doses of BAY 11-7082 (1 μM, 3 μM, 5 μM) for 1 hour, followed by 10 μM Aβ exposure for 48 hours. Cell lysates were analyzed by Western blotting for phosphorylated IκBα (pIkBα) levels (normalized to β-actin). Data are presented as mean ± SEM, with n=3 replicate cultures. Statistical significance was determined by one-way ANOVA followed by Dunnett's *post hoc* test; *p<0.05, **p<0.01. The 3 μM dose of BAY 11-7082 was selected for subsequent experiments due to its efficacy in suppressing Aβ-induced IκBα phosphorylation, indicating optimal NF-κB inhibition.

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CHAPTER 4

GENERAL DISCUSSION

The peripheral nervous system (PNS) consists of all the nerves outside the central nervous system (CNS), transmitting signals between the CNS and the rest of the body. The CNS, which includes the brain and spinal cord, acts as a control center, processing information and sending commands. This chapter provides an integrated discussion of the major findings from Chapters 2 and 3, which examined the effects of estradiol (E2) on adult sensory (DRG) neurons of the PNS and cortical neurons of the CNS, respectively. By comparing these distinct neuronal populations, I've highlighted the central roles of AMPK signaling, mitochondrial function, neuroinflammation, and neuroprotection against injury (relevant to axonal regeneration in neuropathy) and amyloid toxicity (relevant to AD). The following sections comprise key observations and mechanistic insights, discuss broader implications for neuroregeneration and neurodegeneration, and address methodological limitations. I have also proposed future strategies to improve experimental models, enhance translational relevance, and guide therapeutic development. First, I will provide a brief summary of findings from Chapters 2 and 3.

Summary of findings

Chapter 2: In Chapter 2, I investigated the effects of E2 on DRG sensory neurons of the PNS, focusing on AMPK signaling, mitochondrial function, and neurite outgrowth. My findings revealed that E2 activates AMPK in a time-dependent manner via the CaMKK β pathway, leading to the upregulation of downstream targets such as PGC-1 α and ATF3. This activation was rapid, occurring under 60 minutes, and suggests a non-genomic mechanism of E2 action. Understanding the distinction between genomic and non-genomic mechanisms is important, as non-genomic

actions enable rapid cellular responses especially crucial during acute neuronal injury or stress. E2 treatment enhanced mitochondrial function specifically increasing ETC Complex I subunit levels, basal respiration and promoted axonal regeneration/sprouting. Importantly, I established that AMPK activation is essential for E2-mediated PGC-1 α and ATF3 upregulation, and neurite outgrowth. Next, I demonstrated that E2 acts primarily through ER α in adult DRG neurons to mediate these effects, distinguishing the responses from non-neuronal or developmental models. Overall, my findings highlight a novel CaMKK β /AMPK/PGC-1 α signaling axis in adult sensory neurons, with ER α as a key mediator of E2's neurotrophic and mitochondrial effects.

Chapter 3: In Chapter 3, I explored the neuroprotective effects of E2 in primary cortical neurons of the CNS exposed to A β , modeling aspects of AD pathology. E2 robustly activated AMPK and its downstream effector PGC-1 α , enhancing mitochondrial respiration and increasing expression of ETC proteins. E2 also suppressed proinflammatory NF- κ B activity and its nuclear translocation, thereby attenuating neuroinflammatory pathways. Here, it is important to note that I measured indicators of neuroinflammation within isolated neurons, whereas neuroinflammation as seen in an intact brain is a complex, multifactorial process involving additional CNS cell types like microglia and astrocytes. A β exposure impaired AMPK and PGC-1 α signaling, reduced mitochondrial function, and increased NF- κ B activation, resulting ultimately in neuronal death. Importantly, E2 pretreatment reversed these pathological changes and improved neuronal viability. Pharmacological inhibition of AMPK abolished E2's protective effects, establishing AMPK as a pivotal mediator of E2's neuroprotective effects against A β . Notably, inhibition of NF- κ B only partially rescued mitochondrial function and did not prevent neuronal death, indicating that E2's neuroprotection involves AMPK-dependent pathways that extend beyond NF- κ B suppression.

