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Theme

Structural and functional analysis of APP695

Presented on 29/06/2025 before the jury composed of

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DEDICATION

To myself, for the resilience, effort and determination that carried me through every challenge of this journey.

To my dear parents, Fatima and Azzedine for their unconditional love, endless support, and for always believing in me. Your sacrifices and prayers have been my greatest source of motivation

To my sister Aya, whose constant support and constant encouragement have uplifted me at every step. You have been a comfort and an inspiration.

To my little brother Mohammed, whose curiosity, laughter, and quiet pride kept my spirit alive throughout this journey.

To my friends, who stood by my side with patience and reminded me to breathe, to laugh, and to keep going when things got tough.

This accomplishment reflects your love, strength, and belief in me.

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Lastly, I would like to thank everyone who supported me whether from near or afar during this endeavor. Your encouragement, kindness, and contributions, however big or small, have been truly invaluable in helping me reach this milestone

Abstract

APP695 is a neuronal isoform of the amyloid precursor protein, involved in synaptic functions. Post-translational modifications like phosphorylation and ubiquitination play critical roles in regulating APP695 levels and trafficking, which affects its cleavage. Moreover, dysfunction in APP cleavage can lead to the accumulation of neurotoxic fragments associated with Alzheimer's disease. This study used in silico tools including ΔG Predictor, TOPCONS, NetPhos 3.1, and GPS-Uber to perform structural and functional analysis of APP695. Several potential phosphorylation sites were identified. Among them, residues T653, T655, Y682, T686 and Y687 belong to the sorting motifs in the C-terminal region, while T668 is located between them, along with a key ubiquitination site K688 which plays a critical role in trafficking and processing of APP695. Notably, phosphorylation at T668 increases the interaction with β-secretase cleavage and disrupt adaptor protein interactions, shifting APP processing toward amyloidogenic pathways. These findings suggest that the interplay between phosphorylation and ubiquitination could contribute to Aβ production and AD progression. These findings offer insights into how post-translational modifications may regulate APP behavior and contribute to AD pathology.

Keywords : Amyloid precursor protein, APP695, phosphorylation site, ubiquitination site, in silico analysis.

Résumé

APP695 est une isoforme neuronale de la protéine précurseur de l'amyloïde (APP), impliquée dans les fonctions au niveau de la synapse. Les modifications post-traductionnelles, telles que la phosphorylation et l'ubiquitination, jouent un rôle essentiel dans la régulation du taux et du trafic intracellulaire de l'APP695, influençant ainsi son clivage. Par ailleurs, le dysfonctionnement du clivage de l'APP695 peut entraîner l'accumulation de fragments neurotoxiques associés à la maladie d'Alzheimer. Cette étude a utilisé des outils in silico, notamment ΔG Predictor, TOPCONS, NetPhos 3.1 et GPS-Uber, pour réaliser une analyse structurale et fonctionnelle de l'APP695. Plusieurs sites de phosphorylation a potential ont été identifiés, notamment les résidus T653, T655, Y682, T686 et Y687 situés dans la région C-terminale, ainsi qu'un site clé d'ubiquitination K688. La phosphorylation joue un rôle crucial dans le trafic et la modification. APP695 est à noter que la phosphorylation en T668 pourrait augmenter le clivage par la β-sécrétase et perturber les interactions avec les protéines adaptatrices, orientant ainsi l'APP vers des voies amyloïdogènes. Ces résultats suggèrent que l'interaction entre phosphorylation et ubiquitination pourrait contribuer à la production de peptide Aβ et à la progression et de la maladie d'Alzheimer. L'étude apporte ainsi un éclairage sur la manière dont les modifications post-traductionnelles peuvent réguler le comportement de l'APP et participer à la pathogenèse de la maladie.

Mots-clés : protéine précurseur de l'amyloïde, APP695, site de phosphorylation, site d'ubiquitination, analyse in silico.

الملخص

البروتين APP695 هو أحد الأشكال العصبية لبروتين السلَف النشواني (APP)، ويلعب دورًا محوريًا في الوظائف المشبكية. تساهم التعديلات ما بعد الترجمة، مثل الفسفرة واليوبيكويتين، بشكل أساسي في تنظيم مستويات APP695 وحركيته داخل الخلية، مما يؤثر بشكل مباشر على عملية شطره. علاوة على ذلك، فإن خلل شطر APP قد يؤدي إلى تراكم شظايا سامة للأعصاب ترتبط بمرض الزهايمر. في هذه الدراسة، تم استخدام أدوات تحليل حاسوبية (in silico) مثل ΔG Predictor و TOPCONS و NetPhos 3.1 و GPS-Uber لتحليل البنية والوظائف المحتملة لـ APP695. وقد تم تحديد العديد من مواقع الفسفرة ذات الاحتمالية العالية، بما في ذلك ثلاثة مواقع في الطرف الكربوكسيلي T654,T686 وT688، بالإضافة إلى مواقع رئيسية للارتباط باليوبيكويتين مثلK688 .ومن الجدير بالذكر أن الفسفرة في الموقع T668 قد تعزز من شطر APP عبرβ-secretase ,وتُضعف تفاعله مع البروتينات المرافقة، مما يدفع مسار المعالجة نحو المسار النشواني. تشير هذه النتائج إلى أن التفاعل بين الفسفرة واليوبيكويتين قد يسهم في إنتاج بيتا-أميلويد (Aβ) وتطور مرض الزهايمر. وتوفر هذه النتائج رؤى جديدة حول كيفية تأثير التعديلات ما بعد الترجمة في تنظيم سلوك APP ودورها في تطور الأمراض التنكسية العصبية.

الكلمات المفتاحية :بروتين السلَف النشواني، APP695، مواقع الفسفرة، اليوبيكويتين، تحليل حاسوبي(in silico) .

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Abbreviation list

AD: Alzheimer's Disease

APP: Amyloid Precursor Protein

Aβ: Amyloid-beta

AICD: APP Intracellular Domain

AcD: Acidic Domain

CNS: Central Nervous System

CTF: C-terminal Fragment

ΔGapp: Apparent Free Energy of Membrane Insertion

FASTA: Fast Alignment Search Tool for All

CDK5: Cyclin-Dependent Kinase 5

CKI / CKII: Casein Kinase

GPS-Uber: Group-based Prediction System for Ubiquitination Extended

JMR: Juxta Membrane Region

KPI : Kunitz Protease Inhibitor

NetPhos: Neural Network Phosphorylation Prediction Tool

PKC: Protein Kinase C

PTMs: Post-Translational Modifications

sAPP: Soluble APP

TM: Transmembrane Domain

TOPCONS: Consensus Prediction Tool for Membrane Protein Topology

UPS: Ubiquitin–Proteasome System

TGN: Trans-Golgi Network

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Introduction

The Amyloid Precursor Protein (APP) is a type I transmembrane glycoprotein that appears to function like a growth factor and/or an adhesion molecule. It undergoes several post-translational modifications that regulate critical biological processes such as endocytosis, degradation, and trafficking. These modifications include glycosylation, ubiquitination, sulfation, phosphorylation, and palmitoylation (Selkoe, 2001; Bhattacharyya et al., 2013).

Among these modifications phosphorylation and ubiquitination are particularly important for modulating APP behavior. Phosphorylation alters its intracellular trafficking and interactions with other cellular components, while ubiquitination targets APP for proteasomal degradation, thereby determining its intracellular fate (El Ayadi et al., 2012).

The APP is widely expressed in various tissue types. In humans, the APP gene undergoes alternative splicing, resulting in three main isoforms: APP770, APP751, and APP695. Notably, APP695 is predominantly expressed in neurons within the central nervous system. This neuron-specific distribution suggests a specialized functional role for APP695 within the CNS (Kang & Müller-Hill, 1990; Müller *et al*., 2017).

Under physiological conditions, APP has effects on both excitatory and inhibitory neurotransmission. APP can also act through the fragments produced by proteolytic cleavage. Extracellular domain and intracellular domain contribute to gene regulation (Müller et al., 2017; Rice et al., 2019).

In pathological cases, the cleavage of APP leads to the formation of amyloid plaques, primarily composed of β-amyloid peptide, which accumulate in the extracellular space. This accumulation disrupts synaptic transmission, induces oxidative stress, and promotes chronic neuroinflammation, ultimately contributing to neuronal death and disease progression (Vassar et al., 1999; Erkkinen et al., 2017). The amyloid plaque is a hallmark of Alzheimer's disease, the leading cause of dementia in the elderly (Erkkinen et al., 2017).

another key mechanism contributing to its pathological role is the disruption of post-translational modifications by means of phosphorylation and ubiquitination that influence APP’s processing, trafficking, and degradation.

Furthermore, ubiquitination and phosphorylation of APP constitute other mechanisms contributing to its pathological role since they influence APP’s trafficking and degradation (Popovic et al., 2014). Thus, dysregulation in the phosphorylation and/or ubiquitination status

of APP has been implicated in AD pathogenesis. Russo et al., (2001) found that Tyr757 in APP770 has been more phosphorylated in AD patients compared to controls.

In this work, we aim to predict phosphorylation and ubiquitination sites on APP695 to deepen our understanding of how these post-translational modifications influence APP activity. We utilize NetPhos 3.1 to identify potential phosphorylation sites and highlight regulatory hotspots susceptible to kinase action. In parallel, we use GPS-Uber to predict ubiquitination sites. To gain structural insights, we analyze the topology of APP695 using ΔG Predictor and TOPCONS, which help define its transmembrane characteristics. Furthermore, we conduct a sequence alignment between APP695 and APP770 using Clustal Omega, as APP770 is known to be ubiquitous and better studied, with more extensively documented phosphorylation and ubiquitination sites. This comparative analysis helps identify conserved and variable regions, shedding light on isoform-specific properties.

After a general introduction, highlighting the aims of our study, this manuscript consists of two parts:

The first part is a bibliographic summary consisting of two chapters:

• Chapter I presents general information on APP, its structure, the regulation of its activity

• Chapter II presents the central nervous system, the APP function and its expression in CNS as well as their role in neurodegenerative diseases.

The second part is experimental and consists of two chapters:

• Chapter III explains the methods and tools used in this study

• Chapter IV presents the results obtained from the different methods applied during our work, their interpretation and a discussion.

Finally, this dissertation concludes with a general conclusion.

Part I
Bibliographic summary

Chapter I Amyloid protein precursor

1) Amyloid protein precursor family

The APP family expressed in a wide variety of eukaryotes species, its primarily found in vertebrates, specially mammalian species express APP and two additional APP related genes: amyloid precursor like protein 1 (APLP1) amyloid precursor like protein 2 (APLP2) (Poeck *et al*., 2012).

In the human, APLP1 gene is located on chromosome 19 and APLP2 gene is located on chromosome 11, whereas APP gene is located on chromosomes 21 and has 18 exons. The latter can be alternatively spliced into three major isoforms of APP transcript, which are defined based on the number of amino acids, APP 695, APP751 and APP770 (which contain 695 ,751 and 770 amino acids, respectively) **figure 1.1** (Yoshikai *et al*. 1990).

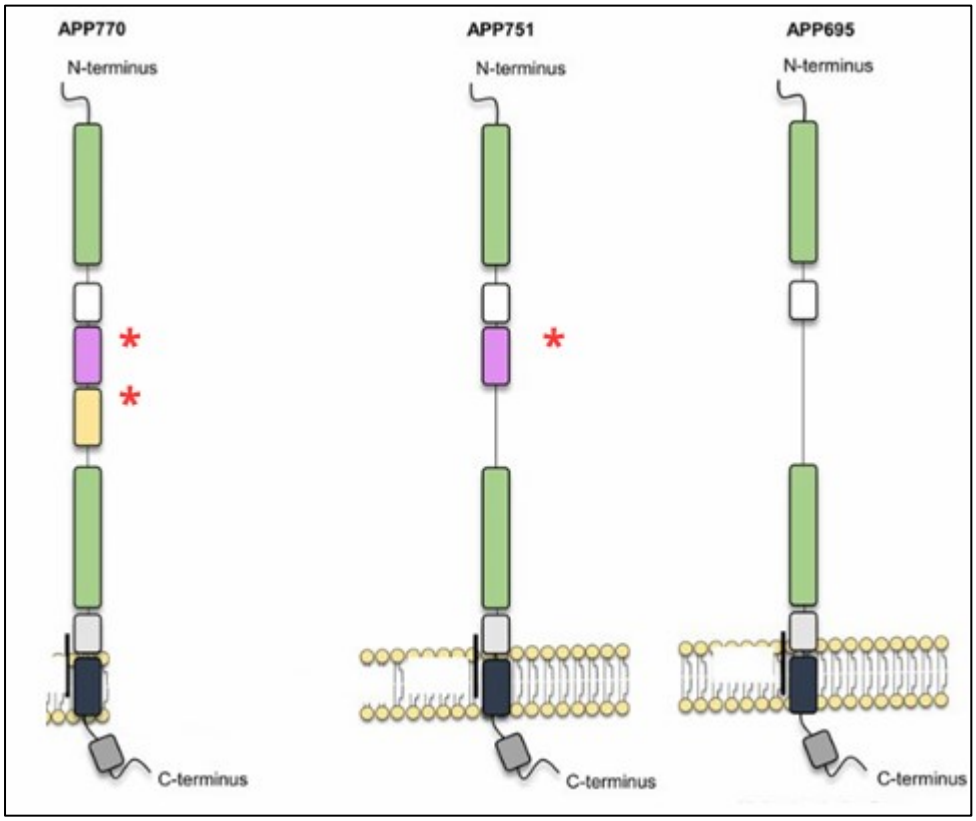

Figure 1.1: APP isoformes (Müller et al., 2017)

The three APP isoforms APP770, APP751 and APP695 differ in their extracellular domains due to alternative splicing. APP695 lacks certain domain present in the other isoforms, the additional domains are shown in red with an asterisk

2) Tissue Distribution of APP

The expression of amyloid precursor protein is ubiquitous, with presence in various systems such as the digestive, muscular, and circulatory systems (Galloway *et al*., 2007; Lee *et al*., 2008).

