GUT MICROBIAL METABOLITE, SODIUM BUTYRATE REGULATES THE BLOOD-BRAIN BARRIER TRANSPORT AND INTRA-ENDOTHELIAL ACCUMULATION OF ALZHEIMER'S DISEASE AMYLOID-BETA PEPTIDES

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ABSTRACT

Alzheimer's disease (AD) is a common type of dementia observed in the elderly with brain amyloid beta (Aβ) deposits as one of its pathological hallmarks. Risk factors contributing to AD include age, genetics, inflammation, gut dysbiosis, and co-morbidities like diabetes, hypertension, and insulin resistance[1]. Recent studies have highlighted the necessity of investigating the combined effect of risk factors on AD onset and progression[2]. In addition, a majority of AD patients are diagnosed with cerebrovascular dysfunction, which is considered to be a significant contributor to the disease progression[3]. Moreover, the gut microbiome diversity was shown to be diminished in AD patients[4]. One of the interactions between the gut and the brain is mediated by gut microbial metabolites through the gut-brain axis[5]. Gut microbial metabolites include mainly short-chain fatty acids (acetate, propionate, butyrate) and trimethylamine N-oxide (TMAO)[6]. Particularly, butyrate treatment was shown to improve impaired cognition and reduce Aβ deposition in the AD brain, although the underlying mechanisms are yet to be characterized[7]. Previously, we reported the impact of insulin signaling on Aβ trafficking between the brain and the blood via the blood-brain barrier (BBB), which lines the cerebrovascular lumen and regulates Aβ levels in the brain[8]. However, the effect of gut microbiome metabolites on Aβ trafficking/accumulation at the BBB and endothelial insulin signaling remains unknown. In this study, we investigated the effect of one of the bacterial metabolites, sodium butyrate (NaBu), on Aβ accumulation at the BBB endothelium and the role of endothelial insulin signaling. The NaBu decreased Aβ40 with 6 h treatment and Aβ42 accumulation upon 2 h and 6 h treatments in BBB cell (hCMEC/D3) monolayers in vitro. Moreover, NaBu increased the phosphorylation of protein kinase B (PKB/AKT) and extracellular signal-regulated kinase (ERK) upon 6 h treatment. Inhibitor studies were conducted to evaluate if

NaBu effect on Aβ accumulation at the BBB is regulated by insulin signaling. Treatment with AKT inhibitor (MK2206) and NaBu increased Aβ42 accumulation compared to the NaBu alone treated group. Similarly, treatment with MEK inhibitor (trametinib) and NaBu increased Aβ42 accumulation compared to the NaBu-treated group. These findings suggest the involvement of AKT and ERK pathways in NaBu-mediated changes in Aβ42 accumulation at the BBB. Also, NaBu affects the expression of transporters and receptors at the BBB. The NaBu treatment increased permeability glycoprotein (P-gp) and decreased receptors for advanced glycated end products (RAGE) compared to the Aβ treated group. Further, studies need to be conducted to elucidate mechanisms underlying NaBu effect on the BBB endothelium in AD.

Keywords: Alzheimer's, Aβ, Blood-brain barrier, dysbiosis, sodium butyrate, Insulin signaling, P-gp, RAGE.

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1. INTRODUCTION

1.1 Alzheimer's disease

Alzheimer's disease is the most common dementia observed in the elderly above 65 years. The AD is an irreversible and progressive neurodegenerative disease[9], clinically characterized by the presence of parenchymal amyloid beta (Aβ) protein plaque deposition and intracellular hyperphosphorylated tau protein. In addition, it is marked by synapse loss, neuronal dystrophy, vascular alterations, etc. Aβ is produced by the proteolytic cleavage of β-amyloid precursor protein (APP) by β and γ secretases through the amyloidogenic pathway[10]. Major isoforms of Aβ include Aβ40 and Aβ42 with Aβ42 dominant in toxic plaque deposition. Based on the onset age and cause of the disease, it is mainly categorized as early onset (familial) and late-onset (sporadic)[11].

i. **Early onset AD**:

Early onset AD is observed in younger individuals with genetic mutations in (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) genes inherited in an autosomal-dominant manner which constitutes 5%[12], [1,13]. The APP is cleaved by PSEN1, and PSEN2 at different regions to produce β-amyloid protein which is majorly deposited in the AD brain. Changes in the genetic sequence or expression of these genes may elevate the risk of disease by increasing the production and aggregation of amyloid beta (Aβ)[14]. APOE4 mutation showed increased accumulation of iron and fibrinogen along with

a decrease in Aβ clearance in AD patient brains[15]. Also, it is associated with BBB disruption[16]. This data suggests that in AD, factors that promote the production of Aβ and reduce its clearance are inherently heightened with acquired genetic mutations from AD affected individuals, rendering them more susceptible to the disease.

ii. Late-onset:

Late-onset AD is observed in adults above 65 years and accounts for 95% of total AD cases. It is believed to result from a combination of events like genetics, environment, lifestyle (gut dysbiosis, co-morbidities-hypertension, diabetes mellitus, obesity), and exposure (cerebrovascular disease, injury). Genome-wide association studies reveal that the genes associated with high risk of AD include Apolipoprotein (APOE) ε4, ε3 isoforms, ABCA7, MS4A6E, TREM2 R47H, Bridging integrator 1 (BIN1), Phosphatidylinositol binding clathrin assembly protein (PICALM). The APOE promotes the clearance of Aβ by coordinating with the LRP1 efflux transporter and is also involved in lipoprotein metabolism[13]. The ABCA7 and the MS4A6E messenger RNA expression correlated with plaque burden and cognitive decline in AD brain[17]. The TREM carriers have increased brain atrophy and correlated with cerebrospinal fluid tau levels compared to control subjects[3]. The BIN1 is involved in endocytosis and trafficking, immune response and single nucleotide polymorph causes dysregulated

APP processing[13]. The PICALM is a part of clathrin assembly mediating lipid endocytosis and its overexpression increased plaque burden in AD transgenic mice[12,18]. Recent advancements in genomic sequencing and bioinformatic analysis allow us to assess the impact caused by these various risk factors on AD onset, severity, and progression.

1.2 Blood-brain barrier Dysfunction in Alzheimer's

The blood-brain barrier (BBB) endothelium is at the interface between the brain parenchyma and plasma. The BBB endothelium works in coordination with pericytes, astrocytes, and neurons to regulate neurovascular regulation and function. The BBB endothelial cells are interconnected with tight junctions and junctional adhesion molecules like claudin, occluding, zona occluding (ZO), and vascular endothelial cadherin (VE-Cadherin) which prevent paracellular transport of substances microbes, microbial peptides, toxins, and xenobiotics[19] (Figure 1). Alternatively, the transport of small nutrients like glucose, oxygen, and amino acids is carried out through a network of polarized transporters as well as the delivery of large endogenous molecules such as lipoproteins and insulin via carrier-mediated endocytosis[20]. Pericytes provide structural support to endothelial cells and assist in controlling blood flow by regulating capillary diameter. Functionally, it helps to phagocyte toxic substances and protects against apoptosis[19]. Loss or injury of pericytes is reported in many neurological diseases including AD[21–23]. Astrocytes are crucial in maintaining ionic homeostasis, pH regulation, neurotransmitter uptake, synaptic activity, etc[24]. Tight junctions like claudin, occludin, and junctional adhesion molecules (VE-

Cadherin), with cytoplasmic proteins zona occludens-1, 2, 3, etc., are connected to cell cytoskeleton actin to maintain the integrity of the barrier[19].

In majority of the AD patients, the BBB is compromised by the loss of pericytes, disruption of astrocytes, tight junctions, and adherent junctions resulting in the infiltration of inflammatory cytokines, immune cells, and microbes into the brain[24]. These conditions aggravate amyloid beta deposition, formation of neurofibrillary tangles, inflammation, neurodegeneration, and ultimately cognitive decline. A decrease in glucose transport, and downregulation of glucose transporter was reported[20]. Aβ accumulation increases the activation of microglia, expression of vascular adhesion proteins, and release of inflammatory cytokines like TNF-, IL-1, and IL-6 nitric oxide synthetase (NOS) which disrupts BBB integrity[25]. In addition, Aβ accumulation disrupts the synthesis and degradation of collagen IV, a basement membrane component, and leads to the loss of microvascular integrity, resulting in microhemorrhages. The Aβ deposits decrease tight junction proteins such as claudin-5 and occludin in endothelial cells and inhibiting RAGE ameliorated the Aβ effect, indicating the role of RAGE in facilitating Aβ deposition[26]. These literature reports suggest that cerebrovascular dysfunction is a leading contributor for AD exacerbation and could serve as major biomarker in disease prognosis.