Together, these findings position AMPK as a central player in E2-mediated neuroprotection against A β -induced mitochondrial dysfunction and neuroinflammation in cortical neurons.

Overall, I found that E2 exerts broad neuroprotective effects by orchestrating a network that integrates AMPK activation, mitochondrial function, axonal regeneration, neuroinflammation, and cell survival across both peripheral and central neurons. In DRG sensory neurons and cortical neurons, E2 rapidly activates AMPK, leading to the upregulation of PGC-1 α , a master regulator of mitochondrial biogenesis and function. This activation enhances mitochondrial respiration, in part through the upregulation of ETC subunits, thereby supporting energy production, and promoting axonal regeneration/sprouting through upregulation of regenerative factors such as ATF3 (Chapter 2). Similarly, in cortical neurons exposed to A β , E2-mediated AMPK activation restores mitochondrial function and suppresses NF- κ B-driven neuroinflammation, ultimately protecting against neuronal death (Chapter 3). Pharmacological inhibition of AMPK abolishes these beneficial effects of E2, preventing the upregulation of ATF3 and neurite outgrowth in DRG neurons, and eliminating the neuroprotective effects of E2 against A β -induced mitochondrial dysfunction and cell death in cortical neurons. Collectively, these findings position AMPK as a central mediator linking my observations of the neurotrophic and neuroprotective actions of E2 across diverse neuronal populations in both the peripheral and central nervous systems.

Thesis objective: The primary objective of this thesis was to investigate the effects of E2 on mitochondrial function and neuroprotection in both the CNS and PNS. Specifically, I examined the impact of E2 on mitochondrial function and its neurotrophic effects in promoting axonal regeneration in DRG neurons. These actions could provide neuroprotection in PNS diseases such as neuropathy, where impaired axonal sprouting/regeneration and mitochondrial dysfunction contribute to disease pathology (Fernyhough, 2015; Sango *et al.*, 2017; Sriwastawa and Kumar,

2024). Furthermore, I explored the role of E2 in modulating mitochondrial function and neuroinflammation in the CNS, particularly in the context of AD, where E2 may offer neuroprotection against CNS neurodegenerative processes. In the following sections, I will discuss the major mechanisms that link E2's actions across both neuronal populations, beginning with a focus on the central role of AMPK.

Broader significance of AMPK beyond this study: AMPK as a central mediator of neuroprotective effects of E2

AMPK is a fundamental metabolic master regulator that maintains cellular energy balance and supports mitochondrial function across multiple cell types, including neurons (Ronnett *et al.*, 2009). AMPK's central role in energy homeostasis makes it a critical mediator of cellular responses to stress and metabolic challenges. Beyond these, AMPK is implicated in neuronal responses to injury, aging, and neurodegenerative diseases such as Alzheimer's and Parkinson's (Cai *et al.*, 2012; Stancu, 2015; Wang *et al.*, 2020). Dysregulation of AMPK signaling contributes to impaired mitochondrial function, heightened inflammation, and increased neuronal vulnerability, underscoring its broader clinical importance (Cui *et al.*, 2023; Dugan *et al.*, 2013).

In this thesis, AMPK consistently emerged as a key mediator linking my observations of E2's neurotrophic and neuroprotective effects across both peripheral and central neuronal populations. E2-induced AMPK activation coordinated critical processes including upregulation of mitochondrial function involving PGC-1 α , and suppression of NF- κ B-mediated neuroinflammation. This multifaceted role was evident in DRG sensory neurons (Chapter 2), where AMPK enhanced PGC-1 α and ATF3, and improved axonal regeneration. Similarly, in cortical neurons challenged with A β (Chapter 3), AMPK activation restored mitochondrial function and suppressed NF- κ B-driven inflammatory signaling. While AMPK activation was a