Notably, APP is highly expressed in the nervous system, with the predominantly APP695 isoform found in neuron cells of central nervous system (CNS) (Kang & Müller-Hill, 1990), While the APP751 and APP770 isoforms are expressed in glial cells especially astrocytes **figure** 1.**2** (Belyaev *et al*., 2010).

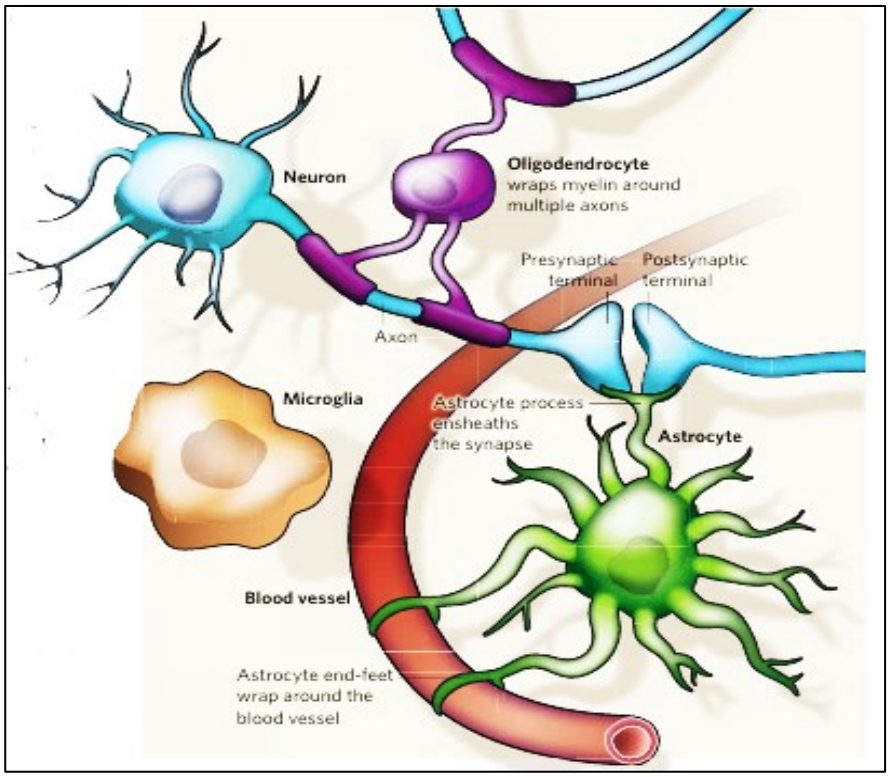

Figure 1.2: Glia-neuron interaction (Allen and Barres 2009

3) Amyloid protein precursor structure

APP is a type I transmembrane protein that has a conserved topology, shared by the three isoforms. It has a large extracellular N-terminal domain that represents about 88% of the whole protein mass, a single-pass transmembrane domain and a small cytoplasmic C-terminus (O'Brien & Wong, 2011; Chen *et al*., 2017).

The extracellular region of APP contains two key functional domains E1 and E2.

E1 is a cysteine-rich domain and consists of two distinct regions. The first is the growth factor-like domain (GFLD), which contains heparin-binding domain (HBD). The second region is a copper-binding domain (CuBD). The HBD is structurally composed of an α-helix and anti-parallel β-sheet, which contribute to its binding properties, while the CuBD contains single α -helix and a short β-sheet (Dahms *et al*., 2016). The E2 domain is predominantly helix-rich and has the ability to form dimers. It includes an HBD and several putative metal-binding sites, which are thought to stabilize its relatively rigid conformation **figure 1.3** (Wang *et al*., 2014).

The three isoforms share the same flexible region that connects E1 and E2, named acidic domain (AcD) which is most likely unfolded. However longer isoforms APP770 and APP751 contain two additional functional domains that are located just after the AcD, Kunitz-type protease inhibitor (KPI) and an Ox-2 antigen domain, while the APP695 lacks them (Menéndez-González *et al*., 2006).

The unique transmembrane domain spans the cell membrane and is important for the stability of APP. This domain includes a GxxxG sequence motif that has been implicated in homodimerization and in non-covalent cholesterol binding (Munter *et al*. 2007; Barrett *et al*. 2012).

The APP intracellular domain (AICD) can vary in length and contains important regulatory elements and sorting motifs, including YTSI and YENPTY in all three isoforms (Zhang *et al*., 2011; Müller *et al*., 2017).

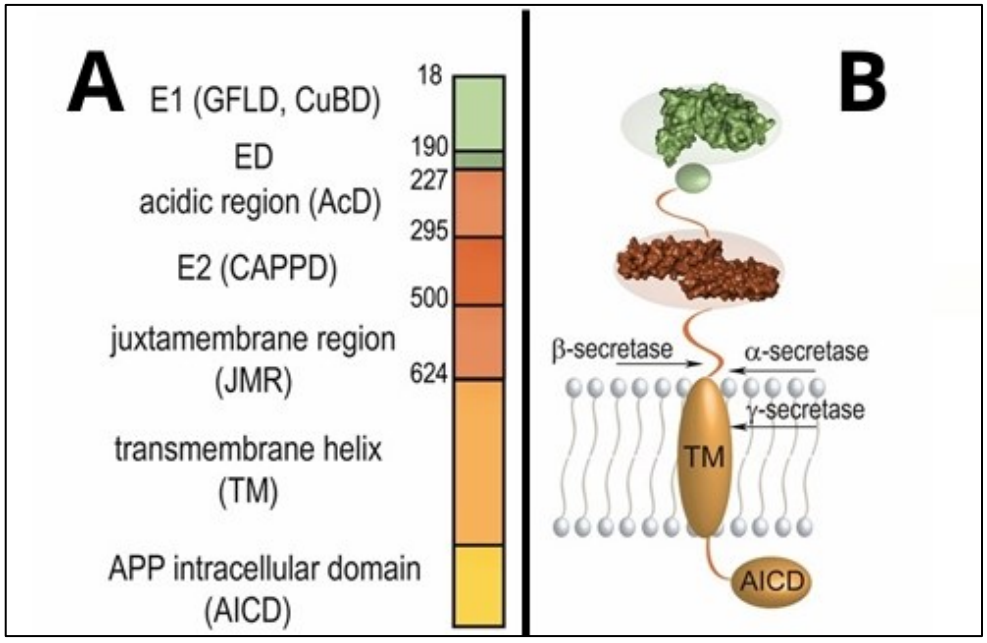

Figure 1.3: domains structure of APP (Coburger et al., 2013).

A represents the different domains of APP; **B** shows APP structure embedded in the cell membrane

GFLD: growth factor-like domain; HBD: heparin binding domain; CuBD: copper-binding domain; SP: peptide sequence; AcD: acidic domain; KPI: Kunitz-type protease inhibitor; Ox-2: Ox-2 antigen domain; AICD: APP intracellular domain.

4) Regulation of APP activity

When the APP polypeptide is synthesized, it undergoes post-translational modification, that involved in the regulation of numerous processes. such as endocytosis, degradation and

trafficking. These modifications include glycosylation, ubiquitination, sulfation, phosphorylation and palmitoylation (Selkoe, 2001; Bhattacharyya *et al*., 2013).

1. Regulation of APP by phosphorylation

Phosphorylation is the most prevalent post-translational modification that regulates many cellular processes by covalently attaching phosphate groups to specific amino acids serine/threonine and tyrosine. This reversible post-translational modification is a key regulatory switch for protein function, localization, and protein – protein interactions.

The regulation of protein phosphorylation is dependent on the crosstalk between protein kinases and protein phosphatases via complex signaling pathways. **figure 1.4**

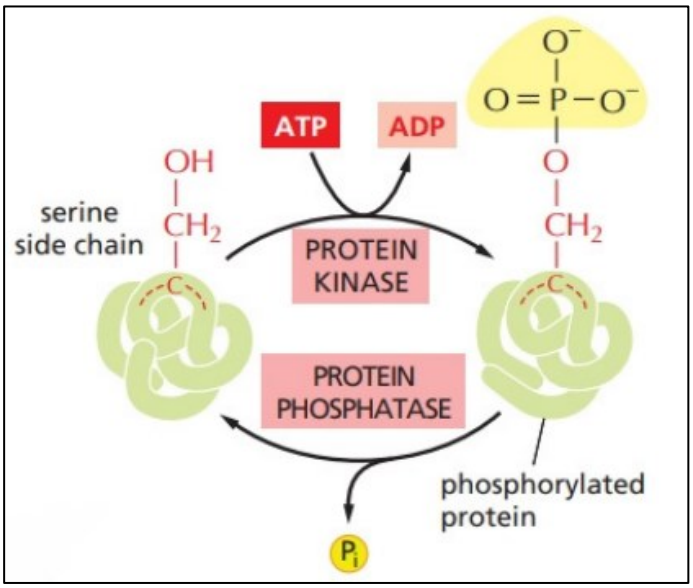

Figure 1.4: Illustration of the phosphorylation reaction catalyzed by kinases and phosphatase (Alberts et al., 2015).

Phosphorylation of APP770 occurs at multiple domains, the experimentally confirmed site is T743, which appears to bind to prolyl isomerase (PIN1) that can change protein conformation, this phosphorylation-dependent conformational change might act as a switch to control APP binding to diverse interactors proteins (Stukenberg and Kirschner, 2001)

2. Regulation of APP by ubiquitination

Ubiquitination serves as a fundamental regulatory mechanism that targets proteins for proteasomal degradation through poly-ubiquitination, as represented by the "ubiquitin code" (Komander & Rape, 2012). Ubiquitin (Ub) is a 76-amino acid protein. When it is activated, Ub is attached to target proteins at lysine residues through a three-step process, E1 enzymes activate Ub, E2 enzymes transfer it, and E3 ligases attach it to the target protein (Weissman,2001;

Glickman & Ciechanover, 2002). Distinct ubiquitin modifications modulate how proteins are routed to distinct degradation systems. One of these routs is the ubiquitin-proteasome system (UPS), which is a key mechanism for maintaining protein quality in cells and eliminate misfolded or unwanted proteins. **figure 1.5**

Studies demonstrate that APP and its fragments undergo poly-ubiquitination. leading to their degradation by the UPS pathway. In addition, ubiquitination not only signals APP for degradation but also affects its subcellular trafficking and is implicated in regulating APP endocytosis, a process that determines whether APP is recycled to the plasma membrane or directed toward lysosomal or proteasomal degradation pathways (El Ayadi *et al*., 2012). This intracellular trafficking of APP is regulated through interactions with different factors that recognizes and attaches to sorting motif YENPTY and YTSI during endocytosis. The sorting motifs on the cytosolic domain of APP isoforms have key roles during its trafficking while each of adaptors have different roles (Kerr and Small., 2005).

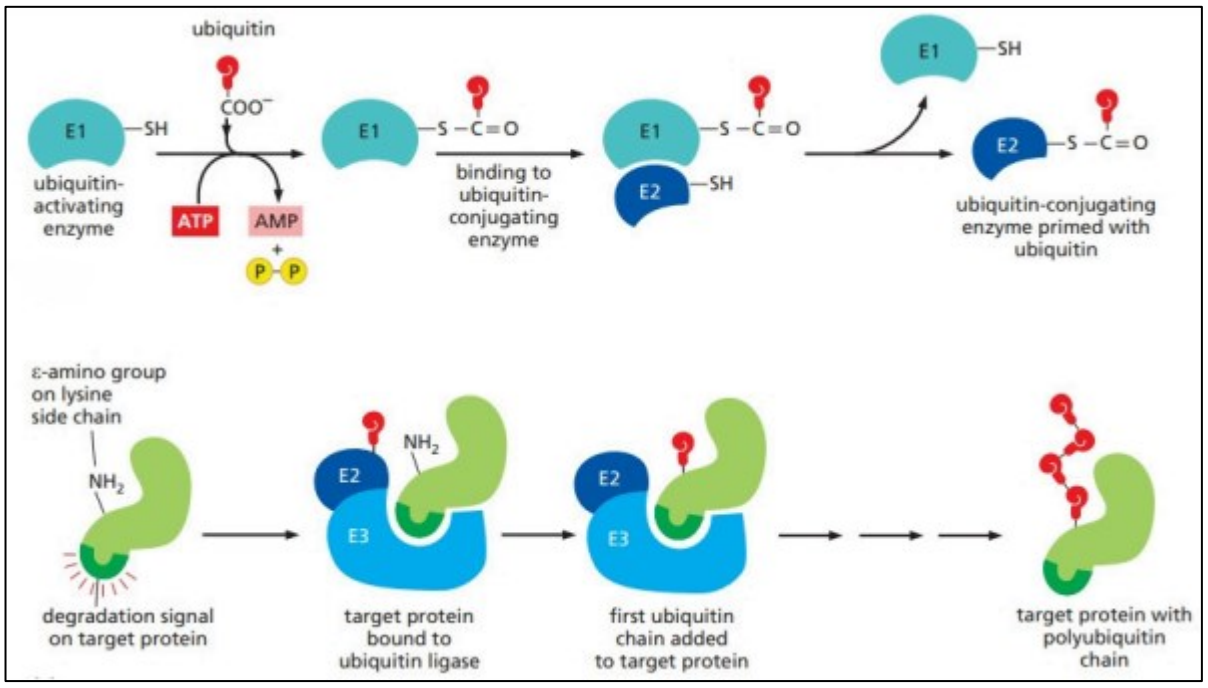

Figure 1.5: The marking of proteins with ubiquitin (Alberts et al., 2015).

