ผลของการพร่องซีโรโทนินต่อการเปลี่ยนแปลงของ blood brain barrier ที่ถูกกระตุ้นด้วยภาวะ cortical spreading depression

นางสาวชลลวัลย์ แสงเจริญธรรม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาวิทยาศาสตร์การแพทย์

คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2555

THE EFFECT OF SEROTONIN DEPLETION ON THE CORTICAL SPREADING DEPRESSION INDUCED ALTERAION OF BLOOD BRAIN BARRIER

Miss Chonlawan Saengjaroentham

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Sciences
Faculty of Medicine
Chulalongkorn University
Academic Year 2012

Thesis Title	Effect of serotonin depletion on the cortical spreading depression induced alteration of blood brain barrier
By	Miss Chonlawan Saengjaroentham
Field of Study	Medical Sciences
Thesis Advisor	Assistant Professor Supang Maneesri le Grand, Ph.D.
Thesis Co-advisor	Professor Anan Srikiatkhachorn, M.D.

Accepted by the Faculty of Medicine, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

..... Dean of the Faculty of Medicine
(Associate Professor Sophon Napathorn, M.D.)

THESIS COMMITTEE

..... Chairman
(Associate Prefessor Wilai Anomasiri, Ph.D.)

..... Thesis Advisor
(Assistant Professor Supang Maneesri le Grand, Ph.D.)

..... Thesis Co-advisor
(Professor Anan Srikiatkhachorn, M.D.)

..... Examiner
(Assistant Professor Thananya Thongtan, Ph.D.)

..... External Examiner
(Professor Pansiri Phansuwan, Ph.D.)

ชลลวัลย์ แสงเจริญธรรม : ผลของการพร่องซีโรโทนินต่อการเปลี่ยนแปลงของ blood brain barrier ที่ถูกกระตุ้นด้วยภาวะ cortical spreading depression (THE EFFECT OF SEROTONIN DEPLETION ON THE CORTICAL SPREADING DEPRESSION INDUCED ALTERAION OF BLOOD BRAIN BARRIER) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร. สุภางค์ มณีศรี เลอกรองค์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.นพ. อนันต์ ศรีเกียรติขจร, 87 หน้า.

ภาวะพร่อง สารสื่อประสาท ซีโรโตนินเป็นหนึ่งในพยาธิกำเนิดที่สำคัญของโรคไมเกรน ซึ่งสารสื่อประสาทนี้มีความเกี่ยวข้องกับการควบคุมทั้งเซลล์ประสาทและหลอดเลือด การศึกษาก่อนหน้านี้พบว่าการชักนำให้เกิดปรากฏการณ์คอร์ติคัล สเปรดดิ้ง ดีเพรสชั่น (CSD) ในสัตว์ทดลองที่มีภาวะพร่องซีโรตินิน สามารถ ส่งผลให้เซลล์ประสาทมีความไวต่อการกระตุ้นมากขึ้น และ เนื่องจาก blood brain barrier (BBB) มีการ ทำงานที่ประสานกันอย่างใกล้ชิดกับ เซลล์ประสาท ดังนั้นภาวะพร่องซีโรโตนินจึงน่าจะมีผลต่อการเปลี่ยนแปลง ความสมบูรณ์ของ BBB ได้ แต่อย่างไรก็ตาม ยังไม่มีการศึกษาถึงกลไลที่เกี่ยวข้องกับภาวะดังกล่าว ดังนั้น การทดลองนี้จึงศึกษาผลของภาวะพร่องซีโรโตนินต่อความสมบูรณ์ของ BBB ที่มีการชักนำให้เกิดปรากฏการณ์ CSD และการแสดงออกของนิวโรเปปไทด์ substance P (SP) and calcitonin gene related peptide (CGRP) ในปมประสาทไตรเจอมินัล โดยแบ่งหนูทดลองสายพันธุ์วิสต้าออกเป็น 4 กลุ่ม ประกอบด้วย กลุ่มควบคุม กลุ่มที่มีปรากฏการณ์ CSD กลุ่มที่มีภาวะพร่องซีโรโตนิน และกลุ่มที่มีภาวะพร่องโซโรโตนินร่วมกับการกระตุ้นด้วยปรากฏการณ์ CSD ซึ่งกลุ่มพร่องซีโรโตนินจะได้รับการฉีด parachlorophynylalanine (PCPA) ทางช่องท้อง 3 วันก่อนการทดลอง และกลุ่มที่มีปรากฏการณ์ CSD จะได้รับการวางโพแทสเซียมคลอไรด์บนผิวสมอง จากการศึกษาพบว่า เซลล์เอนโดธีเลียมของหลอดเลือดสมอง ในภาวะพร่องซีโรตินินที่มีการ กระตุ้นร่วมกับปรากฏการณ์ CSD มีจำนวน microvili และ pinocytic vesicle มากกว่ากลุ่มควบคุมและกลุ่ม CSD และพบการบวมบริเวณปลายแขนงของเซลล์แอสโตรไซต์มากกว่ากลุ่ม CSD การวิเคราะห์การวางตัวของ tight junction แสดงให้เห็นถึงการเพิ่มขึ้นของ BBB permeability มากกว่ากลุ่ม CSD ซึ่งการเปลี่ยนแปลงของ ultrastructure นี้สอดคล้องกับผลวิเคราะห์ปริมาณโปรตีนด้วยวิธี western blotting โดยการแสดงออกของ occludin ZO-1 และ ZO-2 ซึ่งเป็นโปรตีน ที่สำคัญของ tight junction พบว่าลดลงทั้งหมดในกลุ่ม พร่องซีโรตินินที่มีการกระตุ้นร่วมกับปรากฏการณ์ CSD นอกจากนี้ภาวะพร่องซีโรโตนินสามารถชักนำให้มี การแสดงออก ของ CGRP และ SP ในปมประสาทไตรเจอมินัล มากขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มที่มีระดับซีโรโตนินปกติ

จากการศึกษานี้แสดงให้เห็นว่าการเกิดปรากฏการณ์ CSD ในภาวะพร่องซีโรโตนิน ส่งผลให้มีการเพิ่มขึ้นของ BBB permeability และการแสดงออกของนิวโรเปปไทด์ที่กระตุ้นหลอดเลือด (CGRP และ SP) ในปมประสาทไตรเจอมินัล ซึ่งความผิดปกติของ BBB ในภาวะพร่องซีโรโตนิน นี้สามารถอธิบายถึงความสัมพันธ์ใกล้ชิดของไมเกรนและโรคหลอดเลือดสมองได้

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2555 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5274767530 : MAJOR MEDICAL SCIENCES

KEYWORDS: BLOOD BRAIN BARRIER / CORTICAL SPREADING DEPRESSION / SEROTONIN / TIGHT JUNCTION PROTEIN

CHONLAWAN SAENGJAROENTHAM: EFFECT OF SEROTONIN DEPLETION ON THE CORTICAL SPREADING DEPRESSION INDUCED ALTERAION OF BLOOD BRAIN BARRIER. ADVISOR: ASST.PROF. SUPANG MANEESRI LE GRAND, Ph.D., CO-ADVISOR: PROF. ANAN SRIKIATKHACHORN, M.D. 87 pp.

Serotonin (5-HT) depletion is one of the important pathogenesis involved in migraine pathophysiology. This neurotransmitter is involved in both neural and vascular controls. Previous studies in the cortical spreading depression (CSD) migraine animal model demonstrated that depletion of serotonin could induce the hyper-excitability of cortical neurons. Since the tight association between the neuron and the blood brain barrier (BBB), the alteration of the BBB integrity in 5-HT depleted state can be expected. However, the alteration of BBB induced by 5-HT depletion and the mechanism underlying of this event have never been studied. In order to clarify this point, this study aims to investigate the effect of 5-HT depletion on the CSD induced alteration of the BBB integrity and the expression of neuropeptides SP and CGRP in the trigeminal ganglion in 5-HT depleted state. Wistar rats were divided into four groups: control, CSD, 5-HT depletion, and 5-HT depletion with CSD group. 5-HT was depleted by intraperitoneal injection with parachlorophenylalanine (PCPA) 3 days before the experiment. The CSD was induced by KCl application on the cortical surface. The results from this study demonstrated that the induction of CSD in the 5-HT depletion can significantly increase the number of endothelial pinocytic vesicles and microvilli compared with those observed the control and CSD group. The degree of astrocytic foot plate swelling in 5-HT depletion with CSD group was more severe than that in the CSD group. The analysis of tight junction demonstrated that alignment of tight junction between the endothelial cells in the 5-HT depleted with CSD group demonstrate the increased of the BBB permeability compared with those observed in the CSD group. The results from the ultrstructural study are in line with the result from protein analysis by western blotting. The expression of occludin, ZO-1, and ZO-2, which are the important tight junction proteins, are all decreased in the 5-HT depleted with CSD group. In addition, we have found that the depletion of serotonin could induce an significant increment of CGRP and SP expression in TG as compared with those observed in rats with a normal level of 5-HT.

Altogether, it can be concluded that CSD activation in the serotonin depletion can result in both the increment of the BBB permeability and the expression of vasoactive neuropeptide (CGRP and SP) in TG. These abnormalities of the BBB observed in 5HT depletion from this study might be one explanation for the close association between migraine and cerebrovascular disease.

Field of Study: Medical Sciences Student's Signature .....

Academic Year: 2012 Advisor's Signature .....

Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I wish to express my gratitude and appreciation to my kind advisor, Asst Prof. Supang Maneesri le Grand, for her excellent instruction, guidance, encouragement and support during the long working process, which enable me to carry out this study. Her kindness will be long remembered.

I would like to express my gratitude to my co-advisor, Prof. Anan Srikiatkachorn, MD, for vulnerable advises and helps me more deeply understand my research.

I also would like to express my gratitude to Asst. Prof. Thananya Thongtan for her invaluable suggestions and training about western blot technique.

I would like give a special vote to thank to Dr. Weera Supornsilpchai, Miss Waranurin Yisarakun, and Miss Chattraporn Chantong, for their cheerfulness and support throughout this study. My special thanks are also extending to Mr. Peter le Grand for his valuable help and cheerfulness.

I wish to express my sincere thank to Mr. Preecha Reuangwechvorachai for his help and advice in immunohistochemical study and Miss Wilawan Ji-au for her help in electron microscope technique.

My thanks also go to all my teachers in Medical Sciences Program and Department of Pathology, Faculty of Medicine, Chulalongkorn University who are not mentioned here for all their helps during the time I was study.

My appreciation is also devoted to my dear father, mother, brother and all members of my family for their love, kindness and supporting throughout this study.

CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C = degrees celsius

µm = micrometer

5-HIAA = 5-Hydroxyindoleacetic acid

5-HT = serotonin

BBB = blood brain barrier

BSA = bovine serum albuzmin

BW = body weight

C_1 = cervical spinal cord segment 1

C_2 = cervical spinal cord segment 2

Ca^{2+} = calcium ion

CGRP = calcitonin gene related peptide

CGRP-IR = calcitonin gene related peptide immunoreactive

CSD = cortical spreading depression

DAB = 3,3-diaminobenzidin

K^+ = potassium ion

KCl = potassium chloride

kg = kilogram

mg = milligram

mm = millimeter

NaCl = sodium chloride

NO = nitric oxide

NSS = normal saline

PBS = phosphate buffer saline

PCPA = parachlorophenylalanine

SP = substance P

SP-IR = substance P immunoreactive

TG = trigeminal ganglion

TNC = trigeminal nucleus caudalis

VIP = vasoactive intestinal polypeptide

ZO = zona occluden

CHAPTER I

INTRODUCTION

The central nervous system (CNS) is considered one of the most important and vital systems of the human body. This system requires high oxygen consumption (approximately 20% of total oxygen consumption) (1). In order to work properly, this system needs to be in the stable environment without alteration in the chemical or ion concentration. Therefore, it requires a special circulation system which is capable of supplying nutrients and oxygen to the brain, protecting the brain from harmful substances, and at the same time pumping the toxic substance from the brain out through this circulation. The combined function of all these special properties of the circulation in the brain is quite unique and known as the blood brain barrier (BBB) (2).

The BBB is a neurovascular unit which is composed of various cellular elements such as endothelial cells, astrocyte end-feet, microglial cells, pericytes, and neurons (3). The endothelial cells of the brain capillary are different from others as it is the continuous type of endothelial cells connected to each other with tight junctions, which comprise of various tight junction proteins such as occludin, zona occluden, and claudin (4). With such a variety of relevant functions, abnormality of the BBB can easily lead to several pathological conditions in the brain, including Parkinson's disease, Alzheimer's disease, infectious disease, and stroke (5).

Migraine is one of the neurovascular diseases, which involved in the activation of the trigeminovascular system. This system comprises of the tight association between the trigeminal neurons and cerebral circulation (6). It is known that activation of the trigeminovascular system can result in the release of numerous vasoactive neurotransmitters including nitric oxide (NO), calcitonin gene related peptide (CGRP), and substance P (SP) from the perivascular neurons (7). This phenomenon is believed to be the cause of the neurogenic inflammation leading to the sensitization of the cortical neuron and activation of the trigeminal nociceptive pathway, respectively (8). Among those neurotransmitters released at the perivascular neurons, CGRP and SP are of particular interested to several researchers due to their

capability to induce the neuro-inflammation in the brain as well as their vasodilating effect. The previous studies reported that the accumulation of vasoactive neurotransmitters around the cerebral vessels can induce the neurogenic inflammation and finally lead to the alteration of the BBB (6,9).

Several previous studies also provided supporting evidences indicating the association between migraine and alteration of the BBB. In their study, it was found that through the activation of cortical spreading depression (CSD) in the animal model, matrix metalloproteinase 9 (MMP-9), a proteolytic enzyme, which breaks down the extracellular matrix, was up-regulated within 6 hours after activation (10). In addition, Gupta had demonstrated in 2009 that the CSD during a migraine attack was correlated with the cerebrovascular dilatation and BBB disruption (11). This result was in agreement with the magnetic resonance image (MRI) study by Rotstein and colleague They found that the vascular permeability in patients with migraine with aura was increased as compared with the case-control (12). With several evidences indicating the relation between a migraine attack and the alterations of the BBB integrity, migraine was recently recognized as the risk factor for the cerebrovascular disease, especially the migraine with aura (13,14). However, the mechanism underlying this relationship is still unclear.

Referring to migraine pathophysiology, serotonin (5-HT) depletion is one of the important mechanisms underlying the migraine attack. Several clinical studies have revealed abnormality of the 5-HT system in migraine patients. The study in 1961 by Sicuteri and colleague had demonstrated that the level of 5-HT in platelets has decreased and 5-hydroxyindole acetic acid (5-HIAA), the metabolite of 5-HT, in the urinary excretion has increased in patients with migraine (15). Several studies indicated that the reduction of 5-HT synthesis in the brain resulted in less 5-HT neurotransmission which consequently increased the symptoms of migraine (16–18).

The previous studies in the headache animal model have demonstrated that the induction of CSD in a low 5-HT condition could result in the hyper-excitation of the cortical neurons (19). These alterations were observed in parallel with an increase of the trigeminovascular nociception.

Based on accumulative evidence, we did hypothesize that the depletion of 5-HT could induce an increase in the cerebrovascular responses to CSD activation as well. The CSD might alter the BBB integrity in a 5-HT depleted state more than what it did in a normal 5-HT animal. As mentioned earlier that the CGRP and SP are important neuropeptides involved in the BBB changes. The CSD activation in 5-HT depletion might increase these two neuropeptide expressions in the trigeminal ganglion as well.

In order to prove our hypothesis, this study was performed to investigate the effect of 5-HT depletion on the alteration of the BBB integrity and the expression of neuropeptides (CGRP, SP) in the CSD animal model. The alteration of the BBB integrity was monitored by measuring the changes in the expression of the tight junction proteins and IgG extravasation by using western blot analysis. The ultrastructural changes of the endothelial cell were determined by electron microscopy. In addition, since the release of neuropeptides from the trigeminal ganglion is involved in the alteration of the BBB integrity, the expression of CGRP and SP in the trigeminal ganglion was monitored by using the immunohistochemical study.

We hope that the results obtained from this study will let us understand more in the relationship between migraine and cerebrovascular damage. The knowledge might explain why a migraine patient especially with CSD (aura) has a higher risk for stroke or cerebrovascular disease.

CHAPTER II

LITERATURES REVIEW

2.1 Blood brain barrier and tight junction

The central nervous system (CNS) is the most important system in the human body. In order to work normally, all the neurons need to be in the stable environment with the restricted of ion concentration. Based on the constantly high metabolic activity of this system, the CNS utilized about 20% of total oxygen consumption for the whole body (1). Hence for this system, the blood circulation is not only for supplying the nutrient and oxygen, but also protecting the neural tissue from the variations in ion concentration and toxic substances. The special property of the circulation in the brain is named Blood Brain Barrier "BBB". Because of the sensitivity of brain, this barrier shields the brain from harmful substances and filtering detrimental compounds from the brain back to the blood circulation (2).

The essential functions of the BBB are (3,5):

1. Control the ionic composition in the brain to the optimal concentration for the synaptic signaling function.
2. Supply the nutrients and important compounds for neural tissue through its transport system.
3. Shield the brain from the harmful molecules and toxic substances from blood circulation.
4. Introduce the inflammatory cells to the inflammation area of the brain.

The BBB is a neurovascular unit which composes of various cellular elements including endothelial cells, microglias, astrocytes, pericytes, and neurons (3). All BBB cellular components need to working together in order to maintain the BBB integrity. Endothelial cells are lining inside along the vessels with the special cell to cell junction (tight junction). These lining cells are surrounded by the basement membrane attached with variety of perivascular neurons terminal and astrocytic foot plates (20,21).

Anatomical description of BBB

The understanding of the BBB functions was more evident since the study in 1967 by which clarified that BBB was the microvessels with specific type of endothelial cells characterized by the presence of cell-cell tight junctions, lacked fenestrations and selective diffusion barrier (22). Compared to the endothelial cells obtained from other organs, the endothelial cells of the BBB demonstrate no fenestrations (23), higher number of mitochondria (24), fewer of pinocytotic vesicles (25), and presence of tight junctions (26). Pericytes which classified as granular and filamentous subtypes attaches to the abluminal membrane of the endothelial cell (27). The basement membrane comprises of laminin, fibronectin, collagen type IV and the extracellular matrix (28). Astrocytes processes the end-feet to cover the capillaries (Figure 2-1).

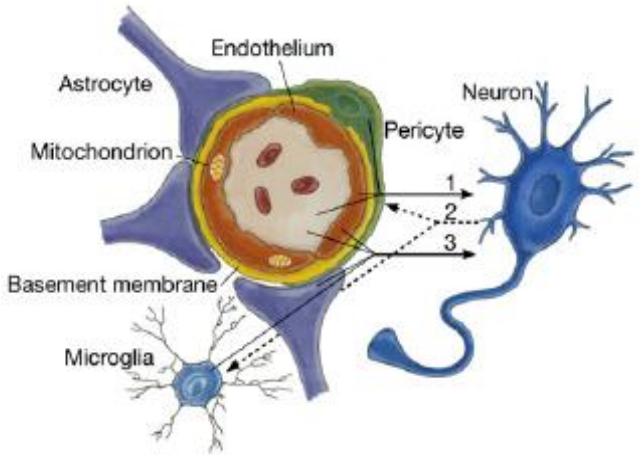

Figure 2-1 Schematic picture of the neurovascular unit composed of endothelial cells, pericytes, microglia, neurons, and astrocytic endfoot (21)**.**

2.1.1 Cellular elements of BBB

- Endothelial cells

The cerebral endothelial cells in CNS revealed a special morphology and function which is different from those present in peripheral tissues. The ultrastructural examination demonstrated the connection of endothelial cell plasma membranes at junctional complex by tight junctions and adherent junctions (29). The important

characteristic of endothelial cells are: [1] TJs established by the connection of several transmembrane proteins with tight sealing and limit the transportation including the lacking of fenestrations of cytoplasm (30,31). [2] Fewer pinocytotic vesicles indicating the restriction of trancytosis (32), and [3] Massive mitochondria in endothelial cells representing the high metabolic function of these cells.

- Astrocytes

Based on the anatomical and morphological information, astrocytes were divided into two types, protoplasmic and fibrous astrocytes. Protoplasmic astrocytes were found in gray matter, exhibiting several stem branches that gave rise to many finely branching processes whereas fibrous astrocytes are found in white matter, exhibiting many long fiber-like processes. In addition, the process of astrocyte extensive attached with blood vessels. Both subtypes of astrocytes form gap junctions between processes of neighboring astrocytes (33).

Several studies indicated that astrocytes involve in BBB properties regulation (34). Astrocytes envelop more than 99% of BBB endothelial cells (2). Interaction between astrocytes, endothelial cell of brain capillaries and neurons are important for maintaining the neurovascular unit (34). The co-culture of astrocytes and brain endothelial cells indicated that astrocytes are important in the maintenance of BBB tightness and function (35,36). The temporary loss of astrocytes demonstrated the loss and restoration capability of BBB integrity (37).

- Pericytes

Pericytes are undifferentiated, contractile connective tissue cells which send out a cellular projection to spread over capillary walls. A previous study found that contractile proteins in pericytes may involve in the capillary blood flow regulation (38). In addition, pericytes correlating with the endothelial cell indicated gap junction between them (39). The co-culture of pericytes, endothelial cells, and astrocytes represented the capillary-like structure formation (40). In the pathological condition, the migration of pericytes from the brain endothelial cell indicated the association of pericytes and the increased BBB permeability (41,42).

- Microglia

Microglia are cells which contact cerebral blood vessels serving as a perivascular macrophage. These cells involve in the immune response of the brain and BBB modulation (4). Perivascular microglia have molecules which involve in recognition and presentation of antigen indicating the important role of these cells in the inflammatory process in the brain (43). In the pathological condition, microglia are found in the perivascular space inducing the increase of BBB properties and the activation of microglial cell transformation from the ramified to the amoeboid shape (44). However, the mechanisms of microglia and BBB permeability are still unclear.

- Neurons

The brain highly requires nutrient and oxygen which transported through blood circulation to support neural activity and metabolism. Blood flow regulation is the one mechanism which facilitates for this function (45). Interestingly, the change of cerebral blood flow, hemorrhage, and traumatic injury in the pathological condition correlates with the BBB integrity disruption (46,47). In addition, anatomical evidences show the direct innervations of microvascular endothelial cells by neurotransmitters containing neurons such as noradrenergic (48), serotonergic (49), cholinergic (50,51), and GABAergic neurons. The study in Alzheimer's disease found that the loss of cholinergic innervations of cortical microvessels may lead to cerebrovascular impairment (51). However, the involvement of neuron activity and BBB alteration has not yet been clearly demonstrated.

