THE DIFFERENCE PATTERNS OF ELECTROPHYSIOLOGICAL PROPERTIES AND EFFECTS OF KYNURENIC ACID IN TRIGEMINAL GANGLION NEURONS ISOLATED FROM RATS MIGRAINE MODELS

Miss Naeemah Haji-a-sa

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รูปแบบความแตกต่างของคุณสมบัติทางสรีรวิทยาไฟฟ้าและผลของคินูรีนิกแอซิดในเซลล์ประสาทไตรเจอมินัลแกงเกลียนซึ่งแยกออกมาจากหนูแรทที่เป็นแบบจำลองโรคไมเกรน

น.ส.นาอีมะฮ์ หะยีอาซา

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By	Miss Naeemah Haji-a-sa
Field of Study	Medical Sciences
Thesis Advisor	Associate Professor SAKNAN BONGSEBANDHU-PHUBHAKDI, Ph.D.
Thesis Co Advisor	Sekh Thanprasertsuk, M.D.

Accepted by the Faculty of Medicine, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the Faculty of Medicine
(Professor SUTTIPONG WACHARASINDHU, M.D.)

THESIS COMMITTEE

..... Chairman
(Professor VILAI CHENTANEZ, M.D.)

..... Thesis Advisor
(Associate Professor SAKNAN BONGSEBANDHU-PHUBHAKDI, Ph.D.)

..... Thesis Co-Advisor
(Sekh Thanprasertsuk, M.D.)

..... Examiner
(Associate Professor POONLARP CHEEPSUNTHORN, Ph.D.)

..... External Examiner
(Professor Banthit Chetsawang, Ph.D.)

นาอีมะฮ์ หะยีอาซา : รูปแบบความแตกต่างของคุณสมบัติทางสรีรวิทยาไฟฟ้าและผลของคินูรีนิกแอซิดในเซลล์ประสาทไตรเจอมินัลแกงเกลียนซึ่งแยกออกมาจากหนูแรทที่เป็นแบบจำลองโรคไมเกรน. (THE DIFFERENCE PATTERNS OF ELECTROPHYSIOLOGICAL PROPERTIES AND EFFECTS OF KYNURENIC ACID IN TRIGEMINAL GANGLION NEURONS ISOLATED FROM RATS MIGRAINE MODELS) อ.ที่ปรึกษาหลัก : รศ. ดร.ศักนัน พงศ์พันธุ์ผู้ภักดี, อ.ที่ปรึกษาร่วม : นพ. เสกข์ แทนประเสริฐสุข

อาการปวดศีรษะไมเกรนที่เกิดขึ้นในระบบการรับความรู้สึกเจ็บปวดไตรเจอมินัล ซึ่งถูกกระตุ้นโดยปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชัน นอกจากนี้พยาธิกำเนิดของไมเกรนสัมพันธ์กับการเปลี่ยนแปลงของสารสื่อประสาทบางชนิด ซึ่งรวมถึงภาวะพร่องซีโรโทนินในระบบการรับความรู้สึกเจ็บปวดไตรเจอมินัล โดยไตรเจอมินัลแกงเกลียนมีส่วนของเส้นประสาทรับความรู้สึกลำดับที่หนึ่ง เป็นส่วนประกอบของระบบการรับความรู้สึกเจ็บปวดไตรเจอมินัล นอกจากนี้การเปลี่ยนแปลงของสารสื่อประสาทเช่น ซีโรโทนิน หรือกลูตาเมทอาจทำให้มีการทำงานของตัวรับเพิ่มมากขึ้น เช่นตัวรับชนิด NMDA อย่างไรก็ตามสารชีวโมเลกุลที่มีอยู่ในร่างกายมีความสามารถในการยับยั้งการทำงานของตัวรับได้ เช่นสารคินูรีนิกแอซิด ซึ่งเป็นอนุพันธุ์หนึ่งของสารสื่อประสาทซีโรโทนินและมีผลยับยั้งการทำงานของตัวรับ NMDA โดยในงานวิจัยนี้มีจุดประสงค์เพื่อศึกษาผลการเปลี่ยนแปลงทางไฟฟ้าสรีรวิทยาของไตรเจอมินัลแกงเกลียนที่แยกออกมาจากหนูแรทในแบบจำลองไมเกรนและผลของสารคินูรีนิกแอซิดต่อแบบจำลองไมเกรน ผลการศึกษาพบว่าค่าศักย์ไฟฟ้าในระยะพักมีการลดลงอย่างมีนัยสำคัญทางสถิติในกลุ่มที่เกิดปรากฏการณ์คอร์ติคัลสเปรดดิ้งดีเพรสชัน และในกลุ่มที่เกิดภาวะพร่องซีโรโทนินร่วมกับเกิดคอร์ติคัลสเปรดดิ้งดีเพรสชันด้วย จึงสรุปได้ว่าคอร์ติคัลสเปรดดิ้งดีเพรสชันเป็นโมเดลเกิดขึ้นที่ผิวสมองแต่มีผลต่อเซลล์ประสาทตัวรับที่หนึ่งของระบบไตรเจอมินัล ดังนั้นคอร์ติคัลสเปรดดิ้งดีเพรสชันมีศักยภาพที่จะเปลี่ยนแปลงค่าศักย์ไฟฟ้าในระยะพัก เป็นผลให้เกิดการเพิ่มขึ้นของการตอบสนองต่อสิ่งกระตุ้นในระดับเซลล์ประสาทระบบไตรเจอมินัลที่รับรู้การปวดหัวในโรคไมเกรน

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

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Naeemah Haji-a-sa : THE DIFFERENCE PATTERNS OF ELECTROPHYSIOLOGICAL PROPERTIES AND EFFECTS OF KYNURENIC ACID IN TRIGEMINAL GANGLION NEURONS ISOLATED FROM RATS MIGRAINE MODELS . Advisor: Assoc. Prof. SAKNAN BONGSEBANDHU-PHUBHAKDI, Ph.D. Co-advisor: Sekh Thanprasertsuk, M.D.

Migraine headache generates in trigeminal nociceptive system which is activated by cortical spreading depression (CSD) phenomenon. There are also some neurotransmitter changes relating to pathogenesis of migraine, one of representative examples is serotonin (5-HT) depletion that may predispose to migraine development. Trigeminal ganglion (TG) is a structure containing cell body of neurons that undertakes a role as first order neuron in trigeminal nociceptive system. Moreover, the alteration of glutamate may contribute to hyperactivity of its specific receptors, for instance N-methyl-D-aspartate (NMDA) receptor. Endogenous biomolecule, such as kynurenic acid (KYNA) which is derived form of 5-HT can inhibit NMDA receptor. Basically, KYNA plays a role as NMDA antagonist, and at the same time, it is one of metabolites of tryptophan pathways as well as 5-HT. This study aimed to explore the electrophysiological properties change of TG neuron isolated from rat migraine models (CSD and 5-HT depletion), and the effects of KYNA on them. The results revealed that resting membrane potential (RMP) of TG neurons had significant depolarization shift in CSD and combined model of CSD and 5-HT depletion, while this alteration was not observed in 5-HT depletion only. In conclusion, CSD is a cortical model of migraine that affects to TG neuron. Thus, CSD has a potential to alter RMP of TG neurons that may results to increase of neuronal excitability in trigeminal nociceptive system.

Field of Study:	Medical Sciences	Student's Signature .....
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Naeemah Haji-a-sa

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Chapter 1

INTRODUCTION

BACKGROUND AND RATIONALE

Migraine is a non-communicable disease (NCD), which affects many ranges of ages and genders. Migraineurs have suffered from their disease and have impairment in activities of daily living. According to disease cause of years lived with disability (YLDs) in Thailand, migraine is in the fifth rank. [1] Migraine usually characterized by severe unilateral throbbing or pulsating headache. The International Classification of Headache Disorders (ICHD), 3rd edition, divided migraine into two major subtypes, which were migraine without aura and with aura. Migraine without aura is characterized by unilateral headache and attacks for 4-72 hours. The transient neurological symptoms that occurs in one-third of migraineurs are called aura symptoms such as flashing lights, zig zag lines, or tunnel vision, etc. These symptoms generate in migraine with aura. [2] Headache in migraine is generated in trigeminal nociceptive system which is activated by cortical spreading depression (CSD) phenomenon, which is described as cortical neuronal depolarization followed by repression activities and excitabilities of neurons. CSD was provoked and leading to release of inflammatory substances around the nerves and blood vessels of the head, such as substance P (SP), calcitonin gene-related peptides (CGRP) and nitric oxide (NO). [3] In addition to CSD, there are some neurotransmitter changes related to migraine pathogenesis, for instance, serotonin (5-HT) depletion or excessive releases of glutamate. The previous study revealed that KCl-induced CSD or 5-HT depleted or a combination of CSD and 5-HT depleted rats, had increased Fos protein expression in trigeminal nucleus caudalis (TNC) and cortical neurons. [4] The changes of 5-HT and glutamate levels may contributed to hyperactivity of some receptors, for instance N-methyl-D-aspartate (NMDA) receptor. [5] However, the regulation of central nervous system is amazing, as it has a biomolecule which can suppress or inhibit NMDA receptor suchlike kynurenic acid (KYNA). [6] Actually, KYNA is a metabolite of tryptophan, as well as 5-HT. Previous study revealed that both 5-HT

and KYNA were decreased in migraine.[7] Thus, KYNA may have an association with migraine pathogenesis. However, it is still unclear if there is interaction of KYNA to the electrophysiological properties of trigeminal ganglia (TG) neurons in rats with KCl-induced CSD or 5-HT depletion. Additionally, if KYNA has some physiologic effect to trigeminal nociceptive system is rat migraine model, it may have potential for developing as anti-migraine agent in the future.