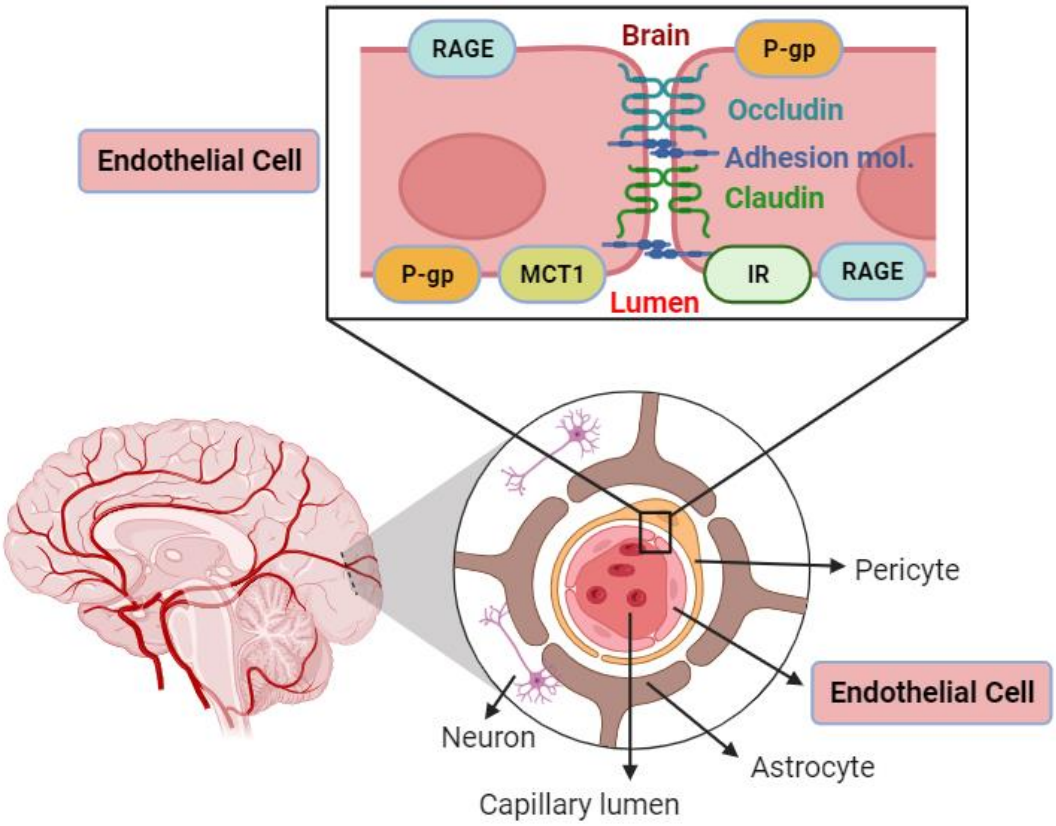


Figure 1: Blood Blood-brain barrier (BBB) structure. BBB is the physiological barrier separating blood from the brain parenchyma. The BBB is primarily made of polarized endothelial cell monolayer, which works in coordination with pericytes, astrocytes, and neurons. Endothelial cells are connected with tight junctions and adherent junctions. These junctions are regulated by tight junction proteins (claudin, occludin), cytoplasmic accessory proteins (zona occludens-1, 2, 3, cingulin), adherent junctional protein (vascular endothelial cadherin, VE-Cadherin) connected to the actin cytoskeleton[19]. The luminal side of the BBB endothelium expresses efflux transporters such as P-gp, which contributes to the barrier integrity of the BBB. In addition, the BBB endothelium has transporters and

receptors and transporters to transport solutes between plasma and the brain selectively. Created with BioRender.com.

1.3 Risk Factors

The most common risk factors for Alzheimer's include old age, genetic mutations, gut dysbiosis, insulin resistance, and inflammation. Moreover, recent evidence from epidemiological and clinical studies suggests metabolic syndrome as one of the major risk factor factors for AD. [27–30]

1.3.1. Age, Genetics

Primarily, the population above 65 years is highly prone to Alzheimer's. Aging causes several physiological changes in the body including genetic susceptibility, decreased immune response, increased inflammation, endothelial dysfunction, and decreased gut microbiome diversity. Changes in DNA methylation is highly observed in the elderly and are correlated with age and cognitive decline. Histone acetylation declined in human and mouse models with age and inhibiting it restored cognitive deficits[31,32]. Brain aging is also characterized by altered signaling and senescent organelles[33].

Genetic factors play a role in the inheritance of both early and late-onset AD. Techniques to understand genetic susceptibility include- Next-generation sequencing and genome-wide association studies. These studies

revealed the genes associated with processes like changes in β-amyloid precursor protein (APP), lipid metabolism (ABCA7), immune regulation (CR1, CD33, MS4A, TREM2), etc[13,34]. The next prominent factor is the inheritance of mutated APP, PSEN1, PSEN2, and APOE ε4, ε4 genes[12].

1.3.2 Dysbiosis

The human gut is the residence of thousands of microbial species with 7000 strains collectively called gut microbiome. Firmicutes and Bacteroidetes are dominant bacterial families[35]. Firmicutes have gram-positive and negative species and Bacteroidetes comprises gram-negative bacteria[36]. The gut microbiome is widely known to be involved in the digestion of ingested fibers in the colon. The gut microbiome has been acknowledged to regulate intestinal and BBB integrity and promote anti-inflammatory as well as antioxidant properties. Increase in *Verrucomicrobia,* and *Akkermansia* are correlated with reduced inflammation and metabolic dysfunction[33,37]. In addition, the gut microbiota impacts various gut functions including digestion of carbohydrates, vitamins, neurotransmitter synthesis, xenobiotic metabolism, and strengthening barrier function by releasing metabolites into the systemic circulation to maintain the gut-brain axis[5]. Imbalance in the diversity and proportion of microbiome due to diet, sedentary lifestyle, sleep deprivation, and oxidative stress can result in dysbiosis[37]. A recent study has demonstrated significant differences in the gut microbiome of

younger and elderly Indonesians. Major species in youngsters were *Bifidobacterium* (acetic and lactic acids), *Bacteroides* (acetic acid and propionic acid), *Clostridium* (short chain fatty acids)[38], *and Prevotella* (linked to glucose and insulin sensitivity along with inflammation[39]) and elderly were *Enterobacteriaceae* (inflammation, bile acid dysmetabolism)[40] and *Escherichia coli* (vitamin B1, B2, K)[41]. This indicates the role of age in modulating the gut microbiome species.

1.3.2.1Gut-Brain axis

The gut-brain axis is a bi-directional pathway through which the gut and brain communicate. The gut is controlled by an independent system of neuronal circuitry called the enteric nervous system. The brain also regulates the gut function (motility, mucus production, etc.) via sympathetic or parasympathetic signaling[5]. The gut modulates the brain function by synthesizing and releasing hormones, neurotransmitters/modulators like serotonin, dopamine, and microbial metabolites like short-chain fatty acids into circulation. These neurotransmitters like serotonin, acetylcholine, and noradrenaline regulate sleep and cognition. The activity of other metabolites on CNS is listed in **Table 1**[6,42]. A Healthy gut promotes the release of antioxidative metabolites, whereas, in dysbiosis, inflammatory cytokine production is upregulated leading to disruptive barrier function, which is prevalent in neurodegenerative diseases. Studies conducted in germ-free

mice have implicated the role of gut microbiome in the early brain development of the offspring and its impact on neurogenesis at the later stages[43].

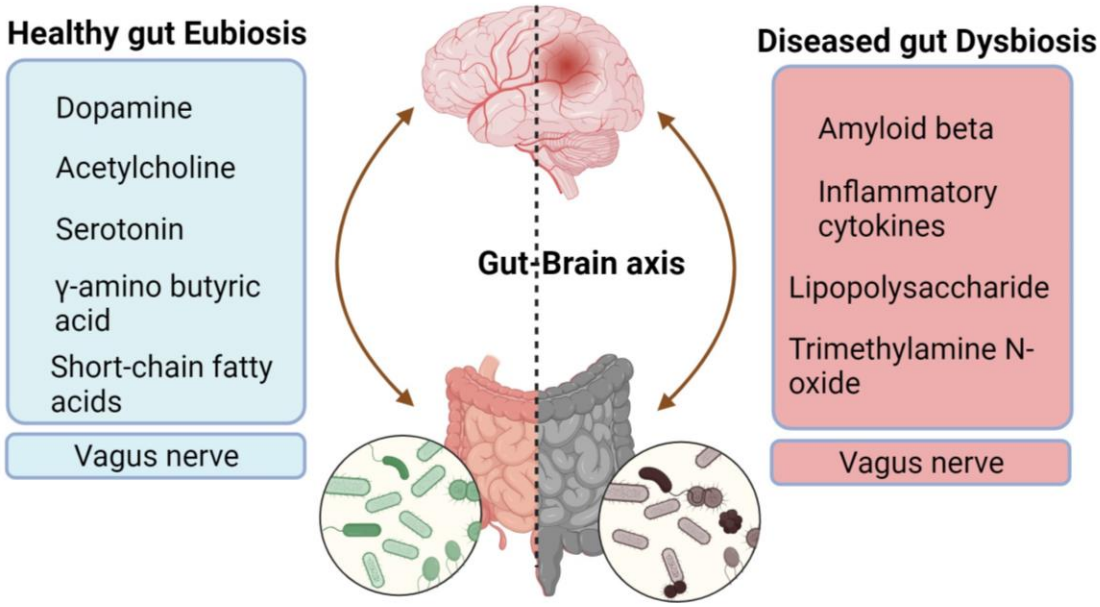


Figure 2: Representation of the Gut-Brain axis in health and disease. The gut and brain exert an effect on each other by direct neural connection, endocrine mediators, and immune mediators via a bi-directional pathway called the Gut-Brain axis (GBA). In the healthy individual, a diverse microbiome (Eubiosis) promotes the release of dopamine, acetylcholine, serotonin, GABA, and SCFA into the systemic circulation, thus enhancing strong intestinal and brain barriers along with a role in sleep, cognition, etc. However, in pathological conditions, the diversity of the gut microbiome is altered promoting the release of amyloid protein, curli amyloid, inflammatory cytokines, lipopolysaccharide

(LPS), trimethyl amine N-oxide (TMAO) into the blood causing leaky gut barrier, dysfunctional BBB, inflammation.