2.1.2 Tight junction

The tight junction is a junctional complex between brain endothelial cells which is the special characteristic for the BBB. The formation of the junction plays an important role in the paracellular diffusion control in the vascular microvessels (52). Effective tight junctions are depended on the modulation and regulation of several tight junction proteins as follows (Figure 2-2)**:**

- Occludin

Occludin is the integral membrane protein, located in tight junctions of endothelial cells in the BBB. Occludin (65 kDa protein) has four transmembrane domains which are the C-terminus and N-terminus, and two extracellular loops that span the intercellular cleft (53). The endothelial cells of CNS have a higher amount of occludin than other vascular endothelial cells (2,20,29,54). There is evidence that tight junctions containing tissues which have occludin, electrical resistance had increased (55). The phosphorylation of serine and threonine to occludin increases the barrier permeability (56). In addition, the cytoplasmic C-terminal is interlacing with the cytoskeleton via accessory proteins [zonula occludens ZO-1 and ZO-2] (57,58). It is controversial that occludin is not major component for the tight junction formation, but the decline of occludin expression correlates with BBB disruption in several diseases (59–61).

- Zona occluden (ZO-1, ZO-2)

Zona occludens are adaptor proteins which belong to the membrane associated with the guanylate kinase (MAGUK) protein family which plays an important role in supporting the structure of the endothelial cell by connecting the C-terminal of ZO-1 and ZO-2 with the cytoskeleton protein (62).

ZO-1 is a phosphoprotein (220 kDa) which is expressed in endothelial and epithelial cells. It links the transmembrane proteins of tight junction to actin cytoskeleton (58). This interaction is important for TJs function stabilization. Abnormality of this junctional complex implicates in vascular permeability (63).

ZO-2 is a phosphoprotein (160 kDa) which has a high homologus sequence to ZO-1 (64). *In vitro* study showed that ZO-2 is located along the cell-cell membrane contact in cultured brain endothelial cells (65). In addition, ZO-2 can replace the function of ZO-1 in formation of normal TJs in cultured epithelial cells lacking ZO-1 (66).

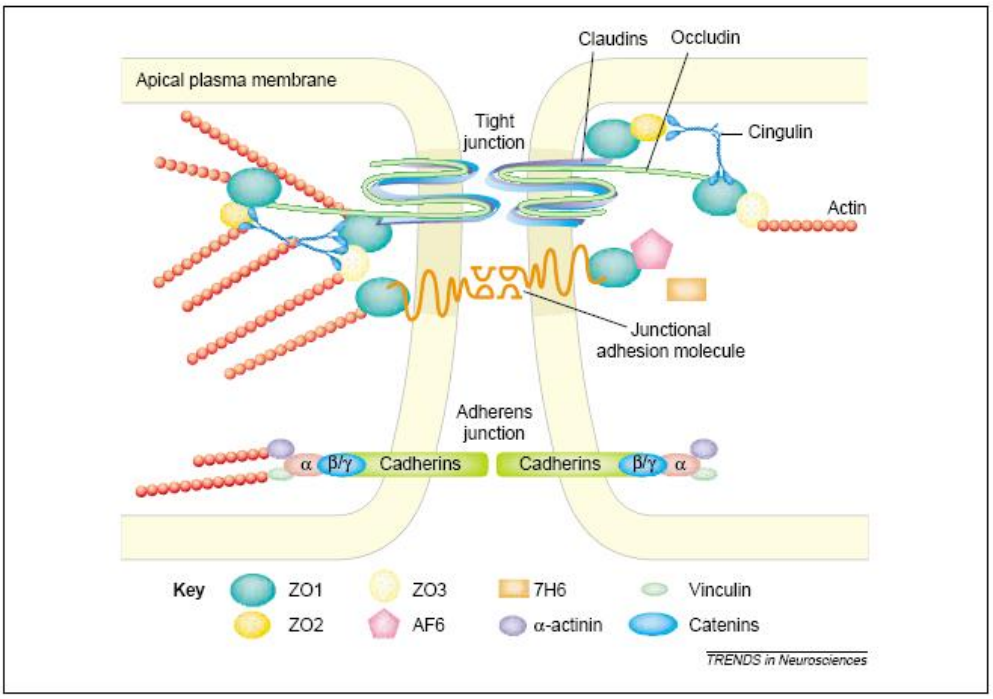

Figure 2-2 Schematic picture of the tight junction protein in association with BBB tight junctions (67).

2.2 The trigeminovascular system

The trigeminovascular system is the neurovascular unit with tight association between cerebral vessels and the trigeminal neurons. The activation of this system is correlated with the pathophysiology of vascular headache including migraine (68).

The activation of the trigeminal neurons which is the pseudounipolar neurons located in the trigeminal ganglion (TG) (69,70) results in the released of several neurotransmitter in both peripheral and central nerve terminals. At the peripheral end, various neuropeptides including CGRP, SP , NO and VIP are released to the cerebral vessels (71) which finally can lead to the dilatation of the cerebral vessels as well as the neurogenic inflammation (72,73). The trigeminal neurons also project the nerve fiber centrally onto second order sensory neurons in C1 and C2-level of trigeminal nucleus caudalis (TNC) in the brainstem (74). The signal transmitted to the thalamus leading to activation of the frontal cortex resulting in the pain consciousness (75). The

trigeminal nerve was activated by various stimuli including cortical spreading depression (CSD).

2.3 Cortical spreading depression theory

Cortical spreading depression (CSD) was first explained by Leào 1944 (76). It was represented as a propagating depolarization wave followed by repolarization. CSD can be initiated by electrical, mechanical, and chemical stimuli (77–79). The wave progresses along the cortical surface from the occipital lope at a speed of approximately 2–5 mm/min (76,78–80). It can occur in several parts of the brain including the cerebral cortex, hippocampus, and cerebellum (81).

From the previous study, it is found that repetitive CSD waves which occur in healthy brains do not induce damage to neural cells (82). but may contribute in the protective role in ischemic stress (83–85). However, in the physiologically impaired condition, CSD can be destructive (86). CSD activation can induce the alteration in neural and vascular compartments (87).The previous study showed that CSD involved in the alteration of neurotransmitters (88), metabolism (89), cerebral blood flow (CBF), and pro-inflammatory cytokines (90). In addition, CSD induced the dramatic increase of K^+ concentration from 3 to more than 50 mM in a short timeframe (91). Likewise, it induces an increase of glutamate levels in extracellular fluid (92,93). During CSD, a short-lasting increase of cerebral blood flow (CBF) occurs, followed by a long-lasting regional hyperemia (94).

CSD is implicated in the pathophysiology of several neurovascular disorders such as trauma, strokes, and exacerbates brain injury (95,96). This phenomenon is also accepted to be closely correlated with migraine with aura (97).

2.3.1 CSD and blood brain barrier permeability

During CSD, several vasoactive neurotransmitters are released from perivascular neurons (2). Those neurotransmitters can directly affect to cerebral vessels. Interaction between neurons and astrocytes also participate in CBF regulation. The cerebral vessel is enclosed by astrocyte endfeet and this contact is important for adjusting the regional CBF in local metabolic activity (98–100). The swelling of astrocyte foot plates has been demonstrated in cerebral vessels after CSD

activation (101,102). Similar to neurons, astrocyte can produce and release many vasodilator substances including carbon monoxide (CO), nitric oxide (NO), hydrogen ion, potassium ion, and lipoxygenase products (103–105). This effect of CSD on BBB was confirmed by Gursoy in 2004. Based on these accumulative data, it can be concluded that CSD activation can lead to the instability of BBB integrity. CSD can initiate the disruption of the BBB via the matrix metalloproteinase (MMP-9) dependent mechanism (10).

2.3.2 CSD and perivascular nerves

The trigeminal pathway is important in regulating and modulating the cerebrovascular responses under physiological and pathological conditions (106,107). The trigeminal afferent stimulation during CSD can finally lead to vasodilation and neurogenic inflammation in the meninges. These phenomenon occurred via axon-reflex activation of collateral trigeminal branches and central-reflex activation of parasympathetic nerves (97,108,109). With CSD activation, perivascular nerves can release various vasoactive neurotransmitters such as NO, endothelin, calcitonin gene related peptide (CGRP) and substance P (SP) (Figure2-3). Among them, the role of CGRP in the alteration of BBB is of particular interest. Several studies have indicated that CGRP is involved in the dilation of cerebral vessels induced by CSD, the blocking of the CGRP receptors can attenuated cerebral hyperemia (110–112). It is found that approximately half of the dilation in CSD comes from CGRP (110,112,113) . The accumulative evidences have demonstrated that serotonin is one of the neurotransmitter released from perivascular nerves which contribute to the overall cerebrovascular response in CSD activation (114).

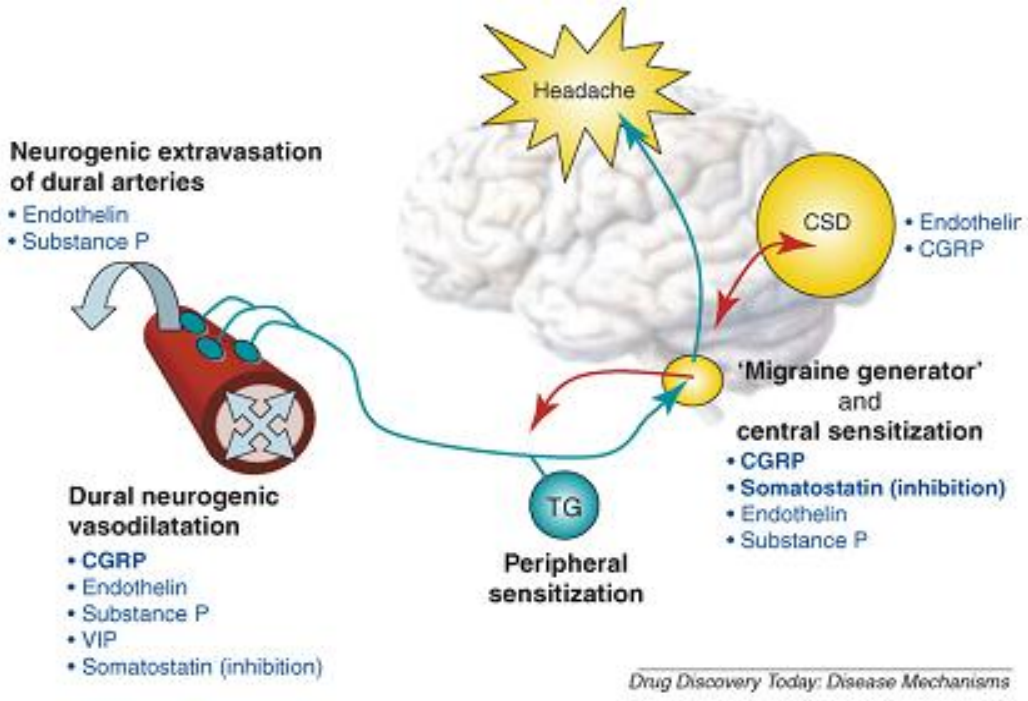

Figure 2-3 Schematic picture of the neurogenic inflammation (7)**.**

2.4 Serotonin

Serotonin, which known as 5-hydroxytryptamine (5-HT), is widely distributed in the central and peripheral nervous system. 5-HT can act as either hormone or neurotransmitter. This neurotransmitter has been implicated in the pathophysiology of mood disorders, systemic hypertension, and migraine (115).

2.4.1 Origin and metabolism of serotonin

5-HT was isolated and characterized in 1948. (116–118). 5-HT is produced from essential amino acid tryptophan, which is hydroxylated to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase and 5-HTP is decarboxylated into 5-HT (119) (Figure 2-4). Tryptophan hydroxylase is considered as a rate-limiting enzyme with low affinity for other amino acids (120), a relatively high K_m (121), and a limited distribution in the serotonin containing tissues (120,122).

In the CNS, the prominent area for 5-HT synthesis is the raphe nuclei which project the ascending serotonergic fibers to the forebrain and descending fibers to the medulla and spinal cord (123). Outside the CNS, the synthesis of 5-HT is very limited in enterochromaffin cells and platelets. In addition, platelets represent a main storage site for 5-HT (124,125). 5-HT is degraded by monoamine oxidase (MAO), especially the MAO-A isoform which is transformed to a reactive aldehyde, followed by further processing by aldehyde dehydrogenase into 5-HT primary metabolite, 5-hydroxyindoleacetic acid (5-HIAA) (126).

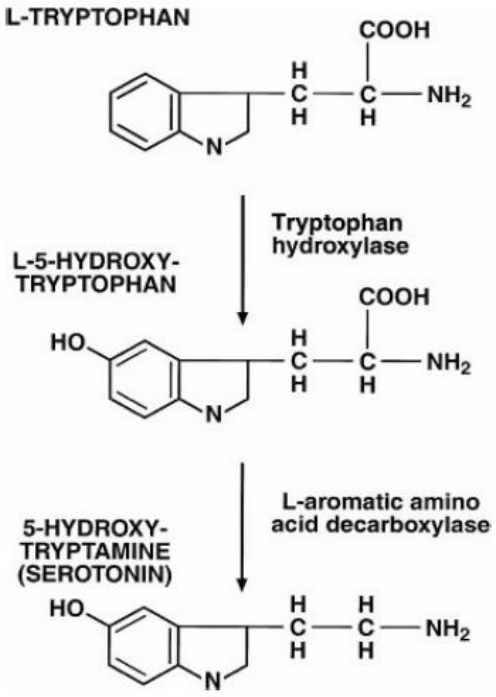

Figure 2-4 The schematic picture represents the synthetic process of 5-HT from tryptophan (127)**.**

2.4.2 5-HT receptor

5-HT receptors are classified into seven subtypes 5-$HT_{1–7}$ (128). Six of these subtypes belong to the G-protein-coupled receptors family. Six of these subtypes belong to the G-protein-coupled receptors family. The 5-HT_3 receptor is only one which involved in a ligand-gated Na^+ /K^+ ion channel (129). The detail of all 5-HT receptor subtypes are demonstrated in Table 2-1.

In the CNS, 5-HT_{1B} receptors can induce the presynaptic inhibition of neurons. It widely distributes in various areas of the brain. The location of this receptor defines its function. In the frontal cortex, the 5-HT_{1B} receptors act as a terminal receptor inhibiting the release of dopamine. In the basal ganglia and striatum, it presents an autoreceptor which inhibits the release of serotonin. In addition, this receptor serves as a heteroreceptor, which control the terminal release of neurotransmitters such as acetylcholine, glutamate, dopamine, norepinephrine, and γ-aminobutyric acid. Moreover, this subtype was found in cerebral and other arteries (130).). In vascular effect, it can induce the vasoconstriction. The 5-HT_{1B} receptors agonist, sumatriptan, is developed to be used as an anti-migraine drugs (131).

The 5-HT_{1D} receptor has a lower expression than the 5-HT_{1B} receptor although they share a structural homology. 5-HT_{1D} receptors function as autoreceptors in the dorsal raphe nuclei and modulate the release of serotonin in the vascular system (132). In the CNS, they induce the vasoconstriction in the brain (133).

2.4.3 Role of 5-HT in vascular tone

5-HT plays an important role in the cerebrovascular control. Both vasoconstriction and vasodilation are side effects of 5-HT which depends on the receptor subtype located on the vessel wall and surrounding smooth muscle tissue (134). The affinity of 5-HT to 5-HT_{1B} and 5-HT_7 are higher than that of the 5-HT_{2A} receptor, therefore, 5-HT constricts large conducting arteries via 5-HT_{1B} receptors and dilates arterioles via 5-HT_7 receptors (135–137). 5-HT is involved in the preserving of vessel wall tone. The decrease of 5-HT in the neurovascular junction can lead to constriction of arterioles, but dilate the large arteries. There are some evidences reporting the change of vessels in the headache phase of migraine (136,138).

2.4.4 Role of 5-HT in pain control and nociception

5-HT modulates pain and nociception in both central and peripheral nervous systems. 5-HT released in inflamed tissue sensitizes the peripheral nerve fibers, which transfer nociceptive information to the CNS. In the brainstem, a descending projection of 5-HT neurons into the spinal cord modulates incoming nociceptive information (139) while ascending projections to cortical and limbic regions modulate the perception of pain (140). Abnormality of the 5-HT system has been reported in various diseases such as mood disorders, pain disorders, and migraine. The alteration of the 5-HT level is reported to correlate with pain perception (140). The involvement of 5-HT in pain control is confirmed by the evidence which demonstrate that activation of 5-HT_{1B} and 5-HT_{1D} receptors by a migraine drugs, triptan, can attenuated the migraine headache (137).

2.4.5 The 5-HT depletion and migraine

5-HT is involved in the pathophysiology of migraine and the 5-HT receptor is distributed throughout the trigeminovascular nociceptive pathway in both neuronal and vascular compartments. The depletion of serotonin has been revealed to associate with the migraine pathophysiology (141,142). The clinical study in 1961 by Sicuteri and college demonstrated that the 5-HT level in platelets has decreased and 5-hydroxyindole acetic acid (5-HIAA) in the urinary excretion has increased in migraine patients (15). Several studies have indicated that the central 5-HT concentration has decreased and 5-HT release in to plasma has increased (143). The reduction of 5-HT synthesis in the brain leads to less 5-HT neurotransmission, which intensifies the symptoms related to migraine (16). The low serotonin transmission in the migraineurs leads to an increase in the sensitivity of the auditory and visual system, which are the characteristic symptoms of migraine (144). The study of $5\text{-HT}_{1B/1D}$ receptor agonists, which activate the inhibitory function of prejunctional autoreceptors on cortical 5-HT afferents, decreased the 5-HT synthesis, which resulted in an increase in the sensitivity of the auditory system in migraine sufferers (145).

In the 5-HT depletion animal model, the previous study has indicated that CSD activation in 5-HT depleted rats resulted in the hyperexcitability of cortical neurons (19). However, the response in vascular compartment and mechanism underlying the alterations in cerebrovascular responses in the 5-HT depleted state is still unclear.

Table 2-1 Families of 5-HT receptors.

Family **Potential/Type**	**Mechanism of action**
5-HT_1 Inhibitor: G_i/G_0-protein coupled	Decreasing intracellular concentration of cAMP
5-HT_2 Excitatory: G_{q11}-protein coupled	Increasing intracellular concentration of IP3 and DAG
5-HT_3 Excitatory: Ligand-gated Na+/K+ Channel	Depolarization of cell plasma
5-HT_4 Excitatory: G_s-protein coupled	Increasing intracellular concentration of cAMP
5-HT_5 Inhibitory: G_i/G_0-protein coupled	Decreasing intracellular concentration of cAMP
5-HT_6 Excitatory: G_s-protein coupled	Increasing intracellular concentration of cAMP
5-HT_7 Excitatory: G_s-protein coupled	Increasing intracellular concentration of cAMP

IP3: inositol trisphosphate; DAG: diacylglycerol; cAMP: cyclic adenosine monophosphate. (146,147).

2.5 Calcitonin gene related peptide (CGRP)

Migraine is a neurological disorder involved with the activation of the trigeminovascular system. The activation of the trigeminovascular system can release calcitonin gene-related peptide (CGRP). This neuropeptide plays an important role in dilation of intracranial blood vessels and transmitting vascular nociception (148). CGRP, a 37-amino acid neuropeptide, which belongs to the calcitonin family (149), was first identified in 1983 in rats through alternative splicing of the calcitonin gene. CGRP exists in 2 forms, α-CGRP and β-CGRP, which both have a high sequence homology (150–152) and similar biological activities (153). α-CGRP is usually found in sensory neurons whereas β-CGRP is found in the enteric nervous system (154).

2.5.1 Distribution and localization of CGRP

CGRP is widely distributed in central and peripheral nervous systems. CGRP is located in posterior horn cells in peripheral nervous systems. In the central nervous systems, CGRP is co-localized with SP in the primary sensory ganglia while co-localized with acetylcholine in the motor neurons (154). In the cardiovascular system, CGRP is stored in nerve fibers which are more abundant around the arteries than around the veins (155).

2.5.2 Physiological functions of CGRP

CGRP-containing nerve fibers and CGRP receptors are wildly distributed throughout the body. It is demonstrated that CGRP plays an important role in various physiological functions. In the central nervous system, CGRP is a neurotransmitter involved in several neuronal responses including the pain perception (156,157). CGRP after binding with its receptor can enhance the release of SP (158) and other excitatory amino acids from primary afferent fibers (157,159).

2.5.3 CGRP receptors

The CGRP receptor is a G-protein coupled receptor (GPCR) family (160) which is classified into $CGRP_1$ and $CGRP_2$ subtypes (153). The structure of the CGRP receptor is composed of seven transmembrane domains protein, termed as receptor activity modifying protein (RAMP) and receptor component protein (RCP) (160) (Figure2-5). The activation of the CGRP receptor can lead to vascular relaxation via both the endothelium-dependent pathway and endothelium-independent pathway. In the endothelium independent pathway, CGRP can have a direct action on the smooth muscle cells via increases in the cyclic adenosine monophosphate. In the previous study, it was found that CGRP receptors coexist with receptors for other neurotransmitters and neuromodulators, such as SP, noradrenaline, neuropeptide Y, vasoactive intestinal peptide in the dorsal root ganglion, and other neurons oho(157,161). In addition, CGRP and its receptor were also found in the trigeminovascular system. CGRP immunoreactivity has been detected in trigeminal nerve fibers and ganglion cells (162) (Figure 2-6)**.**

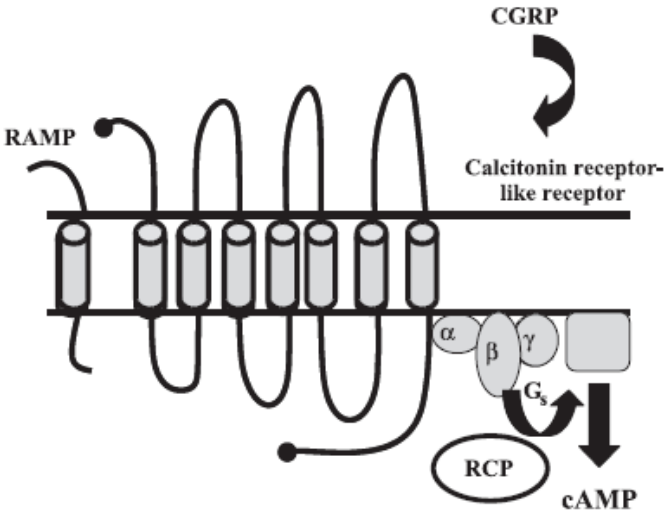

Figure 2-5 Schematic picture represents the CGRP receptor, which interacts with receptor activity modifying protein (RAMP1) and a receptor component protein (RCP) (163)**.**

2.5.4 CGRP and its role in migraine pathophysiology

The pathophysiology of migraine demonstrates the close relationship between migraine attack and the activation of the trigeminovascular system. Stimulation of the trigeminal ganglia and sensory nerves induces the release of CGRP, which further dilates blood vessels and stimulates sensory nerve transmission (164). Previous study showed that CGRP-like immunoreactivity was expressed in trigeminal nuclei and trigeminal sensory nerve fibers (165). Clinical studies had reported that during the migraine headaches, the concentration of CGRP in plasma obtained from jugular venous blood had increased (166). Pathophysiology has been strongly confirmed from the result which demonstrates that CGRP infusion can provoke migraine-like headaches (167) should add the result about the CGRP receptor antagonist, Recently the CCRP receptor antagonist has been develop to be an anti-migraine drug (Figure 2-6).

