Exploring Na^+/K^+-ATPase in Astrocytes to Uncover Therapeutic Insights into Dementia Pathophysiology

M.Tech. Thesis

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **Exploring Na^+/K^+–ATPase in Astrocytes to Uncover Therapeutic Insights into Dementia Pathophysiology** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF TECHNOLOGY** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July 2023 to May 2025 under the supervision of Dr. Sivaraj Mohana Sundaram, Assistant Professor.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Jayant Kashyap

(JAYANT KASHYAP)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

M.S. Sivaraj
16-05-2025

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JAYANT KASHYAP has successfully given his M.Tech. Oral Examination held on **5 May 2025**.

M.S. Sivaraj

Signature of Supervisor of M.Tech. thesis
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Date:

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for mother

Abstract

Dementia, a progressive neurodegenerative syndrome which is characterised by cognitive decline, currently affects over 55 million people globally and is a major public health challenge. Recent research highlights the pivotal role of astrocytes in the instances of dementia-related disorders, particularly Alzheimer's disease (AD). Among the various astrocytic ion channels, the sodium-potassium ATPase (Na^+/K^+–ATPase) is of particular interest due to its critical role in regulating ionic gradients, neurotransmitter uptake, and neuroprotection. This study investigates the direct interaction between amyloid-beta (Aβ), an important protein in AD pathology, and astrocytic NA^+/K^+–ATPase, and explores therapeutic strategies aimed at rescuing astrocyte function.

The study employed U87 and LN229 astrocyte-like cell lines to address a key question looking at astrocyte recovery through enhanced NA^+/K^+–ATPase activity. Approach included assessing NA^+/K^+–ATPase isoform expression and localisation, confirming Aβ-NA^+/K^+–ATPase binding, cell viability and cytotoxicity assays, and more. Key findings reveal that Aβ42 colocalises with NA^+/K^+–ATPase α1 isoform in astrocytes, Aβ treatment reduces cell viability and leads to significant morphological simplification of astrocytes. Treatment with oleic acid rescued Aβ-induced deficits in astrocyte branching when co-administered with Aβ.

Overall, these results underscore the significance of Na^+/K^+–ATPase dysfunction in Aβ-mediated astrocyte pathophysiology and highlight the therapeutic potential of targeting astrocytic ion channels to mitigate neurodegeneration. Future research ought to elucidate the molecular mechanisms underlying astrocyte–Aβ interactions and expand the repertoire of astrocyte-targeted interventions.

Keywords: astrocytes, amyloid-beta, dementia pathophysiology, neuroprotection, ion homoeostasis

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NOMENCLATURE

Acronym	Expansion
Aβ	Amyloid beta
AD	Alzheimer's disease
AMOG	Adhesion molecule on glia
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BSA	Bovine serum albumin
CNS	Central nervous system
DMEM	Dulbecco's Modified Eagle's Medium
FHM2	Familial hemiplegic migraine type 2
GABA	Gamma-aminobutyric acid
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GS	Glutamine synthetase
KI	Knock-in
KO	Knock-out
LTP	long-term potentiation
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCX	Na^+/Ca^{2+} exchanger
NA^+/K^+–ATPASE	Na^+/K^+–ATPase
NKCC1	$Na^+/K^+/2Cl$ cotransporter 1
NMDA	N-methyl-D-aspartic acid receptors
ORO	Oil Red O staining
PLL	Poly-L-lysine
PSD	Post-stroke dementia
ROS	Reactive oxygen species
TRP	Transient receptor potential
VCIDs	Vascular cognitive impairments

SYMBOLS

Symbol	Meaning
α	Alpha
β	Beta
Ca^{2+}	Calcium ion
Cl^{-}	Chloride ion
°C	Degrees Celsius/centigrade
K^{+}	Potassium ion
H^{+}	Proton
≤	Less than / equal to
µ	Micron
µM	Micromolar
µm	Micrometre
mg/mL	Milligram/millilitre
mM	Millimolar
M	Molar (moles per litre)
Na^{+}	Sodium ion
pg/ml	Picogram/millilitre
%	Percentage

Chapter 1

Introduction

Na^+/K^+–ATPase is an enzyme present in the eukaryotic plasma membrane. Na^+/K^+–ATPase, because of its role in maintaining cellular homoeostasis (regulation of sodium and potassium ions in and out of the cell), is a critical membrane protein. It has an important role in maintaining proper ion balance, particularly extracellular K^+ or intracellular Na^+ homeostasis (through an active transport of 3 Na^+ ions out of the cell and 2 K^+ ions into the cell, thus maintaining an ionic balance), and for facilitating neurotransmitter reuptake (Pietrobon and Conti, 2024).

Na^+/K^+–ATPase is composed of three subunits, *viz.* α, β, and γ subunits. These are further divided into α1-4 and β1-3 isoforms (Palmgren, M., 2023). The functions of the subunits and isoforms are presented below.

i. α subunit, which performs catalytic activities, and is responsible for ion transport and hydrolysis of ATP. It is divided into four isoforms, which are as follows:
   a. α1, which is ubiquitous, and expressed in most of our body cells (Bossyut *et al.*, 2009),
   b. α2 is majorly present in muscle cells, astrocytes and developing neurons (Yuen *et al.*, 2017),

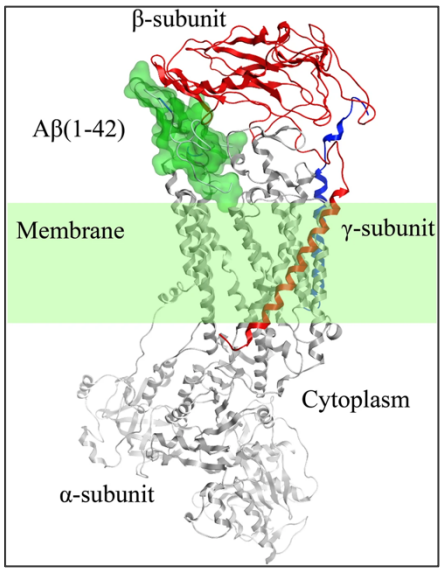

Figure 1. The representative structure of NKA with attachment region for Aβ(1-42) (**Petrushanko *et al.*, 2019**)

c. α3 is present in neurons, is widely studied, and is known to essentially partake in neuronal firing (Clausen *et al*., 2017), and
d. α4, which is specifically expressed in the testes and is essential for the fertility of sperm (Syeda *et al*., 2020).

ii. β subunit is the glycoprotein required for α subunit trafficking to plasma membrane (Stoops *et al*., 2014). It is divided into three isoforms, identified as:
   a. β1, which is a major β isoform found in the heart (Clausen *et al*., 2017),
   b. β2, which was initially called Adhesion Molecule on Glia (AMOG) because it was originally found in glia, as the name suggests, where it is involved in cell-cell contacts, and
   c. β3, which has expression and localisation similar to β1 (Blanco and Mercer, 1998), is primarily the human blastocyst's trophectoderm (TE) cells, and is found in the testis and central nervous system (Hirakawa *et al*., 2022; Suhail, M., 2010).

iii. γ subunit, which has not been very widely studied and the exact function of the γ is unclear. FXYD2 (Yap *et al*., 2021), or γ subunit has often been considered non-essential, but through studies has been seen as an important regulator of the sodium pump by modulating the affinity of Na^+/K^+–ATPase for ATP and Na^+ in an alternate manner (Rivard *et al*., 2005).

1.1. Roles of Na^+/K^+–ATPase

Na^+/K^+–ATPase, which in itself is a ubiquitous transmembrane protein, is involved in the maintenance of the Na^+ and K^+ gradients across the cell membrane by transporting 3 Na^+ out and 2 K^+ into the cell (Suhail, M., 2010).

The role of Na^+/K^+–ATPase in neurons is to execute the following crucial processes:

i. Maintaining ionic homoeostasis across the neuronal membrane (supporting neuronal function) (Bellot-Saez *et al*., 2017),
ii. Indirectly supporting neurotransmitter uptake, particularly glutamate (Rose *et al*., 2009). Na^+/K^+–ATPase pumps Na^+ and K^+ ions to create a sodium gradient, which is then used to facilitate the movement of glutamate across the cell membrane;
iii. Participation in signalling cascades essential for neuronal function (Pivovarov *et al*., 2019), such as participating in the production of reactive oxygen species (ROS), and
iv. Protection against neurodegeneration through regulated neurotransmitter uptake (Huang *et al*., 2024), which is performed through the maintenance of the electrochemical gradients across cell membranes, influencing neuronal excitability (Nagaoka *et al*., 2022); and by inhibiting any induced neurotoxicity, which is also performed through maintaining ion homeostasis and supporting neuronal and glial cell function (Kinoshita *et al*., 2016).

1.2. Aβ as a Key Player in Alzheimer's Disease

Amyloid beta (Aβ) is a protein playing a key role in the development of Alzheimer's disease, which is the most prevalent form of dementia, and generally affects people older than 60 years of age. Alzheimer's disease is characterised by the formation of multiple senile (or amyloid) plaques in the brain, where Aβ42 gets deposited in unusually high concentrations (above 800 pg/ml, from Sturchio *et al.*, 2021) as a consequence of the dysregulation of the amyloid-beta (Aβ) levels.

As it is, a healthy brain is generally capable of breaking down and eliminating Aβ with the help of blood vessels (facilitated via the blood-brain barrier) and insulin-degrading enzyme (IDE) (Wang *et al.*, 2021). This is because IDE as a primary protease degrades soluble Aβ, Aβ42 in

particular, in neurons, microglia, and brain interstitial fluid (Miners *et al*., 2008). As a result of breaking soluble Aβ, IDE also prevents its aggregation into neurotoxic oligomers and plaques. This suggests that Aβ can also be cleared through either enzymatic or non-enzymatic brain pathways, including neuronal and glial cells, the blood-brain barrier (BBB), interstitial fluid bulk flow, perivascular drainage, and cerebrospinal fluid absorption-mediated pathways.

This enzymatic degradation as complemented by non-enzymatic clearance pathways follows:

- Vascular routes: BBB uses transporters (*e.g.*, LRP-1) to shuttle Aβ into systemic circulation, while perivascular drainage flushes Aβ along blood vessel walls into CSF and lymphatic systems.
- Cellular mechanisms: Neurons and glial cells absorb Aβ via phagocytosis or receptor-mediated uptake, with microglia further degrading it intracellularly.
- Fluid dynamics: Bulk flow of ISF and CSF absorption physically remove Aβ from the brain.

Meanwhile, enzymatic pathways involve IDE and proteases that directly degrade Aβ. IDE's dual role – clearing soluble Aβ and inhibiting aggregation – works synergistically with these structural and cellular systems to maintain Aβ homeostasis and prevent neurotoxic buildup (Sahoo *et al*., 2021; Vingtdeux *et al*., 2015; Yoon and Jo, 2012).

