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KOÇ UNIVERSITY
GRADUATE SCHOOL OF HEALTH SCIENCE

**THE INTERACTION IN BETWEEN
CAVEOLIN-1 AND TRANSFORMING
GROWTH FACTOR BETA RECEPTOR I ON
BLOOD-BRAIN BARRIER INTEGRITY IN
HYPERTENSIVE ANIMAL MODEL**

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Cellular And Molecular Medicine
PhD Programme

İSTANBUL- 2019

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**Cellular and Molecular Medicine
PhD Programme**

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DECLARATION

I hereby declare that this thesis is my work, planning of the thesis is made under ethical conditions, all the information in this thesis has followed academic and ethical rules, the results and work of others are duly cited, and citations were listed at the end of this thesis, and lastly, in the progress of this thesis, copyrights and patents were not violated.

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ABSTRACT

Title of Thesis: The Interaction in between Caveolin-1 and Transforming Growth Factor Beta Receptor I on Blood-Brain Barrier Integrity in Hypertensive Animal Model

The blood-brain barrier (BBB) is disrupted in both acute and chronic hypertension. Transforming growth factor beta (TGF β) increases in circulation in hypertensive conditions, however little is known about its possible effects on brain microvessels. We investigated the impact of TGF β on BBB integrity in hypertension induced by angiotensin II (ANGII) in mice. Hypertensive animals were treated with SB-431542 that inhibits TGF β RI, and dynasore that blocks plasmalemmal vesicle formation. The alterations in the expression of glucose transporter (Glut)-1, claudin-5, caveolin-1, and glial fibrillary acidic protein (GFAP) were evaluated by immunofluorescence method. Alexa Fluor594-labeled bovine serum albumin was used to assess BBB permeability. ANGII increased arterial blood pressure in animals ($P<0.01$). Increased BBB permeability to albumin-Alexa594 in hypertension was worsened by dynasore but alleviated by SB-431542 ($P<0.01$). The expression levels of Glut-1 increased in the cerebral cortex in ANGII, while dynasore and SB-431542 treatments in the same condition decreased even under sham values ($P<0.01$). The expression of Glut-1 in the hippocampus was found to decrease in ANGII, ANGII+dynasore, and ANGII+SB-431542 groups compared to sham animals ($P<0.01$). Claudin-5 fluorescence intensity increased in ANGII and decreased in ANGII+SB-431542 groups in the hippocampus ($P<0.01$). Fluorescence intensity of caveolin-1 dramatically increased in ANGII and ANGII+dynasore groups, however severely decreased in the hippocampus in ANGII+SB-431542 group ($P<0.01$). GFAP fluorescence intensity decreased in the hippocampus in ANGII+dynasore, and ANGII+SB-431542 groups ($P<0.01$). Our study suggests that TGF β signaling could play an important role in BBB integrity owing to our observations of decreased BBB permeability by SB-431542 under hypertensive conditions.

Keywords: Hypertension, Transforming growth factor β , Blood-brain barrier, Alexa Fluor 594-labeled bovine serum albumin, Caveolin-1, Claudin-5.

ÖZET

Tez başlığı: Hipertansif Hayvan Modelinde Kaveolin-1 ile Transforming Büyüme Faktörü Beta Reseptör Tip I Etkileşiminin Kan-Beyin Bariyeri Üzerine Etkileri

Akut ve kronik hipertansiyonda kan-beyin bariyeri (KBB) geçirgenliğinin arttığı bilinmektedir. Hipertansif koşullarda dolaşımdaki dönüştürücü büyüme faktörü beta (TGF β)'nın artışı bilinmesine rağmen, bu artışın beyin kan damarlarına olan etkileri hala bilinmemektedir. Bu çalışmada, anjiyotensin (ANG) II ile farede indüklenmiş hipertansiyon koşulunda TGF β 'nın KBB bütünlüğüne olan etkisi araştırılmıştır. Hipertansiyon oluşturulan hayvanlarda, kaveolar vezikül oluşumunu engelleyen dinazor (dynasore) inhibitörü ve TGF β RI inhibitörü olan SB-431542 kullanılmıştır. Glukoz taşıyıcısı (Glut)-1, klaudin-5, kaveolin-1 ve glial fibrilar asidik proteini (GFAP) proteinlerinin düzeyleri immünofloresan yöntem kullanılarak belirlenmiştir. Alexa Flor 594 ile işaretlenmiş serum albumin KBB geçirgenliğini göstermek için kullanılmıştır. ANGII uygulanması arteriyal kan basıncı değerlerini yükseltmiştir (P<0.01). Hipertansiyonda artan albumin-Alexa Flor 594 geçirgenliği dinazor ile daha da artmış, ancak SB-431542 uygulanmasında anlamlı bir şekilde azalmıştır (P<0.01). Beyin korteksinde Glut-1 yapımı artarken, dinazor ve SB-431542 kullanılan hipertansif hayvanlarda floresan yoğunluğu sham grubu değerlerinin altına düşmüştür (P<0.01). Hipokampustaki Glut-1 immunboyanma şiddeti ANGII, ANGII+dinazor ve SB-431542+ANGII gruplarında azalmıştır. ANGII grubundaki hayvanların hipokampus bölgelerinde klaudin-5 floresan yoğunluğu artmış, ancak ANGII+SB-431542 grubunda azalmıştır (P<0.01). ANGII ve ANGII+dinazor gruplarındaki kaveolin-1 floresan yoğunluğu hipokampusta dramatik bir şekilde artarken, ANGII+SB-431542 grubunda ciddi bir azalma gözlenmiştir (P<0.01). ANGII+dinazor ve ANGII+SB-431542 gruplarındaki hayvanlarda hipokampus GFAP floresan yoğunluğu azalmıştır (P<0.01). Bu çalışmada, bir TGF β reseptör inhibitörü olan SB-431542'nin hipertansiyonda gözlenen KBB geçirgenlik artışını azaltması, TGF β sinyal yolağının KBB bütünlüğü üzerine önemli bir etki yaptığını ortaya koymaktadır.

Anahtar Kelimeler: Hipertansiyon, Dönüştürücü büyüme faktörü β , Kan beyin bariyeri, Alexa Flor594-işaretli serum albumin, Kaveolin-1, Klaudin-5.

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NOMENCLATURE

AD	Alzheimer's Disease
A β	Amyloid beta
AGEs	Advance glycation end products
AJs	Adherence junctions
ANG	Angiotensin
ANG II- R1	ANG II receptor type 1
BBB	Blood-brain barrier
BCSFB	Blood-cerebrospinal fluid barrier
BSA	Bovine serum albumin
BP	Blood pressure
Ca ²⁺	Calcium ion
Cav-1	Caveolin-1
CBF	Cerebral blood flow
CNS	Central nervous system
CSF	Cerebrospinal fluid
CVOs	Circumventricular organs
DBP	Diastolic blood pressure
DPBS	Dulbecco's phosphate buffered saline
eNOS	Endothelial nitric oxide synthase
ESC/ESH	European Society of Cardiology/European Society of Hypertension
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
Glut-1	Glucose transporter-1
IL-1 β	Interleukin-1 β
JAM	Junctional adhesion molecule
JNC VII	Seventh report of the joint national committee
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NGS	Normal goat serum
NO	Nitric oxide

NVC	Neurovascular coupling
NVU	Neurovascular unit
RAAS	Renin-angiotensin-aldosterone system
RAS	Renin- ANG system
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rats
SHRSP	Stroke- prone SHR
TGF β	Transforming growth factor beta
TGF β RI	TGF β -receptor-I
TGF β RII	TGF β -receptor-II
TJs	Tight junctions
TNF- α	Tumor necrosis factor- alpha
WKY	Wistar- Kyoto
VEGF	Vascular endothelial growth factor
ZO-1	Zonula occludens-1

Chapter 1

1. INTRODUCTION

Hypertension means high blood pressure; therefore, it causes pressure on the walls of blood microvessels. Hypertension as a disease affects nearly 1 billion people worldwide, 50 million people in the United States according to the seventh report of the joint national committee (JNC VII) on the prevention, detection, evaluation, and treatment of high blood pressure [2].

According to one study, individuals aged 90+ and survivors from the Leisure World Cohort Study are monitored 20 years earlier, and 559 participants who were not suffering from dementia were followed every six months for ten years. As a result, 224 participants developed dementia during the experiment. Interestingly, while comparing participants who do not suffer from hypertension and suffer from hypertension with onset age of 80-89 years had lower risk, and participant with onset age of 90+ years had also lower risk for dementia [3]. Therefore, development of hypertension at older ages may have protected effects for dementia. Another study was carried on with 667 Japanese- American men with their post-mortem brain tissue from the Honolulu Heart Program/Honolulu Asia Study (1965-2000). Their midlife blood pressure (BP) was measured starting from at the age of 58 years, amyloid beta ($A\beta$) sample collection was in 1980-1982, lastly, assessment of dementia and autopsy sample collection started between 1991-1993. Shah and her colleagues suggested that correlation between diastolic BP and plasma $A\beta$ levels showed that $A\beta$ related risk for Alzheimer's Disease (AD) was high when BP was also high. They concluded that midlife diastolic BP modulates $A\beta$ plasma level and the amyloid beta level, which is associated with AD had been decreasing 15 years before AD was diagnosed [4].

In hypertension, increased arterial blood pressure is observed in blood vessels of the brain as well as in periphereal blood vessels [5-7]. Pathologies related to hypertension damage blood microvessels of the brain. For this reason, hypertension is the main risk factor for stroke [8-10]. In conditions of acute and chronic hypertension, BBB, constituted by the endothelial cells of brain microvessels, is also disrupted [11-14]. Studies on an AD's animal models showed that chronic hypertension is related to an

increase in inflammation, impairment in BBB integrity, and a decrease in the number of pericytes [8, 15].

Angiotensin (ANG) II is a peptide hormone and plays an important role in vasoconstriction of blood microvessels. ANG II is a part of renin- ANG system (RAS) which controls blood pressure. Renin is a protein enzyme which is released from the kidney to raise arterial pressure when it falls to under the baseline levels. When renin in the kidney meets with its substrate, angiotensinogen, it converts to ANG I. Angiotensin I splits its amino acids and produces ANG II in the lungs. Endothelium of lung microvessels has angiotensin-converting enzyme. Therefore, the reaction to produce ANG II occurs at a great extent in the lungs. There are three fates of ANG II in the body. First, it can reside 1-2 minutes in the blood because angiotensinase degrades this powerful vasoconstrictor agent. Second and third fates are action mechanisms of ANG II to increase arterial blood pressure. Vasoconstriction is much more effective on arterioles than veins. Constriction of arterioles causes an increase in peripheral resistance. Lastly, ANG II holds salt and water in the kidney, then it increases extracellular fluid volume, and it increased arterial blood pressure for hours and days (Figure 1) [16]. Angiotensin II is associated with hypertension, which is related to increased transcellular transport via caveolar-mediated transcytosis [17-19]. It is suggested that Cav-1 is mainly responsible for activating caveolae-mediated transport [17, 19, 20]. Due to all these information above, it is important to understand the underlying mechanisms of hypertension mediated neurodegenerative diseases, and it is essential to develop therapeutics [21-24].

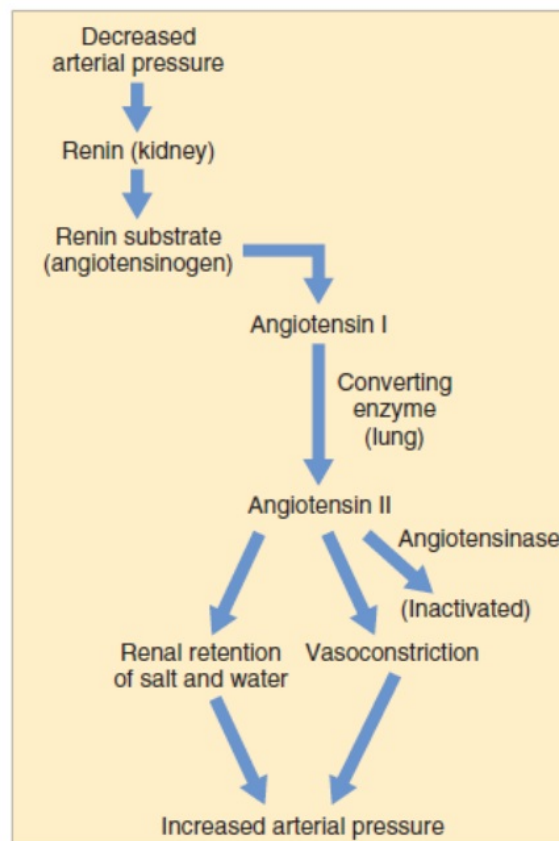


Figure 1. Mechanisms of renin-angiotensin system for arterial pressure control.

Retrieved from Hall, J. et al. 2015 ^[16].

Chapter 2

2. LITERATURE REVIEW

2.1. Hypertension

Hypertension affects people who are over 65-years old and prone to organ damage or cardiovascular diseases [25]. A reason behind those diseases is a failure of the extension of large microvessels [25]. Renal diseases, thyroid disorders, lifestyle, and sex hormones should also be considered as secondary causes of hypertension [25]. Hypertension is considered to affect mostly older people; however, it was reported that children who have a family history of hypertensive conditions, inadequate physical activity and excess adipose tissue in the body are also affected by elevated blood pressure [26].

Pathophysiology of hypertension in elderly people is as follows: stiffness of large microvessels causes enhanced pulse wave velocity, which resulted in late systolic blood pressure increase (SBP) and lack of oxygen for the myocardium. Also, the decrease in forwarding flow limits organ perfusion. Dysregulation of SBP is enhanced by excessive drug-induced diastolic blood pressure (DBP) reduction. Lack of autoregulation of both SBP and DBP cause orthostatic hypotension (a risk for fall or cardiovascular events) or orthostatic hypertension (a risk factor for cerebrovascular diseases and coronary artery diseases) [25].

Some diseases, such as diabetes can be correlated with hypertension in elderly hypertensive patients [25]. Obesity-related hypertension is caused by excess adipose tissue product, which is components of the renin-angiotensin-aldosterone system (RAAS) [25].

Angiotensin II is a multifunctional peptide hormone and mostly shows its activity via ANG II receptor type 1 (ANG II- R1) in pathological conditions [27]. ANG II-R1 affects on a G protein-coupled receptor in the vascular smooth muscle cell. Activation of phospholipase C by AT1 receptor coupled with Gq produce inositol-1,4,5-triphosphate, and diacylglycerol, followed by activation of protein kinase C and calcium (Ca^{2+}) immobilization. This cascade facilitates the vasoconstrictor effects of ANG II. As a result, ANG II controls vascular tone and autoregulation of SBP and DBP [27].

Cerebral microvessels are also affected by fluctuating blood pressure; if mean arterial pressure exceeds 120 mmHg, then cerebral hyperperfusion occurs (Figure 2) [28]. To categorized blood pressure, different guidelines were used (Table 1). Systolic and diastolic blood pressure values were categorized according to JNC VII and the European Society of Cardiology/European Society of Hypertension (ESC/ESH). According to JNC VII, stage II hypertension was reported SBD as ≥ 160 mm Hg, DBP as ≥ 100 mm Hg; whereas, according to ESC/ESH, SBD as 160-179 mm Hg, DBP as 100-1009 mm Hg. This shows that reports can vary between each other; however, there are certain values that indicate hypertensive conditions (Table 1). Therefore, the Joint National Committee and European Society of Hypertension Reports on 2003, blood pressure limits were categorized as “normal”, “prehypertension/high normal”, “hypertension/grade I”, “stage I / grade II”, “stage II / grade III” [1, 2].

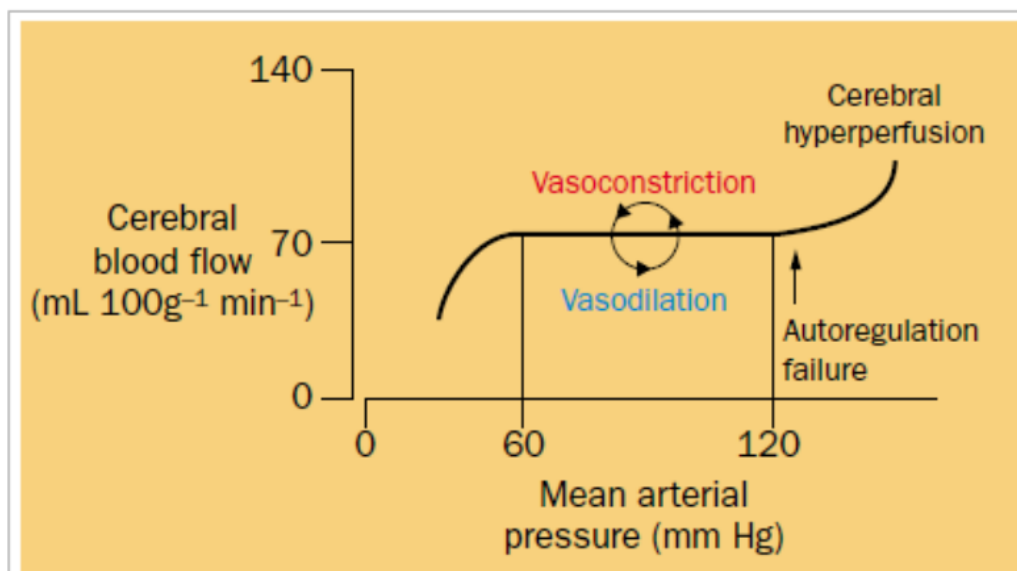


Figure 2. Schematic of autoregulation of cerebral blood flow. Retrieved from Vaughan, C.,J. et. al 2000^[28].

Table 1. Classification of hypertension according to the Joint National Committee on the Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC VII) guidelines and the European Society of Cardiology/European Society of Hypertension (ESC/ESH) guidelines for the management of arterial hypertension). Retrieved from Verdecchia, P. et. al. 2003^[1].

JNC VII				ESC/ESH 2003			
Category	Systolic (mm Hg)		Diastolic (mm Hg)	Category	Systolic (mm Hg)		Diastolic (mm Hg)
Normal	<120		and <80	Normal	120-129		anf 80-84
Prehypertension	120-139		or 80-89	High normal	130-139		or 85-89
Hypertension				Grade I	140-159		or 90-99
Stage I	140-159		or 90-99	Grade II	160-179		or 100-109
Stage II	≥160		or ≥100	Grade III	≥180		or ≥110

Alterations of cerebrovascular autoregulation by hypertension disrupt maintaining constant range of perfusion pressure, which is usually 70 to 150 mm Hg [29]. Therefore, hypertensive conditions set higher limits than previous pressure value for the brain, which is now more susceptible to ischemic injury [30]. As an example, impairments of endothelial relaxation are caused by a drop in perfusion pressure and greater reduction in cerebral blood flow (CBF). Cerebral arterioles are unable to dilate and provide suitable flow to ischemic parts of the brain [30]. Vascular muscle cell ANG II affects cell through AT₁ receptor binding. It starts a cascade binding phospholipase C and Src within a short time. Src and phospholipase C both interact with inositol trisphosphate and contribute muscle contraction by the presence of Ca²⁺. Activation of phospholipase A₂, periodontal ligament, tyrosine kinases, and mitogen-activated protein kinases are early processes of the cascade. They affect nuclear signaling. Generation of reactive oxygen species, proto-oncogene expression, and protein synthesis occurs lately, and therefore, they are late signaling events. At the end cellular growth, extracellular matrix deposition, and growth factor production contributes to vascular remodeling of arterioles (Figure 3) [31].