1.3.2.2 Gut microbial metabolites

The gut microbiome in a healthy individual is diverse and is in symbiosis with the host (eubiosis). The eubiotic microbiome synthesizes various neurotransmitters (Acetylcholine, noradrenaline, gamma amino butyric acid, serotonin), vitamins (K2, B1-3, B5-7, B9, B12), bacterial metabolites (short chain fatty acids, SCFA). However, a dysbiotic microbiome produces harmful substances such as inflammatory cytokines, bacterial amyloids, and endotoxins such as LPS. As mentioned earlier, neurotransmitters regulate CNS responses. The SCFAs like acetate, propionate, and butyrate are produced by the fermentation of ingested fibers by anaerobic bacteria. They play a crucial role in maintaining gut barrier integrity, immune modulation, weight control, etc.[44]. Table 1 shows information about gut microbial species, their metabolites, and their effects on the CNS.

The gut microbiome diversity is decreased under pathological conditions engendered by chronic infections, inflammation, prolonged antibiotic usage, stress, imbalanced diet, chronic intestinal conditions like irritable bowel syndrome, and obesity. In these conditions, the proportion of the microbiome that produces inflammatory cytokines, bacterial amyloid curli, lipopolysaccharide, trimethylamine N-oxide (TMAO), bile acids, and

tryptophan derivatives is increased. Lipopolysaccharide (LPS) is the component of bacterial cell wall in gram-negative bacteria which acts as endotoxin[45]. An increase in the gram-negative bacterial population proportionally increases the LPS in the gut and in the blood, thus promoting systemic inflammation, damage to blood vessels, and septic shock [2,46–48]. The LPS causes elevated brain cytokine levels and disruption of the BBB. TMAO is produced by the fermentation of choline-rich food by the gut microbiome and further transformation in the liver. Elevated levels of TMAO were shown to correlate with an increased risk of cardiac problems, and endothelial inflammation. Bacterial amyloid curli, produced by *Salmonella enterica, and Bacillus subtilis*[49,50] was shown to upregulate amyloid production in the brain neurons[37]. Also, it cross-seeds other amyloid produced in the gut to form β secondary structure mimicking the brain Aβ. High levels of Aβ40, 42 were reported in the gut in the endothelial cells[51–53]. Elevated amyloid burden was reported to initiate pro-inflammatory markers production with an increase in reactive oxygen species levels [54,47,48,55].

Table 1: Gut microbiome species, metabolites, and CNS effect

Gut microbiome	Metabolites	CNS effect	References
Bacteroides, Prevotella	Lipopolysaccharide (LPS)	Promotes pro-inflammatory transcription factor NFκB, in AD progression in microglial cells	[45]
Bacillus, Escherichia, Lactobacillus, Lactococcus, and Streptococcus	Dopamine	Neurotransmitter controls cognition, emotion, locomotion	[56,57]
Escherichia, Enterococcus, Lactobacillus, and Streptococcus	Serotonin	Regulates sleep, cognition	[6]
Lactobacillus and Bacillus	Acetylcholine	Plays role in cognition, attention, involuntary muscle movement	[42]
Bacillus spp.	Noradrenaline/ Norepinephrine	Regulates cognition, stress, attention, and arousal reactions	[58]
Bacteroides, Bifidobacterium, Propionibacterium, Eubacterium, Lactobacillus, Roseburia, Prevotella	Short-chain fatty acids	Promotes synthesis and secretion of neurotransmitters, increases BBB permeability	[6,58]
Lactobacillus, Lactococcus, Streptococcus, and Enterococcus	Histamine	Regulates sleep, cognition, feeding, and energy	[59]
Bifidobacterium and Lactobacillus	γ-aminobutyric acid (GABA)	Inhibitory neurotransmitters required to regulate neuronal activity	[60]

1.3.2.3 Association of AD and Dysbiosis

Recently many studies have shown the importance of healthy gut microbiomes in maintaining brain health. This led to the exploration of microbial species and metabolite relationships in co-morbidities like obesity, diabetes, and cardiovascular disease as they are established risk factors for sporadic AD[36,5]. They led to a renewed focus on investigating the difference in the microbiome of AD vs non-AD subjects[61,62]. Efforts to establish causality are being considered by perturbing the microbiome in AD transgenic mouse models, such as APP/PS1 mice, and investigating alterations in AD pathological features. Such studies have shown that in APP/PS1 mice, a decrease in Aβ plaque deposition and inflammation in the brain were observed following antibiotics and prebiotics administration respectively[63,64]. *Bifidobacterium* improved memory deficits induced by Aβ administration in AD rat model[65]. *Enterobacteria* infection had increased innate gut inflammation in the drosophila AD model[66]. perturbations in the enteric nervous system which regulates the gut have been shown to enhance Aβ accumulation and neuronal loss in APP transgenic mice which makes it more susceptible to inflammation[67]. This suggests the indirect relation between the gut and the brain. Apart from animal studies, even clinical data has shown the correlation between gut microbial composition and AD pathology. In post-mortem AD brains, elevated levels of bacterial LPS were co-localized with Aβ plaque depositions[68]. In live AD patients, a decrease

in *Firmicutes, Eubacterium, Bifidobacterium* and an increase in *Bacteriodetes, Proteobacteria,* etc., were observed[69]. Moreover, an increase in *Porphyromonas gingivalis* is correlated with elevated Aβ plaques and neurofibrillary tangles [70].

The prevalence of dementia and relevant risk factors were compared among various populations. The incidence of dementia is higher in the Japanese-Brazilian elderly population compared to the native Japanese. Japanese have high levels of n-3 polyunsaturated fatty acids (PUFA) in their plasma which inversely correlates with dementia cases[71]. Consequently, PUFA has been linked to a decline in dementia cases attributed to its anti-inflammatory properties. However, closer examination of the intricate details show that PUFA increases the abundance of butyrate-producing bacteria like *Bifidobacterium, Lachnospira, Lactobacillus*, etc. thereby elevating the butyrate levels in the plasma, a phenomenon often overlooked[72]. Also, butyrate enhances insulin sensitivity in mice[73]. These broad-based findings emphasize the complex interplay between gut microbiome and brain health, thus opening opportunities for potential therapeutic interventions in the prevention and management of AD.

1.3.3 Insulin Resistance

According to the Rotterdam study, diabetes nearly doubles the risk of dementia and AD[4]. Also, dementia was strongly associated with increased body mass index or

abdominal obesity with altered neural tissue and decreased blood perfusion to the brain[74–76]. Insulin plays a critical role in metabolic homeostasis by regulating glucose, energy, and fatty acid synthesis. Insulin levels in the brain and plasma are correlated indicating the derivatization of insulin from blood circulation. Peripherally, a decrease in insulin sensitivity leads to elevated blood glucose levels called insulin resistance[77]. Similarly, brain insulin resistance could be due to the incapability of brain cells to respond to insulin either due to downregulation or reduced binding to the receptor[78]. Insulin and insulin-like growth hormone signaling pathways regulate Aβ homeostasis in the brain[9]. Insulin resistance is implicated in augmenting Aβ deposition and Aβ exposure has been shown to worsen insulin resistance[8,79]. Increased accumulation of peripheral adipose tissue releases adipokines and inflammatory cytokines to systemic circulation which enter the brain and lead to insulin resistance and perturbed lipid metabolism resulting in Aβ accumulation and plaque formation[80].

Amyloid beta proteins were shown to decrease cell surface insulin receptors and contribute to insulin resistance[81]. Insulin signaling via MEK and ERK pathways was shown to alter amyloid beta production, clearance, and accumulation in the cells[82]. Insulin degrading enzyme (IDE) degrades Aβ, however, its levels are decreased under insulin resistance leading to higher Aβ accumulation[83]. Insulin resistance and high blood glucose levels can induce inflammation[84]. The intricate role of insulin, Aβ homeostasis and neurodegenerative processes emphasizes the

importance of addressing lifestyle, body weight, and body mass to maintain optimal brain health.

1.3.4 Cerebrovascular Inflammation

The cerebrovascular unit is crucial in maintaining brain homeostasis by regulating the access of pro and anti-inflammatory mediators along with other transport and signaling functions. Elevation of pro-inflammatory cytokines in brain injury or in other conditions can be a potential risk factor for several neurological disorders like AD, and Parkinson's disease[85]. Inflammatory cytokines are found to be co-localized with amyloid beta plaques and are believed to be closely associated with AD pathology[86]. In addition, inflammatory cytokines are known to increase Aβ load by increasing the APP production[87], and tau hyper-phosphorylation by up-regulating CDK5 activation via IL-6[46]. Microbiota influences microglia maturation in germ-free mice potentially through short-chain fatty acids. Similarly, other metabolites from tryptophan metabolism can modulate inflammation in the brain and periphery[5]. Recently in AD mouse studies, Aβ was identified as a protective antimicrobial peptide[88,89], which is it turned disruptive in a dysregulated state resulting in inflammation[88,89]. Amyloid beta plaques activate the surrounding microglia and stimulate the release of inflammatory cytokines through astrocytes. Amyloid beta also activates nuclear factor-κB (NF- κB) to enhance the transcription of inflammatory molecules[25]. Production and deposition of amyloid plaques upregulate the production of inflammatory factor, TNF alpha that exacerbates BBB

disruption [90]. The SCFAs produce anti-inflammatory cytokines like interleukin-10 and repress NF-κB[91] thereby decreasing inflammation. Understanding the relationship between microbial metabolites, Aβ and inflammatory responses provides valuable insights into the potential therapeutic targets for mitigating neuroinflammation in AD.