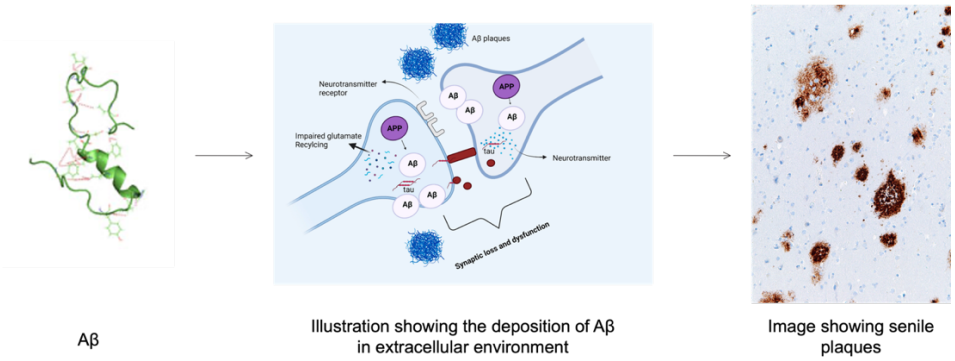

Figure 2. Image showing the deposition of Aβ to produce amyloid/senile plaques

1.2.1. *Involvement of Na^+/K^+–ATPase with Aβ*

Several studies suggest that at higher concentrations (~250 mM, from Raskatov, J.A., 2019), Aβ42 tends to affect Na^+/K^+–ATPase activity directly by forming complexes (Gu and Guo, 2013) with the Na^+/K^+–ATPase α_1 and other isoforms (Adzhubei *et al*., 2022). This is due to binding sites located between the extracellular portions of the α and β subunits, leading to altered Na^+/K^+–ATPase conformational states and reduced activity (Gu and Guo, 2013). However, Aβ accumulation is also associated with neuroinflammatory processes that can indirectly impair Na^+/K^+–ATPase function.

1.3. Dementia

Dementia is an increasingly common syndrome relating to progressive cognitive decline among older people, leading to such results as loss of memory, inability to think, and affected reasoning, even so far as it interferes with the patients' daily lives (Geldmacher and Whitehouse, 1996). However, dementia is also observed in younger people, and it has been understood that it may be caused as a result of any of a number of diseases (Corey-Bloom *et al*., 1995) that damage the brain and affect cognitive function, *e.g.*, Alzheimer's disease (AD), and Lewy body disease. It was estimated in 2019 that there were a total of 57.4 million people around the world with a case of dementia, and this number is expected to increase to 152.8 million cases in 2050 (Nichols *et al*., 2022). In India, this number was 8.8 million among people older than 60 years (Li *et al*., 2023).

Considering the fact that dementia is linked to other diseases that lead to gradual or, in some cases, even an acute cognitive decline, it is only obvious that the glial cells of the central nervous system (CNS), more particularly astrocytes. Astrocytes play roles in regulating synapse formation, maintenance, and elimination through direct interactions with neurons (Hulshof *et al*., 2022), in clearing Aβ via phagocytosis

(Rodríguez-Giraldo *et al*., 2022), in maintaining glutamate homeostasis, and in providing neurons with lactate and glucose for energy (Acosta *et al*., 2017). It is becoming more well acknowledged that any unusual changes in astrocytic activity may play a role in neuro-degenerative illnesses, including various types of dementia (Price *et al*., 2018; Garwood *et al*., 2017). Since astrocytes are present in significant quantity in the brain (making up around 20–40% of all glial cells, as suggested by Verkhratsky *et al.*, 2017), they have the potential to contribute to neurotoxicity in conditions like Alzheimer's and Parkinson's diseases (Chandrasekaran *et al*., 2021). Most of the astrocyte functions can be attributed to their ion channels. This is because astrocytes rely heavily on ion channels to perform their diverse roles in brain homeostasis and disease. These channels, which include calcium channels, potassium channels, and chloride channels, regulate ionic balance, synaptic activity, metabolic support, and neuroinflammation, and through a dysfunction, may lead to reactive astrogliosis (Song *et al*., 2020), resulting in neuroinflammation and oxidative stress, exacerbating cognitive decline (Rahman *et al*., 2024). These ion channels, which serve as regulators of cell function with reference to astrocytes, only allow specific ions and in regulated concentrations, to pass in and out of cells, thus moderating nerve impulse transmission (Panatier *et al*., 2011), muscle contraction (Lee *et al*., 2023), hormone release, and many other cellular processes. Their function also allows astrocytes to provide structural support to neurons and other brain cells, regulate neurotransmitter levels, maintain the blood-brain barrier, and contribute to synaptic plasticity (D Skaper *et al*., 2017), while implying as a certainty that any dysregulation in ion channels may lead to abnormalities in functions.

Recent research into astrocytes has pointed towards possible alteration in astrocytes in patients with dementia (Wilcock *et al*., 2009; Liu *et al*., 2024; Glober *et al*., 2019). This can also be credited to dysregulation of various astrocyte ion channels, resulting in increased calcium influx, reduced potassium channel function, and a change in chloride channel

activity, which may be linked to oxidative stress and neuroinflammation (González-Reyes *et al.*, 2017). These changes further contribute to such disorders as epilepsy (Seifert *et al*., 2010), cardiac arrhythmias, and a number of neurodegenerative disorders (Kumar *et al*., 2016), including dementia. Moreover, given that astrocytes are crucial to modulating the brain's extracellular environment (Sriram *et al*., 2024), changes in ion channel function can have significant consequences on neuronal health and brain function.

In people suffering with dementia, an accumulation of pathological markers, *e.g.*, Aβ, tau proteins, etc., is often linked to impaired astrocytic function (Cisternas *et al*., 2022; Ferrari-Souza *et al*., 2022). This is understood to be the result of astrocyte ion channel malfunction, which can impact ion homeostasis, leading to an increase in inflammatory agents and reactive oxygen species (ROS) production, and can further damage neuronal cells (Rodríguez-Giraldo *et al*., 2022; Wang *et al*., 2022). Abnormalities in aquaporins, for instance, can impede the timely removal of Aβ, which contributes to its buildup in the brain (Xu *et al*., 2015). Increasingly, research looking into the pathophysiology of dementia as a result of various neurological disorders has recommended targeting astrocyte ion channels as an innovative strategy for developing therapies focussed on mitigating the effects of dementia (Gorshkov *et al*., 2018; Rahman *et al*., 2024; Li *et al*., 2024). Restoring normal ion channel function may assist in reducing neuroinflammation, and serve to enhance neuronal health (Rahman *et al*., 2024). Studies suggest addressing astrocyte signalling may serve as a path towards developing broad therapeutic interventions for dementia and dementia-like disorders, ones linked to cerebrovascular diseases in particular (Rodríguez-Giraldo *et al*., 2022; Verkhratsky *et al*., 2023; Sompol *et al*., 2023; Sompol, P., 2024; Nakano-Kobayashi *et al*., 2023).

1.4. Looking into Astrocytes as a Site of Therapeutic Interest

1.4.1. *What Makes Astrocytes a Region of Interest?*

Since astrocytes are seen as being 'actively involved in neurodegenerative diseases' (Phatnani and Maniatis, 2015) and other neurological disorders, including brain tumours, serving as a promoter for neuroinflammation, it could be implied that astrocytes may serve as sites of potential interest with respect to therapeutics (Li *et al*., 2024). Moreover, astrocytes themselves are not a uniform population. The different subtypes of astrocytes tend to express unique and independent characteristics and functions. Studying how these specific subtypes of astrocytes function or are dysfunctional in cases of dementia could provide an interesting avenue inclined towards an improved and more effective therapeutic approach to dementia. Key is to look into restoring normal astrocyte function to reduce neuroinflammation and improve cognitive function (Rodríguez-Giraldo *et al*., 2022; He *et al*., 2024).

1.4.2. *Astrocyte Ion Channel Dysfunction in Dementia*

Since the astrocyte ion channels regulate most astrocytic functions, including neuron-glia signalling and maintaining homoeostasis, they've been studied as part of this project. As suggested by several studies in the past decade, it is the ion channels that allow astrocytes to interact with neurons and regulate synaptic transmission and plasticity (Olsen *et al*., 2015). Some of these channels are Bestrophin-1, hemichannels, and two-pore channels (Min *et al*., 2012). Therefore, astrocytes with their active involvement in ion homeostasis, neuroinflammation, and synaptic regulation, are now seen as key players in the pathogenesis of dementia. The various types of ion channels in astrocytes – *viz*. the typical calcium, chloride, potassium, and sodium channels, and some of the more specialised channels called hemichannels and pannexins – are responsible for the regulation of ionic balance crucial for maintaining the physiological state of our brain (McNeill *et al*., 2021; Lia *et al.*,

2023). Therefore, investigating the molecular pathways resulting in astrocyte activation triggered by the presence of aggregate mal-structured tau protein – as a result of abnormal chemical modifications, such as hyperphosphorylation, *i.e.*, the attachment of too many phosphate groups with tau, resulting in detachment from microtubules, and misfolding, *i.e.*, when detached tau twists into abnormal shapes, *e.g.*, β-sheet structures, instead of staying flexible – and those which lead to modulation of ion channels holds potential for pharmacological developments targeting astrocyte dysfunction in dementia pathophysiology (Lia *et al.*, 2023; Wang *et al*., 2022).

While it has been noted that astrocytes change with age – it is understood that aged astrocytes shift from supportive partners to contributors of synaptic loss and inflammation, creating a permissive environment for dementia (Hulshof *et al.*, 2022) – which may result in a loss of normal astrocyte functions, such as their involvement in providing neuronal support and, therefore, contributing to the dysfunction of the neurovascular unit (Garwood *et al*., 2017), reactive astrogliosis is a major participant in the progression of dementia. It is defined by a series of changes, both morphological or structural, and functional, as a response to unusual events, *e.g.*, disease, injury, etc. (Pekny and Pekna, 2014) Reactive astrogliosis is followed by upregulated production of pro-inflammatory cytokines and reactive oxygen species, altered synaptic transmission. One of the reasons for an altered synaptic transmission could be the higher calcium levels in astrocytes, and decreased potassium channel function, which may result in neuronal hyperexcitability and excitotoxicity (Shah *et al*., 2022; Zhang *et al*., 2018; Wang *et al*., 2022). As it is, unbalances in the regulation of astrocyte calcium and potassium can result in altered synaptic transmission in diseases like as Alzheimer's. Excessive glutamate release from astrocytes with elevated calcium levels over-stimulates neuronal NMDA and AMPA receptors, extending neuronal firing. To further harm neurons, this calcium spike also triggers oxidative stress and inflammatory pathways. At the same time, extracellular potassium

builds up near synapses due to malfunctioning potassium channels (e.g., $K_{ir}4.1$). Depolarisation from this accumulation causes neurons to become hyperexcitable and prone to irregular firing. These disturbances work together to produce a destructive cycle in which overactive neurons use up all of their energy in an attempt to restore ion balance, which results in cellular stress, synaptic damage, and ultimately excitotoxic cell death (McNeill *et al*., 2021). Their significance in causing neurodegeneration is highlighted by the interaction between astrocyte malfunction and neuronal overactivation (Wang *et al*., 2022).