These knowledge gaps lead to the development of our thesis. We aimed to study electrophysiological effects of KYNA in TG neurons, using a patch-clamp recording, to varied different migraine sensitization procedures including KCl-induced CSD, 5-HT depleted or combination between 5-HT depleted and KCl-induced CSD.

RESEARCH QUESTIONS

1. Do TG neurons electrophysiological properties change in different migraine sensitizations?
2. Does KYNA alter electrophysiological properties of TG neurons in different migraine sensitizations?

OBJECTIVE

The objectives of our thesis are to discover electrophysiological properties pattern of TG neurons in different migraine sensitization (5-HT depletion and KCl-induced rats) with KYNA.

HYPOTHESIS

Different migraine-inducing conditions may lead to sensitization of TG neurons. KYNA may reduce the sensitivity of TG neurons in different migraine sensitization procedures.

Chapter 2

Literature review

HEADACHE

Headache is a painful neurological disorder which occurs in many ranges of ages, genders and nationalities. It causes disturbance to activities in daily living (ADLs).[8] There are four types of primary headache syndrome including migraine headache, tension-type headache, trigeminal autonomic cephalalgia and other primary headache syndromes. Migraine has two major subtypes, which are migraine without aura (common migraine) and migraine with aura.[2] Both types of migraine will be described below.

MIGRAINE

Migraine is a common primary headache disorder, occurs 18% in female and 5% in male population. In Thailand there were 11,522 female and 5,298 male migraineurs, according to National Statistical Office Thailand 2016.[9] Migraine without aura usually characterizes by unilateral pulsating head pain, accompanies with nausea and/or vomiting. Duration of the attack is typically 4 to 72 hours. It can be aggravated by ADLs such as walking or climbing stairs and often has a menstrual relationship. No transient neurological symptom associated with this subtype of migraine. While another subtype, migraine with aura, typically collaborates with transient neurological symptoms preceding the pain period called aura, which is found in about 20-30% of patient with migraine headache. Visual aura called “scintillation-scotoma” is reported as the most common form of aura, in which the symptoms consist of flashing lights, zig zag lines, tunnel vision, black spots or partial vision lost. Another common form of aura is the somatosensory aura manifesting by numbness of hand and perioral area. Migraine with aura occurs due to a specific phenomenon in the central nervous system called CSD. [2, 10, 11]

The core pathophysiologic processes in migraine are stimulation and sensitization of trigeminal nociceptive system consisting of cerebral cortex, hypothalamus, TNC and TG.[12] Information about migraine-related structures and detailed pathophysiology are discussed below.

MIGRAINE-RELATED STRUCTURES

Trigeminal nociceptive system

Trigeminal nerve is the fifth cranial nerve (CN V), which consists of three peripheral branches including the ophthalmic branch (V1), the maxillary branch (V2) and the mandibular branch (V3). Three branches of trigeminal nerve (red circle in Fig.1A) received somatosensory and nociceptive information from facial regions and gather together in the TG (also called semilunar ganglion), then projected to the brainstem as a huge sensory root (Fig1B). [13] The terminal nerve branches also innervate and receive signal from meningeal and large cerebral arteries. [14] TNC is a nucleus located in lower brainstem which the TG's nociceptive fibers project to. There are other brainstem structures serving as pain regulation area, which are periaqueductal grey (PAG), Locus coeruleus (LC) and nucleus of raphe magnus (NRM). [15]

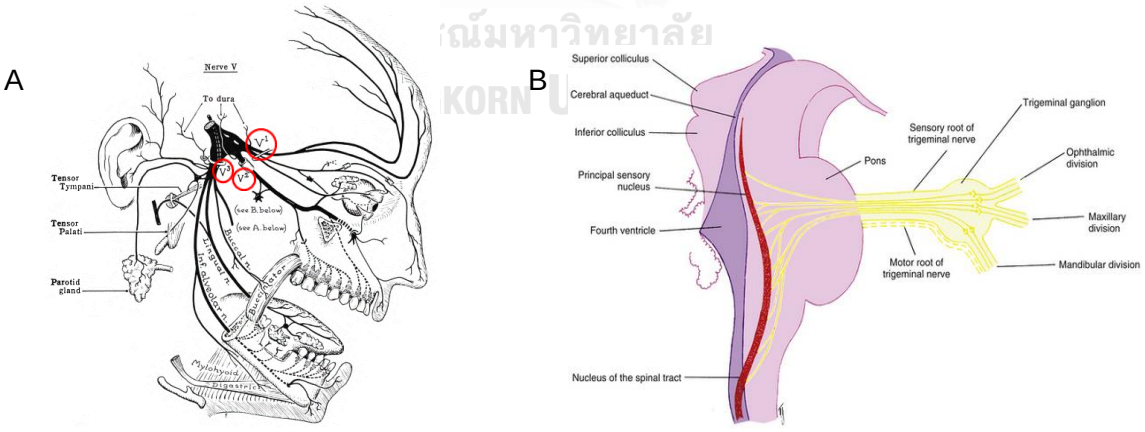


Figure 1 (A) Peripheral distribution of trigeminal nerve (CNV) (B) Projection of nerve root from trigeminal ganglion into brainstem.

Trigeminal ganglion (TG)

TG contains of first order neurons which are pseudounipolar neurons. TG's nociceptive fibers project into TNC which contains second-order neurons [16, 17] TG neurons were classified by size into 3 types: small size (less than 22 micron), medium size (between 22-29 micron) and large size (more than 29 micron). The cell that modulate pain sensation is the small and medium sized neuron. These neurons express and release calcitonin gene-related peptide (CGRP) or substance P (SP) which communicate nociceptive information between neurons at the cell surface receptors. [18]

PATHOPHYSIOLOGY OF MIGRAINE

Migraine pathophysiology associates with neuronal and vascular mechanisms contributing to the development of headache. A pathway which generates migraine is the trigeminovascular pathway.

Trigeminovascular pathway and trigeminocervical complex

Migraine attacks due to activation of nociceptors that innervate cranial, especially meningeal, vasculatures. Immediately after nociceptors activation, pain signal transmits to the TNC via the trigeminal nerve branches and TG (purple line in Fig. 2). This pathway is called trigeminovascular pathway or system. Pain caused by trigeminovascular system can be aggravated by multiple triggers such as stress, certain foods, drugs or injury.[19] Besides the craniofacial pain which is transmitted by trigeminovascular system, pain in migraine is also distributed in occipital and upper cervical region which is transmitted by greater occipital nerve and other upper cervical spinal nerves via cervical ganglion (CG) to the second order neurons in upper cervical spinal cord. These upper cervical spinal cord structures and TNC are collectively known as trigeminocervical complex (TCC). The signal from the TCC are then sent to the third order neurons in higher structures such as hypothalamus and thalamus, which relay pain information before reaching the cerebral cortex.[16, 20] Superior salivatory nucleus

(SSN) is also activated by the repeatedly stimulated TCC. This nucleus is responsible for trigeminal autonomic reflex, which is a causative part of an additional trigeminal nociceptor activation by releasing vasodilatory substances such as vasoactive intestinal peptide (VIP) and nitric oxide (NO), as well as inflammatory substances, at meningeal vessels. [12, 21]

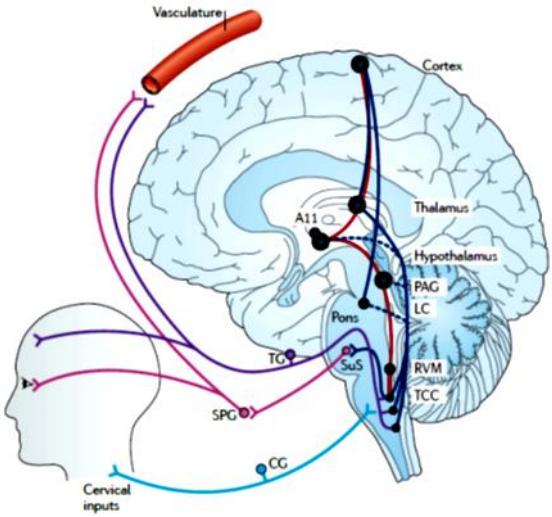


Figure 2 Neuroanatomy of trigeminovascular system in migraine pathophysiology

Cortical spreading depression (CSD)

CSD is a unique electrophysiological phenomenon contributing to the pathophysiology of migraine with aura. It is a wave with velocity of 2-6 mm/min spreading on the cortical surface, usually originating on occipital cortex. It occurs due to neuronal-glia cell depolarization, following by suppression of neuronal activities for about 15-30 minutes. [20, 22] This phenomenon also associates with changes of vascular function [15] and release of local mediators related to pain signal transduction such as potassium, glutamate, NO, ATP, and CGRP. CGRP is a key neuropeptide involved migraine pathophysiology, affects to change in vascular function.[23] These substances (potassium, glutamate, NO, ATP and CGRP) activate and sensitize trigeminal nociceptors

and subsequently cause increased blood flow in the middle meningeal artery, which then activates trigeminal autonomic reflex and cause additional noxious stimulation, then, release of CGRP from TG, and leading to pain development. [21-23]