1.4 Effect of Short-chain Fatty Acid- Butyrate in AD

The SFCAs are some of the immunomodulatory bacterial metabolites secreted by gram-positive anaerobic bacteria belonging to *Firmicutes like clostridial* clusters[7], *Bifidobacterium,* and *Lactobacillus*[92]. Additionally, they are produced by the bacterial anaerobic fermentation of dietary fiber[7]. Short-chain fatty acids mainly constitute acetate, propionate, and butyrate in the proportion of 3:1:1[93]. Early literature suggested the critical role of butyrate in the body, and significant research is being carried out to investigate its role in health and disease states. The majority of the butyrate produced in the gut serves as the main source of energy for the colonocytes[94]. Butyrate is transported by free fatty acid receptors (FFARs), monocarboxylate transporter 1 (MCT1), and sodium-coupled transporters (SMCT1), in gut epithelium and BBB endothelial cells[95,96]. It promotes gut barrier integrity by increasing tight junction proteins. Moreover, it decreases reactive oxygen species and other inflammatory markers in the blood[97]. Through the gut-brain axis butyrate improves the blood-brain barrier integrity by increasing the expression of tight junction proteins like claudin, ZO. It also protects cells from Aβ toxicity and improves cognition in AD mouse models[98]. Moreover, butyrate producing species *Faecalibacterium* abundance is significantly lowered in AD patient's feces compared to

controls which was in turn positively correlated with cognitive decline and disease progression scores[99]. Additionally, it is also known for its Histone Deacetylase (HDAC) inhibition properties *in-vitro* and *in-vivo*. It increases the acetylation of histone H4, which improves cognitive function in AD APP/PS1 mice[32,100]. Butyrate effect in suppressing NF-κB activation and upregulating brain-derived neurotrophic factor (BDNF) might be modulated through HDAC inhibition[101]. On the other hand, the microbiome which produces butyrate ameliorated major AD risk factors such as cerebrovascular dysfunction, metabolic disorders[102,103]. Butyrate is showing protective effect on human brain microvascular endothelial cells by restoring heme oxygenase 2 levels in cerebrovascular dysfunction[102]. Also, butyrate producing microbiota including *R.intestinalis, P.distasonis, B.fragilis* were significantly down regulated in the metabolic syndrome patients, i.e patients with co-morbidities such as hypertension, obesity, diabetes[103]. Although there is information available regarding the role of butyrate in maintaining a healthy BBB and ameliorating the AD symptoms and biomarkers, a definitive mechanism of action has not been established. So, my study aims to elucidate the underlying mechanisms[95].

1.5 Hypothesis

The central hypothesis of the thesis is that gut microbial metabolite, butyrate decreases the accumulation of Aβ42 peptides at the BBB endothelium by modulating the insulin signaling pathway.

1.6 Specific aim

The hypothesis was tested by the following specific aims:

Specific aim 1: Determine the effect of butyrate on the accumulation of Aβ42 peptides at the BBB using cell culture models in-vitro.

Specific aim 2: Investigate the effect of butyrate on insulin signaling pathway constituents that regulate Aβ42 peptide accumulation in polarized BBB endothelial monolayers in-vitro.

1.7 Scientific rationale

In this study, we investigated the mechanism of action of butyrate on amyloid beta accumulation in BBB endothelial cell culture models- human cerebral microvascular endothelial cell (hCMEC/D3) and primary bovine brain microvascular endothelial cell (BBME). Butyrate-producing bacteria is significantly decreased in AD and inoculating butyrate producers in the gut ameliorated plaque deposition, and improved memory deficit in mouse models[5,43]. However, studies also reported no effect or even an adverse effect with butyrate treatment[104]. This suggests the need for further clarity on the mechanism of action of butyrate. Thus, in this study, we investigated the effect of butyrate in modulating Aβ42 accumulation in BBB endothelium.

2. Materials and Methods

2.1 Cell culture:

The Immortalized human cerebral microvascular endothelial cell line (hCMEC/D3) was gifted by P-O Couraud (Institute Cochin, France). The cells were cultured using the procedure previously described (Daniels et al.[105]). Briefly, endothelial cell basal medium (Cell Applications, San Diego, CA) supplemented with 1% v/v lipid concentrate (Thermo Fisher Scientific, Waltham, MA), 1% v/v penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO), 1 ng/mL recombinant human fibroblast growth factor-basic (PeproTech, Rocky Hill, NJ), 1.4 μM hydrocortisone reconstituted in ethanol (Sigma-Aldrich, St. Louis, MO), 5 μg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO), 10 mM HEPES (Millipore Sigma, St. Louis, MO), and 1%, 5% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA) was used to culture the cells. Hereafter, the supplemented medium is referred to as D3 media. The hCMEC/D3 cells used in the studies were of passage# 35 and were grown as polarized monolayers in coverslip-bottomed dishes and cell culture plates coated with collagen (5 μg/ Cm^2) (Thermo Fischer Scientific, Waltham, MA).

Primary bovine brain microvascular endothelial (BBME) cells were acquired from Cell Applications Inc. (San Diego, CA). The cells were cultured on 6-well plates coated with collagen (5 μg/ Cm^2) (Thermo Fischer Scientific, Waltham, MA) followed by 0.01% bovine fibronectin until >90% confluency is attained. The BBME cells were grown in DMEM/F12 medium (Cellgro, Corning, NY) supplemented with donor horse serum

(Cellgro, Corning, NY), gentamycin (25 mg) (Cellgro, Corning, NY), sigma heparin sodium salt (50 mg) (Sigma-Aldrich, St. Louis, MO).

2.2 Aβ40/42 film preparation and reconstitution

The FITC- labeled Aβ40, unlabeled Aβ40, FITC-Aβ42, or unlabeled Aβ42 were supplied by Aapptec (Louisville, KY). The purity of FITC-Aβ40/42, and Aβ42 peptides were 95.21%, 96.52%, and 96.10% respectively as analyzed by liquid chromatography-mass spectrometry. Monomers for each peptide were prepared as previously described by Klein (RI). Briefly, each peptide was resuspended in chilled 1,1,1,3,3,3, -hexafluoro-2-propanol (HFIP) (Sigma-Aldrich, St. Louis, MO) in a chemical fume hood. It was allowed to dissolve at room temperature for 1 h. Later, the peptide solutions were chilled over ice for 30 minutes to prevent evaporation of HFIP during aliquoting. Clear peptide solution was aliquoted into glass vials and dried overnight in the fume hood. The film thus formed was dried under vacuum and stored at -20 ^{o}C over desiccant. The Aβ film was dissolved in DMSO and F-12 (R&D systems, McKinley Place NE, Minneapolis) before the experiment. Later, the Aβ solution was centrifuged for 5 min at 6000 rpm to eliminate high molecular species[106]. The supernatant was then reconstituted in Dulbecco's Modified Eagle Medium, DMEM (Gibco, Grand Island, NY) and treated with cells for 30 min. In the cell culture experiments, precautions were taken to not expose the cells above 0.25% DMSO contained in Aβ solutions.

2.3 Membrane fractionation

The hCMEC/D3 cells were grown in the culture dishes (CELLTREAT scientific products, Pepperell, MA) containing D3 media until they were fully confluent. The day before the experiment, the medium was changed to a low serum D3 medium (1% FBS in D3 medium). The next day, cells were treated with sodium butyrate (NaBu) in D3 media in a low serum medium (1% FBS) for 6 h. Subsequently, the cells were treated with NaBu and Aβ42 (12.5μg/ml) in DMEM for 1 h. After treatment, cells were washed 3 times with ice-cold PBS. Plates were scraped with PBS (1 ml/*3) and lysates were collected into a 15 ml falcon tube. The resultant cell suspension was centrifuged for 5 min at 1200 rpm (Thermo Scientific IEC CL40). Later, the supernatant was aspirated and the pellet was processed for membrane protein isolation (Minute™ Plasma Membrane Protein Isolation and Cell Fractionation Kit, Invent Biotechnologies, Eden Prairie, MN). The pellet was resuspended in the lysis buffer supplemented with RIPA buffer, Protease Inhibitor Cocktail 100x (Sigma-Aldrich, St. Louis, MO), phosphatase inhibitor cocktail 100x (Santa Cruz, Dallas, TX), and Pierce™ nuclease 100x (Thermo Fischer Scientific, Waltham, MA). The cell suspension was filtered through a cartridge and vortexed to separate nuclei. The supernatant was collected and centrifuged for 1 h at 16000*g at 4 ^{o}C to separate the cytosol fraction (supernatant) from the total membrane (pellet). The pellet was dissolved in a Minute denaturing protein solubilization reagent along with a protease and phosphatase inhibitor cocktail. The protein concentration of the total membrane fraction was determined using a Pierce^{TM} BCA assay protein kit (Thermo Fischer Scientific, Waltham, MA). A10-20ug protein aliquot was loaded on the gel for western blot.

2.4 Flow cytometry

The hCMEC/D3 cells were cultured in 6 well plates with 5% D3 media until >95% confluency was attained. The day before the experiment, cells were treated with low serum (1% FBS) D3 medium. The next day, cells were treated with AKT inhibitor, MK2206 (10 μM) or MEK inhibitor, trametinib (0.5 μM), and sodium butyrate (NaBu) in D3 media with 1% FBS for 6 h. Subsequently, the cells were treated with inhibitors, NaBu and Aβ42 in colorless DMEM for 30 minutes. After treatment, cells were washed with chilled PBS. Later, they were trypsinized for 3 min at 37 ^{o}C and quenched with FBS. Then the cell suspension was diluted with chilled PBS and centrifuged for 5 min at 1200 rpm. Later, the cells were resuspended in PBS, followed by fixing with 4% Paraformaldehyde (PFA)[107]. Cells were gently vortexed and analyzed with a Flow cytometer (Laser source: 100 mW Blue, 488 nm, Excitation: 495 nm Emission: 519 nm), and the results were analyzed using FlowJo v10.0.