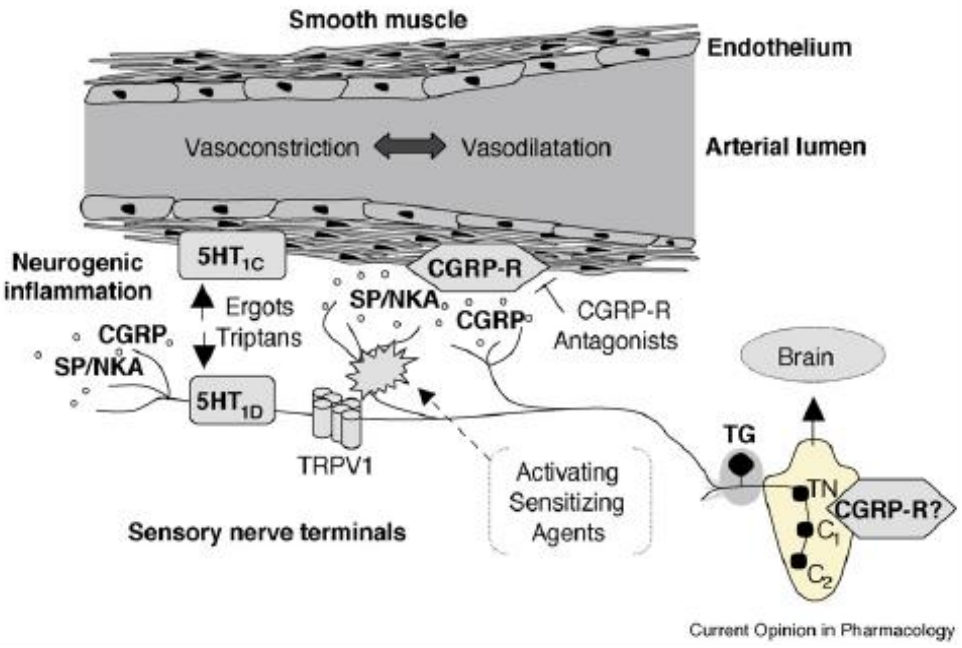

Figure 2-6 Schematic picture represents the innervation of trigeminal neurons around the cranial arterial vessel and the action of various neurotransmitters with its recepter; CGRP, calcitonin gene related peptide; R, receptor; substance P, SP; neurokinin A, NKA; TG; trigeminal ganglion; TRPV1, transient receptor potential vanilloid 1; 5HT, 5-hydroxytryptamine (168)**.**

2.6 Substance P (SP)

Substance P is firstly reported in 1931 and later considered as the neurotransmitter which released from the primary sensory afferent fibers and is involved in pain transmission (169,170). Substance P is one member of the tachykinin family which has a common C-terminal amino acid sequence and a varying N-terminal sequence. The change of N-terminals is correlated with the recognition of a specific receptor (171). The neurokinin 1 (NK-1) receptor has a high affinity with SP (172,173). Both SP and NK-1 are found in various regions of the CNS, especially the dorsal horn (174). The primary nociceptive afferent fibers, which terminate in the dorsal horn, mostly contact with nociceptive fibers, which has SP-containing terminals (170). In addition, SP and NK-1 are present in small-group neurons which are involved in stress, anxiety, and the modulation of pain (173).

2.6.1 Substance P and neurogenic inflammation

The central hyperexcitability and the increased sensitivity of pain are stimulated by the release of SP into the dorsal horn (175). In prolonged noxious stimulation, SP is released from the primary afferent terminal and bind to neuronal NK1 receptors. Excitation occurs by calcium ion influx into neuronal cells. The calcium-dependent nitric oxide synthase is activated and formed NO, which increased the intracellular cGMP level (170).

The production of SP and other tachykinins, which are released from peripheral primary sensory neurons, involve in the neurogenic inflammation. SP prominent effect to the vessels caused the vasodilatation of arterioles, plasma protein extravasation in post-capillary venules, and leukocyte adhesion to endothelial cells (176). Furthermore, SP was found in the terminals which innervate the artery and veins vessel wall which suggests that SP has a role in the regulating blood flow in some tissues (177). The action of inhibitory receptors on sensory nerves limits the neurogenic inflammatory responses. These receptors include adenosine A_1, dopamine D_2, histamine H_3, and 5-$HT_{1B/D}$. Agonists of these receptors were considered as anti-inflammatory agents as well as antagonists of the tachykinin receptor (178). However, the electrical stimulation of the sensory nerve ending effect on vasodilation was caused by the release of CGRP rather than SP (179) .

CHAPTER III
OBJECTIVES

Major objective:

To investigate the effect of 5-HT depletion induced with CSD on the alteration of BBB.

Minor objectives:

This study was divided into two parts as follow:

Part: I Study the effect of 5-HT depletion on the CSD induced the alteration of the BBB.

3.1 To investigate the effect of 5-HT depletion induced with CSD on ultrastructural change in endothelial cells.

3.2 To investigate the effect of 5-HT depletion induced with CSD on the decrease of tight junction (occludin, ZO-1, ZO-2).

3.3 To investigate the effect of 5-HT depletion induced with CSD on IgG extravasation.

Part: II Study the effect of 5-HT depletion on the CSD induced neuropeptides expression in TG.

3.4 To determine the effect of 5-HT depletion induced with CSD on the expression of CGRP and SP in TG.

CHAPTER IV

MATERIALS AND METHODS

4.1 Animals

Wistar rats were supplied by the National Laboratory Animal Center of Salaya Campus, Mahidol University.

4.2 Antibodies

Rabbit anti CGRP and rabbit anti SP were purchased from Sigma, USA. Rabbit anti occludin and rabbit anti ZO-1 were purchased from Invitrogen, USA. ZO-2 was purchased from Santa Cruz Biotechnology, USA. Anti-rat IgG-HRP linked antibody was purchased from Cell Signaling, USA.

4.3 Experimental animals

Adult male Wistar Furth rats weighing 250-300 grams were supplied by the National Laboratory Animal Center of Salaya Campus, Mahidol University. The animals were housed 5 animals per cages in stainless-steel bottom cages. They were kept in a well-ventilated room with the control of temperature (28-32°C) and light/dark cycle (light on from 6.00 AM-6.00 PM). All animals were allowed to have access to food (Purina Laboratory Chow, Premium quality feed, Zuellig Gold Coin Mills Pte., Ltd., Singapore) and water *ad libitum*. To limit the effect of nonspecific stress, all animals were accustomed to daily handling for at least 5 days before experiment. All the protocols in this study were approved by the Chulalongkorn University local ethics committee.

4.4 Study design

In order to study the effect of 5-HT depletion on the alteration of the BBB induced by CSD, the experiment in this study were divided into two major parts as follows:

Part: I Study the effect of 5-HT depletion on the CSD induced the alteration of the BBB.

Part: II Study the effect of 5-HT depletion on the CSD induced neuropeptides expression in TG.

4.4.1 Study the effect of 5-HT depletion on the CSD induced alteration of the BBB (Part I).

In this study rats were divided into 4 groups as follows (Figure 4-1):

- Control group (n=4)
- CSD group (n=4
- 5-HT depletion (n=4)
- 5-HT depletion with CSD (n=4)

Three day before the experiment, serotonin depletion was induced by the i.p. injection of parachlorophenylalanine (PCPA) at the dosage of 100 mg/kg BW to the rat with serotonin depleted state. The 0.9% normal saline (NSS) at the same volume was injected into the rats with normal serotonin.

The CSD was induced in the rat three days after PCPA or NSS injection. CSD activation was generated in the rats by topical application of 3 mg. of potassium chloride (KCl) on the surface of the parietal cortex. For the non-CSD groups, sodium chloride (NaCl) 3 mg was placed on the parietal brain instead. The detail of the CSD activation procedure is described in the method part.

After 2 hours of KCl or NaCl application all animals were humanly killed by decapitation. The frontal cortex was collected from all animals and the tight junction protein expression was detected by western blot analysis. In addition, The perfused brain from the experiment in part I was used to study tight junction protein expression by immunohistochemistry. The ultrastructural tight junction was determined by electron microscopy. The details of these methods are described in the method part.

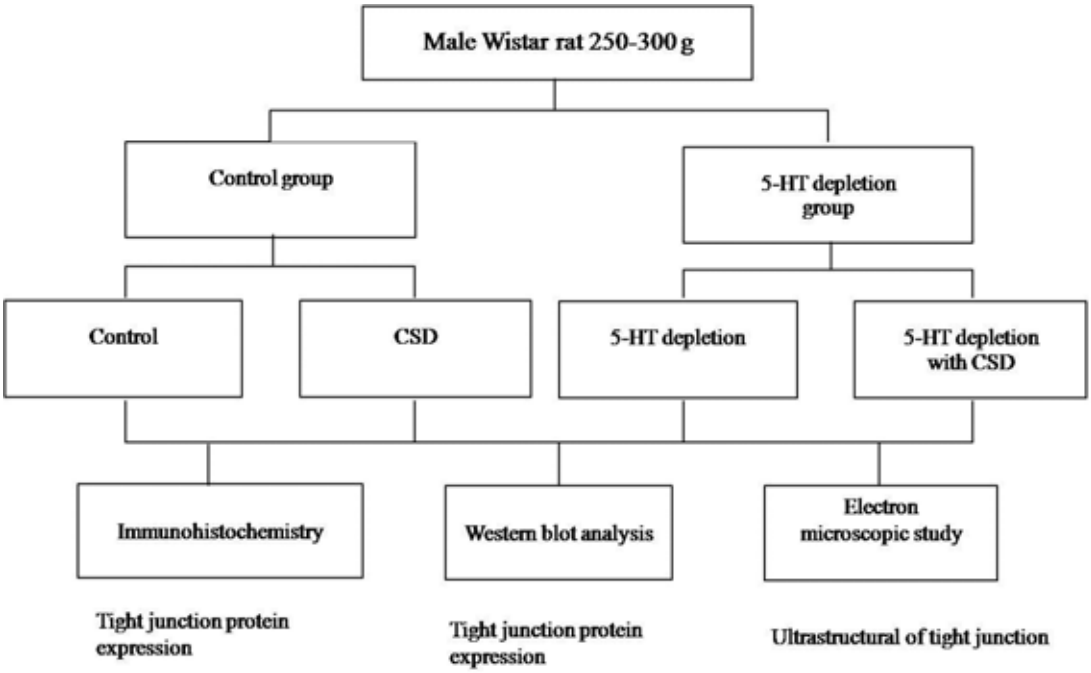

Figure 4-1 Diagram of the study design in part I.

4.4.2 Study the effect of 5-HT depletion on the CSD induced neuropeptides expression in TG. (Part II).

In this study, rats were divided into 4 groups as follows: (Figure 4-2):

- Control group (n=4)
- CSD group (n=4)
- 5-HT depletion (n=4)
- 5-HT depletion with CSD (n=4)

After 2 hours of the KCl or NaCl application, all animals were humanly killed and transcardially perfused with 4% paraformaldehyde. The trigeminal ganglions were collected from all animals. The number of neurons with the CGRP and SP expression was detected by immunohistochemistry. The whole brain was dissected and preserved in 4% paraformaldehyde and 3% glutaraldehyde for immunohistochemical and electron microscopic examination, respectively (Part II).

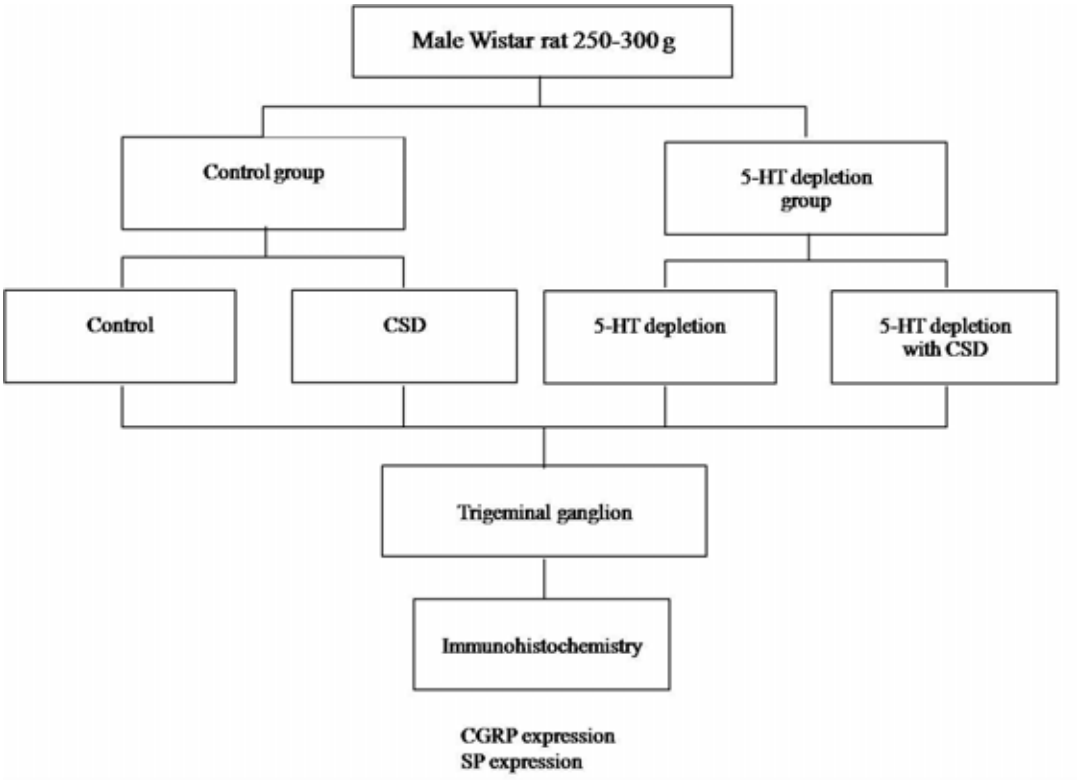

Figure 4-2 Diagram of the study design in part II

4.5 Methods

4.5.1 Animal preparation

All experimental rats were anesthetized for the duration of the experiment by i.p. administration of 60 mg/kg sodium pentobarbital. Additional dosed of anesthetics were given as required to maintain surgical anesthesia based on the testing of corneal reflex and response to tail pinch.

4.5.2 Induction of Cortical Spreading Depression (CSD)

After tracheotomy, the rat was placed on a surgical frame and its head was fixed on a sterotaxic frame. The right parietal bone was exposed by mobilization of skin to both sides side of the midline incision. The anterior craniotomy (diameter about 2 mm.) was performed using a saline-cool drill in the frontal bone at 1 mm. anteriorly and laterally from bregma. The posterior craniotomy (diameter about 2 mm.) was performed in the parietal bone at 7 mm. posteriorly and 1 mm. laterally from bregma. The anterior and posterior craniotomy openings were used for the placement of the electrode and for initiation of CSD, respectively. The dura was opened by using a microneedle. An artificial cerebrospinal fluid was infused in the intracranial space. Solid KCl 3 mg. was placed on the cortical surface to induce a repeated pattern of cortical depolarization characterizing the CSD. However, NaCl application, which had no effect on cortical activity was placed in control and 5-HT depletion without CSD groups.

4.5.3 Immunohistochemical study

- Perfusion and tissue preparation

After 2 hours of KCl or NaCl application, animals were further processed for Immunohistochemical study. The experimental rats were deeply anesthetized with sodium pentobarbital. Laparotomy and thoracotomy were done. A cannula was inserted into the apex of the heart and was advanced just distally to the aortic arch. Then, the vasculature was flushed trancardially with 250 ml PBS, followed by 250 ml of 4% paraformaldehyde in 0.1M PBS, pH 7.4. After perfusion, brains were removed and a small portion of the anterior parietal cortex was taken out and cut into multiple 1x1 mm^2 cubes. Then, these small pieces were immediately immersed in 3%

glutaraldehyde in 0.1M PBS, pH 7.4 for further transmission electron microscopy processing. The brain at 7mm anterior to the bregma and the right trigeminal ganglion were immersed in 4% paraformaldehyde in 0.1M PBS, pH 7.4 overnight at 4°C Next, the tissue was dehydrated and embedded in paraffin block. The longitudinal sections of TG and coronal sections of the brain were cut 3 μm thick by microtome. Sections were picked up in 1 in 6 series, placed on the super-frost plus slide, and heated at 60°C overnight.

- Immunohistochemical study for CGRP, SP, Occludin, ZO-1 and ZO-2

After deparaffinization, the sections were processed for the antigen retrieval, endogenous peroxidase blocking and non-specific binding blocking. The labeling of CGRP and SP were done by incubated the sections with rabbit anti CGRP (1:6000 Sigma, USA), SP (1:2000 Sigma, USA) at 37°C for 30 min. Immunoreactivity of CGRP and SP was detected by using a ultraView Universal DAB Detection Kit (Ventana, USA). After that, sections were dehydrated in ethanol and cleared with xylene. The whole process was operated on the automatic machine for slide staining (Ventana). The stained section was cover slipped with a mounting medium and the expression of CGRP and SP immunoreaction was examined under light microscope.

For occludin and ZO-1 immunohistochemistry, the coronal sections of the brain were deparafinized and processed by a slide staining machine (Ventana). The labeling of occludin and ZO-1 were done by incubation of the sections with rabbit anti occludin (1:100 Invitrogen, USA), and rabbit anti ZO-1 (1:100 Santa Cruz, USA) at 37°C for 30 min. Immunoreactivity of occludin and ZO-1 was detected by using an ultraView Universal DAB Detection Kit (Ventana, USA). The stained section was cover slipped with a mounting medium and the expression of occludin and ZO-1 was examined under a light microscope.

- Data Collection

Measurement of the number of CGRP and SP immunoreactive cells

CGRP immunoreactive (CGRP-IR) neurons were distinguished by their dark stained cytoplasm. The expression of CGRP in TG was determined by counting the CGRP-IR neurons in TG from 3 selected sections per animal. The TG sections with an average of 800-1200 ganglion cells were selected for the study. The data was analyzed and reported as the percentage of CGRP-IR cells per section. The identity of all samples was concealed throughout the counting process.

SP immunoreactive (SP-IR) neurons were distinguished by their dark stained cytoplasm. The expression of SP in TG was determined by counting the SP-IR cells by the same criteria as CGRP expression which is described above.

The expression of occludin, ZO-1, and ZO-2 in the cerebral cortex

The occludin immunoreactive area in the brain section was characterized by their dark stains along the cerebral vessel. The expression of ZO-1 and ZO-2 was presented at cerebral vessel in the same pattern as occludin which is described above.

4.5.4 Electron microscopic study

For the electron microscopic study, the frontal cortex was removed and cut into small cubes sized 1 mm^3. All specimens were immediately fixed in 3% glutaraldehyde overnight and posted-fix with 1% osmium tetroxide. Specimens were dehydrated through 30%, 50%, 70%, 95% and 100% ethanol, respectively. They were passed through propylene oxide two times, infiltrated overnight, and embedded in plastic media (Epon 812; Electron Microscopy Sciences, Ft. Washington, USA) incubated at 60°C for polymerization. Ultramicrotome with a glass knife was used for semi-thin and ultra-thin sectioning. Semi-thin sections (0.5 μm thick) were stained with toluidine blue in order to select the suitable area for investigation by electron microscopy. The ultra-thin sections (70-90 nm thick) were placed on copper grid and stained with uranyl acetate and lead citrate. All of stained specimens were examined under the transmission electron microscope (JEM 1210; JEOL, Tokyo, Japan).

- Quantitative study: alteration of tight junction alignment

In order to evaluate the alteration of the tight junction between endothelial cells, ten capillaries (diameter range between 8-10 μm) per sample were selected. Electron micrographs which cover the area of tight junction were taken from every capillary at the direct magnification of 8,000 times. The angle of the tight junction in the endothelial cell was determined by measuring the angle with vessel lumen (Figure 4-3).

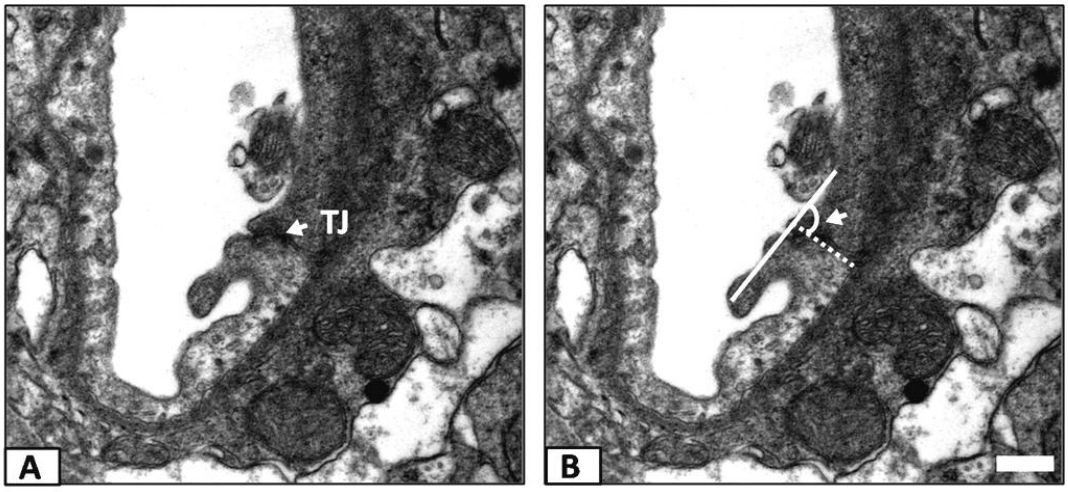

Figure 4-3 The electron micrographs show the criteria of tight junction angle assessment. The tight junction (TJ) is demonstrated in between endothelial cell (A). The alteration of the tight junction alignment is evaluated by monitoring the angle between the tight junction plane of the endothelium (dash line) and the vessel lumen (thick line). In this study, the angle of tight junction was characterized into two groups: normal angle (0-20 degree) and high angle (21-90 degree) (Bar = 300 nm)**.**

4.5.5 Western blot analysis

- Protein extraction

Animals were deeply anesthetized with an excessive dose of sodium pentobarbital and killed by decapitation, brains were promptly removed. The ipsitlateral frontal cortex was dissected on ice, frozen in liquid nitrogen and stored at -80°C until used. Frontal cortex sample were homogenized at 4°C in RIPA buffer (Cell signaling, USA), containing a protease inhibitor cocktail (Thermo, USA) After centrifugation at 12,000 g for 15 min, supernatant were collected as protein fraction and stored at 80°C. Protein concentrations were measured using BCA protein assay kit (Thermo, USA) with bovine serum albumin as standard.