To be precise, increased extracellular potassium level following by influx of sodium and calcium ions accompanied with glutamate release initiate CSD. [21, 22] Glutamate released activates both sodium and calcium channels through NMDA receptors and speedy influx of huge cations result in down concentration gradients, and leading to change normal membrane potential. CSD-induced is one of standard animal model for pathophysiology studies, it sensitized to trigeminovascular system.[23, 24]

Serotonin (5-HT) pathway

Serotonin is a neurotransmitter synthesized from essential amino acid tryptophan. Firstly, tryptophan hydroxylase (TPH) enzyme converts tryptophan to 5-hydroxytryptophan. Then it is converted to 5-HT (5-hydroxytryptamine) by aromatic L-amino acid decarboxylase (AAAD) enzyme. [20] 5-HT is packed in vesicles and found in all part of 5-HT neurons such as dendrites, axon, neuron body and terminals. It released by calcium-dependent exocytosis and reuptake by an energy-dependent membrane transporter. 5-HT neurons located in three raphe nuclei, which are raphe magnus (30,000 neurons), the raphe obscurus, and the raphe pallidus (1,000 neurons), and in the ventral medullary reticular formation lying lateral to the raphe magnus and the pyramids. [25] An action of 5-HT is a vasoactive agents , it binds to 5-HT receptor, which have been classified into 7 different families. 5-HT1B, 1D and 1F receptors were subfamilies associated with migraine pathophysiology discovered in the trigeminovascular system. [26]

Serotonin plays a major role in pathophysiology of migraine. Normally, agonistic effect on 5-HT receptors, laying on trigeminal sensory neurons and around meningeal vessels, cause meningeal vasoconstriction and inhibit releasing of inflammatory

peptide such as CGRP and SP. [27] In migraineurs, both brain and plasma 5-HT was found to be decreased during migraine attacks [28, 29] and decreasing levels of 5-HT are linked to migraine pathophysiology. [30] These findings might associate with meningeal vasodilation and release of inflammatory substances that consequently activate trigeminovascular pathway in migraine patient. Platelet 5-HT depletion might also associate with intracranial vasodilation during migraine attack.[31]

In animal model, 4-chloro-L-phenylalanine (PCPA) injection is a 5-HT-depleted procedure. The injection reduces CNS 5-HT concentrations bilaterally. The effect of endogenous 5-HT reduction is reversible at 3 days after last injection.[32] Previous study suggests that 5-HT depletion enhances CSD-induced trigeminal nociception by increasing cortical excitability and increasing sensitivity of the trigeminal nociceptive system.[4] Correlation between 5-HT depletion and CSD phenomenon play a role in migraine pathophysiology. Mechanisms of 5-HT depletion then leading to upregulate of 5-HT2A receptor in central and increase of cortical excitability collaborate with decreased threshold to initiate CSD and encourage to central sensitization process. In the event that upregulate of 5-HT2A receptor in peripheral, it results in sensitized nociceptor. An activation of this receptor leads to increase nitric oxide synthase expression in trigeminal pathway as well. [33] Dysfunction of 5-HT metabolism in the central nervous system has been implicated in the biogenesis of migraine. [34]

KYNURENINE PATHWAY

Kynurenine pathway was found in the mammalian tissues such as brain. An initial substrate of kynurenine is L-tryptophan which is the same substrate for 5-HT synthesis. Firstly, tryptophan is converted to N-formyl-kynurenine by indoleamine2, 3-deoxygenase (IDO) enzyme. Then, N-formyl-kynurenine is converted into L-kynurenine which can be converted into KYNA or 3-Hydroxykynurenine (3-HK). 3-HK is then changed into many intermediates and is finally converted into nicotinamide adenosine dinucleotide as an end product (figure 3).[35]

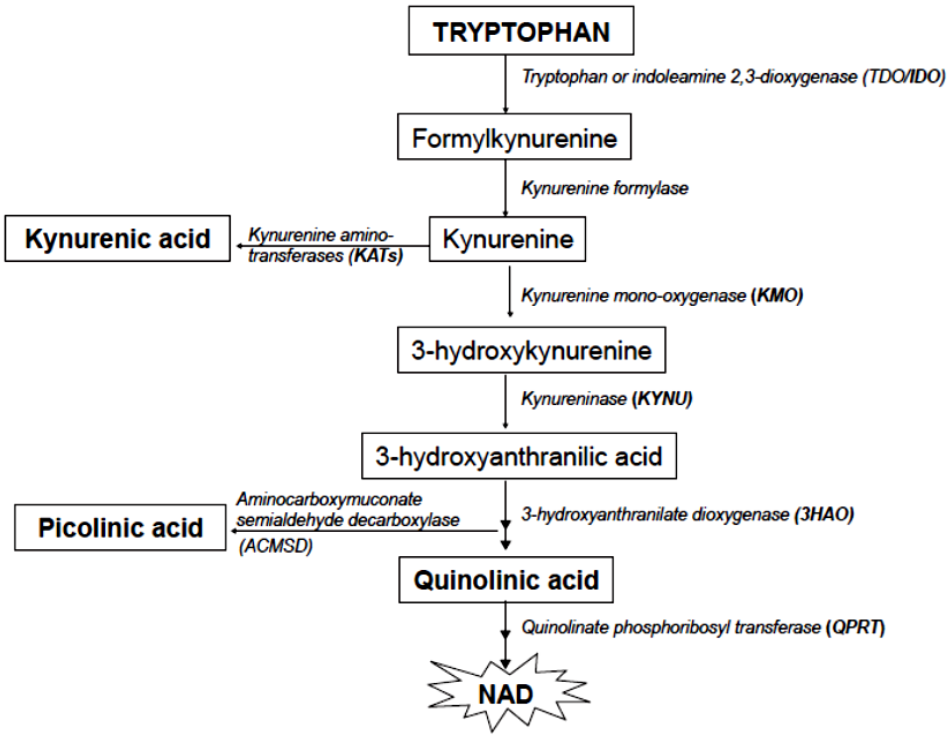


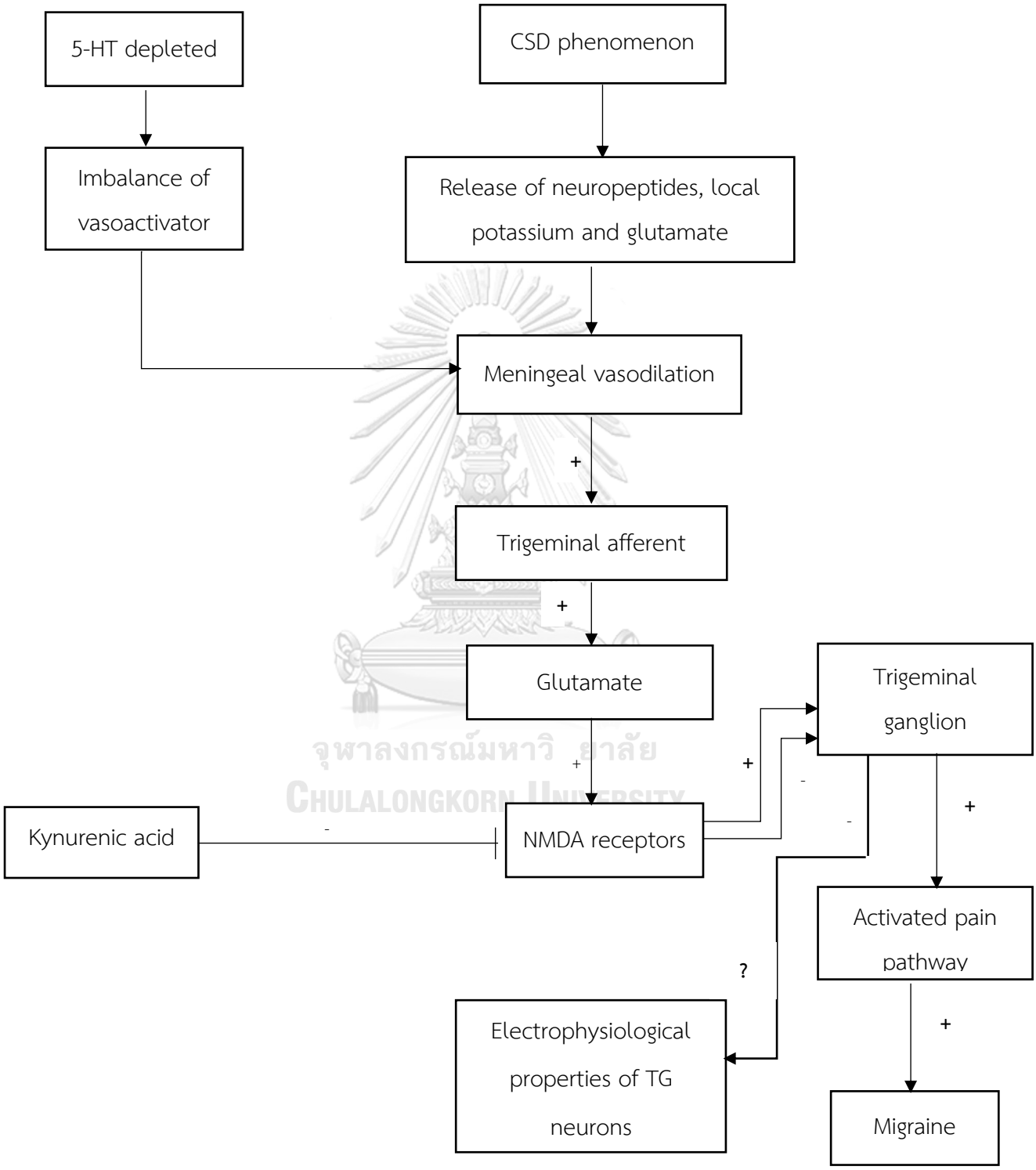
Figure 3 Tryptophan degradation through Kynurenine pathway