2.5 Western blot

Whole-cell lysates (25 ug) and total membrane protein (20 ug) were loaded on 4–12% Criterion™ XT Bis-Tris protein gel (Bio-Rad Laboratories, Hercules, CA) with 20x, XT MOPS running buffer (Bio-Rad, Hercules, CA) for 1.5 h. Later, the gel was transferred to nitrocellulose membrane at 100 V for 30 min using methanol, deionized water, and tris/glycine buffer (Bio-Rad, Hercules, CA) in the ratio of 2:7:1. The membrane was blocked for one hour with 5% v/v blotting grade blocker (Bio-Rad, Hercules, CA) in tris buffered saline (TBS) containing Tween20 (0.1%) (TBST) (Bio-Rad, Hercules, CA). Then, the

membrane was washed with TBST and was incubated overnight at 4 ^{o}C with one of the primary antibodies [MCT1/SLC16A1, P-AKT/Ser473, MDR1/ABCB1/P-glycoprotein, Phospho-ERK1/2, GAPDH (Cell Signaling Technology, Danvers, MA), or receptor for advanced glycation end products (RAGE) (Abcam, Cambridge, MA)] diluted with 5% v/v BSA (Sigma-Aldrich, St. Louis, MO). Antibodies were diluted with 5% BSA in TBST at 1:1000 dilution. The following day, the membrane was washed with TBST and incubated for 1 h at room temperature with a secondary antibody conjugated with near-infrared (800 nm) (Licor; Lincoln, NE) at 1:2000 dilution. Following incubation, the membrane was washed with TBST and TBS and imaged using an Odyssey Licor imager. Protein bands were quantified using Image Studio Lite 5.2. and normalized, with loading protein concentration, GAPDH, or calnexin.

2.6 Statistical analysis

All statistical analyses were performed using GraphPad Prism software (version 10.0). Multiple groups were compared using one-way ANOVA followed by Bonferroni post-test and the differences in the means of the two groups were compared using student T-test. The level of significance is indicated as follows: *p-value<0.05, **p-value<0.01, ***p<0.001.

3.0 RESULTS

3.1 Sodium Butyrate (NaBu) Effect on BBB:

3.1.1 Decreased Aβ40 or Aβ42 accumulation in hCMEC/D3 cells.

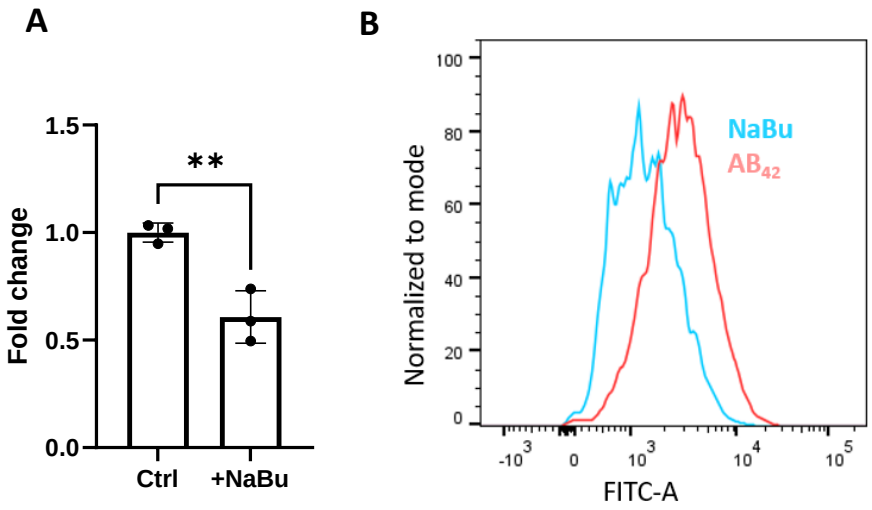
The hCMEC/D3 cells were pre-treated with NaBu and co-incubated with FITC-Aβ40 or Aβ42. Later, cells were processed for flow cytometry as previously described (Wang et, al[107]). Fold change in intracellular accumulation was calculated by dividing butyrate-treated groups with untreated control, FITC-Aβ group. Fold changes of median cellular fluorescence from three replicates in each group were compared. Flow results (Table 2) indicate a significant decrease in FITC-Aβ40 accumulation upon 6 h pretreatment with NaBu (0.89 ± 0.05) compared to the FITC-Aβ40 control group (1 ± 0.02). Other groups with 2 h and 24 h pretreatment have shown no significant difference in accumulation compared to the control group. The FITC-Aβ42 accumulation significantly decreased with 2 h NaBu treatment (0.5 ± 0.04) compared to the FITC-Aβ42 control group (1 ± 0.15). The accumulation of FITC-Aβ42 was decreased compared to the control (1 ± 0.15) after 6 h pre-treatment (0.56 ± 0.14) and no effect was observed with 24 h NaBu pretreatment (**p-value<0.01, one-way ANOVA followed by Bonferroni multiple comparison test).

		Control	2-hr	6-hr	24-hr
Butyrate	F-$A\beta_{40}$ (Mean ± S. D)	1.00±0.02	1.05±0.04	**0.89±0.05***	0.93±0.03
	F-$A\beta_{42}$ (Mean ± S.D)	1.00±0.15	**0.5±0.1****	**0.56±0.14****	0.9±0.1

Table 2: FITC-Aβ40 or Aβ42 accumulation in hCMEC/D3 cells. Cells were treated with 10 µM NaBu for 2 h, 6 h, and 24 h followed by 1 h co-incubation with FITC-Aβ40 (25 µg/ml) or FITC- Aβ42 (12.5 µg/ml). The fold change of median cellular fluorescence from three replicates was assessed by flow cytometry. The standard deviation of fold change is also represented in the above table. *p-value<0.05, **p-value<0.01, One-way ANOVA followed by Bonferroni multiple comparison test.

3.1.2 Decreased Aβ42 accumulation in bovine primary cells.

Bovine primary microvascular endothelial cells were pretreated with NaBu for 6 h followed by 30 min co-incubation with FITC-Aβ42 in DMEM. Fold change of median cellular fluorescence from three replicates were compared. The NaBu treatment decreased FITC-Aβ42 (0.6 ± 0.12) compared to the control (1 ± 0.05) as shown in Figure 3 (**p-value<0.01, student t-test).



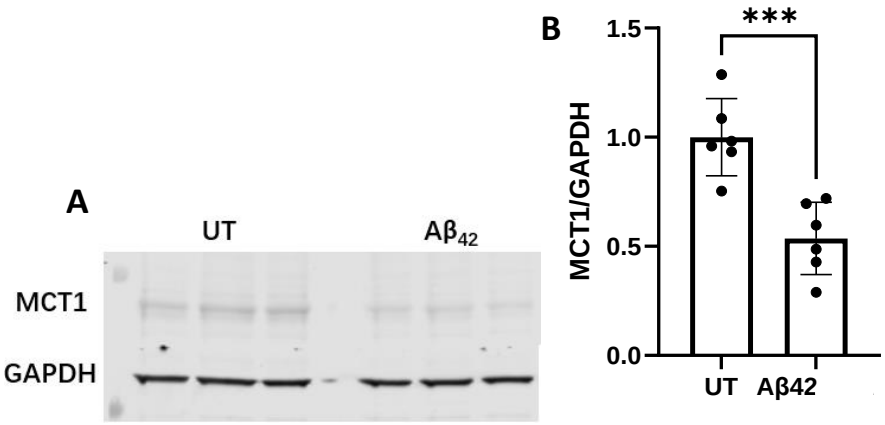
C

FITC-Aβ42 samples (N=3)	Fold change	
	Mean	SD
Control	1	0.05
NaBu	0.6	0.12

Figure 3: **Aβ42 accumulation in BBME cells**. Cells were treated with 10 µM NaBu for 6 h followed by 30 min co-incubation with FITC-Aβ42 (12.5 µg/ml). The fold change of FITC-Aβ42 median cellular fluorescence from three replicates was assessed by flow cytometry. A & B. The NaBu pre-treatment for 6 h decreased FITC-Aβ42 accumulation in BBME cells compared to the control as shown in bar chart and histograms. **C.** The standard deviation of fold change is represented in the above table. **p-value<0.01, student t-test.

3.1.3 Decrease in MCT1 expression by Aβ42.

The hCMEC/D3 cells treated with Aβ42 for one hour showed lower MCT1 expression. The MCT1 expression (0.54 ± 0.17) was decreased compared to untreated control (1 ± 0.18) in whole cell lysates as shown in Figure 4. MCT1 expression was normalized with the expression of loading control, GAPDH. (***p<0.001, student t-test).