Although reactive gliosis may initially serve as a mechanism of protection against possible damage, considering astrocytes particularly act 'by constantly modulating synaptic activity' (Lia *et al*., 2023), it can ultimately contribute to neurodegeneration when the inflammatory response becomes chronic and uncontrolled, implying that the protective mechanisms of reactive gliosis become dysregulated and self-perpetuating, losing their ability to resolve or return to normal (Sidoryk-Węgrzynowicz and Strużyńska, 2021; Gao and Hong, 2008). Over a longer period of time, this might even lead to an exacerbated decline in cognition with ion channel dysfunction playing a decisive role in driving the said process (Liu *et al*., 2024; Li *et al*., 2025; Rahman *et al*., 2024).

Chapter 2

Review of Literature

The exploration of ion channels in astrocytes has emerged as a critical avenue for uncovering therapeutic insights into the pathophysiology of dementia, a complex syndrome characterised by a progressive decline in cognitive function affecting millions worldwide. As of 2019, dementia impacted approximately 55.2 million individuals globally and is projected to affect 139 million by 2050, making it a significant public health concern with substantial economic implications, including anticipated costs of up to $2.8 trillion by 2030 (Preman *et al*., 2021; Verkhratsky *et al*., 2023). Recent research indicates that dysfunction in ion channels, particularly in astrocytes – glial cells crucial for maintaining neuronal health – may play a pivotal role in the onset and progression of dementia-related disorders, including AD and vascular cognitive impairment (Pushparaj *et al*., 2022; Taylor *et al*., 2024; Edison, P., 2024). Astrocytes are essential for regulating extracellular ion concentrations and neurotransmitter dynamics, and their dysfunction has been implicated in various neurodegenerative conditions (Siracusa *et al*., 2019; Wang *et al*., 2022). Ion channels, including sodium, potassium, and calcium channels, are integral to astrocytic function, influencing processes such as calcium signalling, neurotransmitter release, and neuronal excitability. Dysregulation of these channels can exacerbate neuronal damage and contribute to cognitive deficits observed in dementia (Pushparaj *et al*., 2022; Banerjee and Jirsa, 2024; Kumar *et al*., 2016). Notably, alterations in calcium signalling pathways within astrocytes have been identified as early indicators of familial AD, highlighting the urgency of investigating these mechanisms as potential therapeutic targets (Shao *et al*., 2024; Lia *et al*., 2023; Edison, P., 2024). The potential therapeutic implications of targeting astrocytic ion channels are significant, offering promising avenues for intervention in dementia treatment. However, challenges remain, including the

complexity of neurodegenerative pathologies, the need for selective pharmacotherapy, and the difficulties associated with drug delivery across the blood-brain barrier (Monterey *et al*., 2021; Liu *et al*., 2024; Rahman *et al*., 2024). Ongoing research aims to elucidate the mechanisms through which astrocytes regulate neuronal health and to develop innovative strategies for restoring normal ion channel function, ultimately seeking to mitigate the cognitive decline associated with dementia and improve outcomes for affected individuals (Shao *et al*., 2024; Edison, P., 2024; Lia *et al*., 2023).

2.1. Astrocytes

Astrocytes are a morphologically and functionally diverse population of glial cells in the CNS that play critical roles in maintaining brain homeostasis and supporting neuronal function. They are actively involved in synaptic activity, synaptogenesis, and neurogenesis, and are essential, as aforementioned, for regulating extra-cellular ion concentrations and neurotransmitter levels (Siracusa *et al.*, 2019; Liu *et al*., 2024). Due to their multifunctional nature, astrocyte dysfunctions have been linked to various neurodegenerative disorders, including vascular contributions to cognitive impairment and dementia (VCID) and AD (Edison, P., 2024; Wang *et al.*, 2022).

2.1.1. *Functions of Astrocytes and Their Diversity*

Astrocytes contribute significantly to the overall health of the CNS by performing several key functions. They help maintain extracellular homeostasis by buffering ions such as potassium (K^+), sodium (Na^+), and protons (H^+), and also regulate neurotransmitter release, particularly glutamate and gamma-aminobutyric acid (GABA) (Siracusa *et al.*, 2019; Nam *et al*., 2023). Through these actions, astrocytes facilitate synaptic transmission and modulate neuronal excitability.

The diversity of astrocytes, structure- and function-wise (Khakh and Sofroniew, 2015), on the other hand, is observed both across different brain regions and within specific areas, which influences their pathological features in psychiatric disorders (Siracusa *et al.*, 2019). This heterogeneity may be crucial for understanding how astrocyte functions can be selectively targeted in neurodegenerative diseases.

2.1.2. *Ion Channels in Astrocytes*

Astrocytes, in maintaining the homeostasis of the neuronal environment, express various ion channels, transmitter receptors, and transporters, enabling them to sense and respond to neuronal activity effectively (Siracusa *et al.*, 2019). Specifically, weakly rectifying K^+ channels, such as $K_{ir}4.1$, are pivotal for maintaining potassium homeostasis, while two-pore domain K^+ channels have been implicated in astrocytic signalling (Liu *et al*., 2024; McNeill *et al*,, 2021). Additionally, transient receptor potential (TRP) channels are sensitive to various physical and chemical stimuli, further enabling astrocytes to respond to changes in their environment (Villa *et al*., 2020).

The physiological functions of ion channels in astrocytes, their involvement in neurotransmitter uptake, and alterations observed in neurodegenerative diseases, such as AD and glioblastoma, are discussed below.

2.1.2.1. Potassium Channels

Potassium (K^+) channels in astrocytes, particularly $K_{ir}4.1$, are essential for spatial buffering, allowing the clearance of excess K^+ released during neuronal activity. The influx of K^+ through these channels helps prevent neuronal excitability and hyperactivity, and this influx spreads electronically through the cytoplasm before exiting at distant locations (Illarionava *et al*., 2014; Pietrobon and Conti, 2024). Additionally, the

Na^+/K^+–ATPase and $Na^+/K^+/2Cl$ cotransporter 1 (NKCC1) work in concert to import and export K^+ effectively (Pietrobon and Conti, 2024).

2.1.2.2. Calcium Permeable Channels

Calcium (Ca^{2+}) channels play a significant role in astrocytic signalling. Changes in intracellular Ca^{2+} concentrations can influence neurotransmitter release and synaptic activity, impacting neuronal communication and plasticity (Zhang *et al*., 2022).

These channels include TRPA1 and IP3 receptor (IP3R)-dependent pathways, which generate distinct Ca^{2+} signals. TRPA1 channels mediate localised Ca^{2+} microdomains near the plasma membrane, contributing to basal Ca^{2+} levels independently of ER stores. IP3R-dependent pathways, activated by G-protein-coupled receptors (*e.g.*, metabotropic glutamate receptors), trigger widespread Ca^{2+} waves that propagate through astrocytic networks. These Ca^{2+} signals regulate neurotransmitter release (*e.g.*, glutamate, ATP) and influence synaptic plasticity by modulating neuronal NMDA receptor activity (Khakh and McCarthy, 2015). For example, astrocytic Ca^{2+} elevations can induce slow inward currents in neurons *via* extrasynaptic NMDA receptors, enhancing synaptic efficacy or initiating plasticity mechanisms like long-term potentiation (LTP). Dysregulation of astrocytic Ca^{2+} signalling is implicated in neuropathologies, including epilepsy and excitotoxicity (Haydon and Carmignoto, 2006).

2.1.2.3. Anion Channels

Anion channels are involved in regulating the balance of chloride (Cl^-) and bicarbonate (HCO_3^-) ions in astrocytes, which is crucial for maintaining the extracellular ionic environment (Zhang *et al*., 2022). These channels contribute to the overall ionic homeostasis necessary for efficient neuronal signalling.

Several anion channels tend to activate during osmotic stress or cell swelling, enabling efflux of Cl^-, organic osmolytes, and neurotransmitters like glutamate and ATP. This process supports regulatory volume decrease (RVD) and modulates extracellular neurotransmitter levels. Some anion channels, *e.g.*, ClC-3, are also implicated in pH regulation via H^+/Cl^- exchange. Disruption of anion channel function can lead to pathological extracellular Cl^- accumulation, impairing GABAergic inhibition or exacerbating edema during ischemic injury (Kimelberg *et al*., 2006). Additionally, anion channels interact with transporters (e.g., NKCC1) to fine-tune intracellular Cl^- concentrations, influencing astrocyte-mediated synaptic inhibition.

2.1.2.4. Ligand-Gated Ion Channels

Astrocytes also express various ligand-gated ion channels, which can modulate synaptic transmission. These channels respond to neurotransmitters and participate in the dynamic regulation of synaptic activity, highlighting their role in neuronal signalling and plasticity (Zhang *et al*., 2022; Mann *et al*., 2022).

AMPA and NMDA receptors detect glutamate spillover, triggering Na^+/Ca^{2+} influx that modulates astrocyte metabolism and neurovascular coupling. $GABA_a$ receptors mediate Cl^- influx, potentially dampening excitability by shunting membrane potential. P2X receptors, activated by ATP, amplify Ca^{2+} signals and propagate intercellular waves via purinergic signalling (Lia *et al*., 2023). These channels also regulate synaptic plasticity: astrocyte-derived glutamate acting on neuronal NMDA receptors facilitates LTP, while ATP release via VRACs strengthens inhibitory synapses (Haydon and Carmignoto, 2006). Ligand-gated ion channels thus serve as bidirectional communicators, integrating neuronal activity with astrocytic homeostatic and modulatory functions.

2.2. Ion Channel Dysfunction

The dysfunction of ion channels has been implicated in a range of symptoms associated with dementia, such as memory loss and movement disabilities. Studies indicate that alterations in the expression, localization, and function of astrocytic ion channels disrupt ion homeostasis, which can exacerbate neuronal damage and contribute to the progression of neurodegenerative diseases like AD (Ziar *et al*., 2025; Kumar *et al*., 2016). These ion channels, including Na^+, K^+, Ca^{2+}, and Cl^- channels, are essential for regulating the flow of ions across cellular membranes and ensuring proper neuronal excitability (Vitvitsky *et al*., 2012; Monterey *et al*., 2021).