- Western blotting

The extract protein from cerebral cortex (1o µg) was solubilized in SDS sample denatured in 95°C for 5 min and separated on SDS-10% polyacrylamide gels. The protein was transferred at constant 0.35 Amp into nitrocellulose membrane. The membrane was blocked in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% dry milk for 1 h at room temperature then incubated with anti rat IgG-HRP link (1:1000 Cell Signaling, USA), rabbit anti occludin (1:500 Invitrogen, USA), rabbit ZO-1 (1:1000 Invitrogen USA) and rabbit anti ZO-2(1:1000, Invitrogen USA) at 4°C for the detection of IgG, occludin, ZO-1 and ZO-2, respectively. After overnight incubation all membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Activity of peroxidase was detected by enhanced chemiluminescence (ECL) system. Intensity of the bands was measured by densitometry using software and results are expressed as percentage of the housekeeping protein β-actin.

4.6 Statistical analysis

All values were presented as mean ± standard error of mean (SEM). The percentage of CGRP and SP expression in TG were present in mean ± SEM. The ratio of tight junction proteins (occludin, ZO-1, ZO-2), IgG and β-actin were analyzed by Scion Image. One-way ANOVA was used for statistical analysis. Probability values of less than 0.05 were considered to be statistically significant. All statistical analyzes were performed using Prism5.

CHAPTER V

RESULTS

5.1 Study the effect of 5-HT depletion on the CSD induced the alteration of the BBB. (Part: I)

5.1.1 The effect of 5-HT depletion induced with CSD on ultrastructural change of endothelial cells.

Cerebral capillaries are composed of the endothelial cells which are different from other non-neural endothelium cells. They are continuous endothelial cells which connect to each other by tight junction. In physiological condition, they express very few microvilli and pinocytic vesicle which indicate the limitation of transcellular transportation through these microvessels. The damage in the BBB integrity can be indicated by the alteration of all ultrastructures mentioned above. The abnormality of endothelial cells was characterized by the increase in the number of microvilli, pinocytic vesicles, astrocytic footplate swelling, and changing in alignment of tight junction.

A. Alteration in pinocytic vesicles and microvilli

In the control group, endothelial cell demonstrated a few pinocytic vesicles and microvilli formation. In CSD group, an increase in the number of pinocytic vesicles and microvilli in endothelial cells was observed as compared with the control group. In 5-HT depletion, we observed an increase in the number of pinocytic vesicles and microvilli as well. However, the highest density of pinocytic vesicles and microvilli was observed only in endothelial cells obtained from the 5-HT depletion with CSD group. Our study revealed that among those all experimental groups, the endothelial cells obtained from the 5-HT depletion with CSD activation group demonstrated the most prominent ultrastructural alterations (Figure 5-1, Figure 5-2).

B. Alteration of astrocytic footplate swelling

The astrocytic footplate swelling is characterized by clear space around the vessel. The perivascular astrocytic footplate swelling was hardly observed in the control group. In the CSD activation group, astrocytic footplate swelling was rarely found. However, when we observed the capillaries in the rats obtained from the depletion of 5-HT In combination with or without CSD activation, a more severe astrocytic foot pate swelling was observed. This result indicates that the combination of CSD activation and 5-HT depletion increases the severity of astrocytic footplate swelling which can lead to cerebral vessel alteration (Figure 5-2).

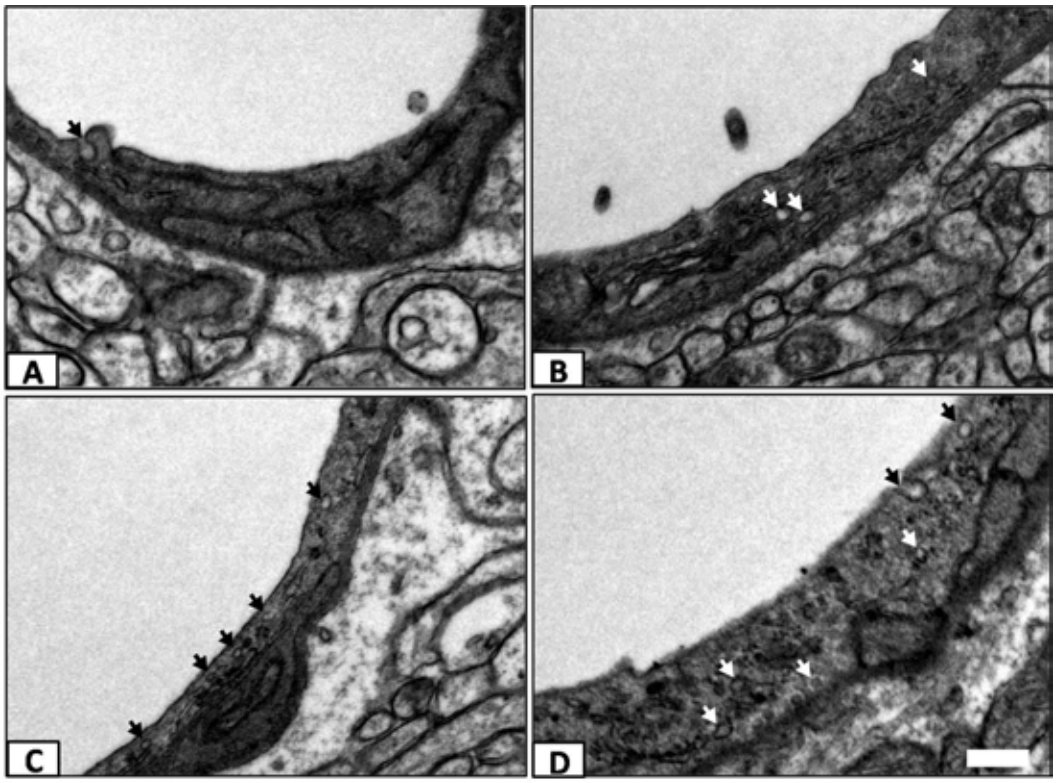

Figure 5-1 The electron micrographs show the pinocytic vesicles in the endothelial cell obtained from the control (A), CSD (B), 5-HT depletion (C), and 5-HT depletion with CSD (D) group. Bar = 300 nm

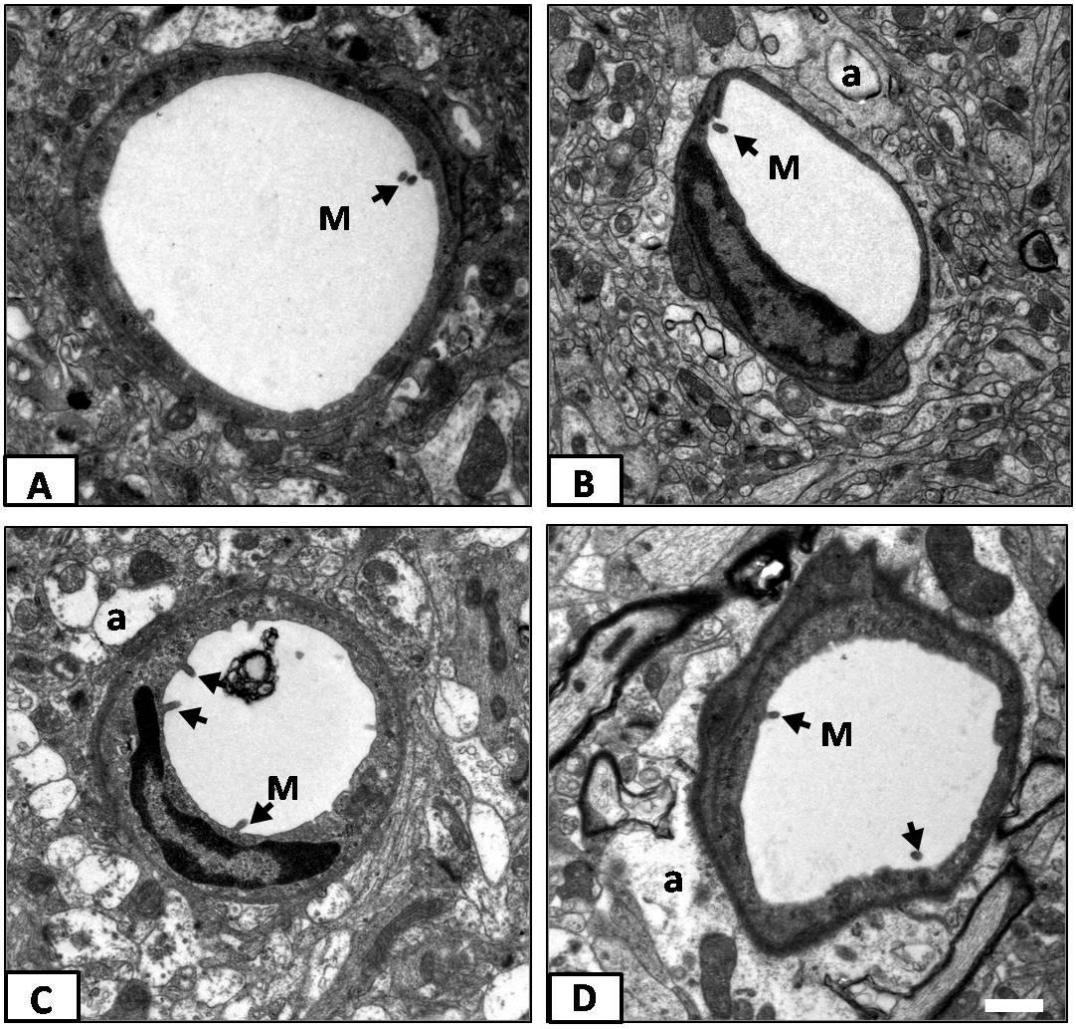

Figure 5-2 The electron micrographs show the microvillus formation (M) and the astrocytic footplate swelling (a) which are observed in the capillary obtained from the control (A), CSD (B), 5-HT depletion (C), and 5-HT depletion with CSD group (D) Bar = 900 nm

C. Alteration in tight junction alignment

The alteration of the tight junction alignment was evaluated by monitoring the angle between the tight junction plane of the endothelium and the vessel lumen which was classified into two categories as normal (the angle between the tight junction and the vessel lumen ranges from 0 to 20 degrees) and high angle (the angle between the tight junction and the vessel lumen ranges from 20 to 90 degree). In this study, the proportion of the normal angle tight junction was highest in the control group and lowest in the 5-HT depletion with CSD activation group. On the other hand, the proportion of tight junction with high angle was lowest in the control and highest in 5-HT depletion combination with CSD group (Figure 5-3, Figure 5-4).

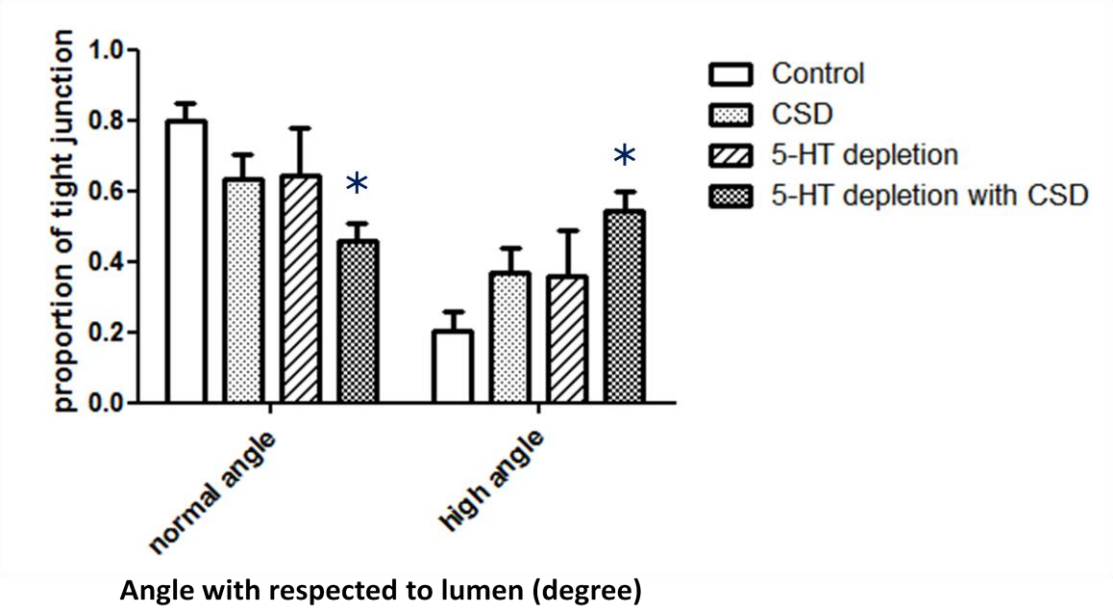

Figure 5-3 The bar graph shows the average of the proportion of endothelial tight junction alignment in normal and high angle obtained from the control, CSD, 5-HT depletion, and 5-HT depletion with CSD groups. The data are mean ± SEM (n=4). *P <0.05 compared with the control group.

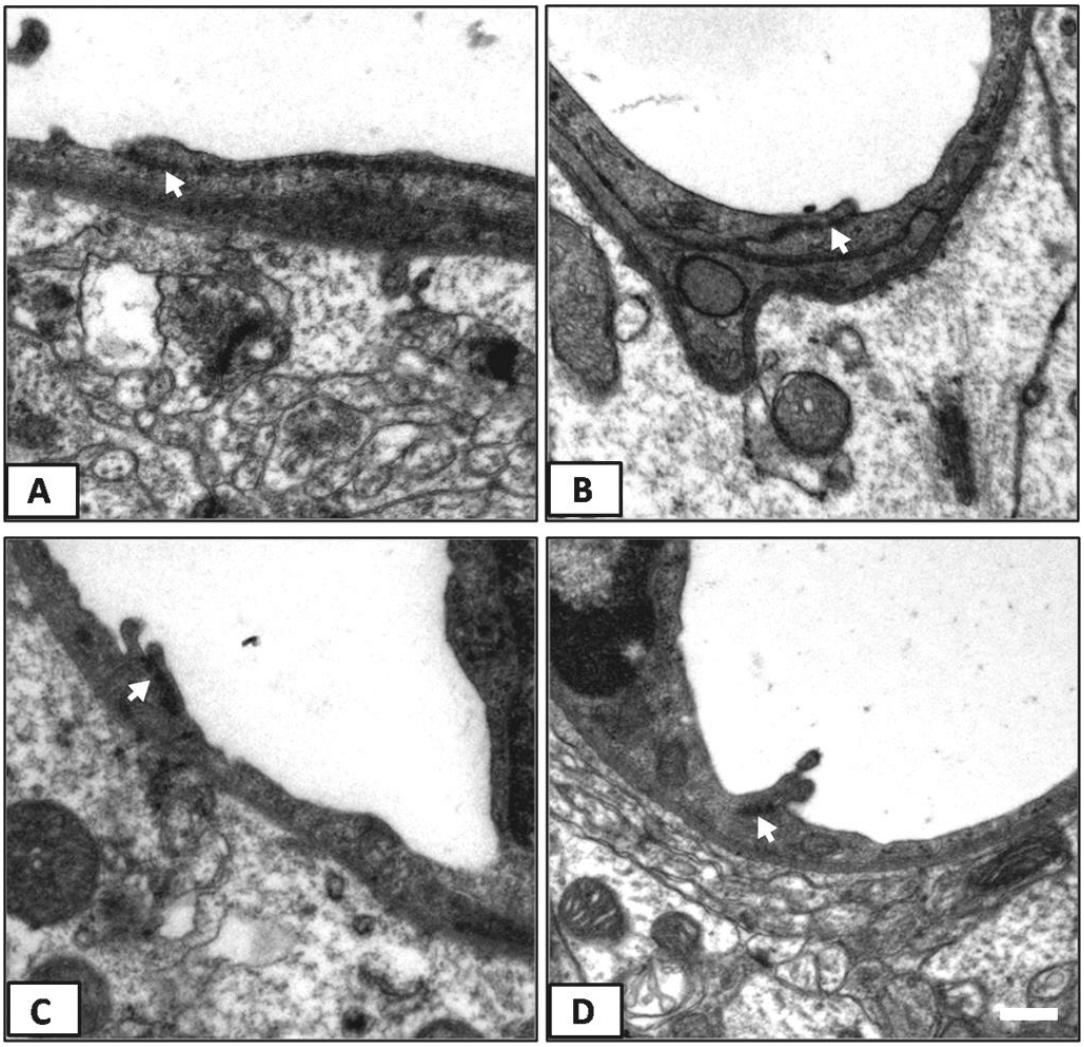

Figure 5-4 The electron micrographs show the alignment of the tight junction between endothelial cells obtained from the control (A), CSD (B), 5-HT depletion (C), and 5-HT depletion with CSD (D) groups. Bar= 300 nm

5.1.2 The effect of serotonin depletion on the CSD induced alteration of tight junction protein (occludin, ZO-1, and ZO-2)

A. Expression of occludin

The quantitative result from western blotting of the expression of occludin had demonstrated that this expression in CSD and 5-HT depletion group was lower than those observed in the control group, but the difference was not statistically significant. Interestingly, induction of CSD in the rat with a 5-HT depleted state resulted in the significant decrease of the expression of the occludin in the cerebral cortex as compared with those measured in the control group (Figure 5-5).

The immunohistochemical study of occludin expression in cerebral cortex demonstrated that occludin located along the microvessels. The qualitative assessment of occludin demonstrated the same pattern in the result as that obtained from western blot analysis. In the control group, an abundance of occludin positive vessels was widespread in the cerebral cortex. Either CSD induction or 5-HT depletion decreased the occludin expression in the cerebral cortex. Interestingly, the lower number of occludin positive vessel in the cerebral cortex obtained from the 5-HT depleted rat with CSD activation was clearly demonstrated (Figure 5-6).

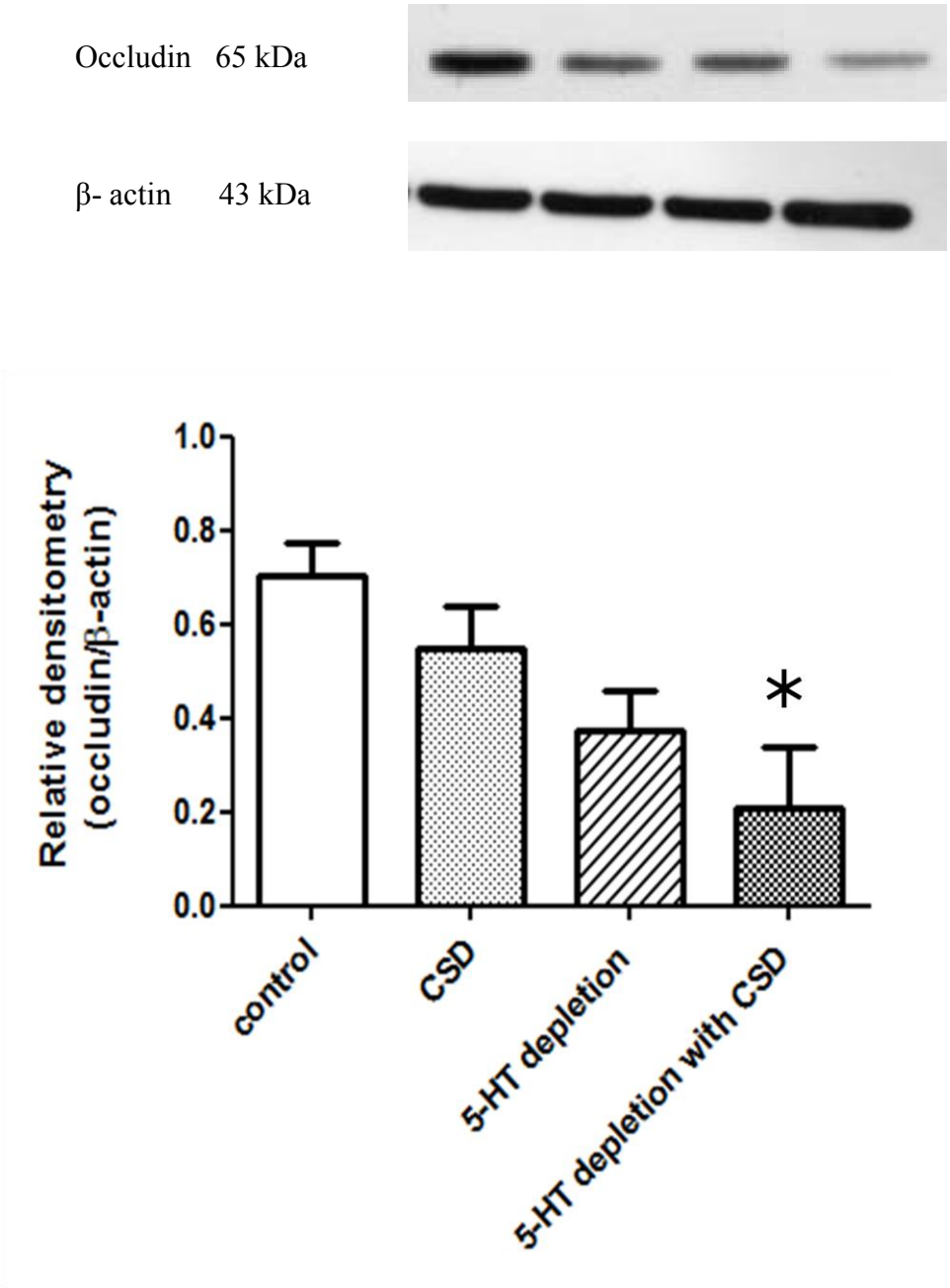

Figure 5-5 Effect of serotonin depletion on the CSD induced expression of occludin. The expression of occludin in the cerebral cortex obtained from all experimental groups was analyzed by western blotting. Quantitative data are expressed as a relative density to β-actin. The data are mean ± SEM (n=4). *P <0.05 compared with the control group.