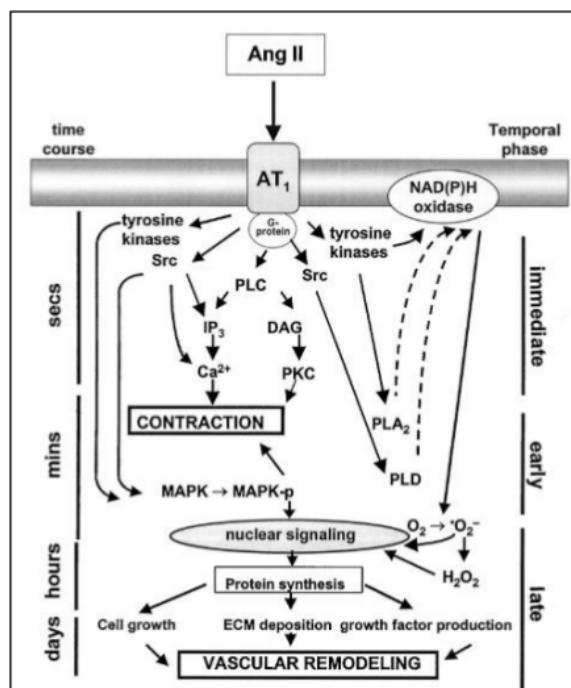


Figure 3. ANG II-mediated signaling. Retrieved from Touyz, R., M. et. al. 2000 [31].

Alzheimer's disease, dementia, stroke, and traumatic brain injury are related to hypertension [32-36]. Dementia and AD are also age-dependent disorders [34]. Moreover, it is stated that hypertension and ischemic brain injury are mostly contributing effects of A β lesions in the brain. Due to the pathological effects of hypertension, A β accumulation could not be cleared in the brain. Cognitive dysfunctions are due to impaired BBB and reduction in synaptic functions [34].

Hypertensive stimuli may have genetic backgrounds that can be triggered through the high salt intake, ANG II, or abnormal pregnancy. All those stimuli cause tissue injury in microvessels, kidney, and brain. Therefore, it activates first neo-antigens and damage-associated molecular pattern molecules and then innate immune cells such as natural killer cells, macrophages, dendritic cells, or myeloid-derived suppressor cells. Lastly, adaptive immunity cells effector memory T cell, helper T cell, regulatory T cell become active. In the end, hypertensive stimuli contribute to the pathophysiology of hypertension (Figure 4) [37].

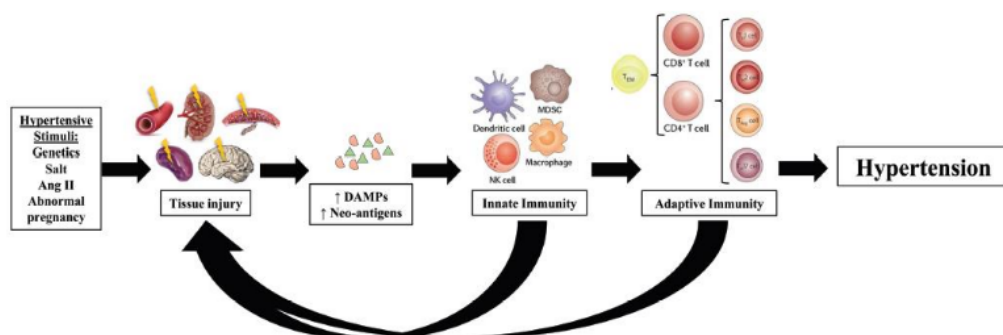


Figure 4. Schematics of hypertension and immunity relations. Abbreviations: ANG II, angiotensin II; DAMPs, danger-associated molecular patterns; NK cell, natural killer cell; MDSC, myeloid-derived suppressor cell; T_{em}, effector memory T cell; Th, helper T cell; Treg, regulatory T cell. Retrieved from Lopez Gelston, C., A., et. al.2017^[37].

2.1.1. Acute hypertension

Acutely elevated arterial pressure causes hypertension [38]. Pathophysiology of acute hypertension in which mechanisms do not completely understand is mainly caused by the failure of the endothelium. However, it was known that disruption of RAAS and vasodilator regulation, increase in activity of proinflammatory mediators including vascular cell adhesion molecules and release of local vasoconstrictors contributes to the pathology of the disease [28]. An animal study showed that acute hypertension contributed forced and long- lasting vasodilation in some part of the brain but some resistant regions to the acute rise managed vascular tone by an increase in the tone [39].

Effects of hypertensive conditions on BBB were studied with albumin extravasation method at many studies [40-45]. Johansson and his colleagues stated that in acute hypertensive conditions which induced by adrenalin, noradrenalin, and angiotensin, adrenalin significantly increased albumin permeability into the brain [43]. They claimed that different effects of drugs on cerebrovascular tone lead different levels of BBB disruption so that protein leakage in the brain was differed according to the level of disruption [43]. A study showed that albumin extravasation was considered as “leaky sites” under control and acute hypertensive conditions [45]. It was reported that these

sites primarily occurred around cerebral venules and veins [45]. It was stated that there was a maximum number of leaky sites during an acute increase in arterial pressure because of those sites became leaky during the progression of acute hypertensive conditions [45]. Another study on the senescence-accelerated mouse shows that serum albumin did not pass the BBB in both young and aged mice [46]. Therefore, acute hypertension affects the permeability of BBB regardless of the age of the mice.

One study shows that other parameter like Ca^{2+} antagonist, nifedipine, can modulate the permeability disruption in acute hypertensive rats by controlling the contraction of vascular muscles [41]. Another study with magnesium sulfate treatment suggested that during acute hypertensive conditions, late-pregnant rats which have reduced BBB permeability [47]. Poulet and colleagues showed that acute hypertension caused an increase, through hyperperfusion, in neurodegenerative factors like oxidative stress, increase in nicotinamide adenine dinucleotide phosphate hydrogen NADPH oxidase enzymatic activity, and inflammation, increase in expression of interleukin- 1β , (IL- 1β), and tumor necrosis factor- α , (TNF- α), which typically occurs also in cerebral hypoperfusion [48]. A recent study showed that messenger ribonucleic acid levels of tight junctions (TJs) (claudins-3, -5, -12) were decreased during acute hypertension [38]. Therefore, the acute elevation of arterial pressure caused damage to brain microvessels by decreasing the gene expression of claudins in the endothelial cells of BBB [38].

Acute hypertension affects brain vasculature and BBB integrity. For example, age or a treatment like magnesium sulfate decreases BBB permeability [47]. On the other hand, in the case of acute hypertension, there was an increase in inflammation and NAD(P)H oxidase activity [48]. Moreover, TJs proteins levels were decreased during the elevation of arterial pressure [38]. Lastly, an agent, nifedipine, calcium channel blocker, was reported to modulate BBB permeability [41]. Overall, in the literature, the effects of acute hypertension on BBB integrity remain unclear.

2.1.2. Chronic hypertension

Chronically hypertensive patients suffer from chronic elevation of intra-arterial pressure [49, 50]. Vascular hypertrophy is a common pathology in chronic hypertensive conditions [51]. It has important physiological effects on limiting blood flow at maximum vasodilatation and increase in vascular responsiveness towards stimuli of constrictor [52]. Chronic hypertension also causes vascular resistance. For example, Figure 5 showed that mesenteric resistance arteries from Wistar- Kyoto (WKY) and spontaneously hypertensive rats (SHR) and SHR showed narrower remodeling than WKY. Confocal images (40x) were taken increased co-localization immunofluorescence for Toll-like receptor 9 (red) and MyD88 (green) [53]. It leads to a reduction in increased CBF as a result of cerebral vascular hypertrophy in the brain [53]. Hart and his colleagues suggested that in the case of protection of small cerebral arteries by large arteries which leads to attenuation of the increase in blood flow, then small cerebral arteries may not undergo hypertrophy during chronic hypertension [52].

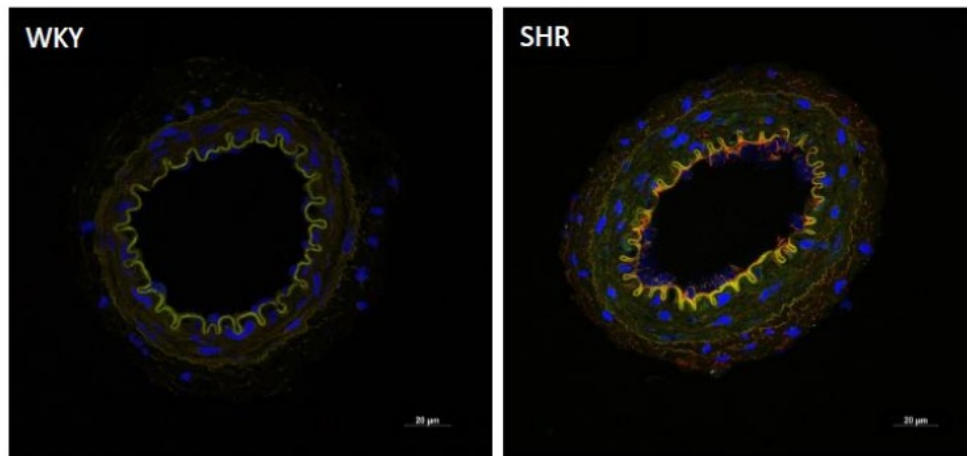


Figure 5. Schematics of arterial remodeling in hypertension. Mesenteric resistance arteries from WKY and SHR. SHR showed narrower remodeling. Confocal images (40x) of increased co-localization immunofluorescence for Toll-like receptor 9 (red) and MyD88 (green). Retrieved from Mueller, S., M. et. al. 1980^[53].

There are many studies on the effects of chronic hypertension on cerebral arteries and BBB integrity. One of the studies shows that the disruptive effect of nitric oxide (NO)- blockage- induced chronic hypertension on BBB permeability was enhanced by exposure of aluminum [42]. Another study examined the cerebral arteries of chronic hypertensive mice model. They created a mouse model by overexpressed human renin and angiotensinogen. In those mice, they showed superoxide-mediated endothelial dysfunction in a genetic model of ANG II-dependent hypertension for the first time [54]. On the other hand, Naessens and her colleagues concluded that chronic hypertension could give more damage to BBB of SHR than normotensive WKY rats of 10 months of age; however, they stated that there was no leakage of a fluorescently labeled lectin at the hypertensive group [55]. Overall, studies showed disruptive effects of chronic hypertension on BBB integrity; however, exact mechanisms still remain elusive.

2.2. Brain barriers

Different types of barriers evolve in the brain to avoid taking up peripheral harmful substances from blood to brain. There are different types of brain barriers, which are BBB, blood-cerebrospinal fluid barrier (BCSFB) and, arachnoid barrier. In addition to these barriers, the meningeal barrier, the fetal cerebrospinal fluid brain barrier, and adult ventricular ependymal are also mentioned.

Mainly, BBB is composed of complex central nervous system (CNS) microvascular endothelial cells, and BCSFB composed of choroid plexus epithelial cells [56-58]. All of these barriers maintain brain homeostasis via creating and protecting brain fluid composition (Figure 6).

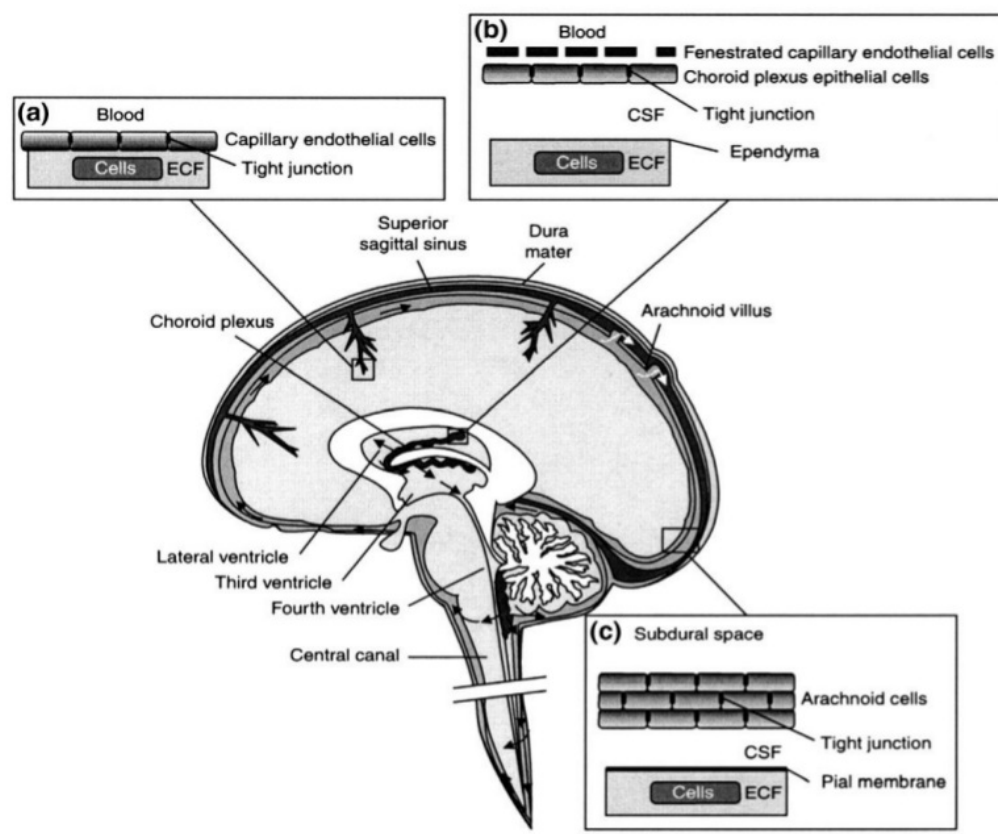


Figure 6. Schematics of brain barriers. Retrieved from Kandel, E., R. 2000^[58].

As well as the protection of the brain from toxic substances which are sourced from peripheral blood, to detect the harmful substance in the blood and cerebrospinal fluid (CSF) is also crucial for keeping brain homeostasis stable. On the other hand, there are regions called “circumventricular organs (CVOs)”, which do not have barrier properties, in the CNS. These organs have fenestrated capillaries, so they are highly permeable to blood-borne products [59, 60]. As a result of this fenestrated capillaries, the brain can communicate with peripheral organs through CSF and blood circulation. CVOs are listed below:

Secretory CVOs:

1. The pineal gland (epiphysis) secretes melatonin and associated with circadian rhythms.
2. Posterior pituitary (neurohypophysis) releases neurohormones, oxytocin, and vasopressin.
3. Median eminence, regulates anterior pituitary through the release of neurohormones.
4. Subcommissural organ secretes glycoproteins into CSF.

Sensory CVOs:

1. Subfornical organ, controls body fluids and salt-water intake.
2. Organum vasculosum of the lamina terminalis, a chemosensory area that detects peptides and other molecules.
3. Area postrema regulates vomiting reflex and feeding.

2.2.1. Blood-brain barrier

The blood-brain barrier is a border which controls the passage of substance and immune cells from the blood into the brain parenchyma in the CNS. It is mainly composed of endothelial cells of microvessels and TJs [61]. Although it does not allow to passage certain size into the brain parenchyma, it allows passing substance in a tightly controlled manner [61-63]. The BBB is essential to regulate the passage of most of the nutrients, oxygen molecules, and ions [62]. Tight regulation of BBB restricts the entry of toxins, pathogens, and immune cells from blood to the brain, and protects CNS from inflammation, injury, and disease for the homeostasis [63]. There is a space between neural body and endothelial cells of capillaries; therefore, the transport of molecules between two side needs to exceed that space by using certain diffusion pathway. Short diffusion pathway between capillaries and neural body in CNS provides rapid delivery (< 1 s) of substances [64]. The only molecular weight of < 400 Da and form of < 8 hydrogen bonds molecules can pass through BBB as small lipophilic molecules [65].

The neurovascular unit (NVU) is composed of vascular endothelial cells, pericytes, basal lamina, astrocytes, oligodendroglia, microglia and neurons (Figure 7) [66, 67].

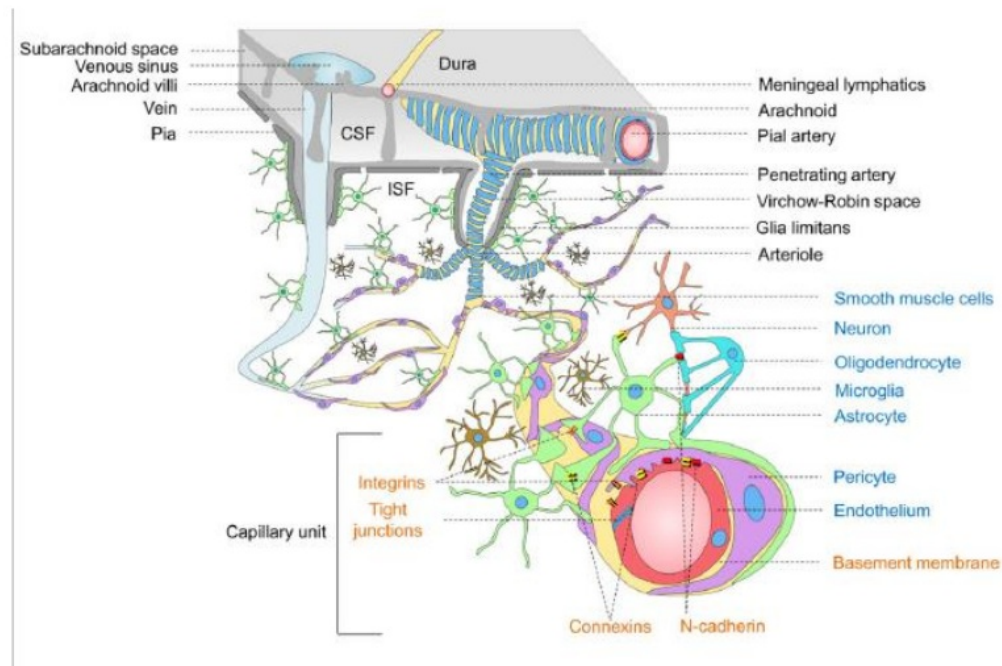


Figure 7. Schematics of the neurovascular unit. Retrieved from Zhao, Z. et. al. 2015^[67].

The BBB is conducted by interendothelial junctions and lack of fenestral formation where paracellular transport taking place across the endothelial cells [68-70]. On the other hand, specific transporters and enzymes contribute to the active exchange of substances between blood and brain tissue [70]. Other components such as basement membrane separate endothelial cells from other compartments of BBB. Pericytes which are attached to basement membrane cover abluminal sides of the endothelium to give physical support. Astrocytes project its endfeet toward the vessel wall [70]. This complex structure of BBB creates essential barrier formation for CNS homeostasis. Beyond the maintenance of homeostasis, negative conditions related to vascular diseases such as uncontrolled cell regeneration, barrier breakdown, apoptosis, cellular migration, and proliferation also affect BBB integrity [71]. Disruption of TJs, increase in transcytosis, change in transport mechanisms, and increase in leukocyte infiltration

are some of the signs of the disease state of BBB [72]. Vasoactive proteins such as vascular endothelial growth factor (VEGF), reactive oxygen species, inflammatory cytokines (IL-1, IL-6, TNF- α , proteases (matrix metalloproteinases -2, -9), and leukocyte adhesion molecules (P-selectin, E-selectin, intracellular adhesion molecule-1, vascular cell adhesion molecule-1) are important markers for pathological state or breakdown of the BBB [72]. Figure 8 summarized cause, characteristics, and consequences of BBB breakdown [73].