2.2.1. *Alterations in Alzheimer's Disease and Glioblastoma*

In Alzheimer's disease, the loss of synaptic function and the decline in synaptic markers correlate more strongly with cognitive impairment than the accumulation of Aβ oligomeric deposits (Ugbode *et al*., 2017; Sontheimer *et al*., 1994). Studies indicate that Aβ soluble oligomers can disrupt synaptic transmission by negatively affecting glutamatergic receptors, thus impairing long-term potentiation (LTP) and overall synaptic integrity and functionality (Ugbode *et al*., 2017). The differential expression of K^+ and Ca^{2+} channels in glioblastoma cells compared to normal astrocytes highlights the potential of targeting these ion channels in developing therapeutic strategies (Zhang *et al*., 2022; Illarionava *et al*., 2014).

2.3. Therapeutic Insights

The exploration of ion channels in astrocytes has revealed significant therapeutic potential for treating cognitive health conditions, particularly post-stroke dementia (PSD), AD, and other vascular cognitive impairments (VCIDs). However, the development of novel treatments encounters several challenges, including the complexity of

multiple comorbidities, diverse neurodegenerative pathologies, and the need to navigate the blood-brain barrier (BBB) while addressing pharmacy issues and disease specificity (Monterey *et al*., 2021; Stoklund Dittlau and Freude, 2024).

2.3.1. *Targeting Ion Channels*

Neuronal and glial cation-chloride cotransporters, especially NKCC1, are identified as promising targets for CNS pharmacotherapy. The therapeutic modulation of these channels may enhance astrocytic functions that are crucial for neuronal health. Notably, astrocytes express various ion channels, including inward-rectifying potassium (K_{ir}) channels, which play vital roles in potassium buffering and maintaining neuronal excitability (Wang *et al*., 2022; Lia *et al*., 2023). Dysfunction in these ion channels can lead to impaired astrocytic support during periods of heightened neuronal activity, contributing to conditions such as seizures and other neurological disorders (Liu *et al*., 2024; Lam *et al.*, 2022; Larsen *et al*, 2014; D'Ambrosio *et al*., 2002; Pietrobon and Conti, 2024).

2.3.2. *Challenges in Drug Development*

Despite the potential benefits of targeting ion channels, significant hurdles remain in drug design. For instance, Bumetanide, often classified as an NKCC1 blocker, poses issues due to its lack of isoform specificity and potential off-target effects, which can lead to complications such as diuresis and hypokalaemic alkalosis. These factors limit the long-term application of Bumetanide for chronic CNS disorders (Monterey *et al*., 2021; Ziar *et al*., 2025; Liu *et al*., 2024). Therefore, the focus is shifting toward dev-eloping more selective compounds that can effectively target NKCC1 without the adverse effects associated with current treatments.

2.4. Na^+/K^+–ATPase in Astrocytes

The Na^+/K^+–ATPase plays a critical role in maintaining ionic homeostasis within astrocytes, which are essential support cells in the CNS. Astrocytes are involved in various functions, including synaptogenesis, neurotransmitter buffering, and maintaining the blood-brain barrier, largely through their ion transport capabilities (Wang *et al*., 2022; Azargoonjahromi, A., 2024). Specifically, the Na^+/K^+–ATPase is integral to astrocytic functions such as K^+ clearance during neuronal activity, thereby sustaining neuronal excitability and overall brain function (Van Eldik *et al*., 2024; Sarkar and Biswas, 2021).

2.4.1. *Mechanism and Function*

This ion transport mechanism is particularly pronounced in astrocytes, where high Na^+/K^+–ATPase activity allows for the regulation of extracellular K^+ concentrations, especially during periods of intense neuronal firing (Wang *et al*., 2022; Verkhratsky *et al*., 2010). Furthermore, astrocytic Na^+/K^+–ATPase has been implicated in modulating glutamate uptake, thus influencing excitatory neurotransmission and glutamatergic signalling (Van Eldik *et al*., 2024; Rajasekhar *et al*., 2015). As it is, astrocytic Na^+/K^+–ATPase, particularly the α2 isoform, acts as a regulator of synaptic health by balancing ion gradients, fuelling glutamate clearance, and linking neurotransmission to metabolic support. Dysregulation of this system is a hallmark of neurodegenerative disorders like Alzheimer's.

2.4.2. *Implications in Disease*

Deficits in Na^+/K^+–ATPase activity, particularly involving the α2 isoform, are increasingly implicated in dementia and Alzheimer's disease. In Alzheimer's, astrocytic α2-NKA is elevated in postmortem brain tissue and tauopathy models, driving neuroinflammation and neurodegeneration (Mann *et al*., 2022). This upregulation disrupts

glutamate homoeo-stasis by impairing astrocytic glutamate uptake, via EAATs, and potassium buffering, via $K_{ir}4.1$ channels, leading to extracellular glutamate accumulation and NMDA receptor overactivation (de Lores Arnaiz and Ordieres, 2014; Kinoshita *et al*., 2016). The resulting excitotoxicity exacerbates synaptic loss, tau hyper-phosphorylation, and Aβ-induced mitochondrial stress, accelerating cognitive decline (Zhang *et al*., 2013; de Lores Arnaiz and Ordieres, 2014).

Key Alzheimer's disease-specific implications include: (a) synaptic energy failure, which is defined by Na^+/K^+–ATPase impairment as reducing ATP availability, worsening Aβ-induced neuronal Na^+/Ca^{2+} overload and oxidative stress (Olivares *et al*., 2012; de Lores Arnaiz and Ordieres, 2014); (b) astrocyte-microglia crosstalk, which is defined as dysregulated α2-NKA-triggered microglial activation in astrocytes, fostering a pro-inflammatory milieu that accelerates tau pathology (Mann *et al*., 2022); and (c) age-related decline, *i.e.*, reduced Na^+/K^+–ATPase activity in ageing brains exacerbates glutamate clearance deficits, predisposing to Alzheimer's disease (Kinoshita *et al.*, 2016).

Therapeutic strategies targeting the glutamatergic system in Alzheimer's disease must balance NMDA receptor modulation (to prevent excito-toxicity) with astrocyte-specific α2-Na^+/K^+–ATPase inhibition (to curb neuroinflammation without disrupting ion homeostasis) (Mann *et al*., 2022; Kinoshita *et al.*, 2016).

Further, the utilisation of immunotherapy represents a promising strategy for modifying the development of Alzheimer's. This approach focuses on harnessing the immune response to target and clear amyloid plaques, thereby reducing neuroinflammation and subsequent neuronal damage (Pietrobon and Conti, 2024). Additionally, the review of current treatment modalities indicates that targeting cholinergic systems and N-methyl-D-aspartic acid (NMDA) receptors could help in alleviating

cognitive symptoms associated with AD (Isaksen and Lykke-Hartmann, 2016).

2.5. Ion Channel Modulation

Understanding how ion channels and ionotropic receptors in astrocytes participate in the pathology of neurodegenerative diseases is essential for developing new therapeutic agents (Mann *et al*., 2022; Illarionava *et al*., 2014). The modulation of Na^+/K^+–ATPase activity could offer mixed effects; for instance, inhibition may reduce sodium efflux from astrocytes but could simultaneously dampen neuronal activity, highlighting the need for careful consideration in therapeutic designs (Illarionava *et al*., 2014; Zhang *et al*., 2013). This duality underscores the importance of designing drugs that can selectively target specific ion channel pathways to achieve the desired therapeutic outcomes without adverse effects on neuronal function.

Furthermore, studies suggest that improving the ion transport mechanisms within astrocytes may enhance their protective roles against neurodegeneration. For example, targeting specific pathways that facilitate ion homeostasis in astrocytes can help maintain the cellular environment crucial for neuronal health (Huang *et al*., 2024; Zhang *et al*., 2022; Mann *et al*., 2022). Thus, elucidating the intricate balance of ion channel function in astrocytes could lead to innovative treatment strategies that harness these cells' potential to counteract the effects of Aβ and support neuronal survival.

Chapter 3

Objectives of Study

In this study, which was performed at the Department of Biosciences and Biomedical Engineering, at the Indian Institute of Technology Indore, the U87 and LN229 glioblastoma cell lines were used to observe the potential of Na^+/K^+–ATPase in astrocytes as therapeutic targets (Qiu *et al*., 2025) in case of dementia.

The following is the key question researched in the study:

a. Can astrocyte pathophysiology be rescued by enhancing Na^+/K^+–ATPase activity?

To answer the project key question, the following are the objectives that were progressively pursued in the study:

a. To observe expression profile of ion channels, particularly the Na^+/K^+–ATPase in astrocytes; and
b. Studying Aβ induced astrocytes pathophysiology upon activating Na^+/K^+-ATPase.

Chapter 4

Materials & Methods

Since the change in the structure is a key factor for characteristic differences, the possible approaches of study may be:

i. interfering with the attachment of Aβ42 to the available Na^+/K^+–ATPase sites, and/or

ii. activating the Na^+/K^+–ATPase in a different manner to rescue astrocyte physiology.

In the second approach, there is a certain need for the binding sites for an activator, *e.g.*, suloctidil, to be different from those for Aβ42 to avoid conditional or competitive inhibition, implying that since colocalisation of Aβ42 is critical to neuroinflammation, no colocalisation means no binding which in turn means reduced chances of neuroinflammation.

The goal here is to see if the α_2 isoform in astrocytes maybe rescued from its silenced state, which will also let us look into probable changes in astrocyte physiology. Understanding the astrocytes' responses to factors surrounding the pathology of a neurodegenerative disease could be key to an effective treatment (Garwood *et al*., 2017). For the same, we procured the Aβ42 proteins to observe the following:

iii. the binding of the Aβ proteins with Na^+/K^+–ATPase,

iv. if certain amyloid activity causes toxicity in astrocytes and, consequently, causing death of the cells.

As a reparatory measure, the focus throughout the study has been on methods to rescue astrocyte physiology, including observing such conditions when Aβ doesn't bind with Na^+/K^+–ATPase isoforms. For Na^+/K^+–ATPase activation, we'll accordingly be looking at varied therapeutic approaches, including certain activators, with focus on the prevention of possible Aβ binding with the α2 isoform

4.1. *Establishing a Model System as Part of the Study*

For the laboratory-based research, we employed the U87 glioblastoma or human astrocytoma cell line. While the cells are widely used in cancer research, especially for studies relating to *glioblastoma multiforme* (GBM), their applications are understood to extend into neuroscience and dementia research. As it is, the glioblastoma cell line can be a perfect choice to study the change in astrocyte function and morphology in response to neurodegenerative disease pathologies because of their structural and functional similarities to the latter. Another very important reason would be their extreme malignancy and the aggressiveness of glioblastoma as a brain tumour. Of most glioblastoma cell lines, U87 has been found to have the highest average migration speed and the longest protrusions into the collagen (Diao *et al*., 2019). This implies the availability of cultures for use in relatively little time with sufficiently observable response.