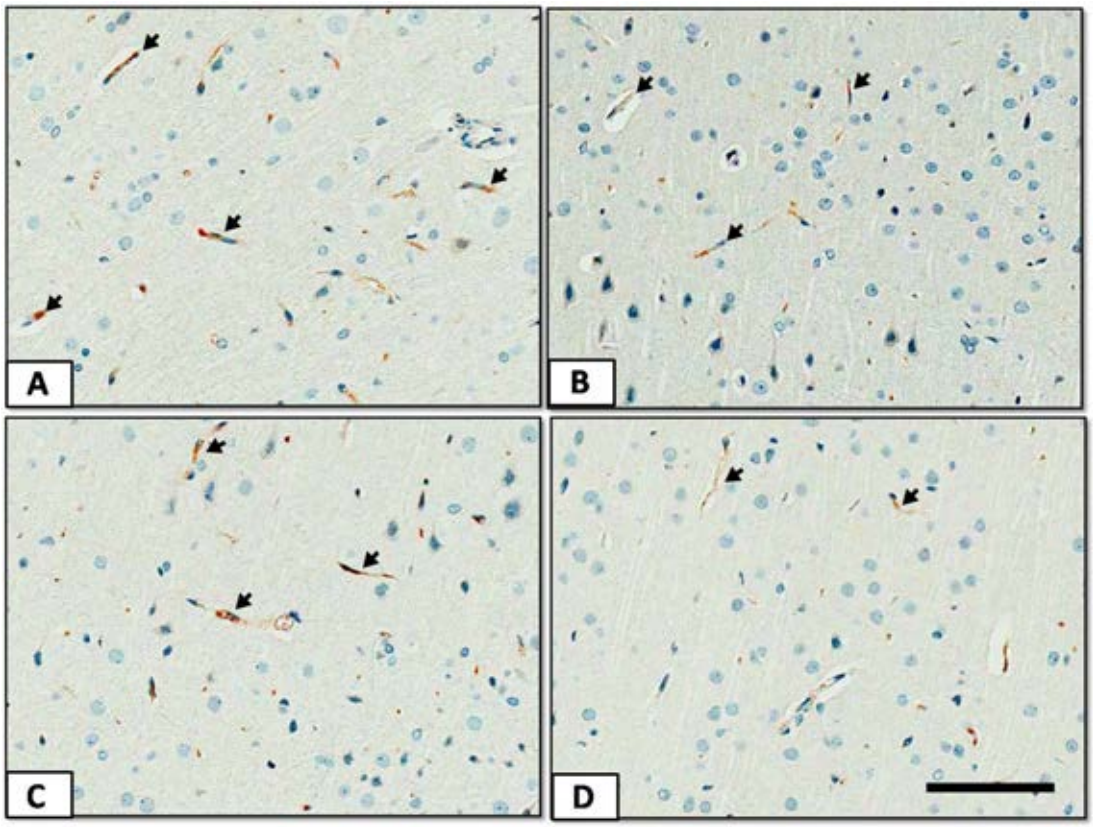

Figure 5-6 The photomicrographs show the expression of occludin immunopositive vessel in the cerebral cortex (ipsilateral to the KCl application) obtained from the control (A), CSD (B), 5-HT depletion (C), and 5-HT depletion with CSD (D) group. Bar = 100 μm

B. ZO-1 Expression

The expression of the TJ protein ZO-1 was detected in the cerebral cortex obtained from all experimental groups. When comparing the level of the expression in all four experimental groups, it was found that the level the level of ZO-1 expression was highest in the control group. The lowest level of ZO-1 expression was obtained from the 5-HT depletion with CSD group. However, all of the differences between groups did not reach statistical significance as shown in the data in Figure 5-7.

The immunostaining of ZO-1 in cerebral cortex shows the result with the same pattern as the result from western blot analysis. ZO-1 was detected along the microvessels similar to the pattern of occludin. Both CSD activation and 5-HT depletion in the normal state slightly decreased the number of ZO-1 positive vessels in the cerebral cortex. However, no significant difference between the levels of the expression of ZO-1 for all groups was observed (Figure 5-8).

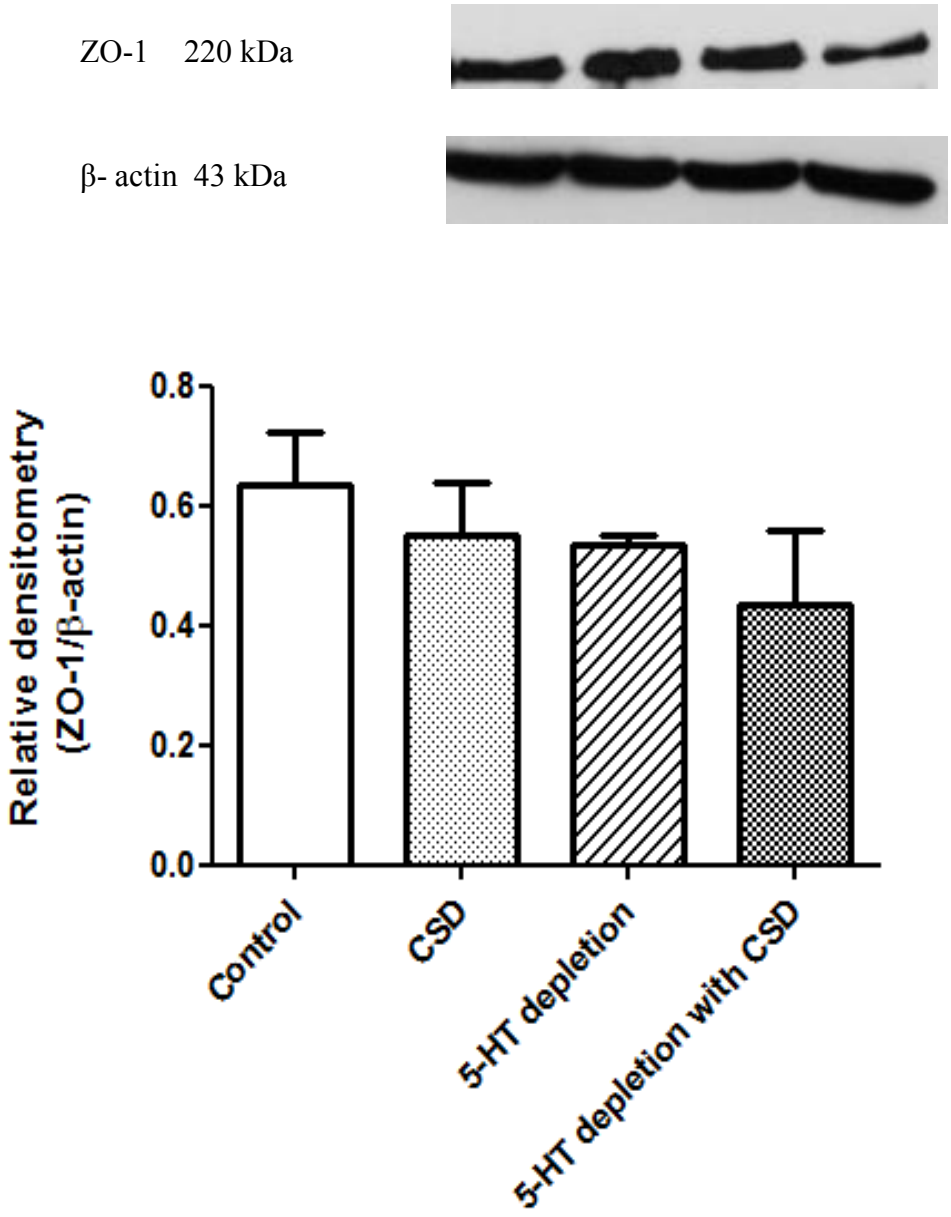

Figure 5-7 Effect of 5-HT depletion on the CSD induced expression of ZO-1. The ZO-1 expression in the cerebral cortex obtained from all experimental groups was analyzed by western blotting. Quantitative data are expressed as a relative density to β-actin. The data are mean ± SEM (n=4). (P>0.05)

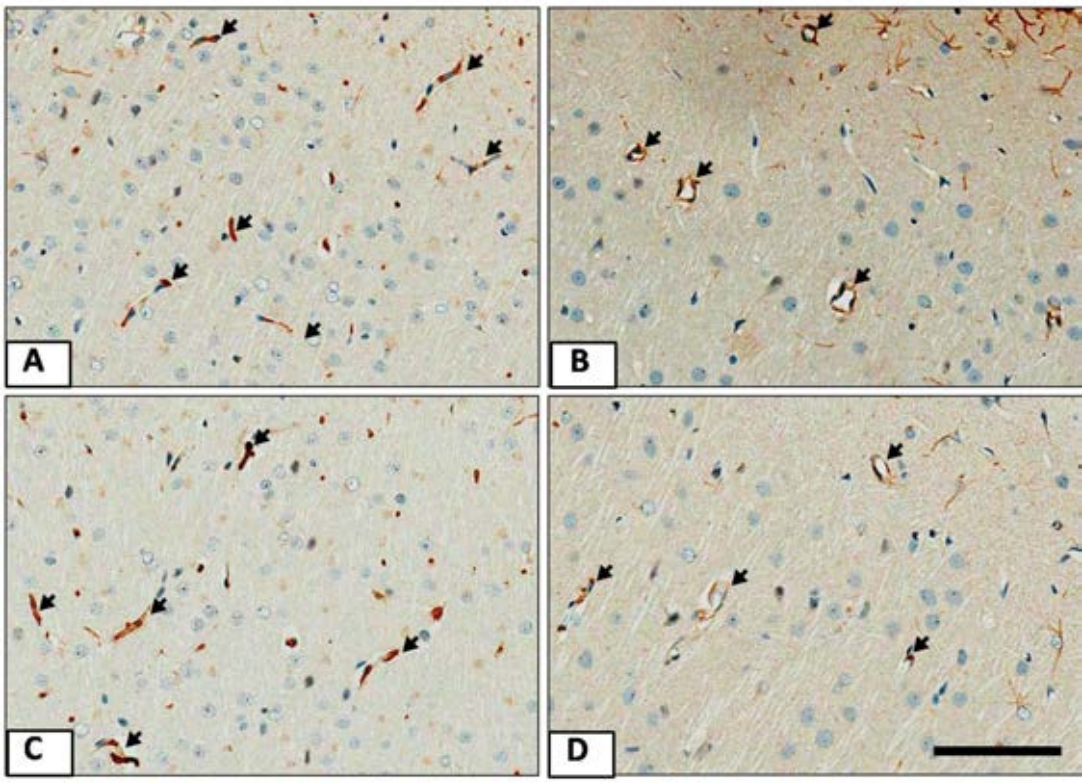

Figure 5-8 The photomicrograph show the expression of ZO-1 immunopositive vessels in the cerebral cortex (ipsilateral to the KCl application) obtained from the control (A), CSD (B), 5-HT depletion (C), and 5-HT depletion with CSD (D) group. Bar =100 μm

C. ZO-2 Expression

The expression of ZO-2 in the cerebral cortex was examined by western blotting. The quantitative result demonstrated that CSD activation or the 5-HT depletion alone had no effect on the ZO-2 expression in the cerebral cortex. The average relative density of ZO-2 to β-actin was 0.72 ± 0.033, 0.58 ± 0.032, and 0.67 ± 0.013 for control, CSD, and 5-HT depletion groups respectively. On the other hand, the integration of the 5-HT depleted state with CSD activation could significantly decrease the expression of ZO-2 as compared with the control group (the average density of ZO-1 was 0.72 ± 0.033 and 0.53 ± 0.047 for the control and 5-HT depletion with CSD activation group, respectively) (Figure 5-9).

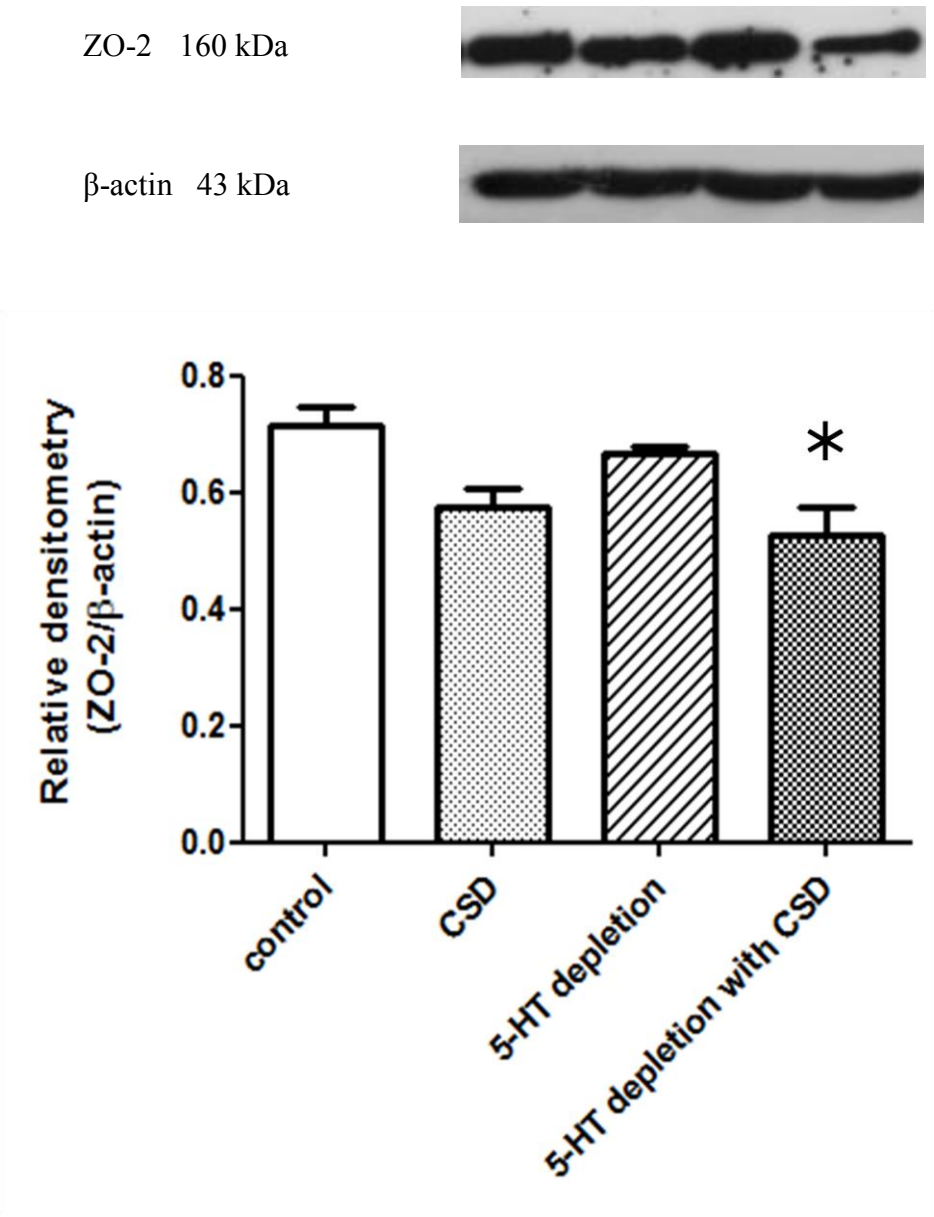

Figure 5-9 Effect of serotonin depletion on the CSD induced expression of ZO-2. The ZO-2 expression in the cerebral cortex obtained from all experimental groups was analyzed by western blotting. Quantitative data are expressed as a relative density to β-actin. The data are mean ± SEM (n=4). *P <0.05 compared with control group.

5.1.3 The effect of 5-HT depletion on the CSD induced IgG extravasation.

IgG extravasation is one marker which indicates the damage of the BBB integrity. IgG was determined by western blotting which can detect both the IgG heavy chain (65 kDa) and the light chain (25 kDa) protein. In this study, In order to detect the IgG extravasation, the expression of IgG light chain was measured in the cerebral cortex obtained from every experimental group. Our results demonstrated that in the rat with normal 5-HT level, CSD activation could significantly increase the level of IgG extravasation as compared with that in the control group. Depletion of 5-HT alone did not have any effect on the IgG level. Interestingly, in combination with CSD activation, the depletion of 5-HT could significant increase the IgG extravasation as compared with that in the control group. However, when comparing the level of IgG extravasation between the normal 5-HT rat and 5-HT depleted rat with CSD activation, no significant difference in the IgG level was observed. These results indicate that CSD activation can increase IgG extravasation which represents the disturbance of BBB integrity (Figure 5-10).

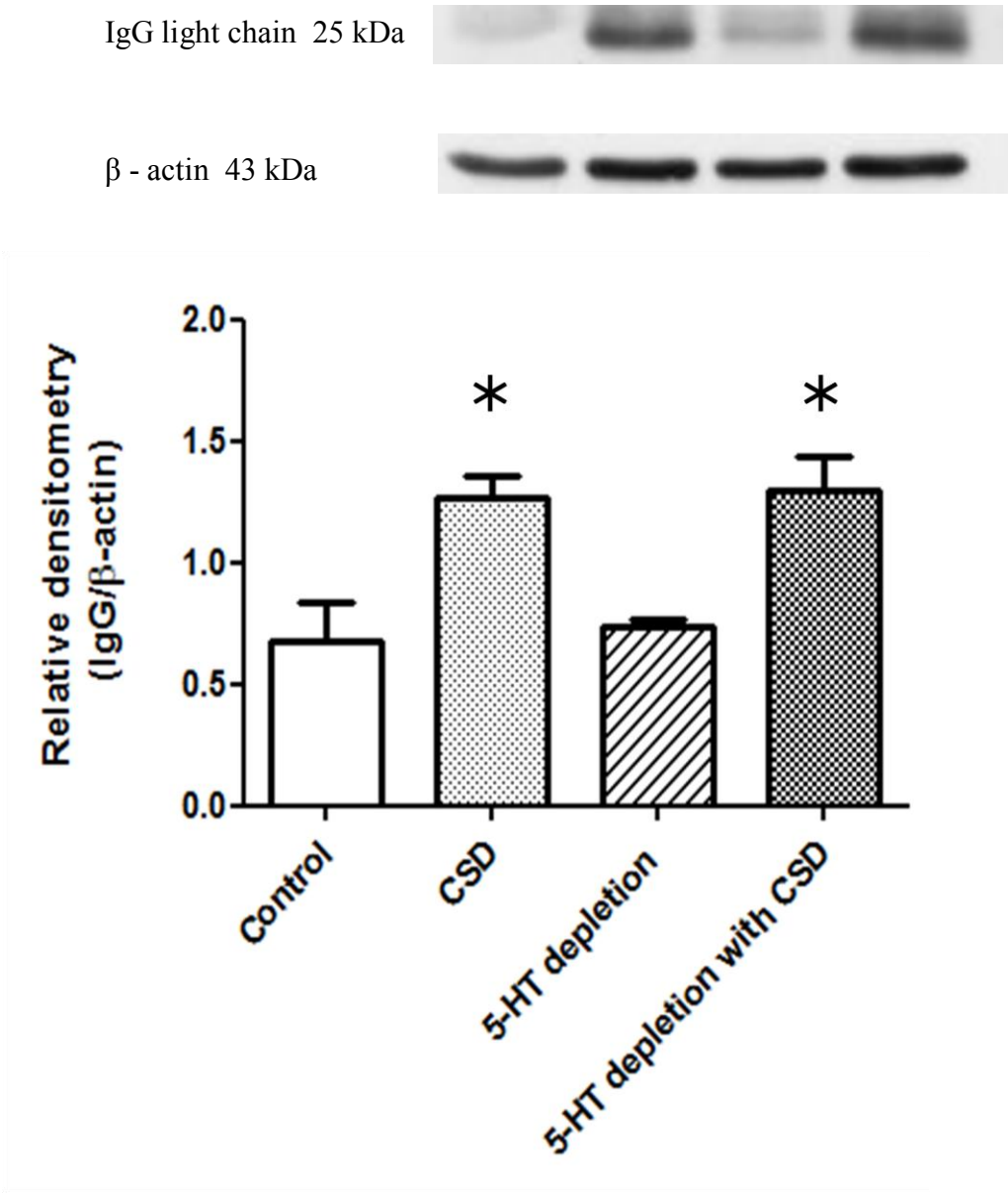

Figure 5-10 Effect of 5-HT depletion on the CSD induced IgG extravasation. The IgG light chain (25 kDa) expression in the cerebral cortex obtained from all experimental groups were analyzed by western blotting. Quantitative data are expressed as a relative density to β-actin. The data are mean ± SEM (n=4). [a]*P* <0.05 compared with the control group.

5.2 The effect of 5-HT depletion on the CSD induced neuropeptides expression of in TG.(Part: II)

In this experiment, all slides with TG sections were examined under the light microscope and scanned with a slide scanner (Aperio ScanScope, Aperio, Vista, CA) using computer software Aperio ImageScope version 12.0.0.5039 (Aperio, Vista, CA). 5 Areas (500 μm x 300 μm) were randomly selected from the TG section from every experimental rat. The results in this part are subdivided into two parts as follows:

5.2.1 The effect of 5-HT depletion on the CSD induced CGRP expression in TG.

The CGRP-immunoreactive (CGRP-IR) neurons were defined as those with either completely dark brown stains in their cytoplasm (homogenous pattern) or dark brown granules in their cytoplasm (granular pattern). The results of CGRP immunostaining had shown that most of CGRP-IR neurons were small to medium sized neurons. The total number of neurons and the number of CGRP-IR neurons were counted in 5 selected areas from each slide. Data were expressed as average number of mean and SEM of the percentage of CGRP-IR neurons per TG section (Tables 5-1, Figure 5-11).

We have found that CSD activation could significantly increase the percentage of CGRP-IR neurons in the TG as compared with those in the control group. The depletion of 5-HT without CSD activation had no effect on the CGRP expression. The number of CGRP-IR neurons in this group was not different from control group. However, the depletion of 5-HT in combination with CSD activation could significantly increase the number of CGRP –IR neurons as compared with CSD and control groups. All of data is shown in Table 5.1 and Figure 5-12.

Table 5.1 The effect of 5-HT depletion on the CSD induced CGRP expression in TG.

