POSSIBLE ROLES OF HISTAMINE RECEPTORS AND RHOA SIGNALLING PATHWAYS IN BLOOD-BRAIN BARRIER

by

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Koc University

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To my beloved dinosaur and his mother “Elf Kızı”

ABSTRACT

Possible Roles of Histamine Receptors and RhoA Signalling Pathways in Blood-Brain Barrier

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Doctor in Philosophy in Neuroscience
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Blood-brain barrier (BBB) which creates a major obstacle for the transport of molecules into the brain can be opened by various treatments for effective drug delivery into the brain parenchyma, however its subsequent closure is also crucial to limit the detrimental alterations that can potentially occur in neuronal microenvironment by circulatory substances. In this study, we tested the efficacy of blocking histamine receptors (HRs) and RhoA in reversing histamine-induced BBB opening. Following intravenous histamine injection (10 mg/kg), mice were treated with HR antagonists, hydroxyzine (H1R, 3 mg/kg), cimetidine (H2R, 10 mg/kg), ciproxifan (H3R, 3 mg/kg) and JNJ-7777120 (H4R, 20 mg/kg), and Rho A inhibitor fasudil (10 mg/kg). Blood-brain barrier permeability to molecules with large and small molecular weights was evaluated by fluorescent detection of intravenously administered albumin-Alexa Fluor-594 (1%) and cadaverine Alexa-Flour-488 (1%), respectively in brain parenchyma. To analyze changes in the immunoreactivity of claudin-5 and caveolin-1 proteins, immunofluorescence staining was performed. The alterations in HR expression were detected by RT-PCR. Treatment with hydroxyzine significantly decreased fluorescence intensity of albumin-Alexa Fluor-594 in brain parenchyma following histamine-induced BBB opening ($p<0.01$), while there were no significant changes in the fluorescence intensity of cadaverine Alexa-Flour-488 by HR antagonists and fasudil in this setting. No significant changes were noted in HR mRNA and protein levels following administration of HR antagonists and fasudil to histamine-treated mice. Our results suggest that treatment with H1R antagonist hydroxyzine restores histamine-induced BBB disruption which emphasizes its potential use as a novel means of closing transiently opened BBB to protect the neuronal microenvironment against potentially toxic blood-borne substances in brain drug delivery applications.

ÖZETÇE

Histamin Reseptörlerin ve RhoA Sinyal Yolaklarının
Kan-Beyin Bariyeri Üzerindeki Olası Rolü

Uğur AKCAN

Nörobilim, Doktoa
18 Kasım 2021

Kan-beyin bariyeri (KBB) beyne ilaç taşınmasının önünde yatan en büyük engellerden biridir. Günümüzde beyne ilaç taşınması için KBB'nin açıldığı birçok yöntem uygulanmakta fakat bu yöntemlerin kısa sürede KBB'yi eski haline geri döndürememesinin kan kaynaklı nörotoksik materyallerin beyne geçerek patolojik durumlara yol açtığı bilinmektedir. Bu çalışmada histamin reseptörleri (HR) ve RhoA'nın bloklanmasının histamin kaynaklı olarak artış gözlenen KBB geçirgenliğini onarıcı etkisini araştırdık. Bu amaçla, farelere histamin enjeksiyonunu (10 mg/kg) takiben, HR antagonistleri hidroksizin (H1R, 3 mg/kg), simetidin (H2R, 10 mg/kg), siproksifan (H3R, 3 mg/kg), JNJ-7777120 (H4R, 20 mg/kg) ve RhoA inhibitörü fasudil (10 mg/kg) intravenöz olarak kuyruk veninden uygulanmıştır. Küçük moleküler ağırlıklı moleküllerin KBB geçişini tayin etmek için kadaverin-Alexa-Flour-488 (%1) ve büyük moleküler ağırlıklı moleküllerin geçişini tayin etmek için ise albümin-Alexa-Flour-594 (%1) intravenöz olarak enjekte edilmiştir. Klaudin-5 ve kaveolin-1 proteinlerinin immünoreaktivitelerindeki değişikiliklerin analiz edilmesi için immünfloresan boyamalar yapılmıştır. Histamin reseptörlerinde oluşan ekspresyon değişimlerini tayin etmek için Western Blot ve RT-PCR metotları uygulanmıştır. Hidroksizin uygulaması, histamin kaynaklı olarak beyne geçişi artan albümin-Alexa Flour-594 floresan ışıma miktarını azaltırken ($p<0,01$), beyne geçen kadaverin-Alexa Flour-488'in miktarında ise gruplar arasında anlamlı bir farklılık gözlenmemiştir. Histamin reseptör antagonistleri ve RhoA inhibitör uygulamaları HR mRNA ve protein düzeylerinde bir değişikliğe yol açmamıştır. Sonuç olarak bulgularımız histamine bağlı KBB açılmasının yol açtığı geçirgenlik artışının H1R antagonisti hidroksizin kullanılarak kısa sürede düzeldiğini göstermekte ve bu yolun beyin ilaç geçişini artırma uygulamalarında geçici olarak açılan KBB'yi kısa sürede kapatarak nöronal mikroçevreyi potansiyel olarak toksik olabilecek kan kaynaklı maddelere karşı korumada yeni bir yaklaşım oluşturabileceğini düşündürmektedir.

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ABBREVIATIONS

AA: Arachidonic acid

ABC: ATP-binding cassette

AC: Adenyl cyclase

AJp: Adherence junction protein

AKT: Protein kinase A

AMT: Adsorptive-mediated transport

AQP: Aquaporin

BBB: Blood-brain barrier

Bcl2: B-cell lymphoma 2

BM: Basal membrane

BMVECs: Brain microvascular endothelial cells

BSA: Bovine serum albumin

BTB: Blood-tumor barrier

Bves: Blood microvessel epicardial substance

Ca^{2+}: Calcium

CaM: Calmodulin

CaMK: Calmodulin kinase

cAMP: Cyclic adenosine monophosphate

CaN: Calcineurin

Cav-1: Caveolin-1

cDNA: Complementary DNA

CNS: Central nervous system

Crb3: Crumb's protein homologs 3

CREB: Cyclic AMP-response binding element protein

CSF: Cerebrospinal fluid

CYP: Cytochrome P450

DAG: Diacylglycerol

DD: Dystroglycan-dystrophin

Dlg1: Drosophila disc large tumor suppressor

DNA: Deoxyribonucleic acid

DPBS: Dulbecco's phosphate-buffered saline

DPBST: Triton-X100/DPBS solution

EAE: Experimental autoimmune encephalomyelitis

ECL: Extracellular loop

FGF: Fibroblast growth factor

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GLUT-1: Glucose transporter-1

GPCR: G protein-coupled receptors

GRIK: G protein-coupled inwardly rectifying potassium channel

GSK-3β: Glycogen synthase kinase 3β

GUK: Guanylate kinase-like domain

H: Histamine

H1R: Histamine-1 receptor

H2R: Histamine-2 receptor

H3R: Histamine-3 receptor

H4R: Histamine-4 receptor

HR: Histamine receptor

HUVEC: Human umbilical vein endothelial cells

ICAM-1: Intercellular adhesion molecule-1

IFNγ: Interferon-gamma

IL-3: Interleukin-3

IL-4: Interleukin-4

IL-6: Interleukin-6

IL-8: Interleukin-8

IP3: Inositol triphosphate

JAMs: Junctional adhesion molecules

MAGI: Membrane-associated guanylate kinase inverted 2

MAGUK: Membrane-associated guanylate kinase-like protein

MAPK: Mitogen-activated kinases

MEF2: Myocyte enhancer factor 2

Mfsd2a: Major facilitator superfamily domain containing 2A

MLC: Myosin light chain

MLCK: Myosin-light chain kinase

MLCP: Myosin-light chain phosphatase

MMPs: Matrix metalloproteinases

MRFT: Myocardin-related transcription factor

mRNA: Messenger RNA

MRP: Multidrug resistance protein

MS: Multiple sclerosis

MYPT: Myosin phosphatase targeting protein

Na^+: Sodium

NFAT: Nuclear factor of activated T-cells

NO: Nitric oxide

NOS: Nitric oxide synthetase

NSS: Saline solution

NVU: Neurovascular unit

P: Phosphate

PFA: Paraformaldehyde

PI3K: Phosphoinositide 3-kinase

PIC: Protease inhibitor cocktail

PIP_2: Inositol diphosphate

PKC: Protein kinase C

PLA_2: Phospholipase A2

PLC: Phospholipase C

PLCβ: Phospholipase β

PVDF: Polyvinylidene difluoride membrane

RAC: Ras-related C3 botulinum toxin substrate

RMT: Receptor-mediated transport

RNA: Ribonucleic acid

ROCK: Rho kinase

RT-PCR: Real time polymerase chain reaction

SDS: Sodium dodecyl sulphate

SEM: Standard error of mean

SLC: Solute carrier transporters

SRF: Serum response factor

TEER: Trans endothelial electrical resistance

TfR: Transferrin

TGFβ1: Transforming growth factor-beta 1

TJ: Tight junction

TNF-α: Tumor necrosis factor-alpha

TRVP: Transient receptor potential cation channels

VCAM-1: Vascular cell adhesion molecule-1

VEGF: Vascular endothelial growth factor

VGCC: Voltage-gated calcium channel

VMAT-2: Vesicular monoamine-transporter

WT: Wild type

ZO: Zonula occludens

1 INTRODUCTION

Neurological disorders/diseases cause a significant mortality and morbidity in humans and socioeconomic costs and therefore, researchers have been trying to develop new therapeutics and treatment approaches. However, they need to deal with many challenges to deliver drugs into the brain effectively because of the presence of barrier systems in the central nervous system. One of the most critical challenges for drug delivery into the central nervous system (CNS) to cure or treat neurodegenerative disorders/diseases is obstacle of the normal blood-brain barrier (BBB) (Banks, 2016; William M. Pardridge, 2020). An intact BBB controls the transport of drugs like molecules, which are present in circulation from the brain parenchyma (Banks, 2016; William M. Pardridge, 2020; W. M. Pardridge, 2015).

Researchers use different methodologies and approaches to deliver drugs into the brain parenchyma in effective doses (Ahlawat et al., 2020). Trojan horse methodologies are used as nanocarriers to transfer drugs into parenchyma effectively. Nanocarriers can be conjugated with their specific ligands that have their transporters located on endothelial cells to increase the transport of drugs into the brain (Ahlawat et al., 2020; Ugur Yilmaz et al., 2020). Using these nanocarriers that were tagged with polyethylene glycol has secondary benefits like increasing the half-life of drugs or bioavailability (Gaillard et al., 2014). Nevertheless, nanocarriers can cause adverse effects on human health depending on their structure, and recent studies have shown that there is still a need for improvement of properties of nanocarriers for using in preclinical studies (Dong, 2018; Egbert et al., 2017).

Another approach to increase drug delivery into the brain is to use ultrasound to open BBB (Abrahao et al., 2019; Fan et al., 2013). This kind of methodology can target lipid bilayer of the barrier type endothelial cell membrane, to enhance drug delivery into the brain (Dasgupta et al., 2016). One of the recent studies using ultrasound to create bubbles for drug delivery can be one of the most potential methodologies, but there is still ~~a~~ need to develop efficient tools to increase drug delivery into the brain (Zhang et al., 2020)

Researchers also develop antibodies like peptides for curing neurological diseases, and antibodies can be targeted with specific ligands similiar as nanocarriers but with lower toxicity. (Gernert & Feja, 2020). This kind of approach is recently developed, and their response is limited or depending on their binding area.

Intranasal and intra-arterial delivery can also be used to transport drugs into the brain parenchyma, but both methodologies have some limitations (Mehta et al., 2012; Pires et al., 2009). For example, the efficacy of nose-to-brain transport is dependent on limited transport capacity of olfactory pathway and the chemical structure of drugs affecting their half-time bioavailability which is crucial for treating neurological disorders (van Woensel et al., 2013). The intra-arterial delivery is mainly based on a device creating a pore on endothelial cells, and after that, the drug is released directly into the brain. However, similar to intranasal delivery, drug transport into the brain parenchyma through the intra-arterial route also depends on the chemical properties of the drugs (Jahangiri et al., 2017; van Woensel et al., 2013).

Moreover, intraarterial delivery of drugs into the brain is also provided by hyperosmolar chemicals like mannitol or arabinose that activate paracellular pathway. For this purpose, drugs are injected into the external carotid artery, and after that, a hyperosmolar solution is injected to open the BBB (Haumann et al., 2020). However, hyperosmolar mannitol can be toxic for brain and drug levels in the brain may not be enough for an effective treatment. Using other chemicals rather than mannitol, which alters BBB integrity can be a valuable asset (Basso et al., 2002; Warren, 2013).

Histamine, a known allergenic molecule that is produced from mast cells, increases BBB permeability, and can be used instead of mannitol to open BBB. Using a histamine-based approach can be a valuable asset for drug delivery into the brain, but there are many unsolved questions for this agent. Understanding which mechanism is responsible for these barrier properties in BBB is crucial for the drug delivery into the brain to treat neurological disorders/diseases such as Alzheimer's disease, brain tumors, and Parkinson's disease.

The blood-brain barrier (BBB) is mainly constituted by brain capillary endothelial cells that separate the circulatory system and brain parenchyma. To maintain CNS homeostasis is crucial for proper neuronal functions. The BBB protects the neural tissue from variations in blood composition and strictly controls transport of ions, toxins, and nutrients into the brain parenchyma; for example, the loss of water content from cerebrospinal fluid (CSF) provokes vertigo (Lay, 2002). On the other hand, hydrocephalus is defined as neuronal tissue loss, caused by increased water content in CSF (Aunan-Diop et al., 2021; Sayore et al., 2021).

The BBB forms physical and enzymatic barriers that prevent the penetrations of circulatory substances and immune cells into the brain parenchyma and maintain normal neuronal function.

The enzymatic barrier of brain microvascular endothelial cells is mainly constituted by efflux pumps and sends back substance into the blood. Physical barrier is mainly constituted by tight junction proteins and seal two adjacent endothelial cells tightly to restrict entry of molecules into the blood (Schaeffer & Iadecola, 2021) (Figure 1.1).

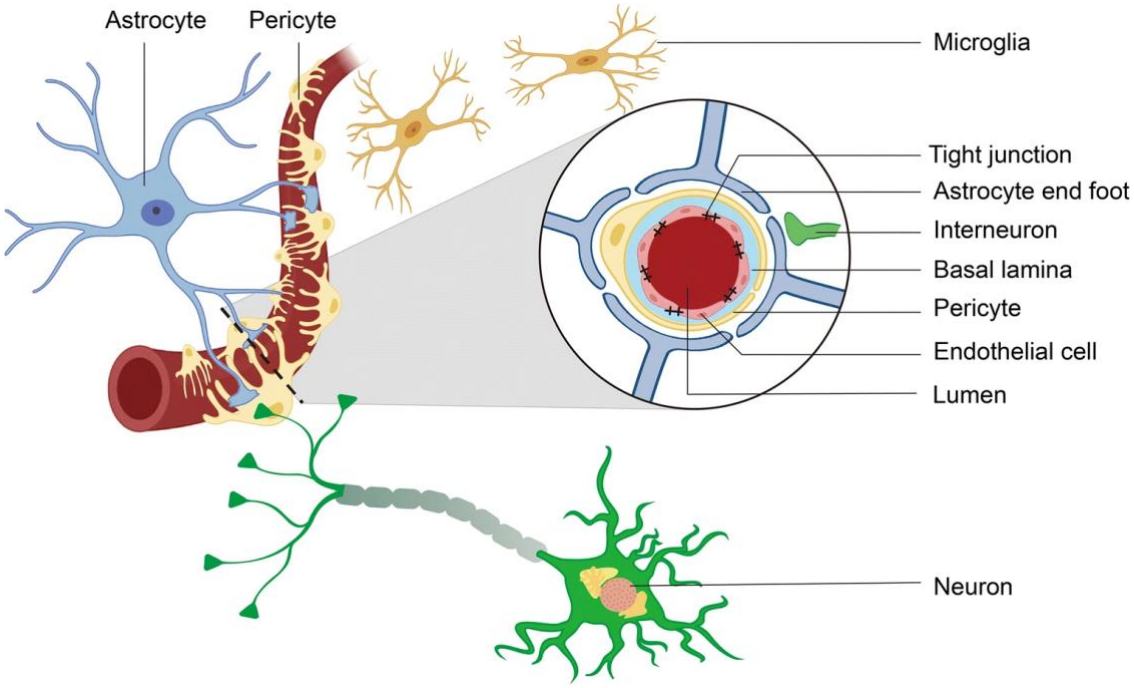

Figure 1.1 The schematic representation of the neurovascular unit. Retrieved from Sun et. al. 2021.

Pericytes and astrocytes provide anatomical and functional support to the BBB. Recent studies show that pericytes, astrocytes, and basement membrane proteins also have a role in the formation and maintenance of BBB (Iadecola, 2017; Schaeffer & Iadecola, 2021). In recent years, researchers have started to use a new term called “neurovascular unit-NVU” to clarify interaction among endothelial cells, pericytes, astrocytes, microglia, and neurons (Schaeffer & Iadecola, 2021). While all these constitute the cellular parts of the NVU, the basement membrane and its proteins can also be considered as parts of the NVU.

1.1 Endothelial Cells

Endothelial cells are primarily thin and elongated cells, but their shape can change depending on their vascular localization. Their size is nearly 30–50 µm in length, 10–30 µm in wide, and a thickness of 0.1–10 µm. Endothelial cells are covered with the basal lamina, and they lay the inside of arteries, veins, arterioles, venules, and capillaries. Tissues are mainly divided by endothelial cells from the circulatory system.

Endothelial cells regulate vascular tone, regional blood flow, and extravasation of fluids and molecules and other cells the interstitial side. Lastly, they can release factors like nitric oxide (NO), endothelin, reactive oxygen species. More importantly, endothelial cells are also accepted as endocrine organs. Recent studies have indicated endothelial cells release factors that govern angiogenesis (Lamalice et al., 2007). During embryonic development, angiopoietin-1 is released from neural progenitor cells (NPCs) and binds to the Tie2 receptor in endothelial cells, and endothelial cells release transforming growth factor-beta 1 (TGFβ1). NPCs phosphorylate the SMAD3 depending on the presence of TGFβ1, and this activation leads to the maturation of oligodendrocytes (Paredes et al., 2021).

The surrounding tissues' needs mainly govern endothelial cell differentiation (Aird, 2007; Monahan-Earley et al., 2013). Barrier type capillary endothelial cells in brain are responsible for creating the BBB phenotype. These cells are sealed by themselves, and their sealing is tighter than other microvascular endothelial cells because the brain endothelial cells are responsible for limited molecular and cellular passage into the brain parenchyma.

Each neuron in CNS quickly communicates with endothelial cells. It has been assumed that brain microvascular endothelial cells (BMVECs) cover up to 95% of neurons. In the brain, the total length of a capillary microvessel is nearly 650 km, and the total transport area of these cells is nearly 20 m^2 for molecular passages (Begley & Brightman, 2003).

Fenestra is defined as a pore or small opening area in the endothelial cell. Fenestrae in the microvascular endothelial cells of peripheral tissues, are responsible for transporting molecules into the tissue (Abbott et al., 2010; Sedlakova et al., 1999).

The tightness of BMVECs is mainly provided by tight junction (TJ) and adherens junction proteins. Tight junction and adherens junction proteins contact each other and

prevent substance transport between the intercellular areas of the endothelial cells. Claudins, occludin, and junctional adhesion molecules are defined as TJ proteins, which are mainly responsible for creating physical barriers, controlling the paracellular permeability in the BBB. On the other hand, adherens junction proteins, such as cadherins, also bind cells, but their protein-protein binding strength is less than TJ proteins (Kaya & Ahishali, 2021).

TJ proteins are mainly located at cell-cell contact sites and bind adjacent that overlap cells. They also connect to the actin cytoskeleton in cells with zonula occludens (ZO) proteins, cingulin, 7H6, AF-6 (Figure 1.2). These connections are one of the crucial bases for carrying out proper barrier functioning. Without accessory proteins, BMVECs represent similar results with a lack of TJ proteins. For example, ZO-1 localization changes in inflammatory conditions. Proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) increase the permeability of human BMVECs. Dose-dependent TNF-α and IL-6 administration to the human BMVECs leads to a gradual decrease in ZO-1 expression and dysregulation of ZO-1 – occludin association (Rochfort & Cummins, 2015).

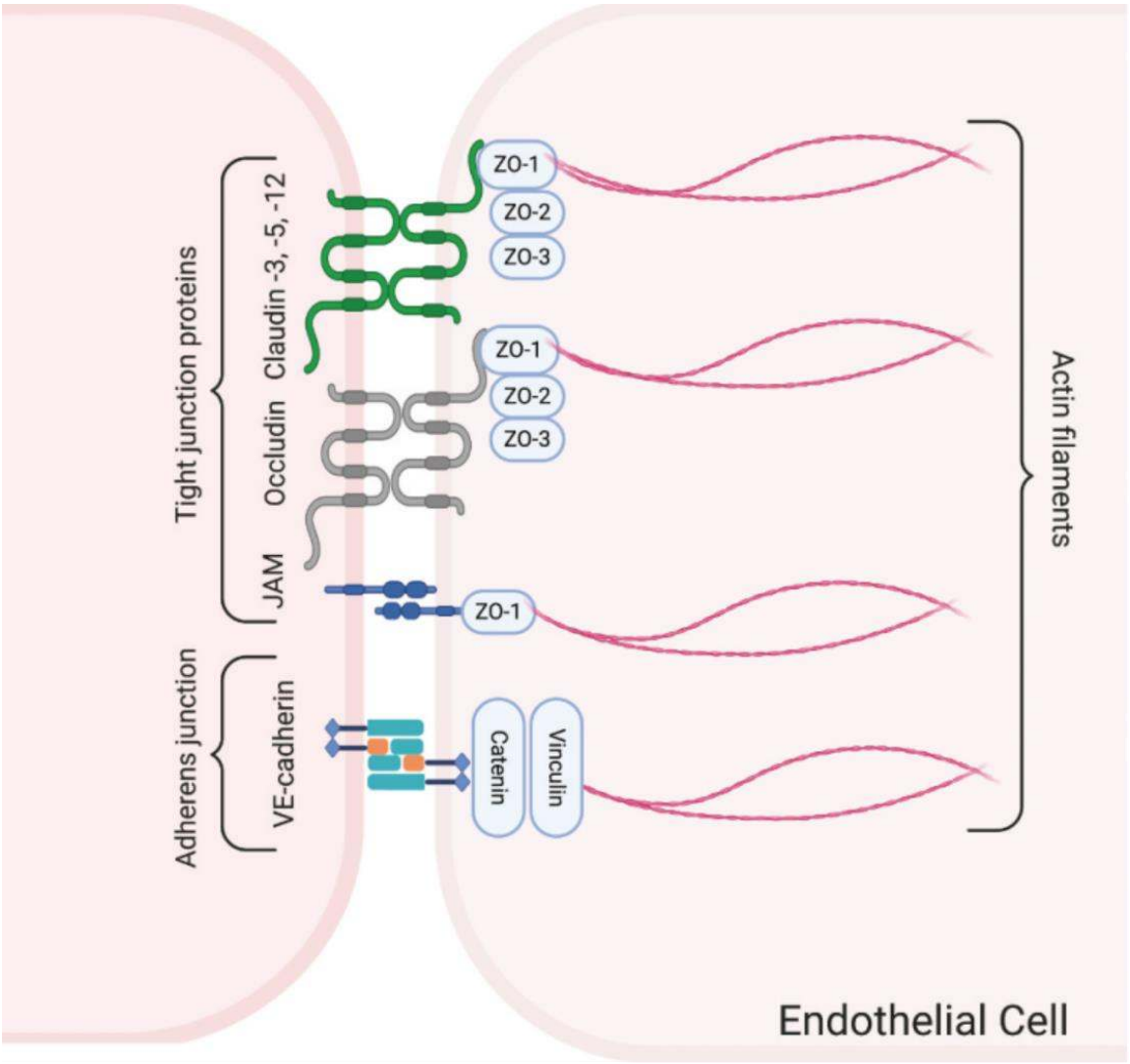

Figure 1.2 The representation of TJ and AJ proteins in the endothelial cells. Retrieved from Kaya and Ahıshalı 2021.