C

FITC-Aβ42 samples (N=6)	Fold change	
	Mean	SD
Control	1	0.18
NaBu	0.54	0.17

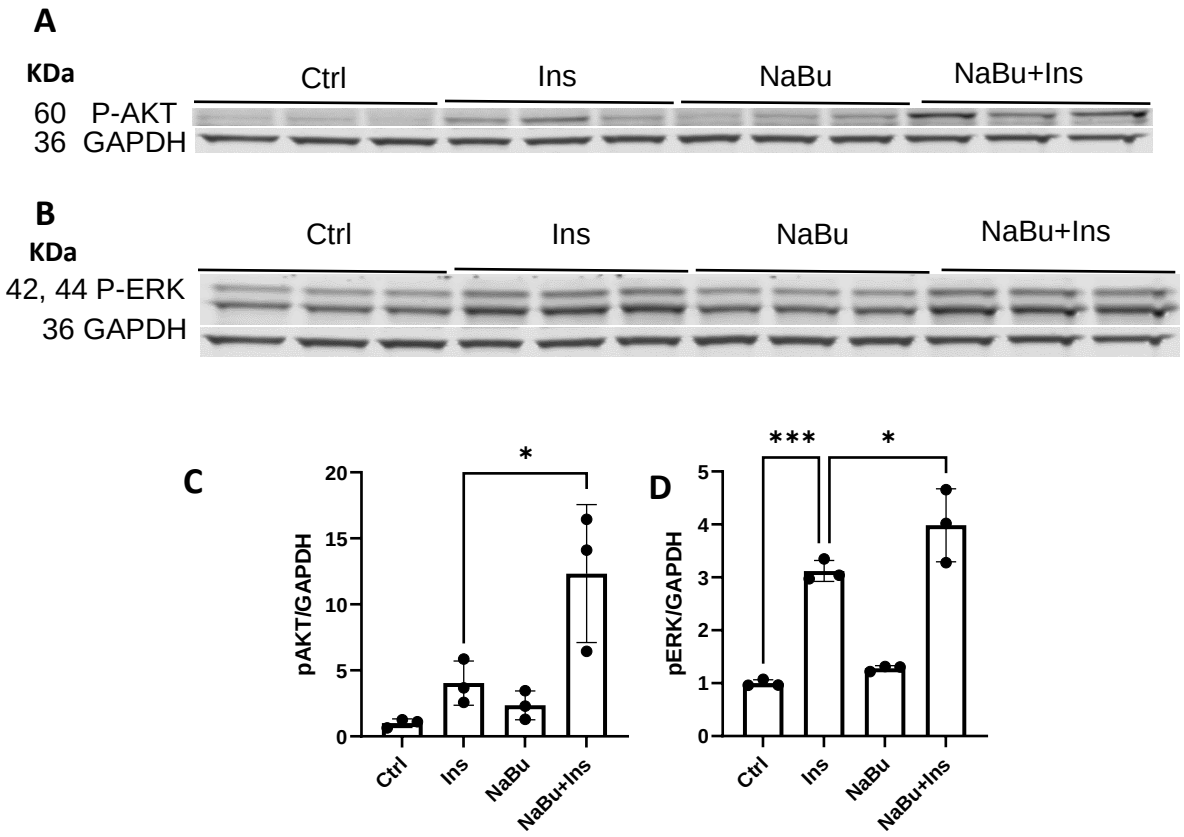
Figure 4: Decreased MCT1 expression in Aβ42 treated hCMEC/D3 cell monolayers as shown by Western blots. The hCMEC/D3 monolayers were treated with 12.5 µg/ml Aβ42 for 1 h. **A**. Immunoblots of MCT1 and GAPDH (loading control). **B** Quantitation of MCT1 normalized with GAPDH in untreated

and Aβ42 treated groups. **C**. The standard deviation of fold change is represented in the above table. ***p<0.001, unpaired student t-test.

3.1.4 Effect of NaBu on phosphorylation of AKT, ERK

The hCMEC/D3 cells were pretreated with NaBu for 6 h and then co-incubated with 50 nM insulin for 5 min. Insulin treatment increased the phosphorylation of AKT (4.03 ± 1.68) compared to the untreated group (1 ± 0.3), however, it was not significant. Insulin and NaBu co-incubation significantly increased the phosphorylation of AKT (12.33 ± 5.22) compared to the insulin-treated group (4.03 ± 1.67) (*p-value<0.05, **p-value<0.01, One-way ANOVA followed by Bonferroni multiple comparison test).

Insulin treatment significantly increased the phosphorylation of ERK (3.12 ± 0.2) compared to the untreated group (1 ± 0.06). The ERK phosphorylation was increased in the NaBu and insulin co-incubation group (3.98 ± 0.69) compared to the insulin-treated group (3.12 ± 0.19) as shown in Figure 4 (*p-value<0.05, **p-value<0.01, One-way ANOVA followed by Bonferroni post-test). Phosphorylated AKT and ERK protein expression were normalized with loading control GAPDH and band intensities were quantified by densitometry using Image studio™ lite software as shown in Fig 5.



E

Expression (N=3)	AKT (FC)		ERK (FC)	
	Mean	**SD**	**Mean**	**SD**
Control	1	0.3	1	0.06
Insulin	4.04	1.68	3.12	0.2
NaBu	2.35	1.09	1.29	0.05
NaBu + Insulin	12.33	5.22	3.98	0.69

Figure 5: Effect of NaBu on insulin signaling pathway in hCMEC/D3 whole cell lysates: Cells were treated with 10 μM NaBu for 6 h with or without 50 nM insulin stimulation for 5 min. **A & B**: Western blots showed increased AKT and ERK phosphorylation with 6 h NaBu pre-treatment followed by 5 min insulin treatment compared to the group that received insulin treatment only. Insulin

significantly increased ERK phosphorylation, however, AKT phosphorylation didn't meet significance. **C & D**: Quantification of AKT and ERK blots. **E**. The standard deviation of fold change (FC) is represented in the above table. *p-value<0.05, **p-value<0.01, One-way ANOVA followed by Bonferroni post-test.

3.2 Effect of Insulin signaling inhibitors on Aβ42 accumulation in hCMEC/D3 cells

The pathogenesis of Alzheimer's disease is associated with metabolic syndrome, which manifests as insulin resistance [1,108–110]. Therefore, we investigated the effect of NaBu on insulin signaling. Primarily, insulin activates AKT (protein kinase B) to modulate energy metabolism and extracellular signal-regulated kinase (ERK) pathway to control cell proliferation, growth, and differentiation[111,112]. Our results show that pretreatment with NaBu followed by co-incubation with insulin increases the phosphorylation of AKT and ERK in hCMEC/D3 cells compared to the insulin-treated group. Therefore, to identify the role of the insulin signaling pathway in intracellular Aβ accumulation, cells were treated with insulin signaling inhibitors (AKT inhibitor, MK2206; MEK inhibitor, Trametinib) for 6 h and then co-incubated with Aβ42 for 30 min as shown in Fig 6.

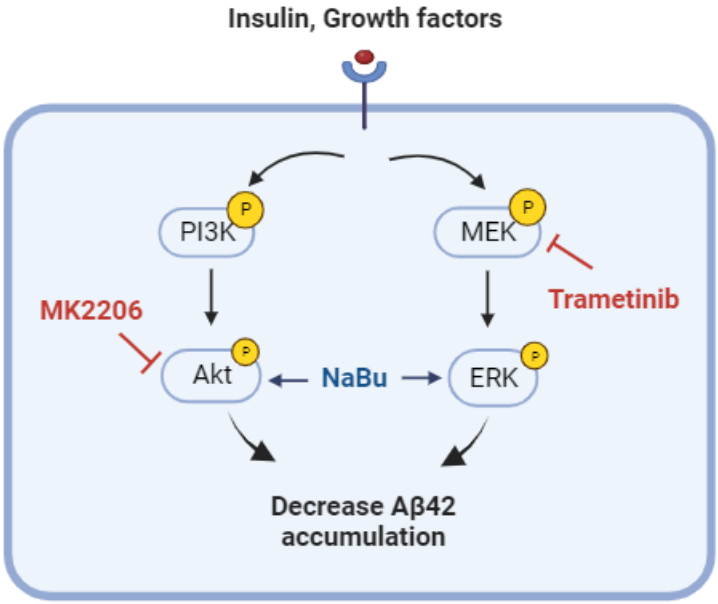
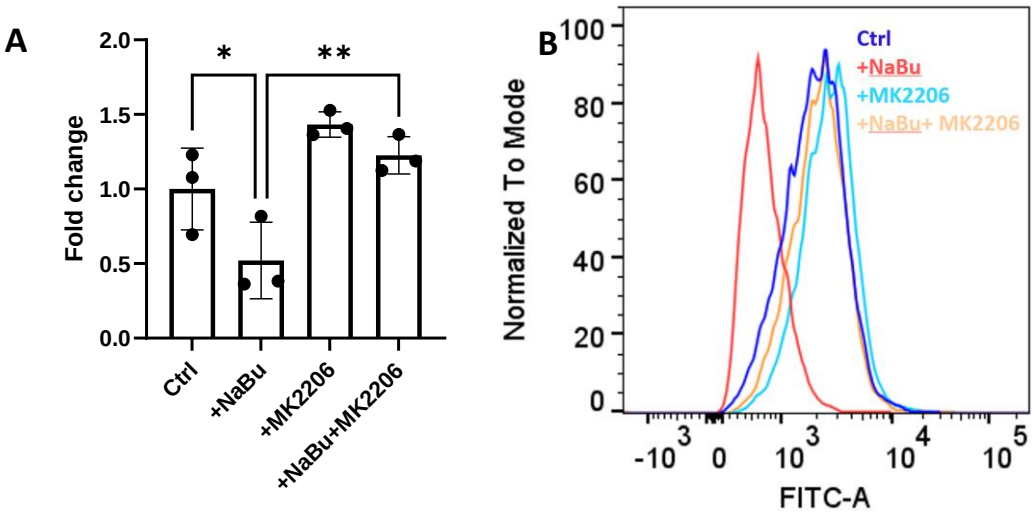


Figure 6: Proposed mechanism for Sodium butyrate (NaBu) action in hCMEC/D3 cells. The NaBu increases the phosphorylation of the AKT and ERK pathway and mediates the decrease in Aβ42 accumulation in the in-vitro BBB polarized monolayer model, hCMEC/D3 cells. Consequently, treatment with AKT inhibitor, MK2206 or MEK inhibitor, trametinib increased the intracellular Aβ42 accumulation.