3. Regulation of APP by Proteolytic Cleavage

Proteolytic cleavage is an important post transitional modification by which proteins are broken down into fragments with distinct biological and pathological functions by specific enzymes called proteases.

APP processing occurs through canonical and non-canonical pathway involving members of the secretase family. In the canonical pathway, APP is cleaved by α-, β-, and γ-secretases,

producing different fragments with distinct biological functions. These secretases are members from the ADAM family (A Disintegrin and Metalloproteinase) (Allinson *et al*., 2003). In other hand, the non-canonical secretases identified, include η-secretase, as well as Meprin β and δ-secretase (Willem *et al*., 2015; Müller *et al*., 2017).

APP undergo biological non-amyloidogenic and pathological pathways that produce the amyloid peptide, these latter depend on cells and secretase types and localization of the protein (O'Brien & Wong, 2011).

In the plasma membrane, non-amyloidogenic processing of APP695 begins, where APP is cleaved by α-secretase at glutamic E613, it generates a N-terminal secreted APP (sAPP) and C-terminal fragment, (CTF). Remaining fragment CTF contains 83 amino acids which is further cleaved by γ-secretase at alanine A638 residue, to generate two products, a 3 kDa peptide (P3) and APP intracellar domain (AICD) **figure 1.6** (Das *et al*., 2016; Chang & Such., 2005)

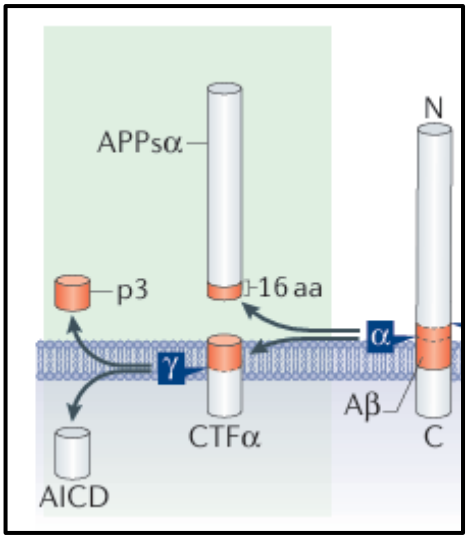

Figure 1.6: APP770 cleavage Products Generated Through Non-Amyloidogenic Processing

APPsα: Amyloid Precursor Protein soluble alpha, **P3**: 3 KDa peptide, **CTFα**: C-Terminal Fragment alpha; **Aβ:** peptide amyloid shown in red

Although the most well-studied cleavage sites are in the juxta membrane and C-terminal regions, the sAPPα fragment (extracellular domain). Coburger *et al*. (2013) demonstrated the presence of cleavages at sites such as E192 and E227 as highlighted in structural analyses **figure1.7**. Particularly at the ectodomain cleavage site, a sequence alignment was performed to show that the cleavage site residues are conserved across different species.

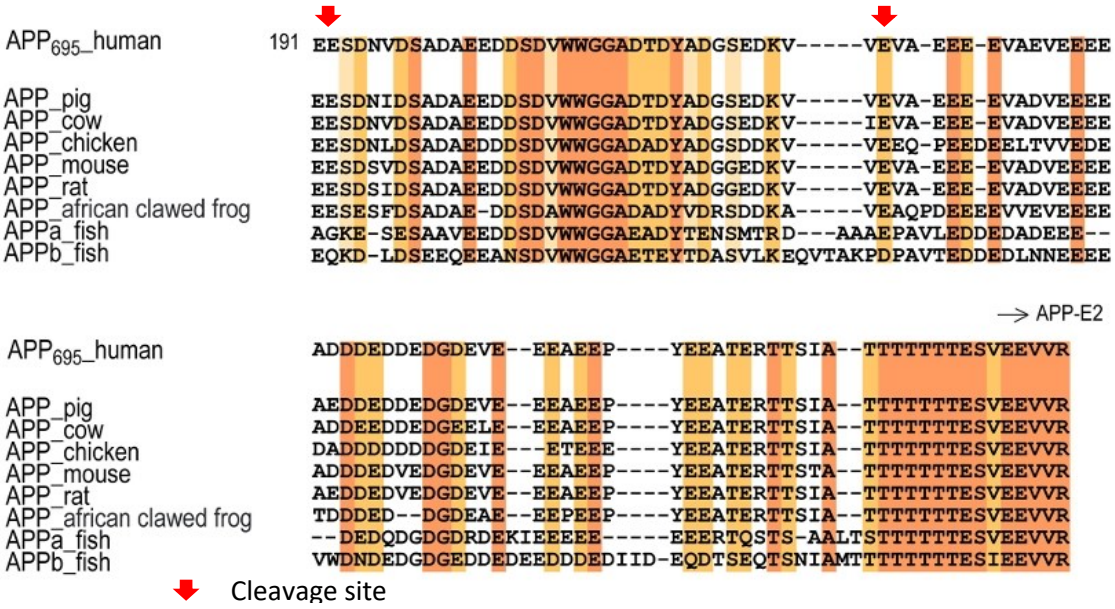

Figure 1.7: sequence alignment of the acidic region among vertebrates (Coburger et al.,2013)

Chapter II APP and Alzheimer Disease

1) Central Nervous System

Two major structures constitute the central nervous system, the brain and the spinal cord. These structures work together to integrate information coming from the peripheral nervous system and sensory organs while sending output signals to the skeletal muscles for voluntary and involuntary movement, as well as to the autonomic nervous system for coordination of the body's internal organs (Waxman., 2017). Structurally, the CNS is differentiated into gray matter, the mainly neuronal cell bodies, and white matter, the mostly myelinated axons (Kandel *et al*., 2013). The cell body receives the information, the axon transmits it via the synapse, which it shares with other neurons. **figure 2.1**

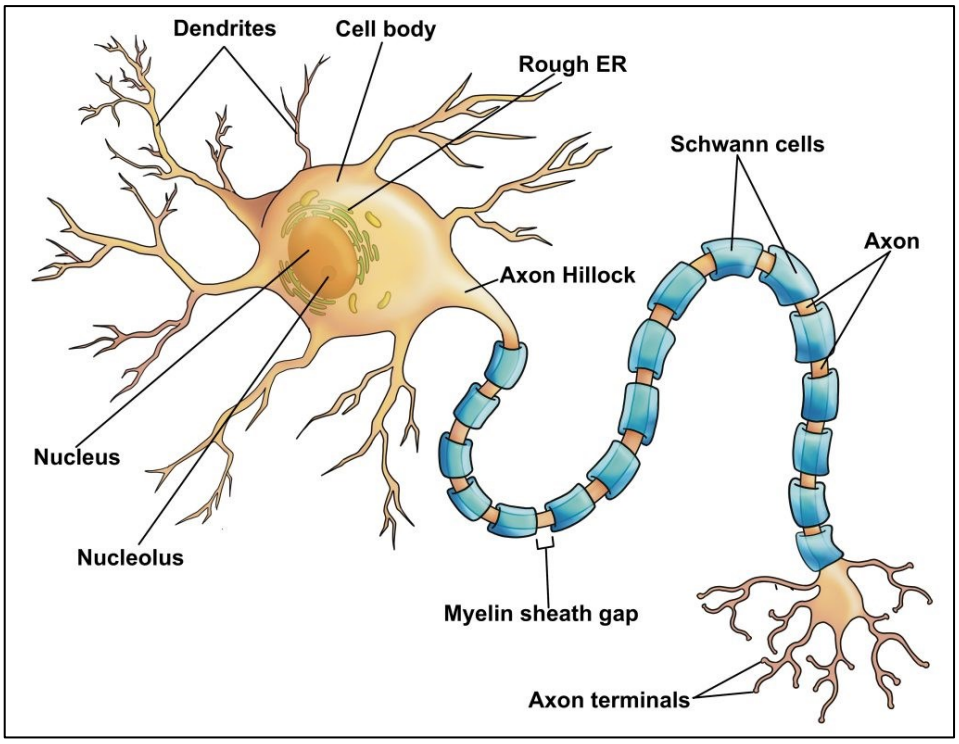

Figure 2.1: The basic parts of a neuron

. To perform these functions and remain viable, neurons need to interact with glia cells, which are the astrocytes, oligodendrocytes, and microglial cells. Glia cells provide important support to neurons like trophic and metabolic assistance. These also help regulate blood flow in response to neuronal activity, known as hyperemia and they help to maintain the homeostasis of the extracellular medium. In addition, astrocytes are involved in the formation and activity of synaptic contacts. Recent work has indicated glial cells may also be involved in higher-order brain functions such as sleep, sensory processing, memory and cognition (Allen and Barres., 2009)

2) APP and CNS

2.1 APP expression in CNS

APP is widely expressed in the CNS, with differential expression patterns among cell types and isoforms that suggest diverse physiological roles. Neurons predominantly express the APP695 isoform. In contrast, glial cells, particularly astrocytes, express APP751 and APP770 isoforms, which contain additional domains that may participate in extracellular matrix interactions **figure 2.2** (Smith *et al*., 2011; Velezmoro & Parpura., 2025).

The cellular localization of APP further reflects its functional specialization. In neurons, APP is distributed in both somatodendritic and axonal compartments. Within axons, APP is transported in specific vesicles distinct from classical synaptic vesicles, indicating a separate trafficking mechanism (Szodorai *et al*., 2009; Groemer *et al*., 2011).

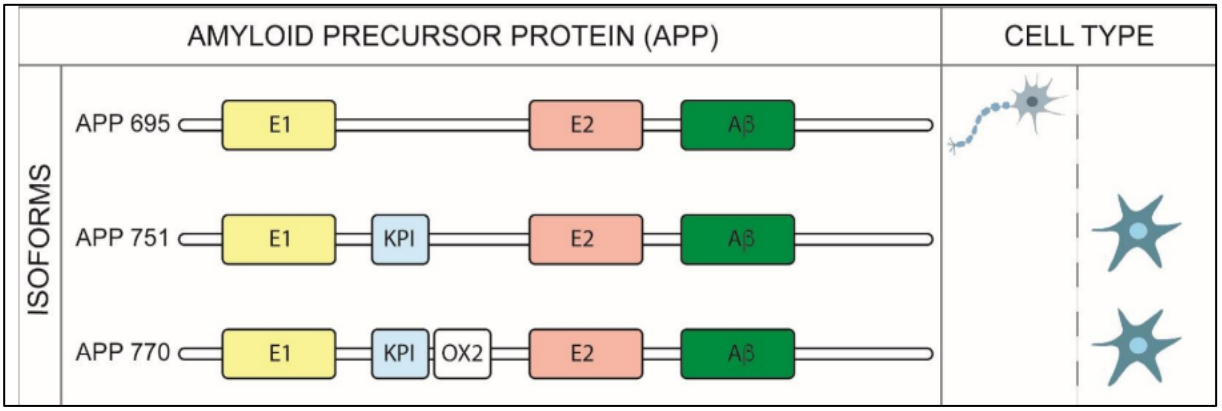

Figure 2.2: APP isoforms and their expression in neuron or astrocyte (Velezmoro & Parpura., 2025)

2.2 Functions of APP695 in nervous system

APP695 carries out a wide array of physiological functions in the nervous system, including synaptic activity, transcriptional regulation and cell adhesion. APP functions act both as receptor and ligand **Figure 2.3** (Müller *et al*., 2017).

At the synapse, APP affects both excitatory and inhibitory neurotransmission. One of its cleavage products, the secreted sAPPα fragment, binds to GABA type B receptors (RCPG) and behaves like a ligand, suggesting it has a specific function in inhibitory signaling (Rice *et al*., 2019).

The AICD of APP695 domain play a role in regulating gene transcription when it interacts with the adaptor protein Fe65, which stabilizes AICD and allows it to translocate into the nucleus where it may modulate gene expression (Borg *et al*., 1996; Cao, 2001).