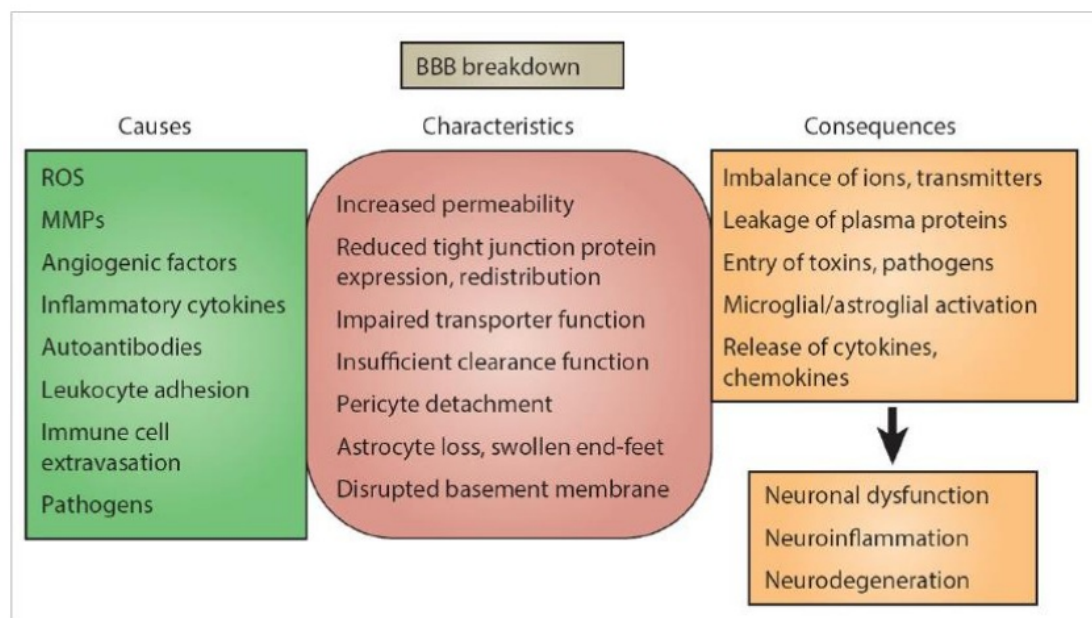


Figure 8. Schematics of cause, characteristics, and consequences of BBB breakdown. Retrieved from Obermeier, B. et. al. 2013^[73].

2.2.1.1. Endothelial cells

Barrier type of endothelial cells regulate the transport of molecules and fluids between blood and brain via not only transporters and receptors but also caveolar-mediated vesicular transport which is called “Transendothelial” or opening of TJ called “Paracellular” route [74-77]. It is well known that TJ proteins, claudin-3, -5 and occludin, work for the paracellular route [75- 78]. Barrier type of endothelial cells of brain microvessels functions as a member of NVU [61, 62, 64, 78].

The transcellular route of endothelial cells is mainly active one for healthy BBB function; however, it was suggested that paracellular route became the dominant one in severe septic conditions and the existence of a brain tumor [79, 80]. Since BBB have a vital role in brain homeostasis, it is the first structure that is affected by pathological conditions related to the CNS. Also, BBB is the main obstruction for transporting several therapeutics of neurodegenerative diseases like Alzheimer's diseases, epilepsy, and hypertensive encephalopathy from blood to the brain. Therefore, it is essential to study pathways affecting BBB integrity and create an option for new therapeutics.

2.2.1.2. Junctional components

Endothelial cells in the brain microvasculature are different from endothelial cells of non- neural tissues, which are mainly like epithelial cells. Because the paracellular gap between the endothelial cells seals by inter-endothelial junctions: TJs and adherence junctions (AJs) in the brain (Figure 9), [61, 62, 70, 72, 81]. Transmembrane TJ molecules are immunoglobulin-like cell junctional adhesion molecule (JAM) is a type I integral membrane protein and presents at extracellular region two domains interchain disulfide bonds [82]. Junctional adhesion molecule is concentrated at the apical region of intercellular junctions, according to TJ [82] and tetrahelical molecules occludin [83], and claudins (claudin-3, claudin -5, and claudin -12) [84]. Intracellular TJ proteins are Zonula occludens-1 (ZO-1), 225 kDa [85], ZO-2 and ZO-3 (membrane-associated guanylate kinase-like, MAGUK, family), and other accessory proteins cingulin, an acidic, heat-stable protein [86], AF-6, and 7H6, 155 kDa (Figure 11), [87].

According to lipophilic, hydrophilic, and molecular weight characteristic, blood-borne substances are eliminated or pass through the paracellular route [61]. Vascular endothelial cadherin and neural cadherin are adherents junctions both acting via homophilic interactions, but vascular endothelial - cadherin also plays a role in sealing endothelial cells, consecutively [88]. Little is known about AJs; however, it is stated that AJs regulate cell to cell contacts and contribute to cell polarity at the brain barriers [56].

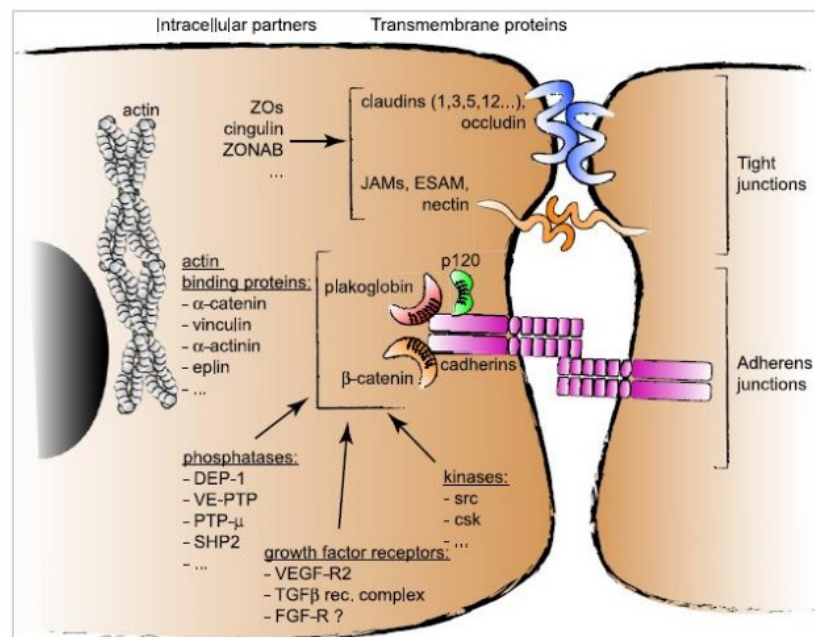


Figure 9. Schematics of TJ and adherens proteins in the endothelial cell. Retrieved from Dejana, E. et. al. 2009 [81] .

On the other hand, TJs have specialized characteristics that conduct electrical resistance on brain vasculature [62]. Tight junctions regulate the passage of solutes and ions through the paracellular route [56]. Also, it controls free movements of proteins and lipids [81]. Transmembrane proteins such as claudins, occludin, JAMs, and zona occludens as cytoplasmic proteins are a member of the TJ proteins [70].

2.2.1.3. Basal lamina

Basal lamina separates endothelial cells and pericytes embedded to the basement membrane from the other cells of the NVU, and astrocytic endfoot touch basal membrane at their contact points. Therefore, basal lamina is an interactive component of BB, which is the connection between astrocyte, pericyte, and neurons [70, 89].

2.2.1.4. Pericytes

Pericytes are vascular mural cells that embedded to the basement membrane of blood microvessels, and they have specific contact points on the endothelium [90]. Pericytes cover 30% to 70% of the capillary wall. Moreover, the pericyte- endothelia ratio in the brain is 1:3 [34]. Recent markers of pericyte are platelet-derived growth factor receptor- β [91] and the neural/glial antigen two proteoglycans [92]. It was revealed that they are essential elements for blood microvessels and important to control development, stabilization, and remodeling of vascularization [90]. Adhesion points between the basement membrane and pericytes formed as holes. These pericyte- endothelial contact points can vary up to 1.000 for a single endothelial cell. Some of them called “peg-socket type,” which are cytoplasmic fingers of pericytes inserted into endothelial invaginations [93, 94]. Another type of contact point is where membranes of two cell types are very close to each other. These close contact points are thought to facilitate anchoring function. The last one is adhesion plaques, which express microfilament bundles from the plasma membrane of pericyte into the cytoplasm of the endothelial cell [93]. Neural-cadherin and connexins are examples of adhesion plaques type connection.

Also, Li and colleagues stated that communication between pericytes and neighboring endothelial cells is facilitated by TGF β and its receptor TGFR β [95]. Pericytes, together with endothelial cells, play a crucial role in the contraction of brain capillaries, arterioles, and small pial arteries. Therefore, the proper functions of neurons and synapses are affected by CBF, BBB integrity, and clearance of toxicity [34].

2.2.1.5. Astrocytes

Astrocytes, as a glial cell, have a variety of functions in CNS [70]. Most of the signals in perivascular space targeting to vasculature should pass through astrocytes to reach the vessel wall [96]. They buffer extracellular space against potassium ions, regulate various neurotransmitters, are a dynamic component of BBB, and supply nutrients such as lactate and growth factors to neurons, immune control regulators in the

CNS, are a source of apolipoprotein E for homeostasis of the brain [97]. The unique process of buffering is related with the ability of astrocytes together with other glial cells, they are connected to each other via gap junctions, and therefore they exchange potassium, sodium or chloride ions between large groups of cells at an astrocyte field [97].

Moreover, it is known that they remove glutamate, gamma-aminobutyric acid (GABA), dopamine, norepinephrine, serotonin, and acetylcholine at extracellular space, so they interact with many different types of neurons [97]. Also, they produce growth factors like epidermal growth factor, fibroblast growth factor, nerve growth factor, and ciliary neurotrophic factor. Since astrocytes have a capability of antigen presenting, they directly affect BBB permeability and circulating T cells [97]. Other than many factors that astrocytes support NVU, it also gave physical support to endothelial cells (Figure 10). The rosette-like structure on endothelium provides controlled diffusion of substance from blood to brain parenchyma [98].

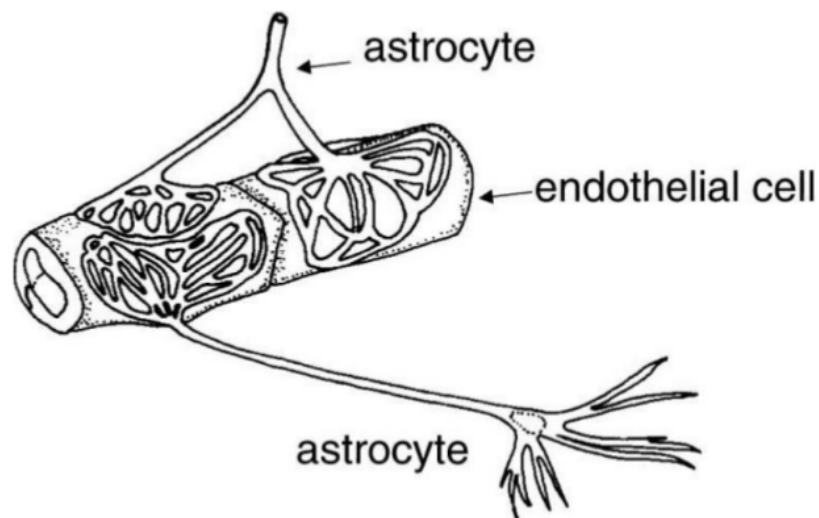


Figure 10. Schematics of astrocyte- endothelial cell relationships and physical support of perivascular astrocyte endfeet to the endothelium. Retrieved from Abbott, N., J. 2002 [98]

2.2.1.6. Microglia

Microglia are parenchymal tissue macrophages and constitute 10% of cells in CNS [99]. They are primary innate immune effector cells in CNS and originated from yolk sac in the early developmental stage, and primitive form migrates to the neural tube. In the adult brain, the main job of microglia is to detect and act on the case of any pathological event. They control the changes in ionic balance, presence of damaged cells, or infectious agents in the neural environment and reside in proximity with endothelial cells [70]. They have two activity states: in the M1 pathway, microglia releases pro-inflammatory cytokines like IL-1 β and TNF- α while in M2 pathway, they involve in tissue repair, phagocytosing damaged neuron, and foreign substance, releasing chemokine, and VEGF [62].

2.2.1.7. Neurons

Within the brain, there are nearly 100 billion neurons, with nearly 1000 synaptic connections per cell [61]. Therefore, neurons create a huge neuronal network and fed by many microvessels. Damage of one blood microvessel can cause damage to several neurons since their energy demand and other substitutes for proper functioning are provided by capillary [70]. The relation between neurons and blood microvessels depends on energy demand of neurons and other functions like regulating CBF is regulated by a positive and negative feedback mechanism, which is called neurovascular coupling (NVC). At each level of arterial branching, artery, arteriole and capillary, neurons and other cells, pericytes and astrocytes are interconnected between each other and also endothelial cells as well as astrocytic endfeet (Figure 11). Local neuronal activation as a result of local needs of nutrients supplied by blood causes increases blood flow at the area [100].

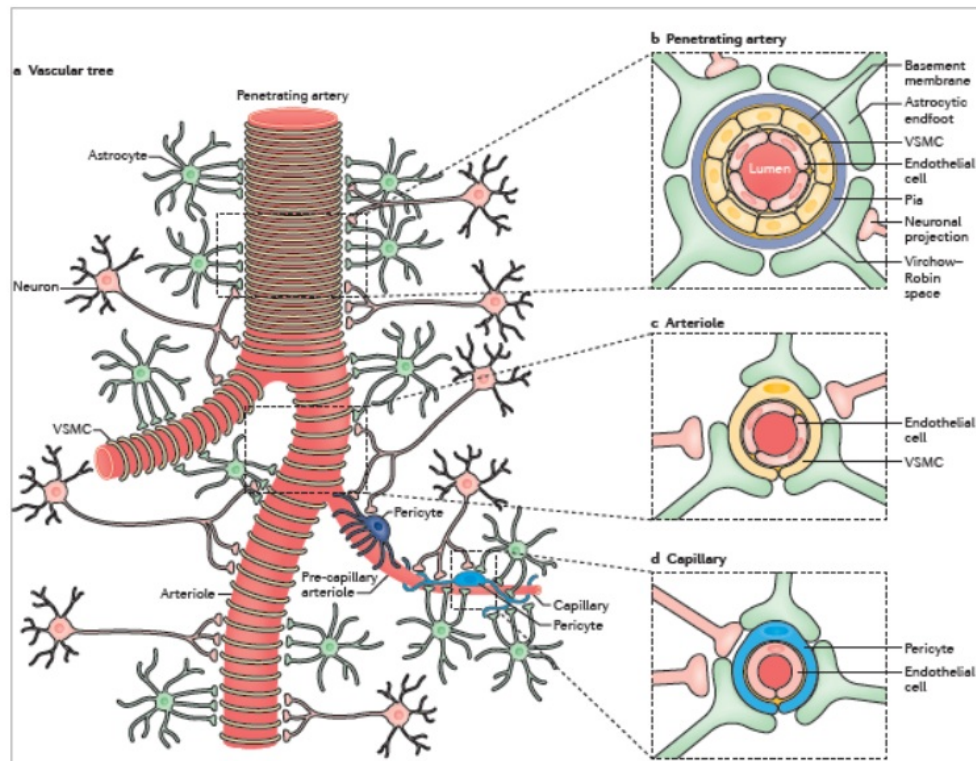


Figure 11. Representation of neurovascular coupling. Retrieved from Kisler, R. et. al. 2017 ^[101].

At first, when neuronal activity changes, local cellular environment such as oxygen partial pressure, pH, temperature, ion balance, neurohormones, and neuropeptides are affected. On the other hand, those changes can have chain reactions which may be independent of vasodilation or vasoconstriction and capillary flow [102]. However, NVC creates a bridge between vascular and neural cell, which in turn make the concept essential for the NVU [100].

2.3. Paracellular and transcellular transport in the endothelium

Transportation between endothelial cells and brain parenchyma are divided into two categories: paracellular and transcellular transport. If the transport occurs between endothelial cells, it is called “*paracellular*”, if it is through the endothelial cell, then it is called “*transcellular*” transport [103].

Paracellular transport is facilitated by TJs. Tight junctions provide highly selective passage of compounds across an intact BBB. Most of the hydrophilic compounds are dependent on this route if it is not possible to transport via active transporter molecule. Therefore, paracellular transport both size dependent and permeability limited [103].

Transcellular transport is facilitated by many mechanisms (Figure 12), [68, 69, 103]. Those mechanisms are simple diffusion, facilitated diffusion, adsorptive mediated endocytosis (pinocytosis), receptor-mediated endocytosis, and include influx/efflux transporters at BBB. In the case of simple diffusion, lipophilicity and size are essential characteristics, and the concentration gradient is dominant at this kind of transport. It is worth to note that the rate of diffusion can be blood-flow limited [103].

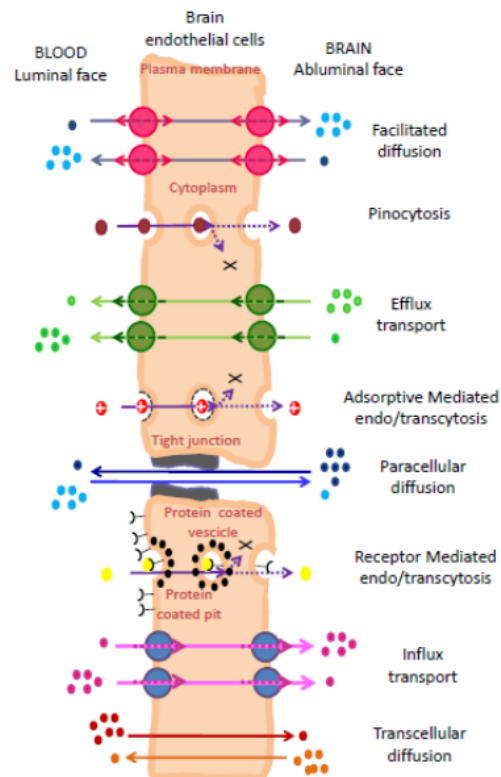


Figure 12. Schematics of transport mechanism at BBB. Retrieved from De Lange, E., C. 2012^[103].

The summary of the transport pathways in the barrier type endothelial cells of brain blood microvessels:

Paracellular Pathway:

It involves TJs, claudin-3,-5, occludin, and JAMs and tight regulation of movement of solutes provide controlled passage.

Transcellular Pathways:

1. **Facilitated diffusion:** It is a passive process because the direction of the diffusion is from high to low concentration. It needs a transporter to facilitate this kind of diffusion.

2. **Pinocytosis:** It is a transcellular pathway that free solute movement is taking place in endothelial cells via plasmalemmal vesicles.
3. **Efflux transport:** Efflux of amino acids, peptides, cations, nucleosides were used that way. The best-characterized efflux transporter is ATP-binding cassette transporters, as P-glycoprotein. Large hydrophobic, positively charged molecules are mostly extruded.
4. **Adsorptive mediated endo/transcytosis:** It involves vesicles of charged substance from outside to the inside of the cell or vice versa.
5. **Receptor-mediated endo/transcytosis:** Vesicle is formed after the binding of a substance to specific receptors.
6. **Influx transport:** Influx of amino acid, organic cations, peptides, monocarboxylic acids from blood to brain side use influx transport. Some drugs such as baclofen, gabapentin, and phenylalanine are the substrates.
7. **Transcellular diffusion:** It is a simple diffusion and direction of the diffusion is from high to low concentration.