4.2. *Designed and Conducted Experiments as Part of the Study*

The following are the steps we completed in the study:

i. First, we cultured U87 and LN229 (National Centre for Cell Science Complex, Pune, India) in Dulbecco's Modified Eagle Medium (HiMedia, Cat No. AL007S) supplemented with 10% (v/v) foetal bovine serum (HiMedia, Cat No. RM10432) and 1% (v/v) penicillin/streptomycin. The cells were maintained in a humidified incubator at 37°C with 5% CO_2 through frequent passaging (2–3-day intervals), and observed frequently following trypsinisation.

ii. After confluent growth of the U87 cells, we performed immunofluorescent staining to observe the expression pattern for β-tubulin and GFAP markers, and for Na^+/K^+–ATPase α1 and Na^+/K^+–ATPase α2, in U87 cells. For the

same, cells were cultured on poly-L-lysine (PLL)-coated 5 mm coverslips. Following treatment, cells were fixed with 4% paraformaldehyde, permeabilized with 0.02% Triton X-100 in PBS for 10 minutes, and blocked with 3% bovine serum albumin (BSA) in PBS for 1 hour. Cells were then incubated overnight at 4°C with primary antibody (Anti-GFAP, Cat No. G9269; Anti Na^+/K^+–ATPase alpha-1, Cat No.05369) diluted in PBS containing 0.2% Triton X-100 (PBST). After three washes with PBS, cells were incubated with fluorescent labelled secondary antibodies for 1 hour at room temperature. Following three washes with PBS, coverslips were mounted on glass slides using a mounting medium fluoromount (Sigma-Aldrich, Cat No. F4680) and imaged using a fluorescence microscope (Thermo Fisher Scientific EVOS M5000 Imaging System).

iii. Furthermore, we worked with our collaborator, Dr Rajnish Kumar, at the Department of Pharmaceutical Engineering & Technology, Indian Institute of Technology (B.H.U.), to obtain the results of molecular docking which note that both Na^+/K^+–ATPase α1 & Aβ42, and Na^+/K^+–ATPase α2 & Aβ42 are probable combinations with respect to Aβ–Na^+/K^+–ATPase attachment/binding.

iv. To visualise fatty acid uptake, in particular that of oleic acid by cells, an assessment was performed. For the same, Oil Red O (ORO) staining was used. ORO staining uses a dye to stain fat in cells and tissues. The principle is that the dye is more soluble in fat than in the dye solution. For this experiment, a stock solution (1 M) of oleic acid (Sigma-Aldrich, Cat. No. 01383) was prepared in ethanol. Sub-stock solutions (1 mM) were prepared from the stock solution in PBS and further diluted in culture media to achieve the desired treatment concentrations.

v. At a later stage, and to better understand and analyse cell activity in presence of oleic acid, we studied another astrocyte-like cell line LN229, alongside the U87 cells already used. Following which, a cell viability test was performed through MTT assay. The principle behind the assay the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide compound is reduced to a coloured formazan dye in the presence of mitochondrial dehydrogenases (particularly succinate dehydrogenase).

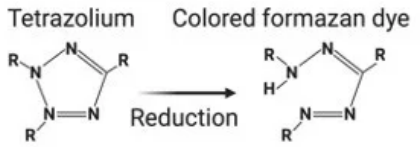

Figure 3. The chemical reaction at the root of MTT assay

Cells were seeded in 96-well plates at a density of 10,000 cells/well. Following treatment and incubation for 12 hours, MTT (Himedia, Cat. No. TC191) solution (5 mg/mL in PBS) was added to the culture media to achieve a final concentration of 0.5 mg/mL. Cells were incubated with MTT for 2 hours at 37°C. Subsequently, the media was removed, and 50 µL of dimethyl sulfoxide (DMSO) was added to each well to solubilise the formazan crystals. The plates were then shaken on an orbital shaker for 15 minutes at room temperature. Absorbance was measured at 570 nm using a BioTek Synergy H1 plate reader.

vi. Following the cell viability assay, live/dead, cytotoxicity assay and ROS assay were performed. Live/dead and cytotoxicity assay followed up after cell viability assay to conclude that oleic acid doesn't show toxicity up to 20µM with respect to U87 and LN229 cells, and following which cell death can be observed in an increasing manner.

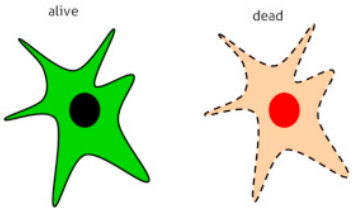

Figure 4. Green fluorescent calcein is seen in live cells, and ethidium homodimer-1 is observed in dead cells

For the same, a Live/Dead Viability/Cytotoxicity kit, (Thermo Fischer Scientific, Cat No. L3224) was used. Cells were seeded and treated in 96-well plates. Following treatment, the media was removed, and cells were incubated with a staining solution containing Calcein AM and ethidium homodimer-1 (2:5 ratio in PBS) at 37°C for 30 minutes. Live (Calcein AM-positive, green fluorescence) and dead (ethidium homodimer-1-positive, red fluorescence) cells were visualised and counted using an EVOS M5000 inverted fluorescence microscope with a 10X objective.

vii. Reactive oxygen species include a number of radicals and charged molecules, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen ($^1O_2$) (Jacubczyk *et al*., 2020). These are highly reactive chemicals containing oxygen that play important roles in both normal cellular processes and disease states, and have a dual nature. At low to moderate levels, ROS act as signalling molecules in various biological processes. However, at high levels, they can cause oxidative stress and cellular damage (An *et al*., 2024). As part of the assay, intracellular reactive oxygen species (ROS) levels were measured using a fluorogenic dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), which is taken up by cells, cleaved by esterases, and then oxidised by ROS into a fluorescent product (DCF). The resulting fluorescence intensity is directly proportional to the ROS levels, which allowed for

ROS activity quantification. Cells were then seeded in 96-well plates at 10,000 cells/well density. Following treatment, the media was removed, and 50 µL of 1X reaction dye (TCI, Cat. No. R0287) was added to each well. After incubation for 30 minutes at 37°C, fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using a BioTek Synergy H1 plate reader. It was crucial to perform ROS assay to understand if ROS are not involved in oleic acid-induced cell stress.

viii. Sholl analysis was performed to assess the complexity and branching patterns of the astrocyte cell processes. For the same, concentric circles (in 2D) were drawn at set distances from the centre of the cell body (soma). The number of times neuronal branches intersect each circle or sphere is counted. For this analysis, The Sholl Analysis plugin (v1.0) for ImageJ was utilised, which generates concentric circles around a user-defined centre point and counts the number of intersections between the astrocyte processes and the circumference of each circle. Before analysis, images were converted to 8-bit grayscale, and the scale was set using the "Set Scale" function in ImageJ, ensuring accurate micron measurements. A point was selected at the centre of the astrocyte soma to serve as the origin for the concentric circles. This analysis, used in quantifying features such as total dendritic length, surface area, volume, and the number of branch points at different distances from the soma, was used to study how astrocytic structure might changes in response to changing environmental conditions during neurodegenerative (dementia-like) disorders. Sholl analysis, performed using the ImageJ application (with open-source SNT plugin installed), makes use of a profile or curve showing the number of intersections versus distance from the soma, which reflects the complexity and spatial distribution

of neuronal branching. The analysis was performed in the following manner:

a. Installation of SNT plugin on ImageJ.
b. Setting scale: Dimension of usable image observed (magnification: 10X) – given, 3 pixel/μm. The global checkbox was selected. This entire step is only performed as required and not every time.
c. Image threshold is adjusted through the 'Adjust' menu (or Ctrl/Cmd + Shift + T). This setting is used to adjust for cell area visibility for a black-and-white cell image against a white background.
d. To remove the salt & pepper noise, "Despeckle" tool was run.
e. The pointer tool was then used to mark cell nucleus, following which Sholl analysis (image analysis) was performed using the following parameters:
    i. Start radius: 5 μm
    ii. Step size: 2 μm
    iii. End radius: 500 μm
    iv. Integration: Mean
    v. Annotations LUT: Grays
    vi. Action: Analyse Image
f. The resulting image was saved as JPG at the end each time, and the accompanying data was stored in an Excel file for further comparative analysis.
g. The resulting data, representing the number of intersections at each radius, were exported for further analysis.

Chapter 5

Results

i. Establishment of experimental model

As the first step of the study, the goal was to see to the culturing of U87 and LN229 cells for further steps and planned analyses in relation to dementia pathophysiology and moving towards possible therapeutic answers.

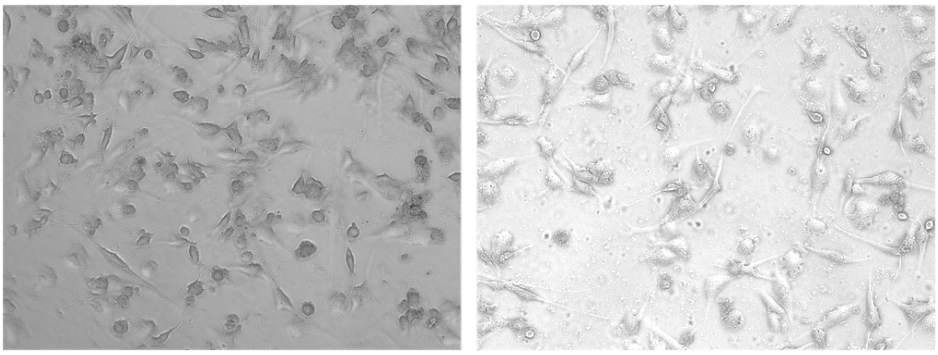

Figure 5. Bright-field images of cultured U87 cells at 20X magnification

ii. Immunofluorescent staining of U87 and LN229 cells

Immunofluorescent staining of the cultured U87 cells was performed to identify live cells and for confirming the presence of astrocytes in the cultured cells. Using immunostaining, the cells were further observed to see the expression pattern for β-tubulin and GFAP markers, and of Na^+/K^+–ATPase α1 and α2 in U87 cells.