Regarding TJ and regulatory proteins, the cytoskeleton has a role in maintaining an ideal BBB formation. Cytoskeleton acts as a basis for TJ proteins and helps to keep them stable for changed environmental conditions. For example, RhoA is also known as Ras homolog family member A protein is responsible for cytoskeleton arrangement in the cell for a cellular response depending on different cellular conditions. Gibson et al (2014) showed that inhibiting RhoA with fasudil, a potent RhoA inhibitor already used for the treatment of hypertension in Japan, improved the integrity of BBB and protected barrier function in the ischemic animal model (Gibson et al., 2014).

Transcellular transport as a function of the barrier type brain microvessel endothelial cells is more frequently used for substance and cell transport between blood and tissue. Transcellular transport is limited in the barrier type capillary endothelial cells

because of molecule characteristics such as solubility in lipid and water, size, and electricity. Ions and solutes cannot pass easily depending on their concentration gradients. On the other hand, lipid-soluble small molecules (<400 – 500 Da) such as ethanol, oxygen, NO, and carbon dioxide can diffuse into the brain by using passive diffusion.

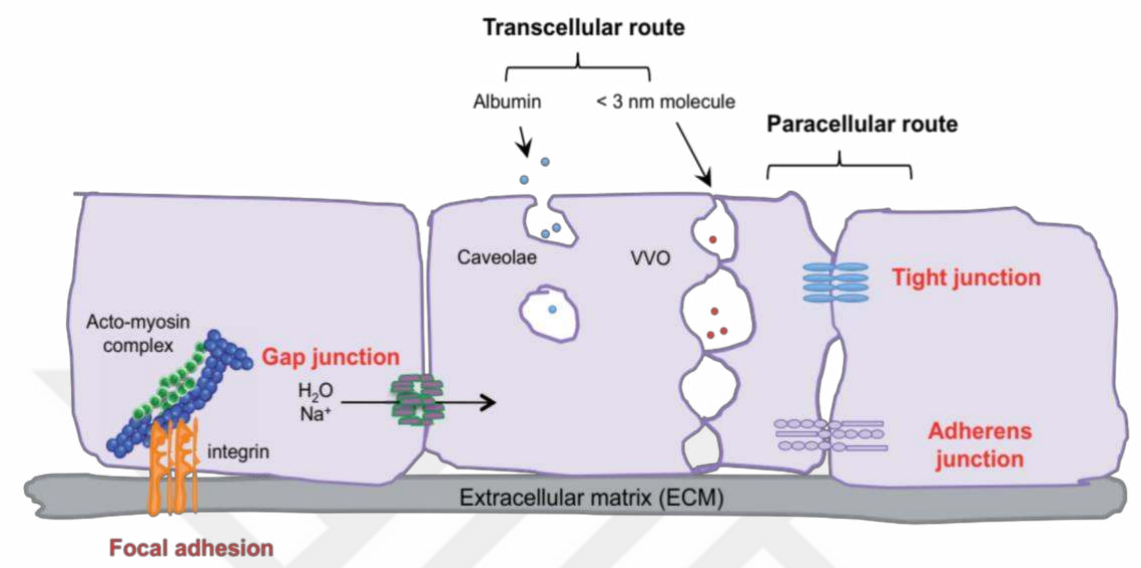

Figure 1.3 Description of transcellular and paracellular pathways in brain microvascular endothelial cells. Retrieved from Azzi et al, 2013.

1.1.1 Tight Junctions

Kissing points located at the edges of endothelial cells and they seal tightly and are mainly responsible for forming the shape of the BBB physical barriers. This physical barrier is also named a paracellular barrier, which TJ proteins regulate. On the other hand, TJ proteins restrain the transfer through the cell membrane between the apical and basolateral sides of the cell, and this phenomenon is called the fence function. However, it remains unclear, and several studies tried to explore this phenomenon, however, further explanation is needed for evaluating the fence (2009 Anderson and Van Itallie; Zihni et al. 2016).

TJ protein complexes mainly regulate paracellular permeability. The substance transport that occurs via paracellular pathway between circulation and perivascular endothelial cells is minimal (Zlokovic, 2008). Nevertheless, some substances can easily pass depending on their molecular size and charges. Small uncharged molecules can pass

through the pores between TJ proteins. TJ proteins' cis-cis interactions allow the penetration of these small and charged ions and molecules (Abbott et al. 2015).

TJ proteins preserve lipids and protein components of apical and basolateral sides of endothelial cells. These proteins limit the movement of lipid leaflet components. Lipid components reverse their movements when they are faced with TJ protein connections. These specialties are not demonstrated, but some experiments have shown that used fluorescent lipids cannot continue their movements in the cellular membrane when they are getting edges of TJ proteins and lipids to turn around (Krug & Fromm, 2020).

Claudins, occludin, tricellulin (MARVELD2), MARVELD3 (occludin and tricellulin can also be named MARVEL domain proteins), and JAMs are listed as TJ proteins. Tricellulin proteins seal three cell connections and these connection spots are considered as passing spots for immune cells (Sweeney et al. 2018). Unlike tricellulin, other TJ proteins seal one or two cells. On the other hand, TJ proteins are classified by their transmembrane domain numbers. JAMs and Crumb's protein homologs 3 (Crb3) consist of a single transmembrane domain and blood microvessel epicardial substance (Bves) consists of a triple transmembrane domain. Claudins and MARVEL proteins have a four-transmembrane domain (Sweeney et al. 2018; Zeniya et al. 2018)

Some proteins can be considered TJ proteins, but they are mainly responsible for supporting TJ proteins instead of sealing cells. They are mainly responsible for anchoring properties, creating bonds between TJ proteins and cytoskeleton proteins (Hashimoto & Campbell 2020). These protein-to-protein bonds provide flexibility and durability to BBB under some cellular stress conditions such as inflammation. ZO-1, -2, -3, cingulin, RhoA, membrane-associated guanylate kinase inverted 2 (MAGI), and Ras-related C3 botulinum toxin substrate (RAC) can be listed as scaffolding proteins (Sweeney et al. 2018).

1.1.1.1 Claudins

The word of claudin is derived from the Latin word ‘claudere’ meaning ‘to close’. First, claudin proteins were defined in 1998 by Shoichiro Tsukita in a chicken liver and showed that claudins are located at the cell membrane. Since then, many claudin proteins have been found and characterized in mammalian tissues and cells. There are 27 claudin

proteins expressed in mammals, but the claudin-13 expression is lacking in human cells. Claudin protein sequences show close similarity with other claudins.

Claudin protein expression is altered in different tissue types. Claudin-2 and -5 are expressed in the stomach and claudin-3 is heavily expressed in the epithelial cell surface of the stomach. In humans, claudin -1 to -5, -7, -8, -10, -15, -18, -20, -21, 23 are expressed in the intestinal system. Depending on the cell type, claudin protein expression patterns can be differentiated in the same tissue. Claudin -1, -3, -5, -11, and -12 proteins expressions have been found in the brain microvessel endothelial cells (Song et al., 2007; Winkler et al., 2021; Zlokovic, 2008). Phylogenetic tree of claudins showed in Figure 1.4.

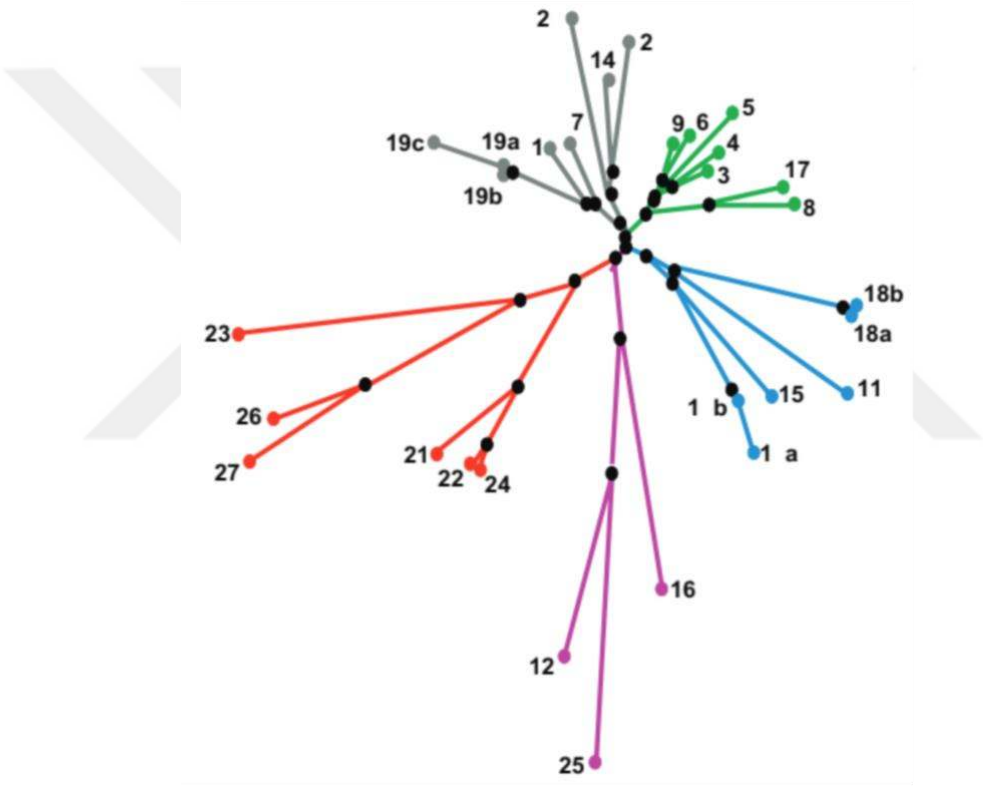

Figure 1.4 The phylogenetic tree of the claudin protein family. Retrieved from Gunzel and Fromm 2012.

Human claudin proteins' amino acid size can be varied between 207 and 305 amino acids and their molecular size can be changed to 21-34 kDa. All claudin proteins have a four-transmembrane domain and consist of an NH2 terminus, one extended COOH terminus, and one short intracellular loop. Variations of this terminus lay down for the differentiation of claudin proteins. For example, claudin-5 protein contains a more extended NH2 terminus than other claudin family proteins. Signature features for a claudin

family protein are the COOH terminal PDZ binding domain that creates spaces for binding scaffolding proteins like postsynaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), ZO-1, -2, -3, MUPP1, and MAGI -1 to -3 proteins.

For the understating role of claudins in the cells, overexpression experiments in fibroblast cells reflect that fibroblast cells form barrier properties like other barrier cells, however their leaky barrier condition because they do not form tight barrier conditions such as brain microvessel endothelial cells (Bhat et al., 2018; Shi et al., 2020). The knockout and knock-down experiments are conducted for the understanding role of claudins in the cells. Based on these experiments, it is undoubtedly known that claudin proteins are the main driving force for tissue barrier-forming properties. Also, mutations in claudin proteins cause several diseases in BBB and other barrier-forming tissues.

Barrier-forming abilities of claudin proteins remain unclear, but claudin proteins must assemble for proper barrier functions. TJ regions contain more than one claudin protein in many cells, but their structures and subunit polymerization are unknown. Following fibroblast transfection with claudin-1 and -2, fibroblast cells display the same fibril formation as the epithelial and endothelial cells under electron microscopy (Furuse, 1998). To better understand individual claudin polymerization ability, Yamazaki 2003 et al expressed different claudin proteins in the epithelial S7 cells that lack most claudin proteins, and they showed that claudin-7, -14, and -19 can manage strands and polymerize, but other claudins do not polymerize. Different claudin proteins form cis heterotypic interactions; for example, claudin-16 and -19 proteins interact, even when claudin-16 is not localized into the TJ (Hou & Goodenough, 2010).

On the other hand, trans interactions also occur in TJ proteins. Claudin-1, -2 and -3 interact homotypically with trans connections. Also, claudin-1 and -3 have trans heterotypic interactions with each other. Extracellular loop 1 (ECL1) mutations on claudin-3 and -4 create heterotypical trans interactions between two proteins unlike they do not have trans heterotypical interactions between them. Besides that, normally claudin-1 and claudin-5 can interact, but mutations on the ECL2 domain of proteins block the trans heterotypical interactions.

L cells fibroblast transfected with claudin-1 form linear strands, but the same cells were also transfected with tricellulin, and they create branches of strands. Expressing tricellulin protein alone does not develop any fibril formation in these cells. When HEK293

cells are transfected with claudin-1 or claudin-5, MARVEL proteins move to cell contact sites and create bridges among TJ strands. However, depending on the type of MARVEL proteins, their binding priority changes, for example, claudin-1 is highly connected with occludin instead of other MARVEL proteins.

In summary, it has been shown that proper barrier properties need claudin interactions and these can be formed by the same claudins and other claudin isoforms. On the other hand, other proteins like occludin, tricellulin, and scaffolding proteins also help to contribute to claudin-claudin connections.

In addition to the barrier-forming properties of claudin proteins, they are also responsible for pore-forming casualties. The knockdown of pore-forming claudin proteins increases the transepithelial resistance, while the knockdown of barrier-forming claudins causes the decrease of resistance. Depending on claudin protein type, pore selectivity is changed. Pore selectivity or pore characteristic is mainly constituted by claudins' extracellular loop one amino acid sequences (ECL1) (Colegio et al., 2003). Claudin-2 is permeable to cations and water, and many studies were conducted on ECL1 of claudin-2 to understand the reason for the altered pore profile (Colegio et al., 2003). Converting on charged amino acids in the ECL1 confirms that D65 residue has an essential role in cation selectivity. These changes increase activation energy for sodium (Na+) ion permeation and these changes do not depend on ionic strength. D65 is crucial for charge selectivity for other claudins too, such as claudin-4, -10a, -15, and -17 (Colegio et al., 2002; Hou et al., 2010; Krug et al., 2012; Van Itallie et al., 2006).

Claudin-1, -3, -5, -11, and -12 have been shown to express in barrier type endothelial cells. However, environmental changes could affect claudin expression patterns. For example, claudin-1 mRNA and protein levels were increased in stroke, and this increased claudin-1 was newly synthesized and gathered at junction complexes. In the same conditions, claudin-5 mRNA levels were decreased and their connections with TJ complexes disappeared. Newly synthesized claudin-1 protein connections replaced these claudin-5 connections. However, in intact conditions, the claudin-1 expression is shallow compared with claudin-5 (300-fold), and claudin-1 protein immunostaining experiments showed that claudin-3 antibodies can also stain claudin-1 protein (Castro Dias, et al., 2019). Despite these findings, there is no clear definition for claudin-1 protein role for barriers in intact conditions.

Claudin-3 is localized in the TJ area of endothelial and epithelial cells only in the embryonic stage and adult choroid plexus (Castro Dias, et al., 2019). Its expression is not observed in epithelial cells of the CSF barrier (Steinemann et al., 2016). On the other hand, recent studies have shown that the role of claudin-3 in BBB is questionable. Claudin-3 knockout mice displayed normal BBB phenotype and commercial claudin-3 antibodies reacted with mysterious membrane protein under claudin-3 knockout conditions (Castro Dias, Coisne, Lazarevic, et al., 2019). He et al reported that single-cell RNAseq data of claudin-3 showed that mRNA levels of this protein were not detected in endothelial cells of the brain (He et al., 2018). These findings postulate that the role of claudin-3 protein in BBB must be reconsidered.

Claudin-12 protein levels are detected in embryonic stages, but not in adults, they remain at deficient levels when compared with claudin-5 protein. In the embryonic stage, claudin-12 is the leading actor for creating barrier properties, and its roles are assigned to claudin-4 in the adult stage (Castro Dias, Coisne, Baden, et al., 2019).

In the brain microvessel endothelial cells, claudin-5 mRNA levels are 600-fold higher than other claudin proteins (Baluk et al., 2007; Daneman, Zhou, Agalliu, et al., 2010), and several studies suggest that claudin-5 is the essential protein for forming BBB. Claudin-5 protein mass is 23 kDa and protein structure contains four transmembrane domains: two ECLs, an intracellular NH2 terminus, a long COOH terminus, and a short intracellular loop and that intracellular loop shows similarity with other claudin family proteins. ECL1 is responsible for the barrier-forming properties of TJs. ECL1 cysteine residue mutations caused increased permeability for mannitol and monosaccharides in MDCK cells (Wen et al., 2004). Although embryonic claudin-5 null mice have intact BBB, 800 Da proteins could pass into the brain parenchyma. In addition to this, mice died 10 h after birth, and the reason for that remains unknown, but the claudin-5 expression is observed in other tissues such as the liver and kidney (Nitta et al., 2003).

Brain microvessel permeability was changed in embryonic stages 11 and 13 in mice depending on claudin-5 protein expression. At this point, brain microvessel endothelial cell permeability is very high, and the claudin protein expression pattern alters this status. After claudin-5 protein expression started, brain microvessel endothelial cell permeability severely reduces and intact BBB develops (H. C. Bauer et al., 1993; Wolburg & Lippoldt, 2002).

In neuroinflammatory conditions, microglial cells release chemoattractant molecules for the migration of leukocytes into the brain parenchyma from the blood. Depending on microglia activation, BMVECs are also activated, and they upregulate cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) for facilitating leukocyte penetration into the inflammation sites. During leukocyte penetration, the actin cytoskeleton is reshaped to reorganize claudin-5 connections because claudin-5 restricts penetrations of leukocytes under intact conditions (Winger et al., 2014). Claudin-5 can be detected in the leukocytes because in the inflammatory state, leukocytes start to express claudin-5 and they cover their surface with claudin-5 to alleviate the trans endothelial migration (Paul et al., 2016). The treatment with glucocorticoids in neuroinflammatory conditions causes a decrease in claudin-5 levels in leukocytes (Mandel et al., 2012; Paul et al., 2016).

Despite other claudin proteins in BBB, claudin-5 is always expressed in brain microvessel endothelial cells, and its expression pattern can be reorganized depending on the conditions. In stroke, claudin-5 is downregulated with other TJ proteins mainly after 48-58 h, and then this causes biphasic changes on BBB (Knowland et al., 2014). In psychiatric diseases, for example in schizophrenia, claudin-5 expression decreases in the frontal cortex of postmortem tissues (Nishiura et al., 2017). However, there is no clear consensus on depression and inflammation relation, but claudin-5 levels are decreased in depression state (S. Lee et al., 2018; Sántha et al., 2015). On the other hand, claudin-5 downregulation in nucleus accumbens may cause depression-like behavior in mice (Menard et al., 2017).

1.1.1.2 Occludin

Occludin is etymologically derived from occlude in the Latin language, meaning to close up. Occludin is the first identified integral TJ protein in chick embryos, and it is a tetraspan of TJ protein and can be counted as one of the MARVEL proteins containing the MAL domain (Furuse et al., 1993; Steed et al., 2009). It contains a long COOH chain for binding ZO-1 and -2 proteins. TJ formation of occludin comes from ECL loops (Y. Chen et al., 1997; Furuse et al., 1994; Yuanhe Li et al., 2005; J. K. Walter et al., 2009; Juliane K. Walter et al., 2009).

There is no clear identification of the roles of occludin on BBB. Occludin knockout mice show intact barrier formation and normal morphological functions. Loss of occludin protein causes testicular atrophy, brain calcifications, which can only be detected under histological examinations (Saitou et al., 2000). On the other hand, embryonic stem cells are differentiated from epithelial cells and show normal barrier function without occludin expression (Saitou et al., 1998). Overexpression of chicken occludin in the MDCK cell line increases trans endothelial electrical resistance (TEER) values (McCarthy et al., 1996). Truncated occludin addition into Xenopus embryo cells increases leakage and insertion of recombinant ECL2 protein of occludin into the cell media resulting in decreased TEER values than usual in Xenopus cells (Balda et al., 1996; Y. Chen et al., 1997; Wong & Gumbiner, 1997). In short, occludin is considered as one of the vital elements for maintaining the BBB phenotype, although there is no clear consensus on the role of occludin on BBB integrity in developmental and mature conditions.

In disease conditions, occludin protein levels are altered. In sepsis, occludin presence in BBB is decreased when compared with healthy conditions but intravenous IgG applications restore these protein levels in the brain (Esen et al., 2012). TNF-α and interferon-gamma (IFNγ) need to occludin to activate cellular signalling, and these two molecules bind the C terminal of occludin and may cause degradation of occludin in septic conditions (Buschmann et al., 2013). Other disease conditions, such as hypertension and irradiations, cause a reduction in occludin levels compared to the healthy subjects (Kalayci et al., 2009; Kaya et al., 2004).

Matrix metalloproteinases (MMPs) are responsible for the degradation of TJ protein structures. In rat models of cerebral ischemia, MMP levels are increased, especially MMP-2 is increased in early conditions of ischemia and are mainly responsible for cleavage of occludin (A. T. Bauer et al., 2010; W. Chen et al., 2009). The causative factor in BBB disruption during the brain edema is mainly MMP-9 dependent degradation of occludin in the barrier type endothelial cells (Higashida et al., 2011). Applying Multiple sclerosis (MS) patients' serum samples to mouse capillary endothelial cell line causes downregulation of occludin and claudin-5 with upregulation of MMP-9 (Blecharz et al., 2010).

1.1.1.3 Other Tight Junction-Associated Proteins

Tricellulin is also named as MARVELD2 protein and its size is nearly 65 kDa (Heinemann & Schuetz, 2019). Tricellulin was first identified in rat intestinal epithelial cells (Staehelin, 1973). This protein is mainly observed in tricellular TJs and less frequently seen in bicellular TJs (Figure 1.5). Tricellulin knockdown causes decreased TEER measurement and increased 4 kDa dextran tracer in the human mammary gland cell line (Ikenouchi et al., 2005). Overexpression of tricellulin in Madin-Darby Canine Kidney cells decreases the passage of macromolecules (Krug et al., 2009). Ion passages show no difference in both conditions (Ikenouchi et al., 2005; Krug et al., 2009).

In other tricellulin knockdown experiments, occludin organizations are altered depending on the absence of tricellulin, and double knockout with occludin and tricellulin conditions tend to be distributed cellular connections (Saito et al., 2021). Tricellulin proteins try to compensate for the loss of occludin protein in the cells (Ikenouchi et al., 2008). In summary, tricellulin can be considered as regulatory elements of TJs rather than mandatory elements of them.