2.3.1. Endothelium in hypertension

Vascular remodeling is a consequence of hypertension. Blood microvessels including capillaries, arterioles, and small arteries (lumen diameter < 300 μm) show peripheral resistance to blood pressure. One of the reasons for the elevated resistance in hypertension is decreased in connected microvessels and narrowing of the lumen of microvessels. Reduced lumen area and increased media-lumen ratio without an increase in the media area in resistance microvessels are consequences of untreated essential hypertension [104]. Major factors that cause endothelial dysfunction reduce NO bioavailability that causes the loss of control of vasodilators over vascular smooth muscle, higher in sensitivity of endothelial cells to vasoconstrictors, elevated vasoconstrictor production, and elevated shear stress. Among them, oxidative stress is counted like as many factors for the pathophysiology of hypertension. Reactive oxygen species contribute to the effects of inflammation and obesity. That oxidative stress elevates the oxidative damage in cellular level by changing DNA molecule, amino acids, proteins, lipids, and carbohydrates (Figure 13), [105].

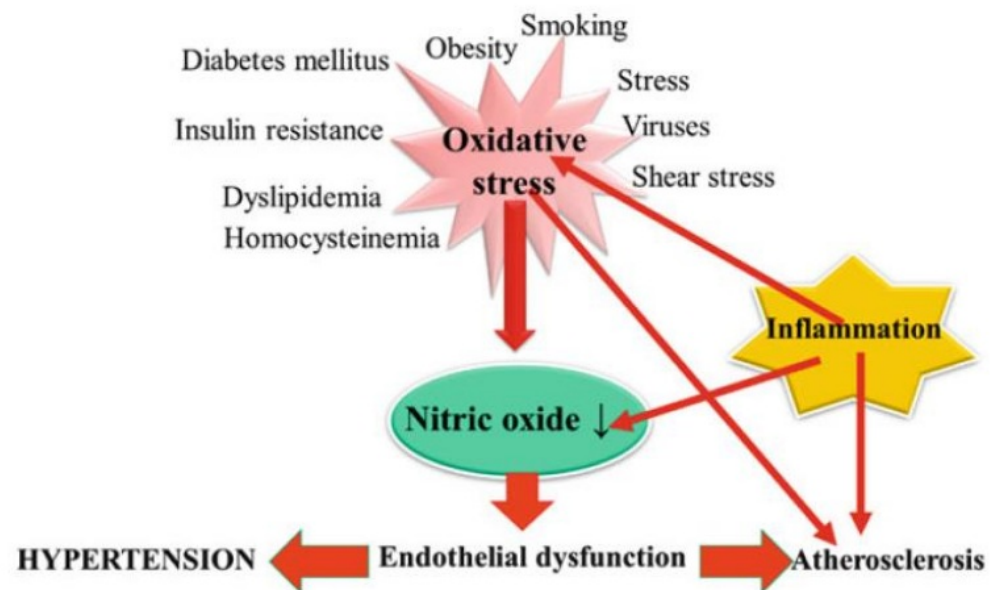


Figure 13. Schematics of the pathophysiology of hypertension caused by oxidative stress. Retrieved from Konukoglu, D. et. al. 2017^[105].

Many local vasoactive agents such as vasodilator molecule NO and the vasoconstrictor peptide endothelin play a vital role in the normal functions of vascular endothelial cells to maintain vasotonus (Figure 14). Dysfunction of endothelium; therefore, results in essential hypertension. Once the hypertensive process has been established, clinically effective antihypertensive therapies could be failed due to the severe consequences of endothelial dysfunction [106].

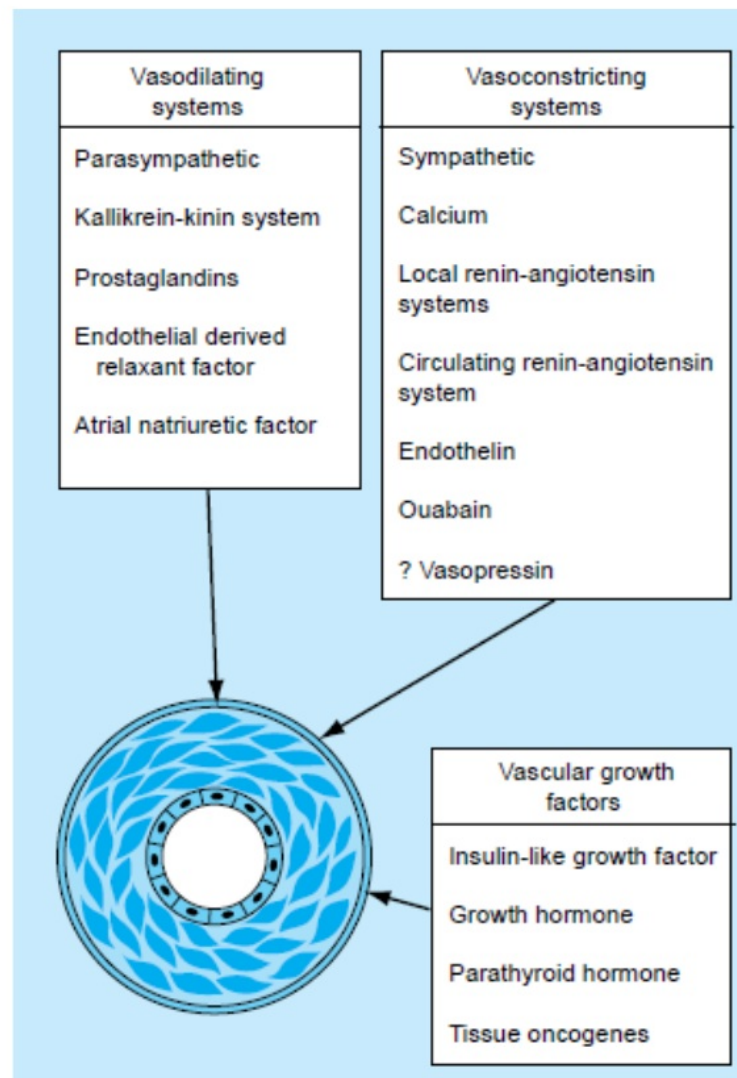


Figure 14. Schematics of control of arteriolar resistance. Retrieved from Beevers, G. et. al 2001^[106].

In the literature, there are opposite statements about the change in endothelial transport at hypertensive conditions [38, 107-109]. To begin with, transport dysfunction at BBB was reported in hypertensive conditions. Studies on chronic hypertensive rats have reported decreased levels of occludin and ZO-1 proteins [109]. Another study mentioned that during short-term acute hypertensive conditions, mRNA expressions of claudins isoforms were reduced. This reduction caused BBB breakdown in response to

oxidative stress on endothelial cells [38]. Moreover, Ueno and his colleagues reported that the hypertensive state increased the BBB permeability more remarkably in the hypothalamus and hippocampus regions of SHR and stroke-prone SHR (SHRSP) [107]. On the other hand, administration of ANG II *in vitro* conditions did not cause a decrease in TJ proteins expressions, claudin-5 and occludin levels [108]; however, hypertensive conditions are mostly associated with a decrease in TJ proteins level (Figure 15).

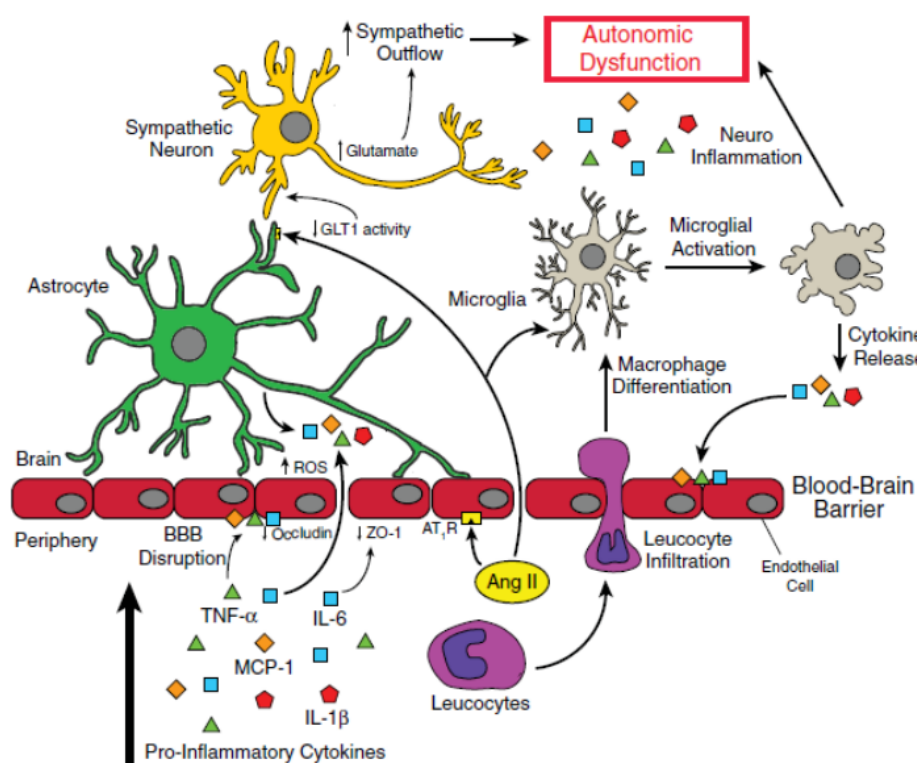


Figure 15. Schematic of dysfunction of BBB in hypertensive conditions. Abbreviations: Ang II, angiotensin II; AT1R, angiotensin II type 1 receptor; GLT1, glutamate transporter 1; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; ROS, reactive oxygen species; TJ, tight junction; TNF- α , tumor necrosis factor- α ; and ZO-1, zonula occludens-1. Retrieved from Setiadi, A. et. al. 2018^[110].

2.4. Transforming growth factor beta in hypertension

Transforming growth factor beta receptors belong to the serine-threonine kinase receptor family. Upon the ligand binding to transforming growth factor β receptor type II (TGF β RII), TGF β RII binds to TGF β RI, and phosphorylates TGF β RI; therefore, they become a receptor complex. Activation of the kinase domain of TGF β RI phosphorylates Smad2 and Smad3. The smad2/smad3 complex binds to Smad4, and Smad4 binding facilitates to enter the nucleus to regulate gene transcription [111]. TGF β has a variety of physiological functions for the body, and the absence of TGF β causes many diseases related to inflammation, atherosclerosis, cancer, and autoimmunity [112]. It is also reported that an increase in TGF β causes fibrotic diseases in kidney, liver, and lung by contributing to the severity of progress [112].

Since there are various mechanisms that participate in BBB dysfunction in both acute and chronic hypertensive conditions, increased production of TGF β RI is one of these mechanisms related to hypertensive conditions [113, 114]. Severe increase in TGF β protein is related to elevated ANG II, increased systemic blood pressure, fluid shear stress, and differential TGF β I gene expression in essential hypertensive conditions [115].

It was reported that TGF β RI signaling pathway with Smad2 and Smad3 proteins play an essential role in the proliferation and angiogenesis of endothelial cells [116]. Syntenin, a multifunctional adapter protein involved in a diverse array of functions including trafficking of a transmembrane protein, induced the activity of TGF β RI through Smad2/Smad3 complex, and it inhibited caveolin-1 (Cav-1) mediated TGF β RI internalization and as a result, disrupts the relationship between Cav-1 and TGF β RI [117]. Another study stated that Cav-1 mediated TGF β RI internalization removes the signaling pathway of TGF β RI [118].

In literature, there are contradictory results about the effects of TGF β on BBB integrity. While some of the studies reported the protective effects of TGF β [119-121], some of them demonstrated the disruptive effects of TGF β on BBB integrity [122, 123]. One study showed that T β RI knock-out hypertensive mice produced TGF β I in parallel to elevated blood pressure [113]. It is suggested that TGF β affects barrier type of endothelial cells, as well as Cav-1 that controls caveolae formation on the endothelial

cell membrane and regulates increased BBB permeability. In an animal model of hepatic encephalopathy, elevated plasma level of TGF β I increased BBB permeability by the loss of claudin-5 integrity, and treatment with recombinant TGF β I reduced BBB permeability [122]. Hawkins and his colleagues suggest that the addition of TGF β I into the 3D cell culture medium, the levels of claudin-5 protein were significantly reduced [124]. In another study, Shimizu and his colleagues suggested that pericytes cause BBB disruption by increasing TGF β signaling in diabetes mellitus type 2 due to the presence of advanced glycation end products (AGEs) [125]. They reported that AGEs decrease TJ production through TGF β I pathway in ischemic conditions, and cilostazol, which inhibits TGF β I signaling pathway, attenuates AGEs related ischemic injury by enhancing TJ integrity [126]. On the other hand, there are studies about the protective effects of TGF β on BBB integrity. For example, TGF β I contributes to the restoration of BBB, decreases the cell death and inflammation in intracerebral hemorrhage conditions in rats [127]. Recombinant TGF β I injections caused to reduce in BBB permeability before λ -carrageenan injection, which caused inflammatory pain in rats [119]. Another study reported that intravenous injection of TGF β I decreased the BBB disruption together with the action of matrix metalloproteinases inhibitor, plasminogen [120]. In an *in vitro* study, oligodendrocyte precursor cells showed that knockdown of TGF β I gene in these cells caused BBB dysfunction; however, in the presence of TGF β signaling pathway, endothelial cells maintain BBB integrity with the intact TJs [121]. Another study shows that TGF β I provided a therapeutic effect of neurogenesis after ischemic conditions in mice [128]. Also, inhibition of TGF β I activity both endothelial and pericytes by cyclosporine A caused an increase in BBB permeability in co-culture [129]. Overall, the debate about the role of TGF β signaling pathway on BBB is still a hot topic, and further investigation is needed to clarify the relationship between the TGF β signaling pathway and BBB.

Since it was known that under hypertensive conditions, there was an increase in gene expression of TGF β I [114, 115, 130]. Under the pathological conditions, Cav-1 and TGF β RI activities change over time [117, 118, 131, 132]. One of the mechanisms that regulates cellular response is Cav-1 mediated endocytotic vesicles in lipid raft rich regions of the cell membrane. The activation of dynamin failed to take off Cav-1 related

vesicles from the membrane, and many signaling molecules, including TGF β RI was degraded after ubiquitination process [117]. It was reported that Cav-1 protein and TGF β RI were placed together in caveolae structure; therefore, Cav-1 regulated TGF β RI in both outside and inside of the cell [131]. It is suggested that Cav-1 suppressed the TGF β signaling pathway by the internalization process of TGF β receptors by Cav-1 mediated endocytosis [118, 132]. At the same time, Cav-1 interacted with Smad2 signaling molecule and other molecules on the TGF β pathway [118]. Also, it was reported that inhibition of Cav-1 activity caused the hyperactivity of the TGF β signaling pathway [131].

By the help of information mentioned above, we aim to provide valuable data regarding the mechanistic effects (Figure 16) of TGF β signaling pathway in hypertensive conditions which affects both TGF β RI and Cav-1 activities on BBB by changing transcellular and paracellular transportation pathways.

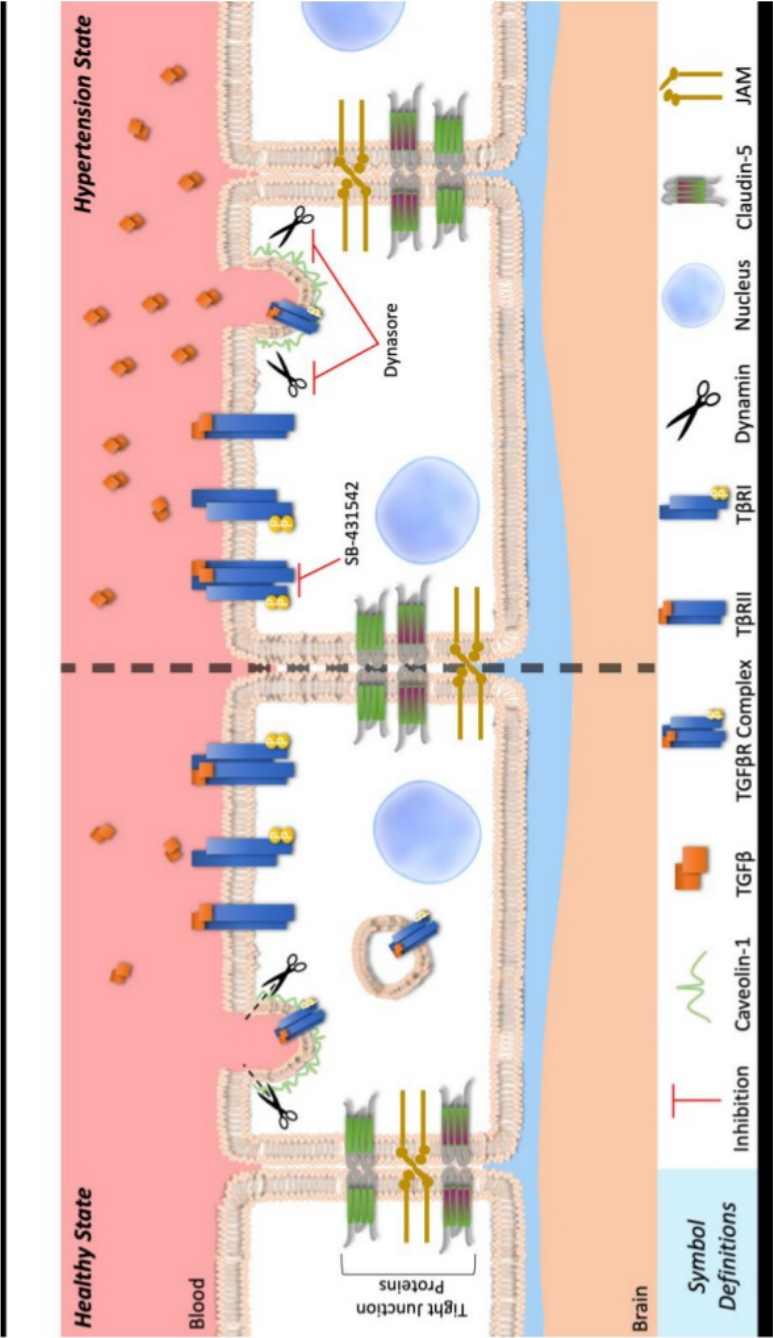


Figure 16. The hypothetical effects of hypertensive conditions on TGF β and Cav-1 proteins and their relationship with caveolar pathway and TJs alterations. Dynasore and SB-431542 are an inhibitor of dynamin and TGF β RI, respectively.

Chapter 3

3. MATERIALS AND METHODS

3.1. Materials

Chemicals, antibodies, and devices that were used in this study were shown in Table 2, 3, and 4, respectively.

