# รูปแบบการเปลี่ยนแปลงของวงจรการเป็นสัดต่อคุณสมบัติทางไฟฟ้าสรีรวิทยาในเซลล์ประสาทเพาะเลี้ยงชนิดไตรเจมินัลแกงเกลี่ยนในระดับปฐมภูมิ

นายวชิรพงศ์ สลีอ่อน





วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
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ปีการศึกษา 2558

# The alternation pattern of estrous cycle modulating electrophysiological properties in primary-cultured trigeminal ganglia neurons

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Science
Faculty of Medicine
Chulalongkorn University
Academic Year 2015

| | |
|---|---|
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วชิรพงศ์ สลีอ่อน : รูปแบบการเปลี่ยนแปลงของวงจรการเป็นสัดต่อคุณสมบัติทางไฟฟ้าสรีรวิทยาในเซลล์ประสาทเพาะเลี้ยงชนิดไตรเจมินัลแกงเกลี่ยนในระดับปฐมภูมิ (The alternation pattern of estrous cycle modulating electrophysiological properties in primary-cultured trigeminal ganglia neurons) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ศักนัน พงศ์พันธุ์ผู้ภักดี, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. นพ. อนันต์ ศรีเกียรติขจร, 50 หน้า.

โรคปวดศีรษะไมเกรนมักเกิดขึ้นบ่อยในผู้หญิงมากกว่าผู้ชาย เพราะการผันผวนของฮอร์โมนเอสโตรเจนในรอบเดือน ซึ่งถูกเรียกว่า อาการปวดศีรษะไมเกรนสัมพันธ์กับการมีรอบเดือน เป็นผลทำให้ความถี่และความรุนแรงของอาการปวดศีรษะไมเกรนเพิ่มมากขึ้น จุดมุ่งหมายการศึกษาครั้งนี้ เพื่อตรวจสอบว่า แต่ละระยะของวงจรการเป็นสัด ซึ่งมีการผันผวนของฮอร์โมนเอสโตรเจนนั้น มีผลกับความถี่และความรุนแรงของอาการปวดศีรษะไมเกรนหรือไม่ โดยเปรียบเทียบการตอบสนองและความไว้ต่อสิ่งกระตุ้นของเซลล์ประสาทเพาะเลี้ยงไตรเจมินัลแกลงเกลี่ยน โดยประเมินจากค่าต่างๆ จากรูปร่างของศักย์ไฟฟ้าทำงานที่วัดด้วยเทคนิคโฮลเซลล์แพทช์แคลมป์รีคอร์ดดิง ผลการทดลองแสดงให้เห็นว่าเซลล์ประสาทในระยะโปรเอสตรัสและระยะเอสตรัส มีเทรสโฮลด์และรีโอเบสทีต่ำลง, ศักย์ไฟฟ้าทำงานมีความสูงมากขึ้น, ระยะเวลาของรีโพลาไรเซชันสั้นลง และในช่วงของไฮเปอร์โพลาไรเซชั่นลึกมากขึ้น นอกจากนี้ผลรวมของการเกิดศักย์ไฟฟ้าทำงาน ยังชี้ให้เห็นว่าเซลล์ประสาทในระยะโปรเอสตรัส และระยะเอสตรัส มีเทรสโฮลด์และรีโอเบสของการกระตุ้นให้เกิดศักย์ไฟฟ้าทำงานที่ต่ำลง และมีการเพิ่มขึ้นจำนวนศักย์ไฟฟ้าทำงาน เมื่อเทียบกับเซลล์ประสาทในระยะไดเอสตรัส ผลของการศึกษานี้ แสดงให้เห็นว่า ความผันผวนของระดับฮอร์โมนเอสโตรเจนในระยะโปรเอสตรัสและระยะเอสตรัสมีผลต่อการเปลี่ยนแปลงคุณสมบัติของศักย์ไฟฟ้าทำงานและมีผลต่อผลรวมการเกิดศักย์ไฟฟ้าทำงานในเซลล์ประสาท ระดับฮอร์โมนเอสโตรเจนที่สูงนั้น อาจก่อให้เกิดการเพิ่มขึ้นของการตอบสนองและความไว้ต่อสิ่งกระตุ้นในเซลล์ประสาท ซึ่งเป็นผลมาจากการปรับสภาพช่องทางไอออนที่เปิดโดยศักย์ไฟฟ้าและความไวผิดปกติที่ประสาทปลาย การค้นพบนี้ สามารถอธิบายความสัมพันธ์จากความผันผวนของระดับฮอร์โมนเอสโตรเจนระหว่างรอบเดือน เชื่อมโยงกับโรคปวดศีรษะไมเกรนที่สัมพันธ์กับการมีรอบเดือน