Variables	percentage of -CGRP IR neuron
Control	26.18 ± 1.84
CSD	35.53 ± 0.44 *
5-HT depletion	34.36 ± 1.20
5-HT depletion with CSD	44.24 ± 2.43 *#δ

The data are expressed as the mean ± SEM

* $P<0.05$ compared with control group

# $P<0.05$ compared with CSD group

δ $P<0.05$ compared with 5-HT depletion group

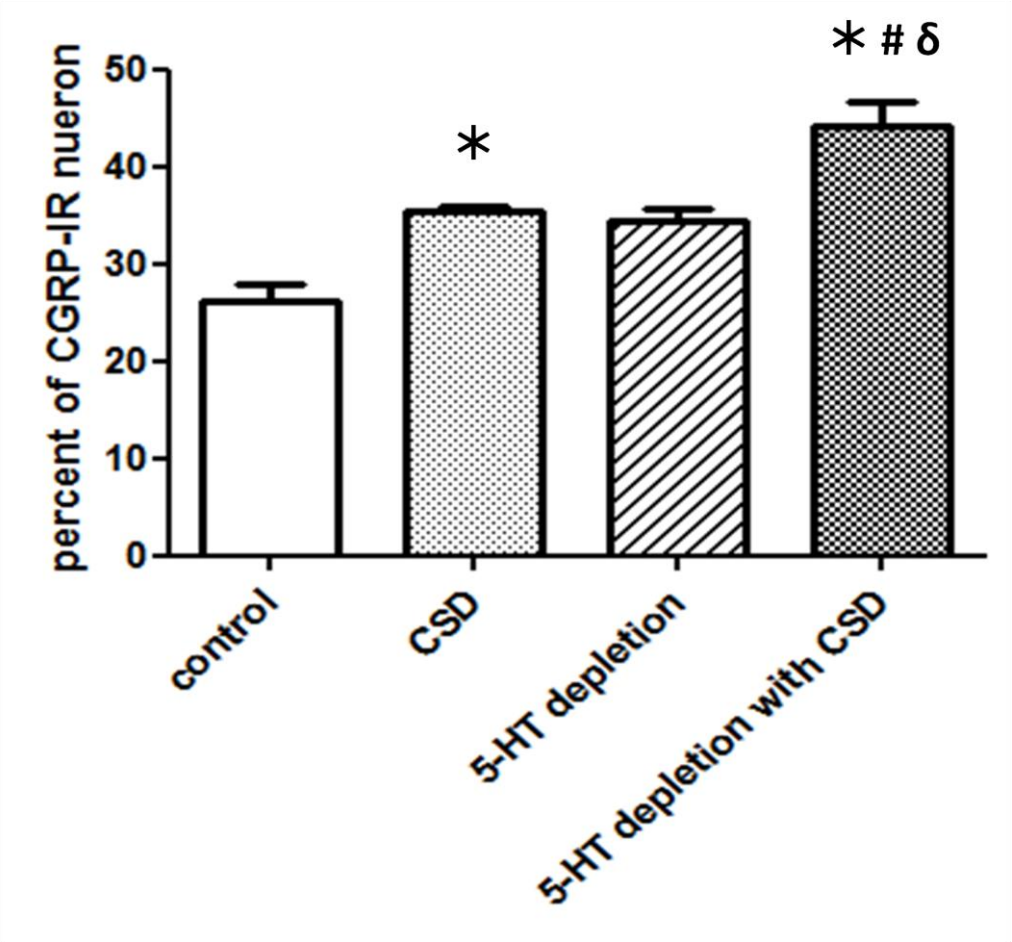

Figure 5-11 The bar graph shows the mean value ± SEM of the percentage of CGRP-IR neurons in the TG sections obtained from the control, CSD, 5-HT depletion, and 5-HT depletion with CSD group. The data are mean ± SEM (n=4). [a] *P*<0.05 compared with the control group. [b] *P*<0.05 compared with the CSD group. [C] *P*<0.05 compared with the 5-HT depletion group.

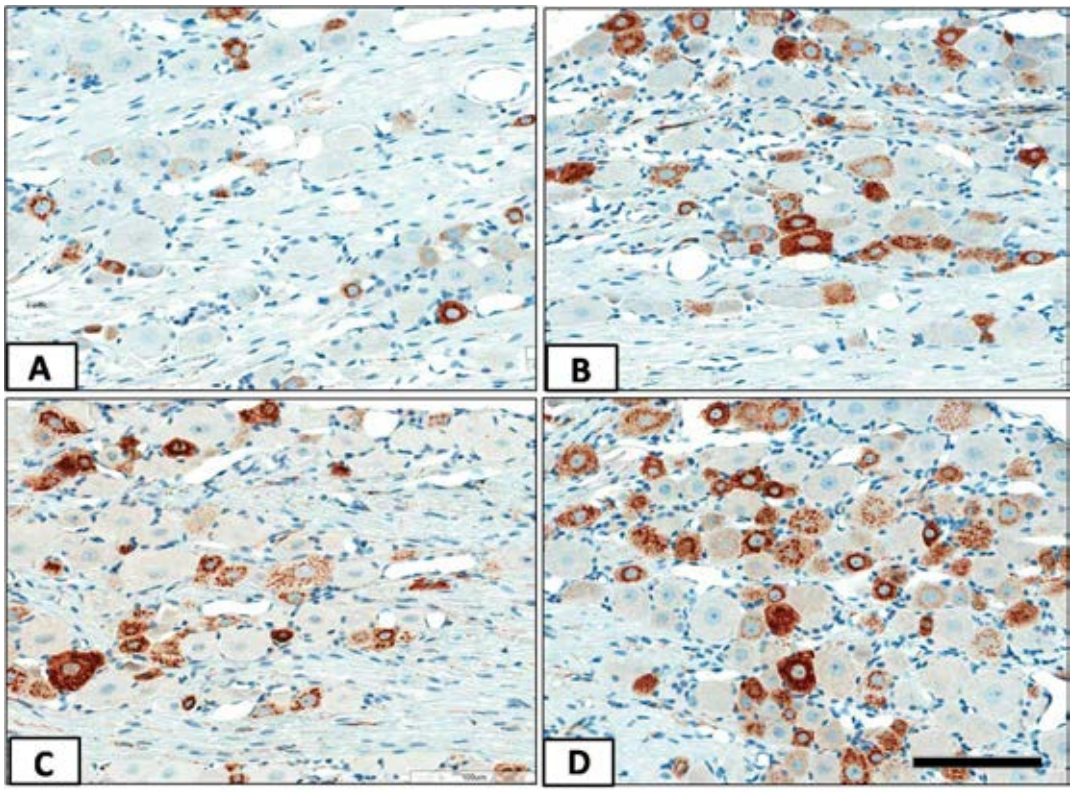

Figure 5-12 The photomicrographs show the CGRP-IR neurons in the TG section (ipsilateral to the KCl application) obtained from the control (A), CSD (B), 5-HT depletion (C), and 5-HT depletion with CSD (D) groups. Bar = 100 μm

5.2.2 The effect of 5-HT depletion on the CSD induced SP expression in TG.

The substance P-immunoreactive (SP-IR) neurons were defined as the neurons with dark brown stains in their cytoplasm. The majority of SP-IR neurons were neurons of small to medium size. The large diameter neurons were usually SP-IR negative. The total number of neurons and the number of SP-IR neurons were counted from each slide. Data were expressed as mean and SEM of the percentage of SP-IR neurons per TG section. (Table 5.2 Figure 5-13)

Substance P immunohistochemistry showed that neither 5-HT depletion nor CSD activation had effect on the SP expression in TG. However, the depletion of 5-HT in the combination with CSD activation could significantly increase the percentage of SP-IR neurons in the TG as compared with the control group (Figure 5-13, Figure 5-14).

Based on these results it can be concluded that with the CSD activation, the depletion of 5-HT can enhance the expression of neuropeptides, CGRP and SP in TG as compared with those observed in rats with a normal level of 5-HT.

Table 5.2 The effect of 5-HT depletion on the CSD induced SP expression in TG.

Variables	percentage of SP-IR neuron
Control	14.82 ± 2.05
CSD	18.83 ± 2.33
5-HT depletion	22.89 ± 4.91
5-HT depletion with CSD	37.55 ± 2.02 $^{*\,\#\,\delta}$

The data are expressed as the mean ± SEM

$^{*}P$ <0.01 compared with the control group

$^{\#}P$ <0.01 compared with the CSD group

$^{\delta}P$ <0.05 compared with the 5-HT depletion group

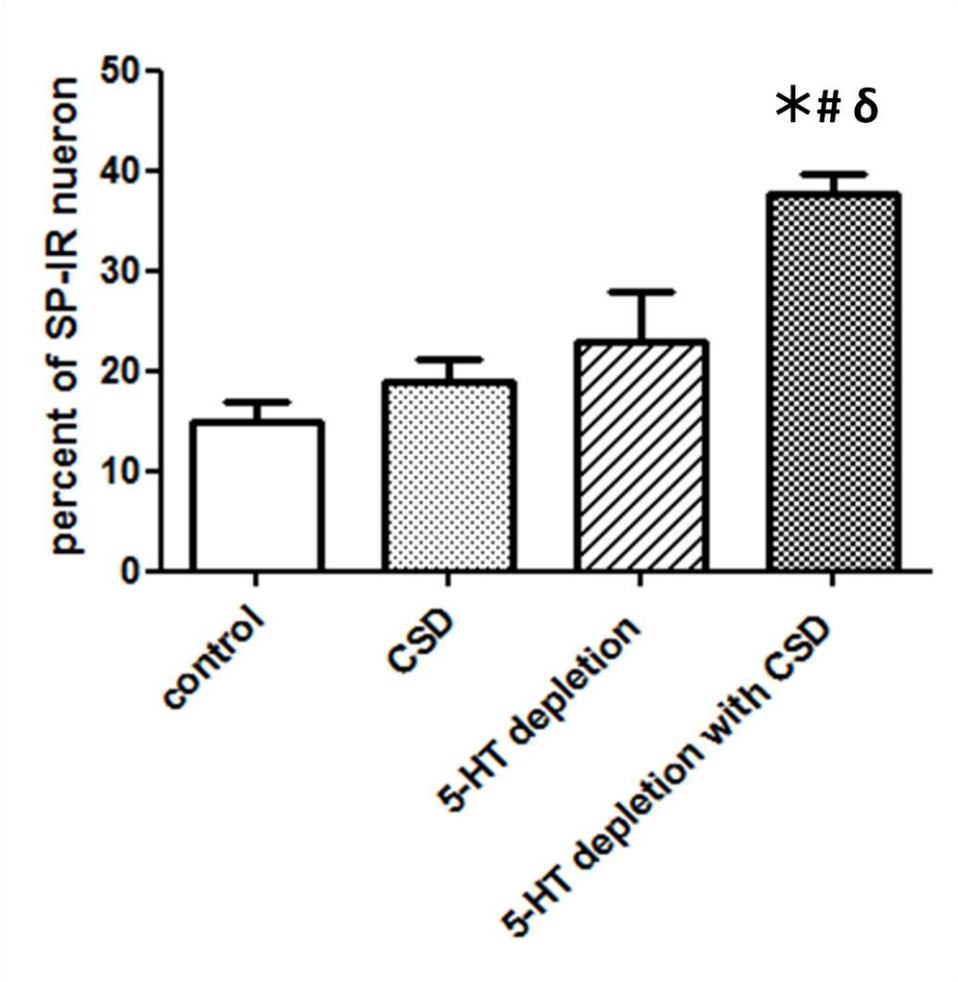

Figure 5-13 The bar graph shows the mean value ± SEM of the percentage of SP-IR neurons in the TG sections obtained from the control, CSD, 5-HT depletion, and 5-HT depletion with the CSD group. The data are mean ± SEM (n=4). *P<0.01 compared with the control group. $^{\#}P$<0.01 compared with the CSD group. $^{\delta}P$<0.05 compared with the 5-HT depletion group.

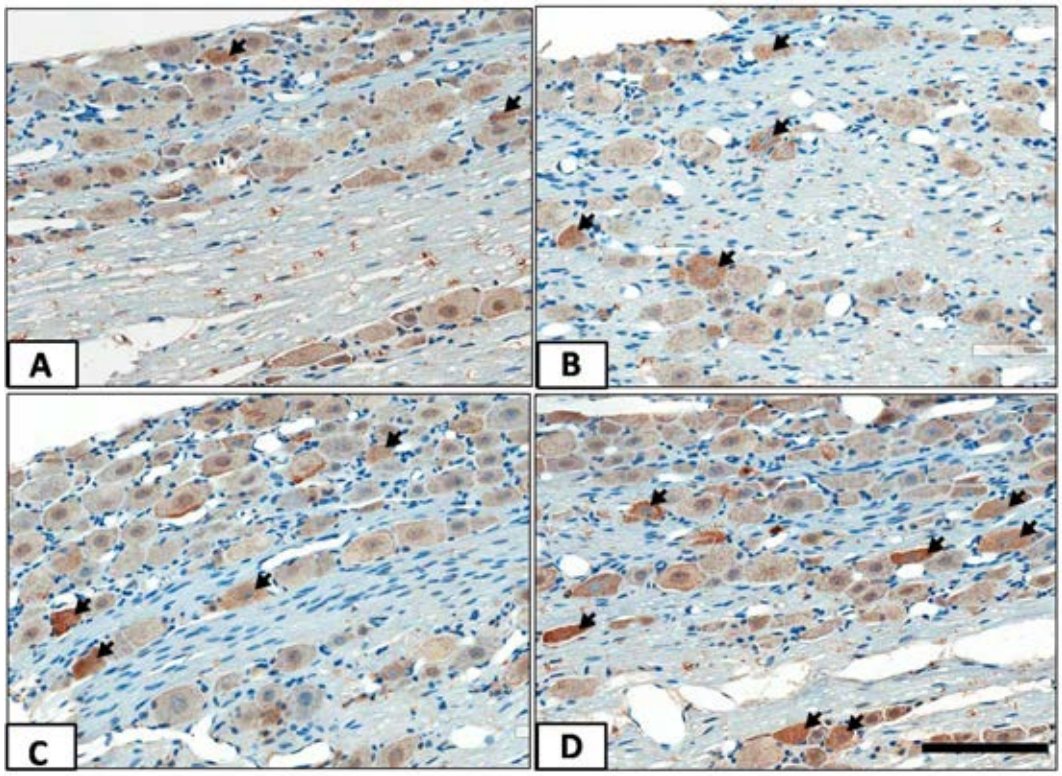

Figure 5-14 The photomicrographs show the SP-IR neurons in the TG section (ipsilateral to the KCl application) obtained from the control (A), CSD (B), 5-HT depletion (C), and 5-HT depletion with the CSD (D) group. Bar = 100 μm

CHAPTER VI

DISCUSSION

The present results demonstrate that the depletion of 5-HT by PCPA injection can induce an increase in the CSD, which in turn induces the alteration the BBB integrity. These abnormalities are demonstrated in parallel with an increase in CGRP and SP expression in TG.

The depletion of 5-HT is recognized as an important factor involved in migraine pathophysiology. Migraine is a neurovascular disorder which has a great impact on public health due to the disability the patient during a migraine attack. Its pathogenesis is tightly associated with the activation of the trigeminovascular system (6) which can result in both neural and vascular responses. In neural compartment, the excitation of cortical neurons can induce several neurotransmitters released from the perivascular nerve terminal such as NO, CGRP, and SP (7). Those vasoactive neurotransmitters can induce the neurogenic inflammation around the cerebral vessels and cause a vasodilatation. The neurogenic inflammation as well as the distension of cerebral vessels can either activate or sensitize the perivascular neurons leading to an increased release of vasoactive neuropeptide and finally can damage the BBB integrity (6,9). Studies in the CSD headache animal model had demonstrated that the induction of CSD can induce the leakage of plasma proteins out from the cerebral vessels, and the increase in the BBB permeability might relate to the up-regulation of the MMP-9, which is a proteolytic enzyme, which has a function to breakdown extracellular matrix after activation (10,180).

Our present study in the CSD animal model has confirmed the previous hypothesis, all parameters indicating the abnormality of BBB integrity are significant increased in CSD group as compared with control group. Interestingly, we have also found that the disruption of BBB integrity induced by CSD activation in the 5-HT depletion group is more prominent than those observed in CSD activation in normal 5-HT.

In this study, the increase in the ultrastructural alterations of the endothelial cell induced by CSD was demonstrated in the 5-HT depletion group. The number of microvilli and pinocytic vesicles and the swelling of perivascular astrocytic footplate were higher in the5-HT depleted group as compared with the CSD group. The study of the alteration of tight junction protein after CSD activation also indicated an increase of BBB permeability in the5-HT depleted state while no significant change in the level of ZO-1 was observed, the occludin and ZO-2, important tight junction proteins, were significantly decreased in 5-HT depletion as compared with the normal 5-HT group. These results indicate that with the CSD activation the depletion of 5-HT can result in the increment of the abnormality of the endothelial cell and BBB disruption compared with those observed in normal 5-HT.

Occludin is the most dominant of all tight junction proteins which plays an important role in the barrier function of the BBB. Accumulative data have indicated that the alteration of occludin leads to BBB disruption in several diseases. Studies in inflammatory pain models have demonstrated that even though no change in the level of ZO-1 present, the decrease in the occludin level was observed in parallel with the increase of BBB permeability (60,181). In addition, Witt and his colleague had demonstrated that induction of hypoxia-reoxygenation, which can induce the rearrangement of tight junction structure, could increase the paracellular diffusion and alteration of occludin expression but had no effect on claudin-3 and ZO-1 (182). Based on these accumulative evidences, the decrease in the ZO-2 and occludin observed in the 5-HT depleted group can suggest that the BBB integrity of the cerebral vessels obtained from this group are more sensitive to the CSD activation than those obtained from normal 5-HT. In this study, increased alteration in the BBB integrity was observed in the animal with 5-HT depletion.

Regarding the BBB structure, the alteration in the tight junction alignment is one of the important markers of BBB disruption. The results obtained from several studies have indicated that in the physiological condition, the tight junction alignment between the cerebral endothelial cells is mostly in the parallel plane with the vessel lumen. With this alignment, the sealing between endothelial cells by tight junction is

tightest. In the pathophysiological condition, the change in the alignment of tight junction had been observed. The higher angle of tight junction alignment to the lumen was, the more increase in permeability of cerebral vessel was observed (183,184). In this study, the observation of the alignment of tight junctions by electron microscope did confirm the result from western blot analysis. The CSD activation could significantly reduce the proportion of tight junction with a normal angle as well as increase the proportion of tight junction with a high angle in the5-HT depleted rat as compared with those observed in the control group.

In addition, the swelling of the astrocyte footplate which is a significant marker for indicating the BBB disruption was prominent in the 5-HT depletion as well. The perivascular endfoot of astrocytes which is closely attached to the microvessel wall is another important structure for maintaining the BBB integrity. The footplate swelling and detachment of, astrocyte, which is revealed by using the transmission electron microscope is recognized as a marker of BBB disruption and brain edema in ischemic models (184,185). The study in the astrocytic endfoot revealed that plenty of water channel aquaporin-4(AQP4) and the K^+ channel, which are the channel involve in ionic and water regulation across the BBB, were demonstrated on those end feet (34). Several studies had indicated that the up-regulation of AQP4 is involved in the astrocyte footplate swelling and BBB disruption (186–188) Based on the endothelial ultrastructural alterations together with the decrease in the tight junction proteins observed in 5-HT depletion with CSD activation group, we can hypothesize here that the CSD activation in 5-HT depletion can induce the increment of BBB permeability more than those observed in the control group.

However, study of the IgG extravasation, the parameter indicating the leakage of plasma protein into the brain parenchyma, could not confirm this hypothesis. The CSD activation could significantly increase the IgG extravasation as compared with the control group. However, there is no significant difference when comparing the CSD induced IgG extravasation between the normal 5-HT and the 5-HT depletion.

Concerning the distribution of the 5-HT receptor in the brain, the receptors are widely distributed on both vascular and neural compartments. The localization of the 5-HT_{1B} receptor on the cerebral vessels has been reported (189). Besides, several studies have demonstrated that the 5-HT_{1B} and 5-HT_{1D} receptors have been observed at the presynaptic nerve terminal including the perivascular nerve terminal. Since these two receptors are G (G_i) protein couple receptors, binding with these receptors will result in the decrease of cAMP (146,147,190), therefore the 5-HT is known as a neurotransmitter which can modulate in both vascular and neural responses. At a vascular response, binding with its receptor can result in the vasoconstriction effect (133). While binding with its receptor at the presynaptic site can result in the decrease release of neurotransmittors (including SP, CGRP and NO) from nerve terminal (130). 1992). Thus, in the condition with low 5-HT, all inhibitory actions from 5-HT are decreased which results in the increase of several vasoactive neurotransmitters released from those nerve terminals.

We have earlier demonstrated that the production of NO was increased in the condition with 5-HT depletion (191). NO is well known as a gas molecule which at a high concentration can damage the BBB integrity via the $ONOO^-$ formation (192). Thus, the increment of the NO production in the brain due to the depletion of 5-HT is at least one mechanism underlying the alteration of the BBB observed in this study. However, the involvement of SP and CGRP, two potent vasoactive neurotransmitters released from the trigeminal ganglion, has been considered in this study as well.

It has been known that CSD activation can activate the trigeminal neurons at the trigeminal ganglion to induce the release of the vasoactive compounds such as NO, VIP, CGRP, and SP from the nerve terminal at the perivascular space (112,193). The results from this study are in agreement with this hypothesis. The CSD activation could enhance an increase in the CGRP and SP production in TG. Interestingly, we have discovered that the CSD activation in the 5-HT depleted state did enhance the expression of both neuropeptides in TG. The number of both CGRP and SP-IR neurons in TG had significantly increased in 5-HT depletion as compared with the

normal 5-HT state. 5-HT receptors are widely distributed throughout the body in both the neuronal and vascular compartment. In TG, it has been demonstrated by the studies in both human and rodent that the 5-HT_1receptor is distributed in the trigeminal ganglion neurons. Using the immunofluorescent technique, co-localization of 5-HT_{1B} and 5-HT_{1D} receptor with CGRP, SP and NOS immunoreactive neurons in both human and rat trigeminal ganglion (194,195). Since the $5\text{-HT}_{1B/1D}$ receptor is a G protein couple (G_i) receptor, these results indicate that 5-HT can modulate the release of those neuropeptides and the activation of those trigeminal neurons by the activation of 5-HT_1 receptor on those trigeminal ganglion neurons. In the condition with a low level of 5-HT, the inhibitory control of 5-HT via the 5-HT_1 receptor activation is decreased, This phenomenon can lead to the hyperexcitation of the trigeminal neurons and result in an increase in the expression of CGRP and SP in TG, which can finally lead to the excessive release of vasoactive neuropeptide at the perivascular area. The accumulation of those neuropeptides can lead to more neurogenic inflammation and finally damage to the BBB integrity.

The schematic diagrams explaining the difference between the activation of trigeminovascular system by CSD in normal 5-HT level and 5-HT depleted state are demonstrated in Figure 6-1 and Figure 6-2, respectively.

Based on these findings, we can conclude that 5-HT depletion affects the instability of the BBB. A decrease in the modulation of 5-HT can lead to a massive release and accumulation of CGRP and SP after CSD activation. In combination with a decrease in the vasoconstriction of vessels, CGRP and SP could increase the dilation of the cerebral vessels and neurogenic inflammation around the blood vessels, which further enhance the BBB alteration. These phenomena can finally result in the damage of BBB.