Kynurenic acid (KYNA)

KYNA is a product in kynurenine pathway which acts as an antagonist on NMDA receptor and α-7 nicotinic acetylcholine (α-7 nACh) receptor. [36] NMDA receptor is an ionotropic glutamate receptor; it has 2 subunits. There are NR1 and NR2 subunits; NR1 subunit contains glycine-binding site, while NR2 subunit contains the glutamate-binding site. KYNA binds to NR1 subunit at glycine binding site, and it inhibits NMDA function that leads to reduction of Ca^{2+} influx to the cell. [37] KYNA is synthesized from astrocytes and neurons via the kynurenine aminotransferases (KATs), or through the aspartate aminotransferase of the mitochondria and hemoperoxidases, or non-enzymatically by reactive oxygen species (ROS). Moreover, KYNA is formed from tryptophan via the enzyme tryptophan aminotransferase and ROS. [38] KYNA was found to be associated with migraine pathophysiology. Previous studies, suggested that combination between L-kynurenine and probenecid in migraine model could influence CSD suppression

which may associate with increased KYNA in cerebral cortex [39, 40] Another study, Gáspár Oláh and colleagues discovered KYNA injection via intraperitoneal route effects to reduce CSD wave.[41] Serum levels of KYNA and 5-hydroxyindoleacetic acid, a metabolite of 5-HT, were also decreased in patients with chronic migraine.[7] Besides migraine, alteration of KYNA level may have roles in several neuropsychiatric disorders such as Parkinson's disease, epilepsy, Huntington's disease, schizophrenia and Alzheimer's disease. [6]

CONCEPTUAL FRAMEWORK



KEY WORDS

Migraine, 5-HT depleted, Cortical spreading depression, Kynurenic acid, Trigeminal ganglion, Patch clamp

RESEARCH DESIGN

Experimental research animal study.

Chapter 3

Research methodology

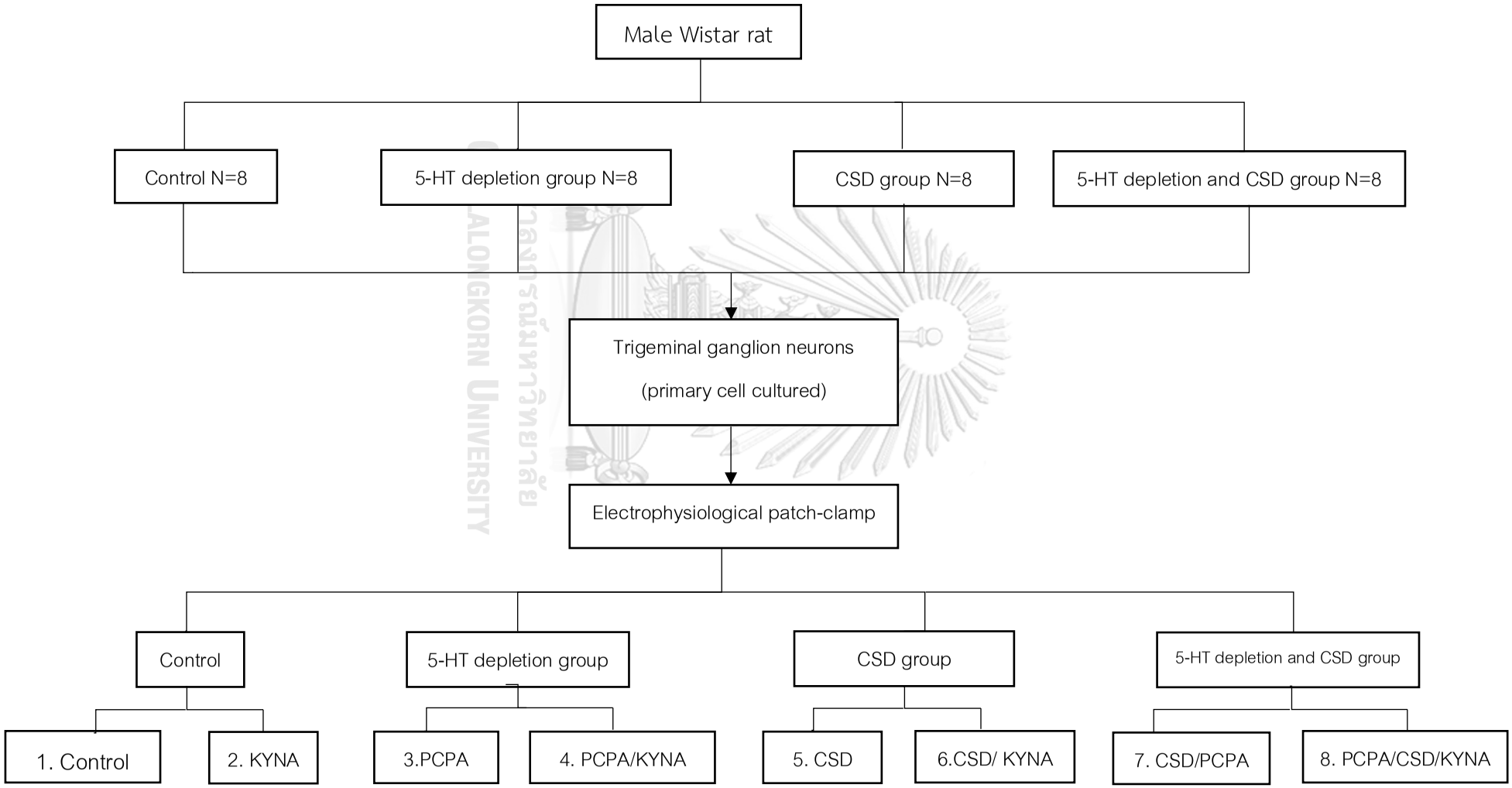
Animal

Adult male wistar rats weighing 200-300 grams were use in all experiments and purchased from Nomura Siam International, Bangkok, Thailand. Rats were housed in stainless cages in the ventilation room under 12-hour dark-light cycle and allowed to food and water *ad libitum*. All protocols were approved by the Animal care and use committee of Faculty of Medicine, Chulalongkorn University, Thailand (019/2561).

Experimental design

The animals were divided into four groups. The first group is a control group. The second group is a 5-HT depleted group, induced by intraperitoneal (i.p.) injection of 100 mg/kg of PCPA for three days. The third group is a KCl-induced CSD and the forth group is 5-HT depleted with KCl-induced group. Once the procedures were applied, the rats were sacrificed with thiopental 70 mg/kg BW and decapitated to remove both sides of TG for primary cultured. We then divided each group into two subgroups. These were control and KYNA group, in which 200 μM of KYNA was added in an external solution via bath application technique for electrophysiological patch-clamp recording.

Experimental design groups

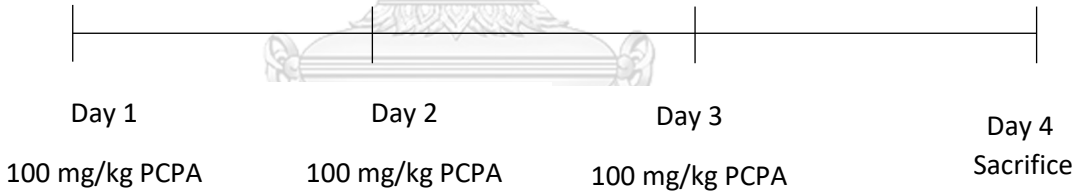


KCl-induced CSD

Rats were placed on a stereotaxic frame. Skull was exposed by removing skin and fascia. To create a burr hole, a dental drill was placed on the skull at 7 mm posterior and 1 mm lateral to bregma. The skull was drilled to expose dura mater, which was subsequently removed. KCl crystal weighed 3 mg was placed on the burr hole for 1 hour. After that, the rat was decapitated, and both sides of TG be removed for primary cell cultured.