shared mechanism in both neuronal types, the upstream pathways of AMPK activation may differ depending on the cellular context. In DRG neurons, using the inhibitor STO-609, I demonstrated that CaMKK β -mediated signaling predominantly activated AMPK to support axonal outgrowth. In cortical neurons, although the exact upstream mechanism was not delineated, it is plausible that, in addition to CaMKK β , LKB1 may also contribute to AMPK activation, consistent with prior evidence suggesting LKB1's critical role in CNS neurons (Huang and Li, 2022). Additionally, A β -induced stress may influence AMPK activity through alternative cellular stress pathways, although this remains to be fully elucidated.

Importantly, these findings underscore the adaptability AMPK and its central relevance to neuroregenerative and neuroprotective strategies. By demonstrating that AMPK plays a pivotal role in the protective effects of E2, my study places AMPK within a broader physiological and clinical context, reinforcing the therapeutic potential of targeting AMPK in neurodegenerative and neuroinflammatory diseases (Day *et al.*, 2017; Yang *et al.*, 2020a). This significance extends beyond my specific findings, as AMPK dysregulation is as a common pathological feature across aging and a wide range of conditions including type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease (CVD) (Jeon, 2016).

Mitochondria as a therapeutic target in neurodegenerative diseases

Mitochondria are essential for neuronal function, not only providing ATP to meet the high energy demands of neurons but also supporting other key processes such as axonal regeneration, calcium buffering, neurotransmitter shuttling into synaptic vesicles, and overall synaptic function (Han *et al.*, 2016; Lee *et al.*, 2018a; Vos *et al.*, 2010; Zhang *et al.*, 2022). Given their central role, mitochondrial dysfunction is implicated in a wide range of diseases affecting both the CNS and PNS (Norat *et al.*, 2020; Tripathi and Ben-Shachar, 2024). Mechanisms contributing to

mitochondrial dysfunction include impaired ETC activity, oxidative stress, altered mitochondrial dynamics, and defective mitophagy (Cai and Jeong, 2020; Chen *et al.*, 2023; Zong *et al.*, 2024). A β can enter mitochondria via the translocase of the outer mitochondrial membrane/ translocase of the inner membrane (TOM/TIM) complex (Pagani and Eckert, 2011), where it disrupts mitochondrial dynamics, impairs oxidative phosphorylation and ATP production, and blocks the import of essential nuclear-encoded proteins (Spuch *et al.*, 2012). Complex I initiates electron transport and drives ATP synthesis (Sharma *et al.*, 2009). Mitochondrial dysfunction also triggers inflammatory responses via the release of mtDNA and ROS (Li *et al.*, 2024; Sharma *et al.*, 2009), activating immune pathways and accelerating neurodegeneration. As a result, mitochondria have emerged as a crucial therapeutic target in neurodegenerative diseases.

Recognizing this, I investigated mitochondrial function across different models. In Chapter 2, I explored the effects of E2 on mitochondrial function in DRG sensory neurons. My findings showed that E2 enhances mitochondrial respiration and upregulates ETC Complex I subunit, key factors for axonal regeneration, as the regeneration process requires high levels of ATP. This improvement in mitochondrial function supports the neurotrophic effects of E2 in the PNS, contributing to enhanced neurite outgrowth and potentially promoting axonal survival and regeneration in peripheral neuropathies, which are typically characterized by distal axonal degeneration. These findings are consistent with previous work by Chakrabarty and colleagues, who showed that estrogen stimulates axon sprouting in neonatal DRG neurons, although their model represented developmental rather than adult systems (Chakrabarty *et al.*, 2008). Building on this, I next examined the CNS. In Chapter 3, I assessed the impact of E2 on mitochondrial function in cortical neurons exposed to A β , modeling AD pathology. My results demonstrated that E2 improved mitochondrial function by increasing ETC protein expression and respiration,

counteracting the mitochondrial dysfunction induced by A β . Restoration of mitochondrial function is critical for preventing neuronal death in AD, where mitochondrial deficits contribute heavily to synaptic failure and neurodegeneration (Bhatia *et al.*, 2022; Wang *et al.*, 2024b).