Table 2. Table of Chemicals

Name	Brand name	Catalog number
Angiotensin II	Merck/Calbiochem	05-23-0101
Ketamine (Ketasol 10%)	Richerpharma Ag	-
Xylazine (Rompun 2%)	Bayer	-
Ethanol	Merck Millipore	1009862500
DMSO	Sigma Aldrich	D2650
Glutaraldehyde	Merck Millipore	354400
Paraformaldehyde	Sigma Aldrich	158127
Phosphate Buffer Tablet	Sigma Aldrich	P4417
Saline (NaCl)	Sigma Aldrich	31434
Dynasore	Tocris	2897
SB-431542 (4-[4-(1,3-benzodioxol-5-yl)-pyridinyl]-1H-imidazol-2-yl]-benzamide)	Tocris	1614
DPBS no Ca no Mg	Gibco	14190-169
Triton™ X-100	Fisher Bioreagent	BP151-100
Tween- 20	Sigma Aldrich	P9416
Sodium Citrate Dihydrate	Fisher Scientific	BP327
Normal Goat Serum	Sigma Aldrich	G9023
Hoechst 33342	Thermo Fisher	62249
Glycerol	Sigma Aldrich	49782

Table 3. Table of Antibodies

Name	Brand name	Catalog numb	Lot number
GLUT1 Polyclonal Antibody	Thermo Scientific	PA1-21041	SL2486592B
Anti-Caveolin-1 antibody	Abcam	Ab2910	GR3241952-1
GFAP (GA5) Mouse mAb	Cell signaling	3670	-
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Life Technology	A11029	1745855
Goat Anti-Rabbit IgG (H+L), Alexa	Jackson ImmunoResearch	111-605-003	-
Albumin from Bovine Serum (BSA), Alexa Fluor 594 conjugate	Thermo Fisher	A13101	-

Table 4. Table of devices

Name	Brand name
Micro-Osmotic Pump	Alzet Model 1004
Confocal Microscopy	Leica Dmi8/Cs/Sted
Cryostat	Leica Cm 1950
Noninvasive Blood Pressure System	CODA® High Throughput System Kent Scientific Corporation

3.2. Animal experiments

In the study, 25-30 gr, 10-12 weeks old male Balb/c mice were used. All animal experiments were held at Koç University Research Center for Translational Medicine “KUTTAM”, and this study was approved by the Local Animal Ethics Committee of Koç University under the approval number 2018.HADYEK.014. Experimental design of the study is shown in Figure 17. The total animal number for the study is n=24.

Experimental Groups:

1. Sham ($\Sigma n=6$)
2. ANG II ($\Sigma n=6$)
3. ANG II + Dynamin inhibitor (Dynasore) ($\Sigma n=6$)
4. ANG II + Transforming growth factor β receptor type I inhibitor (SB-431542) ($\Sigma n=6$)

Sub-group:

1. Immunofluorescence: Albumin-Alexa Fluor 594, Glut-1, claudin-5/Cav-1, and Cav-1/GFAP colocalization.

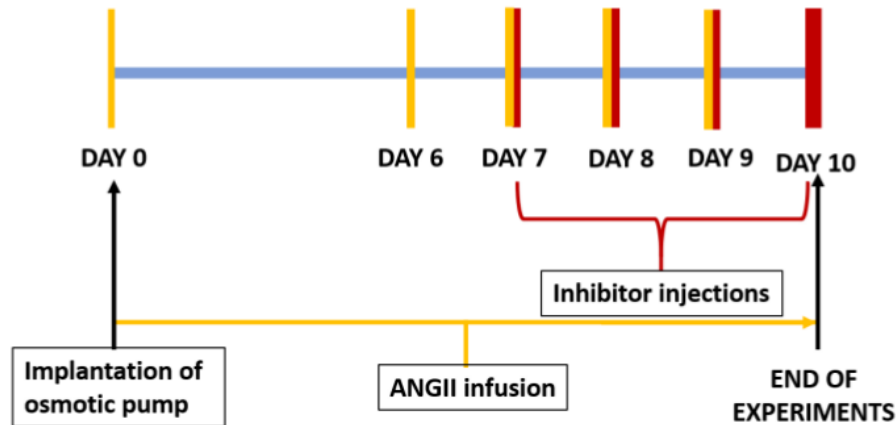


Figure 17. Experimental design of the study.

3.2.1. Hypertension model

Alzet osmotic pump (Model 1004) were filled with angiotensin II (ANG II; 2000 ng/kg/min) which was dissolved in saline, and the pump delivers 0.11 $\mu\text{L/h}$ for a 10 days period [133]. To induce hypertension model in mice, the procedure was as follows: mice were anesthetized with ketamine (80 mg/kg)/xylazine (10 mg/kg). The lower back region of mice was shaved and disinfected with 70% ethanol over the implementation

site, and then 0.5 cm long skin incision was made to insert the osmotic pump. Following these steps, a hemostat was inserted into the incision area and proceed to reach the subcutaneous tissue in order to make a pocket for the pump. When the filled pump was inserted into the pocket with correct orientation (delivery portal part of the pump should position as pointed at the back of mice), it reduced the interaction between the compound delivered and the incision. Lastly, the wound was closed with surgical stamps (wound clips).

3.2.2. Inhibitor injections

In this study, we used two different inhibitors; dynasore and SB-431542. After following one week of ANG II infusion, on the 7th day of ANG II infusion, dynamin inhibitor dynasore and TR β I inhibitor SB-431542 were intraperitoneally injected every 12 hours for three days by continuing of ANG II infusion. To make an injectable dynamin inhibitor, dynasore, as an inhibitory agent, Reid's method was used. According to this method, dynasore was dissolved in 5 mM ethanol and then diluted the concentration of inhibitor to 30 mg/kg with physiological saline solution [134]. SB-431542 (4-[4-(1,3-benzodioxol-5-yl)- 5-(2-pyridinyl)-1H-imidazol-2-yl]-benzamide) was used for TR β I/ALK-5 inhibitor and was dissolved in DMSO (20 mg/1 mL DMSO) and diluted with physiological saline solution. The inhibitor at the dosage of 0.3 mL (0.2 mg/kg body weight) was intraperitoneally injected every 12 hours or three days [135].

3.2.3. Non- invasive blood pressure measurements

The non-invasive blood pressure system (Kent Scientific Corporation, CT, USA) was used to measure indirectly arterial blood pressure. All the measurements were held at 08.00 a.m. Before measurements, the tail of mice kept warm to 32°C to 35°C and checked by laser thermometer provided by CODA system (Figure 18). To measure indirect arterial blood pressure, the volume pressure recording (VPR-cuff) apparatus were used by determining the tail blood volume (Figure 19). Other apparatus, an occlusion tail-cuff (O-cuff) measurement was used for the total blood volume in the tail without the need to obtain the individual pulse signal (Figure 19). Then, the program

was adjusted to following set-up (Figure 20, 21, 22): Acclimation cycles: 5, number of sets:1, the time between sets: 30 sec., cycles per set:10, the time between cycles: 5 sec., deflation time: 15 sec., minimum volume: 15 μ l. According to the purpose of the experiment, mice were trained for three times a week before starting the experiment. Representation of measurements and the result table were shown in Figure 23, and 24, respectively. Arterial blood pressures were measured four times a week before starting the experiment. Therefore, the indirect blood pressure was measured for all the mice without using any anesthetic agent.

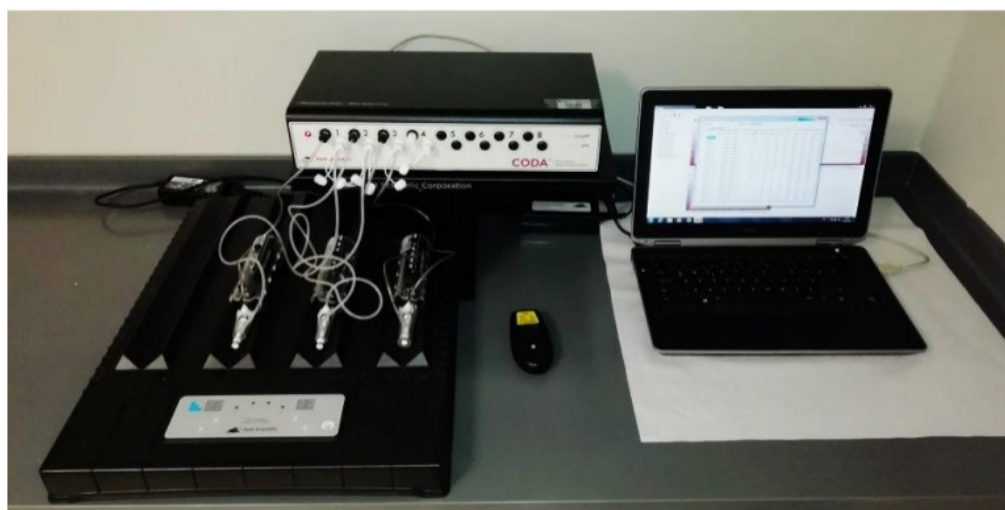


Figure 18. Schematics of Noninvasive Blood Pressure System, CODA® High Throughput System Kent Scientific Corporation.

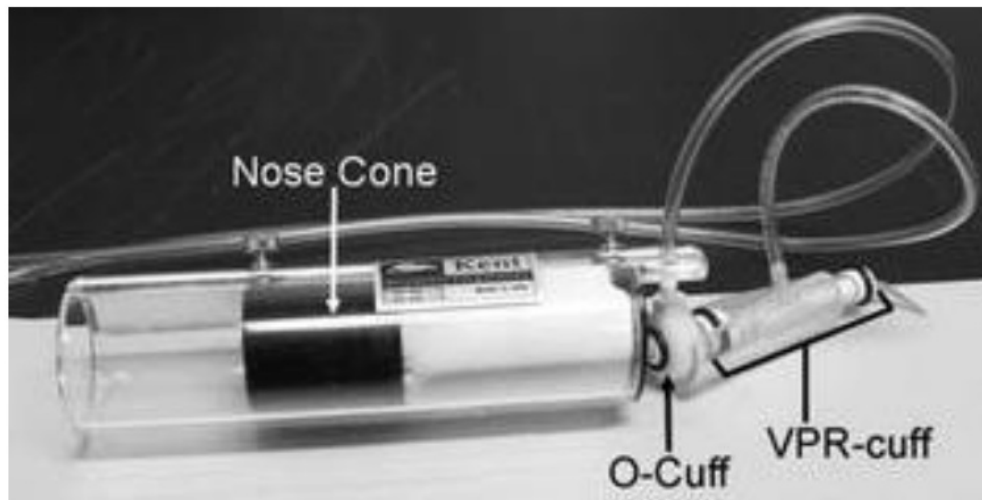


Figure 19. Representation of mice holder, nose cone, the volume pressure recording, and occlusion tail-cuff. Noninvasive Blood Pressure System, CODA® High Throughput System Kent Scientific Corporation.

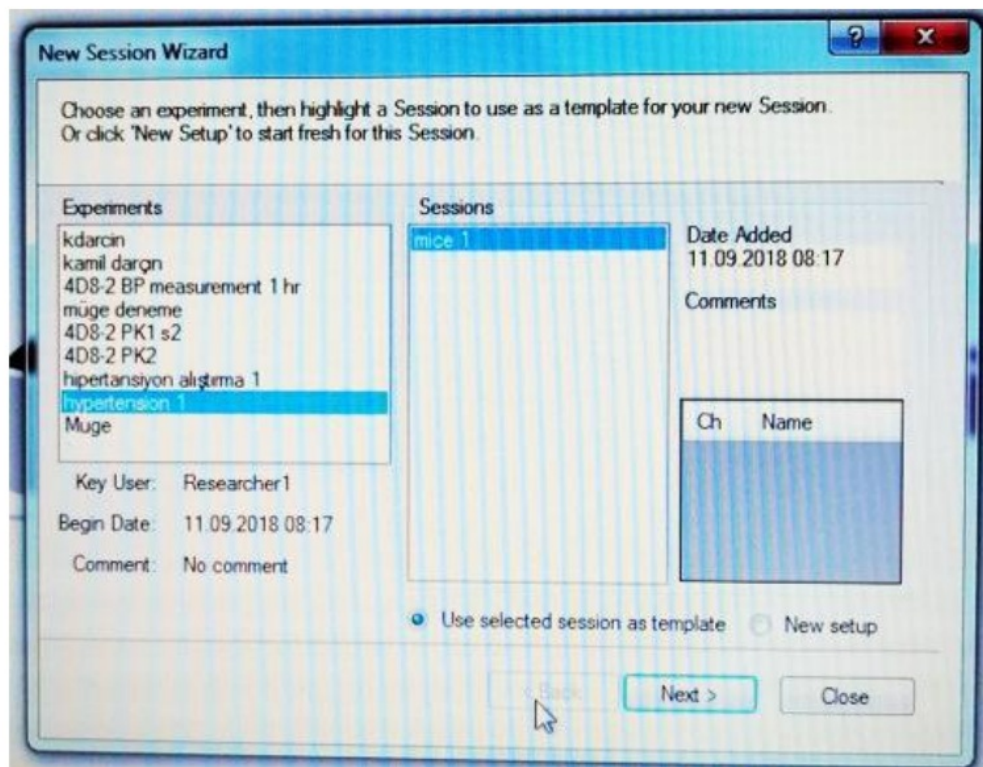


Figure 20. Program adjustment step 1.

New Session Wizard

Basic Session Information
Enter a name for this Session and set its Cycle information.

Session Name:

Acclimation Cycles:

Number of Sets:

Time between Sets:

Cycles per Set:

Time between Cycles:

Max Occlusion Pressure (mm Hg) = 250

Deflation Time (seconds):

Minimum Volume (microliters):

Display Style:

Warning! You must start the experiment for it to be saved in the Experiment Manager.

< Back **Next >** Close

Figure 21. Program adjustment step 2.

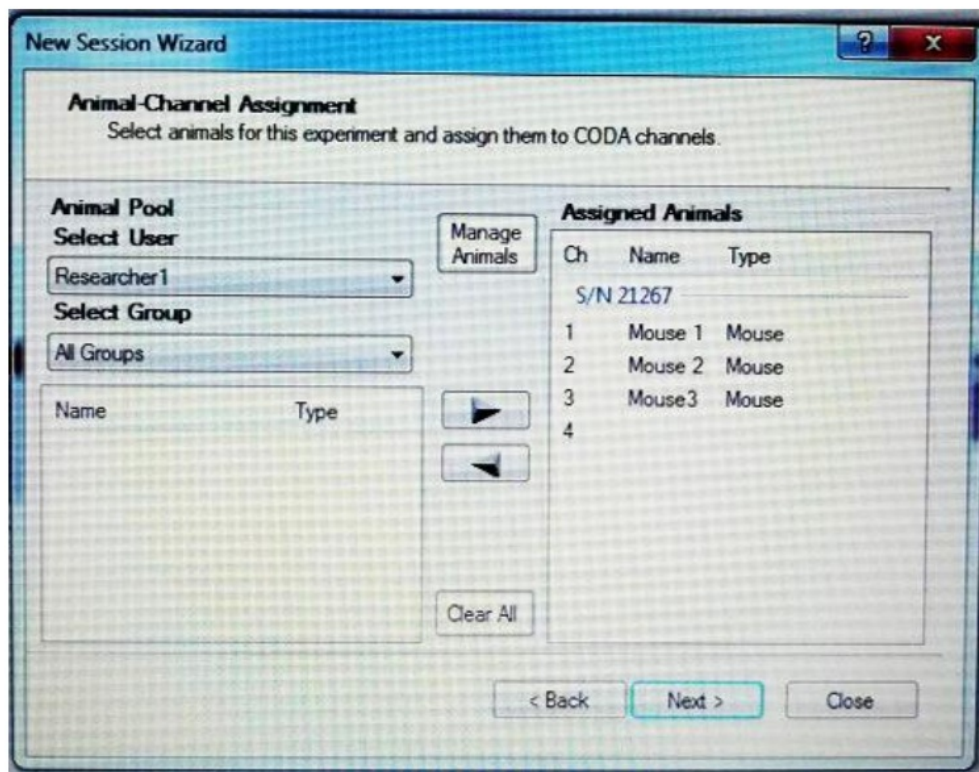
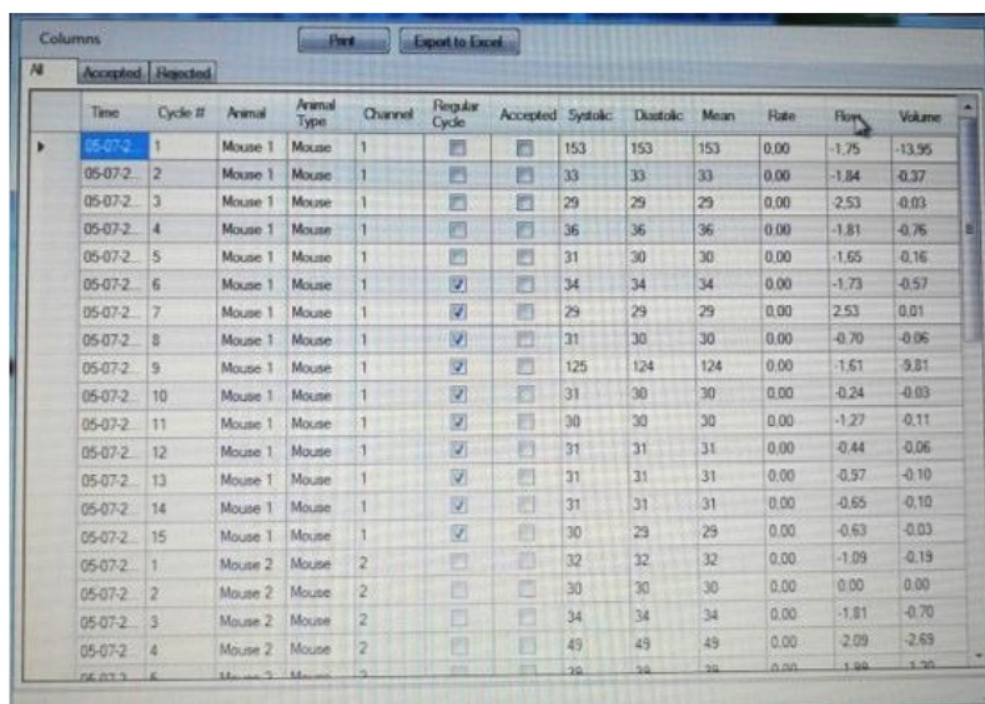


Figure 22. Program adjustment step 3.



Figure 23. Representation of measurements.



Time	Cycle #	Animal	Animal Type	Channel	Regular Cycle	Accepted	Systolic	Diastolic	Mean	Rate	Flow	Volume
05-07-2	1	Mouse 1	Mouse	1	☐	☐	153	153	153	0.00	-1.75	-13.95
05-07-2	2	Mouse 1	Mouse	1	☐	☐	33	33	33	0.00	-1.84	-0.37
05-07-2	3	Mouse 1	Mouse	1	☐	☐	29	29	29	0.00	2.53	-0.03
05-07-2	4	Mouse 1	Mouse	1	☐	☐	36	36	36	0.00	-1.81	-0.76
05-07-2	5	Mouse 1	Mouse	1	☐	☐	31	30	30	0.00	-1.65	-0.16
05-07-2	6	Mouse 1	Mouse	1	☒	☐	34	34	34	0.00	-1.73	-0.57
05-07-2	7	Mouse 1	Mouse	1	☒	☐	29	29	29	0.00	2.53	0.01
05-07-2	8	Mouse 1	Mouse	1	☒	☐	31	30	30	0.00	-0.70	-0.06
05-07-2	9	Mouse 1	Mouse	1	☒	☐	125	124	124	0.00	-1.61	-9.81
05-07-2	10	Mouse 1	Mouse	1	☒	☐	31	30	30	0.00	-0.24	-0.03
05-07-2	11	Mouse 1	Mouse	1	☒	☐	30	30	30	0.00	-1.27	-0.11
05-07-2	12	Mouse 1	Mouse	1	☒	☐	31	31	31	0.00	-0.44	-0.06
05-07-2	13	Mouse 1	Mouse	1	☒	☐	31	31	31	0.00	-0.57	-0.10
05-07-2	14	Mouse 1	Mouse	1	☒	☐	31	31	31	0.00	-0.65	-0.10
05-07-2	15	Mouse 1	Mouse	1	☒	☐	30	29	29	0.00	-0.63	-0.03
05-07-2	1	Mouse 2	Mouse	2	☐	☐	32	32	32	0.00	0.00	0.00
05-07-2	2	Mouse 2	Mouse	2	☐	☐	30	30	30	0.00	-1.81	-0.70
05-07-2	3	Mouse 2	Mouse	2	☐	☐	34	34	34	0.00	-2.09	-2.63
05-07-2	4	Mouse 2	Mouse	2	☐	☐	49	49	49	0.00	1.99	1.70

Figure 24. Representation of result table.