Junctional adhesion proteins (JAMs) are members of the immunoglobulin superfamily and are expressed in various cell types of mainly leukocytes, endothelial and epithelial cells. Generally, they are located between the cell connection sites and are mainly found in regions of TJs (Klaus Ebnet et al., 2004). They have a single transmembrane domain, two extracellular loops, and one PDZ domain for cell skeleton interactions (Garrido-Urbani et al., 2014; Martìn-Padura et al., 1998). There are three types of JAM family; JAM-A, JAM-B, and JAM-C, and they are mainly located in TJ regions (Klaus Ebnet et al., 2004). JAM-B and JAM-C localization are not mandatory for establishing proper BBB functions. Knockout of JAM-B and -C represent intact barrier formations (Mochida et al., 2010; Tietz et al., 2018; Wyss et al., 2012). Unlike JAM-B and -C, JAM-A has a certain role in BBB functioning. JAM-A transfection develops barrier formation in the Chinese hamster ovary cell line (Otani et al., 2019; Padden et al., 2007). At the same time, JAMs have roles in cell motility, shape, and polarity. JAM-A-deficient endothelial cells form more protrusions than normal endothelial cells do (Gianfranco Bazzoni et al., 2005). On the other hand, JAMs are closely associated with inflammation because JAMs are expressed in circulating inflammatory cells, except JAM-A and JAM-B are not observed in leukocytes (Martìn-Padura et al., 1998; Palmeri et al., 2000; Williams et al., 1999). TNF-α and INF-ɣ cause reorganization of JAMs in endothelial cells, and JAM-A

facilitates migration of leukocytes into the brain parenchyma under these conditions (Ostermann et al., 2002; Ozaki et al., 1999).

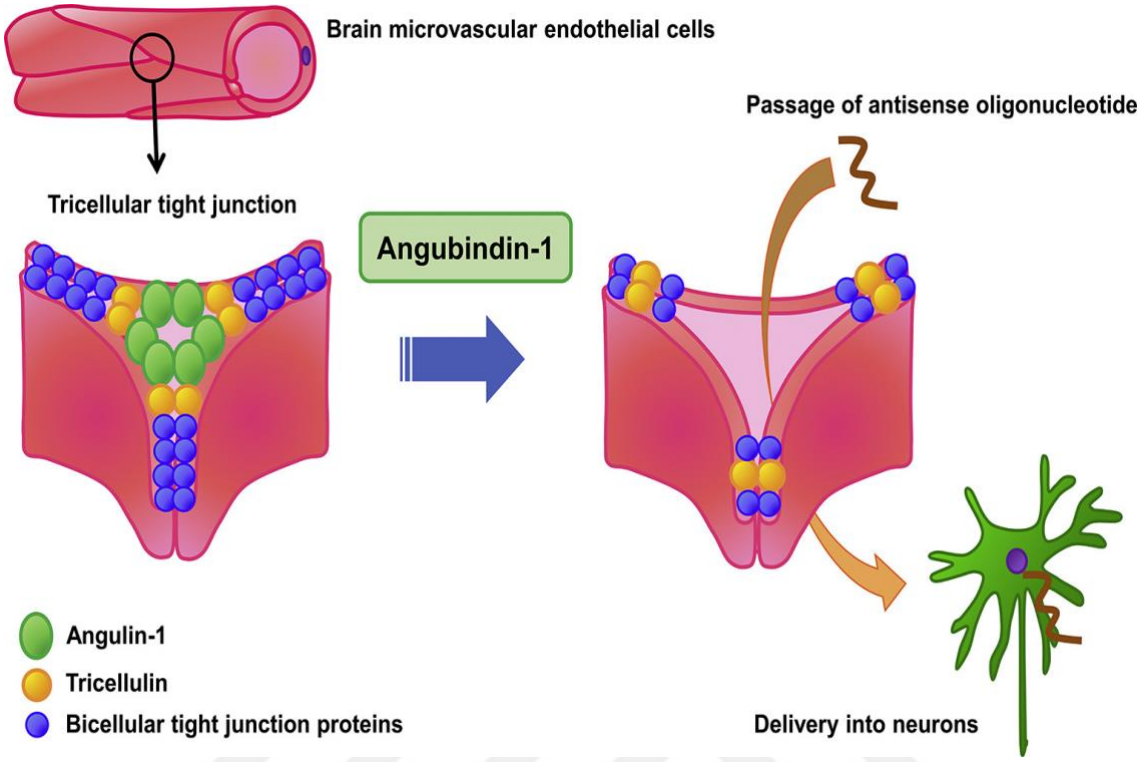

Figure 1.5 The representation of tricellular junctions of the BBB. Retrieved from Zeniya et al., 2018.

1.1.1.4 Regulatory Tight Juntion Proteins

Tight junction proteins bind each other and seal the cellular space, but these connections are not sufficient to maintain barrier function without cell skeleton bindings. For this purpose, some proteins are specialized for building bridges between TJ proteins and cell skeleton. Zonula occludens proteins are the most important protein family for the stabilization of BBB (Kadry et al., 2020; Kaya & Ahishali, 2021).

Zonula occludens proteins can be listed under the membrane-associated guanylate kinase-like protein (MAGUK) and located in the inner surface of the cell membrane. Zonula occludens proteins contain three PDZ (PDZ-1, PDZ-2, and PDZ-3) domains, one SH3 domain, and one guanylate kinase-like (GUK) domain (K. Ebnet et al., 2000; Itoh et al., 1999; Mitic et al., 2000). PDZ domains act as binding sites for TJ proteins and actin binds to -COOH terminal of Zos (Haskins et al., 1998). Zonula occludens-1 (220 kDa), ZO-2 (160 kDa), and ZO-3 (130 kDa) belong to the ZO family protein, and they have the

same responsibilities in the cell (Kaya & Ahishali, 2021). However, ZO-3 expression was not observed in brain barrier type endothelial cells, however ZO-2 regulates ZO-1 connections on TJ proteins in the barrier type of endothelial cells. Moreover, ZO-1 can be considered as the main regulator of TJ-actin connections, and the absence of ZO-1 results in a leaky BBB phenotype. On the other hand, the knockdown of ZO-1 and ZO-2 is lethal for animals (Katsuno et al., 2008; Phua et al., 2014; J. Xu et al., 2008).

JAM-A, occludin, and claudin-5 have binding sites on ZO-1 (G. Bazzoni et al., 2000; K. Ebnet et al., 2000; Nomme et al., 2011; Ooshio et al., 2010; Tash et al., 2012). Loss of ZO-1 disturbs the binding of these TJ proteins and causes leaky barriers as claudin-5 deficiency (Otani et al., 2019). Knockdown of ZO-1in human dermal microvascular endothelial cells shows that JAM-A binding is distributed, and claudin-5 stabilizing is damaged due to the absence of ZO-1 (Tornavaca et al., 2015). Under pathological conditions, ZO-1 levels are correlated with disease severity. Stroke, encephalopathy, and cerebral hemorrhage conditions cause the decrease of ZO-1 protein expression in barrier-type endothelial cells (Ding et al., 2014; Huang et al., 2013; Zehendner et al., 2013; Zhou et al., 2014). Other *in vivo* studies conducted in epilepsy or irradiation in rats reported a decrease in the immunolabeling of ZO-1 in brain slices (Arican et al., 2006; Kaya et al., 2004). The addition of endogenous ZO-1 to the MDCK cell line disrupts the continuous TJ proteins line between the two adjacent cells (Beutel et al., 2019).

1.1.2 Adherens Junctions

Adherens junctions' protein (AJp) can be found in nearly all cells in the human tissues. These junctional proteins generally locate the edges of the cells and help to seal two cells (Gavard, 2013). Depending on the tissue, their expression pattern changes. Vascular endothelial cadherin (VE-cadherin) is the main AJp in BBB, however, N-cadherin and E-cadherin expressions are seen in the barrier type endothelial cells of the brain (Abbruscato & Davis, 1999; Luo & Radice, 2005). In addition to these AJps, neural cadherin expression is detected in barrier type endothelial cells of the brain, but it is reported that their roles differ from other AJps (Gerhardt et al., 2000; Salomon et al., 1992). Their interaction with each other AJp frail than TJs. AJp's can interact with the ZO protein family, but they mainly interact with α-catenin, p120-catenin, ß-catenin, vinculin, and eplin for stabilizing cadherins (Broman et al., 2006; Dejana, 2004).

VE-cadherin is an endothelial cell-specific protein, and it has been used for the detection of the endothelial cells in transgenic animal models (Carmeliet et al., 1999; Vittet et al., 1997). Knockout of VE-cadherin is fatal for animals, and newborn animals cannot continue their life (Carmeliet et al., 1999; Vittet et al., 1997). Same studies showed that the loss of VE-cadherin altered the angiogenesis of endothelial cells and increased the endothelial cell death with increased VEGF releases (Carmeliet et al., 1999; Vittet et al., 1997). The loss of BBB catenins reflects the same result with VE-cadherin (Herron et al., 2011). AJps are not required to maintain BBB, but their roles in the BBB are mainly associated with governing angiogenesis and regulating permeability changes in the barrier type endothelial cells (Harris & Nelson, 2010).

1.1.3 Transcytosis in BBB

A molecule can use two different mechanisms for passing through endothelial cells (Kaya & Ahishali, 2021). The first one is the paracellular route, which is controlled by TJ proteins for molecules except the small molecules such as carbon dioxide and NO (Abbott et al., 2006; Kaya & Ahishali, 2021). Due to tight control of TJ proteins, molecules generally use a transcellular route for achieving transport into the brain parenchyma and also into the blood. The second one is the transcellular route can be defined as absorbing molecules located outside of the cells, and then transferring them to the other side of the cells. With the help of transcellular route, cells can easily control the entry of molecules into the brain parenchyma. Barrier type endothelial cells of the brain have different routes for different molecules or cell transportation (Figure 1.6.) (Pulgar, 2018).

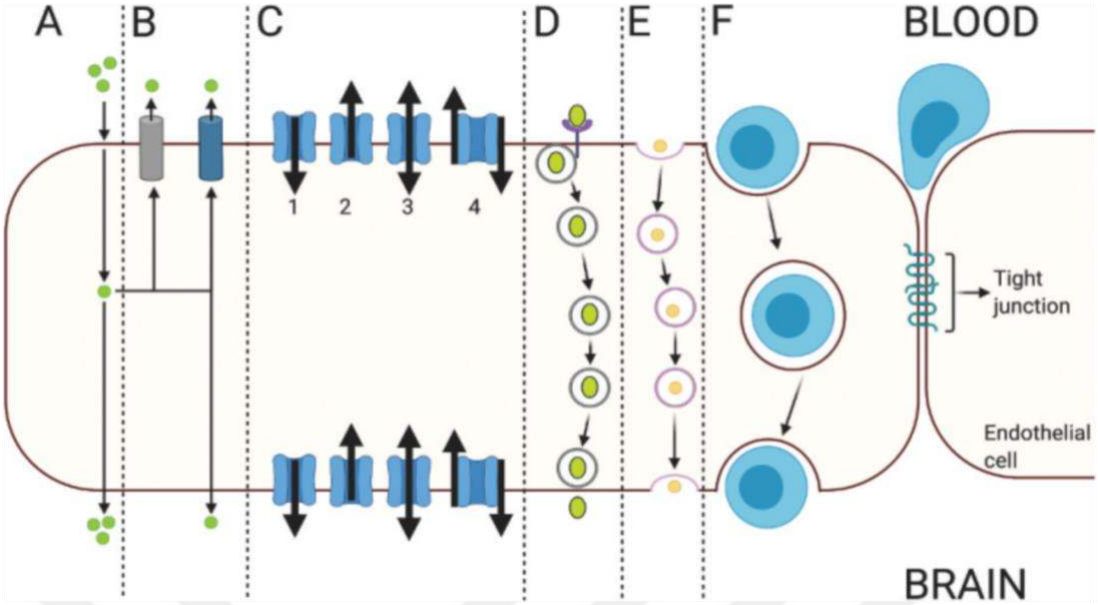

Figure 1.6 The schematic representation of transcytosis in the BBB. Retrieved from Kaya and Ahıshalı 2021.

1.1.3.1 Passive Transport

Thermodynamic law shows that molecules move from areas of high concentration to low concentration (Skene, 2015). Brain barrier type endothelial cells generally use facilitated diffusion for molecular entry into the cell and out of cell. Gases and low weight lipophilic molecules (<400 Da) can freely diffuse through barrier-type endothelial cells (Abbott et al., 2010; Daneman & Prat, 2015; William M. Pardridge, 2015) (Figure 1.6. A).

1.1.3.2 Efflux Transport

Barrier type of brain microvessel endothelial cells also have efflux transporters which are responsible for pumping xenobiotics, such as a drug, pesticide, or carcinogen back circulation to protect the brain. Efflux transporters use energy that comes from the hydrolysis of ATP for transporting molecules against their concentration gradient, and they are mainly located on the luminal side of the barrier type endothelial cell membrane (Abbott et al., 2010; Löscher & Potschka, 2005; Sweeney et al., 2019). However, in some cases,

efflux pumps also limit the entry of neuroprotective agents or drugs using in treatment of neurological diseases/disorders.

ATP-binding cassette (ABC) transporters and multidrug resistance-related transporters (MRPs) are the most known efflux transporters, and they are expressed in the luminal side of barrier type endothelial cells. ABC transporters are expressed in almost all cell types, but they are mainly specialized to endothelial and epithelial cells that are responsible for creating a barrier (BBB, blood-cerebrospinal fluid barrier, blood-testis barrier) (Campos et al., 2012; A. M. S. Hartz & Bauer, 2011; Robillard et al., 2012) (Figure 1.6. B).

P-gp (MDR1), multidrug resistance-associated protein (ABCC – MRPs), and breast cancer resistance protein (BCRP - ABCG2) are the main efflux transporters in BBB. Their expressions can change depending on the disease phenotype. In epilepsy, p-gp, MRPs, and BCRP are increased, which is why nearly 30% of epilepsy patients cannot be treated well (Löscher & Potschka, 2005). In Alzheimer's disease, p-gp expression is decreased, as well as in Parkinson's disease (Anika M. S. Hartz et al., 2010; van Assema et al., 2012; Westerlund et al., 2008). In addition, the expression levels of efflux proteins are increased in the certain type of brain tumors (Adkins et al., 2013; Demeule et al., 2002; Fletcher et al., 2010).

1.1.3.3 Carrier-Mediated Transport

Barrier type capillary endothelial cells restrict the entry of most of the molecules into the brain, but neurons demand specific molecules for their normal working conditions (Figure 1.7.). For example, ion concentration is one of the most critical factors for the neuronal environment, and also glucose can be considered in that manner. For that purpose, barrier type endothelial cells have a specific transport system to deliver these molecules into the brain parenchyma (Daneman & Prat, 2015). These transport systems are called solute carrier (SLC) transporters. The SLC gene family consists of more than 300 genes for the same targeted molecules (Lin et al., 2015). For example, glucose transporter-1 (GLUT-1), which is also known as SLC2A1, transports glucose from blood to the brain (Kaya & Ahishali, 2021) These SLC transporters can be located on both sides of barrier type endothelial cells and they can work bidirectional or unidirectional or act as pumps that

allow one molecule into cells and release other molecules to the outside of the cells (Sweeney et al., 2019). SLC transporters carry amino acids, carbohydrates, ions, nucleic acids, vitamins, fatty acids, and hormones into the brain and each transporter has unique properties to achieve (Abbott et al., 2010; Pardridge, 2015) (Figure 1.6. C).

Loss of any one of these SLC transporters can cause neurologic deficits. For example, some developmental neurologic disorders are linked with loss of T3 thyroid hormone transporter (SLC16A2) (Kersseboom et al., 2013). On the other hand, inhibition of GLUT-1 activity does not affect BBB permeability but causes neurologic disorders such as epilepsy (Benarroch, 2014). These SLC transporters are also targeted for drug delivery against neurological diseases (William M. Pardridge, 2015). Conjugating desired drugs with glucose can be used for efficient drug delivery to the brain parenchyma (Ugur Yilmaz et al., 2020).

1.1.3.4 Receptor-Mediated Transport

Larger molecules have specific mechanisms for bypassing BBB and these mechanisms are called receptor-mediated transport (RMT) (Figure 1.7.). In this context, when a ligand binds to its receptor, it gets surrounded by a vesicle and enters into the barrier type endothelial cells. After that vesicle reaches the other side of the cell, so it releases its cargo into the brain parenchyma. Most of the RMT uses clathrin-coated vesicles, but their ligand specificity decreases by aging (William M. Pardridge, 2005; Pulgar, 2018). Well-known RMTs are transferrin (TfR), insulin, and lipoprotein receptors (LRP1 and LRP2). Trojan horses' drug delivery methodology mainly targets TfR and insulin receptors to increase the transport rate of the drugs into the brain parenchyma (William M. Pardridge, 2015) (Figure 1.6. D).

1.1.3.5 Adsorptive-Mediated Transport

Once the negatively charged cell membrane can interact with positively charged peptides, the adsorptive-mediated transport (AMT) begins (Abbott et al., 2010; Tsuji &

Tamai, 1999). AMT mainly uses the caveolae for endocytotic processes (Pulgar, 2018) (Figure 1.6. E).

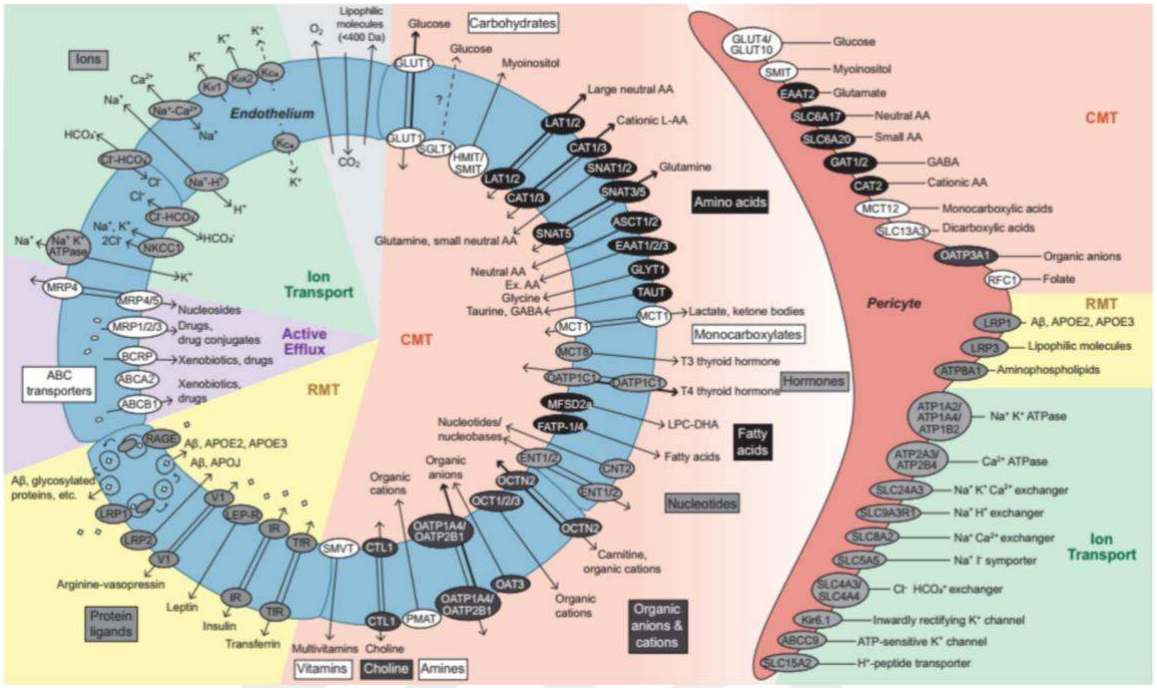

Figure 1.7 Schematic representation of transcytotic mechanisms in brain microvascular endothelial cells and pericytes. Retrieved from Sweeney et al., 2018

1.1.3.5.1 Caveolae

Endocytosis is required for transcytosis. In the BBB, caveolae is the main endocytosis mechanism. Endothelial cells contain caveolar pits which are 50-80 nm in diameter (Tuma & Hubbard, 2003).

Caveola was first described in 1953 by G.E. Palade under electron microscope and then named by Yamada in 1955 (Palay & Palade, 1955; Yamada, 1955). Caveolae are named after their cave-like-shaped formation in cellular membranes (Figure 1.8.). In 1992, Rothberg et al defined caveolin-1 (Cav-1) as one of the components of caveolae (Rothberg et al., 1992). After the discovery of Cav-1, researchers found that Cav-2 and Cav-3 are the other components of caveolae, but Cav-1 is considered as the main protein for caveolae (Chidlow & Sessa, 2010). Cav-1, Cav-2, and Cav-3 are not only the required proteins for transcytosis, but also, they have a regulatory role in the caveolar process. The family of cavin proteins is cavin-1, cavin-2, cavin-3 and cavin-4 (Bastiani et al., 2009; Gustincich et al., 1999; Izumi et al., 1997; Vinten et al., 2005; X. L. Xu et al., 2001).

Caveolin-1 expression is steadily controlled in barrier type endothelial cells in the brain unless a pathological condition occurs. Under pathological conditions, Cav-1 expression is increased, which is correlated with enhancement of BBB permeability (Andreone et al., 2017; Gu et al., 2012; Zhao et al., 2011). Caveolin-1 also has a role in the TJ structure of the barrier. Many studies reflect that TJ proteins are affected by changes in Cav-1 protein levels in barrier (Ares et al., 2019; Yao Li et al., 2015; Song et al., 2007; S. Xu et al., 2016)

Increased Cav-1 protein levels could be a sign of a pathological condition in the CNS (Gu et al., 2012). In the aging mouse studies, it has been shown that uplifted Cav-1 level results in increased BBB permeability (Andreone et al., 2017; Gu et al., 2012; Zhao et al., 2011). On the other hand, under some pathological conditions, Cav-1 expression is upregulated in barrier type endothelial cells of the brain (Ahishali et al., 2010; Atış et al., 2019).

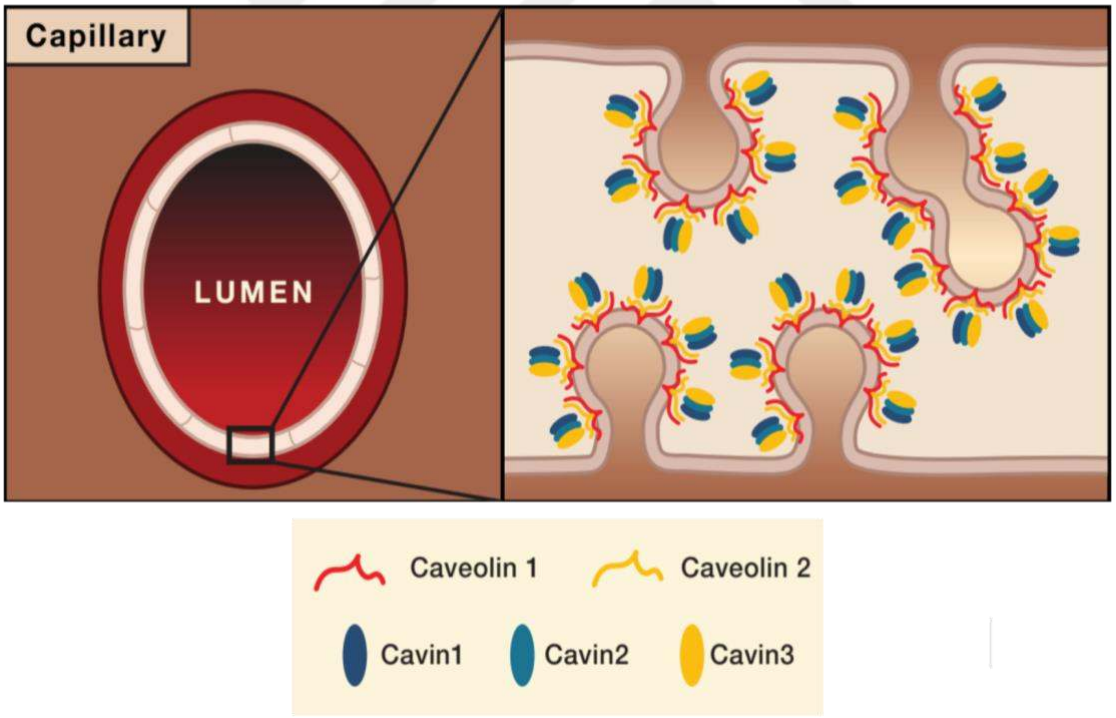

Figure 1.8 The schematic representation of caveolin protein in the brain microvascular endothelial cell. Retrieved from Ariotti and Parton 2013.