Together, these chapters demonstrate that maintaining optimal mitochondrial function is vital across neurodegenerative diseases, whether in peripheral neuropathy or AD. My work shows that E2 enhances mitochondrial function through AMPK activation in both DRG and cortical neurons. In DRG neurons, improved mitochondrial respiration supports axonal regeneration, while in cortical neurons, it offers protection against A β -induced toxicity. These findings highlight the therapeutic potential of targeting mitochondrial health in neurodegenerative diseases. Moreover, the identification of mitochondrial dysfunction as a shared pathological mechanism strengthens the rationale for targeting mitochondria therapeutically. Approaches aimed at enhancing mitochondrial biogenesis and function, such as AMPK activators or PGC-1 α agonists, may offer broad therapeutic benefits not only across aforementioned neurodegenerative conditions but also in other diseases characterized by mitochondrial impairment, including CVDs, cancer, multiple sclerosis, and muscular dystrophy (Barcelos *et al.*, 2019; Chen *et al.*, 2023b; Hsu *et al.*, 2016; Yang *et al.*, 2022). Furthermore, the ability of E2 to modulate mitochondrial health suggests that estrogenic compounds or SERMs could be explored for neuroprotection in both CNS and PNS disorders.

Neuroinflammation as a therapeutic target: Insights from the PNS and CNS

Neuroinflammation is a critical pathological feature in both the PNS and CNS, contributing to disease progression and neuronal damage (Stüve and Zettl, 2014). In the PNS, neuroinflammation refers to a dynamic immune response involving resident and infiltrating immune cells particularly macrophages and neutrophils that are activated following nerve injury. These cells can accumulate

in the DRG and injured nerve regions, where they play a role not only in axonal regeneration but also in the initiation and persistence of neuropathic pain (Yu *et al.*, 2020; Zigmond and Echevarria, 2019). In addition, Schwann cells respond to nerve injury by releasing pro-inflammatory mediators, contributing to the development of neuropathic pain (Wei *et al.*, 2019). Together, these immune and glial responses drive neuroinflammation which plays a central role in the development of neuropathic pain and contributes to various neuropathies, including Guillain–Barré Syndrome, chronic inflammatory demyelinating polyneuropathy, and diabetic neuropathy (Ellis and Bennett, 2013; Martini and Willison, 2016; Sandireddy *et al.*, 2014). Given the significant role of inflammation in these conditions, it is essential to address it therapeutically. Although my studies in the PNS (Chapter 2) primarily focused on mitochondrial function and axonal regeneration, I acknowledge the potential of E2 to regulate and possibly suppress inflammation in peripheral neurons. Pain is a common feature across multiple neuropathic conditions (Mitsikostas *et al.*, 2022), and accumulating evidence suggests that estradiol can counteract neuropathic pain by modulating inflammatory and immune responses (Chen *et al.*, 2021; Vacca *et al.*, 2016). For instance, E2 attenuates neuropathic pain following spinal cord injury (Lee *et al.*, 2018b), and in the DRG of ovariectomized rats, indicating its broader anti-inflammatory potential (Xu *et al.*, 2019).