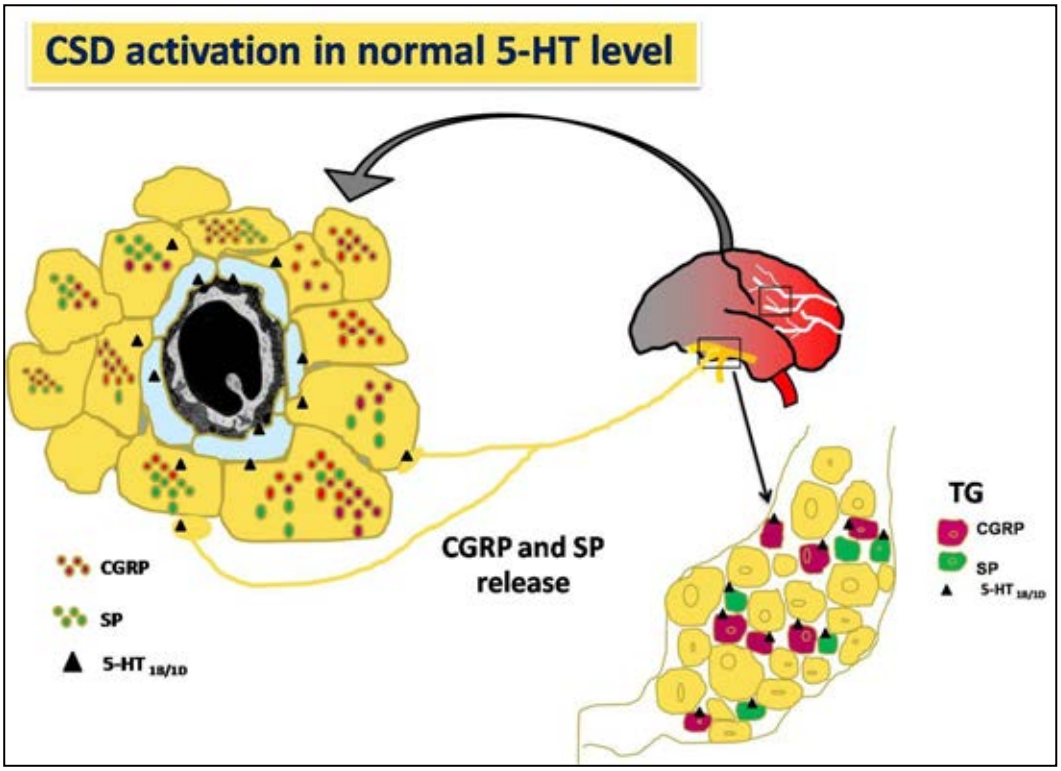

Figure 6-1 Schematic diagram of trigeminovascular system activation by CSD in the condition with normal 5-HT level.

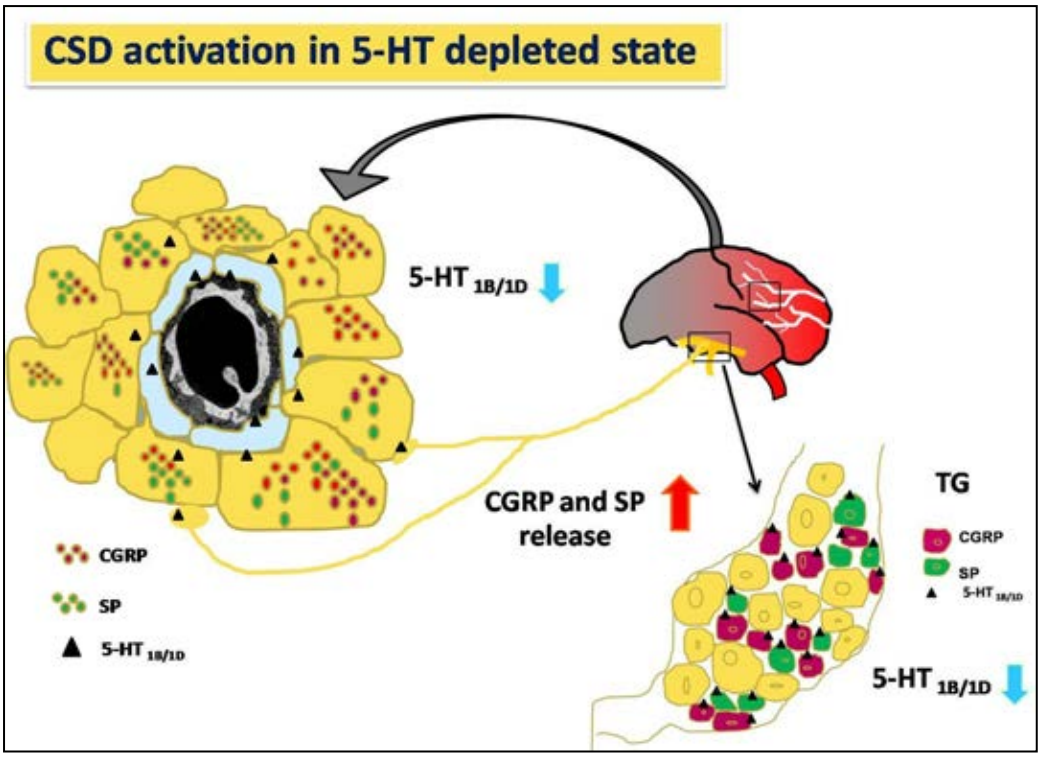

Figure 6-2 Schematic diagram of trigeminovascular system activation by CSD in the condition with 5-HT depletion.

CHAPTER VII

CONCLUSION

The results obtained from this study demonstrated that the depletion of 5-HT could induce an increase in the CSD-induced alteration of the BBB integrity. Those abnormalities were demonstrated in parallel with an increase of CGRP and SP expression in TG. It can be suggested that the depletion of 5-HT alters the stability of the BBB. In combination with CSD activation, the BBB in the condition with 5-HT depletion can be more damaging than in abnormal 5-HT condition. Since the 5-HT depletion is tightly associated with migraine, our results can suggest that a low level of 5-HT can be one explanation for a high risk of cerebrovascular disease in migraine patients.

REFERENCES

(1) Rolfe, D.F., and Brown, G.C. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol. Rev. 77 (1997) : 731–758.

(2) Hawkins, B.T., and Davis, T.P. The blood-brain barrier/neurovascular unit in health and disease. Pharmacol. Rev. 57 (2005) : 173–185.

(3) Cardoso, F.L., Brites, D., and Brito, M.A. Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches. Brain Res Rev. 64 (2010) : 328–363.

(4) Correale, J., and Villa, A. Cellular elements of the blood-brain barrier. Neurochem. Res. 34 (2009) : 2067–2077.

(5) Persidsky, Y., Ramirez, S.H., Haorah, J., and Kanmogne, G.D. Blood-brain barrier: structural components and function under physiologic and pathologic conditions. J Neuroimmune Pharmacol. 1 (2006) : 223–236.

(6) Edvinsson, L., Villalón, C.M., and MaassenVanDenBrink A. Basic mechanisms of migraine and its acute treatment. Pharmacol. Ther. 136 (2012) : 319–333.

(7) Just, S., Arndt, K., Weiser, T., and Doods, H. Pathophysiology of migraine: A role for neuropeptides. Drug Discovery Today: Disease Mechanisms. 3 (2006) : 327–333.

(8) Lauritzen, M. Pathophysiology of the migraine aura. The spreading depression theory. Brain. 117 (1994) : 199–210.

(9) Wang, H.H., Hsieh, H.L., and Yang, C.M. Nitric oxide production by endothelin-1 enhances astrocytic migration via the tyrosine nitration of matrix metalloproteinase-9. J. Cell. Physiol. 226 (2011) : 2244–2256.

(10) Gursoy-Ozdemir, Y., et al. Cortical spreading depression activates and upregulates MMP-9. J. Clin. Invest. 113 (2004) : 1447–1455.

(11) Gupta, V.K. CSD, BBB and MMP-9 elevations: animal experiments versus clinical phenomena in migraine. Expert Rev Neurother. 9 (2009) : 1595–1614.

(12) Rotstein, D.L., Aviv, R.I., and Murray, B.J. Migraine with aura associated with unilateral cortical increase in vascular permeability. Cephalalgia. 32 (2012) : 1216–1219.

(13) Kurth, T., Chabriat, H., and Bousser, M.G. Migraine and stroke: a complex association with clinical implications. Lancet Neurol. 11 (2012) : 92–100.

(14) Caminero, A.B., and Sánchez Del Río González, M. [Migraine as a cerebrovascular risk factor]. Neurologia. 27 (2012) : 103–111.

(15) Sicuteri, F., Testi, A., and Anselmi, B. Biochemical Investigations in Headache: Increase in the Hydroxyindoleacetic Acid Excretion During Migraine Attacks. Int Arch Allergy Immunol. 19 (1961) : 55–58.

(16) Drummond, P.D. Tryptophan depletion increases nausea, headache and photophobia in migraine sufferers. Cephalalgia. 26 (2006) : 1225–1233.

(17) Schuh-Hofer, S., et al. Increased serotonin transporter availability in the brainstem of migraineurs. J. Neurol. 254 (2007) : 789–796.

(18) Chugani, D.C., et al. Increased brain serotonin synthesis in migraine. Neurology. 53 (1999) : 1473–1479.

(19) Supornsilpchai, W., Sanguanrangsirikul, S., Maneesri, S., and Srikiatkhachorn, A. Serotonin depletion, cortical spreading depression, and trigeminal nociception. Headache. 46 (2006) : 34–39.

(20) Wolburg, H., and Lippoldt, A. Tight junctions of the blood-brain barrier: development, composition and regulation. Vascul. Pharmacol. 38 (2002) : 323–337.

(21) Zlokovic, B.V. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron. 57 (2008) : 178–201.

(22) Reese, T.S., and Karnovsky, M.J.. Fine structural localization of a blood-brain barrier to exogenous peroxidase. J. Cell Biol. 34 (1967) : 207–217.

(23) Fenstermacher, J., Gross, P., Sposito, N., Acuff, V., Pettersen, S., and Gruber, K. Structural and functional variations in capillary systems within the brain. Ann. N. Y. Acad. 529 (1988) : 21–30.

(24) Oldendorf, W.H., Cornford, M.E., and Brown, W.J. The large apparent work capability of the blood-brain barrier: a study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat. Ann. Neurol. 1 (1977) : 409–417.

(25) Sedlakova, R., Shivers, R.R., and Del Maestro, R.F. Ultrastructure of the blood- brain barrier in the rabbit. J. Submicrosc. Cytol. Pathol. 31(1999) : 149–161.

(26) Kniesel, U., and Wolburg, H. Tight junctions of the blood-brain barrier. Cell. Mol. Neurobiol. 20 (2000) : 57–76.

(27) Tagami, M., Nara, Y., Kubota, A., Fujino, H., and Yamori, Y. Ultrastructural changes in cerebral pericytes and astrocytes of stroke-prone spontaneously hypertensive rats. Stroke. 21 (1990) : 1064–1071.

(28) Farkas, E., and Luiten, P.G. Cerebral microvascular pathology in aging and Alzheimer's disease. Prog. Neurobiol. 64 (2001) : 575–611.

(29) Vorbrodt, A.W., and Dobrogowska, D.H. Molecular anatomy of intercellular junctions in brain endothelial and epithelial barriers: electron microscopist's view. Brain Res. Brain Res. Rev. 42 (2003) : 221–242.

(30) Brightman, M.W., and Reese, T.S. Junctions between intimately apposed cell membranes in the vertebrate brain. J. Cell Biol. 40 (1969) : 648–677.

(31) Lane, N.J., Reese, T.S., and Kachar, B. Structural domains of the tight junctional intramembrane fibrils. Tissue Cell. 24(1992) : 291–300.

(32) Dorovini-Zis, K., Prameya, R., and Bowman, P.D. Culture and characterization of microvascular endothelial cells derived from human brain. Lab. Invest. 64 (1991) : 425–436.

(33) Peters, A., Palay, S.L., and Webster, H.D. The fine structure of the nervous system: Neurons and their supporting cells. (3ed.) New York : Oxford University Press; 1990.

(34) Abbott, N.J., Rönnbäck, L., and Hansson, E. Astrocyte-endothelial interactions at the blood-brain barrier. Nat. Rev. Neurosci. 7 (2006) : 41–53.

(35) Tao-Cheng, J.H., Nagy, Z., and Brightman MW. Tight junctions of brain endothelium in vitro are enhanced by astroglia. J. Neurosci. 7 (1987) : 3293–3299.

(36) Tao-Cheng, J.H., and Brightman, M.W. Development of membrane interactions between brain endothelial cells and astrocytes in vitro. Int. J. Dev. Neurosci. 6 (1988) : 25–37.

(37) Willis, C.L., Leach, L., Clarke, G.J., Nolan, C.C., and Ray, D.E. Reversible disruption of tight junction complexes in the rat blood-brain barrier, following transitory focal astrocyte loss. Glia. 48 (2004) : 1–13.

(38) Bandopadhyay, R., Orte, C., Lawrenson, J.G., Reid, A.R., De Silva, S., and Allt, G. Contractile proteins in pericytes at the blood-brain and blood-retinal barriers. J. Neurocytol. 30 (2001) : 35–44.

(39) Lai, C.H, and Kuo K.H. The critical component to establish in vitro BBB model: Pericyte. Brain Research Reviews. 50 (2005) : 258–265.

(40) Ramsauer, M., Krause, D., and Dermietzel, R. Angiogenesis of the blood-brain barrier in vitro and the function of cerebral pericytes. FASEB J. 16 (2002) : 1274–1276.

(41) Gonul, E., Duz, B., Kahraman, S., Kayali, H., Kubar, A., and Timurkaynak, E. Early pericyte response to brain hypoxia in cats: an ultrastructural study. Microvasc. Res. 64 (2002) : 116–119.

(42) Dore-Duffy, P., Owen, C., Balabanov, R., Murphy, S., Beaumont, T., and Rafols, J.A. Pericyte migration from the vascular wall in response to traumatic brain injury. Microvasc. Res. 60 (2000) : 55–69.

(43) Fabriek, B.O., et al. CD163-positive perivascular macrophages in the human CNS express molecules for antigen recognition and presentation. Glia. 51 (2005) : 297–305.

(44) Choi, Y.K., and Kim, K.W. Blood-neural barrier: its diversity and coordinated cell-to-cell communication. BMB Rep. 41 (2008) : 345–352.

(45) Buxton, R.B., and Frank, L.R. A model for the coupling between cerebral blood flow and oxygen metabolism during neural stimulation. J. Cereb. Blood Flow Metab. 17(1997) : 64–72.

(46) Petty, M.A., and Wettstein, J.G. Elements of cerebral microvascular ischaemia. Brain Res. Rev. 36 (2001) : 23–34.

(47) Hatashita, S., and Hoff, J.T. Brain edema and cerebrovascular permeability during cerebral ischemia in rats. Stroke. 21 (1990) : 582–588.

(48) Ben-Menachem, E., Johansson, B.B., and Svensson, T.H. Increased vulnerability of the blood-brain barrier to acute hypertension following depletion of brain noradrenaline. J. Neural Transm. 53 (1982) : 159–167.

(49) Cohen, Z., Bonvento, G., Lacombe, P., and Hamel, E. Serotonin in the regulation of brain microcirculation. Prog. Neurobiol. 50 (1996) : 335–362.

(50) Vaucher, E., and Hamel, E. Cholinergic basal forebrain neurons project to cortical microvessels in the rat: electron microscopic study with anterogradely transported Phaseolus vulgaris leucoagglutinin and choline acetyltransferase immunocytochemistry. J. Neurosci. 15 (1995) : 7427–7441.

(51) Tong, X.K., and Hamel, E. Regional cholinergic denervation of cortical microvessels and nitric oxide synthase-containing neurons in Alzheimer's disease. Neuroscience. 92 (1999) : 163–175.

(52) Wolburg, H., Noell, S., Mack, A., Wolburg-Buchholz, K., and Fallier-Becker, P. Brain endothelial cells and the glio-vascular complex. Cell Tissue Res. 335 (2009) : 75–96.

(53) Furuse, M., et al. Occludin: a novel integral membrane protein localizing at tight junctions. J. Cell Biol. 123 (1993) : 1777–1788.

(54) Hirase, T., et al. Occludin as a possible determinant of tight junction permeability in endothelial cells. J. Cell. Sci. 110 (1997) : 1603–1613.

(55) McCarthy, K.M., et al. Occludin is a functional component of the tight junction. J. Cell. Sci. 109 (1996) : 2287–2298.

(56) Sakakibara, A., Furuse, M., Saitou, M., Ando-Akatsuka, Y., and Tsukita, S. Possible involvement of phosphorylation of occludin in tight junction formation. J. Cell Biol. 137 (1997) : 1393–1401.

(57) Furuse, M., et al. Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. J. Cell Biol. 127 (1994) : 1617–1626.

(58) Fanning, A.S., Jameson, B.J., Jesaitis, L.A., and Anderson, J.M. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. J. Biol. Chem. 273 (1998) : 29745–2953.

(59) Bolton, S.J., Anthony, D.C., and Perry, V.H. Loss of the tight junction proteins occludin and zonula occludens-1 from cerebral vascular endothelium during neutrophil-induced blood-brain barrier breakdown in vivo. Neuroscience. 86 (1998) : 1245–1257.

(60) Huber, J.D., Hau, V.S., Borg, L., Campos, C.R., Egleton, R.D., and Davis, T.P. Blood-brain barrier tight junctions are altered during a 72-h exposure to lambda-carrageenan-induced inflammatory pain. Am. J. Physiol. Heart Circ. Physiol. 283 (2002) : H1531–1537.

(61) Brown, R.C., and Davis, T.P. Hypoxia/aglycemia alters expression of occludin and actin in brain endothelial cells. Biochem. Biophys. Res. Commun. 327 (2005) : 1114–1123.

(62) Haskins, J., Gu, L., Wittchen, E.S., Hibbard, J., and Stevenson, B.R. ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin. J. Cell Biol. 141 (1998) : 199–208.

(63) Abbruscato, T.J., Lopez, S.P., Mark, K.S., Hawkins, B.T., and Davis, T.P. Nicotine and cotinine modulate cerebral microvascular permeability and protein expression of ZO-1 through nicotinic acetylcholine receptors expressed on brain endothelial cells. J Pharm Sci. 91 (2002) : 2525–2538.

(64) Gumbiner, B., Lowenkopf, T., and Apatira, D. Identification of a 160-kDa polypeptide that binds to the tight junction protein ZO-1. Proc. Natl. Acad. Sci. U.S.A. 88 (1991) : 3460–3464.

(65) Mark, K.S., and Davis, T.P. Cerebral microvascular changes in permeability and tight junctions induced by hypoxia-reoxygenation. Am. J. Physiol. Heart Circ. Physiol. 282 (2002) : H1485–1494.

(66) Umeda, K., et al. Establishment and characterization of cultured epithelial cells lacking expression of ZO-1. J. Biol. Chem. 279 (2004) : 44785–4494.

(67) Huber, J.D., Egleton, R.D., and Davis, T.P. Molecular physiology and pathophysiology of tight junctions in the blood-brain barrier. Trends Neurosci. 24 (2001) : 719–725.

(68) Buzzi, M.G. Trigeminal pain pathway peripheral and central activation as experimental models of migraine. Funct. Neurol. 16 (2001) : 77–81.

(69) Link, A.S., Kuris, A., and Edvinsson, L. Treatment of migraine attacks based onthe interaction with the trigemino-cerebrovascular system. J Headache Pain. 9 (2008) : 5–12.

(70) Eftekhari, S., Salvatore, C.A/, Calamari, A., Kane, S.A., Tajti, J., and Edvinsson, L. Differential distribution of calcitonin gene-related peptide and its receptor components in the human trigeminal ganglion. Neuroscience. 169 (2010) : 683–696.

(71) Goadsby, P.J., Edvinsson, L., and Ekman, R. Release of vasoactive peptides in the extracerebral circulation of humans and the cat during activation of the trigeminovascular system. Ann. Neurol. 23 (1988) : 193–196.

(72) Edvinsson, L., and Uddman, R. Neurobiology in primary headaches. Brain Res. Rev. 48(2005) : 438–456.

(73) Edvinsson, L., and Krause, D.N. Neuropeptide Y. In Edvinsson L., and Krause D.N. (ed.), Cerebral Blood Flow and Metabolism, pp 212-221. Philadelphia : Lippincott Williams & Wilkins, 2002.

(74) Edvinsson, L., Eftekhari, S., Salvatore, C.A., and Warfvinge, K. Cerebellar distribution of calcitonin gene-related peptide (CGRP) and its receptor components calcitonin receptor-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1) in rat. Mol. Cell. Neurosci. 46 (2011) : 333–339.

(75) Goadsby, P.J. Recent advances in understanding migraine mechanisms, molecules and therapeutics. Trends Mol Med. 13 (2007) : 39–44.

(76) Lea˜o, A.A.P. Pial circulation and spreading depression of activity in the cerebral cortex. J. Neurophysiol. (7) 1944 : 391–396.

(77) Ayata, C., and Moskowitz, M.A. Cortical spreading depression confounds concentration-dependent pial arteriolar dilation during N-methyl-D-aspartate superfusion. Am. J. Physiol. Heart Circ. Physiol. 290 (2006) : H1837–1841.

(78) Shibata, M., Leffler, C.W., and Busija, D.W. Cerebral hemodynamics during cortical spreading depression in rabbits. Brain Res. 530 (1990) : 267–274.

(79) Somjen, G.G. Mechanisms of spreading depression and hypoxic spreading depression-like depolarization. Physiol. Rev. 81(2001) : 1065–1096.

(80) Grafstein, B. Mechanism of spreading cortical depression. J. Neurophysiol. 19 (1956) : 154–171.

(81) Busija, D.W., Bari, F., Domoki, F., Horiguchi, T., and Shimizu, K. Mechanisms involved in the cerebrovascular dilator effects of cortical spreading depression. Prog. Neurobiol. 86 (2008) : 379–395.

(82) Nedergaard, M., and Hansen, A.J. Spreading depression is not associated with neuronal injury in the normal brain. Brain Res. 449 (1988) : 395–398.

(83) Horiguchi, T., Kis, B., Rajapakse, N., Shimizu, K., and Busija, D.W. Cortical spreading depression (CSD)-induced tolerance to transient focal cerebral ischemia in halothane anesthetized rats is affected by anesthetic level but not ATP-sensitive potassium channels. Brain Res. 1062 (2005) : 127–133.

(84) Otori, T., Greenberg, J,H,, and Welsh, F.A. Cortical spreading depression causes a long-lasting decrease in cerebral blood flow and induces tolerance topermanent focal ischemia in rat brain. J. Cereb. Blood Flow Metab. 23 (2003) : 43–50.