3.2.4. Immunofluorescence

3.2.4.1. Albumin-Alexa Fluor 594 injection

At the 10th day of ANG II infusion, 100 μ L of 1% albumin-Alexa Fluor 594 injected through the tail vein under ketamine (80 mg/kg)/xylazine (10 mg/kg) anesthesia, and the conjugate was allowed to circulate for 1 hour. Then, under the high dose of ketamine (200 mg/kg) and xylazine (30 mg/kg) anesthesia, the chest of animals was opened. A small cut to the right atrium was performed to remove intravascular blood from vascular circulation. Cardiac perfusion was started by using a bolus infusion of 25 mL saline to the left ventricle of mice. Then the fixation process was performed by using a 100 mL fixative solution [0.1 M phosphate buffer (pH: 7.4) in 4% paraformaldehyde] for 15 min. At the end of perfusion/fixation process, the brains were removed and transferred to the same fixative for 2 hours. Afterward, the brains were put into a 30% sucrose solution for cryoprotection until they fell down to the bottom of the tube. They were frozen on dry ice, then stored at -20°C overnight and -80°C, respectively. Frozen

sections were taken as 15 μm to each histology glass slide by using cryostat (Leica CM 1950, Leica Biosys., Germany). Each slide had 3 sections which were from a frontal, middle, and posterior part of the brain (Figure 25). Tissue sections were held at the dark room, and immediately after sectioning, imaging of sections was done with confocal microscopy.

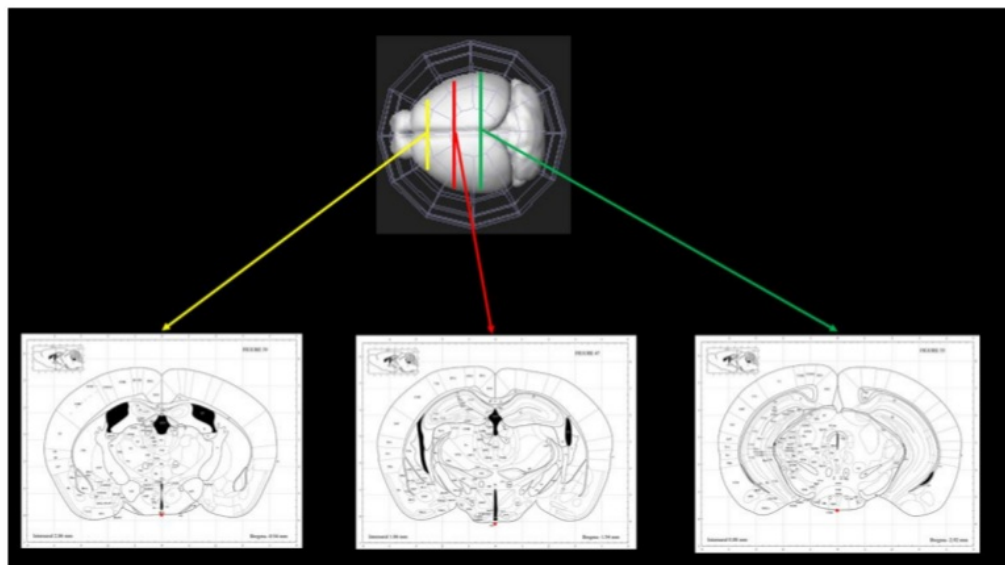


Figure 25. Representation of brain sections for each level (Adapted from the web site: <http://labs.gaidi.ca/mouse-brain-atlas/?ml=0&ap=0&dv=0> on 13 May 2019)

3.2.4.2. Stainings

3.2.4.2.1. Recipes

Citrate buffer:

1. 2.94 gr trisodium citrate dihydrate was dissolved in 800 mL distilled H_2O .
2. pH was adjusted to 6.00 with HCl.
3. 0.05% tween-20 was added (500 μl).
4. Complete the volume to 1 liter.

Blocking solution:

1. 0.1% triton-x / Dulbecco's phosphate buffered saline (DPBS) was prepared.

2. 1 mg of Bovine serum albumin (BSA) was weighted and dissolved in triton-x / DPBS solution.
3. 10% Normal goat serum (NGS) was dissolved in 0.1% triton-x / PBS.
4. 1 mg in 100 μ L was added the solution to finalize.

Primary antibodies:

1. 2% NGS, 2 μ L.
2. Single staining: Glut-1 (1:100), primary antibody.
3. 0.3% triton-x/DPBS, 97 μ L in total 100 μ L solution.
4. Double staining: claudin-5 (1:50), Cav-1 (1:50), GFAP (1:150). Prepare separately with 2% NGS and 0.3% triton-x/PBS in total 50 μ L solution. Then, mix the volume into one Eppendorf tube for each claudin-5/Cav-1 and Cav-1/GFAP stainings.

Secondary antibodies:

1. 2 % NGS.
2. Single staining, 1:100 seconder antibody.
3. 0.3% triton-x/DPBS dilution solution in total of 100 μ L of secondary antibody solution.
4. Double staining: 1:50 for each seconder antibody. Prepare separately with 2% NGS and 0.3% triton-x/DPBS in total 50 μ L solution and mix the volume into one Eppendorf tube for each claudin-5/Cav-1 and Cav-1/GFAP stainings.

Nucleus dye:

1. 1 mL of 1x DPBS was mixed with 1 mL glycerol.
2. 10 μ L Hoechst was added into the solution and cover with aluminum foil in order to protect from light.

3.2.4.2.2 Procedure

1. Before the staining period, mouse brain was cut into 15 μ m sections by using cryostat (Leica CM 1950, Leica Biosys., Germany). Then, sections were stored at -80°C until the staining day.
2. Histology glass slides were incubated at room temperature for 30 minutes.

3. Then slides were washed with 0.1% triton-x/DPBS solution for 2x5 minutes.
4. Citrate buffer (10mM, pH: 6) wash was applied at the microwave (600 Watt) for 3x5 minutes. Citrate buffer was used for all the stainings for antigen retrieval.
5. Slices were washed again with 0.1% triton-x/DPBS solution for 2x5 minutes and enclosed the sections with pappen.
6. Block solutions were prepared. Blocking solution was dropped 100 µl at each section, then incubated the sections for 1 hour at room temperature.
7. Primary antibody solutions were prepared.
8. Blocking solutions were removed by swinging slide up and down. Without washing step, 50 µl of the primary solution was dropped at each section and incubated at 4°C overnight.
9. The day after, sections were washed again with 0.1% triton-x/DPBS solution for 3x5 minutes.
10. Secondary antibody for claudin-5 was Alexa488 goat/mouse (1:100); Glut-1, GFAP Alexa488 goat/rabbit (1:100); Cav-1 Goat Anti-Rabbit IgG (H+L), Alexa 647 (1:100).
11. The secondary antibody solution was dropped at each section and incubated for 1 hour. At the last time, the slides were washed with 0.1% triton-x/DPBS solution for 3x5minutes.
12. Nucleus dye was prepared manually as follows: 5 ul of nucleus dye was dropped and close the sections with a coverslip.

Confocal microscopy was used for imaging, and the analysis of images was evaluated by ImageJ software using the script for colocalization, and single staining was evaluated by using ImageJ software.

Statistical Analysis:

Arterial blood pressure analysis:

Graphpad Prism 7 (GraphPad Software, CA, USA) were used for statistical analysis. Two-way ANOVA method was used with Tukey's multiple comparison test for comparison of arterial blood pressure value on day zero, five, and ten. Differences for the data from the experimental groups were considered statistically significant with a $P < 0.05$.

Albumin-Alexa Fluor 594 staining analysis:

Five images from each frontal, middle, and posterior sections were taken from each animal. Each group had four animal, and there were four experimental groups. The maximum intensity of each image was taken by using Image J software (USA). Graphpad Prism 7 (GraphPad Software, CA, USA) were used for statistical analysis. Ordinary One-way ANOVA method was used with Tukey's multiple comparison test for the total brain fluorescence intensity analysis of albumin-Alexa Fluor 594 staining.

Glucose transporter-1 staining analysis:

The cerebral cortex and hippocampus regions of animals were used for the evaluation of immunostaining intensity. From each region, ten images were taken. In total, 420 images were taken from all experimental groups. The maximum intensity of each image was taken by using Image J software (USA). Graphpad Prism 7 (GraphPad Software, CA, USA) were used for statistical analysis. Ordinary One-way ANOVA method was used with Tukey's multiple comparison test.

Claudin-5/Cav-1 and Cav-1/GFAP colocalization analysis:

The cerebral cortex and hippocampus regions of animals were used for the evaluation of immunostaining intensity. From each region ten images were taken. In total, 420 images were taken from all experimental groups. The maximum intensity of each image was taken by using Image J software (USA) using colocalization macro. For statistical analysis, ordinary One-way ANOVA method was used with Tukey's multiple comparison test.

Chapter 4

4. RESULTS AND DISCUSSION

4.1. Results

The systolic, diastolic, and mean arterial blood pressure values of animals are shown in Table 5. ANG II treatment for 10 days increased the levels of arterial blood pressure of animals. When diastolic, systolic, and mean arterial blood pressures of day 0 were compared with 5th and 10th days' values, there was a significant increase in systolic, diastolic and mean arterial blood pressure values in experimental groups, except sham ($P<0.01$) (Table 5). For example, ANG II and ANG II plus Dynasore groups were reached to 147.08 ± 17.22 from 112.52 ± 11.78 and to 147.86 ± 13.99 from 115.70 ± 6.52 systolic arterial blood pressure, respectively, mentioned in Table 5.

Table 5. The arterial blood pressure levels of animals in the experimental groups. Comparing with day 0, the systolic, diastolic, and mean values of arterial blood pressure statistically increased on day 10 ($*P<0.01$).

Arterial Blood Pressure (mmHg)									
Groups	Systolic			Diastolic			Mean		
	Day 0	Day 5	Day 10	Day 0	Day 5	Day 10	Day 0	Day 5	Day 10
Sham	119.11±8.51	118.1±16.42	124.81±6.99*	95.86±4.18	97.46±12.29	99.33±6.35*	96.06±6.60	95.21±12.18	96.79±10.9*
ANG II	112.52±11.78	131.81±18.36	147.08±17.22*	94.46±6.44	102.53±14.74	115.52±13.62*	93.21±5.73	106.62±15.28	116.85±10.94*
ANG II + Dynasore	115.70±6.52	137.23±19.74	147.86±13.99*	91.77±4.76	109.83±17.54	118.41±12.23*	95.18±6.71	111.90±14.96	119.26±15.22*
ANG II + SB431542	113.54±10.78	130.84±7.22	136.99±18.53*	94.89±17.56	95.26±15.08	106.72±15.48*	96.16±13.13	100.26±14.22	108.54±14.75*

On day 0, systolic arterial blood pressures did not show significance among experimental groups (Figure 26 A). On the 5th day, systolic arterial blood pressure of ANG II with dynasore administration group significantly increased compared to the sham group ($P<0.05$), (Figure 26 A). On day 10, systolic arterial blood pressure of both ANG II and ANG II plus dynasore group significantly increased compared to the sham group ($P<0.01$), (Figure 26 A).

Diastolic arterial blood pressure on day 0, there was no significance between experimental groups (Figure 26 B). On day 5, diastolic arterial blood pressure value of ANG II plus dynasore significantly increased compared to ANG II plus SB-431542 ($P<0.05$), (Figure 26 B). Diastolic arterial blood pressure on day 10, ANG II plus dynasore group increased significantly compared to the sham group ($P<0.05$), (Figure 26 B).

Mean arterial blood pressure on day 0; there was no significance between experimental groups (Figure 26 C). Mean arterial blood pressure of ANG II plus dynasore significantly increased compared to ANG II plus SB-431542 group on day 5 ($P<0.05$), (Figure 26 C). On day 10, only mean arterial blood pressure of ANG II plus dynasore group significantly increased compared to the sham group ($P<0.05$), (Figure 26).

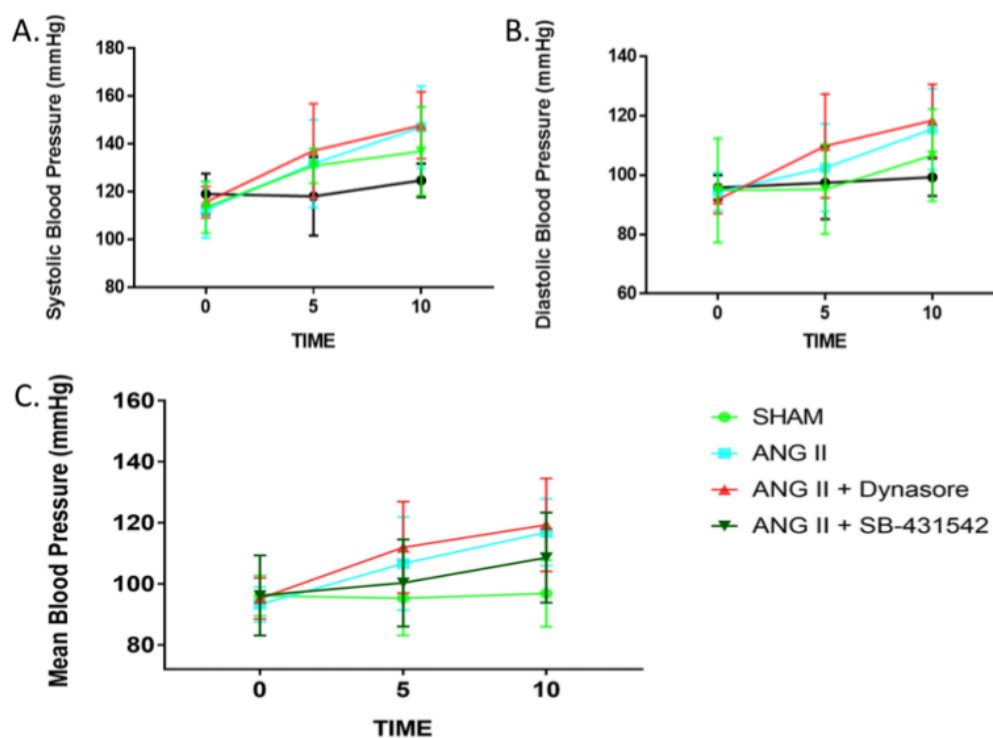


Figure 26. Systolic, diastolic, and mean arterial blood pressure values. The number of animals used in each group was 26.

We demonstrated that due to the BBB leakage, albumin-Alexa Fluor 594 in the brain parenchyma significantly increased in all hypertensive groups compared with the sham and between each group ($P<0.01$), (Figure 27). Fluorescence images and intensity of albumin-Alexa Fluor 594 were shown in Figure 27. Pictures were taken from regions that had maximum albumin-Alexa Fluor 594 permeability. ANG II with dynasore showed the maximum albumin-Alexa Fluor 594 permeability compared to the rest of the experimental groups ($P<0.01$).

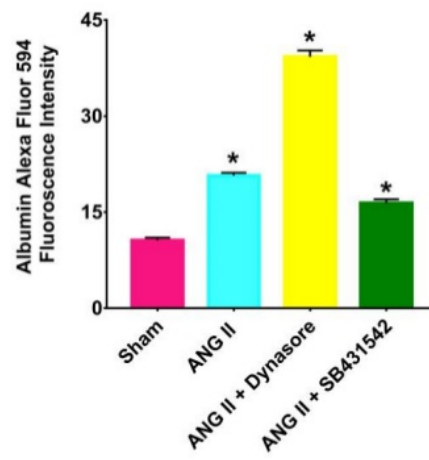
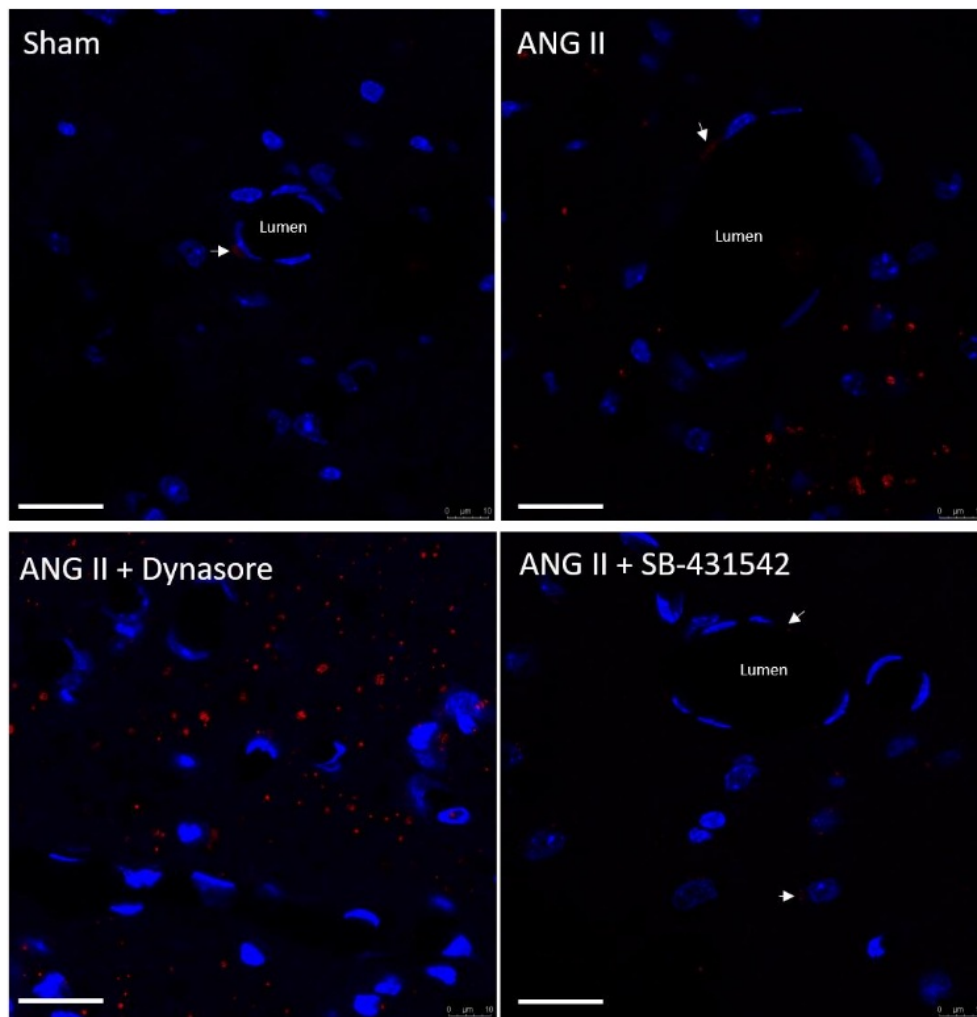


Figure 27. The fluorescence intensity of albumin-Alexa Fluor 594 tracer (Red), a barrier permeability marker, in the cerebrum of animals in the experimental groups, and representation of Albumin-Alexa Fluor 594 extravasation in Sham; ANG II; ANG II plus Dynasore and ANG II plus SB-431542 experimental groups. Pictures were from regions that had maximum permeability. White arrows ($\rightarrow$) showed that Albumin-Alexa Fluor 594 was present inside the endothelial cells of vessels. ANG II with dynasore administration group showed maximum permeability compared to other groups ($P < 0.01$). Note that blue color presents nuclei in images. Data represent mean $\pm$ SD. Arbitrary unit (A.U). Scale bar: 10 μ m.