In contrast, in my CNS studies (Chapter 3), I directly examined the role of inflammation, specifically focusing on the NF- κ B pathway. NF- κ B is a key transcription factor that regulates the expression of numerous pro-inflammatory genes (Shih *et al.*, 2015). Under pathological conditions, activation of NF- κ B leads to the amplification of inflammatory responses, contributing to neuronal dysfunction and death. Studies have demonstrated that inhibition of NF- κ B-mediated inflammation offers neuroprotection. For example, blockade of NF- κ B reduces damage in

ischemic stroke (Xu *et al.*, 2024) and serves as a promising strategy in the management of AD (Sivamaruthi *et al.*, 2023). In my study, A β exposure led to activation of NF- κ B signaling, evidenced by increased phosphorylation of I κ B α and elevated nuclear levels of NF- κ B p65. Further confirmation was obtained through a NF- κ B DNA binding assay, which showed enhanced transcriptional activation. Notably, NF- κ B activation can further promote the assembly of inflammasomes, such as NLRP3, leading to caspase-1 activation and subsequent maturation of major pro-inflammatory cytokines like IL-1 β and IL-18 (Sutterwala *et al.*, 2014). Consistent with this pathway, I observed increased levels of caspase-1, IL-1 β , and IL-18 following A β treatment, indicating robust inflammasome activation. Importantly, E2 pretreatment effectively prevented A β -induced NF- κ B activation, suppressed downstream inflammasome signaling, and reduced the expression of caspase-1, IL-1 β , and IL-18. This highlights E2's strong anti-inflammatory role in the CNS. Given the widespread presence of ERs in the brain (Sato *et al.*, 2023), these findings suggest that targeting estrogen signaling could be a viable strategy to mitigate neuroinflammation. Moreover, studies have shown that brain-derived estrogen exerts potent anti-inflammatory and neuroprotective effects (Zhang *et al.*, 2014), further supporting my findings. Together, my data suggests that E2's anti-inflammatory actions could have significant therapeutic relevance across both the PNS and CNS. By enhancing mitochondrial function and suppressing inflammatory pathways, E2 holds potential as a dual therapeutic agent for combating neurodegenerative and neuroinflammatory diseases.

Role of E2 in promoting neuronal survival

E2 plays a significant role in promoting neuronal survival across both the PNS and CNS. In the PNS, although I did not directly assess cell survival, E2's ability to enhance neurite outgrowth in DRG neurons suggests a potential protective effect against neuropathic conditions characterized by dying back of nerve fibers (Cashman and Höke, 2015). This is supported by previous studies showing that E2 increases the survival of cultured embryonic DRG neurons deprived of nerve growth factor (NGF) (Patrone *et al.*, 1999). In contrast, within the CNS, E2 alone did not significantly impact neuronal viability. However, it effectively preserved cortical neurons against A β mediated neurotoxicity, which is particularly relevant in AD (Chapter 3) (Whitwell, 2010). This protective effect aligns with other studies demonstrating the ability of E2 to prevent A β -induced cell death in various neuronal models, including cholinergic cell lines, SH-SY5Y neuroblastoma cells, and hippocampal neurons (Marin *et al.*, 2003; Mateos *et al.*, 2012; Nilsen *et al.*, 2006).

Estradiol-mediated neuroprotection extends beyond AMPK

E2 exerts neuroprotective effects by regulating multiple signaling pathways and homeostatic molecules. In my PNS study, E2 enhanced axonal regeneration/sprouting in DRG neurons, and inhibition of AMPK abolished this effect, implicating AMPK as a key mediator. However, it is likely that additional mechanisms also contribute to E2's actions. Previous studies have highlighted that endogenous BDNF is critical for sensory neuron survival and regeneration following injury (Acheson *et al.*, 1995). Inhibition of endogenous BDNF reduced the expression of regeneration-associated genes (RAGs) and impaired neurite extension (Geremia *et al.*, 2010). Interestingly, an estrogen response element (ERE) has been identified within the BDNF gene (Sohrabji *et al.*, 1995), suggesting that E2 may enhance neurite outgrowth by upregulating

endogenous BDNF expression and release. Another potential contributor is neuroglobin (Ngb), which is upregulated by E2 (Barreto *et al.*, 2021), and has neuroprotective actions. Ngb is found in neurons including localization to neuronal growth cones and mitochondria (Xiong *et al.*, 2018), and may represent an additional E2-regulated pathway supporting nerve repair (Barreto *et al.*, 2021).