3.2.1 Effect of AKT inhibitor, MK2206

The hCMEC/D3 cells were treated with NaBu and AKT inhibitor for 6 h followed by co-incubation with FITC-Aβ42 for 30 min. Fold change was calculated by comparing the individual group with the untreated control, FITC-Aβ group. The NaBu-treated group has significantly lower FITC-Aβ42 accumulation (0.51 ± 0.26) compared to the untreated control (1 ± 0.27). The FITC-Aβ42 accumulation is increased in the NaBu and MK2206 co-incubation group (1.22 ± 0.12) compared

to those treated with NaBu (0.5 ± 0.26). However, FITC-Aβ42 uptake differences between MK2206 and NaBu+MK2206 groups were not statistically significant (Figure 7). **p-value<0.01, one-way ANOVA followed by Bonferroni multiple comparison test.



C

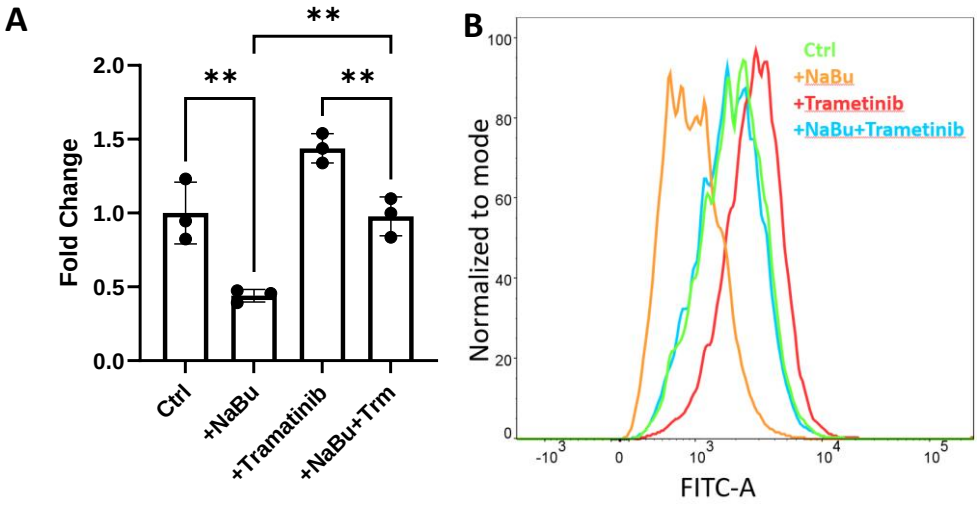
FITC-Aβ42 samples (N=3)	Fold change	
	Mean	SD
Control	1	0.27
NaBu	0.51	0.26
MK2206	1.43	0.09
NaBu + MK2206	1.22	0.12

Figure 7: Effect of AKT inhibitor, MK2206 on NaBu-mediated intracellular Aβ42 uptake in hCMEC/D3 cells: The cells were treated with 10 µM AKT inhibitor, MK2206 for 6 h followed by co-incubation with FITC-Aβ42 for 30 min. **A**. Bar chart shows fold change of intracellular FITC-Aβ42 median fluorescence intensity (MFI) in the cells treated with FITC-Aβ42, NaBu, MK2206, or

NaBu+MK2206. Accumulation of FITC-Aβ42 decreased with NaBu pretreatment but increased upon co-incubation with MK2206. **B**. Histograms, which were normalized to mode, depict FITC- Aβ42 uptake in various treatment groups. **C**. The standard deviation of fold change (FC) is represented in the above table. **p-value<0.01, one-way ANOVA followed by Bonferroni multiple comparison test.

3.2.2 Effect of MEK inhibitor, Trametinib

The hCMEC/D3 cell monolayers were treated with NaBu and/or MEK inhibitor, trametinib for 6 h followed by co-incubation with FITC-Aβ42 for 30 min. Fold change was calculated by comparing the individual group with the untreated control and FITC-Aβ treatment group. The NaBu-treated group has significantly lower FITC-Aβ42 accumulation (0.44 ± 0.04) compared to the untreated control (1 ± 0.20). In contrast, the NaBu, trametinib, and FITC-Aβ42 group (0.98 ± 0.13) has increased FITC-Aβ42 accumulation within the cells compared to the NaBu group (0.44 ± 0.04). The FITC-Aβ42 accumulation in the trametinib group decreased significantly (1.4 ± 0.10) compared to NaBu co-incubation (0.98 ± 0.13) as shown in figure 8. **p-value<0.01, one-way ANOVA followed by Bonferroni multiple comparison test.



C

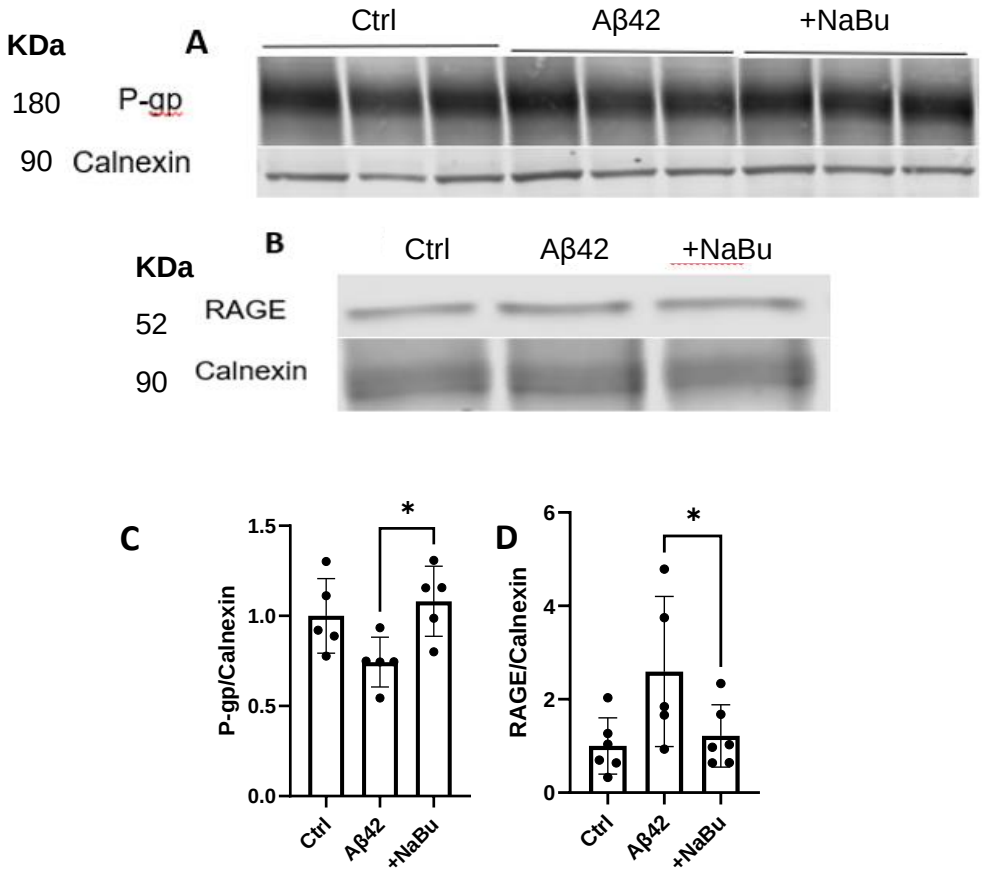
FITC-Aβ42 samples (N=3)	Fold change	
	Mean	SD
Control	1	0.2
NaBu	0.44	0.04
Trametinib	1.4	0.1
NaBu + Trametinib	0.98	0.13

Figure 8: Effect of MEK inhibitor, Trametinib on NaBu-mediated intracellular Aβ42 uptake in hCMEC/D3 cells: The hCMEC/D3 monolayers were treated with 0.5 µM MEK inhibitor, trametinib, for 6 h followed by co-incubation with FITC-Aβ42 for 30 min. **A**. Bar chart shows fold change of intracellular FITC-Aβ42 median fluorescence intensity (MFI) in the cells treated with FITC-Aβ42, NaBu, trametinib, or NaBu with trametinib. Accumulation of FITC-Aβ42 decreased with NaBu pretreatment compared to the control group but it increased upon co-incubation with trametinib. The FITC-Aβ42 accumulation decreased with NaBu co-incubation compared to the trametinib-treated group. **B**. Histograms, which were normalized to mode, depict FITC- Aβ42 uptake in various

treatment groups. **C**. The standard deviation of fold change (FC) is represented in the above table. **p-value<0.01, one-way ANOVA followed by Bonferroni multiple comparison test.

3.3 Effect of NaBu on transporters/proteins at BBB in the hCMEC/D3 monolayers

The hCMEC/D3 monolayers were treated with NaBu for 6 h followed by co-incubation with Aβ42 in DMEM for 1 h. Whole cell lysates were subjected to membrane fractionation to isolate total membrane protein which was used for the western blot. The fold change of P-gp in the NaBu pretreated group (1.08 ± 0.19) is significantly decreased compared to the Aβ42 treated group (0.74 ± 0.14). Fold change of RAGE expression in the NaBu pretreated group (0.87 ± 0.37) is significantly increased compared to the Aβ42 treated group (2.45 ± 1.58) as shown in figure 9. *p-value<0.05, one-way ANOVA followed by Bonferroni multiple comparison test.