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

# # 5674067230 : MAJOR MEDICAL SCIENCE
KEYWORDS: MENSTRUAL MIGRAINE, ESTROUS CYCLE, TRIGEMINAL GANGLION (TG) NEURONS, VOLTAGE-GATED ION CHANNELS, PERIPHERAL SENSITIZATION, WHOLE-CELL PATCH CLAMP RECORDING

WACHIRAPONG SALEEON: The alternation pattern of estrous cycle modulating electrophysiological properties in primary-cultured trigeminal ganglia neurons. ADVISOR: ASSOC. PROF. SAKNAN BONGSEBANDHU-PHUBHAKDI, Ph.D., CO-ADVISOR: PROF. ANAN SRIKIATKHACHORN, M.D., 50 pp.

Migraine occurs more frequently in women than men. Many women suffer from migraine attacks during menstruation, which are known as menstrual migraine. The aim of this study was to investigate whether different stages of the estrous cycle are involved in migraine development by comparing the excitability and sensitivity of trigeminal ganglion (TG) neurons in four different stages of the estrous cycle by using action potential (AP) parameter assessments performed with whole-cell patch clamp recordings. The result showed that TG neurons in the proestrus and estrus stage had lower AP threshold, lower rheobase, higher AP height, shorter AP falling time and deeper after-hyperpolarization (AHP) depth. In addition, The summation of AP development indicated that TG neurons in proestrus and estrus stages exhibited significantly lower thresholds and rheobase of stimulation, and significant increase of total spikes compared with the TG neurons in the diestrus stage. Our results revealed that fluctuation of estrogen levels in the proestrus and estrus stages altered the single AP properties and summation of AP development of TG neurons. High estrogen levels in proestrus and estrus stages of the estrous cycle may induce increases of neuronal excitability and sensitivity in TG neurons that result in modulation of voltage-gated ion channels and peripheral sensitization. These findings may provide an explanation for the correlation of estrogen fluctuations during the menstrual cycle with the pathogenesis of menstrual migraine.



Field of Study: Medical Science
Academic Year: 2015

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## ACKNOWLEDGEMENTS

The research was supported by the Research Unit and Center of Excellence for Neuroscience of Headache, Integrated Innovation Academic Center, the 2012 Chulalongkorn University Centenary Academic Development Project, the National Research University Project, the Office of Higher Education Commission (WCU-008-HR-57), the Government Research Budget 2014-2019 and the Ratchadapiseksompoj Fund from the Faculty of Medicine, Chulalongkorn University.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| 5HT | 5-hydroxytryptamine receptor |
| µl | microlitre |
| µm | micromete |
| ANOVA | analysis of variance |
| AP | action potential |
| AHP | after-hyperpolarization |
| CGRP | calcitonin gene related peptide |
| CMIA | Chemiluminescent Microparticle Immunoassay |
| DRG | dorsal root ganglion |
| D | diestrus |
| E | estrus |
| E1 | estrone |
| E2 | estradiol |
| E3 | estriol |
| ER | estrogen receptor |
| ERE | estrogen responsive element |
| ERK | extracellular-signal-regulated kinase |
| FSH | Follicle stimulating hormone |
| GnRH | gonadotropin-releasing hormones |
| HBSS | hank's Balance Salt Solution |
| ICHD II | the International Classification of Headache Disorders-II |
| K | potassium |
| LH | luteinizing hormone |
| M | metestrus |
| ms | millisecond |
| mV | millivolt |
| pA | picoampere |
| MAP | mitogen-activated protein kinase |
| SEM | standard errors of the mean |
| NMDA | N-methyl-D-aspartate |
| Na | sodium |
| NGF | nerve growth factor |

| | |
|---|---|
| nNOS | neuronal nitric oxide synthase |
| P | proestrus |
| PNS | peripheral nervous system |
| PKA | protein kinase A |
| PKC | protein kinase C |
| RMP | resting membrane potential |
| SD | standard deviation |
| SP | substance P |
| SRE | serum response element |
| TG | trigeminal ganglion |
| TNC | trigeminal nucleus caudalis |
| TPH | tryptophan hydroxylase |
| TRPV1 | transient receptor potential cation channel V1 |