In my CNS studies, E2 provided significant protection against A β -induced toxicity, again with AMPK inhibition abolishing its protective effects. However, while E2 suppressed A β -induced NF- κ B activation, inhibition of NF- κ B alone did not fully rescue neuronal survival, suggesting involvement of other signaling mechanisms. Indeed, E2 activates additional pro-survival signaling cascades, such as the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and phosphatidylinositol 3-kinase (PI3K/Akt) pathways (Saraceno *et al.*, 2018; Singh *et al.*, 1999). These pathways upregulate antiapoptotic proteins such as Bcl-2 (Saraceno *et al.*, 2018) and Bcl-xL (Pike, 1999), while suppressing proapoptotic molecules like Bax (Nilsen *et al.*, 2006). Supporting this, A β exposure downregulates Bcl-2 and promotes Bax-mediated mitochondrial dysfunction, while E2 pretreatment restores Bcl-2 levels and prevents Bax translocation and cytochrome c release, associated with cell survival (Nilsen *et al.*, 2006). Furthermore, E2 protects against A β toxicity by inhibiting death domain-associated protein (Daxx) mediated apoptotic responses (Pike, 1999). Beyond apoptosis regulation, E2 also promotes A β clearance. Studies have demonstrated that E2 upregulates neprilysin, a major A β -degrading enzyme, through activation of ER α and ER β (Liang *et al.*, 2010). Additionally, E2 also reduces A β production itself, further decreasing neurotoxic burden in various neuronal models (Xu *et al.*, 1998). Together, these findings highlight that E2's neuroprotection is multifactorial, involving not

only AMPK activation but may also involve modulation of BDNF, Ngf, anti-apoptotic pathways, and A β clearance.

Strengths and Limitations

Strengths: This study offers several key strengths that enhance its scientific value and translational relevance. By investigating the effects of E2 on both peripheral (DRG) and central (cortical) neurons, the study addresses two interconnected systems both of which express ERs and are affected in neurodegenerative conditions. This dual focus provides a broader understanding of E2-mediated neuroprotective and neurotrophic potential across the nervous system. A major strength lies in the mechanistic insights into E2-mediated signaling. My study demonstrates consistent activation of AMPK signaling in both neuronal models, and modulation of the NF- κ B pathway in cortical neurons. These findings, particularly the role of the AMPK–PGC1 α axis, underscores the importance of mitochondrial regulation by E2 and strengthens the molecular rationale for its use as a therapeutic agent. While whole animal rodent disease models (including well characterized transgenic approaches) were not used, both *in vitro* studies employed disease relevant stressors: A β peptide to mimic Alzheimer's pathology and axotomy to model peripheral nerve injury. These approaches reproduce some of the key pathological features of neurodegeneration and provide a valuable entry point for therapeutic investigation.

Another significant strength is the demonstration of mitochondrial rescue by E2. Given the central role of mitochondrial dysfunction in diseases (Li *et al.*, 2023a; Melli and Höke, 2009), this finding supports the therapeutic relevance of targeting mitochondrial health. Methodologically, the use of Western blotting for protein level validation, Sholl analysis for quantifying neurite complexity, Seahorse assays for assessing mitochondrial function in live cells, and primary neuronal cultures rather than immortalized cell lines adds robustness and physiological relevance

to my study's conclusions. Finally, although the experiments were conducted *in vitro*, my findings establish a solid foundation for future *in vivo* studies (discussed in next sections). Together, these strengths position my study as a promising step toward developing estrogen-based interventions for neurodegenerative disorders affecting PNS and CNS.