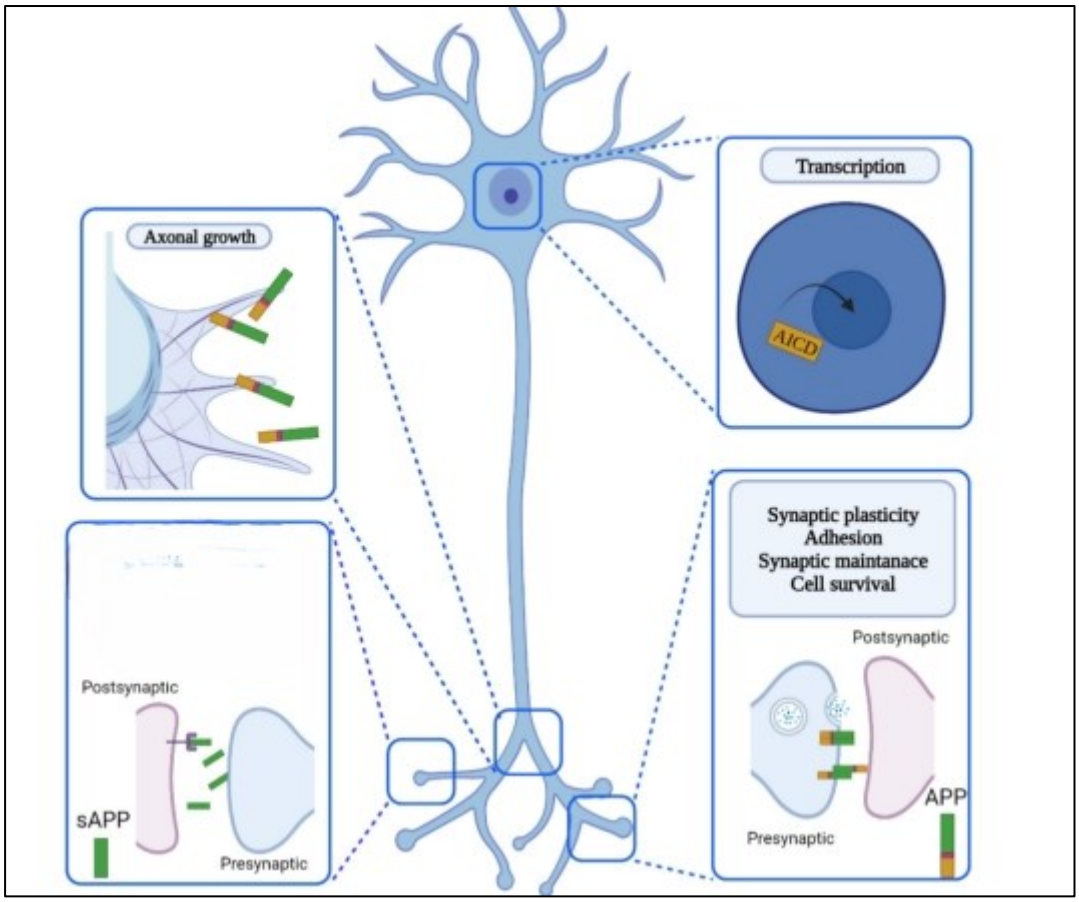

Figure 2.3: APP biological functions (Gabriele et al.,2022)

AICD: APP intracellular domain; sAPPα: soluble APP. APP plays a role in many biological processes including maintenance of synapse, transcriptional regulation and neuroprotection

The extracellular and cytoplasmic regions of APP have essential cell adhesion molecule (CAM) characteristics, similar to cadherins and integrin β1. In cell–extracellular matrix adhesion, APP may serve as a CAM capable of linking the extracellular ligand such as collagen and heparin to the cytoskeleton via its HBDs (Liu *et al*., 2019; de Jager *et al*., 2013).

Moreover, in cell–cell adhesion APP is also able to form both cis- and trans-dimers, either homophilic (APP-APP) or heterophilic (APP-APLP1/2). This enables to mediate synapse stabilization **figure 2.4** (Rice *et al*., 2022).

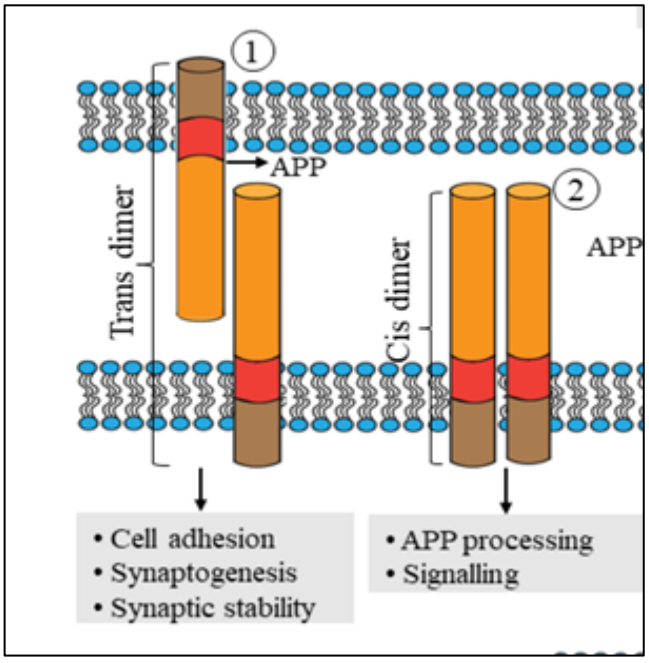

Figure 2.4 : APP dimerization (Müller et al., 2017)

1: Trans dimers; **2**: cis dimers. APP can form dimers in both cis (within the same cell membrane) and trans (between adjacent cells) configurations. These dimers may be homophilic (between two APP molecules), heterophilic (between APP and other proteins)

3) APP695 and Neurodegenerative disease

3.1 Neurodegenerative disease

Neurodegenerative diseases pose significant risks to human health, particularly in the older adult population, as neurodegenerative diseases typically entail progressive dysfunction or loss of neurons structure or function.

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease. The next most common is Parkinson's disease, which involves the death of the dopaminergic neurons of the nigrostriatal pathway and the appearance of Lewy bodies, which are intracytoplasmic inclusions in neurons comprised of insoluble fibrillated aggregates that include α-synuclein (Erkkinen *et al*., 2017; Sanchez *et al*., 2020).

AD is characterized by two pathological hallmarks first, the accumulation of extracellular amyloid plaques derived principally from the β-amyloid peptide (Aβ) and second the deposition of neurofibrillary tangles in the cytosol of neurons derived from hyperphosphorylated species of tau, a microtubule-associated protein (Erkkinen *et al*., 2017). With the rapid increase in the aging

population, AD has become a major cause of senile dementia. The cognitive dysfunctions caused by AD diminish patient's quality of life significantly, often leading to hospitalization and ultimately death attributed to its complications, it has been recognized as one of the most challenging medical conditions, placing a substantial economic and social burden on individuals, caregivers, and healthcare systems (Wimo *et al*., 2010).

3.2 APP695 in Alzheimer disease

The amyloidogenic processing APP is a central mechanism in Alzheimer's disease pathology. This pathway begins with the internalization of APP into endosomal compartments, in which case, where β-secretase (BACE1) cleaves APP at the N-terminus of the extracellular region next to juxta-membrane domain named Aβ sequence, releasing two products a soluble ectodomain fragment (sAPPβ) and a C-terminal fragment (CTFβ) of 99AA (Gervais *et al*., 1999; Müller *et al*., 2017). This C99 fragment is cleaved by γ-secretase, an enzymatic complex that releases AICD and amyloid-beta (Aβ) peptides of varying lengths of 38-43 AA (Das *et al*., 2016). Among these, Aβ40 and Aβ42 are the most prevalent, with Aβ42 being more prone to aggregation and neurotoxicity (Burdick *et al*., 1992). Aβ peptides are produced either in the late endosome/lysosomal compartment or in the TGN compartment (Vieira *et al*., 2010; Choy *et al*., 2012). Aβ peptides can be transported to the plasma membrane in vesicles and released via exocytosis. Additionally, it secret through vesicles, particularly exosomes, which originate from multivesicular bodies (MVBs) that fuse with the plasma membrane to release their contents and

are secreted into the extracellular space, where they can aggregate into oligomers, protofibrils, and eventually senile plaques **Figure 2.5** (Soriano *et al*.,2001; Rajendran *et al*., 2006)

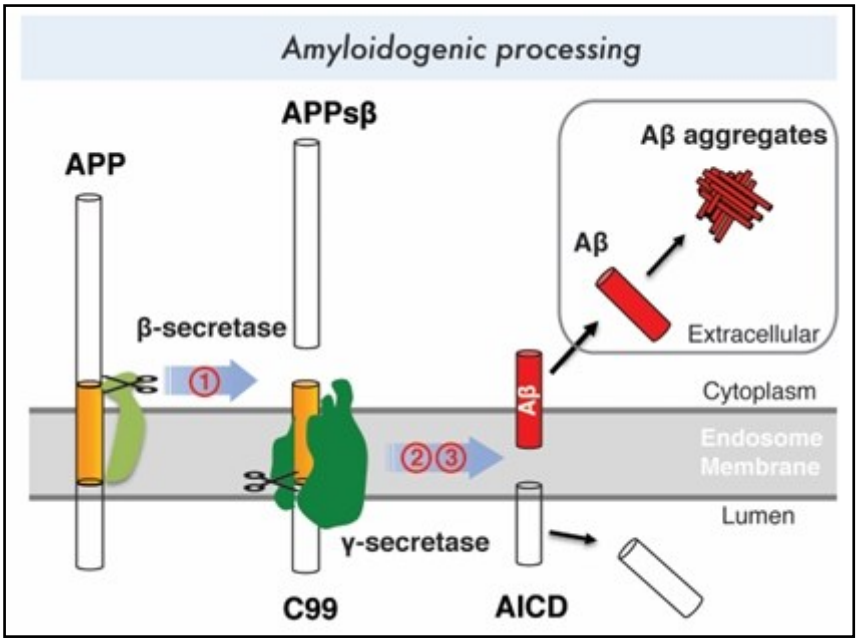

Figure 2.5: Amyloidogenic processing of APP (Zhao et al,.2020)

Notably, Aβ oligomers are believed to be more neurotoxic than fibrillar plaques because they are soluble and mobile, allowing them to diffuse through brain tissue and interact directly with neuronal membranes, synapses, and receptors. So, may play a more direct role in synaptic dysfunction and neurodegeneration (Das *et al*, 2016).

Dysregulated processing of APP can result from a variety of genetic, cellular, and environmental factors that shift its cleavage toward the amyloidogenic pathway. On the other hand, the brain's mechanisms for clearing Aβ become less efficient with age or disease. As a result, Aβ accumulates in the extracellular space. This accumulation triggers a cascade of oxidative stress, which cause neuronal damage and leads to chronic neuroinflammation. These effects disrupt neuronal function, promote synaptic loss, and ultimately cause neurodegeneration, which are hallmark features of Alzheimer's disease pathology (Vassar, 2004; Vassar *et al*., 1999).

Part II
Experimental work and Results

Chapter III Materials and methods

1. Selection choice of APP

In this study, we chose to focus on APP695, the predominately expressed isoforms in neuron. Despite its physiological relevance, APP695 remains less studied compared to the other isoforms, particularly in terms of its post-translational modifications and structure-function relationships.

We focused on this isoform because of its central role in the neurodegenerative mechanisms associated with Alzheimer's disease, particularly its involvement in the production Aβ peptides through sequential cleavage by β- and γ-secretases.

The APP695 sequence was retrieved from the UniProtKB/Swiss-Prot database https://www.uniprot.org/ , which provides protein sequences manually curated by expert scientists and several pieces of information such as the full nomenclature of the protein, the accession number and the organism

The text file downloaded from this database is in a specific format called 'FASTA, which represents the sequence using a single-letter code **Figure 3.1**. This format is supported by all the programs used in this study


FASTA

A4 amyloid protein precursor [Homo sapiens]

GenBank: CAA31830.1

GenPept Identical Proteins Graphics

```
>CAA31830.1 A4 amyloid protein precursor [Homo sapiens]
MLPGLALLLLAAWTVWALEVPTDGNAGLLAEPQIAMFCGRLNMHMNVQNGKWDSDPSGTKTCIDTKEGIL
QYCQEVYPELQITNVVEANQPVTIQNWCKRGRKQCKTHPHFVIPYRCLVGEFVSDALLVPDKCKFLHQER
MDVCETHLHWHTVAKETCSEKSTNLHDYGMLLPCGIDKFRGVEFVCCPLAEESDNVDSADAEEDDSDVWW
GGADTDYADGSEDKVVEVAEEEEVAEVEEEEADDDEDDEDGDEVEEEAEEPYEEATERTTSIATTTTTTT
ESVEEVVRVPTTAASTPDAVDKYLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWEEAERQAKNLPK
ADKKAVIQHFQEKVESLEQEAANERQQLVETHMARVEAMLNDRRRLALENYITALQAVPPRPRHVFNMLK
KYVRAEQKDRQHTLKHFEHVRMVDPKKAAQIRSQVMTHLRVIYERMNQSLSLLYNVPAVAEEIQDEVDEL
LQKEQNYSDDVLANMISEPRISYGNDALMPSLTETKTTVELLPVNGEFSLDDLQPWHSFGADSVPANTEN
EVEPVDARPAADRGLTTRPGSGLTNIKTEEISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGL
MVGGVVIATVIVITLVMLKKKQYTSIHHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN
```

Figure 3.1: APP695 sequence in FASTA format

2. Protein alignment by Crustal Omega

Clustal Omega is an open-access web tool used for multiple sequence alignment in bioinformatics. It can also be used for nucleotide sequences. (Madeira *et al*., 2024). https://www.ebi.ac.uk/jdispatcher/msa/clustalo **figure 3.2**. We aligned the protein sequences of APP695 and APP770 to compare sequences to pinpoint matching regions. These segments may

reveal shared functional roles or structural features, helping us understand how different isoforms of the APP protein relate to each other biologically.