1.2 Astrocyte

In the 19th century, Virchow described neuroglia as the connective tissue of the brain. At the end of the 19th century, Lenhossék defined a new cell type as a star-shaped glial cell and termed it as an astrocyte (Lenhossek, 1898). Astrocytes can interact with neurons, other glial cells, pericytes, and endothelial cells to create equilibrium for keeping the exact brain functioning. On the other hand, astrocytes can be differentiated based on their localization which gives them specific functioning (Zalc, 2002).

Depending on the neuronal activity, astrocytes can sense the demands of adjacent neurons, and thereafter adjust the blood volume and molecular entry into the brain parenchyma (Figley & Stroman, 2011). Astrocytes can be used as an indirect blood regulatory system for neurons.

They cover blood microvessels via their astrocytic end feet process and their coverage ratio is nearly 99% of blood vessels surface (Mizee & de Vries, 2013). Astrocytes bind to basal lamina on NVU instead of binding directly to barrier type endothelial cells. Cytoskeletons of astrocytes are related to an agrin protein of basal membrane (BM), which acts as a docking site via dystroglycan-dystrophin (DD) complex (Noell et al., 2011; Wolburg et al., 2011). The binding of astrocyte end feet with BM is important for the regulation of specific transporters. For example, aquaporin (AQP)-4, a water channel, is mainly responsible for water transport in astrocytes, and its function can rely on proper DD and agrin links (Noell et al., 2011; Wolburg et al., 2011). Other transporters, such as p-gp, GLUT-1, and Kir4.1 K+ channels, also embody astrocytes (Leino et al., 1997; Mercier et al., 2004; Olsen & Sontheimer, 2008). On the other hand, these end feet and basal lamina connections also create glia limitans, which block the penetration of some molecular passage into the brain parenchyma.

Astrocytes have detrimental roles in BBB integrity. In 1967, Davson and Oldendorf grew brain microvessels outside of the brain and they showed these microvessels lack barrier properties, but the growth of these brain vessels in the brain tissue grafts represented tight barriers (Davson & Oldendorf, 1967). The opposite of this work was also investigated, when astrocytes were transplanted into the areas that contain leaky vessels, the ability of the astrocytes to constitute a barrier phenotype from that leaky endothelium was attenuated (Vinten et al., 2005).

In addition to these *in vivo* studies, monolayer brain microvascular endothelial cells (BMVECS) are not capable of establishing tight barrier formation in *in vitro* like under normal conditions (Reinhardt & Gloor, 1997). In some aspects, they lack some transporter receptors and TJ proteins, leading to decreased TEER values (Abbott, 2002; Testa et al., 2001). The addition of astrocytes upregulates TJ proteins, and as a result, TEER values become much higher than under monolayer conditions. This can also be discussed for the transporter systems. For example, GLUT-1 is important for balancing energy consumption and astrocyte upregulates GLUT-1 transporters in barrier type endothelial cells (Abbott, 2002; el Hafny et al., 1996; Testa et al., 2001). The addition of an astrocyte-conditioned medium instead of astrocytes also induces barrier properties in the BMVECs with the help of secreted proteins. For supporting the BBB phenotype, astrocytes secrete sonic hedgehog (Shh), retinoic acid (RA), angiopoietin-1 (Ang-1), TGFβ, VEGF, and fibroblast growth factor (FGF) (Alvarez et al., 2011; Gurnik et al., 2016; Haseloff et al., 2005). All these secreted proteins promote the tightness of the BBB. On the other hand, barrier type endothelial cells also secrete leukemia inhibitory factor-1 for astrocyte differentiation (Engelhardt & Liebner, 2014). Astrocytes are not required for just controlling molecular passage, but also, they have a role in controlling cellular passage into the brain parenchyma (Abbott et al., 2006). All these previous data show that astrocytes are related to the formation and maintenance of BBB and the presence of astrocytic factors is crucial for proper BBB integrity and function.

1.3 Pericyte

Pericytes can be defined as the cell type located adjacent to the capillary endothelial cells. They were first described by Rouget in 1870 and named after their localization by Zimmermann (Rouget, 1870). Pericytes, except the CNS residents, are originated from mesoderm; however, CNS pericytes are derived from neural crests (Majesky, 2007). This is not the only difference between peripheral and CNS pericytes. In the peripheral tissue, the pericyte ratio is 1:100, however, the pericyte which contributes to the NVU ratio is 1:1 to 1:30 (Majesky, 2007; Shepro & Morel, 1993).

Pericytes share the same basal lamina with barrier type endothelial cells and they cover nearly all abluminal surfaces of endothelial cells (Shepro & Morel, 1993; von Tell et al., 2006). Pericyte and endothelial cells use different integrin proteins for attaching to the

basal lamina (Díaz-Flores et al., 2009; Stratman et al., 2009). When pericyte coverage reaches the zone without basal lamina, the pericyte makes direct peg-and-socket contact points with endothelial cells for transferring nutrients, metabolites, ions, and messenger molecules for the precise interactions to keep BBB healthy. Adherens junction proteins, N-cadherin and connexin-43 hemichannel, are responsible for transferring these molecules between two cells of NVU (Bobbie et al., 2010; Díaz-Flores et al., 2009; F. Li et al., 2011; Ornelas et al., 2021).

In addition to supporting the mechanical stability of endothelial cells, pericytes also help to regulate cerebral blood flow, basal lamina production, differentiation of NVU cells, transcytosis, and immune cell passage into the brain parenchyma (Armulik et al., 2005; Hartmann et al., 2021; Török et al., 2021; von Tell et al., 2006). Pericyte depletion studies show how pericytes are important for the proper functioning of the BBB. The total loss of the pericytes in the developmental state causes vascularization is damaged and death at birth. On the other hand, both maturation and adulthood depletion of pericyte increases immune cell migration into the brain parenchyma (Armulik et al., 2010; Daneman, Zhou, Kebede, et al., 2010). To understand the role of pericytes in cerebral blood flow, Hartmann and colleagues depleted a single pericyte with laser and measured the change of cerebral blood flow, which is adjacent to the endothelial cells, and they found that cerebral blood flow increased after pericyte depletion (Hartmann et al., 2021). In addition to these findings, the loss of pericyte due to aging can decrease the expression levels of TJ proteins and increase transcytosis into the brain parenchyma, eventually leading to BBB breakdown and increasing the susceptibility of neurodegenerative disorders/diseases (Bell et al., 2010).

1.4 Microglia

Microglial cells make up 10-15% of the CNS glial cell population and they differentiate into non-neuronal tissue. They penetrate the brain parenchyma during embryogenesis just before the proper BBB is constructed. Their main function is defined as creating a response to brain injury including inflammation, but their role is just not limited to that. They also help with BBB functioning, neuronal circuit development, synaptic pruning, and neurogenesis and astrogenesis (Kabba et al., 2018; Pelvig et al., 2008).

Microglia are not directly contacted with barrier type endothelial cells, but they secrete chemokines, cytokines, and growth factors to change the BBB behavior. After microglial activation, microglia release proinflammatory cytokines that can cause BBB disruption (Thurgur & Pinteaux, 2019). During inflammation, microglial cells secrete MMP-9 that can disturb BBB (J. Y. Lee et al., 2015). Besides that, endothelial cells can activate microglial cells (Thurgur & Pinteaux, 2019).

1.5 Neuron

Neurons use 20% of the energy which is produced in the whole body and BBB disruption is correlated with neuronal disorder or disease. For example, the extravasation of albumin into the brain parenchyma results in hyperexcitability of neurons, furthermore, the loss of neutral amino acid transporter (Slc7a5) causes changes in the brain and leads to autism spectrum disorder (Pelvig et al., 2008; Senatorov et al., 2019; Tărlungeanu et al., 2016).

Neurons can send the required substances directly to the endothelial cells for their functioning, including toxic removal, via contact sites that also regulate vascular blood flow through their connection with other cell types of NVU (Chow et al., 2020; Hawkins & Davis, 2005; Kaplan et al., 2020). On the other hand, neuronal cues during the angiogenesis process help vasculogenesis and develop barrier phenotypes (Canfield et al., 2019; Zlokovic, 2008).

1.6 Basement Membrane

Basement membrane (BM) is located between endothelial cells and pericytes, separating them. Basal membrane is mainly constituted by agrins, laminins, collagen type IV, fibronectin, elastin, and heparan sulphate, which are produced by endothelial cells and pericytes (Rempe et al., 2016). These proteins are used for docking sites of the cells and also give them physical support. There is another membrane that is mainly formed by an astrocyte end-feet process, lays on between pericyte and astrocyte, and it is called glia limitans or parenchymal BM. These two types of BM have differences in their structure, for example, the laminin subtype represents different localization (Thomsen et al., 2017).

Matrix metalloproteinases (MMPs) are not directly located at the BM, but they govern BM stability, which can help the repair process. Besides increased production of MMPs, during inflammatory or disease conditions BM composition is disturbed, leading to alteration in BBB permeability (Sorokin, 2010).

1.7 Histamine

Histamine was described by Windaus and Vogt in 1907, but after that their biological roles were defined by Sir Henry Dale and Laidlaw in 1910 (Barger & Dale, 1910). Dale and Laidlaw found that histamine can act as a vasodepressor during anaphylaxis state (Dale & Laidlaw, 1910). Histamine etymologically comes from the Greek word "histos", which means tissue for isolating nearly all kinds of tissue, but its chemical name is "2-(1*H*-Imidazole-4-yl)ethanamine or imidazolyl ethylamine".

Until 1932, there was no clear definition of the role of histamine in allergic reactions. But after that time point, researchers identified that histamine is one of the main actors for governing allergic reactions (Lieberman, 2011). On the other hand, histamine can be considered as one of the main mediators for inflammatory response (Moriguchi & Takai, 2020). The tuberomammillary nucleus is located in the hippothalamus region of the brain expressing a histamine receptor subtype, which is responsible for governing the sleep-wake cycle of the body, and histamine is also associated with memory and learning functions (Panula & Nuutinen, 2013).

Histamine, produced by various cell types, is only stored in basophils and mast cells. There is clear evidence of the production of histamine in immune cells, such as T cells, dendritic cells, and other cell types including, muscle cells, neurons, astrocytes, and endothelial cells (Akdis & Blaser, 2003; Jurič et al., 2016; Panula & Nuutinen, 2013).

Histamine is a monoamine synthesized from the amino acid histidine through a reaction catalysed by the enzyme histidine decarboxylase enzyme and histidine amino acid can be carried into the cell via L-amino acid transporters (Haas, 1974). Generally, released histamine is methylated by N-methyltransferase for inactivation and turned to t-methyl-imidazole acetic acid (Bowsher et al., 1983; Matuszewska & Borchardt, 1983). On the other hand, diamine oxidase is another enzyme that converts histamine to imidazole acetic acid and is responsible for clearance of histamine that is secreted to the extracellular space

(Mondovi et al., 2013). In the brain, histamine acts as a neurotransmitter and its clearance is very important. For that purpose, histamine is carried from extracellular space into the cell for degradation by vesicular monoamine-transporter (VMAT-2).

How histamine acts in a cell depends on histamine receptor subtypes, and the action of a histamine receptor depends on the stimulation by histamine. There are four different types of histamine receptors in the body. These are called histamine-1 receptor (H1R), histamine-2 receptor (H2R), histamine-3 receptor (H3R), and histamine-4 receptor (H4R). All of these receptors are G protein-coupled receptors (GPCR), but their subtypes are different from each other. Histamine-1 receptor was first discovered in 1937 (Bovet & Staub, 1937). Black et al described H2R, H3R (Arrang et al., 1983; Black et al., 1972). Several groups discovered that there is also another type of histamine receptor called H4R in 2000 (Liu et al., 2001; Nakamura et al., 2000; Oda et al., 2000).

1.7.1 Histamine-1 Receptor

The H1R gene is located on chromosome 3 and contains 487 amino acids. H1R expression is seen in a wide variety of cell types including, lung vascular smooth muscle cells, neurons, astrocytes, microglia, endothelial cells, dendritic cells, neutrophils, monocytes, T and B cells. IL-3, IL-4 and histamine upregulate H1R expression (Horio et al., 2010). H1R is a Gq/11 type GPCR, and its activation triggers the phospholipase C (PLC) signalling pathway (Le Coniat et al., 1994). In addition to the PLC pathway, H1R also activates phospholipase A2 (PLA2) and phospholipase D (Figure 1.9.).

Activation of the PLC signalling pathway leads to degradation of membrane phosphatidyl inositol diphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). DAG activation can culminate in diverse cellular actions. Releasing DAG can induce mitogen-activated kinases (MAPK) similar to H4R actions, upregulation of proinflammatory genes, and also expression of cytokines such as IL-4 (Beermann et al., 2015) Furthermore, DAG can trigger protein kinase C (PKC) to release intracellular Ca^{+2} ions from the storages. IP3 moves to the endoplasmic reticulum to bind its own receptor and be released. Activation of both pathways results in change of gene expression patterns in the cells, such as of nuclear factor kappa B (NF-κB) (Carmeliet et al., 1999). On

the other hand, other signalling pathways, already activated by H1R, result in production of NO (Horinouchi et al., 2020).

Studies have shown that H1R activation is also associated with RhoA activation ((Kugelmann et al., 2018). Releasing Ca^{2+} activates RhoA while inhibiting MLC phosphatase activity, which results in producing stress fibers in cells (Kugelmann et al., 2018).

H1R antagonists are divided into two groups depending on their ability to pass BBB. First generation H1R antagonists can penetrate into the brain and cause drowsiness due to inhibition of H1R located in the neurons. Second generation of H1R (SGH1R) antagonists are not able to penetrate the brain because they can be substrate for p-gp and also metabolized by cytochrome P450 (CYP). In addition to these non-side effect benefits, SGH1Rs gave longer half-time, and specificity compared with the first generation of H1R antagonists (Kugelmann et al., 2018; M. Walter & Stark, 2012).

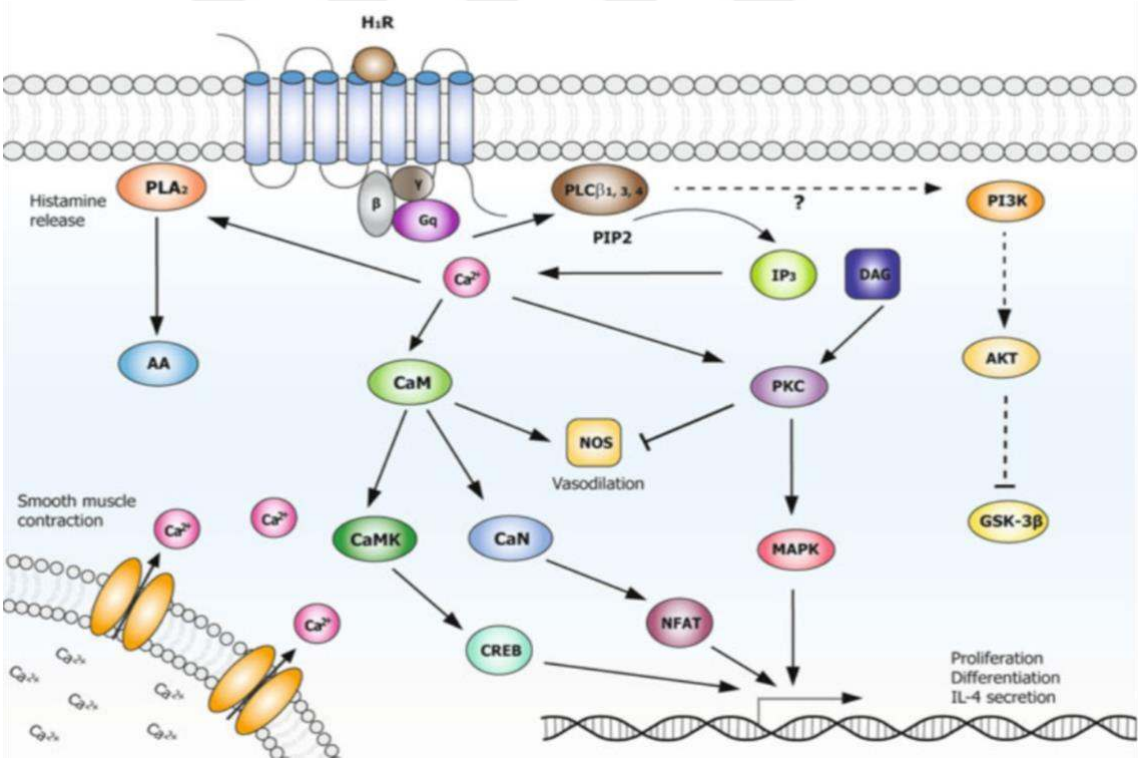

Figure 1.9 The schematic representation of H1R activity. Retrieved from Mocking et al., 2016.
PLA_2: Phospholipase A2, AA: Arachidonic acid, CaM: Calmodulin, CaMK: Calmodulin kinase, CREB: cyclic AMP-response binding element protein, CaN: Calcineurin, NFAT: Nuclear factor of activated T-cells, NOS: Nitric oxide synthetase, PLCβ: Phospholipase β, PIP_2: Phosphatidylinositol 4,5-bisphosphate, DAG: Diacylglycerol, PKC: Protein kinase C, MAPK: Mitogen activated protein kinase, PI3K: Phosphoinositide 3-kinase, AKT: Protein kinase A, GSK-3β: Glycogen synthase kinase 3β, Ca^{2+}: Calcium

1.7.2 Histamine-2 Receptor

The existence of H2R was first suggested in 1966, but it was definitively proven in 1972 by the Nobel laureate Sir James Black (Ash & Schild, 1966; Black et al., 1972). The H2R gene is located on chromosome 5 and contains 358 amino acids (Traiffort et al., 1995). Like H1R, H2R is also expressed in various tissues, including brain, heart and stomach (Gantz et al., 1991). H2R is Gαs type GPCR and induced cyclic adenosine monophosphate (cAMP) pathway, which triggers the cAMP-dependent protein kinase (PKA) (Dove et al., 2004), but there is a report that shows H2R also uses PLC for signalling (Traiffort et al., 1995) (Figure 1.10.).

Activation of cAMP pathway by H2R stimulates the transcription factor cAMP-response element-binding protein (CREB) and leads to promotion of survival, inhibition of IL-12, proliferation, and differentiation of cells (Wellner-Kienitz et al., 2003). Downstream signals of cAMP pathway phosphorylate L-type Ca^{2+} channels, increasing Ca^{2+} influx and causing relaxation of smooth muscle cells (Cheng et al., 2008). PKA also initiates H+/K+ exchanger and is responsible for gastric acid secretion in the stomach (Tricco et al., 2012). H2R activation decreases glycogen synthase kinase (GSK)-3αβ and this makes H2R a potential therapeutic target for acute myeloid leukemia (Wang et al., 1996). In addition, H2R also decreases production of TNF-α by reducing MAPK activity (Wang et al., 1996).

First H2R antagonist, burimamide, was found by Black et al, but then, following studies showed that burimamide acts as an H3R antagonist and an H4R agonist as well (Black et al., 1972; Ghamari et al., 2019). After that, cimetidine was discovered and used against gastric ulcers as a H2R antagonist (Brittain & Jack, 1983).

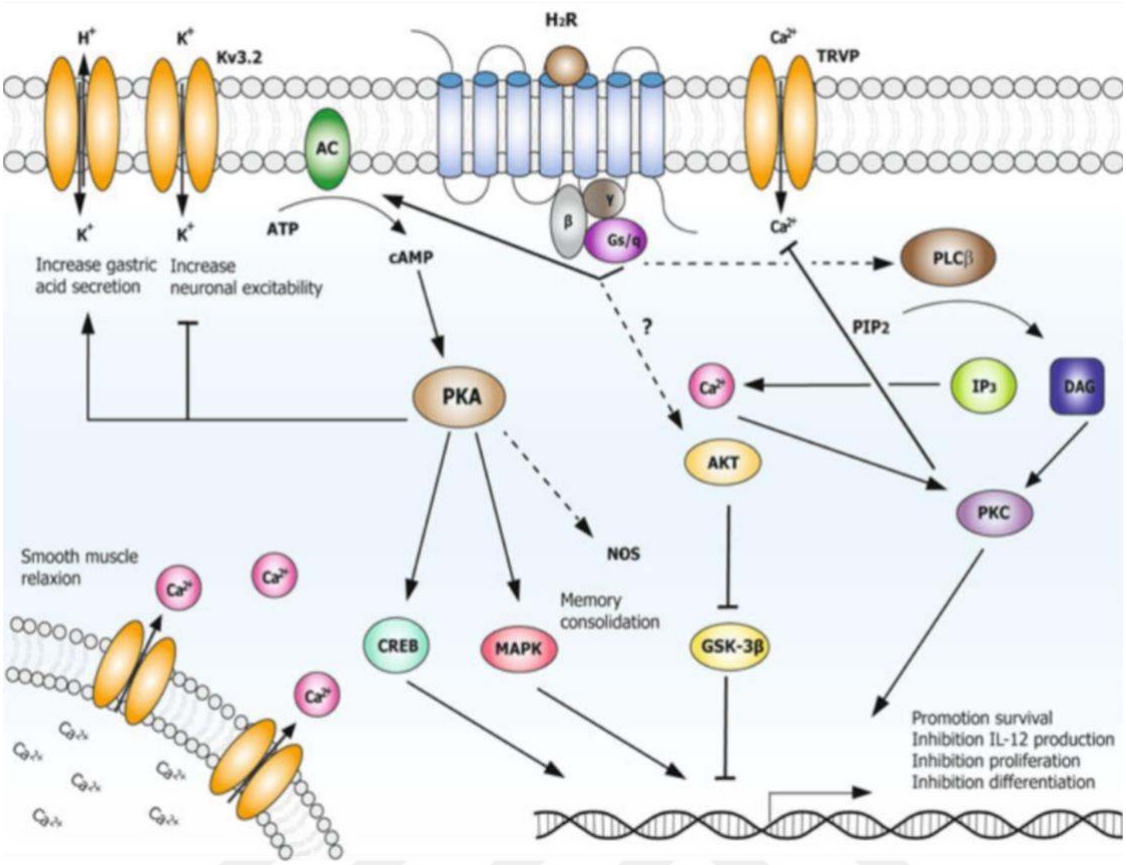

Figure 1.10 The schematic representation of H2R activity. Retrieved from Mocking et al., 2016.
TRVP: transient receptor potential cation channels, AC: Adenyl cyclase, AKT: Protein kinase A, GSK-3β: Glycogen synthase kinase 3β, PLCβ: Phospholipase Cβ, DAG: Diacylglycerol, PKC: Protein kinase C, PIP_2: Phosphatidylinositol 4,5-bisphosphate, PKA: Protein kinase A, MAPK: Mitogen activated protein kinase, CREB: cyclic AMP-response element binding protein, Ca^{2+}: Calcium.