5-HT-depleted rat

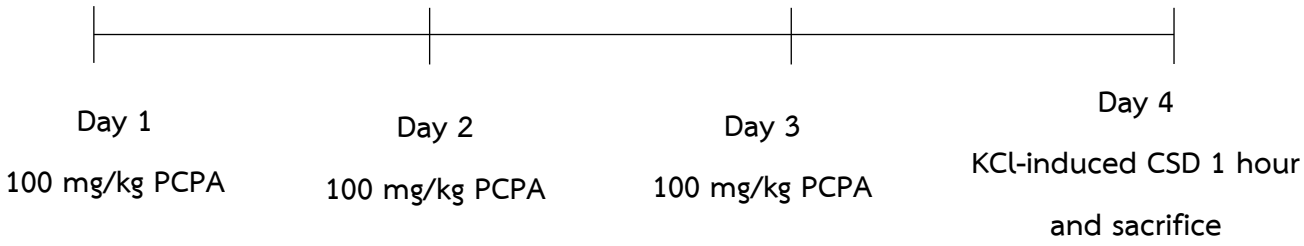
Serotonin depletion was induced by i.p. injection of 100 mg/kg PCPA for three consecutive days. [42] Three days after PCPAs were injected, the rats were sacrificed with thiopental overdose and decapitated 24 hours after last dose of PCPA. Both sides of TG will then be removed and merged into Hank's balanced salt solution for primary cell cultured.



Timeline PCPA injection

5-HT depleted rat and KCl-induced CSD

At first, rats had underwent on 5-HT depleted procedure for three consecutive days using the same technique as described above. Twenty-four hours after completing 5-HT depleted procedure, KCl-induced CSD procedure was applied using the same technique as described above. After 1 hour of KCl-induced CSD procedure, the rats will be decapitated, and both sides of TG will then be removed for primary cell culture.



Timeline PCPA injection and KCl-induced CSD

Primary culture of TG neuron

Both sides of TG were removed into a dish containing of Hank's balance salt solution integrated with penicillin/streptomycin and will be washed for 2 times. TG tissue was cut into small pieces in Hank's balance salt solution and collagenase enzyme was subsequently added. Then the samples were filtrated with 0.2 μm filter, incubated at 37°C for 20 minutes, and centrifuged for 1 minute at RCF 400g. After that, papain was added. The samples were filtrated again with 0.2 μm filter, were incubated at 37°C for 20 minutes, were added with 2 ml L-15 medium and were centrifuged for 8 minutes at RCF 400g. Next, the samples were washed with F-12 completed medium 400 μl and were pipetted into Laminin / PDL coated dish, then were maintained in an incubator (37°C, 5% CO_2 for 3 hours). Lastly, F-12 completed medium was further changed for 2 times and the samples were maintained in an incubator for 18-24 hours before using in electrophysiological patch-clamp study.

Electrophysiological patch-clamp recording

TG neuron dishes were perfused with an external solution containing 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose and 10% HEPES; which the pH was adjusted to 7.40 with 1 M NaOH and the osmolality was adjusted to 320±5 mOsm/kg by using D-glucose. For KYNA groups, 200 μM of KYNA (Sigma Aldrich, UK) was added into the external solution. Microelectrodes were filled with an internal solution containing 140 mM K-gluconate, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES and 10 mM ATP; which the osmolality was adjusted to 290±5 mOsm/kg with D-glucose.

Then, microelectrodes were inserted to the head stage of an Axopatch amplifier (Axon, Sunnyvale, CA, USA). The dishes were then placed on sample stand of microscopy (Olympus BX51WI microscope, Olympus, USA). Patch pipettes had tip resistances between 6-8 MΩ. Membrane currents including resting membrane potential (RMP) and action potential (AP) were recorded using an Axopatch 200B amplifier (Axon instruments, Foster City, CA) and clampex 10.2 software (Molecular devices).

To determine single AP properties; short period current was injected in current clamp recording mode for evaluating AP properties. Membrane potential was manually held at -60 mV. Then we injected with currents of 10 pA/step with 100 ms duration. The criteria for successful recording was a minimum 10 minutes recording time, with a stable RMP, an amplitude of action potential; and an input resistance that was higher than 100 mega-ohms. The protocol was adapted from previous studies. [43, 44]

To determine summation of AP development; long period current was injected in current-clamp recording mode for evaluating AP properties. Membrane potential was manually held at -60 mV. Then we injected with currents of -30 to 70 (5 pA/step) in 21 steps of 500 ms duration.

The size of TG neurons used in these experiments were small size (less than 22 micron) and medium size (22-29 micron). The experiments were performed at room temperature.

Data collection

Data were analyzed by Clampfit 10.2 software (Molecular Devices). Cell capacitance (Cm), resistance (Re) and resting membrane potential (RMP) were recorded at resting state.

Parameters of single AP after injection short duration current pulses from a holding potential at -60 mV were threshold, rheobase, AP height, AP overshoot, rising time, falling time after-hyperpolarization (AHP) depth and AHP duration. Threshold (mV) is a lowest membrane potential that triggered first depolarization phase of AP.

Rheobase (pA) is a minimal current injection was able to cause the depolarization phase of an AP. AP height (mV) was measured from the holding potential to peak amplitude of the AP. The overshoot of AP (mV) was measured from 0 mV to peak of AP. The rising time (msec) is a rapid of depolarization time, measuring from threshold to the peak of amplitude of an AP. The AP falling time (msec) is repolarization time, measuring from the peak of amplitude of AP to the holding potential (Fig.4). The AHP depth (mV) was measured from the holding potential to the lowest point of AP. The AHP duration (msec) was measured from the negative peak of an AHP to 50% of the recovery of the holding potential. (Fig.5)

Parameters of AP summation that were recorded by current-clamp mode included threshold, rheobase and total spikes. Total spike is number of peak lead to AP by injecting step current from 1-21 step (5pA/step) (Fig. 6)

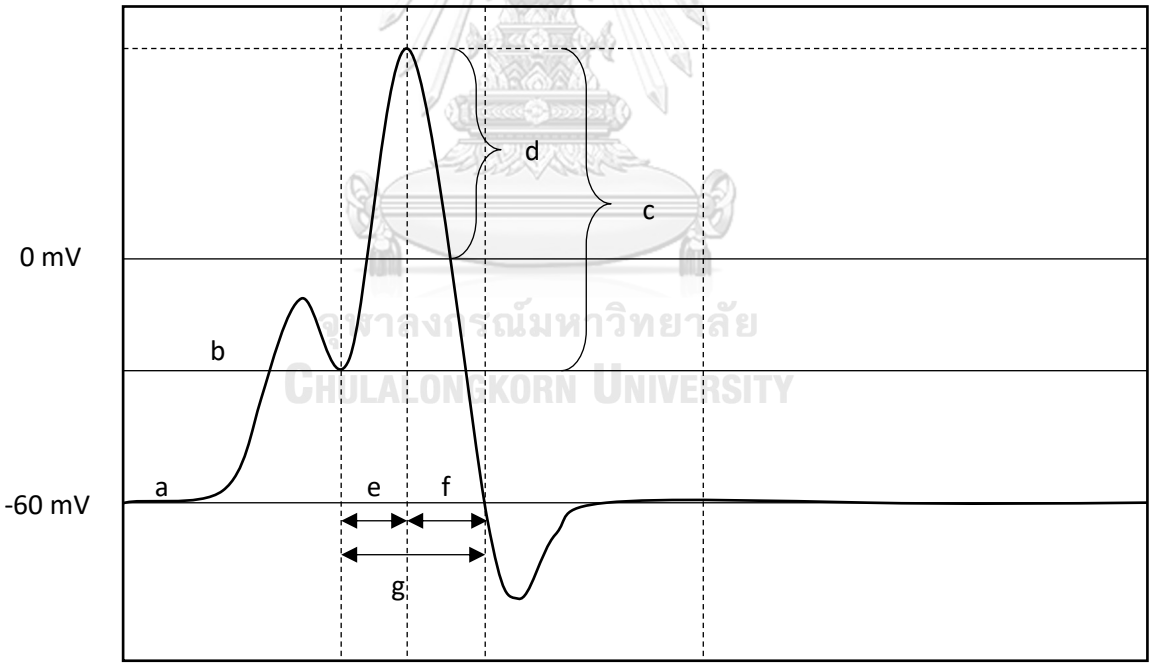


Figure 4 An Action potential parameters; a= holding potential, b= Threshold (mV), c= Action potential height (AP height), d= Action potential overshoot (AP overshoot), e= Depolarization time (ms), f= Repolarization time (ms), g= Action potential duration (ms)

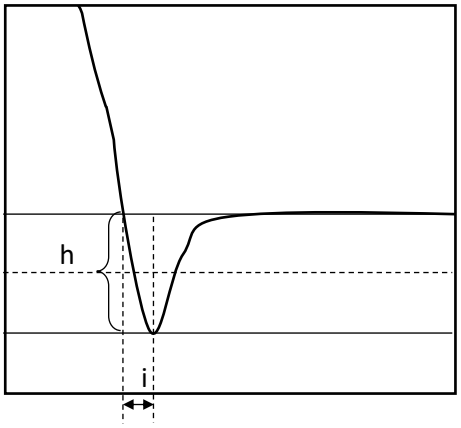


Figure 5 An after-hyperpolarization (AHP) parameters: h= After-hyperpolarization depth and i = A half of after-hyperpolarization duration.