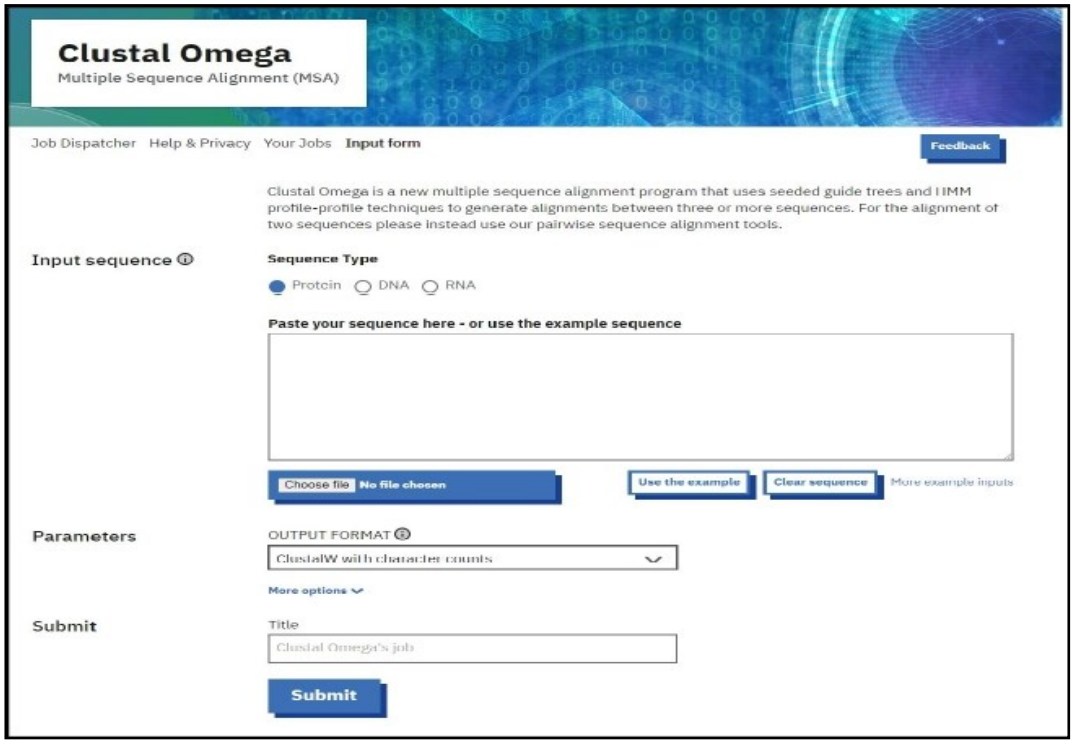

Figure 3.2: Clustal Omega online server user interface.

The online server returned a result viewer option; however, we chose to use Jalview to get a more manageable visual representation of the aligned sequences of the two proteins APP695 and APP770 for better editing capability. Jalview is a free program that allows for the editing, visualization and analysis of multiple sequence alignments **figure 3.3** (Waterhouse *et al*., 2009).

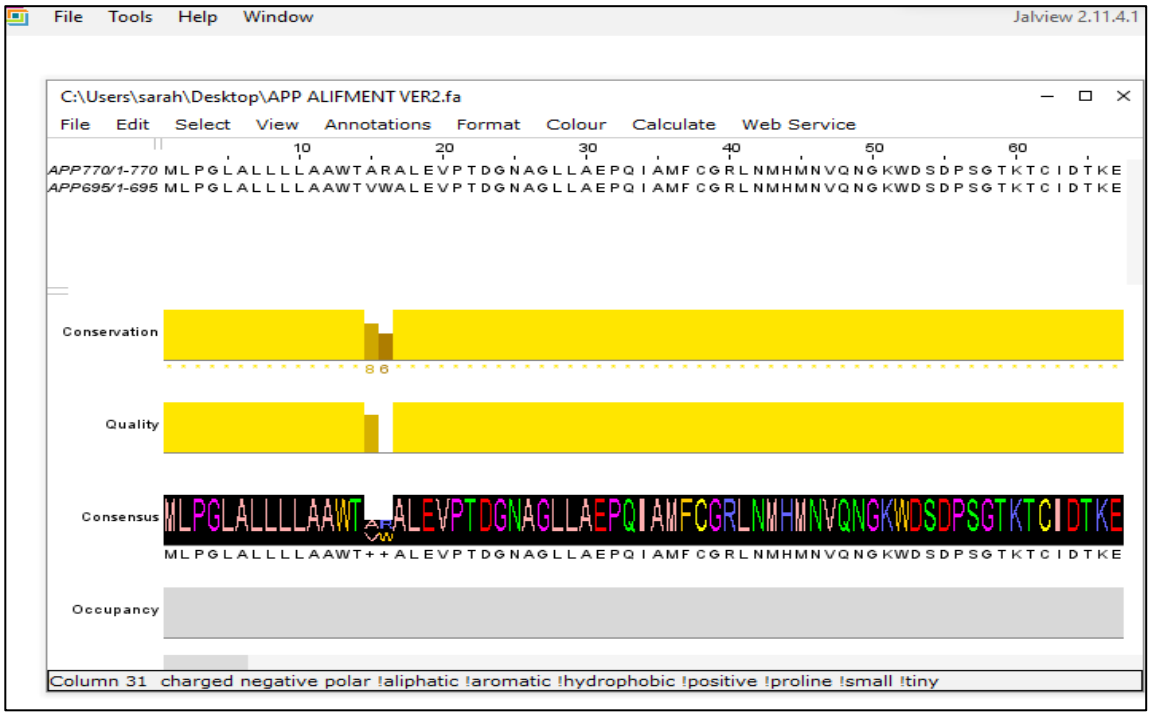

Figure 3.3: Jalview's user interface

3. Topology prediction

3.1 ΔG predictor

ΔG predictor is a validated bioinformatic pipeline designed to generate two-dimensional hydrophobicity plots and identify α helicoidal transmembrane segments within protein sequences, using the biological scale demonstrated by Hessa *et al*. (2005).

The program establishes the apparent free energy ΔGapp of membrane insertion segments, plotting these values on the ordinate (Y-axis) against residue positions on the abscissa (X-axis) for variable segment lengths. It is built upon the findings of Hessa *et al*. (2007), who measured membrane segments insertion thermodynamics using the Sec61 translocon in the endoplasmic reticulum. The negative (ΔGapp) values demonstrate sequences recognized as transmembrane helices, whereas positive values associate with cytosolic or luminal domains.

The prediction algorithm utilizes a sliding window approach to analyze protein sequence of APP695 examining contiguous segments of 19-23 amino acid residues. This systematic scanning generates multiple overlaid hydrophobicity profiles.

The program features two prediction modes, the first is "ΔG prediction", which calculates position-specific insertion energies to identify high probability transmembrane segments and the second is the "Full protein scan", a comprehensive analysis that maps all potential membrane-spanning regions across the entire sequence.

We used "Full protein scan" mode https://dgpred.cbr.su.se/index.php?p=fullscan **figure 3.4**

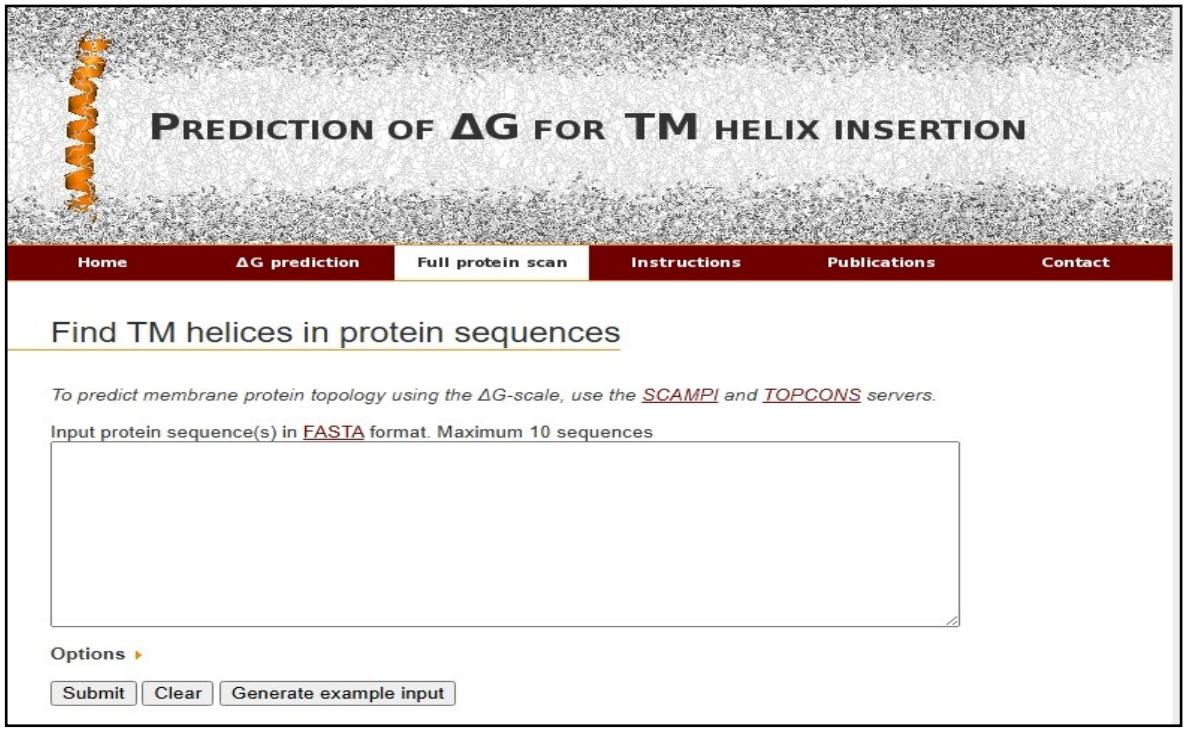

Figure 3.4: ΔG predictor user interface for "Full protein scan" mode.

3.2 TOPCONS

TOPCONS enables precise prediction of protein topology http://topcons.net/. This tool outperforms functionally analogous predictors programs by uniquely differentiating hydrophobic signal peptides from true transmembrane segments while simultaneously determining the orientation of transmembrane domains. Its dual capability to discriminate and topologically map structural elements makes TOPCONS particularly valuable for membrane protein characterization, resolving ambiguities that challenge conventional prediction methods.

TOPCONS employs five distinct topology prediction methods, one of which analyzes protein sequences directly in FASTA format. The other four methods require sequence profiles. For each residue in the protein sequence, the program predicts one of four possible localizations inside (i), outside (o), transmembrane (m), or signal peptide (s). By integrating results from all five methods, TOPCONS generates a final consensus topology **figure 3.5** (Brensel *et al*., 2009).