Cimetidine inhibits binding of histamine to H2R completely and also inhibits certain CYP 450 enzymes (Larsson et al., 1982). These motilities give cimetidine for use against peptic ulcer and as neoadjuvant therapy (Pino & Azer, 2021).

1.7.3 Histamine-3 Receptor

After identification of H3R, histamine is considered as a neurotransmitter. H3R is located on chromosome 20 and contains nearly 445 amino acids (Arrang et al., 1983). 20 isoforms of H3R have been detected. Despite other HRs, H3R is mainly located in the CNS, including neurons, astrocytes and microglia. H3R expression is detected in low amounts in

other peripheral tissues (Pollard et al., 1993). Due to its localization, H3R has a great role in cognition, memory, learning and behavior (Martinez-Mir et al., 1990). Activation of H3R causes release of other neurotransmitters, such as serotonin, dopamine, gamma-aminobutyric acid, and glutamate because of its heteroreceptor structure. H3R is a Gαi/o GPCR and decreases PKA activity, but there is no clear statement on how H3R acts on the cell (Chazot et al., 2001; Martinez-Mir et al., 1990; West et al., 1990) (Figure 1.11.).

Activation of H3R negatively regulates cAMP and decreases cAMP level in the cell. This decrease also leads to lower PKA and CREB activity. On the other hand, H3R activates MAPK and phosphoinositol 3-phosphate pathways (Francis et al., 2007). Pharmacological properties of H3R may depend on its isoforms, localization, and protein-protein interactions (Hancock et al., 2003).

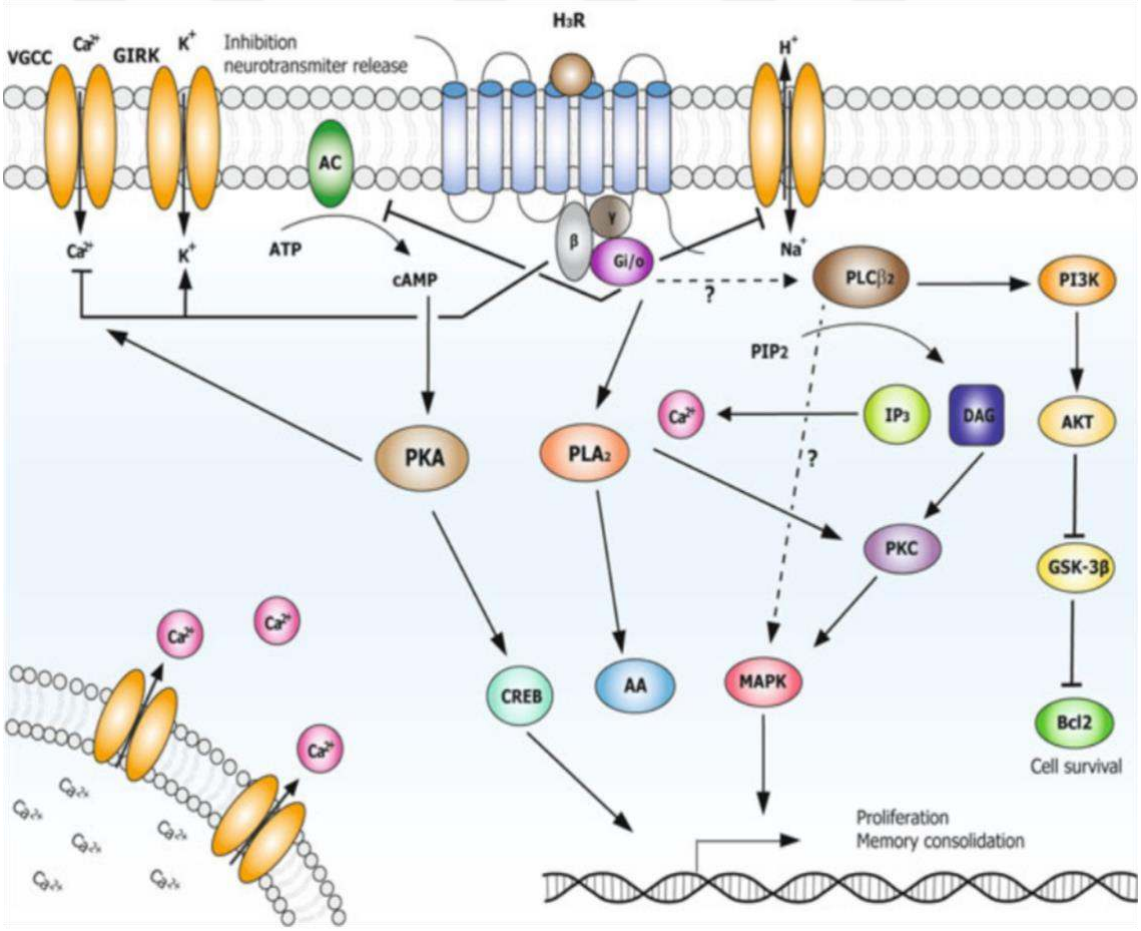

Figure 1.11 The schematic representation of H3R activity. Retrieved from Mocking et al., 2016.
AC: Adenyl cyclase, ATP: Adenosine triphosphate, cAMP: cyclic adenosine monophosphate, VGCC: Voltage gated calcium channel, GRIK: G protein-coupled inwardly rectifying potassium channel, PKA: Protein kinase A, PLA_2: Phospholipase A2, PLCβ2: Phospholipase B2, PI3K: Phosphoinositide 3-kinase, AKT: Protein kinase A,

GSK-3β: : Glycogen synthase kinase 3β, Bcl2: B-cell lymphoma 2, IP_3: Inositol triphosphate, MAPK: Mitogen activated protein kinase, AA: Arachidonic acid, CREB: cyclic AMP-response element binding protein, Ca^{2+}: Calcium.

Antagonists of H3R are divided by their chemical structure and they are called imidazole-based or non-imidazole-based H3R antagonists. Imidazole-based H3R antagonists can bind to other HRs as well. Imidazole, one of the main structures of histamine, can be the cause of why imidazole based H3R antagonists are unable to have specificity against H3R.

Ciproxifan is an imidazole-based H3R antagonist and inverse agonist that has high affinity against H3R (Hancock et al., 2003; Witkin & Nelson, 2004) (Witkin & Nelson, 2004). Ciproxifan blocks H3R activity in the brain and increases the release of histamine. Moreover, this leads to activation of H1R and increases awakeness of all kinds of species (Passani et al., 2007).

1.7.4 Histamine-4 Receptor

In 1994, Raible et al suggested that there must be another HR subtype expressed in humans and in 2000, researchers were able to describe the latest HR subtype and they named it as H4R (M. Walter & Stark, 2012). H4R is located on the chromosome 18 and contains 390 amino acids. H4R expression is observed in bone marrow, spleen, thymus, and other cell types including BMVECs (M. Walter & Stark, 2012). Like H3R, H4R is also a Gαi/o GPCR.

Activation of H4R causes Ca^{2+} release from intracellular storages and induces chemotaxis (M. Walter & Stark, 2012). H4R governs actin polymerization via Akt activation (Zampeli & Tiligada, 2009). Inflammation-related several kinases and AP-1 transcription factor are activated by H4R (Desai & Thurmond, 2011) (Figure 1.12.).

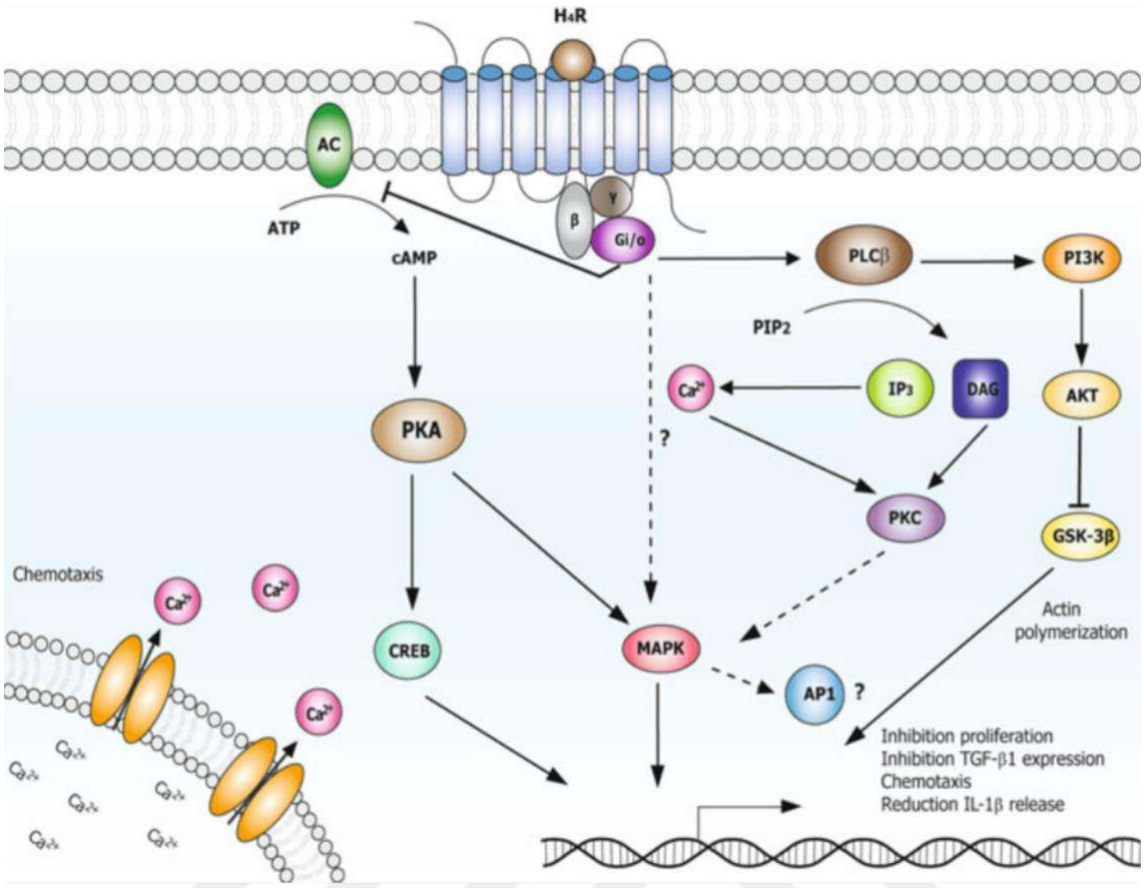

Figure 1.12 The schematic representation of H3R activity. Retrieved from Mocking et al., 2016.
AC: Adenyl Cyclase, PLCβ: Phospholipase Cβ, PI3K: Phosphoinositide 3-kinase, AKT: Protein kinase B, GSK-3β: Glycogen synthase kinase 3β, DAG: Diglyceride, PKC: Protein kinase C, IP_3: Inositol triphosphate, MAPK: Mitogen-activated protein kinase, PKA: Protein kinase A, CREB: cyclic AMP-response element binding protein, Ca^{2+}: Calcium.

JNJ 7777120 is a selective antagonist for H4R. Using JNJ 7777120 prevents cell migration after blocking H4R activity (Desai & Thurmond, 2011; Stark, 2014). JNJ 7777120 is not used in clinical conditions due to its limited half-life and bioavailability (Desai & Thurmond, 2011).

1.7.5 RhoA/Rho Kinases

RhoA and Rho Kinase (ROCK) are members of Rho GTPase, and they are part of G-proteins of the Ras superfamily (Brakebusch, 2021). Their main function is regulation

of the actin cytoskeleton, but they are also involved in cell migration, proliferation, polarity and stemness (Brakebusch, 2021).

Rho Kinase has two different isoforms, ROCK1 and ROCK2. ROCK1 is mainly found in non-neuronal tissues, and ROCK2 represented in the neuronal tissues (Seccia et al., 2020). Activation of ROCK mainly depends on the binding of RhoA, but other pathways can initiate ROCK activity, too (Kempers et al., 2021; Seccia et al., 2020). To activate RhoA, signals must come from Gq type GPCR. Angiotensin II, endothelin, and histamine can activate RhoA signalling, and these led to changes of cell actin cytoskeleton. In addition to these molecules, growth factors and cytokines directly affect ROCK activity (Kempers et al., 2021).

Activation of ROCK phosphorylates to myosin targeted phosphate protein (MYPT) and inactivates myosin light chain (MLC) (Del Re et al., 2007). Inactivation of MLC results in stress fiber formation in the cells and leads to contraction of cells. ROCK mediated changes also regulate Ca^{2+} efflux and this efflux activates transcription factors in the cell (Hodge & Ridley, 2016) (Figure 1.13.).

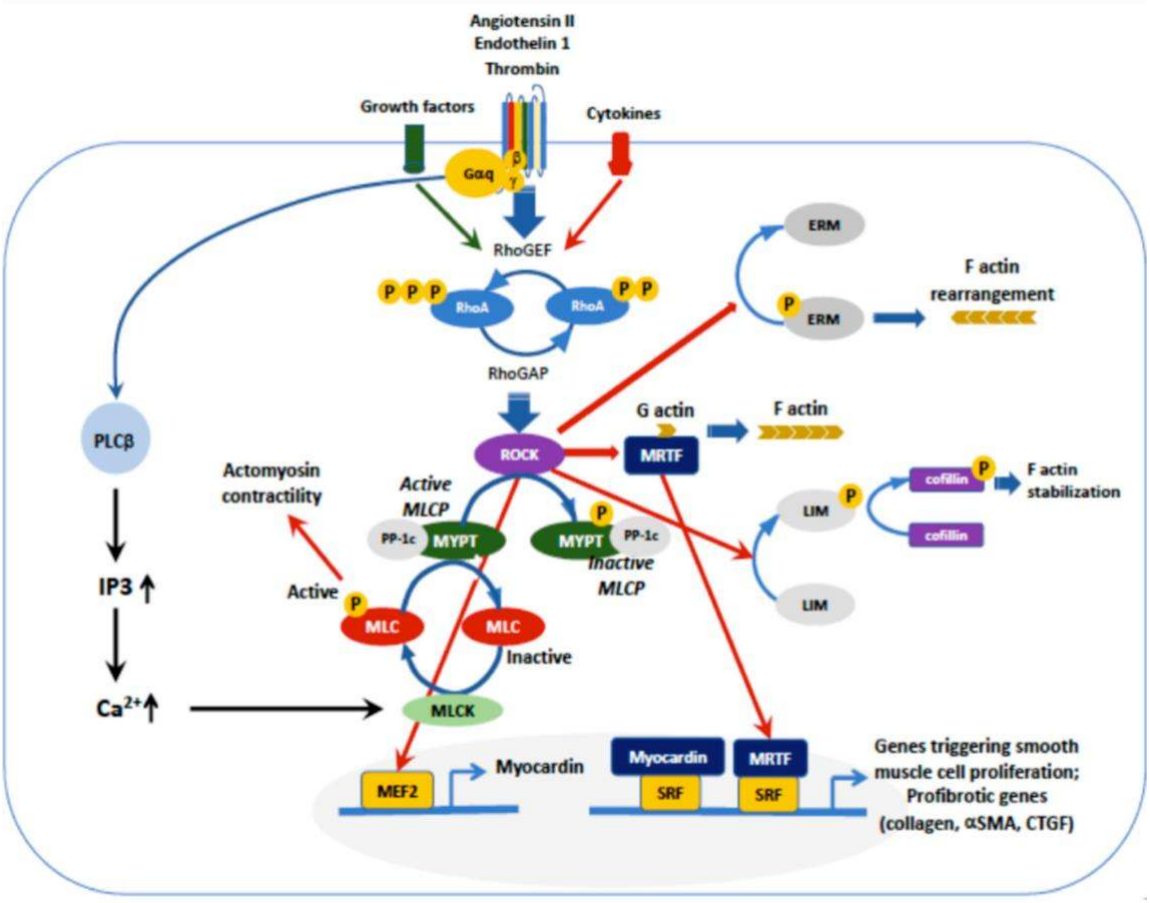

Figure 1.13 The schematic representation of regulation of ROCK signalling pathway. Retrieved from Seccia et all., 2020.
PLCβ: Phospholipase Cβ, IP3: Inositol triphosphate, MYPT: Myosin phosphatase targeting protein, MLC: Myosin-light chain, MLCK: Myosin-light chain kinase, MLCP: Myosin-light chain phosphatase, MEF2: myocyte enhancer factor-2, MRFT: Myocardin-related transcription factor, SRF: Serum response factor, Ca^{2+}: Calcium, P: Phosphate.

Fasudil is a highly selective ROCK inhibitor that reverses stroke mediated effects observed vascular structure and function (Al-Hilal et al., 2021). Moreover, fasudil also causes vasodilation in blood microvessels (Yamashita et al., 2007).

1.8 Histamine and Blood-Brain Barrier

Histamine affects cell polarization, motility, and cell to cell contacts. As a result of the HR subtypes, these effects are regulated at the cell level. Barrier type endothelial cells in the brain express all four HR subtypes (Karlstedt et al., 2013).

Many studies have reported that histamine increases endothelial cell permeability (Flynn & Owen, 1979; Rozenberg et al., 2010; Van de Voorde & Leusen, 1983). For

keeping neuronal activity in the homeostasis state, permeability of endothelial cells must be controlled tightly, but the administration of histamine altered BBB permeability (Rigor et al., 2013; Yuan, 2000). Both transcellular and paracellular transport regulate BBB permeability, and histamine can affect both pathways (Carmeliet et al., 1999; Dubroca et al., 2007). Beside direct impact on BBB integrity, histamine also acts on secondary structures of BBB such as ZO-1 and AJ proteins (Gardner, 1995; Orsenigo et al., 2012).

Before finding evidence of H3R and H4R expression on endothelial cells, histamine-mediated BBB studies focused on H1R and H2R dependent activities. Activation of these two receptors increases BBB permeability (Kugelmann et al., 2018; Meyrick & Brigham, 1984).

Activation of PKC by downstream signalling of H1R increases Ca^{2+} and causes RhoA/ROCK mediated stress fiber formation (Kugelmann et al., 2018; van Nieuw Amerongen et al., 1998). Especially creating these changes on actin fiber results in disruption of TJ protein in BBB (Rigor et al., 2013). On the other hand, PKC also activates src kinases that play an important role on cav-1 activity and results in increased cav-1 vesicle formation in BMVECs (Hu et al., 2008; Waschke et al., 2006).

In addition to the H1R activity, H2R also causes BBB disruption via Ca^{2+} changes in the cell, therefore H1R activity governs to H2R activity and increases BBB permeability (Garbarg & Schwartz, 1988). Moreover knock-out models of H3R and H4R also resulted in increased BBB leakage, but how H3R and H4R regulates BBB remains unknown (del Rio et al., 2012; Teuscher et al., 2007).

On the flipside, RhoA/ROCK activity, affected by Ca^{2+} signalling in the cell, disrupts BBB integrity (Mikelis et al., 2015). All HR subtypes change Ca^{2+} release in the cell, and it may be one of the mechanisms that underlies how histamine affects BBB integrity (Sedeyn et al., 2015).

1.9 Aim

Researchers have mainly focused on transiently opening of the TJs and/or increase transcytosis on barrier type of endothelial cells of brain capillaries to enhance brain-targeted delivery. Various substances such as hyperosmolar solutions (Neuwelt EA, 1981; Neuwelt and Rapoport 1984; Fortin D, 2007; D'Amico RS, 2020), histamine (Nomura T,

1993 Inamura T, 1994; Wang Z, 2016), sphingosine-1-phosphate (Wang Z., 2020) and techniques such as focused ultrasound (Chu P.-C., 2016; Mainprize T, 2019; Dubey S, 2020; Wei HJ, 2021) have been tested for opening of BBB. But most of these approaches that open BBB did not have a chance to be used in daily practice since they also caused the entry of blood-sourced immune cells, microorganisms, and neurotoxins which cause potentially toxic effects in the brain.

Histamine is known to enhance BBB permeability by binding to four different histamine H1, H2, H3, and H4 receptors (R) which are expressed by brain microvascular endothelial cells (Akdis and Simons, 2006; Karlstedt K, 2013; Borriello F, 2017). Accumulated data show that histamine impacts on barrier type of endothelial cells through the activation of H1R (Rotrosen and Gallin, 1986; Carson MR, 1989; Lu C, 2010; Mikelis CM 2015). Histamine-induced vascular hyperpermeability in human dermal microvascular endothelial cells was attenuated by inhibition of H1R-mediated signaling (Ashina K, 2015). On the other hand, intracarotid infusion of histamine increased the number of pinocytotic vesicles in endothelial cells and this effect was mainly provoked by binding of histamin to H2R (Joo F 1981). In addition, intracarotid histamine infusion increased brain-tumor barrier permeability via H2R (Nomura T, 1994), and this action was inhibited by H2R antagonist cimetidine in an in vitro blood-tumor barrier (BTB) model (Wang Z 2016). Intracerebroventricular administration of AβP (1–40) resulted in a pronounced breakdown of the BBB, and it was significantly reduced by histamine H3 receptor reversed agonist BF 2649 and H3 receptor antagonist with partial H4 agonist clobenpropit (Patnaik R, 2018).

The above-mentioned studies examining the effects of histamine on BBB integrity have reported contradictory results, and the precise molecular mechanisms of how histamine regulates BBB permeability are still not fully understood. The signaling pathways regulating the opening and closing of BBB are of great importance in terms of protection of brain cells from the side effects of blood-borne toxic substances. In this study, we investigated the efficacy of blocking histamine receptors and RhoA in reversing histamine-induced BBB opening in in vitro and in vivo conditions (Figure 1.14).

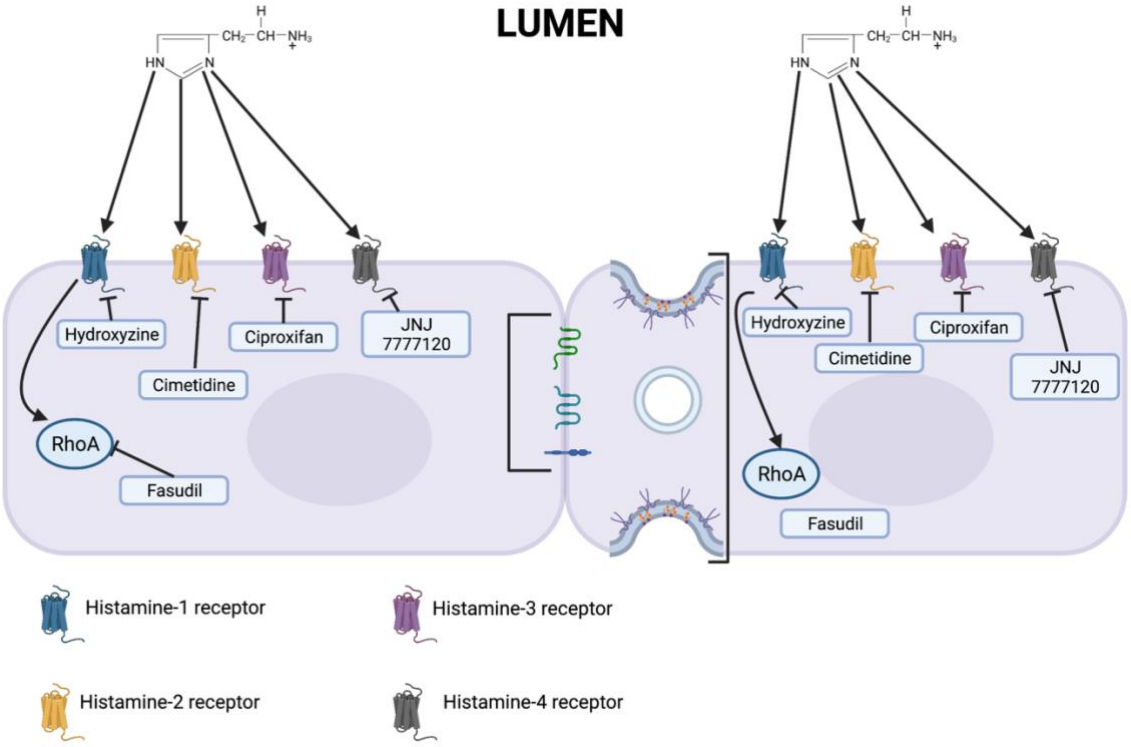

Figure 1.14 Schematic representation of the histamine signaling pathways and the inhibitor and antagonist

2 MATERIALS AND METHODS

2.1 Materials

All chemicals, devices, antibodies, and primers that were used in these experiments are shown in tables 4.1, 4.2, 4.3, and 4.4.