Limitations: While my study provides valuable insights into the neurotrophic and neuroprotective effects of E2 on mitochondrial function and neuroinflammation in both peripheral and central neurons, several limitations must be acknowledged. Firstly, the study utilized *in vitro* cultures of DRG neurons and primary cortical neurons to model peripheral regeneration and A β -induced toxicity, respectively. Although these systems are widely accepted for mechanistic exploration (Chiang *et al.*, 2005), they do not fully capture the complexity of neuropathy nor AD pathology. Clinical conditions involve additional regions such as skin-innervating nerves in neuropathy (Ishii and Iadecola, 2015; Rao *et al.*, 2022) and the hippocampus or hypothalamus in AD (Rzepecki *et al.*, 2019; Spencer *et al.*, 2008) that were not represented here. These areas also express ERs (Rzepecki *et al.*, 2019; Spencer *et al.*, 2008) and their exclusion from *in vitro* models may overlook region-specific responses to estradiol.

Secondly, the neurons used in Chapter 3 were taken from fetal mice, which is a common practice because these cells survive well in culture (Cetin *et al.*, 2022). However, they do not represent the aged neurons affected in AD. Aging alters receptor expression, mitochondrial function, and inflammatory responses, all of which influence disease progression and treatment outcomes (Adams *et al.*, 2002; Lanza and Nair, 2009; Li *et al.*, 2023b; Mehra *et al.*, 2005; Somasundaram *et al.*, 2024). In Chapter 2, we used an adult DRG axotomy model, which is a well-established approach for studying peripheral nerve injury and regeneration. However, it models a direct injury rather than the slow, progressive axonal degeneration typically observed in chronic neuropathies

such as diabetic or inflammatory neuropathy (Zaino *et al.*, 2023). A β toxicity in my study was induced by exogenous peptide application, bypassing the crucial step of sequential APP proteolysis that leads to neurotoxic A β formation in AD (O'Brien and Wong, 2011). This approach overlooks upstream mechanisms and gradual pathology accumulation. While useful for mechanistic insight, older transgenic models may offer a more physiologically relevant context. Additionally, our focus on A β excluded tau aggregation, another key hallmark of AD, which must be targeted alongside A β for effective therapeutic outcomes. Furthermore, in the CNS study, I only measured markers of neuroinflammation within isolated neurons, not the broader inflammatory process. True neuroinflammation involves a complex interplay of multiple cell types, including astrocytes and microglia in the CNS, and macrophages, Schwann cells, and neutrophils in the PNS (Singh, 2022; Wei *et al.*, 2019; Yu *et al.*, 2020). This limitation underscores the need for future studies to include these diverse cell types to fully understand inflammatory responses in neurodegenerative diseases.

A significant translational concern is the oncogenic potential of estradiol. Long-term exposure has been linked to increased risks of breast and uterine cancers (Johansson *et al.*, 2022). While this study focused on neuronal effects, future evaluations must carefully assess safety, especially in postmenopausal women. SERMS may offer a safer alternative by providing tissue-specific estrogenic effects without stimulating other estrogen-sensitive tissues. Another limitation is the lack of consideration for sex differences. Estrogen levels and receptor expression vary between sexes and change with age (Yu *et al.*, 2022), influencing both disease susceptibility and treatment response. Notably, women are at higher risk for AD in older age (Fratiglioni *et al.*, 1997), partly due to postmenopausal estrogen decline (Paganini-Hill and Henderson, 1994). These differences may require sex-specific dosing of E2, although I examined the effects of E2 in female DRG neurons, a detailed comparison between male and female responses was not conducted,

particularly in the CNS portion of this study. Additionally, the study did not investigate the role of male sex hormones, such as testosterone and dihydrotestosterone (DHT), which have been implicated in neuroprotection and potentially delaying AD progression (Bianchi, 2022; Kang *et al.*, 2014; Yang *et al.*, 2020b). Collectively, these limitations highlight the need for more comprehensive, age and sex inclusive *in vivo* models that address multiple facets of neurodegeneration.