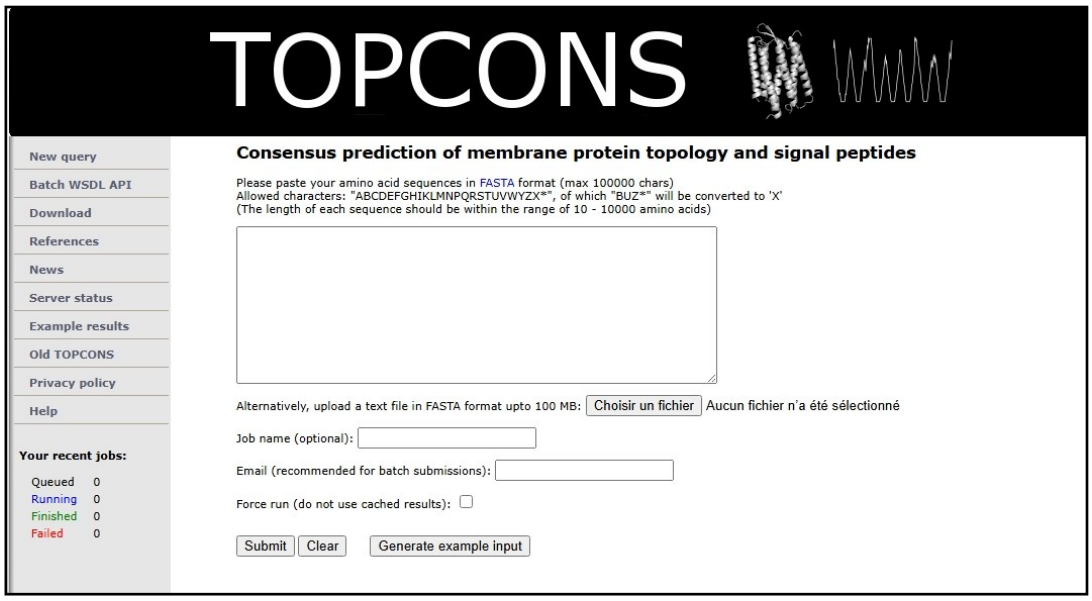

Figure 3.5: TOPCONS Interface Features

4. Phosphorylation sites prediction by Netphos

To identify potential phosphorylation sites in APP695, we employed NetPhos 3.1, a publicly accessible prediction server that utilizes neural networks to analyze S, T and Y residues for kinase-specific modifications. This tool generated probabilistic scores (ranging from 0 to 1) for each candidate site, facilitating targeted experimental validation. As the latest version of this platform, NetPhos 3.1 utilizes an artificial neural network algorithm to predict phosphorylation sites with high accuracy, supporting analysis for 17 kinases including ATM, CKI, CKII, CaM-II, DNAPK, EGFR, GSK3, INSR, PKA, PKB, PKC, PKG, RSK, SRC, cdc2, cdk5, and p38MAPK (Blom *et al*., 2004).

While the interface permits customization of multiple settings **figure3.6**, we modified only the "Display only the scores higher than" parameter, setting it to a threshold of 0.6.

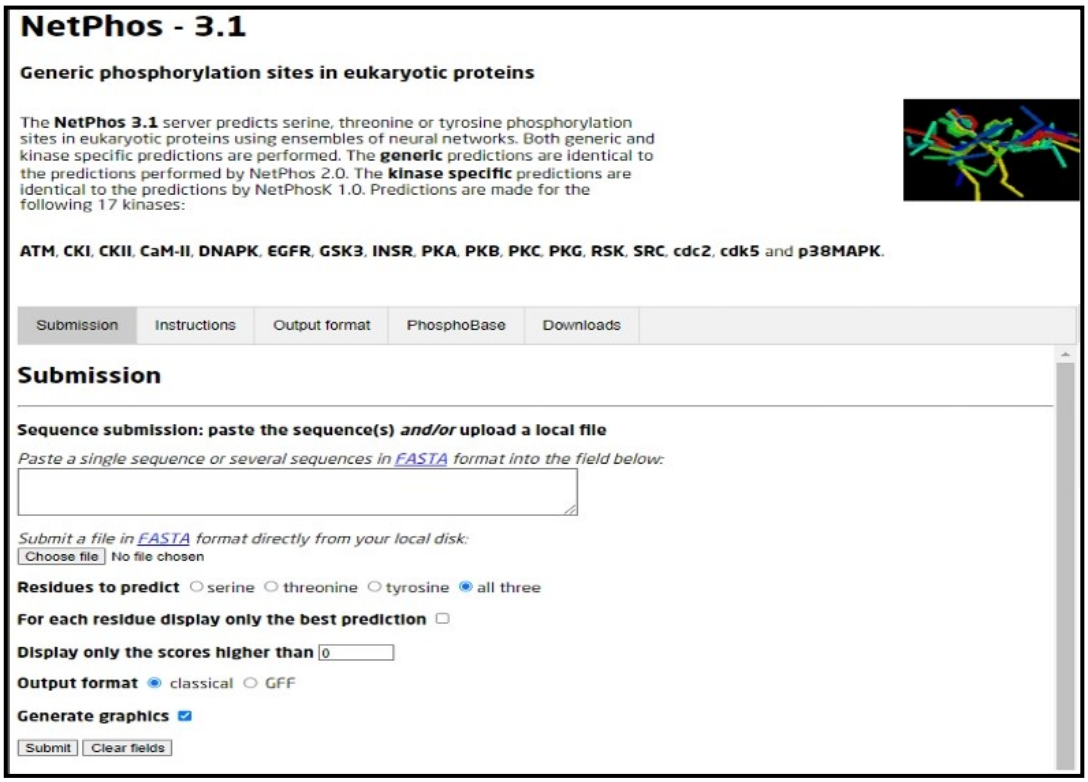

Figure 3.6: Netphos 3.1 user interface

5. Ubiquitination Site Prediction by GPS-Uber

To determine possible ubiquitination sites in APP695, we used GPS-Uber, a web-based prediction program to identify lysine ubiquitination sites. This method uses artificial intelligence for prediction based on experimental data. GPS-Uber is an advanced version of GPS (Group-based Prediction System) figure **3.7** (Wang *et al*., 2022).

The protein sequence for APP695 was obtained from UniProt and submitted in FASTA format. The server returned candidate lysine residues with scores indicating their potential to become ubiquitinated.

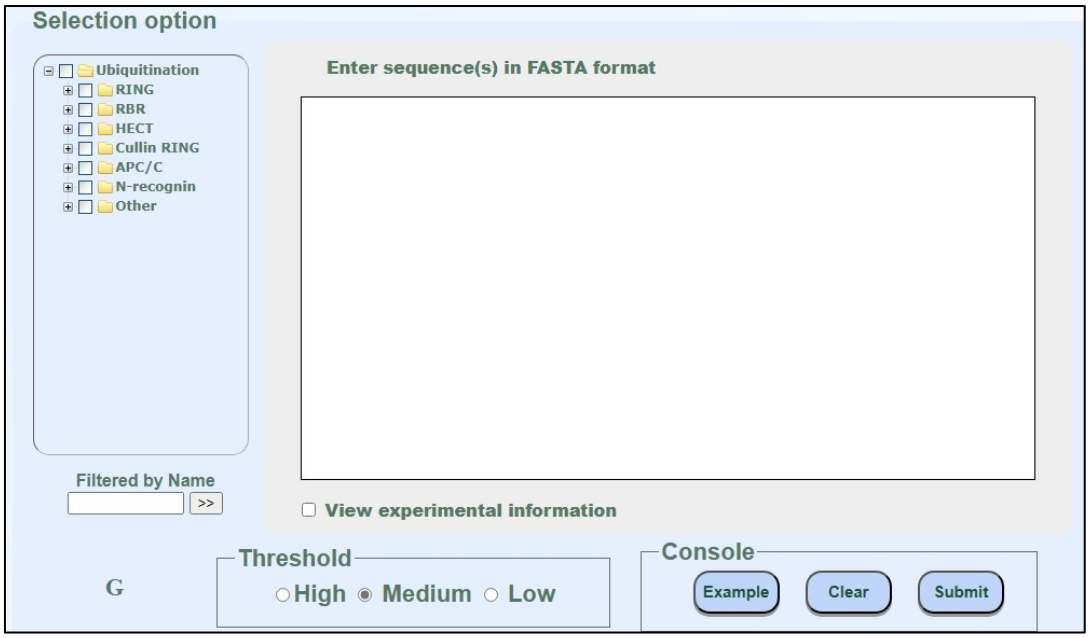

Figure 3.7: GPS-uber interface

6. Protter

Protter is a versatile web tool that creates customized visualizations of protein membrane topology, allowing researchers to dynamically display key features such as post-translational modifications (e.g., phosphorylation, glycosylation) and functional motifs (Omasits *et al*., 2014). With an intuitive interface, that integrates experimental and predictive data, making it invaluable for structural and functional analysis of membrane proteins, this tool streamlines the interpretation of complex protein attributes through interactive, annotated diagrams **figure 3.8**. http://wlab.ethz.ch/protter

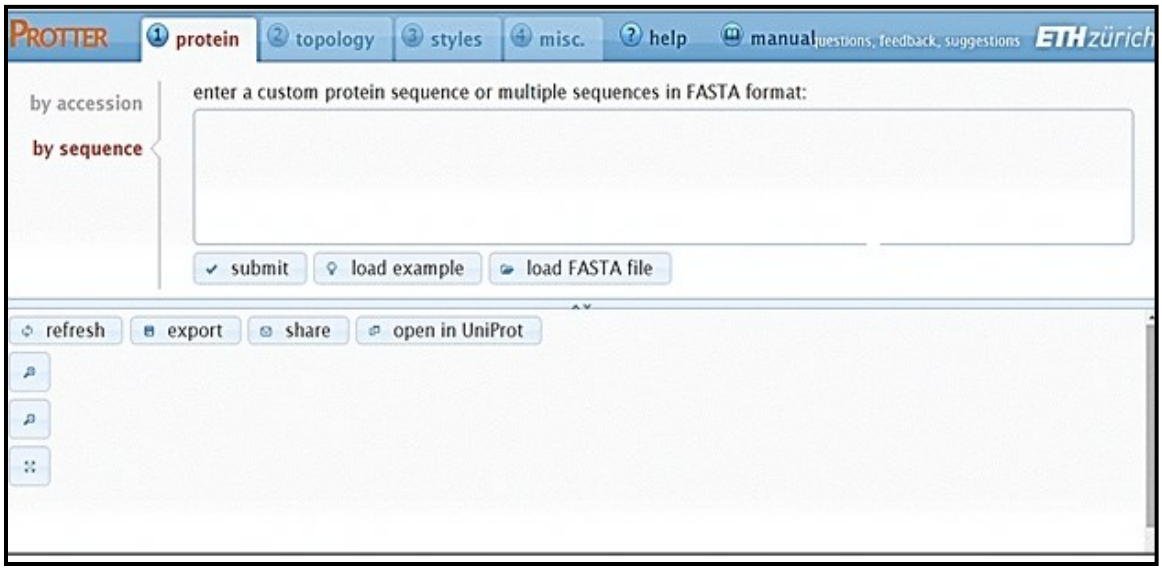

Figure 3.8: Protter Interface Features

Chapter IV Results and Discussion

1. Multisequence alignment of APP770 and APP695

A multisequence alignment between APP695 the main isoforms in our study and APP770 was performed with Clustal Omega and viewed in "Jalview", as shown in the figure **figure 4.1**

The alignment showed that the two isoforms share a high degree of sequence identity, however we observe in the alignment a gap in APP695 corresponding to the two domains in, about 75 amino acids located between residues arginine R288 and valine V362 in APP770, the amino acid sequences of APP695 and APP770 are identical, indicating that both isoforms share the same functional domains and structural features, including the transmembrane domain and intracellular domain.

The gap observed in the alignment is due to the presence of the KPI and OX-antigen domains in the APP770 isoform (Sandbrink et al., 1994), which are involved in protein-protein interactions, suggesting cell-specific APP biology (Menéndez-González et al., 2006). These two domains are absent in APP695, which agrees with our results.

From a structural standpoint, the conservation of key functional sequence including the transmembrane domain, sorting motifs and AICD across both isoforms suggests that the core processing and signaling functions of APP are preserved. These conserved features are essential for APP cleavage site by α-, β-, and γ-secretases, and for the generation of amyloid-beta peptides and AICD fragments that regulate transcriptional activity.

Genetic analysis by Sandbrink *et al* (1994) for the APP gene determinate that APP contains 18 exons, the differential splicing of exons 7 and 8 of APP results in the generation of three major APP isoforms. APP695 excludes exons 7 (Kunitz protease inhibitor domain) and 8 (Ox-2 antigen domain) while APP770 expresses all the exons (Nalivaeva & Turner, 2013).