Table 2.1 List of chemicals used in the experiments.

Name	**Brand**	**Catalog Number**
0.1M DTT	Invitrogen	Y00147
10X Tris/Glycine Buffer	Bio-Rad	1610771
10X Tris/Glycine/SDS Buffer	Bio-Rad	1610772
2-Mercaptoethanol	Bio-Rad	1610710
2-propanol	Merck Millipore	1.09634.2500
4X Laemmli Sample Buffer	Bio-Rad	1610747
5X First-Strand Buffer	Invitrogen	Y02321
Albumin Alexa Fluor 594	Invitrogen	A13101
Bovine Serum Albumin (BSA)	Thermo Scientific	A9418-500G
Cadaverine Alexa Fluor 488	Invitrogen	A30676
Cimetidine	Sigma Aldrich	C4522-5G
Ciproxifan Hydrochloride	Sigma Aldrich	C5492-5MG
Citrate Buffer 10X	Sigma Aldrich	C9999-100ML
cOmplete ULTRA tablets	Roche	05892970001
Dimethyl Sulfoxide	Sigma Aldrich	D2650
dNTP Mix	Thermo Scientific	R0192
DPBS	Biowest	L0615-500
Ethanol	Merck Millipore	1009862500
Fasudil Hydrochloride	Sigma Aldrich	CDS021620-10MG
Glycerol	Sigma Aldrich	49782
Histamine Hydrocholoride	Sigma Aldrich	H7250-5G

Hoechst 33342	Thermo Fischer	62249
Hydroxyzine Hydrochloride	Sigma Aldrich	BP841
JNJ 7777120	Tocris Biosciences	4021
Ketamine	Richter Pharma	-
LightCycler 480 SYBR Green	Roche	04887352001
M-MLV	Invitrogen	28025-013
NaCl	Sigma Aldrich	31434
Normal Goat Serum	Sigma Aldrich	P9416
Nuclease-free Water	Thermo Fisher	R0582
Paraformaldehyde	Sigma Aldrich	158127
Pierce BCA Protein Assay Kit	Thermo Scientific	23225
Pierce ECL Substrate	Thermo Scientific	32106
Polysine Slides	Thermo Scientific	J2800AMNZ
Polyvinylidene Difluoride (PVDF) Membrane	Bio-Rad	1620177
Quick RNA MiniPrep	Zymo Research	R1058
Random Hexamer	Jena Bioscience	PM3015
RIPA Lysis Buffer	Eco-Tech	RIPA-100
RNasin	Promega	N2518
TritonX-100	Fisher Bioreagent	BP151-100
Tween-20	Sigma Aldrich	P9416
Xylazine	Bayer	-

Table 2.2 List of antibodies used in the experiments.

Antibody Name	Brand	Catalog Number	Host	Dilution
Caveolin-1	Abcam	ab192869	Rabbit	1:100
Claudin-5	Invitrogen	35-2500	Mouse	1:100

GAPDH	Abcam	ab8245	Rabbit	1:1000
Histamine-1 Receptor	Alomone Lab	AHR-001	Rabbit	1:100
Histamine-2 Receptor	Alomone Lab	AHR-002	Rabbit	1:100
Histamine-3 Receptor	Alomone Lab	AHR-003	Rabbit	1:100
Histamine-4 Receptor	Bioss Antibodies	bs-10993R	Rabbit	1:100
Mouse Alexa Fluor 488	Life Technology	A11029	Mouse	1:1000
Mouse HRP	Abcam	ab97021	Mouse	1:5000
Rabbit Alexa Fluor 488	Life Technology	A11021	Rabbit	1:1000
Rabbit HRP	Abcam	ab97051	Rabbit	1:5000
RhoA	Abcam	ab187027	Rabbit	1:1000

Table 2.3 List of devices used in the experiments.

Name	**Brand**
-80ºC Refrigerator	Eppendorf
Centrifuge	Thermo Scientific
Confocal Microscope	DMI8 SP8
Cryostat	Leica
LightCycler 480 II	Roche
Pipettes	Thermo Scientific
Power Supply	Bio-Rad
Sonicator	Branson
Thermal Cycler	Bio-Rad
Ultra Turrax	IKA
Western Blot Imaging Device	Li-Cor

Table 2.4 List of primers used in the experiments.

Gene	Primer
Caveolin-1_Forward	CTT GAC CAC GTC GTC GTT GAG A
Caveolin-1 Reverse	GGG CAA ATA CGT AGA CTC CGA GG
GAPDH_Forward	TGA CTC CAC TCA CGG CAA AT
GAPDH_Reverse	TGG AAG ATG GTG ATG GGC TT
Histamine 1 Receptor_Forward	CAC TGG AGG CTG CCC TTG TGC
Histamine 1 Receptor_Reverse	CAC CAG CAG GTT GAG GCC CAC
Histamine 2 Receptor_Forward	TCC TAA GCG ACC CGG TAC AGC
Histamine 2 Receptor_Reverse	ATG GAG ACT GAG GCA CTG CTG G
Histamine 3 Receptor_Forward	TTC GAG CCT CCG CAC CCA GAA
Histamine 3 Receptor_Reverse	GGT CCA ACG GCC GGT CAG C
Histamine 4 Receptor_Forward	TGC TCA GGT CCC CTT GGC ATT T
Histamine 4 Receptor_Reverse	ACG TGA GGG ATG TAC AGA GGA ATG G
Occludin_Forward	GGA CCC TGA CCA CTA TGA AAC AGA CTA C
Occludin_Reverse	ATA GGT GGA TAT TCC CTG ACC CAG TC
RhoA_Forward	AGC TTG TGG TAA GAC ATG CTT G
RhoA_Reverse	GTG TCC CAT AAA GCC AAC TCT AC

Zonula Occludens-1_Forward	AGC TCA TAG TTC AAC ACA GCC TCC AG
Zonula Occludens-1_Reverse	TTC TTC CAC AGC TGA AGG ACT CAC AG

2.2 Animal Experiments

In this study, 144 10 – 12-week-old Balb/c mice weighing 25 - 30 gr were used. All animal experiments were held at Koç University Research Center for Translational Medicine "KUTTAM" and approved by the Local Animal Ethics Committee of Koç University (2018.HADYEK.032). The project was granted by TUBİTAK. The experimental design of the study is shown in Figure 2.1.

Experimental Groups:

1- Sham (∑n=6)

2- DMSO (∑n=6)

3- Histamine (∑n=6)

4- Histamine + Hydroxyzine (H + Hydroxyzine) (∑n=6)

5- Histamine + Cimetidine (H + Cimetidine) (∑n=6)

6- Histamine + Ciproxifan (H + Ciproxifan) (∑n=6)

7- Histamine + JNJ 7777230 (H + JNJ7777120) (∑n=6)

8- Histamine + Fasudil (H + Fasudil) (∑n=6)

Sub-Groups:

1- BBB permeability measurements: Albumin Alexa Fluor 594 & Cadaverine Alexa Fluor 488

2- Immunofluorescence: Claudin-5, Caveolin-1

3- Western Blot: H1R, H2R, H3R, H4R, RhoA

4- RT-PCR: Caveolin-1, Occludin, ZO-1, H1R, H2R, H3R, H4R, RhoA

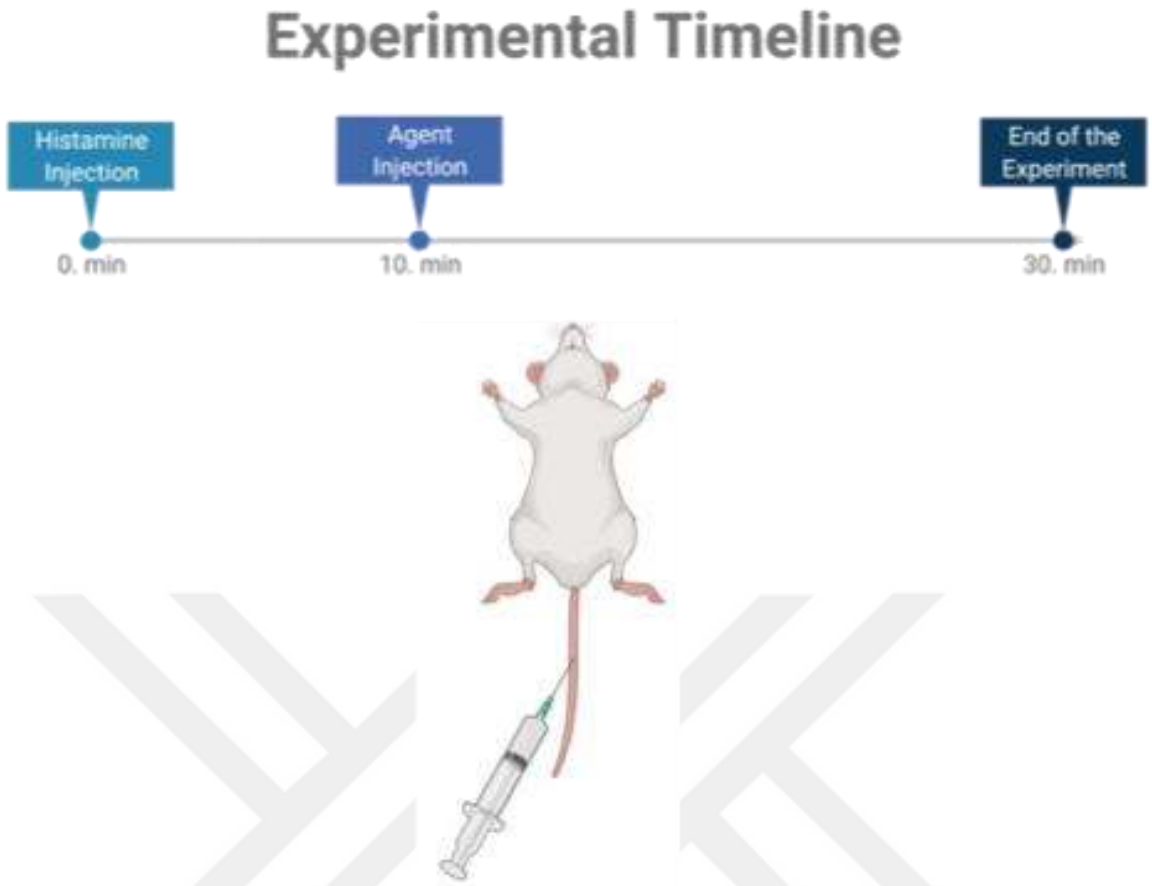

Figure 2.1 Experimental design of the study.

2.2.1 Histamine, Histamine Receptor Antagonist, and Inhibitor Administration

All chemicals were injected via the tail vein. Histamine (10 mg/kg), hydroxyzine (10 mg/kg), cimetidine (10 mg/kg), ciproxifan (3 mg/kg), fasudil (10 mg/kg) were dissolved in normal saline solution (NSS). JNJ 7777120 was dissolved in 50 mM DMSO and then the concentration of the antagonist was adjusted to 10 mg/kg with SF. Histamine injection was considered as the start of the experiments and therefore was called zero point. Ten minutes after histamine injection, the inhibitor or antagonists were injected, and the experiments were terminated 20 min later.

2.2.2 BBB Permeability Measurements

Five minutes before the histamine injection to animals, albumin Alexa Fluor 594 (1 mg in 100 µL saline per animal) and cadaverine Alexa Fluor 488 (1 mg in 100 µL saline

per animal) were injected and allowed to circulate in the blood. At the end of the experiment, the animals were anesthetized with intraperitoneal injection of ketamine (200 mg/kg) and xylazine (30 mg/kg). The chests of the animals were opened, and 25 mL NSS was administered in the left ventricle. The right atrium was simultaneously cut to remove intravascular blood and 100 mL of fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH: 7.4) was administered. Then, the brains were removed and incubated in the same fixative for 3 hours. After incubation, the brain was transferred to 30% sucrose solution and kept in until the brain sank to the bottom of the flasks. The brains were then transferred to dry ice and stored at -80ºC until experiments started.

Ten µm thick sections were taken from the three different parts, frontal, middle, and posterior of the frozen brains using a cryostat and placed on polysine coated slides. Images were then taken by a confocal microscope and positive pixel values were calculated by ImageJ software using a script for each image.

2.2.3 Immunofluorescence

At the end of the experiment, the animal was anesthetized with an intraperitoneal injection of ketamine (200 mg/kg) and xylazine (30 mg/kg). Animal chests were opened, and the right atrium was cut. 25 mL SF was administrated in the left ventricle, intravascular blood was removed, and 100 mL 0.1 M phosphate buffer (pH: 7.4) in 4% paraformaldehyde was administered to the animal. The brain was removed and incubated in the same fixative for 3 hours. After incubation the brain was transferred to 30% sucrose solution and waited until brains fell to the bottom of the flasks. Brain samples were transferred to dry ice and stored at -80oC until experiments started. Ten µm thickness sections were taken from frozen brain samples using a cryostat and placed on polysine coated slides.

2.2.3.1 Staining Procedure

- Sections were incubated at room temperature for 30 minutes.
- Sections were washed with 0.1% Triton-X100/DPBS solution (DPBST) 2x5 minutes.

- Sections were heated in the microwave (600 W, 15 minutes) in citrate buffer for antigen retrieval process.
- Sections were washed with DPBST 3x5 minutes.
- Blocking solution was applied to the sections for 1 hour at room temperature.
- Sections were incubated with primary antibody at 4°C overnight.
- Sections were washed with DPBST 3x5 minutes.
- Sections were incubated with secondary antibody at room temperature for 1 hour.
- Sections were washed with DPBST 3x5 minutes.
- Sections were mounted with glycerol/PBS (1:1) containing Hoechst 33342 for nuclear staining and sealed with a coverslip.

Images were taken by confocal microscopy, and positive pixel values were calculated by ImageJ software using the script for each image.

2.2.4 Western blot

At the end of the experiment, the animal was anesthetized with an intraperitoneal injection of ketamine (200 mg/kg) and xylazine (30 mg/kg). The animal chest was opened, and the right atrium was cut. 25 mL NSS was administrated in the left ventricle, and intravascular blood was removed. The brain was removed and transferred into liquid nitrogen. A protease inhibitor cocktail (PIC) was prepared using a manufacturer's recipe, and 1 mL PIC was used for each brain sample. Brain samples were transferred into tubes containing PIC and homogenized with hand thorax. Then samples were sonicated (10%, 5 seconds pulse, 5 seconds stop total 30 seconds pulse). Samples were centrifuged at 13.000 rpm for 10 minutes at 4°C, and supernatants were collected. Protein levels were measured with the Pierce BCA assay, and the manufacturer's protocol was followed.

After measuring, protein levels for each sample were mixed with 4X loading dye and boiled at 95°C for 10 minutes. Samples were loaded into SDS gels and ran at 90V for 50 minutes. Proteins were transferred into the PVDF membranes with a wet transfer methodology (150 mA 1 hour + 200 mA 1 hour at 4°C). Membrane was blocked with 5% nonfat dry milk for 1 hour and shook gently at room temperature. Then, blocking solutions

were replaced with primary antibodies and incubated overnight at 4°C. Membranes were washed with DPBST 3x5 minutes, and secondary antibodies were applied at room temperature for 1 hour. Membranes were washed with DPBST 3x5 minutes. Membranes were incubated with ECL substrate for 2 minutes at dark, and images were taken with the Odyssey-Fc imaging system. Band intensities were calculated by ImageJ and normalized according to GAPDH band intensities.

2.2.5 RT-PCR

At the end of the experiment, the animal was anesthetized with an intraperitoneal injection of ketamine (200 mg/kg) and xylazine (30 mg/kg). The animal chest was opened, and the right atrium was cut. 25 mL NSS was administrated in the left ventricle, and intravascular blood was removed. The brain was removed and transferred into liquid nitrogen and stored at -80oC until experiments were conducted. Total RNAs were isolated from brain samples via kit according to the manufacturer's instructions. RNA samples were stored at -80oC until cDNA production. 500 ng RNA was mixed with 2.5 µL of dNTP mix, one µL of the random hexamer, and nuclease-free water up to 16.5 µL for cDNA production. The mixture was incubated at 65oC for 5 minutes and was put into the ice. 5 µL 5X First-Strand Buffer, 2 µL of DTT, and 0.5 µL RNAs were added to the tubes and incubated at room temperature for 5 minutes. After 5 minutes, one µL M-MLV RT enzyme was added into the tubes and incubated in a thermal cycler with the following conditions: 37oC for 1 hour, 70oC for 15 minutes. cDNAs stored in -20oC until RT-PCR experiments were conducted.

For RT-PCR experiments, 2 µL cDNA, 10 µL SYBR green, 7 µL nuclease-free water, and 1 µL primer mix (Table 4.4.) are transferred into a 96-well plate. Plates were sealed and centrifuged at 1300 rpm for 2 minutes. RT-PCR was conducted with the following conditions: 95oC for 5 minutes, 45 cycles of (95oC for 10 seconds, 60oC for 30 seconds, and 72oC for 30 seconds), and 4oC ∞ in RT-PCR device. Relative gene expression was calculated by using the ΔΔCT method, and GAPDH was used as a reference gene.

2.3 Statistical Analysis

All charts were plotted by GraphPad Prism v7.0 software. Two-way analysis of variance analysis and Tukey's post hoc test was performed for BBB permeability measurements, immunofluorescence, and western blot experiments. For the RT-PCR experiments Kruskal-Wallis test was performed. All analyses were performed in GraphPad Prism software. All data represented Mean ± Standard Error of Mean (SEM) values. $p<0.05$ was considered statistically significant.

3 RESULTS

3.1 BBB Permeability Assay

To assess the roles of HRs' antagonists and RhoA inhibitor on the histamine-induced BBB disruption in mice, we used two different BBB tracers, albumin Alexa fluor 594 and cadaverine Alexa fluor 488 which are in different molecular weight to show BBB permeability changes. In histamine group, the content of both tracers in brain parenchyma increased compared to sham group (Figure 3.1.).

We found that administration of cimetidine (H2R antagonist), JNJ 7777120 (H4R antagonist) and fasudil (RhoA inhibitor) after histamine injection increased BBB permeability to albumin compared to histamine group ($p<0.01$). However, hydroxyzine (H1R antagonist) administration after histamine treatment decreased albumin passage into the brain compared to histamine group alone ($p<0.01$; Figure 3.1.).

Cadaverine Alexa fluor 488 was used for evaluating of low molecular weight molecules passage into the brain under our experimental set up. Applying all antagonist and inhibitors after histamine injection increased the content of cadaverine in brain parenchyma compared to histamine group alone ($p<0.01$). Hydroxyzine and cimetidine applications showed the most increased cadaverine intensity levels in the brain parenchyma compared with histamine. Other HRs' antagonist and inhibitors also slightly increased cadaverine level in the brain versus histamine group (Figure 3.1.).

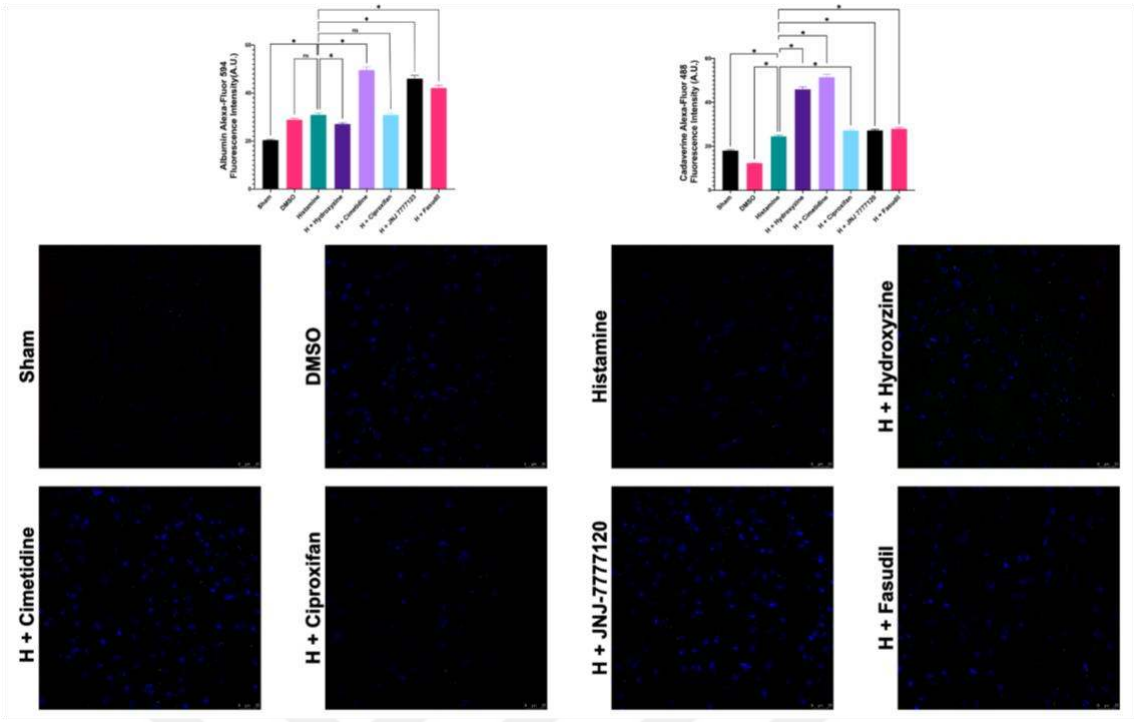

Figure 3.1 The fluorescence intensity of cadaverine Alexa fluor 488 and albumin Alexa fluor 594 in the cerebrum of the animals and representation of cadaverine Alexa Fluor and albumin Alexa Fluor extravasation in experimental groups.
Albumin Alexa fluor 594 and cadaverine Alexa fluor 488 levels were highest in the cimetidine groups treated with histamine. Blue: Nucleus, Green: Cadaverine Alexa fluor 488, Red: Albumin Alexa fluor 594. * means $p<0.01$. Data represent mean ± SEM. Arbitrary unit (A.U). Scale bar: 25 µm.

3.2 Immunofluorescence

3.2.1 Claudin-5

In our experimental setup, claudin-5 immunostaining was performed for evaluating TJ structure in cerebral cortex and hippocampus regions of the brain.