(85) Kiss, C., Shepard, P.D., Bari, F., and Schwarcz, R. Cortical spreading depression augments kynurenate levels and reduces malonate toxicity in the rat cortex. Brain Res. 26 (2004) : 129–135.

(86) Somjen, G.G. Is spreading depression bad for you? Focus on “repetitive normoxic spreading depression-like events result in cell damage in juvenile hippocampal slice cultures”. J. Neurophysiol. 95 (2006) : 16–17.

(87) Parsons, A.A., and Strijbos, P.J. The neuronal versus vascular hypothesis of migraine and cortical spreading depression. Curr Opin Pharmacol. 3 (2003) : 73–7.

(88) Davies, J.A., Annels, S.J., Dickie, B.G., Ellis, Y., and Knott, N.J. A comparison between the stimulated and paroxysmal release of endogenous amino acids from rat cerebellar, striatal and hippocampal slices: a manifestation of spreading depression? J. Neurol. Sci. 131 (1995) : 8–14.

(89) Mayevsky, A., and Weiss, H.R. Cerebral blood flow and oxygen consumption in cortical spreading depression. J. Cereb. Blood Flow Metab. 11 (1991) : 829–836.

(90) Jander, S., Schroeter, M., Peters, O., Witte, O.W., and Stoll, G. Cortical spreading depression induces proinflammatory cytokine gene expression in the rat brain. J. Cereb. Blood Flow Metab. 21 (2001) : 218–225.

(91) Hansen, A.J., Quistorff, B., and Gjedde, A. Relationship between local changes in cortical blood flow and extracellular K+ during spreading depression. Acta Physiol. Scand. 109 (1980) : 1–6.

(92) Fabricius, M., and Lauritzen, M. Transient hyperemia succeeds oligemia in the wake of cortical spreading depression. Brain Res. 602 (1993) : 350–353.

(93) Iijima, T., Shimase, C., Iwao, Y., and Sankawa, H. Relationships between glutamate release, blood flow and spreading depression: real-time monitoring using an electroenzymatic dialysis electrode. Neurosci. Res. 32 (1998) : 201–207.

(94) Sanchez-Del-Rio, M., Reuter, U., and Moskowitz, M.A. New insights into migraine pathophysiology. Curr. Opin. Neurol. 19 (2006) : 294–298.

(95) Dreier, J.P., et al. Delayed ischaemic neurological deficits after subarachnoid haemorrhage are associated with clusters of spreading depolarizations. Brain. 129 (2006) : 3224–3237.

(96) Fabricius, M., et al. Cortical spreading depression and peri-infarct depolarization in acutely injured human cerebral cortex. Brain. 129 (2006) : 778–790.

(97) Dalkara, T., Zervas, N.T., and Moskowitz, M.A. From spreading depression to the trigeminovascular system. Neurol. Sci. 27 (2006) : S86–S90.

(98) Xu, H.L., and Pelligrino, D.A. ATP release and hydrolysis contribute to rat pial arteriolar dilatation elicited by neuronal activation. Exp. Physiol. 92 (2007) : 647–651.

(99) Jakovcevic, D., and Harder, D.R. Role of astrocytes in matching blood flow to neuronal activity. Curr. Top. Dev. Biol. 79 (2007) : 75–97.

(100) Iadecola, C., and Nedergaard, M. Glial regulation of the cerebral microvasculature. Nat. Neurosci. 10 (2007) : 1369–1376.

(101) Takano, T., et al. Cortical spreading depression causes and coincides with tissue hypoxia. Nat. Neurosci. 10 (2007) : 754–762.

(102) Tomita, M., et al. Initial oligemia with capillary flow stop followed by hyperemia during K^+-induced cortical spreading depression in rats. J. Cereb. Blood Flow Metab. 25 (2005) : 742–747.

(103) Zonta, M., et al. Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. Nat. Neurosci. 6 (2003) : 43–50.

(104) Filosa, J.A., et al. Local potassium signaling couples neuronal activity to vasodilation in the brain. Nat. Neurosci. 9 (2006) : 1397–1403.

(105) Leffler, C.W., Balabanova, L., Fedinec, A.L., and Parfenova, H. Nitric oxide increases carbon monoxide production by piglet cerebral microvessels. Am. J. Physiol. Heart Circ. Physiol. 289 (2005) : H1442–H1447.

(106) Wei, E.P., Moskowitz, M.A., Boccalini, P., and Kontos, H.A. Calcitonin gene-related peptide mediates nitroglycerin and sodium nitroprusside-induced vasodilation in feline cerebral arterioles. Circ. Res. 70 (1992) : 1313–1319.

(107) Macfarlane, R., Tasdemiroglu, E., Moskowitz, M.A., Uemura, Y., Wei, E.P., and Kontos, H.A. Chronic trigeminal ganglionectomy or topical capsaicin application to pial vessels attenuates postocclusive cortical hyperemia but does not influence postischemic hypoperfusion. J. Cereb. Blood Flow Metab. 11 (1991) : 261–271.

(108) Bolay, H., Reuter, U., Dunn, A.K., Huang, Z., Boas, D.A., and Moskowitz, M.A. Intrinsic brain activity triggers trigeminal meningeal afferents in a migraine model. Nat. Med. 8 (2002) : 136–142.

(109) Edvinsson, L. Novel migraine therapy with calcitonin gene-regulated peptide receptor antagonists. Expert Opin. Ther. Targets. 11 (2007) : 1179–1188.

(110) Colonna, D.M., Meng, W., Deal, D.D., and Busija, D.W. Calcitonin gene-related peptide promotes cerebrovascular dilation during cortical spreading depression in rabbits. Am. J. Physiol. 266 (1994) : H1095–H1102.

(111) Wahl, M., Lauritzen, M., and Schilling, L. Change of cerebrovascular reactivity after cortical spreading depression in cats and rats. Brain Res. 411 (1987) : 72–80.

(112) Reuter, U., et al. Perivascular nerves contribute to cortical spreading depression-associated hyperemia in rats. Am. J. Physiol. 274 (1998) : H1979–H1987.

(113) Bari, F., Paprika, D., Jancsó, G., and Domoki, F. Capsaicin-sensitive mechanisms are involved in cortical spreading depression-associated cerebral blood flow changes in rats. Neurosci. Lett. 292 (2000) : 17–20.

(114) Gold, L., et al. Cortical spreading depression-associated hyperemia in rats: involvement of serotonin. Brain Res. 783 (1998) : 188–193.

(115) Mohammad-Zadeh, L.F., Moses, L., and Gwaltney-Brant, S.M. Serotonin: a review. J. Vet. Pharmacol. Ther. 31 (2008) : 187–199.

(116) Rapport, M.M., Green, A.A., and Page I.H. Serum vasoconstrictor, serotonin; isolation and characterization. J. Biol. Chem. 176 (1948) : 1243–1251.

(117) Rapport, M.M., Green, A.A., and Page I.H. Partial purification of the vasoconstrictor in beef serum. J. Biol. Chem. 174 (1948) : 735–741.

(118) Rapport, M.M., Green, A.A., Page, I.H. Crystalline Serotonin. Science. 108 (1948) : 329–330.

(119) Clark, C.T., Weissbach, H., and Udenfriend S. 5-Hydroxytryptophan decarboxylase: preparation and properties. J. Biol. Chem. 210 (1954) : 139–48.

(120 Noguchi, T., Nishino, M., and Kido, R. Tryptophan 5-hydroxylase in rat intestine. Biochem. J. 131 (1973) : 375–380.

(121) Tong, J.H., and Kaufman, S. Tryptophan hydroxylase. Purification and some properties of the enzyme from rabbit hindbrain. J. Biol. Chem. 250 (1975) : 4152–4158.

(122) Champier, J., et al. Evidence for tryptophan hydroxylase and hydroxy-indol-O-methyl-transferase mRNAs in human blood platelets. Life Sci. 60 (1997) : 2191–2197.

(123) Dahlstroem, A., and Fuxe, K. Evidence for the existence of Monoamine-containing neurons in the central nervous system I demonstration of momoamines in the cell bodies of brain stem neurons Acta Physiol Scand Suppl. Suppl. 232 (1964) : 1–55.

(124) TOH, C.C. Release of 5-hydroxytryptamine (serotonin) from the dog's gastrointestinal tract. J. Physiol. (Lond.). 126 (1954) : 248–254.

(125) Hardistry, R.M. and Stacey, R.S. 5-Hydroxytryptamine in normal human platelets. J. Physiol. (Lond.). 130 (1955) : 711–720.

(126) McIsaac, W.M. and Page I.H. The metabolism of serotonin (5-hydroxytryptamine). J. Biol. Chem. 234 (1959) : 858–864.

(127) Gwaltney-Brant, S.M., Albretsen, J.C., and Khan, S.A. 5-Hydroxytryptophan toxicosis in dogs: 21 cases (1989-1999). J. Am. Vet. Med. Assoc. 216 (2000) : 1937–1940.

(128) Hoyer, D., et al. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). Pharmacol. Rev. 46 (1994) : 157–203.

(129) Derkach, V., Surprenant, A., and North, R.A. 5-HT3 receptors are membrane ion channels. Nature. 339 (1989) : 706–709.

(130) Jin, H., et al. Characterization of the human 5-hydroxytryptamine1B receptor. J. Biol. Chem. 267 (1992) : 5735–5738.

(131) Morelli, N., et al. Do $5HT_{1B/1D}$ receptor agonists have an effect on mood and anxiety disorders? Cephalalgia. 27 (2007) : 471–472.

(132) Pullar, I.A., et al. The role of the $5\text{-}HT_{1D}$ receptor as a presynaptic autoreceptor in the guinea pig. Eur. J. Pharmacol. 493 (2004) : 85–93.

(133) Hamblin, M.W., and Metcalf, M.A. Primary structure and functional characterization of a human $5\text{-}HT_{1D}$-type serotonin receptor. Mol. Pharmacol. 40 (1991) : 143–148.

(134) Kaumann, A.J., and Levy, F.O. 5-hydroxytryptamine receptors in the human cardiovascular system. Pharmacol. Ther. 111 (2006) : 674–706.

(135) Martin, G.R. Vascular receptors for 5-hydroxytryptamine: distribution, function and classification. Pharmacol. Ther. 62 (1994) : 283–324.

(136) Saxena, P.R. Serotonin receptors: Subtypes, functional responses and therapeutic relevance. Pharmacology & Therapeutics. 66 (1995) : 339–368.

(137) Hamel, E. Serotonin and migraine: biology and clinical implications. Cephalalgia. 27 (2007) : 1293–1300.

(138) Villalón, C.M., De Vries, P., and Saxena, P.R. Serotonin receptors as cardiovascular targets. Drug Discovery Today. 2 (19970 : 294–300.

(139) Braz, J.M., and Basbaum, A.I. Genetically expressed transneuronal tracer reveals direct and indirect serotonergic descending control circuits. J. Comp. Neurol. 507 (2008) : 1990–2003.

(140) Jann, M.W., and Slade, J.H. Antidepressant agents for the treatment of chronic pain and depression. Pharmacotherapy. 27 (2007) : 1571–1587.

(141) Hardebo, J.E. Migraine--why and how a cortical excitatory wave may initiate the aura and headache. Headache. 31 (1991) : 213–221.

(142) Anthony, M., Hinterberger, H., and Lance, J.W. The possible relationship of serotonin to the migraine syndrome. Res Clin Stud Headache. 2 (1969) : 29–59.

(143) Ferrari, M.D., Odink, J., Tapparelli, C., Van Kempen, G.M., Pennings, E.J., and Bruyn, G.W. Serotonin metabolism in migraine. Neurology. 39 (1989) : 1239–1242.

(144) Hegerl, U., and Juckel, G. Intensity dependence of auditory evoked potentials as an indicator of central serotonergic neurotransmission: a new hypothesis. Biol. Psychiatry. 33 (1993) : 173–187.

(145) Proietti-Cecchini, A., Afra, J., and Schoenen, J. Intensity dependence of the cortical auditory evoked potentials as a surrogate marker of central nervous system serotonin transmission in man: demonstration of a central effect for the $5HT_{1B/1D}$ agonist zolmitriptan (311C90, Zomig). Cephalalgia. 17 (1997) : 849–854; discussion 799.

(146) Berger, M., Gray, J.A., and Roth, B.L. The expanded biology of serotonin. Annu. Rev. Med. 60 (2009) : 355–366.

(147) Pytliak, M., Vargová, V., Mechírová, V., and Felšöci, M. Serotonin receptors - from molecular biology to clinical applications. Physiol Res. 60 (2011) : 15–25.

(148) Arulmani, U., Maassenvandenbrink, A., Villalón, C.M., and Saxena, P.R. Calcitonin gene-related peptide and its role in migraine pathophysiology. Eur. J. Pharmacol. 500 (2004) : 315–330.

(149) Rosenfeld, M.G., et al. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. Nature. 304 (1983) : 129–135.

(150) Amara, S.G., Arriza, J.L., Leff, S.E., Swanson, L.W., Evans, R.M., and Rosenfeld, M.G. Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. Science. 229 (1985) : 1094–1097.

(151) Hopper, R.A., and Garthwaite, J. Tonic and phasic nitric oxide signals in hippocampal long-term potentiation. J. Neurosci. 26 (2006) : 11513–11521.

(152) Steenbergh, P.H., Höppener, J.W., Zandberg, J., Lips, C.J., and Jansz, H.S. A second human calcitonin/CGRP gene. FEBS Lett. 183 (1985) : 403–407.

(153) Poyner, D., and Marshall, I. CGRP receptors: beyond the CGRP(1)-CGRP(2) subdivision? Trends Pharmacol. Sci. 22 (2001) : 223.

(154) Wimalawansa, S.J. Calcitonin gene-related peptide and its receptors: molecular genetics, physiology, pathophysiology, and therapeutic potentials. Endocr. Rev. 17 (1996) : 533–585.

(155) Bell, D., and McDermott, B.J. Calcitonin gene-related peptide in the cardiovascular system: characterization of receptor populations and their (patho)physiological significance. Pharmacol. Rev. 48 (1996) : 253–288.

(156) Powell, K.J., Ma, W., Sutak, M., Doods, H., Quirion, R., and Jhamandas, K. Blockade and reversal of spinal morphine tolerance by peptide and non-peptide calcitonin gene-related peptide receptor antagonists. Br. J. Pharmacol. 131 (2000) : 875–884.

(157) Van Rossum, D., Hanisch, U.K., and Quirion, R. Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. Neurosci Biobehav Rev. 21 (1997) : 649–678.

(158) Oku, R., Satoh, M., Fujii, N., Otaka, A., Yajima, H., and Takagi, H. Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of substance P from the spinal dorsal horn in rats. Brain Research. 403 (1987) : 350–354.

(159) Kangrga, I., Larew, J.S., and Randic, M. The effects of substance P and calcitonin gene-related peptide on the efflux of endogenous glutamate and aspartate from the rat spinal dorsal horn in vitro. Neurosci. Lett. 108 (1990) : 155–160.

(160) McLatchie, L.M., et al. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature. 393 (1998) : 333–339.

(161) Ohtori, S., Takahashi, K., Chiba, T., Yamagata, M., Sameda, H., and Moriya, H. Substance P and calcitonin gene-related peptide immunoreactive sensory DRG neurons innervating the lumbar intervertebral discs in rats. Ann. Anat. 184 (2002) : 235–240.

(162) Lennerz, J.K., et al. Calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1), and calcitonin gene-related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: differences between peripheral and central CGRP receptor distribution. J. Comp. Neurol. 507 (2008) : 1277–1299.

(163) Kapoor, K. Novel potential antimigraine compounds: carotid and systemic haemodynamic effects in a porcine model of migraine. Doctoral dissertation. Department of Pharmacology, Erasmus Medical Center, Erasmus University Rotterdam, 2003.

(164) Edvinsson, L. Blockade of CGRP receptors in the intracranial vasculature: a new target in the treatment of headache. Cephalalgia. 24 (2004) : 611–622.

(165) Welch, K.M.A. Concepts of migraine headache pathogenesis: insights into mechanisms of chronicity and new drug targets. Neurol. Sci. Suppl.2 (2003) : S149–S153.

(166) Goadsby, P.J., Lipton, R.B., and Ferrari, M.D. Migraine--current understanding and treatment. N. Engl. J. Med. 346 (2002) : 257–270.

(167) Lassen, L.H., Haderslev, P.A., Jacobsen, V.B., Iversen, H.K., Sperling, B., and Olesen, J. CGRP may play a causative role in migraine. Cephalalgia. 22 (2002) : 54–61.

(168) Benemei, S., Nicoletti, P., Capone, J.G, and Geppetti, P. CGRP receptors in the control of pain and inflammation. Curr Opin Pharmacol. 9 (2009) : 9–14.

(169) Iversen, L. Substance P equals pain substance? Nature. 392 (1998) : 334–335.

(170) Radhakrishnan, V., and Henry, J.L. Electrophysiology of neuropeptides in the sensory spinal cord. Prog. Brain Res. 104 (1995) : 175–195.

(171) Vaught, J.L. Substance P antagonists and analgesia: a review of the hypothesis. Life Sci. 43 (1988) : 1419–1431.

(172) Kerdelhué, B., et al. Stimulatory effect of a specific substance P antagonist (RPR 100893) of the human NK1 receptor on the estradiol-induced LH and FSH surges in the ovariectomized cynomolgus monkey. J. Neurosci. Res. 50 (1997) : 94–103.

(173) Stahl, S.M. Peptides and psychiatry, Part 3: substance P and serendipity: novel psychotropics are a possibility. J Clin Psychiatry. 60 (1999) : 140–141.

(174) Kramer, M.S., et al. Distinct mechanism for antidepressant activity by blockade of central substance P receptors. Science. 281 (1998) : 1640–1645.

(175) De Felipe, C., et al. Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. Nature. 392 (1998) : 394–397.

(176) Geppetti, P., and Holzer, P. Neurogenic Inflammation. Boca Raton, FL: CRC Press USA, 1996.

(177) Chan-Palay, V., and Palay, S.L. Ultrastructural identification of substance P cells and their processes in rat sensory ganglia and their terminals in the spinal cord by immunocytochemistry. Proc. Natl. Acad. Sci. U.S.A. 74 (1977) : 4050–4054.

(178) Maggi, C.A. The pharmacology of the efferent function of sensory nerves. J. Auton Pharmacol. 11 (1991) : 173–208.

(179) Kawasaki, H., Takasaki, K., Saito, A., and Goto, K. Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat. Nature. 335 (1988) : 164–167.

(180) Moskowitz, M.A. Genes, proteases, cortical spreading depression and migraine: impact on pathophysiology and treatment. Funct. Neurol. 22 (2007) : 133–136.

(181) Brooks, T.A., Hawkins, B.T., Huber, J.D., Egleton, R.D., and Davis, T.P. Chronic inflammatory pain leads to increased blood-brain barrier permeability and tight junction protein alterations. Am. J. Physiol. Heart Circ. Physiol. 289 (2005) : H738–H743.

(182) Witt, K.A., Mark, K.S., Hom, S., and Davis, T.P. Effects of hypoxia-reoxygenation on rat blood-brain barrier permeability and tight junctional protein expression. Am. J. Physiol. Heart Circ. Physiol. 285 (2003) : H2820–H2831.

(183) Daneman, R., Zhou, L., Kebede, A.A., and Barres, B.A. Pericytes are required for blood-brain barrier integrity during embryogenesis. Nature. 468 (2010) : 562–566.

(184) Castejón, O.J. Ultrastructural pathology of endothelial tight junctions in human brain oedema. Folia Neuropathol. 50 (2012) : 118–129.

(185) Zhang, J., et al. Anti-high mobility group box-1 monoclonal antibody protects the blood-brain barrier from ischemia-induced disruption in rats. Stroke. 42 (2011) : 1420–1428.

(186) Tomás-Camardiel, M., et al. Blood-brain barrier disruption highly induces aquaporin-4 mRNA and protein in perivascular and parenchymal astrocytes: protective effect by estradiol treatment in ovariectomized animals. J. Neurosci. Res. 80 (2005) : 235–246.

(187) Zador, Z., Bloch, O., Yao, X., and Manley, G.T. Aquaporins: role in cerebral edema and brain water balance. Prog. Brain Res. 161 (2007) : 185–194.

(188) Grafstein, B. Subverting the hegemony of the synapse: complicity of neurons, astrocytes, and vasculature in spreading depression and pathology of the cerebral cortex. Brain Res Rev. 66 (2011) : 123–132.

(189) Kovács, A., Hársing, L.G., and Szénási, G. Vasoconstrictor 5-HT receptors in the smooth muscle of the rat middle cerebral artery. Eur. J. Pharmacol. 689 (2012) : 160–164.

(190) Hoyer, D., Hannon, J.P., and Martin, G.R. Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacol. Biochem. Behav. 71 (2002) : 533–554.

(191) Le Grand, S.M., Supornsilpchai, W., Saengjaroentham, C., Srikiatkhachorn, A. Serotonin depletion leads to cortical hyperexcitability and trigeminal nociceptive facilitation via the nitric oxide pathway. Headache. 51 (2011) : 1152–1160.

(192) Darvesh, A.S., Yamamoto, B.K., and Gudelsky, G.A. Evidence for the involvement of nitric oxide in 3,4-methylenedioxymethamphetamine-induced serotonin depletion in the rat brain. J. Pharmacol. Exp. Ther. 312 (2005) : 694–701.

(193) Wahl, M., Schilling, L., Parsons, A.A., and Kaumann, A. Involvement of calcitonin gene-related peptide (CGRP) and nitric oxide (NO) in the pial artery dilatation elicited by cortical spreading depression. Brain Res. 637 (1994) : 204–210.

(194) Hou, M., Kanje, M., Longmore, J., Tajti, J., Uddman, R., and Edvinsson, L. 5-$HT_{(1B)}$ and 5-$HT_{(1D)}$ receptors in the human trigeminal ganglion: co-localization with calcitonin gene-related peptide, substance P and nitric oxide synthase. Brain Res. 909 (2001) : 112–120.

(195) Ma, Q.P., Hill, R., and Sirinathsinghji, D. Colocalization of CGRP with 5-$HT_{1B/1D}$ receptors and substance P in trigeminal ganglion neurons in rats. Eur. J. Neurosci. 13 (2001) : 2099–2104.

BIOGRAPHY

NAME	Miss Chonlawan Saengjaroentham
DATE OF BIRTH	14th March 1987
PLACE OF BIRTH	Samutsongkhram, Thailand
INSTRUCTIONS ATTENDED	Kasetsart University (2005-2008) Bachelor of Science (Biology)
SCHOLARSHIPS	Development and Promotion of Science and Technology talents project (DPST)