# CHAPTER I

# INTRODUCTION

Migraine more often occurs in women than men. More than 50% of women experiences migraine that is associated with menstrual cycle. The menstrual cycle augments various conditions of migraine, such as the severity, duration and frequency of painful migraine attacks. (1-4). Previous study revealed that menstrual cycle fluctuates hormone levels during the progress of menstrual migraine. Migraine attacks always reach a peak before or after the onset of menstruation (5). Estrogen level fluctuates during the menstrual cycle that is separated to follicular and luteal phases. In female rats, the estrous cycle is the recurring physiologic changes that are induced by reproductive hormones in female rats. Estrous cycle has major four stages; diestrus, proestrus, estrus and metestrus stages(6). Estrous cycle has shorter duration than human menstrual cycle (4-5 days and 28 days, respectively), but hormonal fluctuation of rats estrous cycle is roughly similar to human menstrual cycle. In both cycles, there are marked augment followed by rapid decline of estrogen levels before the initiation of ovulation at the latter half of preovulatory phase. During postovulatory phase, estrogen level fluctuates again, and then returns to an initial level at the end of ovarian cycle (7). Thereby, the rat estrous cycle is suitable to be an animal model for study the effects of estrogen level in the menstrual cycle women.

Cyclical fluctuation of estrogen level occurs during estrous cycle progression. Estrogen level steadily rises during the diestrus stage and peak during the proestrus stage. Subsequently, estrogen level rapidly drops and then slowly rises to reach a plateau at the estrus stage. From the plateau during the estrus stage, the estrogen level steeply decreases during the metestrus stage and then increases during the progression towards the diestrus stage. Estrous cycle has been implicated as a model of menstrual migraine because high levels of estrogen during the proestrus and estrus stage can enhance the excitability of neurons in the

trigeminal nucleus caudalis (TNC), which resembles migraine development in menstrual migraine (8). Furthermore, estrogen exposure also increases the sensitization of the temporomandibular branch, which is innervated by TG neurons. Previous studies showed that the high levels of estrogen related to the induction of migraine attacks by increasing pain perception in the trigeminal nociceptive pathway. Estrogen probably heightens the activation of TG neurons via estrogen receptors (ERs) that results in peripheral sensitization. Furthermore, the examination of electrophysiological has shown that the total numbers of APs in TG neurons are enhanced by chronic administration of estrogen.

In the trigeminal nociceptive system, ERs expressing on TG neurons are activated by estrogen via either genomic or non-genomic pathways. Estrogen modulates neuronal activity through the expression of ion channels or intracellular cascades, such as extracellular-signal-regulated kinase (ERK) signaling, which phosphorylates various types of ion channels. (9, 10). In addition, previous studies demonstrated that exogenous estrogen activates voltage-gated Na channels and voltage-gated K channels in dorsal root ganglion (DRG) cells (11). In other words, activation of voltage-gated ion channels affects AP development in TG neurons, which is a major nociceptive signal from the periphery to higher cortical neurons. Thus, fluctuation of estrogen level during the estrous cycle may correlate to the change of nociceptive inputs from trigeminal system by altering AP development in TG neurons.

Thus, our study aimed to investigate whether the estrous cycle is involved in neuronal excitability and sensitivity of trigeminal nociceptive system by comparing electrophysiological properties of TG neurons in four different stages of estrous cycle. The single AP properties reflect the activation of voltage-gated ion channels that involves in neuronal excitability of TG neurons, while summation of AP development reflects sensitivity of TG neurons that involves in peripheral sensitization. Our results suggested that alteration pattern of estrogen level in estrous cycle modulates electrophysiological properties of TG neurons that may underlie the pathophysiology of menstrual migraine.

## Research questions

Does the estrous cycle have an effect on electrophysiological properties of TG neurons in four different stages of the estrous cycle?