For cerebral cortex, injection of histamine decreased to claudin-5 staining compared with sham group ($p < 0.01$). Hydroxyzine, cimetidine, JNJ 7777120, and fasudil increased claudin-5 immunofluorescence intensity in the cerebral cortex compared to histamine group ($p<0.01$). On the other hand, ciproxifan (H3R antagonist) decreased to the immunofluorescence intensity of claudin-5 levels in the cerebral cortex ($p<0.01$; Figure 3.2.).

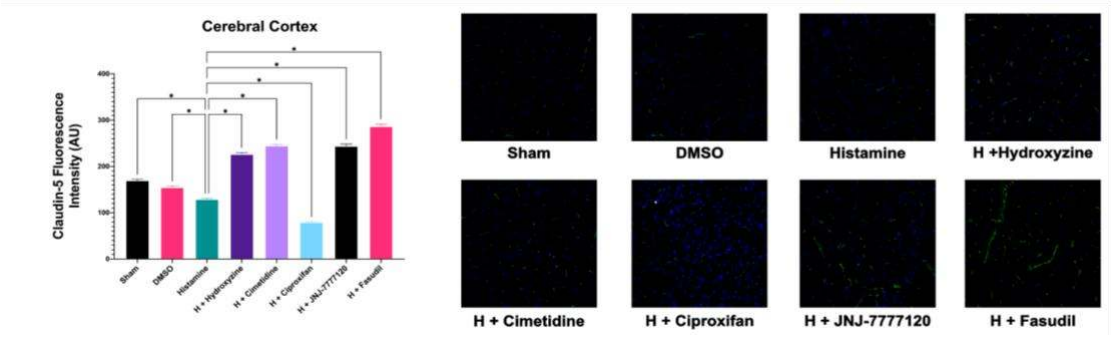

Figure 3.2 Claudin-5 immunofluorescence intensity in the cerebral cortex. Fasudil application caused most increased claudin-5 fluorescence signal in the cerebral cortex compared with other groups. Histamine and ciproxifan applications resulted decreased claudin-5 signalling. Hydroxyzine, cimetidine and JNJ 7777120 represented increased claudin-5 immunoreactivity compared histamine. Blue: Nucleus, Green: Claudin-5. * means $p<0.01$. Data represent mean ± SEM. Arbitrary unit (A.U). Scale bar: 25 µm.

For hippocampus, histamine decreased claudin-5 immunostaining intensity compared to sham group ($p < 0.01$). Treatment with ciproxifan showed the highest claudin-5 immunoreactivity in the hippocampus $p < 0.01$). Applying other antagonist and inhibitor after histamine injection also increased the claudin-5 immunofluorescence intensity in the hippocampus ($p < 0.01$; Figure 3.3.).

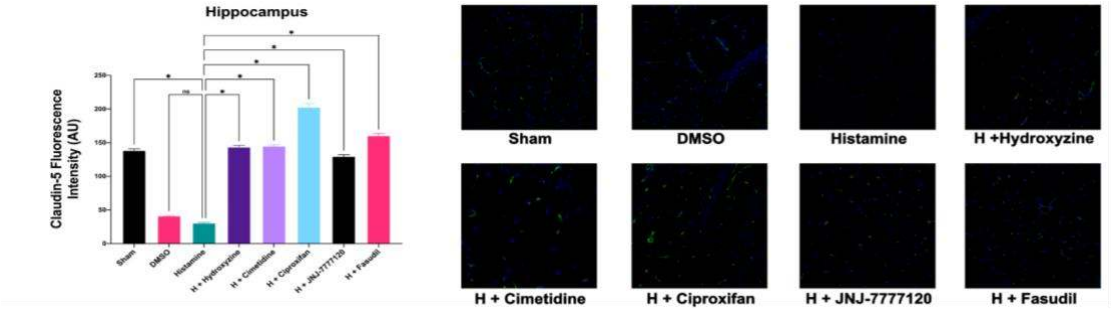

Figure 3.3 Claudin-5 immunofluorescence intensity in the hippocampus. Ciproxifan caused increased claudin-5 immunofluorescence intensity in the hippocampus compared to other groups. Hydroxyzine, cimetidine, JNJ 7777120 and fasudil also represented increased claudin-5 signalling in hippocampus region versus histamine applications. Blue: Nucleus, Green: Claudin-5. * means $p<0.01$. Data represent mean ± SEM. Arbitrary unit (A.U). Scale bar: 25 µm.

3.2.2 Caveolin-1

Immunostaining of caveolin-1 was used to evaluate the production of caveolar vesicles in the barrier type of endothelial cells in cerebral cortex and hippocampus regions of the brain in our experimental set up. For cerebral cortex, histamine group showed the highest immunofluorescence intensity of caveolin-1 protein ($p < 0.01$). Applications of agents after histamine administration decreased the caveolin-1 immunoreactivity in the cerebral cortex region of the brain ($p < 0.01$). Hydroxyzine, ciproxifan, JNJ 7777120 and fasudil application decreased caveolin-1 intensity more than sham group (($p < 0.01$; Figure 3.4.).

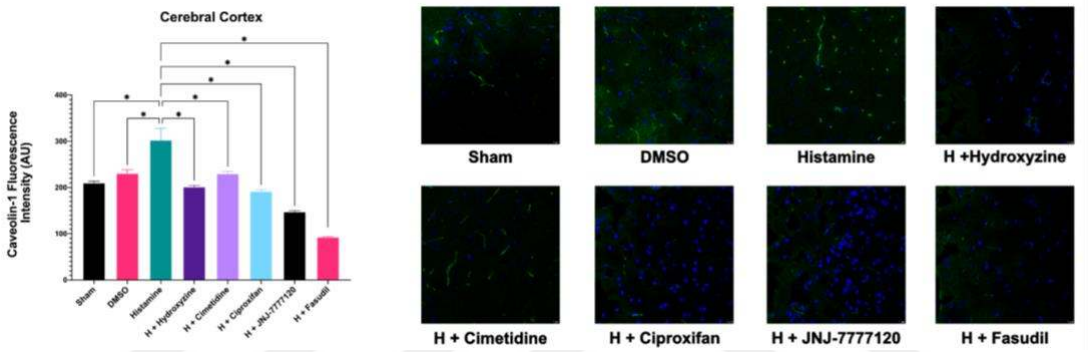

Figure 3.4 Caveolin-1 immunofluorescence intensity in the cerebral cortex. Fasudil represented most decreased caveolin-1 immunofluorescence intensity in the cerebral cortex of brain compared to other groups. All agents also decreased caveolin-1 fluorescence signalling in cerebral cortex. Histamine causes increased caveolin-1 levels in the cerebral cortex. Blue: Nucleus, Green: Caveolin-1. * means $p<0.01$. Data represent mean ± SEM. Arbitrary unit (A.U). Scale bar: 25 μm.

Histamine increased caveolin-1 immunofluorescence intensity in hippocampus region ($p < 0.01$). Cimetidine resulted the highest immunofluorescence intensity when compared to the rest of experimental groups ($p < 0.01$). Hydroxyzine, ciproxifan, JNJ 7777120, and fasudil decreased caveolin-1 immunostaining intensity in the hippocampus region of brain compared to histamine group alone ($p < 0.01$; Figure 3.5.)

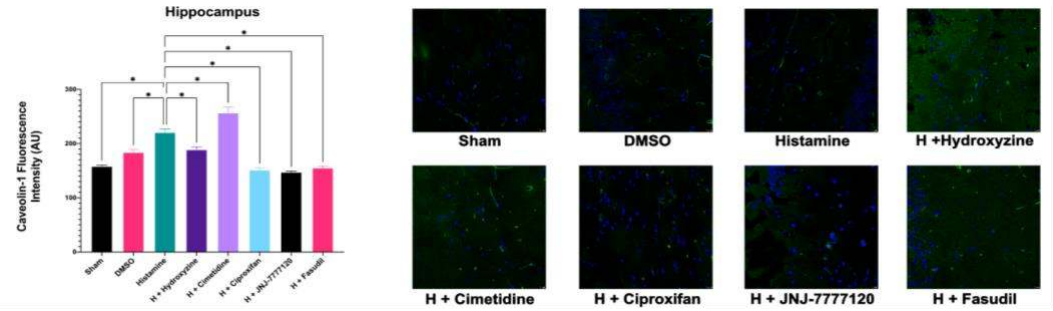

Figure 3.5 Caveolin-1 immunofluorescence intensity in the hippocampus. Ciproxifan and JNJ 7777120 decreased caveolin-1 immunoreactivity in the hippocampus region of the brain compared to other groups. Blue: Nucleus, Green: Caveolin-1. * means $p<0.01$. Data represent mean ± SEM. Arbitrary unit (A.U). Scale bar: 25 μm.

3.3 Western Blot

The protein levels of H1R, H2R, H3R, H4R, and RhoA were measured to evaluate group differences in our experimental setup (Figure 3.6.).

There were no significant changes among groups for H1R protein. Only JNJ 7777120 applications increased H1R levels in the brain compared with histamine group ($p < 0.01$; Figure 3.6. B)

Histamine-2 receptor protein levels did not alter significantly among experimental groups. (Figure 3.6. C).

Histamine increased H3R protein level compared to rest of the groups ($p < 0.01$). Administration of all HR antagonists and ROCK inhibitor decreased H3R protein levels compared to histamine alone ($p < 0.01$; Figure 3.6. D).

Applications of all HR antagonists and ROCK inhibitor did not cause any significant changes in H4R protein levels in brain for all groups (Figure 3.6. E)

We did not observe any significant changes in RhoA levels for all groups (Figure 3.6. F).

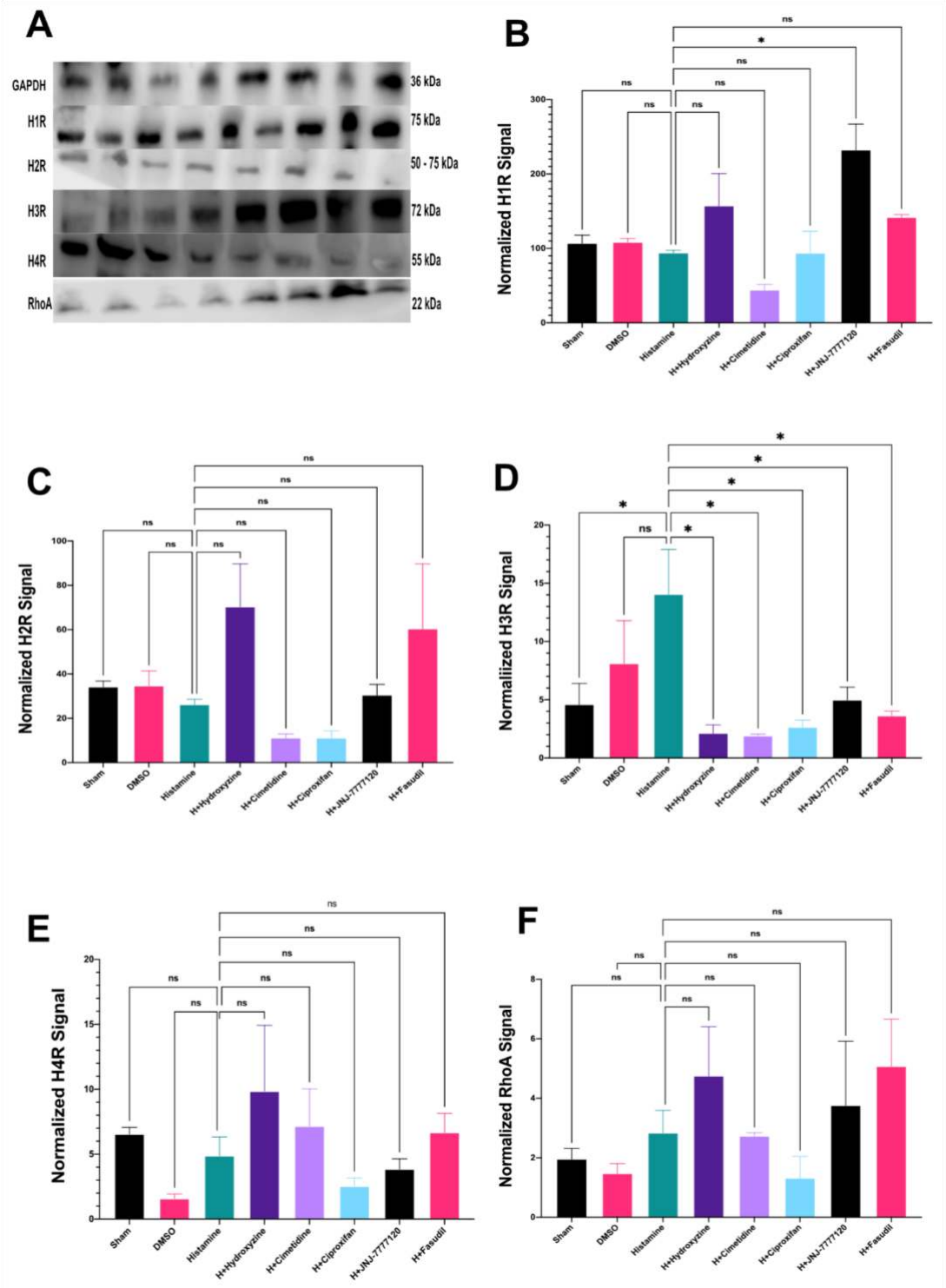

Figure 3.6 Western Blot Analysis.

A) Western Blot images for each protein.
B) H1R levels increased only JNJ777120 application. C) Applications all agents did not cause any significant changes in H2R protein levels between experimental conditions. D) Application of histamine increased H3R levels compared with other groups. E) Applications of all agents did not result any significant changes between groups for H4R levels. F) Applying all agents to animals did not cause any significant changes in RhoA levels. * means $p<0.01$. Data represent mean ± SEM. All signals normalized GAPDH signals.

3.4.RT-PCR

We detected the expression levels of H1R, H2R, H3R, H4R, RhoA, ZO-1, caveolin-1, occludin genes in the brains in our experimental set up using RT-PCR technique.

For detecting H1R, H2R, H3R, H4R, RhoA, ZO-1, caveolin-1, occludin genes expression changes occurred during experimental condition RT-PCR experiments conducted.

H1R mRNA levels did not changed significantly among our experimental groups but Fasudil application increased H1R mRNA levels compared to other groups (Figure 3.7. A).

H2R mRNA levels did not significantly differ among groups but fasudil increased H2R mRNA levels compared with other groups (Figure 3.7. B)

H3R mRNA results represent that there are no significant alternations among experimental groups (Figure 3.7. C)

H4R RT-PCR experiments indicated that changes did not showed significant difference among groups (Figure 3.7. D)

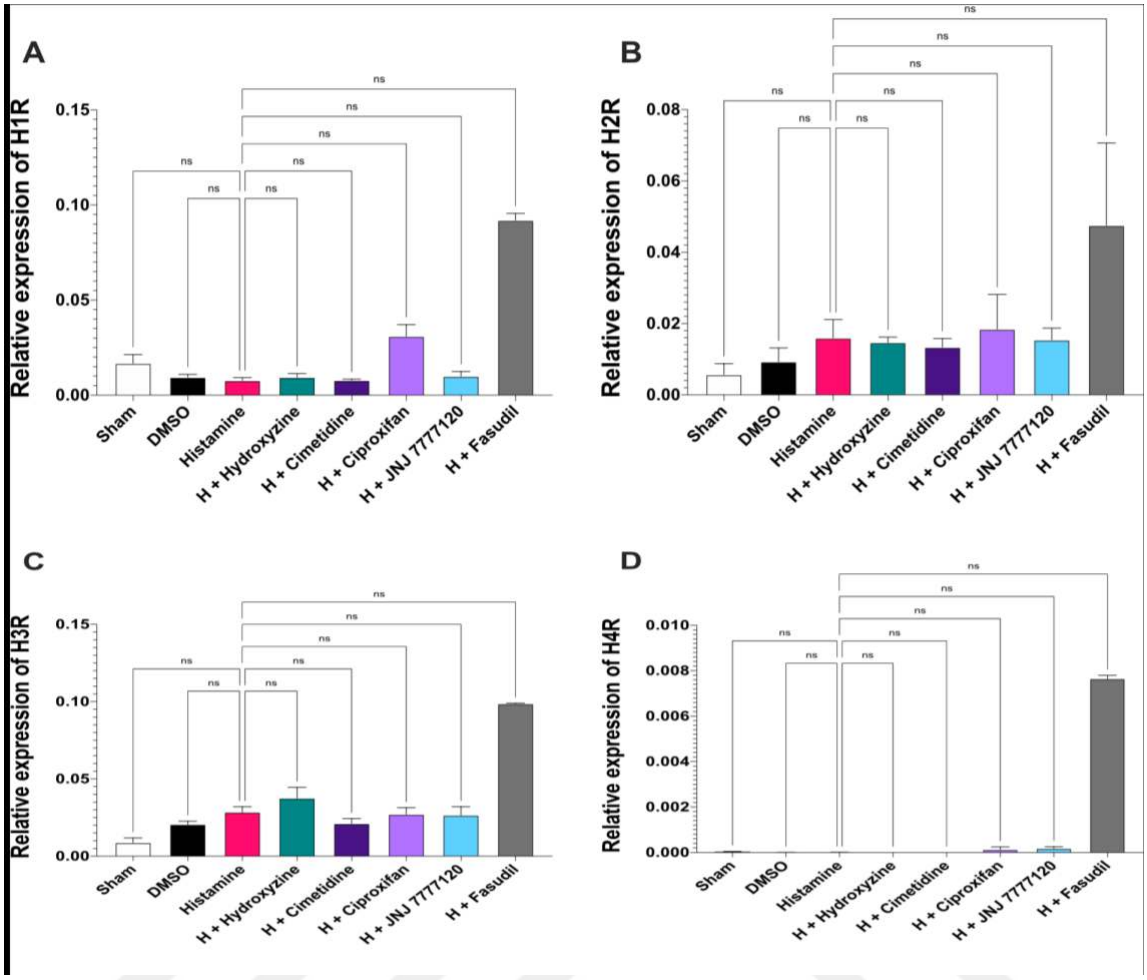

Figure 3.7 RT-PCR result for H1R, H2R, H3R and H4R genes in experimental groups. A) H1R gene expression did not showed any significant differences among experimental groups. B) Expression of H2R did not change among groups. C) Applications of all agents did not result in any significant changes among groups. D) H4R expression levels did not alter among experimental groups. * means $p<0.01$. Data represent mean ± SEM. All gene expression levels were normalized GAPDH expression.

RhoA expression was altered among groups, but these alterations did not show any significant differences (Figure 3.8. A).

Occludin mRNA levels did not make significant changes among experimental groups (Figure 3.8. B).

We did not observe any significant changes on ZO-1 expression among experimental conditions (Figure 3.9. C).

Caveolin-1 mRNA increased in fasudil group compared with other groups ($p<0.01$) and we did not detect any significant changes among other groups (Figure 3.9. D).

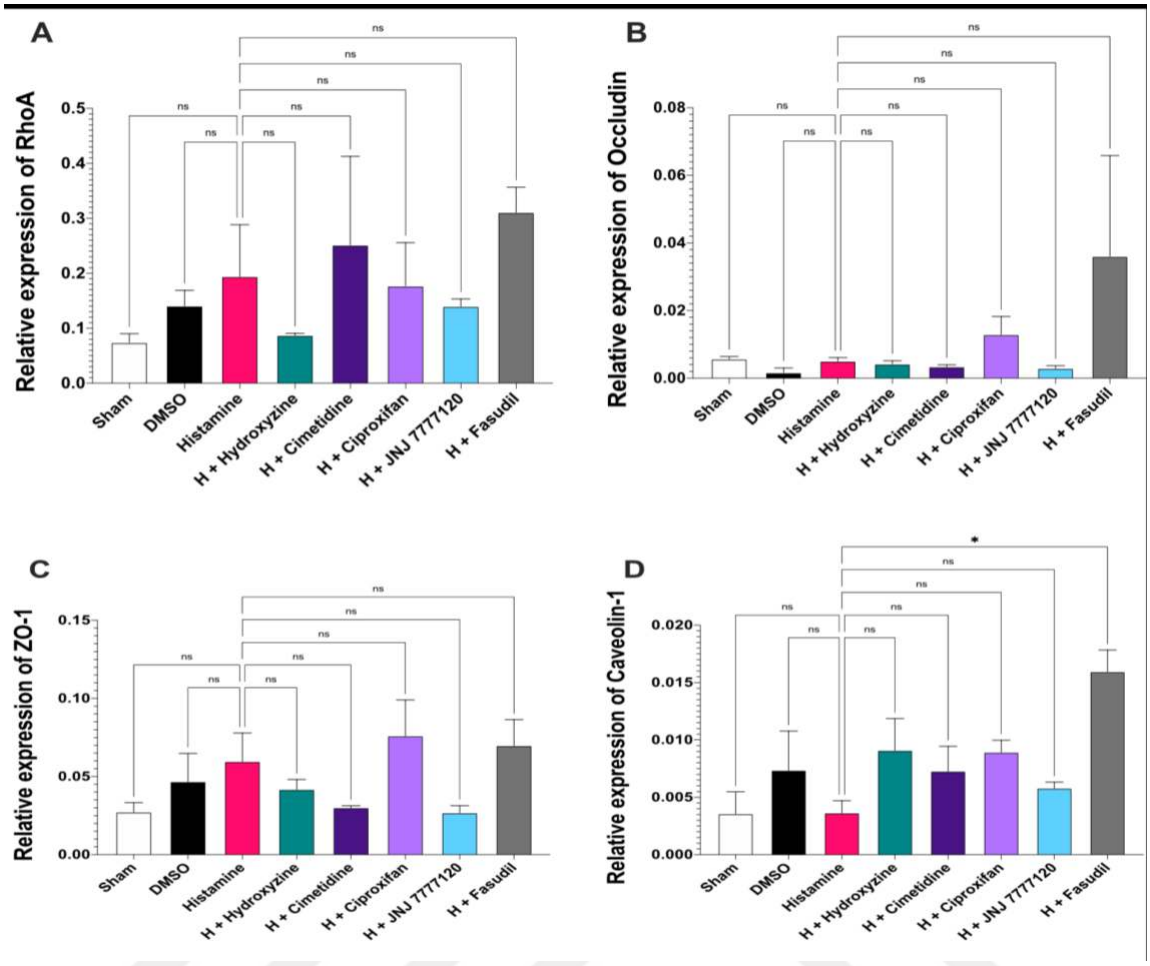

Figure 3.8 RT-PCR results for RhoA, Occludin, ZO-1 and Caveolin-1 genes in experimental groups.
A) RhoA levels did not change among experimental groups. B) Experimental conditions did not shift any significant change among groups. C) ZO-1 levels were not altered by any applications of agents. D) Fasudil increased caveolin-1 expression against histamine applications. * means $p<0.01$. Data represent mean ± SEM. All gene expression levels were normalized GAPDH expression.

4 DISCUSSION

In this study, we aimed to explore the BBB response to histamine receptor antagonists and RhoA inhibitor after opening by histamine. Therefore, this study has focused on developing a novel histamine-based therapeutic approach to establish transiently open/close BBB mechanism for efficient drug delivery and to minimize blood-borne toxic substances and immune cells into the brain. For this purpose, we investigated alterations in BBB integrity under histamine and or histamine receptor antagonists and RhoA inhibitor conditions and examined changes in transport pathways across BBB. Our results showed that using an H1R antagonist reverses histamine-mediated BBB opening at 30 minutes, and we suggest that HIR antagonist could be an efficient agent to control BBB integrity against neuropathological conditions and used for controllable drug delivery into the brain.