Glut-1 staining among groups changes significantly both in the cerebral cortex and hippocampal regions of the brain (Figure 28 and 29). In the ANG II group, cerebral cortex region showed increased fluorescence intensity compared to the sham group, whereas ANG II with dynasore and ANG II with SB-431542 groups had reduced fluorescence intensity ($P < 0.01$), (Figure 28).

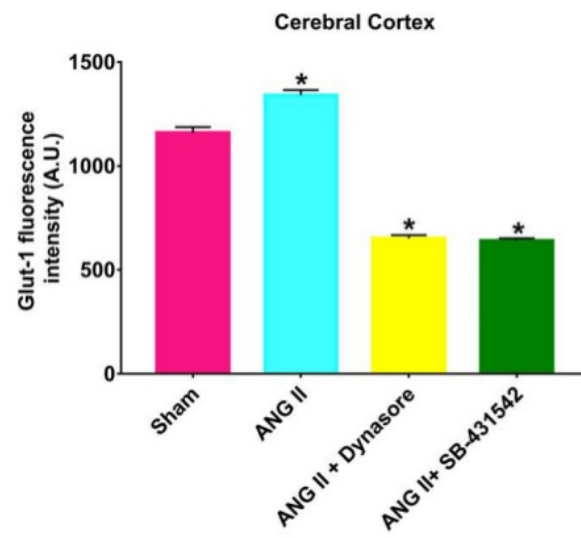
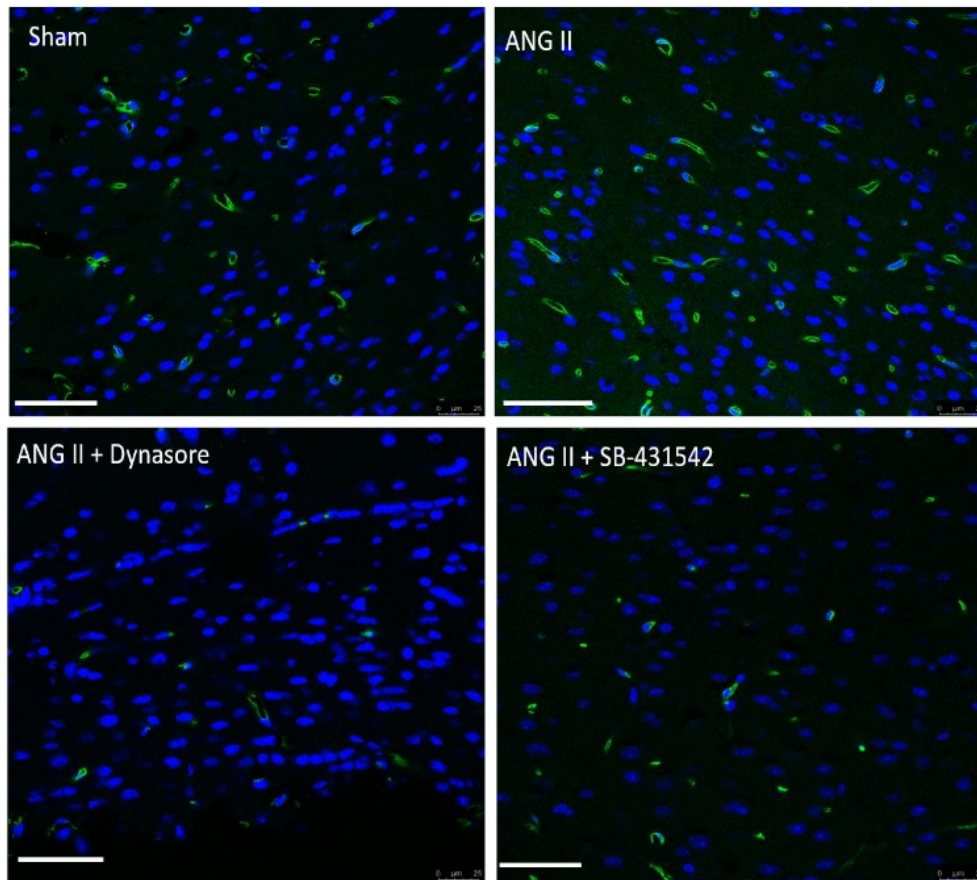


Figure 28. Representation of Glut-1 fluorescence staining (Green) and intensity of all experimental groups. Note that Glut-1 fluorescence intensity increased in the ANG II group. However, it decreased in ANG II with dynasore administration and ANG II with SB-431542 administration groups in the cerebral cortex ($P<0.01$). Note that blue color presents nuclei in images. Data represent mean $\pm$ SD. Arbitrary unit (A.U). Scale bar 25 μm .

Figure 29 shows the fluorescence images and the intensity values from all experimental groups. The hippocampus region in the ANG II conditions showed decreased fluorescence intensity of Glut-1 compared to the sham group. The fluorescence intensity of Glut-1 in ANG II with dynasore was found to be lower than observed in ANG II and ANG II with SB-431542 ($P<0.01$), (Figure 29).

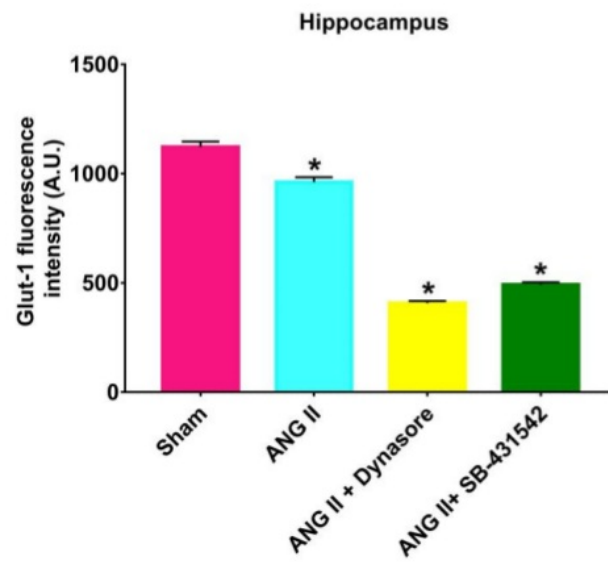
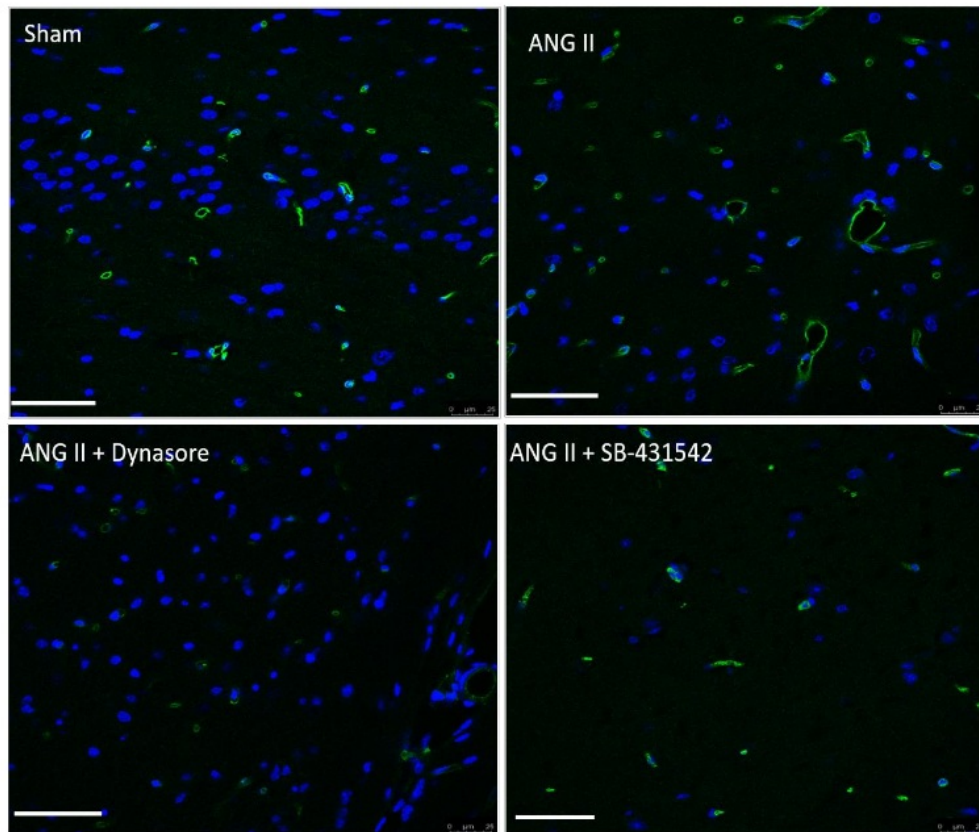


Figure 29. Representation of Glut-1 fluorescence staining (Green) and intensity of all experimental groups. Note that a dramatical decrease in the fluorescence intensity was observed in ANG II with dynasore administration group compared with the rest of the groups in the hippocampus ($P<0.01$). Note that blue color presents nuclei in images. Data represent mean $\pm$ SD. Arbitrary unit (A.U). Scale bar: 25 μ m.

Images of claudin-5 and Cav-1 colocalization of cerebral cortex and hippocampus are shown in Figure 30. Claudin-5 fluorescence intensity of all the groups did not change significantly in the cerebral cortex (Figure 31). The fluorescence intensity of claudin-5 decreased in the hippocampus of ANG II plus SB-431542 group, however it significantly increased in only ANG II compared to sham ($P<0.01$), (Figure 31). The Cav-1 fluorescence intensity at cerebral cortex did not show any significant changes among experimental groups (Figure 32). The fluorescence intensity of Cav-1 in hippocampus significantly increased in ANG II group compared to ANG II plus dynasore and sham groups ($P<0.01$). The fluorescence intensity of Cav-1 in the hippocampus significantly decreased ANG II plus SB-431542 group compared to ANG II and ANG II with dynasore groups ($P<0.01$), (Figure 32). Lastly, the Cav-1 fluorescence intensity of ANG II with dynasore group increased significantly compared to ANG II with SB-431542 group in the hippocampus ($P<0.01$), (Figure 32).

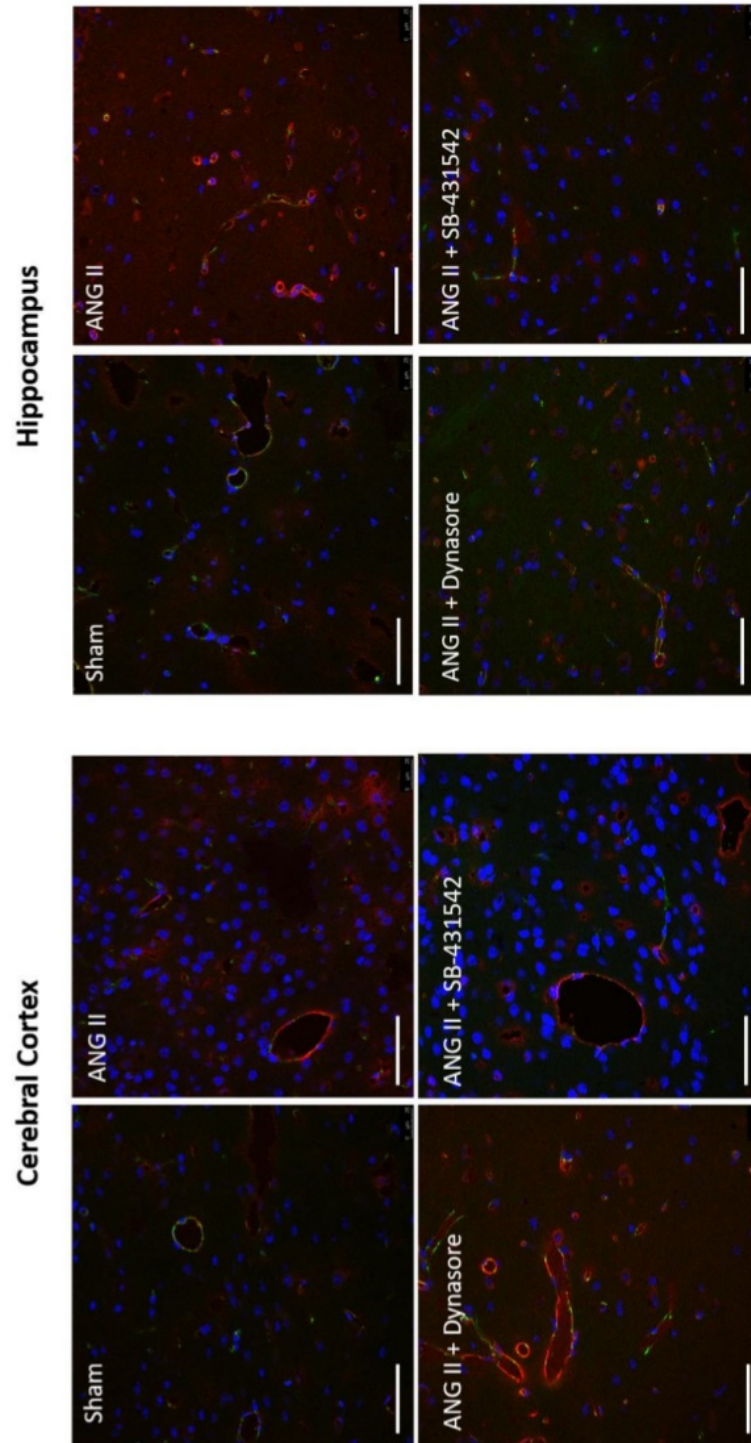


Figure 30. Claudin-5 (Green) and Cav-1 (Red) colocalization stainings of cerebral cortex and hippocampus. Note that nuclei in images are blue color. Scale bar: 25 μ m.

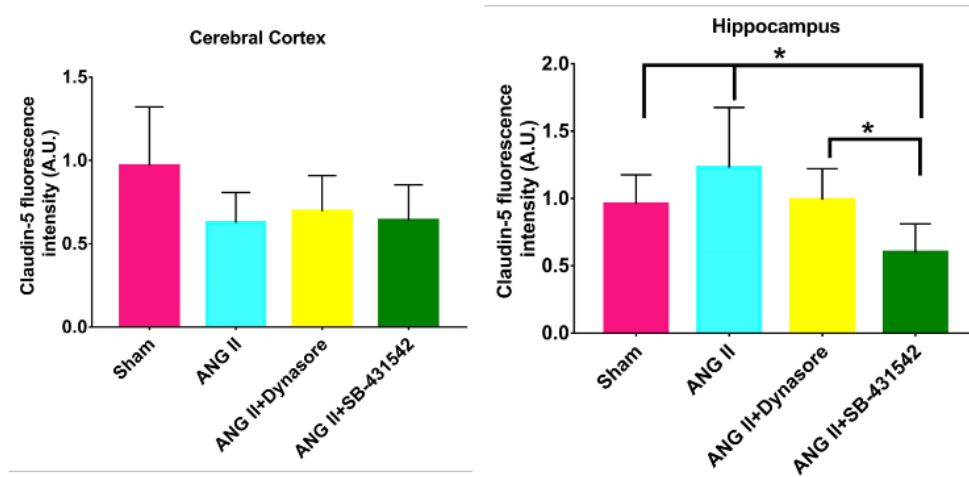


Figure 31. Claudin-5 fluorescence intensity in both cerebral cortex and hippocampal regions from colocalization staining of claudin-5/Cav-1.

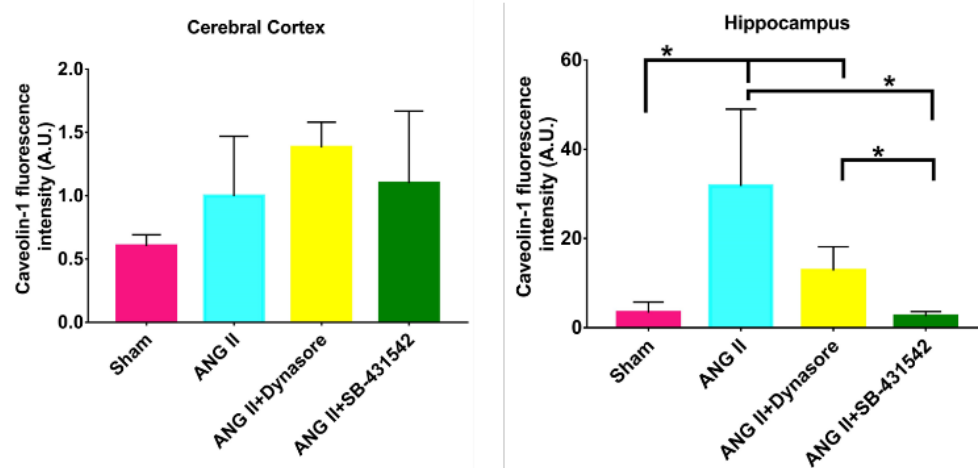


Figure 32. Caveolin-1 fluorescence intensity in both cerebral cortex and hippocampal regions from colocalization staining of claudin-5/Cav-1.

Images of Cav-1 and GFAP colocalization stainings of cerebral cortex and hippocampus were shown in Figure 33. The fluorescence intensity of the GFAP in cerebral cortex did not show significant differences among groups (Figure 34). GFAP immunostaining intensity in the hippocampus in ANG II group increased compared to ANG II plus dynasore and ANG II plus SB-431542 groups ($P<0.01$). The immunostaining intensity of GFAP in hippocampus significantly decreased in ANG II with dynasore and ANG II with SB-431542 groups compared to sham and ANG II groups ($P<0.01$), (Figure 34). The Cav-1 fluorescence intensity of experimental groups did not show significant differences in cerebral cortex region (Figure 35). However, the Cav-1 fluorescence intensity at the hippocampal region of ANG II group increased compared to both sham and ANG II with dynasore groups significantly ($P<0.01$). Lastly, the Cav-1 fluorescence intensity significantly increased in ANG II with SB-431542 compared to ANG II with dynasore group ($P<0.01$), (Figure 35).