## Objectives

To investigate the effect of estrous cycle in excitability of TG neurons by determining AP properties those reflect activation of voltage-gated ion channels.

To investigate the effect of estrous cycle in sensitivity of TG neurons by determining summation of AP development that reflects peripheral sensitization.

## Hypothesis

The alteration pattern of estrogen level in estrous cycle may modulate excitability and sensitivity of trigeminal nociceptive system that can examine by changes of electrophysiological properties in primary-cultured TG neurons.

# CHAPTER II

# LITERATURE REVIEWS

## Menstrual migraine

Migraine is a primary headache disorder that is important to public health. Migraine is remarkable characterized by unilateral location lasting for 4 to 72 hours, pulsating or throbbing pain, moderate or severe intensity, and worsened by physical activity. Migraine is divided into two major subtypes; migraine without aura and migraine with aura. Migraineurs are the people who are suffering by migraine. Migraineurs has been estimated that about 14.70% of population that are 10.68% of male and 18.79% of female (1). Epidemiological studies have documented its high prevalence and high socio-economic and personal impacts. Migraine is now ranked by the World Health Organization as number 19 among all diseases world-wide causing disability. In the United States, the prevalence rate of migraine attacks per one year are 17.5 % in female, 8.6 % of male and 13.2% in overall, and the prevalence rate is highest between the age of 25 to 55 (2). In Europe, 14% of population are suffered from migraine that are 7.5 % of adult male and 16.6 % of adult female (3). Asia female are suffered by migraine more than male in the similar prevalence rate of United States and Europe (4). The percentages of 60-70 of these women are suffered from migraine during menstruation, which is called “menstrual migraine”. Menstrual migraine always associates with fluctuation of estrogen level in menstrual cycle. Migraines attacks of female often decrease in a pregnancy period. However, some female still had a migraine attack during the three months of pregnancy, and usually disappear after the third month of pregnancy. The mechanism of menstrual migraine is still unclear. However, it is believed to be a neurovascular disorder that relates to an increase of neuronal excitability during menstruation.

## Classification of menstrual migraine

Menstrual migraine is classified into two subtypes following The International Headache Society (12). Menstrual related migraine have an onset during the peri-menstrual time period (2 days before to 3 days after the onset of menstruation), and this pattern must be confirmed in 2/3 of menstrual cycles, but other migraine attacks may occur at other times of the menstrual cycle. Attacks of pure menstrual migraine are similar to the above criteria except that migraine headaches are strictly limited to the peri-menstrual time period and do not occur at other times of the month. The prevalence of menstrual related migraine without aura ranges from 35-51% of females, while that of pure menstrual migraine without aura varies from 7-19%. There are many explanations for pathophysiology of menstrual migraine, but it is not fully understood. Migraine is a complicated phenomenon that has multiple causes. However, it should include dysfunctions of cerebral blood vessels. It is believed that an abnormal activity of cortical neuron may initiate migraine attack and the aura phase. (13). Migraine may result from activation of nociceptors in the terminal afferent that correlates to releases of substance P (SP), serotonin, and calcitonin gene related peptide (CGRP) from sensory synaptic fibers (14, 15).

## Menstrual cycle and migraine

Migraine that occurs in female specifically at the time of menstruation is called menstrual migraine. The International Classification of Headache Disorders-II (ICHD II) describes a new criteria of menstrual migraine in 2004, that divided menstrual migraine into two subtypes, pure menstrual migraine that occurs at a period on day 1 ± 2 of menstruation, and menstrual related migraine that occurs at the other period of the cycle (12, 16). Although migraine prevalence in boys and girls is similar until puberty, girls show a significant migraine development in puberty in the ratio of 3:1 compared with boys. The tendency continues to the age of 50 in the time of menopause (17, 18). (17, 18). In female, the most prevalent of migraine development presents in aged 30-40 years old (19, 20), and about 60% of migraine attacks relates to menstrual cycle that they happen at the beginning of menstruation (21). Female sex hormones are noted as the most important trigger in menstrual migraine. Indeed, menstruation is one of the most significant risk factors of migraine without aura. In healthy subjects, pain thresholds and pain tolerance are examined by various stimuli such as heat, pressure and chemical irritants that reveals lower threshold of pain in female compared with male (22-24). Hormonal events, such as pregnancy and post menopause, associated with high or low hormone states. High estrogen states may associate with an increase of migraine with aura during the normal menstrual cycle.