E

Expression (N=5)	P-gp (FC)		RAGE (FC)	
	Mean	**SD**	**Mean**	**SD**
Control	1	0.2	1	0.5
Aβ42	0.7	0.14	2.45	1.58
Aβ42 +NaBu	1.08	0.19	0.87	0.37

Figure 9: Butyrate effect on transporters/receptors in hCMEC/D3 total membrane lysates: The hCMEC/D3 cells were treated with NaBu for 6 h followed by co-incubation with 12.5 µg/ml Aβ42 for 1 h **A& B:** Immunoblots show increase in P-gp and decrease in RAGE expression after 6 h with NaBu pretreatment. **C& D**: Quantification of P-gp and RAGE levels normalized to calnexin (loading control). **E.** The standard deviation of FC is represented in the above table. *p-value<0.05, one-way ANOVA followed by Bonferroni multiple comparison test.

4. Discussion

The prevalence of AD is increasing in the elderly population across the world, and it poses a significant social and financial burden on the families of patients. Therefore, substantial resources are being invested to decipher the mechanisms underlying disease onset, progression, and cognitive impairments resulting from AD. Moreover, various strategies are being developed for the early diagnosis and treatment of AD. It is widely recognized that AD is characterized by the presence of intracellular amyloid beta (Aβ) plaques and extracellular neurofibrillary tangles in the brain and is also associated with cerebrovascular pathologies. The brain blood vessel lumen is lined by a monolayer of endothelial cells that constitutes the blood-brain barrier (BBB)[113]. The intercellular spaces between BBB endothelial cells are held together by tight junctions that restrict the passive diffusion of solutes between plasma and the brain. The BBB protects the brain from toxic substances and selectively transports essential nutrients to the Brain from plasma. Moreover, the BBB endothelial cells work in coordination with pericytes, astrocytes, and neurons, which together form the neurovascular unit[19]. In pathological conditions, endothelial tight junctions are compromised facilitating the entry of harmful substances into the brain leading to edema and neurovascular dysfunction.

Research conducted thus far primarily focused on establishing causality between the cognitive changes and the most visible AD biomarkers such as amyloid beta plaques and neurofibrillary tangles[114]. However, the treatment with anti-amyloid beta monoclonal antibodies yielded only modest results[115]. Hence, it may be likely that there may be other pathophysiological and environmental risk factors that need to be considered for early diagnosis and comprehensive treatment of AD. Literature evidence suggests that some of these risk factors may include BBB

disruption[116], neuroinflammation[117], insulin resistance[118–120], gut dysbiosis[57,104,121–123], and the presence of other comorbidities. The impact of neurovascular inflammation and the involvement of metabolic syndrome in increasing AD risk is being widely investigated[118,119]. Recent studies have also shown the influence of diet on the composition and diversity of gut microbiome[124,125], and its association with mild cognitive impairment and AD[124–126]. Substantial diet changes resulting from urbanization across the world have shown an increase in the incidence of metabolic disorders, which is believed to elevate the risk for cardiovascular diseases. Recent studies have shown that these risk factors also engender neurodegenerative diseases including AD[124,125,127–129]. The gut microbiome secretes several molecules like γ-aminobutyric acid (GABA), serotonin, norepinephrine, dopamine, and short-chain fatty acids which act on the brain through the gut-brain axis[130]. The gut-brain axis is a bi-directional communication pathway between the brain and gut mediated by the vagal nerve, and other chemical messengers including neurotransmitters and short-chain fatty acids [69,131] like acetic acid, propionic acid, and butyric acid. The impact of butyrate on vascular and brain functions is currently being investigated by various researchers and is also the main topic of my thesis project. Butyrate is known to play a key role in modulating the integrity of the intestinal barrier by regulating the expression of tight junction proteins. In addition, it acts as a histone deacetylase inhibitor (HDACi) promoting histone acetylation to induce gene expression of anti-inflammatory molecules[132]. Butyrate also exerts an effect on brain function through the gut-brain axis[69,128,131,133,134].

In the 5xFAD mouse model of AD, sodium butyrate (NaBu) reduces Aβ levels in the brain[135]. Also, it was shown to be protective against Aβ induced cytotoxicity in mouse neuroblastoma cells[136]. The butyrate was shown to reduce excessive reactive oxygen species

induced by high cholesterol in neuronal cells[123]. Moreover, butyrate was reported to improve BBB integrity in lipopolysaccharide (LPS) treated endothelial/glial cell co-cultures, which reduces the trans-endothelial electrical resistance (TEER)[137]. Alternatively, there have also been claims made that SCFAs derived from microbiota promote Aβ plaque deposition in germ-free AD mice[138]. Hence, to understand butyrate's role in AD pathophysiology, it is critical to explore its actions on the BBB. In this project, we have investigated the effect of sodium butyrate (NaBu) on the intracellular Aβ accumulation in the polarized hCMEC/D3 monolayer in-vitro, a widely used BBB model.

In this study, we pre-treated hCMEC/D3 monolayers with a physiological concentration of NaBu (10 uM) for various durations of time and then treated with Aβ40 (25 μg/ml) or Aβ42 (12.5 μg/ml) to investigate the effect of NaBu on intracellular Aβ accumulation in endothelial cells. Treatment with NaBu with 2 h or 6 h has decreased the accumulation of Aβ42. However, 24 h treatment showed no significant effect compared to the controls. This could be due to a reduction in the concentration of NaBu during 24 h most likely due to the utilization of NaBu as an energy source by the endothelial cells similar to that observed in colonocytes[139]. On the other hand, Aβ40 accumulation decreased only at the 6 h time point. To confirm that the NaBu effect is consistent across various BBB endothelial cell models, Aβ42 uptake in the presence of NaBu was also investigated using primary bovine brain microvascular endothelial (BBME) cells. Similar results were observed in both hCMEC/D3 and BBME cell monolayers. These findings can explain how butyrate reduced Aβ induced toxicity in the published literature on diverse cell types and in-vivo models[7,128,129,140].

We have further investigated molecular mechanisms driving NaBu's effects on Aβ uptake by BBB endothelial cells. The impact of Aβ as well as NaBu on the expression of monocarboxylic acid transporter 1 (MCT1), which serves as the primary butyrate transporter in BBB endothelial cells, was investigated. Moreover, expression of the receptor for advanced glycation end products (RAGE), which handle luminal-to-abluminal Aβ transport as well as the permeability glycoprotein (P-gp) that promotes Aβ efflux in the abluminal-to-luminal direction were assessed. In addition, the effect of NaBu on caveolin-1 expression which is essential for the caveolae-mediated endocytosis of Aβ42 was determined [26,141,142]. The Aβ exposure was shown to decrease MCT1 expression, P-gp expression, and increased RAGE expression. On the other hand, NaBu increased P-gp and decreased RAGE expression in total membrane cell lysates upon 6 h treatment. According to the literature, increased Aβ deposition is correlated with increased RAGE expression, and decreased P-gp expression in the human AD brains compared to age-matched controls. As NaBu is decreasing the Aβ accumulation, it is logical to observe decreased RAGE expression and increased P-gp expression in hCMEC/D3 cell monolayers[26,141]. Since Aβ enters the cell through RAGE and is expelled via P-gp, the downstream mediators of NaBu's action, which regulate RAGE and P-gp expression remain unknown and require further investigation.

The metabolic syndrome that manifests insulin resistance is implicated in the pathogenesis of AD [1,108–110]. Therefore, we investigated the effect of NaBu on insulin signaling. Insulin mainly activates AKT (protein kinase B) to regulate energy metabolism and extracellular signal-regulated kinase (ERK) pathway to regulate cell proliferation, growth, and differentiation[111,112]. The western blots of the BBB endothelial cell lysates treated with NaBu and insulin showed an increase in the phosphorylation of both AKT and ERK compared to insulin insulin-alone group after 6 h. Then,

additional studies were conducted to explore the involvement of AKT and ERK in the regulation of Aβ uptake by BBB endothelial cells. The BBB endothelial monolayers were treated with NaBu alone and NaBu with AKT inhibitor, MK2206 (10 μM); the Aβ accumulation decreased with NaBu treatment and increased upon co-incubation with MK2206. These results suggest the involvement of the AKT pathway in the NaBu-mediated reduction of Aβ accumulation in the endothelial cells. Similarly, BBB endothelial cells were treated with NaBu alone or in co-incubation with MEK inhibitor, trametinib (0.5 μM). Like in the case of AKT inhibitor, the BBB endothelial cells treated with NaBu and trametinib increased Aβ accumulation compared NaBu alone group. These results demonstrate the involvement of MEK in Aβ accumulation. These findings provide a strong basis to speculate that NaBu modulates insulin signaling pathways to alter Aβ uptake by the BBB endothelium. Therefore, it is highly likely that this uptake mechanism is disrupted under insulin resistance, promotes Aβ transport dysregulation at the BBB, and augments AD pathogenesis.

To obtain a comprehensive insight into the NaBu effect on AKT and ERK pathways in regulating Aβ endocytosis in the blood-brain barrier endothelial cells, it is imperative to investigate various mediators involved in the insulin signaling pathway. Thus, further studies to elucidate the NaBu effect on BBB integrity and function, specifically their role in regulating Aβ kinetics at the BBB need to be conducted.

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