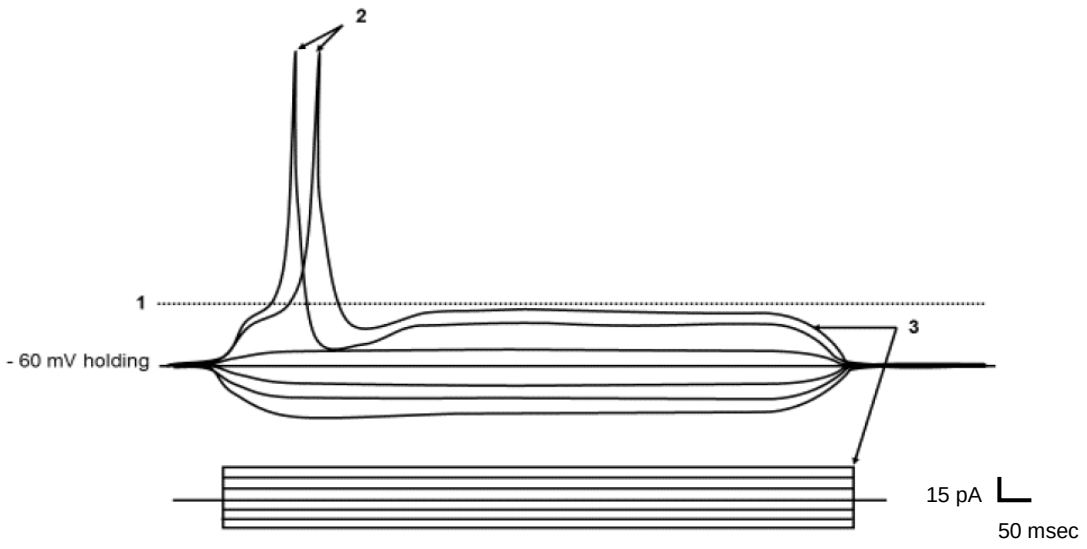


Figure 6 An illustration shown electrophysiological parameters, current in each step are 5 pA (7 steps, 2 spikes). 1= Threshold, 2=Total spikes, 3= Rheobase (15 pA).

Statistical analysis

All data were shown as means ± standard deviation (SD). Statistical analysis was done using one-way ANOVA and Tukey's post hoc test. A *p*-value of < 0.05 is accepted as indicative of a statistically significant difference

Equipment needed for the project

All experiments in this thesis were applied on electrophysiological instrument at room 920, Pattayapattana building, Faculty of medicine, Chulalongkorn University.

Benefits of this study

1. Know and understand how electrophysiological properties of TG neurons changes after applying migraine-inducing procedures
2. Know and understand how KYNA affects electrophysiological properties of TG neurons before and after applying migraine sensitization procedures.
3. Determine the potential of KYNA as anti-migraine agent based on its effect on the sensitivity of TG neurons.

Chapter 4

Results

Effect of serotonin-depleted and KCl-induced CSD on rats' TG neurons properties and effects of KYNA

Electrophysiological patch clamp recordings were obtained from 112 neurons small-to-medium size. In control group (Control) n=14 neurons, KYNA group (KYNA) n= 11 neurons, PCPA (PCPA) n= 11 neurons and PCPA with KYNA (PCPA/KYNA) n=16 neurons were included. Additionally, KCl-induced CSD group (CSD) n= 20 neurons, KCl-induced CSD group with KYNA (CSD/KYNA) n=13 neurons, PCPA with KCl-induced CSD group (PCPA/CSD) n=14 neurons and PCPA combined with KCl-induced CSD with KYNA group (PCPA/CSD/KYNA) n=13 neurons were included.

The Diameter of neurons and morphology were observed, and then measured under upright microscope. The diameter of neurons in each group were not significantly different. (*p*-value<0.831)

Table 1 Neurons diameter (micron) and resting membrane potential (RMP; mV) of neurons included in each group. Data is shown in mean±SD. (Italic value represents *p*-value<0.05 by one-way ANOVA, * *p*-value<0.05 by Tukey's post-hoc test comparing to control group)

	Control	KYNA	PCPA	PCPA with KYNA	CSD	CSD with KYNA	PCPA/CSD	PCPA/CSD with KYNA	*p*-value
n	14	11	11	16	20	13	14	13	
Diameter (micron)	24.52±2.79	25.36±2.62	24.80±2.98	24.89±2.01	24.19±2.25	25.34±2.22	24.16±2.62	24.80±2.02	0.831
RMP (mV)	-58.01±11.08	-54.75±16.42	-51.25±7.99	-47.48±9.69	-42.48±11.61*	-49.03±12.64	-42.95±9.29*	-48.20±8.70	***0.003***

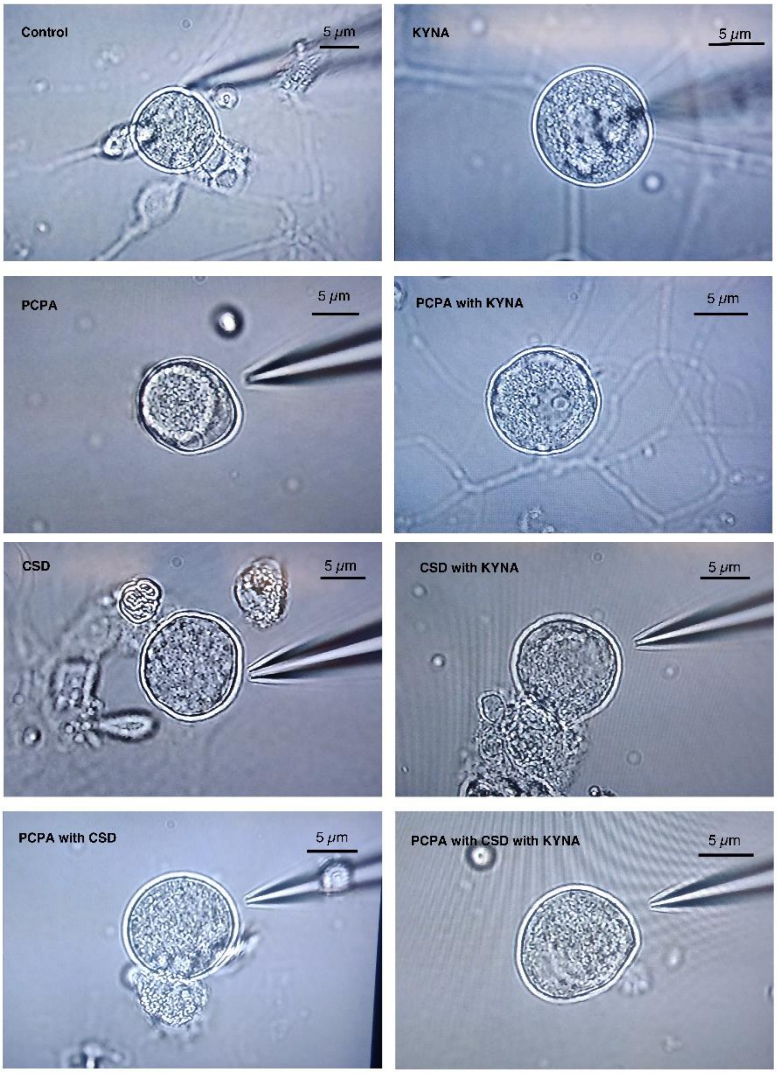


Figure 7 Morphology of TG neurons in 8 groups have shown no significantly difference in diameter of neuron, p-value=0.831, respectively.

Results in this research that had significant difference among eight groups by one-way ANOVA and Tukey's post-hoc test was presented in Table 1. Resting membrane potential (RMP) was significantly different (one-way ANOVA, *p*-value=0.003), and Tukey's post-hoc test comparing to control group (-58.01±11.08 mV) was reported. CSD group (-42.48±11.61 mV) and PCPA/CSD group (-42.95±9.29 mV) had depolarized RMP (more positive RMP) comparing to control group (*p*-value=0.003 and 0.012 respectively) (Table 1).

The results in this research have been shown as mean±SD. An action potential parameter contained threshold (mV), action potential height (APheight), action potential overshoot (APovershoot), rising time (Risetime), falling time (Falltime), action potential duration (APduration), afterhyperpolarization depth (AHPdepth) and afterhyperpolarization duration (AHPDuration).

To determine an action potential (AP) in short period of stimulation (100 ms), neurons were stimulated until cells reach their threshold. An amount of electricity that use to stimulate neurons was called rheobase, the results of rheobase in short period had no significantly differences between groups (p-value=0.183). However, it tended to be decreased in groups with KYNA (PCPA with KYNA=70.00±17.51 pA, CSD with KYNA =66.92±17.97 pA and PCPA/CSD with KYNA =53.84±15.56 pA) when comparing to each control groups (PCPA =62.72±20.04 pA, CSD =60.00±24.92 pA and PCPA/CSD =52.85±21.27 pA). When neurons reached the threshold to generates an AP, threshold had no significant differences among any group of experiments (*p*-value =0.168, Table 2). An amplitude of AP (AP height) had not any significant difference between groups (*p*-value=0.420). AP overshoot also revealed no significant difference (Table 2, *p*-value=0.581). Duration of an AP was recorded, and it was divided in rising time (depolarization time), falling time (repolarization time), nonetheless there were not any significant differences in each parameter (*p*-value=0.391, 0.681, 0.694 respectively). Afterhyperpolarization depth (AHP depth) revealed no significantly difference (*p*-value=0.338, and AHP duration also had no significant difference (*p*-value=0.638) (Table 2).