Menstrual migraine is associated with oscillation of estrogen concentrations and abnormal change of neurotransmitters such as serotonin or CGRP (15). mRNA expression of tryptophan hydroxylase (TPH) test reveals increasing of TPH mRNA in estrogen administration group, while progesterone administration group is not statistically different. In animal, there is several studies report that female rats are more sensitive to acute pain than male rats. These results which suggest that fluctuation of estrogen level may increase the expression of peripheral NMDA receptors in TG neurons of female rats. In estrous cycle, the c-fos expression in TG neurons increases at proestrus stage

suggesting that pain perception reaches the peak at proestrus. However, progesterone has not been shown to significantly influence to pain and inflammation in male and female rats.

## Estrogen (Hormonal change effects in migraine)

Estrogen is one of a steroid hormone. Although the level of estrogen fluctuates during the menstrual cycle, estrogen level is always higher in female than in men. Estrogen is a basic regulator of several organs of the body. Estrogen controls the growth of cells, regulates the differentiation of cells, influences cellular metabolism and modulates cardiovascular function, bone formation, hemostasis, water retention, salt balance and metabolism. (25). In female, estrogen is primarily produced by the ovary. Follicle stimulating hormone (FSH) stimulates granulosa cells of the ovarian follicles and corpora lutea. Some estrogens are also produced in other tissues such as brain, liver, adrenal glands and breast. Estrogen is synthesis from cholesterol. The precursors of estrogen are estrone and testosterone which are converted to 17beta-estradiol by 17beta-HSD and aromatase, respectively. The specific source of 17beta-estradiol synthesis is at the ovary; however, aromatase can be found in the brain and partially can cross the blood brain barrier from other source. In CNS, specific cell, such as neurons and astrocytes can synthesis estrogen in the brain. In generally, estrogen is neuroprotective peptide that prevents neuronal cell death in response to hypoxia (26).

Estrogen is divided into three major types that are estrone (E1), estradiol (E2), and estriol (E3). The action of estrogen can regulate several biological functions which are triggered by both genomic and non-genomic mechanisms (27, 28). Estrogen acts via the estrogen receptor (ER).The intracellular estrogen receptors (ER-alpha and ER-beta) are widely expressed throughout the central nervous system. Binding of estrogen with its receptors induces an estrogen responsive element (ERE) and initiates gene transcription. Moreover, estrogen activates intracellular pathways, ion channels and other membrane receptors **(Figure 1)**. ER expresses in various parts of the nociceptive

pathway. Several studies have shown that ER-alpha is expressed only in the small nociceptive neurons in rat DRG, while ER-beta is expressed in all DRG neurons (29). Indeed, small DRG neurons contain neuropeptides that involves in nociceptive process, such as SP and CGRP. While SP expresses mainly in small neurons of the DRG, CGRP has a wider distribution and is found in both small and medium sized neurons (30). The level of estrogen increases neuronal excitability by up-regulating N-methyl-D-aspartate (NMDA) receptor gene expression (10). Estrogen may affect neuronal excitability and decrease the threshold of migraine attack. Furthermore, the role of estrogen receptors involving in the trigeminal nociceptive system may be the important key of migraine pathogenesis.