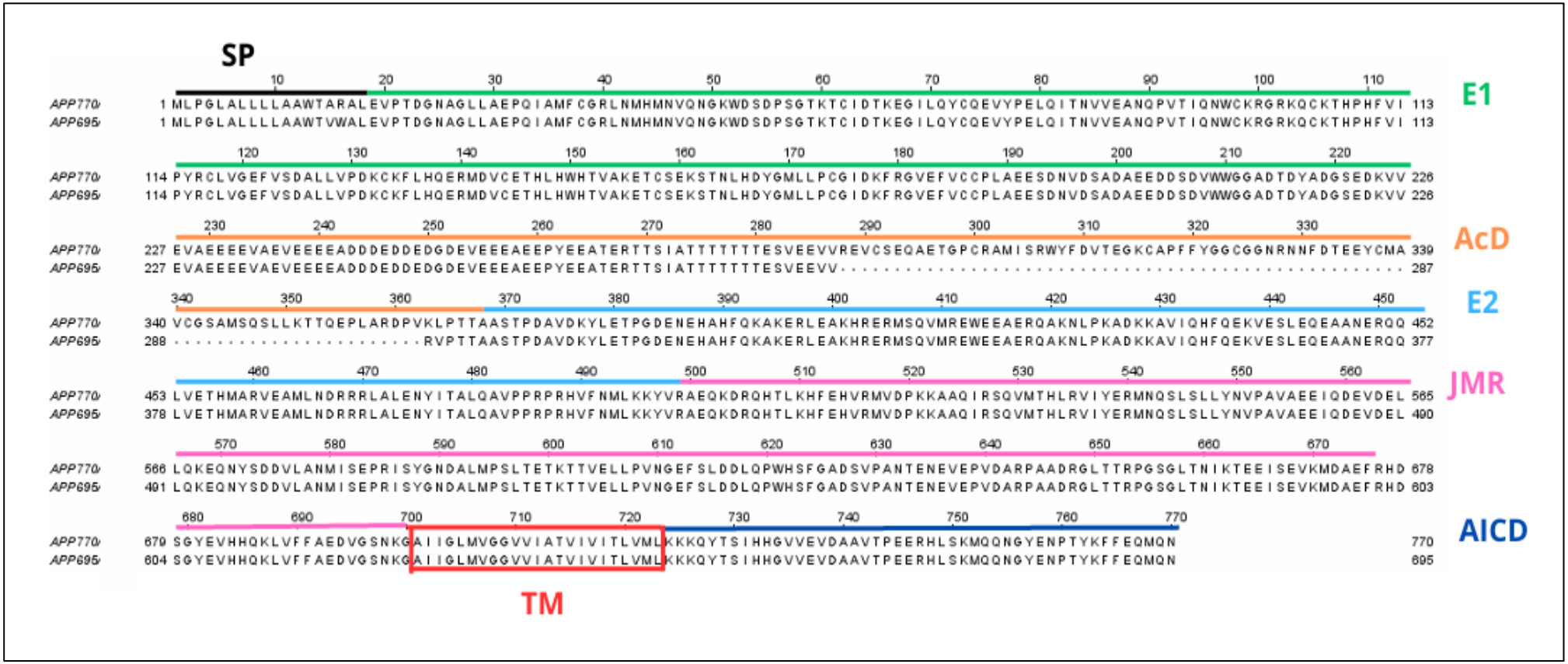

Figure 4.1: alignment of APP770 and APP695

The different domains, **SP:** sequence peptides; **E1**: extracellular region 1; **AcD**: acidic domain; **E2**: extra cellular region 2; **JMR**: juxta membrane domain **TM**: transmembrane domain; **AICD**: APP Intracellular Domain

2. Topological analysis of APP695

2.1.ΔG predictor

In this study, we performed an analysis of the APP695 which are predominately expressed in neuron. The sequences provided from Uniprot data bank are used to characterize the transmembrane domains using the ΔG predictor program.

The results are presented in the form of a diagram called a hydrophobicity plot. ΔGapp are values in the ordinate axis and position in sequence in the abscissa axis for different segment lengths. The hydrophobic regions of the hydropathy profile present negative values of ΔGapp, they correspond to transmembrane domain. However, it should be noted that the C-terminal segments are associated to positive values of ΔGapp. These results will allow us to predict the topology of APP695

The analysis of diagrams provided by ΔG predictor shows the presence of two hydrophobic helicoidal segments. The predicted segment (**Figure 1A**) for APP695 corresponds to the sequence:^{1}M-V^{20} and ^{626}A-L^{648} (**Figure 1B)**

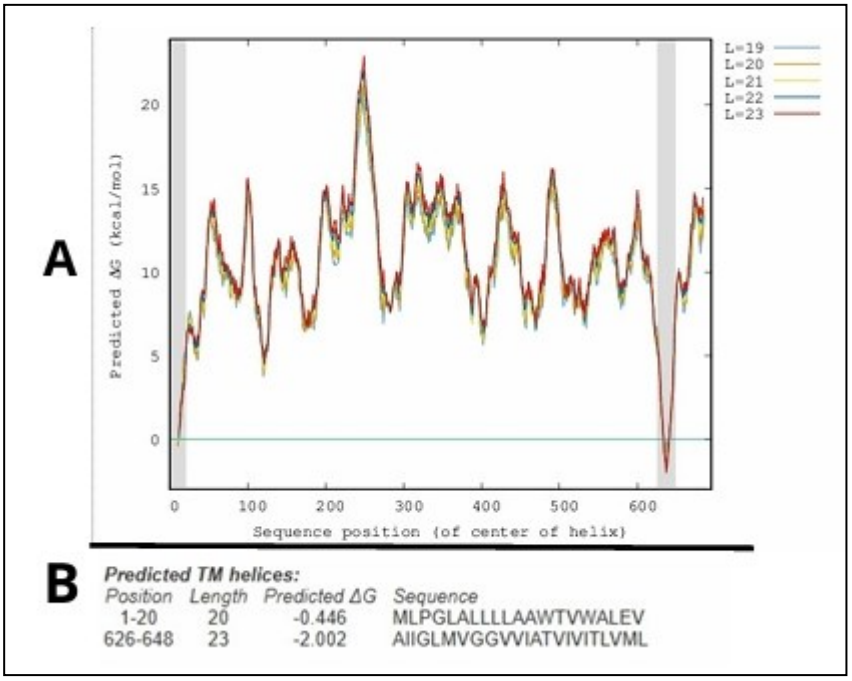

Figure 4.2: Hydropathy diagrams provided by ΔG predictor for APP695

2.2.TOPCONS

In this study, we performed a topology prediction analysis of APP695 using the TOPCONS program. The sequence data obtained from the UniProt database was analyzed to characterize the sequence signal from the transmembrane domains and overall membrane topology of APP695

The TOPCONS prediction for APP695 presents the black box at the N-terminus around position 1–20 indicating the presence of a signal peptide. The blue line positions 21 to 626 represents the region predicted to be outside the membrane extracellular. The white box near the C-terminus position 626-646 indicates a single transmembrane helix (TM-helix, OUT→IN) and a red line at the C-terminal end in position 647-695 indicates the cytosolic region (inside). **figure 4.3**

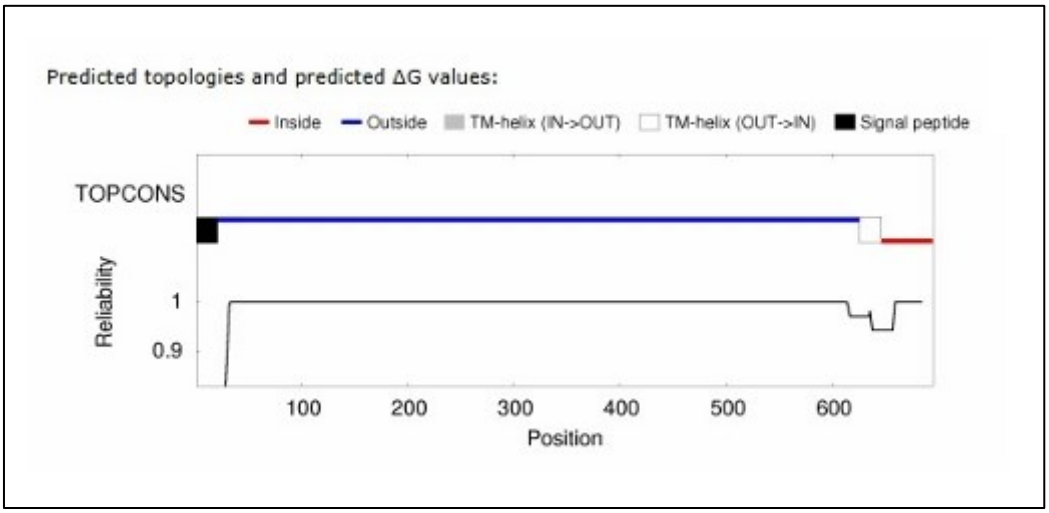

Figure 4.3: TOPCONS prediction results

The results of ΔG predictor and TOPCON allowed us to characterize the different domains for APP695 which we compared below in **Table 4.1**

Table 4.1: Comparative table of the Δ G predictor and TOPCONS results of APP695

Domain	ΔG Predictor	TOPCONS
Sequence peptide	1-20	1-20
Extracellular region	21-625	21-625
Transmembrane domain	626-648	626-646
Intracellular region	649-695	647-695

The TOPCONS prediction complements the ΔG predictor results by confirming that APP695 is a single-pass transmembrane protein with a large extracellular domain, a single transmembrane helix, and a short cytosolic tail. We conclude that the two programs target the same TM region.

Furthermore, the analysis of **Figure 4.1** shows and confirms this region in both APP770 (^{700}G-L^{723}) and APP695 (^{626}G-L^{649}).

3. Phosphorylation sites prediction of APP695

In this work, we used another program, NetPhos 3.1, which allows for the prediction of potential phosphorylation sites based on the sequence for APP695. The results of predicted phosphorylation sites are provided under the form of a sequence of nine amino acids centered around the phosphate acceptor site (P-4 S* P+4). Each result is associated with a probability score. The threshold value was set to 0.6 in our work.

Netphos 3.1 results are presented in a table, where Uniprot identifier is used as name for APP695, for each amino acid residue (x) of the sequence, Netphos provides its position indicated with #, the type of phosphorylated amino acid with T/S/Y. The sequence containing the predicted site is also given as well as possible kinases which may be responsible for this phosphorylation. The program only approves predictions with scores higher than 0.6 labeling them with "YES", otherwise it labels them with "." **figure 3.1**

```
>CAA31830.1     695 amino acids
#
# netphos-3.1b prediction results
#
# Sequence                 # x   Context     Score   Kinase    Answer
# ---------------------------------------------------------------------
# CAA31830.1               57 S   DSDPSGTKT   0.745   unsp       YES
#
# CAA31830.1               61 T   SGTKTCIDT   0.604   unsp       YES
#
# CAA31830.1              107 T   KQCKTHPHF   0.742   PKC        YES
```

Figure 4.4: Netphos 3.1 prediction results for APP695

NetPhos 3.1 predicted more than 50 potential phosphorylation sites on APP695, with a wide distribution across the length of the protein. These sites included serine, threonine and tyrosine. A score of 0.6 was used to define confidence sites, noted that several sites had a site prediction score of > 0.9, indicating high confidence in prediction.

The prediction results are given in **Table 4.2** for E1, **Table 4.3** for AcD domain, juxta membrane domain and E2, **Table 4.4** for C-terminal domain.

Table 4.2: E1 domain Predicted phosphorylation sites by NetPhos3.1

	Site	Kinase	Sequence	Score	Experimental confirmed
E1	S57	Unsp	DSDPS*GTKT	0.745	-
	T61	Unsp	SGTKT*CIDT	0.604	-
	T107	PKC	KQCKT*HPHF	0.742	-
	S159	Unsp	KETCS*EKST	0.9	-
	S162	Unsp	CSEKS*NLH	0.835	-
	S198	CKII	DNVDS*ADAE	0.650	+/ CK-1and CK-2-like ectoprotein kinases
	S206	Unsp	EEDDS*DVWW	0.604	+/ CK-1 and CK-2-like ectoprotein kinases
	T215	Unsp	GGADT*DYAD	0.670	-
	Y217	Unsp	ADTDY*ADGS	0.981	-

Unsp: Unspecified; The phosphorylation sites are shown in red with an asterisk, experimentally confirmed sites and their kinases were obtained from Walter *et al*., (2000)

The results predict nine phosphorylation sites located in the E1 domain, which are S57, T61, T107, S159, S162, S198, S206, T215, and Y217. Their scores are high, range from 0.6 to 0.9. In the work of Walter *et al*. (2000), residues S198 and S206 in the ectodomain were observed to be phosphorylated under physiological conditions. It has been found that two distinct mechanisms of ectodomain phosphorylation of APP can take place. In the first mechanism, the ectodomain of APP is phosphorylated by ectoprotein kinases in Golgi compartments. However, mature APP proteins on the cell surface may also experience phosphorylation at the extracellular domain by membrane-bound ectoprotein kinases. Furthermore, casein kinase CK-1- and CK-2-like ectoprotein kinases are responsible for the ectodomain phosphorylation of both membrane-anchored and secreted forms of APP. This type of ectodomain phosphorylation might be significant for APP localization, as well as for its biological function (Walter *et al*., 2000; Tsatsanis *et al*., 2019).

Table 4.3: Predicted phosphorylation sites of AcD, E2 and JMR by NetPhos3.