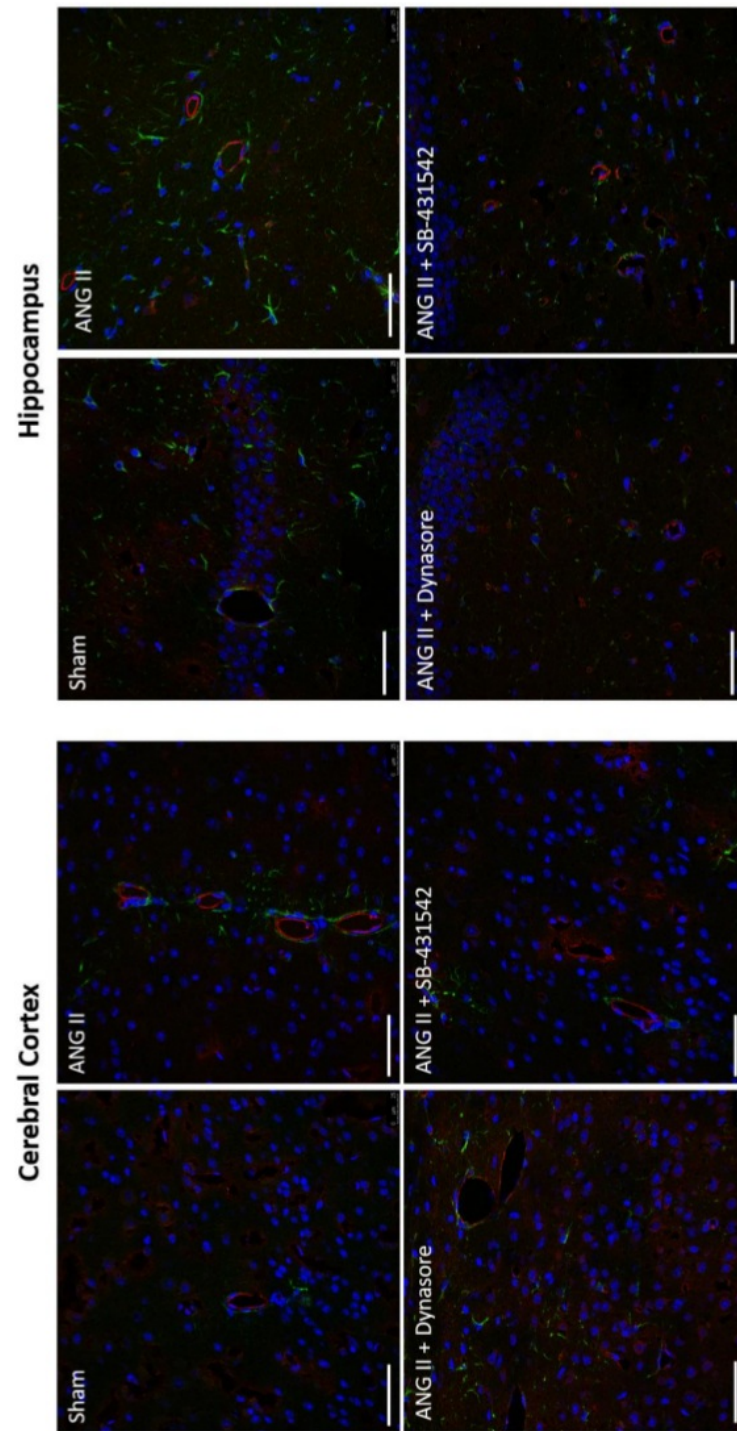


Figure 33. Caveolin-1 (Red) and GFAP (Green) colocalizations in cerebral cortex and hippocampus regions of animals in the experimental groups. Note that blue color represents nuclei in images. Scale bar: 25 μm.

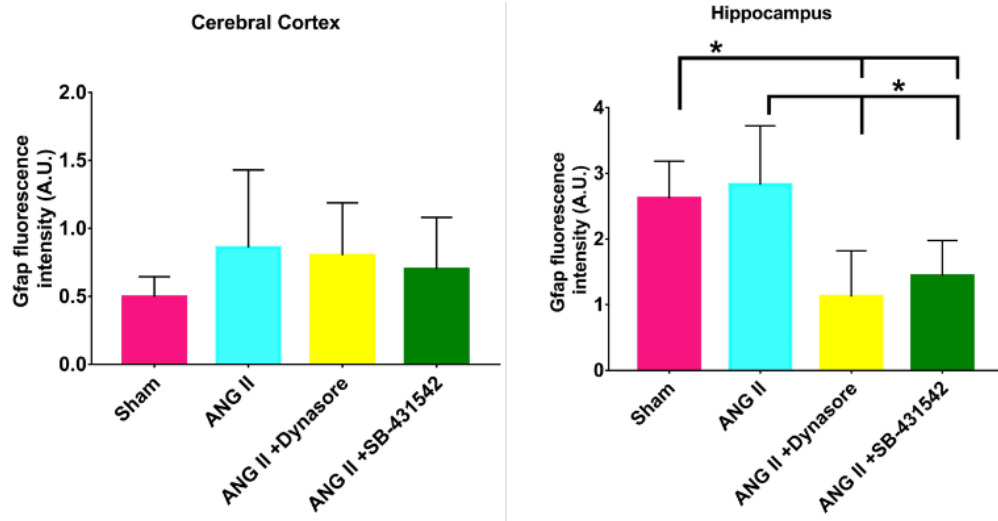


Figure 34. The fluorescence intensity of GFAP in both cerebral cortex and hippocampal regions in experimental groups.

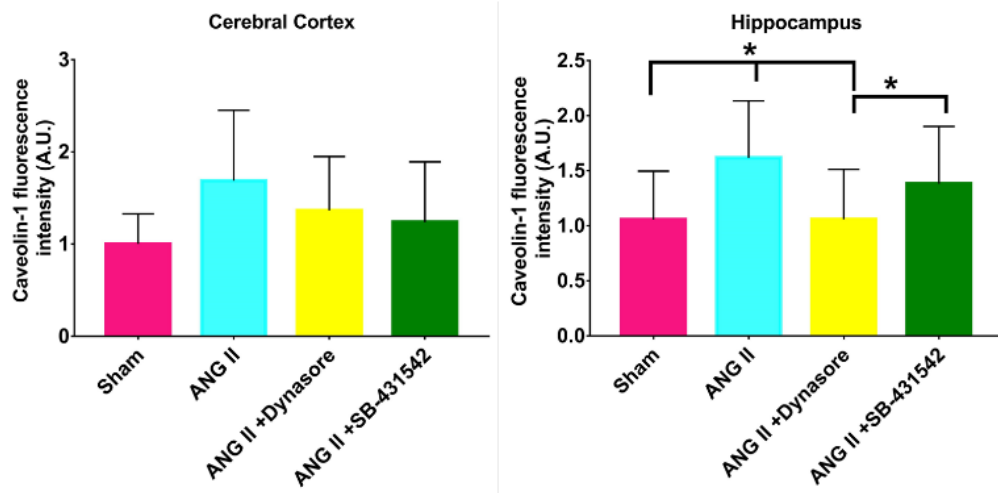


Figure 35. Caveolin-1 fluorescence intensity in both the cerebral cortex and hippocampal regions in the experimental groups.

4.2. Discussion

The BBB integrity in ANG II-induced hypertensive mice was studied to understand the roles of TGF β RI and Cav-1 on paracellular and/or transcellular transport pathways. We aimed to investigate the effects dynasore and SB-431542 on transport pathways that are functional in barrier type of brain microvessels.

In our study, we infused ANG II for 10 days in order to acquire long term hypertension in mice. The arterial blood pressure levels increased on day 5 and day 10 following ANG II administration with or without both dynasore and SB-431542. Related with our data on the arterial blood pressure, a recent study reported that the tail-cuff blood pressure measurements that obtained from Balb/CJ strain which was treated with ANG II and salt combined treatments for 5 days revealed an increase in systolic, diastolic, and mean arterial blood pressure values [136]. In the mentioned study, ANG II and salt caused much more increase in arterial blood pressure at day 5 compared to our results at day 5. In addition, in mice that were exposed to a certain amount of ANG II for two weeks, the Authors observed an increase in mean arterial blood pressure [137]. It is also reported that ANG II infusion for two weeks resulted in a maximum increase in systolic blood pressure [133]. Therefore, our results related to arterial blood pressure demonstrated that the long term (10 days) infusion of ANG II increased arterial blood pressure to hypertensive levels. Acute and/or chronic hypertension that represent an increased in the arterial blood pressure severely impact on the vascular structures, including endothelial cells and lead to BBB impairment. Moreover, a recent study showed that an increase in pulse pressure in hypertension causes BBB impairment [138].

In our study, we observed that the increase in arterial blood pressure could affect caveolar transport by increasing caveolar production in the endothelial cells of brain microvessels. The dynasore, as a dynamin inhibitor, inhibits dynamin protein which facilitates caveolar internalization on the plasmalemma. Our results showed that dynasore enhanced arterial blood pressure observed in hypertensive conditions in mice. A study reported that internalization of caveolae inhibited in response to dynamin inhibitor [139]. In the parallel with the above-mentioned mechanism, one study showed that caveolar production decreased, but arterial blood pressure did not change in cavin-1 deficient mice [140]. Interestingly, in another study, it is suggested that Cav-1 had an

inhibitory effect on endothelial nitric oxide synthase (eNOS), and the mutation of Cav-1 prevented the inhibitory effect of Cav-1 on the eNOS that produces NO in aortic endothelial cells [141]. We suggest that the decreased internalization of caveolae by dynasore could reduce the capacity of eNOS related to caveolar production, while we did not measure the capacity of eNOS. Therefore, it is quite hard to make a conclusion on how dynasore rises up arterial blood pressure levels over hypertension in this experimental setup.

It is known that Cav-1 that locates on an early endosome form regulates TGF β signaling and TGF β receptor internalization [142]. It was reported that TGF β RI signaling pathway with Smad2 and Smad3 proteins play an essential role in proliferation and angiogenesis in endothelial cells [116]. Therefore, the interaction between Cav-1 and TGF β RI may be affected by the hypertensive conditions, and after the interaction, Cav-1 induces internalization of TGF β RI [117]. In a study in which TGF β RI inhibitor (SB-525334) was used in dietary high- salt intake in rat, it is reported that TGF β RI inhibitor decreased the arterial blood pressure observed in hypertensive conditions, and they suggest that further dysfunction of the endothelium could be prevented due to SB-525334 stops the intake of high salt [143]. In another study which SB-525334 inhibitor was intracerebroventricularly used, this inhibitor enhanced the arterial blood pressure observed in the hypertension-induced by subcutaneous infusion of ANG II in mice [144]. However, our results regarding the effects of TGF β RI inhibitor on arterial blood pressure in hypertensive conditions do not conform with the results of Li and Feng. Therefore, our data suggest that inhibiting the TGF β RI decreases arterial blood pressure in hypertensive conditions even if it does not reach to the intact baseline levels. However, the mechanism(s) how inhibitor decreased the arterial blood pressure in the hypertensive conditions is/are not clear yet.

At day 10 of ANG II infusion, the arterial blood pressure reached its highest levels. The increase in arterial blood pressure in ANG II plus dynasore conditions could be an important factor causing to the endothelial dysfunction due to inhibition of caveolar internalization. One study stated that dynasore contributed a diastolic blood pressure increase in acute ischemia/perfusion injury, and they suggested the role of dynasore that inhibits mitochondrial stress by maintaining oxidative phosphorylation [145]. Another

study stated that Cav-1 deficient mice infused with ANG II showed higher diastolic blood pressure compared to control mice [146]. Caveolin-1 may not function properly since dynasore inhibits caveolar internalization [147]. Therefore, in our study, dynasore can be an additive factor on top of the effects of hypertension for a significant increase in diastolic arterial blood pressure at the 10th day.

Due to the BBB disruption, we expected to observe more albumin-Alexa Fluor 594 extravasation into the brain parenchyma in hypertensive conditions. In this context, increased levels of albumin-Alexa Fluor 594 in brain parenchyma obtained from ANG II was enhanced by the dynasore administration. However, TGF β R1 inhibitor, SB-431542, markedly decreased the transport of albumin-Alexa Fluor 594 into the brain parenchyma in hypertensive conditions. On the other hand, we observed the highest arterial blood pressure values in ANG II with dynasore administration group. The role of dynasore the increased BBB permeability in the hypertensive conditions could be both inhibiting of caveolae internalization and enhancement of arterial blood pressure. Related with our assumption mentioned above, a study using *in vitro* human endothelial cells reported that internalization of caveolae inhibited in response to dynamin inhibitor, dynasore, and inhibiting transcytosis by this inhibitor caused to the extravasation of FITC-conjugated albumin into the brain parenchyma via activation of the paracellular pathway [139].

The main function of the Glut-1 transporter is to carry glucose into the brain from the circulation. In our study, fluorescence imaging of both cerebral cortex and hippocampus showed different results for Glut-1 expression of ANG II group. Our results are that Glut-1 expression in the cerebral cortex region of the animals in ANG II group increased, while hippocampus Glut-1 levels decreased. Contrary to our results on the Glut-1 in the cerebral cortex, one study reported that Glut-1 expression lowered in the cerebral cortex region of SHRSP animals [148]. We suggest that glucose intake by the brain parenchyma could increase the production of Glut-1 during the hypertensive conditions. As for hippocampus, Glut-1 expression in the hippocampus could decrease because of both the most vulnerable region of the brain and the presence of hypertensive conditions. The fluorescence intensity of Glut-1 decreased in both cerebral cortex and hippocampus of animals in ANG II plus dynasore and ANG II plus SB-

431542 groups. Both inhibitors deteriorate the reduced expression levels of Glut-1 observed in the hypertensive conditions.

Claudin-5, as one of the TJ protein, functions to tight regulation of barrier characteristics of endothelial cells related to the paracellular pathway to control the passage of the substances and blood sources immune cells into the brain. In this study, the claudin-5 fluorescence intensity in cerebral cortex did not change significantly. Whereas, the claudin-5 fluorescence intensity in ANG II with SB-431542 administration group showed a decrease in fluorescence intensity significantly compared to sham in the hippocampus region. The inhibition of TGF β RI pathway by SB-431542 can be the first reason of decreased intensity of claudin-5. It is demonstrated that TGF β I induced TJs disruption [149]. Another study stated that the addition of TGF β I into the 3D cell culture, the levels of claudin-5 were significantly reduced [124]. TGF β levels which increased in a mouse model of hepatic encephalopathy decreased the expression of claudin-5 and increased BBB permeability, however in the mentioned study, using recombinant TGF β 1 reversed these conditions *in vivo* and *in vitro* BBB culture to the normal levels [122]. In our study, the absence of TGF β signaling in the presence of the ANG II plus SB-431542 decreased the claudin-5 expression in the hippocampus, except cerebral cortex, however, increased only in hypertension. Claudin-5 data regarding cerebral cortex are correlated to the study showing SB-431542 had beneficial effects on TJ proteins (paracellular transport) [149]. Therefore, we suggest that the inhibition of TGF β RI could be an important factor playing a role in both transcellular and also the paracellular pathway in the hypertensive conditions.

One of the transcellular transport mechanisms for BBB is a caveolar pathway that is mainly regulated by Cav-1. We did not observe any significant changes in the expression levels of Cav-1 at the cerebral cortex among experimental groups, whereas the expression levels of Cav-1 dramatically decreased in the hippocampal region of ANG II plus SB-431542 group even under the sham levels, while it increased only in ANG II. A significant increase in the expression levels of Cav-1 in ANG II group explains how BBB permeability increased because of Cav-1 increased caveolae production in this setup. On the contrary, dramatically decreased expression levels of Cav-1 in ANG II plus SB-431542 group could play a crucial role in the reducing of

BBB permeability conditions. ANG II plus dynasore group also showed a decrease in the expression of Cav-1 that is correlated to claudin-5 levels in the hippocampus. Dramatically increased permeability to albumin-Alexa Fluor 594 in ANG II plus dynasore group could be explained via the decreasing claudin-5 expression in both cerebral cortex and hippocampus and increasing Cav-1 expression in the cerebral cortex. Therefore, we suggest that both paracellular and transcellular pathways could be important players in the severe BBB disruption in the ANG II plus dynasore group. This consumption is supported by the study showing the effects of dynasore on permeability in primary human microvascular endothelial cells *in vitro* conditions and in that study, the Authors suggest that under dynamin conditions there is a critical link between paracellular and transcellular pathways [139]. The expression levels of Cav-1 dramatically decreased to even under sham levels in ANG II plus SB-431542. This dramatic decrease may have a protective effect to decrease BBB permeability increased in hypertensive conditions. Consequently, BBB permeability of albumin-Alexa Fluor 594 was decreased by the inhibitory action of SB-431542 on TGF β RI in ANG II/hypertension conditions.

Astrocytes, which are dynamic components of NVU, contribute to the maintenance of the integrity of BBB with their endfeet [150, 151]. In our study, we observed that the expression levels of GFAP in the cerebral cortex regions of animals increased, but did not show any significance among the experimental groups. The increased expression levels of GFAP in the cerebral cortex of animals are well correlated with a study reporting that hippocampi of SHRs showed an increased GFAP staining intensity [152]. In one of the recent studies, dynasore was shown to inhibit astrocyte proliferation at 3 days after spinal cord injury by inhibiting the expression of GFAP [153]. A study on human glioma cell lines showed that TGF β has an inhibitory effect on the proliferation of normal astrocytes [154]. Our results obtained from ANG II plus dynasore and ANG II plus SB-431542 groups show decreased expression levels of GFAP. Specifically, the decreased expression levels of GFAP in the ANG II plus dynasore group could be in line the increased extravasation levels of albumin-Alexa Fluor 594 tracer in the brain parenchyma.

There are contradictory reports in literature regarding the expression levels of GFAP in certain experimental conditions that could impact on the BBB integrity. In a study, the Authors investigated the relation between BBB integrity and albumin intake by astrocytes in patients with a brain tumor, and they showed that there was no significant albumin uptake by astrocytes [155]. Another study showed that an agent, DI-3-n-butylphthalide, caused increased expression of GFAP in a cerebral infarction model in mice, and they stated that DI-3-n-butylphthalide improved BBB integrity under cerebral infarction experimental model [156]. In spinal cord injuries, BBB is damaged and has failed to repair in the absence of reactive astrocytes [157]. In addition, astrocytes increase blood-retinal barrier properties by increasing the expression levels of ZO-1 protein [158]. Moreover, astrocytes can also induce tight junction formation in primary HUVEC cultures [159].

Therefore, we suggest that the observation of the most markedly decreased expression levels of GFAP in ANG II plus dynasore group could be related to the the highest BBB permeability increases in the same group. The above mentioned data indicate that GFAP could be an important factor for the BBB integrity.

Chapter 5

5. CONCLUSION

The blood-brain barrier has dynamic properties with the interaction of neuronal and non-neuronal cells in brain parenchyma. Any disease condition in the body or brain parenchyma could surely affect BBB integrity. First, the endothelial cells of brain microvessels face with those blood-borne substances and blood-source cells, and BBB functions to control and regulate the transport of the substances both sided using a variety of transport mechanisms. In disease conditions, paracellular and/or transcellular transport mechanisms do not function properly; therefore, the brain homeostasis is lost. An impairment in the endothelial function is seemed to be the main responsible factor in the loss of brain homeostasis. For example, in hypertension, it is known that ANG II changes BBB integrity and increases BBB permeability via the enhancement of caveolar transport and/or may be via the impairment of the TJs, paracellular pathway.

The main objective of our study was to clarify the effects of TGF β on BBB integrity in hypertensive conditions. According to our results, 10 days of ANG II infusion via micro-osmotic pump is sufficient to have a hypertensive mice model. The long term hypertension increases BBB permeability to albumin-Alexa Fluor 594 tracer via increased caveolar production and/or possibly the opening of TJs. Decreased expression of the Glut-1 in experimental groups could be related to the disruption degree of BBB integrity. The alteration in claudin-5 expression that leads to the opening of TJs could be an important factor for the disruption degree of BBB integrity in the experimental groups. The alterations in GFAP expression could also be another important factor for the disruption degree of BBB integrity in the experimental groups.

In conclusion, we suggest that TGF β plays a crucial role in the disruption of BBB in hypertensive conditions. We propose that SB-431542 would be the most important agent, rather than dynasore, to eliminate the detrimental effects of TGF β on both transcellular and paracellular pathways in this experimental setup.

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END

PhD THESIS BY MÜGE ATIŞ

GRADEMARK REPORT

FINAL GRADE

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