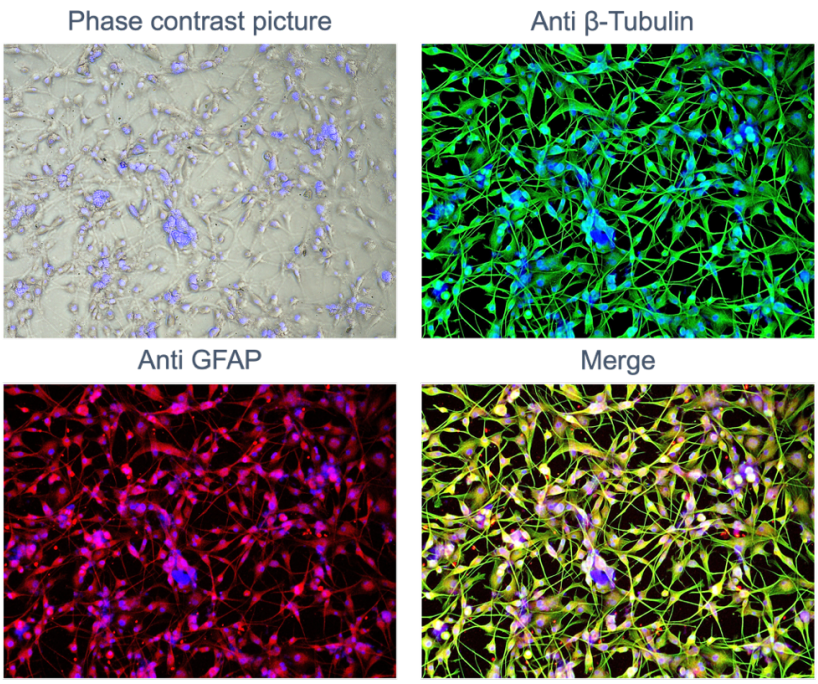

Figure 6. **Immunofluorescent identification of U87 glioblastoma cells using cytoskeletal and astrocyte markers:** Representative fluorescence images show U87 cells stained for β-tubulin (a cytoskeletal marker) and GFAP (glial fibrillary acidic protein, an astrocyte marker). The co-expression confirms the presence and astrocytic identity of live U87 cells in culture.

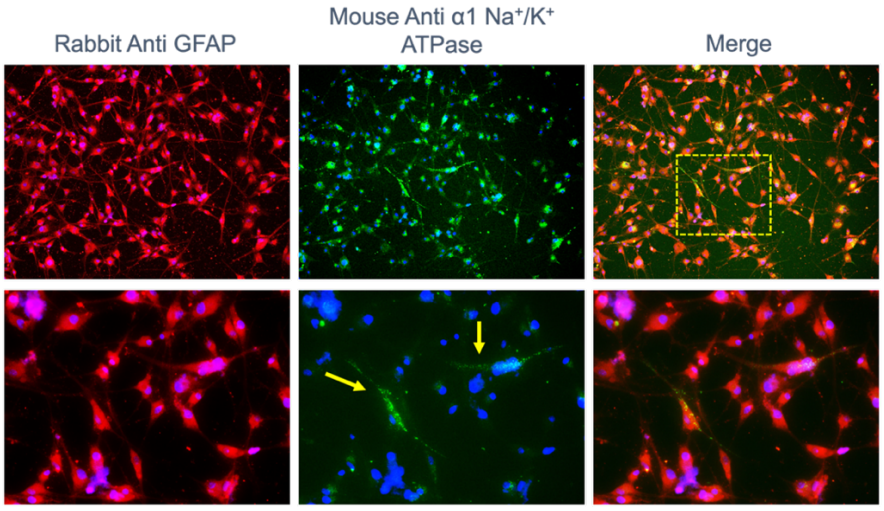

Figure 7. **Expression of Na^+/K^+–ATPase α1 isoform in U87 cells:** Immunofluorescence images display U87 cells stained with anti-GFAP (astrocyte marker) and mouse anti-Na^+/K^+–ATPase α1 antibodies. The observed fluorescence indicates successful expression of the α1 isoform in astrocyte-like U87 cells (marked by arrows).

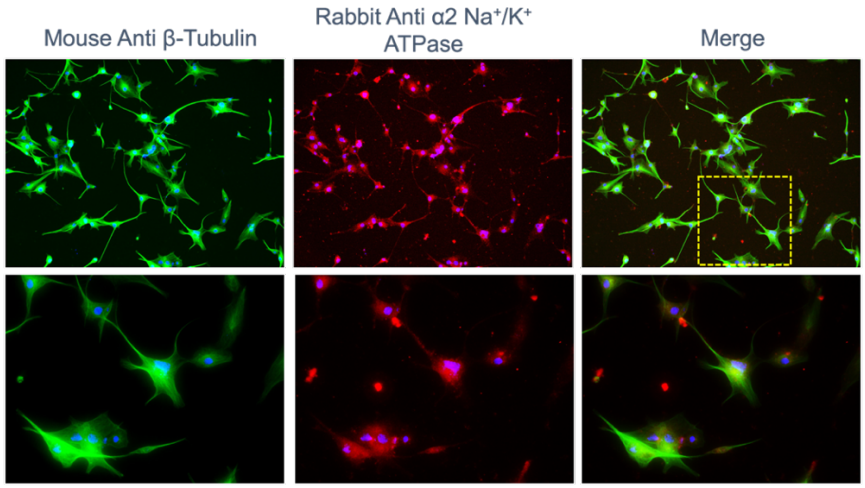

Figure 8. **Expression of Na^+/K^+–ATPase α2 isoform in U87 cells:** Immunofluorescent staining demonstrates the presence of the Na^+/K^+–ATPase α2 isoform in U87 cells, confirming expression of this astrocyte-enriched isoform.

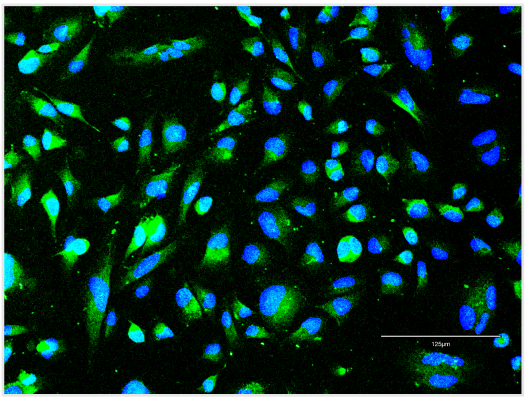

Figure 9. **Immunostaining of LN229 glioblastoma cells for β-tubulin:** LN229 cells were stained with anti-β-tubulin antibody to visualise cytoskeletal structure and confirm cell identity. Fluorescence microscopy reveals robust β-tubulin expression in the LN229 cell line.

Respective tests performed showed the expression pattern of Na^+/K^+–ATPase α1 and α2 in U87 cells, which established the U87 glioblastoma cell line as our test cell line. Moving further, the interactions between Na^+/K^+–ATPase α1 and α2 isoforms and Aβ42 were studied to observe the effect of Aβ42 on U87 cells.

iii. Investigating the interactions between Aβ42 and Na^+/K^+–ATPase α1 and α2

Through our lab's collaboration with Dr Rajnish Kumar at the Department of Pharmaceutical Engineering & Technology, at the Indian Institute of Technology (B.H.U.), we sought support from our collaborators for obtaining the results of molecular docking, which note that both (i) Na^+/K^+–ATPase α1 & Aβ42, and (ii) Na^+/K^+–ATPase α2 & Aβ42 are probable combinations with respect to Aβ–Na^+/K^+–ATPase attachment/binding.

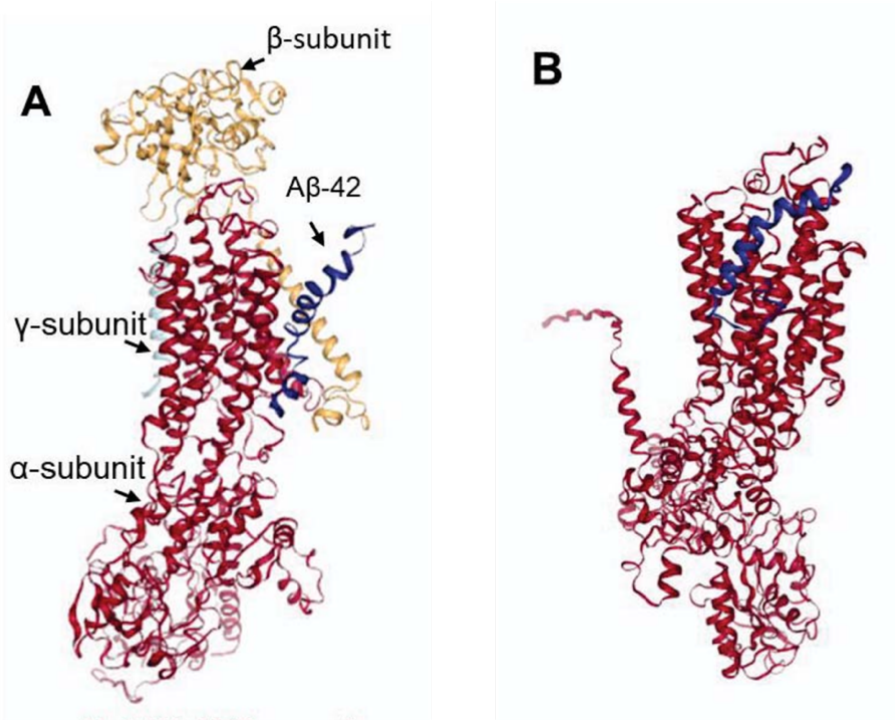

Figure 10. **Molecular docking of Aβ42 with Na^+/K^+–ATPase α1 and α2 isoforms:** (**A**) Structural model showing the binding interface of Aβ42 with the Na^+/K^+-ATPase α1 isoform. (**B**) Structural model showing the binding interface of Aβ42 with the Na^+/K^+-ATPase α2 isoform. Docking studies reveal probable interaction sites, suggesting both isoforms can bind Aβ42, which may underlie pathophysiological effects in astrocytes.

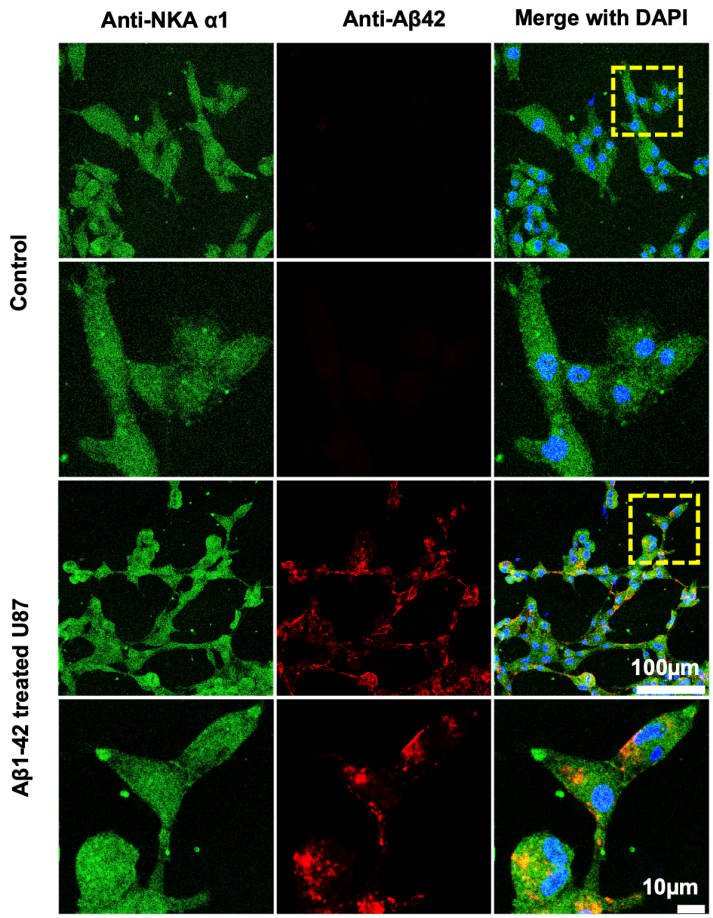

Figure 11. **Colocalisation of Aβ42 and Na^+/K^+-ATPase α1 in astrocytes:** Immunofluorescence images illustrate the spatial overlap of Aβ42 and Na^+/K^+–ATPase α1 signals in U87 astrocyte-like cells, indicating potential direct interaction relevant to Alzheimer's disease pathophysiology.