Future directions

To extend the translational value of my findings, several critical areas warrant further investigation. Most importantly, *in vivo validation* is essential to confirm the neuroprotective and neurotrophic effects of E2 observed in my *in vitro* models. Peripheral nerve injury models, such as sciatic nerve crush or diabetic neuropathy, can be utilized to assess axonal regeneration and functional recovery. Using targeted E2 delivery via intrathecal or nerve-directed routes could help evaluate its localized therapeutic benefits while reducing the risk of systemic side effects. In the CNS, aged AD models such as the triple-transgenic (3xTg), transgenic CRND8 (TgCRND8), and five familial AD mutations (5xFAD) mice (Zhong *et al.*, 2024) are highly relevant for investigating E2's effects on cognitive function and neuropathology. Combining ovariectomy with E2 replacement in these models can simulate postmenopausal estrogen decline and allow for the evaluation of estradiol's potential in mitigating AD-related cognitive deficits. *Behavioral and functional assessments* such as the Morris water maze or Barnes maze should be used to determine whether the molecular improvements observed with E2 treatment translate into meaningful cognitive outcomes *in vivo*.

Another important direction is the *investigation of sex differences*. E2 levels and receptor expression vary significantly between males and females and change dramatically with age

especially after menopause in women (Yu *et al.*, 2022). These differences influence susceptibility to neurodegeneration and responsiveness to hormonal therapies. Notably, research using the 3xTg-AD mouse model has shown that female mice exhibit early mitochondrial dysfunction, while similar changes appear later in males (Djordjevic *et al.*, 2020). These findings underscore the importance of incorporating sex, brain region, and age as variables in future studies, which could reveal distinct therapeutic windows and optimize treatment strategies. Given the oncogenic risks associated with long-term E2 use, *SERMs* represent a promising alternative. SERMs offer tissue-specific modulation of estrogen receptors, potentially preserving the beneficial effects of E2 in neurons while avoiding adverse outcomes in estrogen-sensitive tissues like the breast and uterus. Evaluating different SERMs in both sexes and across age groups could refine their therapeutic application and improve safety profiles.

Finally, my findings in Chapters 2 and 3 suggest that E2's protective actions are mediated, at least in part, through the *AMPK signaling pathway*, opening the door for *combination therapies targeting AMPK* alongside E2 or SERMs. One approach could involve using ovariectomized (OVX) mice to mimic estrogen loss in females, and aromatase knockout (ArKO) mice or orchidectomized males to reduce estrogen levels, in established animal models of AD or peripheral neuropathy. This would allow assessment of the combined effects of E2 and pharmacological AMPK activators like metformin or AICAR under disease-relevant conditions. Intracerebroventricular (ICV) delivery could be used for CNS targets, while intramuscular injection may be suitable for peripheral tissues. This strategy would help determine whether AMPK activation synergizes with E2 in restoring neuronal function and mitigating neurodegeneration.

My results also underscore mitochondria as a key therapeutic target. Modulating mitochondrial dynamics such as fusion and fission could further improve mitochondrial health and neuronal resilience. Together, these approaches can better support axonal regeneration, and counter A β toxicity than monotherapies alone. In summary, future research should focus on *in vivo* validation, sex-specific responses, safer estrogen-based alternatives, and combination strategies to fully harness estradiol's therapeutic potential in both peripheral and central neurons.

Concluding remarks: In summary, this thesis demonstrates that estradiol exerts robust neurotrophic and neuroprotective effects in both peripheral and central neurons by targeting AMPK signaling, mitochondrial function, and neuroinflammation. Through distinct yet convergent mechanisms in DRG and cortical neurons, E2 enhances mitochondrial health, promotes axonal regeneration, and confers protection against A β toxicity. These findings establish AMPK as a central mediator of E2's actions and underscore the therapeutic potential of targeting mitochondrial dysfunction and inflammatory pathways in neurodegenerative diseases. While *in vitro* models and experimental limitations exist, the insights gained provide a strong foundation for future *in vivo* studies and the development of targeted therapies. Collectively, this work advances our understanding of estradiol's multifaceted neuroprotective roles and highlights promising avenues for intervention in neurodegenerative disease.

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