4.1 Histamine increased BBB permeability

In our project, we intended to find which primary transport mechanism is responsible for histamine-mediated BBB leakage. Our findings show that the application of histamine opened both transport pathways of BBB. In HUVEC cells, histamine applications caused tyrosine kinase-related AJ protein degradation and increased permeability in endothelial cells (Andriopoulou et al., 1999). Unfortunately, we did not have the opportunity to measure AJ protein changes after histamine applications in our experimental setup.

Transcellular transport is one of the most important mechanisms for keeping BBB in healthy conditions, and it is mainly regulated with TJ proteins (Schaeffer & Iadecola, 2021). Wang et al. (2016) found that histamine application opens BTB via degradation of TJ proteins (Gschwandtner et al., 2013; Wang et al., 2016). Our immunofluorescence experiments showed that histamine decreased claudin-5 fluorescence intensity in the cerebral cortex and hippocampus regions of the brain. Histamine-mediated decrease in claudin-5 levels in the brain might be related to inflammation caused by histamine application. Histamine can increase IL-8 release, which causes downregulation of claudin-5 (Laakkonen et al., 2017; Yu et al., 2013). On the other hand, histamine also activates angiogenic factors that downregulate claudin-5 in the BBB (Laakkonen et al., 2017). In our experiments, decreased claudin-5 fluorescence intensity may result in histamine-

activated IL-8 release and increased expression of transcription factors responsible for angiogenesis, similar to Laakkonens' findings. Our RT-PCR experiments showed that the expression of occludin, another TJ protein responsible for barrier maintenance, did not significantly change in the histamine group compared with sham animals. There are two studies on occludin-histamine relation, but BBB is not their primary emphasis. Wang and colleagues focused on BTB, and Gschwandtner et al. evaluated the skin barrier. Both of them represented the downregulation of occludin in different types of barriers after histamine administration. (Gschwandtner et al., 2013; Wang et al., 2016). Our results do not correlate with their findings due to the differences in our approach. They apply histamine for more than 24 hours, but we aim to investigate acute changes of BBB after histamine application, and BBB might have a distinct mechanism for regulation, unlike BTB and skin barrier.

In another aspect, it is known that histamine activates RhoA signalling pathway (Kugelmann et al., 2018; van Nieuw Amerongen et al., 1998). Inhibiting RhoA/ROCK2 signalling pathway reverses the degradation of TJ proteins, such as claudin-5, occludin, and ZO-1 in inflammatory conditions (Feng et al., 2018). For that purpose, we wanted to examine whether histamine changes RhoA mRNA and protein expression levels. We found that RhoA protein levels and mRNA expression increased, but we did not observe any significant changes against the sham group following histamine application. It is mainly associated with the short period of our experimental design.

Another transport mechanism responsible for proper BBB functioning is caveolar transcytosis. Caveolin-1 is the core protein of cell permeability, regulating caveolae formation and trafficking (Andreone et al., 2017; Gu et al., 2012; Zhao et al., 2011). Caveolin-1 levels can be affected by PKC activity. All HR subtypes are GPCRs, and their interaction with histamine can change PKC levels (Mocking et al., 2016). PKC is located inside the caveolae at the cell membrane, and DAG activation results in PKC activation, which triggers Ca2+ release inside the cell. (Glukhov et al., 2020; Ocharan et al., 2005; Prakash et al., 2007; Sathish et al., 2011). Our results showed that increased caveolin-1 levels might be the result of activated PKC signalling.

Caveolin-1 upregulation is associated with impaired BBB, and it is suggested that inflammation or neuropathologic conditions are associated with this caveolin-1 increase (Ahishali et al., 2010; Zhao et al., 2011; Atış et al., 2019). In an EAE experimental model, decreased activity of the caveolin-1 protein is closely associated with a reduced number of

infiltrated Th1 cells in the brain (Lutz et al., 2017). Increased caveolin-1 fluorescence intensity could be related to inflammatory conditions activated following histamine applications.

On the other hand, Sommer et al. showed that the Rho-kinase pathway is also essential for histaminergic activity. Increased caveolin-1 intensity levels detected in our experiments might be related to the Rho pathway (Sommer et al., 2009).

4.2 Histamine receptor in BBB leakage

We injected histamine through the tail vein to examine BBB integrity, and we showed that histamine increased both tracers, albumin Alexa Fluor 594 and cadaverine Alexa Fluor 488, transport into the brain parenchyma. Our findings are correlated with other studies working on BBB integrity in histamine exposure (Andriopoulou et al., 1999; Kugelmann et al., 2018; Sedeyn et al., 2015; Sommer et al., 2009; Wang et al., 2016). Increased BBB permeability can result from the histamine-activated inflammatory conditions because mast cell-mediated histamine release causes BBB disruption (Shelestak et al., 2020). On the other hand, the inhibition of H1R helps to prevent T helper cell diapedesis into the brain in EAE conditions (Lu et al., 2010).

We intended to find out which of the primary transport mechanisms is primarly responsible for histamine-mediated BBB disruption. Our findings show that the application of histamine altered both paracellular and transcellular transport pathways of BBB. In HUVEC cells, histamine caused tyrosine kinase-related AJ protein degradation and increased permeability in endothelial cells (Andriopoulou et al., 1999). Unfortunately, we did not have the opportunity to measure AJ protein changes after histamine in our experimental setup.

Paracellular transport is one of the most important mechanisms for maintaining BBB in healthy conditions, and it is mainly regulated by TJ proteins (Schaeffer & Iadecola, 2021). Wang et al (2016) found that histamine opens the BTB via degradation of TJ proteins (Gschwandtner et al., 2013; Wang et al., 2016). Our immunofluorescence data showed that histamine decreased claudin- 5 immunofluorescence intensity in the cerebral cortex and hippocampus regions of the brain. Histamine-mediated decrease in the immunostaining intensity levels of claudin- 5 in the brain might be related to inflammation

caused by histamine. Histamine can increase IL-8 release, which causes downregulation of claudin- 5 (Laakkonen et al., 2017; Yu et al., 2013). On the other hand, histamine also activates angiogenic factors that downregulate claudin- 5 in the BBB (Laakkonen et al., 2017). In our experiments, decreased claudin- 5 immunofluorescence intensity may result in histamine-activated IL-8 release and increased expression of transcription factors responsible for angiogenesis similar to Laakkonens' findings. Our RT-PCR experiments showed that the expression of occludin, another TJ protein responsible for barrier maintenance, did not significantly change in the histamine group compared with sham animals. There are two studies on occludin-histamine relation, but BBB is not their primary emphasis. Wang et al have focused on BTB, and Gschwandtner et al. evaluated the skin barrier. Both of them represented the downregulation of occludin in different types of barriers after histamine administration (Gschwandtner et al., 2013; Wang et al., 2016). Our results do not correlate with their findings due to the differences in our approach. They apply histamine for more than 24 hours, but we aim to investigate acute changes (30 minutes) of BBB after histamine, and BBB might have a distinct mechanism for regulation unlike BTB and skin barrier.

In another aspect, it is known that histamine activates RhoA signalling pathway (Kugelmann et al., 2018; van Nieuw Amerongen et al., 1998). Inhibiting RhoA/ROCK2 signalling pathway reverses the degradation of TJ proteins, such as claudin- 5, occludin, and ZO-1 in inflammatory conditions (Feng et al., 2018). For that purpose, we wanted to examine whether histamine changes RhoA mRNA and protein expression levels. We found that RhoA protein levels and mRNA expression increased, but we did not observe any significant changes compared to histamine group. We assumed that this could be associated with the short period of our experimental design.

Another transport mechanism responsible for proper BBB functioning is caveolar transcytosis. Caveolin- 1 is the core protein of cell permeability, regulating caveolae formation and trafficking (Andreone et al., 2017; Gu et al., 2012; Zhao et al., 2011). Caveolin- 1 levels can be affected by PKC activity. All HR subtypes are GPCRs, and their interaction with histamine can change PKC levels (Mocking et al., 2016). PKC is located inside the caveolae on the cell membrane, and DAG activation results in PKC activation, which triggers Ca^{2+} release inside the cell (Glukhov et al., 2020; Ocharan et al., 2005; Prakash et al., 2007; Sathish et al., 2011). We assumed that increased caveolin- 1 levels might be the result of activated PKC signalling.

Caveolin- 1 upregulation is associated with impaired BBB, and it is suggested that inflammation or neuropathologic conditions are associated with caveolin- 1 increase (Ahishali et al., 2010; Zhao et al., 2011; Atış et al., 2019). In an EAE experimental model, decreased activity of the caveolin- 1 is closely associated with a reduced number of infiltrated Th1 cells in the brain (Lutz et al., 2017). Increased caveolin- 1 immunofluorescence intensity could be related to inflammatory conditions activated following histamine. On the other hand, Sommer et al. showed that the Rho-kinase pathway is also essential for histaminergic activity (Sommer et al., 2009). Increased caveolin- 1 immunostaining intensity levels detected in our experiments might be related to the Rho pathway.

4.2.1 H1R activity is the primary mechanism for increased BBB leakage

We showed that using H1R antagonist hydroxyzine decreased the passage of albumin into brain parenchyma after exposure of histamine. Niimi et al. reflect that blocking H1R activity with its antagonist decreased BBB permeability (Niimi et al., 1992). Furthermore, before the histamine administration, treating cells with thiazolyl ethylamine preserved TEER values in the HUVEC monolayer (van Nieuw Amerongen et al., 1998). In addition to these results, endothelial cell-specific knockout of H1R in the mouse increases BBB permeability. Likewise, endothelial cell-specific overexpression of H1R decreases BBB permeability (Lu et al., 2010). We conclude that inhibition of H1R activity decreased the BBB permeability to high molecular weight tracer into the brain. However, the same antagonist increased the passage of cadaverine into the brain compared with histamine alone. The molecular weight of cadaverine is 641.61 Da, and we applied this tracer for evaluating the transport of the low molecular weight substances. Mäe and colleagues knocked out the Mfsd2a gene, which is the primary regulator of caveolin- 1, and the increased expression levels of caveolin- 1 did not represent any significant change between knock-out and wild-type (WT) animals.

One of the recent studies suggested that cadaverine mainly uses paracellular pathway instead of the transcellular in BBB (Mäe et al., 2021). On the other hand, Heithoff et al. demonstrate that cadaverine leakage has continued by barrier repairs (Heithoff et al.,

2021). Cadaverine levels in the brain could be reduced because it can easily pass-through cell-cell connections by diffusion following histamine alone. Nevertheless, the H1R inhibition restored claudin- 5 levels in the endothelium and increased claudin- 5 expression blocks removal of cadaverine to interstitial area from the cytosol. Increased cadaverine immunofluorescence intensity in the brain could have resulted from this mechanism. Although cadaverine might use different routes for entry to the cells, one study demonstrated that cadaverine could be used against breast cancer (Kovács et al., 2019). Cadaverine could decrease endothelial-to-mesenchymal cell transition via decreasing cell movement and invasion, which might be related to the blocking of H1R-mediated angiogenic effects (Kovács et al., 2019).

We wanted to evaluate the response of the transport mechanisms after hydroxyzine application. H1R antagonist increased claudin- 5 immunoreactivity in the cerebral cortex and hippocampus regions of the brain compared with the histamine alone. Especially in the cerebral cortex, hydroxyzine elevated the levels of claudin- 5 immunofluorescence intensity higher than the sham group. Our result suggest that histamine-mediated downregulation of claudin- 5 might be associated with the activation of H1R. Inhibition of H1R-mediated PLC and PKC signalling might desensitize the release of Ca^{2+} from intercellular storage, which is already known as one of the causes for claudin- 5 downregulation.

On the other hand, RhoA/ROCK pathways are also associated with the downregulation of TJ proteins, and H1R has distinct signalling within RhoA/ROCK pathway. Inhibition of H1R-mediated Rho/ROCK signalling may cause the same result with inhibition of Ca^{2+} release inside the cell. RhoA mRNA level was decreased by hydroxyzine when compared to the histamine group, but we did not observe any significance between these two groups. However, the RhoA protein level increased in the H1R group compared to the histamine alone. We did not obtain any significant changes between these two groups. Our experimental design might be the reason for the increased RhoA level. In our protocols, ten minutes after histamine application, we injected the antagonists or the inhibitor to animals to see any changes on BBB permeability. We expected RhoA might be activated during this period, but it reduced after hydroxyzine application to animals.

Other TJ proteins, ZO-1, and occludin mRNA levels decreased, but there was no significance between the histamine and sham groups. We conclude that applications of H1R antagonists could reverse histamine-mediated degradation of TJ protein.

4.2.2 H2R has a protective role on BBB

Using an H2R antagonist increased the immunofluorescence intensity levels of both tracers compared to histamine alone. In 1986 Rotrosen and Gallin showed that the administration of H2R agonist reversed histamine-mediated BBB opening, but using cimetidine worsened histamine-mediated BBB opening (Rotrosen & Gallin, 1986). Cortical superfusion of histamine also caused leaky BBB, and H2R antagonist increased dextran transport into the brain (Schilling & Wahl, 1994). Our data on cimetidine are correlated with those findings (Rotrosen & Gallin, 1986; Schilling & Wahl, 1994). However, H2R activates cAMP, and activating cAMP with forskolin increases BBB permeability (Zhu et al., 2010). H2R antagonist, metiamide, reverses cAMP-mediated BBB leakage, and the same study showed that applying H2R antagonist also increases the BBB permeability (Gulati et al., 1985). In addition, using impromidine, an H2R agonist, also increased the BBB permeability to albumin in rats (Boertje et al., 1990). The data obtained from these two studies oppose our findings, because they used H2R antagonists before histamine application, which may cause the main difference between their results and ours.

It is known that H2R activation causes elevated cAMP which is associated with protecting BBB properties (Furihata et al., 2015; Patterson et al., 2000; Wolburg et al., 1994). Elevated levels of both tracers may be resulted from inhibiting the cAMP-mediated BBB protection mechanism in our experiments. We suggest that H2R provides protective effects on histamine-mediated BBB disruption.

In our experiments, we found that blocking H2R activation with cimetidine increased claudin- 5 immunoreactivity in the cerebral cortex and hippocampus regions of the brain. Unlike our permeability results, increased claudin- 5 levels cause tight BBB integrity (Aijaz et al., 2006; Luissint et al., 2012). Despite that other studies conducted on BTB showed that cimetidine reversed the downregulation of claudin- 5 and other TJ proteins such as ZO-1 and occludin (Nomura et al., 1993, 1994; Wang et al., 2016). Our results showed similarities with BTB studies, and we think this is the main reason we found

increased claudin- 5 immunofluorescence intensity on barrier type of endothelial cells in hydroxyzine group compared to the histamine group.

Caveolin- 1 data regarding immunoreactivity showed the same decreasing pattern in both brain regions compared to histamine alone. However, in the hippocampus, caveolin-1 immunoreactivity increased more than histamine group, which is correlated with our permeability experiments for both tracers. A previous study revealed that the localization of H2R receptor were limbic and striatal regions (Vizuete et al., 1997). This localization might be the main event that shows why H2R-mediated caveolin- 1 immunofluorescence intensity changes were observed among different brain regions. On the other hand, H2R antagonist increased caveolin- 1 mRNA levels, but we did not detect any significant changes between histamine alone group (Razani et al., 1999). Caveolin- 1 activation inhibits protective H2R-mediated cAMP/PKA signalling, and it might result in increased BBB permeability for our experiments.

H2R protein levels were increased after hydroxyzine injection in the animals, but there were no significant changes observed between histamine and other groups in our experimental setup. A previous study showed that H2R signalling is also regulated by H1R activation (Alonso et al., 2013). Due to this phenomenon, H2R levels might be increased to compensate for decreased H1R activation.

4.2.3 H3R opens the BBB, and it is irreversible

The H3R is mainly expressed in different brain regions, especially in the tuberomammillary nucleus and its neuronal projections reach other parts of the brain (Pollard et al., 1993). Inhibition of H3R did not make any significant difference for albumin passage into the brain compared to histamine group. Contrary, ciproxifan increased cadaverine transport into the brain compared to histamine. H3R activation decreases histamine release from neurons however, decreasing H3R activity leads to more histamine release (Witkin & Nelson, 2004). In addition, H3R knock out animals represent a leaky barrier compared to WT animals (Teuscher et al., 2007). Champion and Kadowitz showed that H3R is expressed in hindlimb and mesenteric arteries, in which vascular leakage is increased via NO release after H3R activation (Champion & Kadowitz, 1997, 1998). We

suggest that H3R inhibition causes BBB disruption via increased production of NO in barrier-type endothelial cells due to histamine that releases from neuronal structure.

The protein level of H3R increased after histamine administration, while all antagonists and inhibitor decreased H3R protein amounts. Our data suggest that H3R levels were upregulated by histamine when compared to rest of the groups. Ciproxifan administration did not alter H3R mRNA levels compared with the histamine group under our experimental conditions.

For evaluation of changes in the H3R blocking-mediated transport mechanism, we stained brain sections with a claudin- 5 antibody. H3R represented the most decreased claudin- 5 immunoreactivity levels in the cerebral cortex region of the brain. Nonetheless, claudin- 5 immunofluorescence intensity in the hippocampus was the elevated mostly in the ciproxifan group compared to other groups. A study showed that using an H1R antagonist resulted in the same activity with similar to H3R antagonist, and H1R is mainly located in the hippocampus part of the brain region (Aceves et al., 1985; Parmentier et al., 2007; Tangri et al., 1989). For example, most of the H1R antagonists, especially hydroxyzine, which is commercially sold as Atarax^{TM} and mainly clinically used for the treatment of anxiety-like behaviors, clinically causes changes in sleep-wake cycles, memory, and learning similar to H3R antagonists (Parsons & Ganellin, 2006; Sangalli, 1997; Timmerman, 1989). In our study, we showed that inhibiting H1R activity also resulted in increased claudin- 5 immunofluorescence intensity in the hippocampus. Blocking H3R concurrently may cause inhibition of H1R-mediated changes, and this process might be the reason for the upregulation of claudin- 5 in the hippocampus. We found that ciproxifan increased ZO-1 and occludin mRNA levels compared to the histamine group, but there is no significant difference among the groups.

Caveolin- 1 immunofluorescence intensity increased by ciproxifan compared to the sham group in the cerebral cortex, which may be related an underlying mechanism seen in the increased permeability of both tracers. However, caveolin- 1immunofluoescence intensity level remained same as the sham group and represented different patterns compared to BBB leakage, caused by inhibition of H3R in the hippocampus. The same co-activation of H3R and H1R might mediate decreased caveolin- 1 production after histamine administration. In addition to these results, caveolin- 1 mRNA levels did not show any changes after ciproxifan administration.

4.2.4 Blocking H4R increases BBB leakage

JNJ 7777120 is an H4R-specific antagonist, and we used it to evaluate H4R-mediated changes in BBB permeability in our experiments. JNJ 7777120 injection increased albumin and cadaverine transport into the brain compared to histamine alone. Based on our results, we suggest that blocking H4R has protective effects similar to H2R inhibition.

Current knowledge of the H4R-mediated changes in BBB permeability contains enormous unclarities. In our study, we used an H4R-specific antagonist that only regulates H4R activation for evaluating H4R roles in BBB. Unfortunately, we could not perform experiments to illuminate H4R-mediated protective effects represented in the BBB to explain which signalling pathway was used.

Total H4R protein and mRNA levels did not change significantly among experimental groups. It can be associated with our experimental design.

We found that JNJ 7777120 administration increased claudin- 5 immunostaining intensity in the cerebral cortex and hippocampus. H4R activation is generally associated with decreased cAMP production and increase MAPK levels (Mocking et al., 2016). Blocking H4R increased claudin- 5 levels, while and ZO-1 and occludin mRNA levels did not differ among groups. H4R mechanisms did not exceed the capacity to block the passage of molecules into the brain parenchyma, but it restores TJ protein levels.

Applications of JNJ 7777120 decreased the caveolin- 1 immunoreactivity in both brain regions. Caveolin- 1 mRNA levels were increased by JNJ 7777120 compared with histamine, but it did not reach to significant levels. H4R decreased caveolar trafficking in which caveolin-1 controls, in BBB. We suggest that blocking H4R leads to increased BBB permeability, different pathways could be responsible regarding these changes.

4.2.5 RhoA/ROCK pathway does not reverse histamine-mediated BBB opening

In the literature, inhibiting RhoA with fasudil decreases BBB permeability and upregulates TJ proteins (Cui et al., 2013; Huang et al., 2011). However, we found that using fasudil worsened BBB integrity and increased BBB permeability compared to histamine

groups. After histamine administration, amount of both tracers in the brain parenchyma excessively increased by fasudil.

Western blot and rt-PCR results show that RhoA levels did not change among all groups, and these results are found to be correlated previous study (Büyükafşar & Un, 2003). It is also suggested that the application of fasudil needed longer time to decrease RhoA efficiently (Chen et., 2017).

Fasudil, despite its slow-acting response against RhoA, showed increased claudin-5 immunofluorescence intensity in the brain. Inhibition of RhoA increased claudin- 5 levels higher than that of hydroxyzine group. Our results are correlated with the previous studies, (Kugelmann et al., 2018; van Nieuw Amerongen et al., 1998; Feng et al., 2018) and we suggest that inhibiting Ca^{2+} release and MYPT activity preserves claudin- 5 integrity in the brain. ZO-1 and occludin mRNA levels did not change significantly under our experimental conditions, and we suggest that it is due to the exact mechanism underlying RhoA-mediated regulation.

The immunofluorescence intensity levels of caveolin- 1 decreased in both groups with fasudil injection compared to other groups, and RhoA-mediated effects were also related to vesicular trafficking. Inhibition of stress fiber formation after fasudil injection may cause caveolin- 1 inhibition (Xiong et al., 2017). In contrast to the literature, our data showed that caveolin- 1 mRNA levels increased after RhoA inhibition. We speculate that cells compensate caveolin- 1 reduction upon RhoA inhibition by stimulating transcription of caveolin- 1 mRNA.

4.3 Conclusion

In conclusion, our study showed that H1R is the leading factor for histamine-mediated BBB changes, and H1R can be used as a tool that can effectively deliver drugs into the brain to treat neuropathological conditions. Our study also showed that H1R-mediated effects did not occur through RhoA/ROCK pathways only, and they might trigger other mechanisms for BBB alterations. We showed that inhibition of RhoA causes different results compared to H1R blocking.

On the other hand, our study also demonstrates the effects of H4R on BBB permeability for the first time, and we showed that H4R has a protective effect on histamine-mediated BBB leakage, similar to H2R activity. There is still a need to explain which mechanisms are responsible for H4R effects on BBB.

Another aspect of our focus was to explain H3R activity on BBB, and we showed that H3R levels are brain is not mediated only by itself. H3R activity on BBB is altered with other HR subtypes.

RhoA protects the TJ structure and inhibits caveolin- 1-mediated vesicular transport. However, RhoA inhibition did not cause a decrease in BBB permeability. We suggest that RhoA-mediated effects preserve TJ proteins, but this protection is insufficient to reverse histamine-mediated BBB leakage.

Thus far, the thesis has argued that histamine-mediated BBB leakage is decreased with H1R antagonist, and H1R can be used as an efficient tool for drug delivery systems.

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