iv. Treatment with Aβ42 shows its toxic effect on the viability of U87 glioblastoma cells

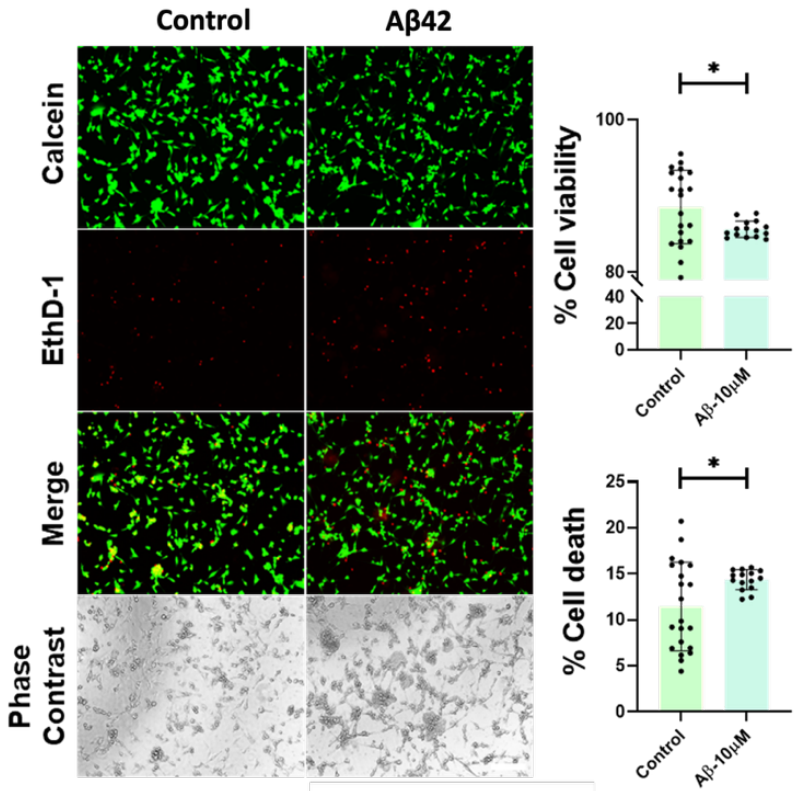

Figure 12. **Cytotoxic effect of Aβ42 on U87 glioblastoma cell viability:** Bar graph (and representative images) showing a significant reduction in U87 cell viability following Aβ42 treatment compared to control, demonstrating the cytotoxic potential of Aβ42 in U87 glioblastoma cell line.

On Aβ42 treatment, the U87 glioblastoma cell line showed an increase in cell death and, therefore, a consistent decrease in cell viability. For potential rescue in this case, a number of activators were identified through a review of existing literature in the field (Obradovic *et al.*, 2023; Vejrazka, M., 2014). Oleic acid was selected as the activator for this project to look into its impact on the growth and viability of the U87 glioblastoma cell line (Cornelius, F., 1991).

v. Oleic acid uptake

It is understood that astrocyte cells release an amount of oleic acid by themselves. However, through Oil Red O staining, it was understood that oleic acid uptake was still happening in astrocyte cells since the intensity of colour under the microscope was seen as increasing in a proportional manner with the treatment concentration of oleic acid.

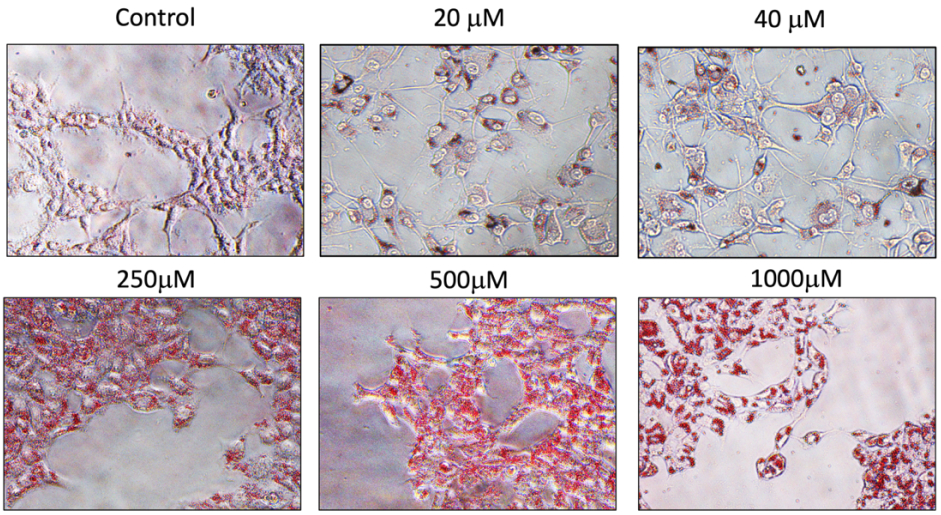

Figure 13. **Oleic acid uptake by U87 cells visualized by Oil Red O staining:** Microscopy images display increased Oil Red O staining intensity in U87 cells with rising oleic acid concentrations, indicating dose-dependent uptake of oleic acid.

vi. Cell viability assay with respect to both U87 and LN229 cells

An assessment of cell viability was performed as an essential part of the entire procedure, considering studies suggest that a high concentration of oleic acid can induce cytotoxicity (Giulitti *et al.*, 2021). Our studies concluded that cell viability is not considerably affected by treatment of cell lines with oleic acid up to 40µM.

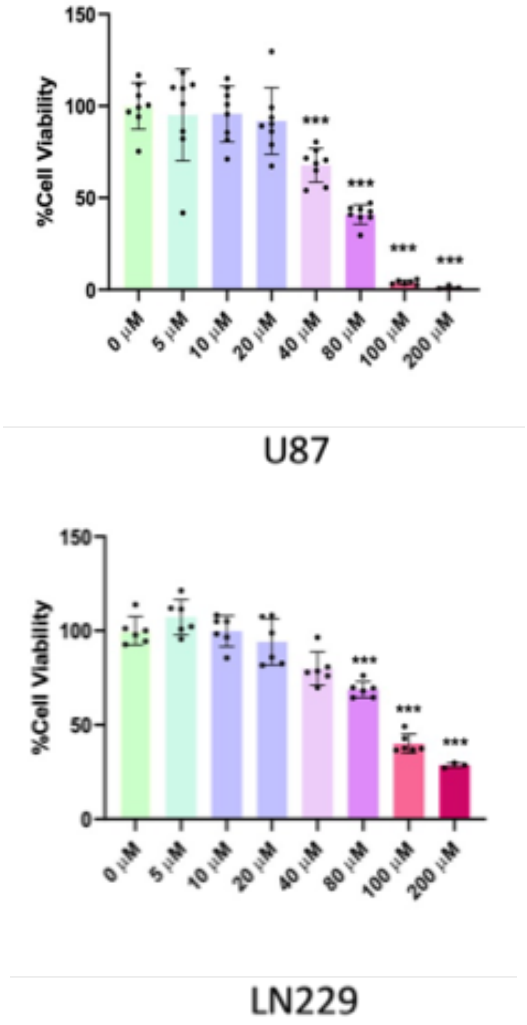

Figure 13. **Cell viability following oleic acid treatment in U87 and LN229 cells:** Bar graphs show the percentage of viable U87 (left) and LN229 (right) cells after exposure to varying concentrations of oleic acid. Viability remains unaffected up to 40 μM, suggesting low cytotoxicity at these doses.

vii. Live/dead and cytotoxicity assay

Following on from the previous assay, cells were treated with calcein-AM, a non-fluorescent compound form of calcein, a green fluorescent protein, and the red fluorescent ethidium homodimer-1. Calcein-AM breaks down in presence of esterases produced by live cells to release the highly fluorescent calcein, which leads to the colour of the live cells being green under a microscope and that of dead cells being red.

Following the assay, it was concluded that oleic acid doesn't show toxicity up to 20µM, following which cell death can be observed.

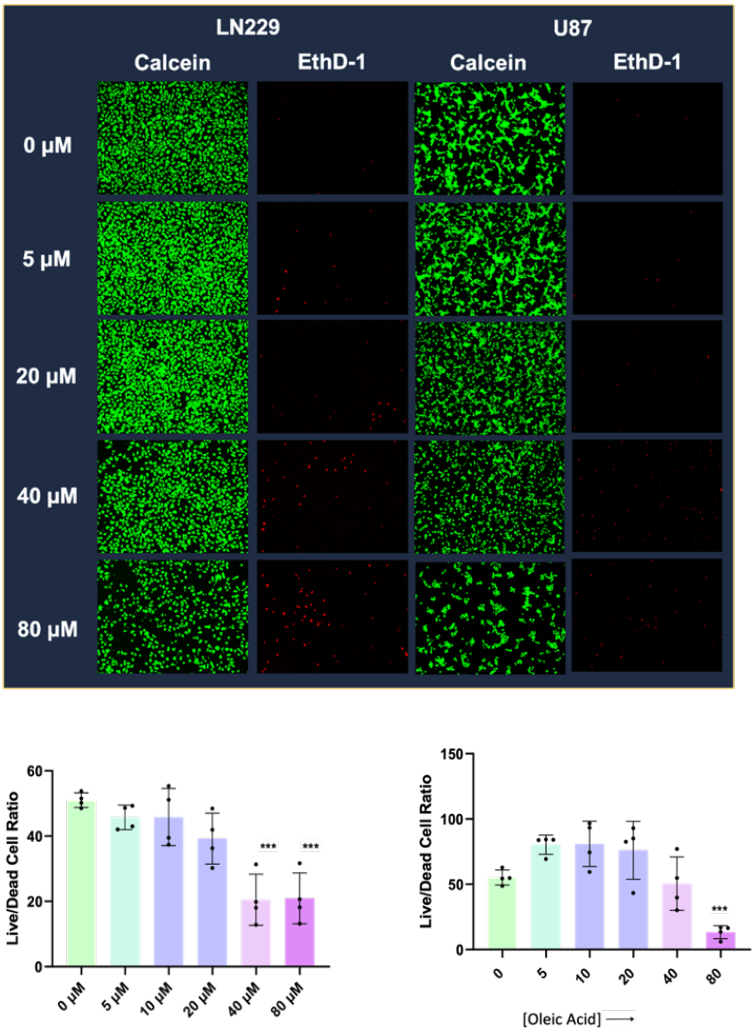

Figure 14. **Live/dead and cytotoxicity assay in U87 and LN229 cells treated with oleic acid:** Fluorescence microscopy images show live cells (green, Calcein AM-positive) and dead cells (red, ethidium homodimer-1-positive) after treatment. Cell death increases above 20 µM oleic acid, indicating threshold-dependent cytotoxicity.

viii. Experiment to understand the involvement of ROS in cell stress condition

Through the assay, we concluded that ROS are not involved in oleic acid-induced cell stress.