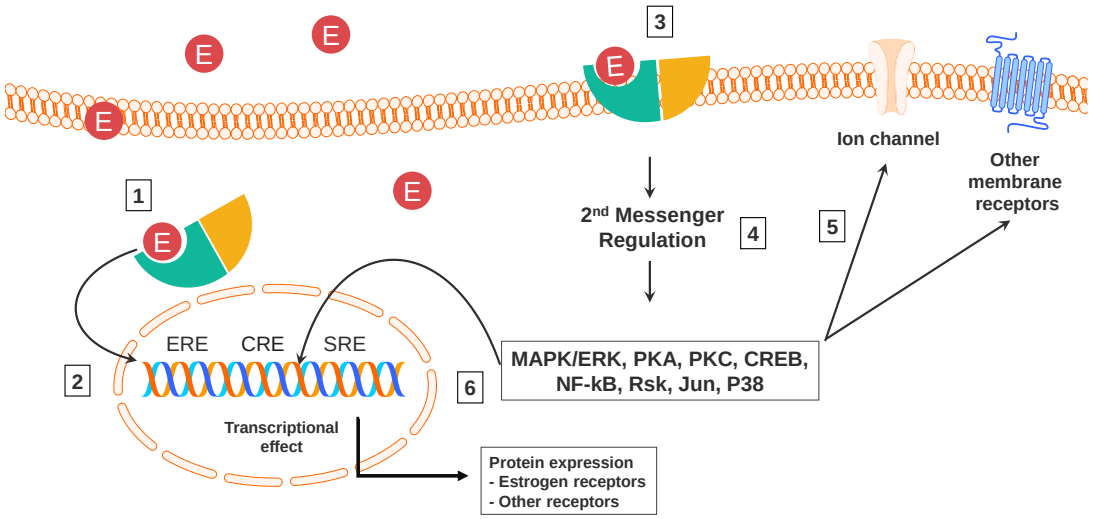


**Figure 1** Genomic and non-genomic effects of estrogen. Estrogen exerts an effect on the nervous system by genomic and non-genomic mechanisms. Genomic mechanism occur with the following steps; 1. Estrogen binds to an intracellular estrogen receptor (ER), 2. ER complex translocates to the nucleus and then binds to the estrogen response element (ERE) leading to transcription. Non-genomic mechanism occur with the following steps; 3. Estrogen binds to ER on a membrane, 4. Second messengers, such as protein kinase A (PKA), protein kinase C (PKC) are activated. 5. These second messengers phosphorylate ion channels and other membrane receptors. 6. Second messengers activate cAMP response element (CRE) and serum response element (SRE) regions of DNA that starts the process of transcription and protein expression.

## Estrous cycle; an animal model of menstrual cycle

The hormonal fluctuation in human reproductive cycle is similar to rat model. Estrogen level markedly increases in the preovulation and then rapid falling down before initiation of ovulation. At the early period after ovulation, estrogen level continuously falling down and stable in proestrus stage. At the estrus stage, estrogen level gradually increases and falling down again. There are same points of reproductive cycle between human and rat, while the shorter cycle in rat compared with in human (4-5 days and 28 days, respectively)(31, 32) **(Figure 2)**. Both menstrual cycle and estrous cycle include events that are controlled by a hormonal secreted from the pituitary gland and ovary. In human, one menstrual cycle is usually 28 days. Menstrual cycle begins when cells in the hypothalamus neurons secrete gonadotropin-releasing hormones (GnRH) which stimulate the production and release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by anterior pituitary gland. Menstrual cycle is divided into follicular and luteal phases. The follicular phase begins from the first date of menstruation to early ovulation, while the luteal phase starts from early ovulation to the end of menstruation. Each phase is subdivided into early, middle and late stages correlated with serum ovarian hormone levels. Estrogen is one of the ovarian hormones that increase its level to peak from middle to late stages of follicular phase and then it will slightly decrease.

Female rats, must have a 4 or 5 days of estrous cycle that is the most rapid ovarian cycles in mammals. We can determine estrous cycle phase by vaginal smear, as well as the direct method by measuring of estrogen concentration in the serum. However, hormonal changes associated with ovarian cycle also causes changes of cellular characteristics in vagina. Thus, the observation of changes in vaginal epithelial cells can lead us to know the period of ovarian cycle. In the rat estrous cycle, estrogen level is highest at proestrus, when progesterone level is lowest. At the diestrus stage, estrogen level is lowest while progesterone level is highest. In the estrus stage, estrogen level arises, but it drops precipitously as the day progresses. The estrous cycle is divided

in of four stages; metestrus (M), diestrus (D), proestrus (P), and estrus (E). Each of these stages has varying lengths (M, 6–8 h; D, 55–57 h; P, 12–14 h; and E, 25–27 h, respectively)(32), when the female rat is in proestrus, mostly nucleated and some cornified epithelial cells are present. Some leukocytes present if the female is in early proestrus stage. As the stage of the cycle advances to estrus stage, mostly cornified epithelial cells present. If the cycle is not interrupted by pregnancy, pseudopregnancy, or other phenomena, metestrus stage will begin. Metestrus stage is a brief stage when the corpora lutea forms but fails to fully luteinize due to a lack of progesterone. The uterine lining will begin to slough and evidence of this is seen in the form of cornified epithelial cells and polymorphonuclear leukocytes presented in vaginal swabs. Some nucleated epithelia cells will also present in late metestrus stage. Diestrus stage is the longest stage of rat estrous cycle lasting more than 2 days. Vaginal swabs during diestrus stage show primarily polymorphonuclear leukocytes and a few epithelial cells during late diestrus stage. Leukocytes remain the predominant cell type having removed cellular debris.