	Site	Kinase	Sequence	Score	Experimental confirmed
AcD	Y262	unsp	AEEPY*EEAT	0.986	-
	T266	unsp	YEEAT*ERTT	0.970	-
	T270	unsp	TERTT*SIAT	0.884	-
	T270	PKC	TERTT*SIAT	0.613	-
	S271	Unsp	ERTTS*IATT	0.973	-
	S271	PKC	ERTTS*IATT	0.806	-
	T276	Unsp	IATTT*TTTT	0.621	-
	T278	Unsp	TTTTT*TTES	0.787	-
	T280	Unsp	TTTTT*ESVE	0.661	-
	S282	Unsp	TTTES*VEEV	0.996	-
	T291	PKC	VRVPT*TAAS	0.618	-
	S295	Unsp	TTAAS*TPDA	0.992	-
E2	T296	Unsp	TAAST*PDAV	0.911	-
	Y303	Unsp	AVDKY*LETP	0.912	-
	T306	Unsp	KYLET*PGDE	0.827	-
	S332	Unsp	RERMS*QVMR	0.948	-
	S332	DNAPK	RERMS*QVMR	0.627	-
	S366	Unsp	EKVES*LEQE	0.906	-
	T433	Unsp	DRQHT*LKHF	0.953	-
	T433	PKC	DRQHT*LKHF	0.606	-
	S453	DNAPK	AQIRS*QVMT	0.612	-
	S469	PKA	RMNQS*LSLL	0.808	-
	S471	PKC	NQSLS*LLYN	0.618	-
	Y497	Unsp	KEQNY*SDDV	0.786	-
	S512	Unsp	EPRIS*YGND	0.994	-
	S512	PKA	EPRIS*YGND	0.787	-
	S521	Unsp	ALMPS*LTET	0.958	-

JMR	T527	Unsp	TETKT*TVEL	0.781	-
	S539	Unsp	NGEFS*LDDL	0.868	-
	T558	Unsp	VPANT*ENEV	0.924	-
	T558	CKII	VPANT*ENEV	0.670	-
	T576	Unsp	DRGLT*TRPG	0.989	-
	T576	PKC	DRGLT*TRPG	0.666	-
	T577	PKC	RGLTT*RPGS	0.672	-
	S581	Unsp	TRPGS*GLTN	0.953	-
	S592	Unsp	TEEIS*EVKM	0.795	-
	S604	Unsp	FRHDS*GYEV	0.963	-
	S604	PKA	FRHDS*GYEV	0.727	-
	Y606	Unsp	HDSGY*EVHH	0.870	-
	S622	Unsp	EDVGS*NKGA	0.787	-

Unsp: Unspecified; The phosphorylation sites are shown in red with an asterisk

The **table 4.3** presents predicted phosphorylation sites in the APP ectodomain, determined by NetPhos 3.1. It includes sites across the AcD, E2, and JMR segments of APP695. Among the 40-residue predicted, S282, S512 and S576 demonstrate particularly high scores superior of 0.9 suggesting a strong potential for phosphorylation.

Although in the work of Hung *et al* (1994), it was previously thought this region to be less frequently phosphorylated. However, the high scoring sites indicate there may be numerous opportunities for post-translational modifications to affect APP.

Table 4.4: AICD Predicted phosphorylation sites by NetPhos3.1

	Site	Kinase	Sequence	Score	Experimental confirmed
AICD	Y653	UNSP	KKKQY*TSIH	0.820	-
	S655	UNSP	KQYTS*IHHG	0.888	+/ ROCK1; PKC
	T668	UNSP	DAAVT*PEER	0.977	+/ JNK1; Cdk1
	S675	PKA	ERHLS*KMQQ	0.710	+
	Y682	UNSP	QQNGY*ENPT	0.939	+/ Abl; Src
	T686	PKA	YENPT*YKFF	0.668	+/ PKC
	Y687	UNSP	ENPTY*KFFE	0.945	+

Unsp: Unspecified; The phosphorylation sites are shown in red with an asterisk, experimentally confirmed sites and their kinases were obtained from Zhang, Chen, & Lee, 2019

Our study predicts seven potential phosphorylation sites in the APP C-terminal domain, two threonine, two serine and three tyrosine **(figure 4.4)**, T668, T686, S655, S675, Y653, Y682, Y687. They were obtained with a good score superior than 0.8. This result agrees with the work of Oliveira *et al* (2017). While T654 was not predicted in our work, it is known to be a phosphorylation site.

The reported phosphorylation sites T654 and S655, which are included in ^{653}YTSI^{656} motif, were identified by Gandy *et al* (1988), while their direct impact of phosphorylation on APP processing remains controversial. This residue lies within a sequence that is crucial for proper sorting and transport of APP (Lai *et al*., 1995). Therefore, modifications at these sites may influence APP's cellular fate.

Furthermore, three phosphorylated site Y682, T686 and Y687 were found in the second motif ^{682}YENPTY^{687}, their phosphorylation is known to disrupt the binding of important adaptor proteins such as Fe65 and X11. which are involved in APP's trafficking and processing (Thinakaran & Koo, 2008; La Rosa *et al*., 2015).

In addition, residue T686 which is predicted with high confidence score of 0.977 is found between these two motifs, this site has been experimentally validated, and is well-studied as a site known to modulate both the distribution and function of the APP intracellular domain

(AICD). Phosphorylation at T668 induces a conformational change in the ^{667}V**T**PEER^{672} region which T668 increases the interaction with β-secretase cleavage and disrupts adaptor protein interactions, shifting APP processing toward amyloidogenic pathways (Slomnicki *et al*., 2008).

4. Ubiquitination by GPS Uber

We utilized the GPS-Uber server, which predicts lysine residues likely to undergo ubiquitination. The analysis identified five lysine residues in APP695 with moderate scores, suggesting possible ubiquitination sites. These are K60, K66, K132, K676, and K688, each associated with "General" E3 enzyme classes and scores ranging from approximately 0.57 to 0.68. Among these, K676 had score 0.6833, making it the most likely candidate for ubiquitination, followed by K688 score 0.5848. These results are shown in **table 4.5.** Both of these residues are located near the C-terminal cytoplasmic region, a domain enriched with regulatory motifs.

Table.4.5: Ubiquitination prediction by GPS uber

Position	E 3 enzymes	Score	Experimental confirmed
K60	General	0.5957	-
K66	General	0.5689	-
K132	General	0.5758	-
K676	General	0.6833	-
K688	General	0.5848	+/ Ubiquilin-1

It is interesting that the score of K688 is moderate, even though it has been confirmed experimentally by El Ayadi *et al*. (2012). He demonstrated that ubiquitination at K688 in the intracellular domain of APP plays a critical role in its trafficking and processing. The positioning of K676 and K688 is particularly important as these residues lie in proximity to the YENPTY motif, which is known to mediate interaction with adaptor proteins such as Fe65 and X11. Ubiquitination at these sites could potentially interfere with APP's ability to bind these partners, thereby influencing its intracellular trafficking, endocytosis, or proteolytic processing. Moreover, the cytosolic tail of APP is also the site of γ-secretase cleavage, and ubiquitination in this region

may influence accessibility or stability of the cleavage substrate (Thinakaran & Koo, 2008; La Rosa *et al*., 2015).

GPS-Uber predictions indicate that APP695 is a potential target for ubiquitin-mediated regulation, especially in its C-terminal region. These modifications may work in coordination or competition with phosphorylation, adding a layer of complexity to APP's regulation.

5. Topology of APP695

The Protter software allowed us to visualize APP695 membrane topology according to the ΔG Prediction, NetPhos 3.1 and ubiquitination results. It Enabled us to bring together post-translational data to visualize APP695's structure. **Figure 4.5**

The topology analysis of APP695 reaffirms that it is a type I transmembrane protein with a transmembrane domain crossing the membrane near the C-terminus, a large N-terminal extracellular domain, and a smaller intracellular C-terminal tail.

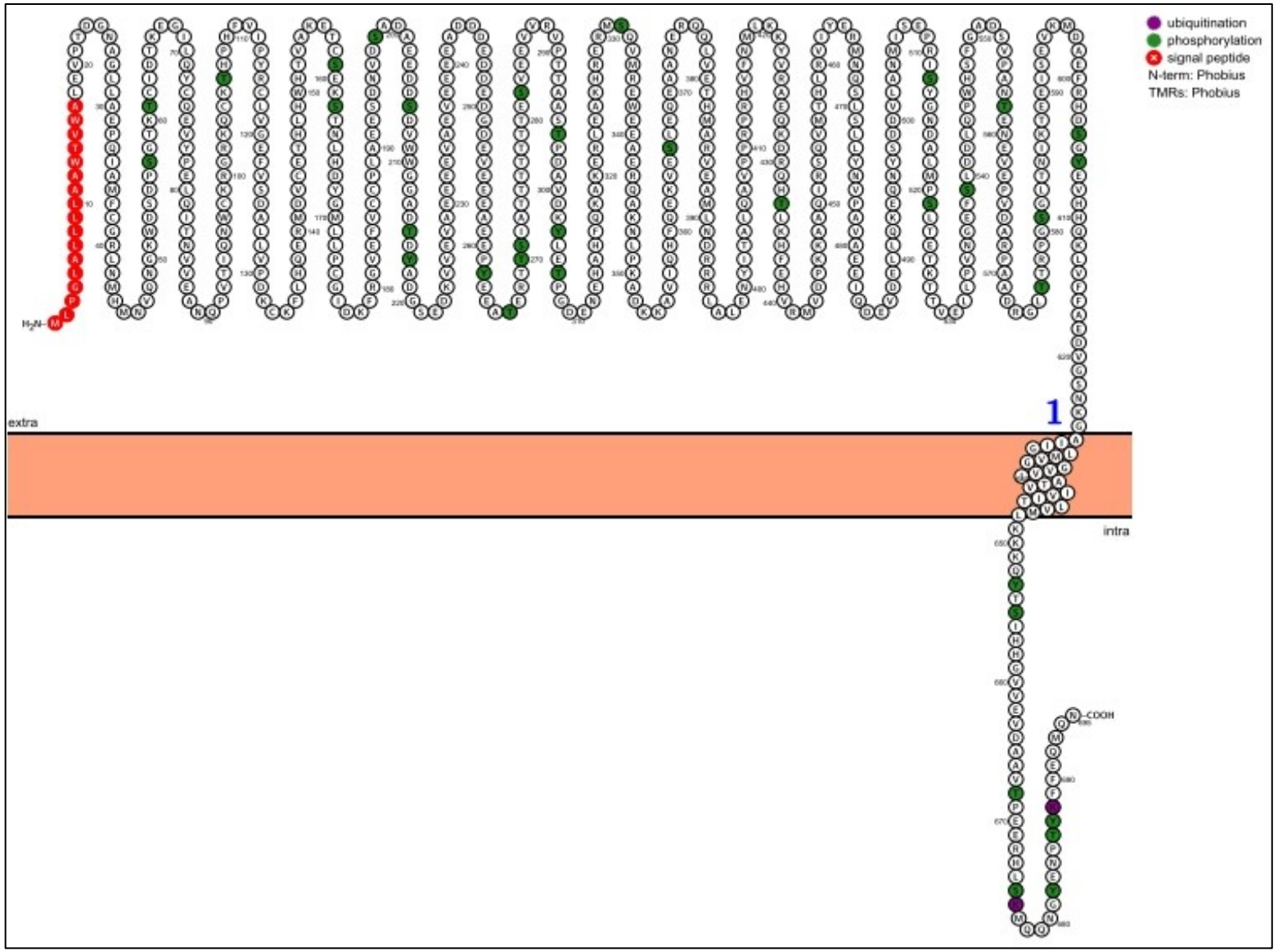

Figure 4.5: Protter topology

Conclusion

In this study, we focused on the structural and functional analysis of the APP695 isoform, which is predominantly expressed in neurons and plays a central role in neuronal signaling, development, and synaptic adaptability. Researches implicate APP in the pathophysiology of neurodegenerative diseases, particularly AD. One of the key mechanisms contributing to its pathological role is the disruption of post-translational modifications especially phosphorylation and ubiquitination that influence APP's processing, trafficking, and degradation.

Using in-silico approach, we carried out a prediction of phosphorylation, ubiquitination of APP695. Sequence alignment with APP770 confirmed the absence of KPI and Ox-antigen domains in APP695, corresponding to its neuron-specific expression. Topology predictions through ΔG Predictor and TOPCONS validated the presence of a single transmembrane domain, a large extracellular domain and a small intracellular domain.

Our phosphorylation site prediction with NetPhos 3.1 revealed over 50 potential sites. Among them several sites were identified for the first time, located in the ectodomain of APP695. In addition, seven residues in the C-terminal region have been experimentally validated and are involved in APP trafficking and endocytosis. Several high-probability phosphorylation sites were identified. Among them, residues T653, T655, Y682, T686 and Y687 belong to the sorting motifs in the C-terminal region, while T668 is located in between them. Phosphorylation at T668 not only changes APP's conformation and disrupts its interaction with adaptor proteins such as Fe65, but also promotes β-secretase cleavage, increasing the production of toxic intermediates such as CTFβ. While T668 phosphorylation may inhibit γ-secretase cleavage, it promotes conditions that worsen amyloidogenic processing, including endosomal accumulation and Fe65 dissociation both linked to elevated Aβ levels.

The ubiquitination site analysis using GPS-Uber identified five candidate sites, with lysine 688 (K688) being the most functionally significant. K688 ubiquitination regulates APP's stability and degradation via UPS. Failure in this process results in APP accumulation and a shift toward amyloidogenic cleavage.

Furthermore, phosphorylation Y687 may affect the accessibility of E3 ligases to K688, indicating a dynamic interplay between phosphorylation and ubiquitination in determining APP's cellular fate.

Finally, visualizing APP695 with Protter allowed us to integrate these findings into a structural context, highlighting how specific post-translational modifications are spatially positioned to influence APP's intracellular trafficking and processing pathways.

In perspective, these results open the horizon to initiate other studies such as:

- Experimental confirmation of the predicted sites.
- Investigation of how post-translational modifications affect APP695's structure and trafficking, particularly in the context of Alzheimer's disease.
- Exploration of other post-translational modifications (e.g., glycosylation, palmitoylation) and their cooperative or competitive interaction

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