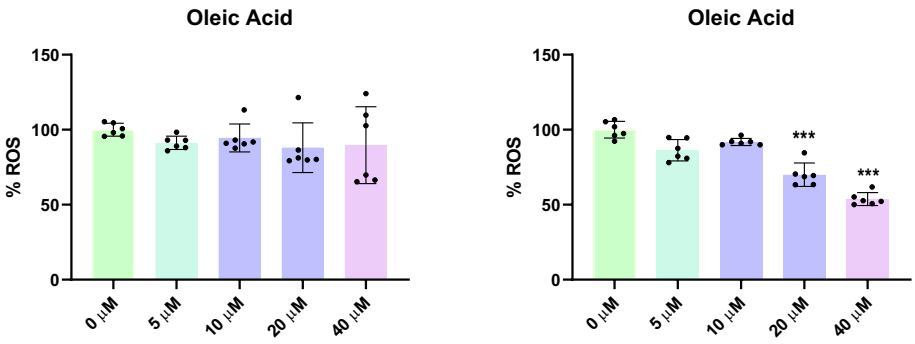

Figure 15. **Reactive oxygen species (ROS) levels following oleic acid treatment:** Bar graph data demonstrate that oleic acid treatment does not significantly elevate ROS levels in U87 cells, indicating that oleic acid-induced cell stress is not mediated by ROS.

ix. Sholl analysis

Sholl analysis helped quantify branching complexity by counting intersections at increasing radial distances from the cell body. The following is a set of representative images from the four treatments that were generated through ImageJ (using the SNT plugin).

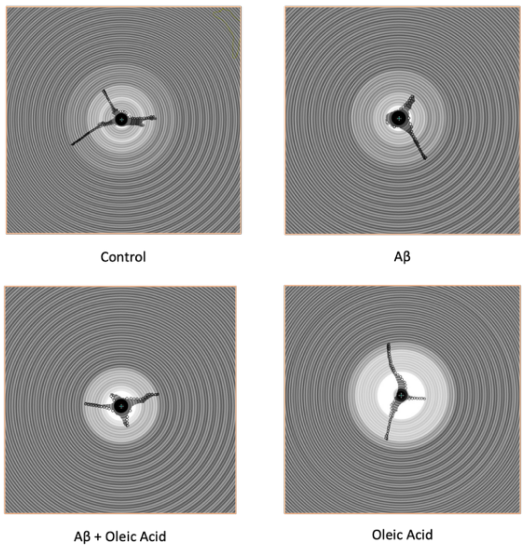

Figure 16. **Representative Sholl analysis images of astrocyte morphology under different treatments:** Images generated using the SNT plugin in ImageJ show the complexity and branching of astrocyte processes for each treatment group, as visualized by concentric circles and intersections.

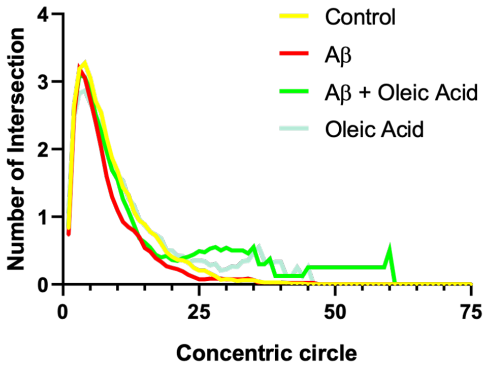

Figure 17. **Quantitative Sholl analysis of astrocyte process complexity:** The line graph depicts the number of intersections between astrocytic processes and concentric circles at increasing radial distances from the soma (start radius: 5 µm, step difference: 2 µm, end radius: 500 µm). Treatment with Aβ42 reduces branching complexity, while oleic acid alone or in combination with Aβ42 partially rescues or enhances branching.

Several conclusions were drawn, which are as follows:

a. Aβ-treated astrocytes observed reduced branching complexity, fewer intersections at intermediate distances (10-30 µm radius), suggesting Aβ induces simplified morphology via toxicity or retraction of processes.
b. In case of treatment with Aβ + Oleic Acid, partial restoration of branching compared to Aβ-only group, observed through increased intersections at 15-25 µm radius. This may be because of oleic acid's counteraction against Aβ-induced structural deficits through lipid-mediated neuroprotection.
c. Oleic acid alone, on the other hand, seeming enhanced branching vs control (especially at 20-35 µm), with maximum intersections shifted outward, indicating pro-growth effects of fatty acids on astrocyte morphology.

x. AC/AP ratio and cell projections

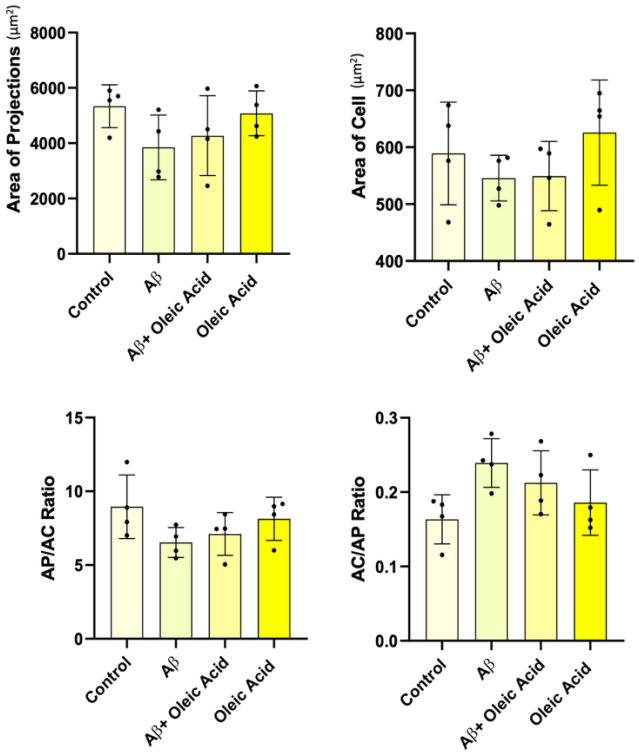

Figure 19. **Analysis of cell area, projection area, and AC/AP and AP/AC ratios in treated astrocytes:** Bar graphs illustrate differences in total cell area, projection area, the area of cell to projection (AC/AP) ratio and that of projection to cell (AP/AC) among treatment groups. Aβ42 increases the AC/AP ratio (indicative of process retraction), while oleic acid treatment decreases the ratio, suggesting process regeneration or stimulation.

The graphs produced as a result were drawn following Sholl analysis data, which observed the varying quantities of intersections at varying distances, allowing us to conclude the effects of treatment with Aβ and oleic acid individually, and with both at once. The results derived from the same are the following:

1. Aβ treatment shows reduced ratio, implying process retraction, and reduced branching complexity.
2. Aβ + oleic acid shows an increase in the ratio, implying partial process regeneration.
3. Treatment with oleic acid resulted in further increase in the ratio, implying that oleic acid may have dual roles:
    a. Rescue effect when the cells are also treated with Aβ; and

b. Stimulation effect when used alone.

Chapter 6

Conclusion

Following all the experiments, the key takeaways from this project are as follows:

1. Aβ42 colocalises with Na^+/K^+–ATPase α1 in astrocytes, suggesting direct physical interaction, supporting Aβ's ability to bind extra-cellularly to Na^+/K^+–ATPase, potentially disrupting ion transport.

2. Aβ42 reduces astrocyte viability, linked to Na^+/K^+–ATPase dysfunction, which in turn would cause Na^+ accumulation or impaired Na^+ removal triggering cell death (trigger glutamine synthetase, Na^+/Ca^{2+} exchanger).
    - As it is, elevated Na^+ triggers two critical downstream issues: a.) Na^+/Ca^{2+} exchanger (NCX) dysfunction: High Na^+ reverses NCX activity, forcing astrocytes to import Ca^{2+} instead of exporting it. This calcium overload damages mitochondria, causing oxidative stress and energy deficits. b.) Glutamine synthetase (GS) failure: GS requires ATP to convert glutamate to glutamine.

3. U87 cells uptake oleic acid independent of any BSA conjugate confirmed through Oil Red O' staining.
    - In cell culture experiments, conjugation of fatty acids with BSA before adding them to the culture medium helps mimic a natural system where oleic acid can be taken up by cells in bound state (Alsabeeh *et al*., 2018). This binding also ensures the fatty acid stays in solution and its efficient delivery, preventing the formation of insoluble droplets and allowing for controlled dosing.

- However, our results showed that U87 cells can take up free oleic acid directly, even when it is not bound to BSA. This is notable because it shows the cells are capable of absorbing oleic acid without needing the carrier protein, which is not always the case for all cell types or under all conditions.

4. ROS & cytotoxicity assays suggest oleic acid does not induce ROS or toxicity at ≤20 μM, confirming its potential as an Na^+/K^+–ATPase activator and safety as a therapeutic candidate.

5. Sholl analysis shows that oleic acid rescues Aβ42-induced branching loss through an increase in intersections at 15–25 μm.

6. AP/AC ratio shows that oleic acid reverses Aβ42-induced decrease in ratio (resulting from process retraction) and leads to an increase in the ratio when used alone.

Chapter 7

Future Prospects

Future prospects following the study focussed on 'Exploring Na^+/K^+–ATPase in Astrocytes to Uncover Therapeutic Insights into Dementia Pathophysiology' could look into studying cellular ion concentrations (through Na^+, Ca^{2+} imaging) and their impact on astrocytic activity through concentration changes. Levels of Na^+/K^+–ATPase upon treating U87 cells with Aβ42 +/– oleic acid could be studies to understand their functions and, therefore, the therapeutic implications better. Also, oleic acid is only one of the several activators that could be looked into, with some of the others being linoleic acid, insulin, thyroid hormone (T3), and dopamine. Since a change in the cytoplasmic $[Na^+]$ may affect NCX activity, ultimately leading to oxidative stress, etc., studying the instances of changes in NCX levels, understanding the role of astrocytes in neuroinflammation and neuroprotection better, and moving forward to test the effect of oleic acid in dementia models *in vivo* may pave way to a solution, allowing targeted interventions that could improve cognitive outcomes in affected individuals.

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