## Description of stages of estrous cycle and hormonal considerations

1. Metestrus stage: There are a mix of cell types with a predominance of leucocytes and a few nucleated epithelial and/or cornified squamous epithelial cells. Plasma estrogen concentration is low.

2. Proestrus stage: There are a predominance of nucleated epithelial cells. These cells may appear in clusters or individually. Occasionally, some cornified cells may appear in the sample. This stage corresponds to the preovulatory day, when estrogen increases and consequently, during the night, LH and FSH surge and ovulation occurs.

3. Estrus stage: This stage is distinctively characterized by cornified squamous epithelial cells, which present in clusters. There is no visible nucleus; the cytoplasma is granular; the shape is irregular. Estrogen remains elevating throughout and falls back to basal level.

4. Diestrus stage: This stage consists predominant distribution of leukocytes. During this stage, estrogen levels start to increase. During estrus, metestrus and diestrus stages, the plasma circulation of LH and FSH are continuously lower than proestrus stage.

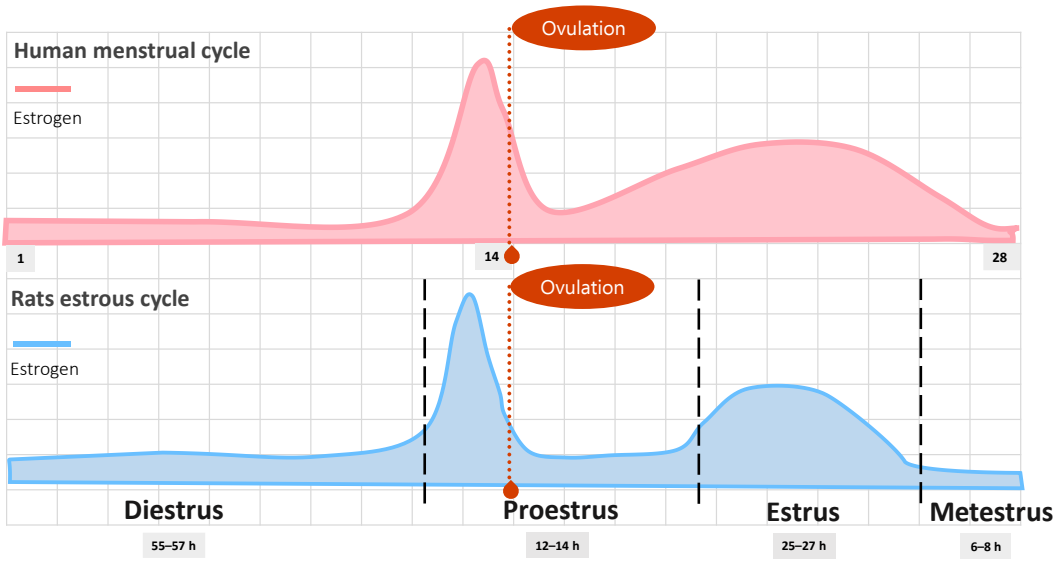


**Figure 2** Estrogen fluctuations during human menstrual cycle and rat estrous cycle. Human menstrual cycle lasts 28 days, while rat estrous cycle is consisting of 4 to 5 days. Estrogen levels are lower and non-fluctuating during the metestrus and diestrus stages, however serum estrogen level abruptly increases and reaches the peak at proestrus stage, and then decline rapidly before ovulation. Estrogen level continuously increases to plateau in estrus stage.