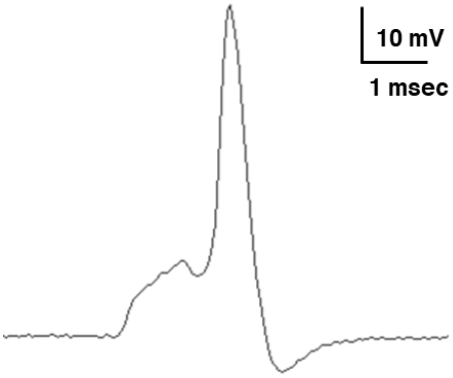


Figure 8 An action potential induced by short period stimulation, scale bar 10 mV and 1 msec.

Table 2 Single AP properties induced by short period stimulation (100ms) of neurons properties. Data is shown in mean±SD

Variables	Control	KYNA	PCPA	PCPA with KYNA	CSD	CSD with KYNA	PCPA/CSD	PCPA/CSD with KYNA	*p*-value
n	14	11	11	16	20	13	14	13	
Rheobase (pA)	57.85±20.44	54.54±8.20	62.72±20.04	70.00±17.51	60.00±24.92	66.92±17.97	52.85±21.27	53.84±15.56	0.183
Threshold (mV)	-36.57±8.98	-34.07±10.43	-33.54±7.28	-28.90±8.70	-35.01±9.92	-35.23±8.43	-32.87±11.79	-39.40±8.09	0.168
AP height (mV)	94.92±12.96	88.69±22.16	97.75±13.76	89.37±24.32	97.85±13.82	84.56±26.79	86.74±16.04	94.17±17.99	0.420
AP overshoot (mV)	58.22±16.31	54.61±16.76	61.00±8.27	60.48±21.56	62.83±13.38	51.37±22.68	53.95±17.75	54.57±18.09	0.581
AP rising time (ms)	1.02±0.61	1.08±0.50	1.13±0.63	1.56±2.19	1.46±1.02	2.14±2.79	1.65±1.64	1.82±2.27	0.681
AP falling time (ms)	1.21±0.78	1.32±0.69	1.06±0.41	1.42±0.77	1.69±1.30	1.71±1.52	1.54±0.96	1.72±1.54	0.694
Duration (ms)	2.23±1.10	2.40±1.13	2.19±0.82	3.00±2.34	3.16±1.95	3.85±3.39	3.20±1.81	3.50±2.67	0.391
AHP depth (mV)	-14.01±10.45	-17.27±11.28	-13.46±8.08	-9.18±7.39	-10.99±8.44	-12.46±8.81	-11.54±7.89	-15.17±7.52	0.338
AHP duration (ms)	0.15±0.16	0.13±0.07	0.39±0.78	0.49±1.44	0.13±0.11	0.13±0.07	0.15±0.07	0.20±0.23	0.638

AP properties that induced by long period stimulation (500 ms) were recorded and analyzed. Rheobase of TG neurons induced by long period stimulation had no significant difference between groups (*p*-value=0.661). TG neurons were stimulated until they developed train of action potentials during long period stimulation, and then threshold in long period stimulation of each neuron was recorded. Threshold potential of TG neurons in long period stimulation had no significant difference (*p*-value=0.167). Total spikes were number of action potentials' train that was developed during long period stimulation. Although total spikes had no significant difference between group (*p*-value=0.350), it tended to be decreased in groups with KYNA (KYNA=131.72±132.12, PCPA with KYNA=121.06±126.54 and CSD with KYNA=85.46±129.53) when comparing to each control groups (Control=150.14±167.89, PCPA=189.90±205.63, CSD= 96.20±119.38) except of PCPA/CSD with KYNA comparing to PCPA/CSD that KYNA increased total spikes (73.42±56.98 and 166.69±166.19 respectively) (Table 3).

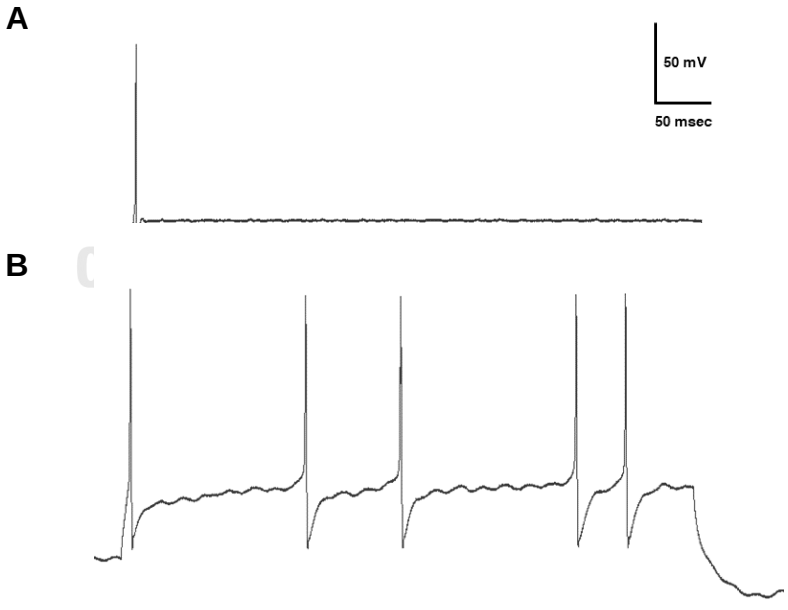


Figure 9 Represented TG responses to long period stimulation (A) showed single action potential and (B) showed multiple action potential, scale bar 50 mV and 50 msec

Table 3 Summation of AP properties induced by long period stimulation (500 ms) has shown in mean± SD, significance level at p-value<0.005.

Variables	Control	KYNA	PCPA	PCPA with KYNA	CSD	CSD with KYNA	PCPA/CSD	PCPA/CSD with KYNA	*p*-value
n	14	11	11	16	20	13	14	13	
Rheobase (pA)	14.28±9.97	11.36±6.36	13.63±11.20	17.62±13.45	13.75±11.22	17.69±11.65	12.14±7.52	11.92±10.90	0.661
Threshold (mV)	-41.50±8.69	-34.70±8.07	-40.01±7.04	-35.60±9.89	-34.26±7.71	-34.04±8.45	-34.49±8.55	-37.39±9.26	0.167
Total spike	150.14±167.89	131.72±132.12	189.90±205.63	121.06±126.54	96.20±119.38	85.46±129.53	73.42±56.98	166.69±166.19	0.350

Chapter 5
Discussion

Our results indicated that resting membrane potential (RMP) in trigeminal ganglion neurons was altered by KCl-induced CSD. RMPs in KCl-induced CSD group and PCPA with CSD group were depolarized comparing to control group, while other parameters were not significantly different. These findings revealed that KCl-induced CSD procedure magnified responses of trigeminal nociceptive pathway whether in the condition of including 5-HT depletion, or not. Since CSD is a cortical phenomenon of animal model that is used for migraine research, it associated pathophysiological changes especially migraine with aura which is consistent with previous studies. Supornsilpchai *et al.* revealed that CSD-induced in rats increased Fos protein expression in trigeminal nucleus caudalis comparing to control group, thus they concluded that effects of CSD phenomenon may increase neuronal activity and excitability through trigeminovascular pathway.[4] On the other hand, induction of 5-HT depletion by PCPA injection procedure is a systemic model. 5-HT depletion has been found in many neurological diseases such as anxiety, multiple sclerosis and migraine. Sokolov *et al.* explained that 5-HT depletion only did not have a potential to initiate migraine, despite of CSD evoked that it had a potential to initiate CSD by itself. [4, 26] Theoretically, KCl-induced CSD escalates glutamate release to be a massive release, and then a massive amount of glutamates causes NMDA receptor hyperactivity. In mammalian, some neurons in peripheral organ, such as TG neurons contains NMDA receptors resulting in the sensitization after CSD that involved by NMDA receptors. Wang *et al.* reported that there was an expression of NMDA receptors in TG neurons, and they composed of NR1 or NR2 subunits. Incidentally, NR1 subunit contains glycine-binding site, while NR2 subunit contains the glutamate-binding site. The activation of NMDA receptors after glutamate binding to NR2 subunit is induced after postsynaptic depolarization resulting in removing of Mg^{2+} block, channel open and positive charge influx (especially Ca^{2+}) through this channel in sequence, which proposed to be the

important event of positive charge increase that may be an explanation of sensitization in TG neurons.[37] As previously described, recent study revealed that CSD activates extracellular signal regulated kinase (ERK) in trigeminal ganglion. [45] Thus, CSD induction may cause ERK phosphorylation that activates transient receptor potential vanilloid 1 (TRPV1) receptor resulting in release of excitatory amino acids and increase of extracellular positive ions. This hypothesis is backed by the data of Iwashita T, et.al. They revealed that extracellular signal-regulated kinases (pERK/ERK) increases in TG neuron after CSD induction. It is known that there is an expression of TRPV1 in primary afferent sensory neurons of the pain pathway. TRPV1 is one of an important factor that activates trigeminal nociceptive system during CSD evoked. Moreover, CSD can occur massive NO release and TRPV1 hyperactivity that increases intracellular potassium concentration. Then, influx of Ca^{2+} or positive ions through TRPV1 receptor channel effects to increase of ERK phosphorylation and this may cause peripheral hypersensitivity throughout transcriptional regulation. [46] So, results of this study presumed that CSD-induced in rat causes altered TG membrane permeability due to TRPV1 activated, then depolarizes RMP in the group of CSD as same as in combined model of 5-HT depleted and CSD induced.

Even though all of AP properties did not have any significantly difference among all experimental groups, these results can be explained that CSD is a cortical model occurring direct activation of intracranial nociceptors. Nevertheless, CSD could alter RMP in TG neuron which assuming a role as a first-order neuron of trigeminal system.[47] Moreover, RMPs of TG neurons in CSD and PCPA/CSD groups were shifted to depolarization whereas, RMP of TG neurons in PCPA group which is systemic neurotransmitter change model was not altered. These findings were consistent with results of Huang F. *et. al.* that revealed RMP of TG neuron in the group of melatonin application did not have any significant alteration, as noted that melatonin is a derivative of serotonin in trigeminal pathway.[48]

In the groups of KYNA applied, my experiment revealed that RMPs shifted to depolarization (more positive) in KYNA and PCPA with KYNA groups whereas RMPs of CSD with KYNA and PCPA/CSD with KYNA groups shifted to hyperpolarization (more negative). Since KYNA is a tryptophan derivative of serotonin, it has a potential to reduce the neuronal excitability. Thus, I observed RMPs hyperpolarization shifts in CSD with KYNA and PCPA/CSD with KYNA groups. These results were consistent with previous studies by Nagy-Grocz *et. Al* and Kortesi T *et al.* that revealed KYNA inhibitory effects on CSD waves propagation. *In vivo* study revealed that pretreatment of KYNA following by electrical stimulation of trigeminal ganglion could reduce PACAP gene expression in TNC. [49, 50]

In the results of this study, all of AP properties did not change. However, it had tend of decreases in some parameters; AP height, AP overshoot of control groups comparing to those KYNA applied groups (Control vs KYNA, PCPA vs PCPA/KYNA, CSD vs CSD/KYNA), excepted the group of combined model (PCPA/CSD vs PCPA/CSD/KYNA). Decreases of AP height and AP overshoot may be caused by KYNA inhibition on NMDA receptor, resulting in less influx of Ca^{2+} or other positive ions through TG cell membrane. It is interesting to note that KYNA reduced AP height in KYNA, PCPA/KYNA, CSD/KYNA with the exception of PCPA/CSD/KYNA group which there was an increase of AP height. The presumption of these findings is that KYNA reduces the peak of action potential in TG neuron by inhibition of NMDA receptor but not in PCPA/CSD group. The results in this study are the first report of TG electrophysiological properties under the 5-HT depleted and/or CSD phenomenon conditions. The mechanism of action on this type of neuron was unclear, and the explanation on this point has been long awaited. The association between CSD and NMDA receptor in migraine has been established in 2012 by Tozzi et.al. Their study revealed that NMDA antagonist reduces CSD area and intensity in rat cortical slices. [51]

In this study, the duration of AP was divided into 2 phases; depolarization phase and repolarization phase. The mean values of depolarization phase in all groups of KYNA applied were increased when compared to those experimental groups (Control vs KYNA, PCPA vs PCPA/KYNA, CSD vs CSD/KYNA, PCPA/CSD vs PCPA/CSD/KYNA). The

mechanism during depolarization phase is open of voltage gated Na^+ channel and allowing Na^+ influx while voltage gated K^+ channel is closed. The results in this study showed that KYNA may have potential to delay an open of NMDA receptor and voltage gated Na^+ channel resulting in gradually increase of positive charge into the cell. Thus, TG neurons in the groups of applied KYNA spent a period of time to rise the depolarization phase more than those experimental groups without KYNA. On the other hand, repolarization phase is developed by opening of voltage gated K^+ channel to allow K^+ ions efflux throughout this channel, and membrane potential returns to resting stage instead of closing voltage gated Na^+ channel. The results in this study revealed that a falling time were increased in groups of KYNA applied comparing to those control groups (Control vs KYNA, PCPA vs PCPA/KYNA, CSD vs CSD/KYNA, PCPA/CSD vs PCPA/CSD/KYNA). A Falling time or repolarising phase tended to increase due to KYNA acting on NMDA receptor, and it may cause of extended time to open and/or close this channel that may affect the open of voltage gated K^+ channel. Moreover, AHP duration tended to increase in groups of KYNA applied with PCPA and PCPA combined with CSD. However, this tendency was not observed in CSD induced group. Since, AHP duration is developed by efflux of K^+ ions until reaching the equilibrium of K^+ and return to resting membrane potential, KYNA may effect on K^+ equilibrium only in the groups of PCPA but not in CSD groups. However, there were paradoxical tendency among the results of long-period stimulation. There were increases of rheobase in the groups of PCPA/KYNA and CSD/KYNA comparing to those control groups without KYNA (PCPA and CSD), but rheobase decreased in KYNA and combined model with KYNA applied comparing to those control groups (Control vs KYNA and PCPA/CSD vs PCPA/CSD/KYNA). Threshold reduced in KYNA applied of KYNA, PCPA/KYNA and CSD/KYNA groups but raised in PCPA/CSD/KYNA comparing to those control groups. The same tendency as threshold was observed in total spikes that decreased in KYNA applied of KYNA, PCPA/KYNA and CSD/KYNA groups but increased in PCPA/CSD/KYNA comparing to those control groups.

Overall, there may be possible effects of KYNA directly toward the CSD-induced groups more than PCPA groups, because KYNA may regulate through pituitary adenylate cyclase-activating polypeptide (PACAP) signalling. NMDA receptor activation results in increase of Ca^{2+} concentration inside of the cell which decreases PACAP gene expression leading to PACAP mRNA degradation. Thus, KYNA has a direct effect to the NMDA receptors leading to inhibition of NMDA function and decrease of Ca^{2+} accumulation inside of the cell, which decreases PACAP gene expression and reduces PACAP mRNA degradation. [50]

An activation of PCPA on tryptophan hydroxylase enzyme reduces all of products in serotonin pathway, while kynurenine pathway is not affected by PCPA at all. Thus, KYNA in kynurenine pathway may still remain as an endogenous substrate in PCPA-induced systemic 5-HT depletion. This study revealed that PCPA does not alter any AP parameters. The explanation of this point is that modulation of endogenous KYNA to TG neurons may compensate in the condition of 5-HT depletion. However, CSD model is different to PCPA-induced systemic 5-HT depletion model. CSD has local and direct effects to cerebral cortex as a central sensitization. Other KYNA injection protocol have to be considered for strengthening its effect. For example, injection of systemic kynurenine with probenecid induces an increase of KYNA concentration in the brain resulting in a reduction of CSD frequency. [40]

Furthermore, the controversial findings in this study can be explained by the facts from previous studies that indicated the lower of NR1 subunit expression in cortical slices of male comparing to female rat. [37] The lower of NR1 subunit expression would create an adverse result in my study, because NR1 subunit is one of the important factors that modulates the neuronal excitability. The differences among groups that may appear in the experiments would be blurred resulting in none of significant AP properties' change. Moreover, I applied KYNA by using bath application technique. It may cause the problems of deficient KYNA concentration that can affect TG responses. Thus, I suggest the further studies conducting in female rats that may reveal an AP properties' change more clearly, in difference stages of estrus cycle especially proestrus and estrus stage which is Saleeon W. *et. al.* indicated that both stages of proestrus and estrus have high level of estrogen which magnifying neuronal

excitability and sensitivity. [52] Additionally, KYNA injection has to be revised. I suggest Gavage feeding with some drugs that can carry KYNA passing blood brain barrier.

Chapter 6

Conclusion

In conclusion, due to significantly change in RMP cause of increasing in neuronal excitability after CSD-induced. Thus, KYNA tended to increase of RMP (more negative) but not seen significant differences. For AP parameters have not significantly differences, it presumed that NMDA expression in TG neuron lower than others CNS tissues.

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9. จำนวนผู้ป่วยใน (หลักประกันสุขภาพถ้วนหน้า และสวัสดิการรักษาพยาบาลข้าราชการและครอบครัว) รวมทุกการวินิจฉัยโรค จำแนกตามเพศและโรค/กลุ่มโรค 298 โรค ตามบัญชีจำแนกโรคระหว่างประเทศ ฉบับแก้ไข ครั้งที่ 10 ทั่วราชอาณาจักร พ.ศ. 2556 - 2559 [Internet]. 2016 [cited 27 February 2018]. Available from: http://statbbi.nso.go.th/staticreport/page/sector/th/05.aspx.

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VITA

NAME	Naeemah Haji-a-sa
DATE OF BIRTH	4 Jan 1993
PLACE OF BIRTH	Yala
INSTITUTIONS ATTENDED	Bachelor of Physical Therapy, School of Health Science, Mae Fah Luang University
HOME ADDRESS	833 Siroros Rd. Sateng, Muang Yala, Thailand 95000