## Involvement of trigeminal system in migraine

The trigeminal system is an important functional area that is included in the pain perception pathways. Trigeminal system is the main sensory information for head and face. The cell bodies are located in TG and give a nerve fiber projecting to peripheral site and another to CNS. TG receives sensory information from three major branches; 1) Ophthalmic branch that receives sensation from cutaneous to upper face, tip of the nose, upper cornea, nasal cavity, frontal sinuses and parts of the meninges, 2) Maxillary branch that receives sensation from middle face (lower lid, side of nose, upper lip and skin under eyelid), upper teeth, palate and nasal cavity, and 3) Mandibular branch that receives sensation from temporal region to side of head and lower face (anterior of tongue, the lower lip, chin, jaw and the lower teeth and gums). Each of these divisions innervates specific region of structures of face and head. These three branches detect specific noxious stimuli (pain, temperature and touch) from the skin of the face, forehead, nose, teeth, cerebral blood vessels, dura mater and in the posterior area of head and neck. All sensory fibers from trigeminal ganglia pass through in the principal sensory nucleus of the brainstem, and then rise to the thalamic nucleus that spread to the terminal point at cerebral cortex (Figure 3) (33).

The trigeminal ganglion (TG) contains the cell bodies in PNS which receive a variety of sensory information from area of the head. This ganglia can classify into nociceptive and non-nociceptive neurons. In diameter of neurons, there are three groups, the nociceptive neurons are defined as small (15-24 um) and medium (25–38 um) size. The non-nociceptive is higher than 38 um (34).

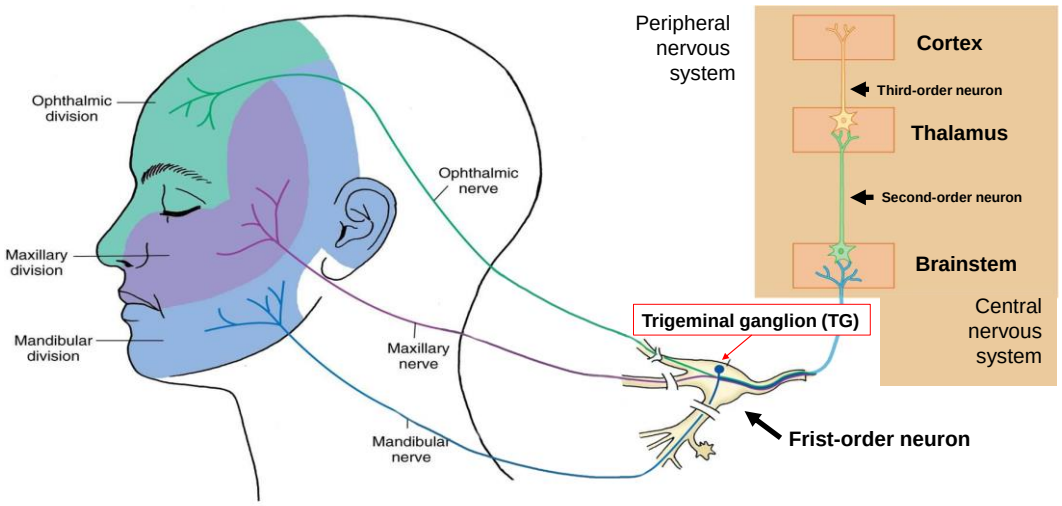


**Figure 3** Trigeminal nociceptive system. Trigeminal system is the main signaling pathway that transmits sensory information from head and face. The first-order neurons are located in the trigeminal ganglion, and give nerve fibers projecting to central nervous system. Trigeminal ganglion receives sensory information from three major branches; the ophthalmic branch that contains sensory information from cutaneous to upper face, the maxillary branch that contains sensory information from middle face, and the mandibular branch that contains sensory information from temporal region to side of head and lower face. These three branches detect specific noxious stimuli; pain, temperature and touch. Sensory fibers from trigeminal ganglion passes through in the principal sensory nucleus of the brainstem that locates second-order neurons of trigeminal system. Subsequently, third-order neurons in thalamic nucleus receives sensory information from brainstem, and transmits them to the terminal point at cerebral cortex.

## Pain transmission in trigeminal pathway

Trigeminal pain pathway starts from peripheral nervous system (PNS) that transmits sensory information from nociceptors to sensory ganglia. Sensory information from specific regions of the face and head is conveyed by sensory fiber passing through TG that contains the first order neurons, and then the first order neurons spread nerve fibers to form synapses with second order neurons that located in TNC. Next, TNC neurons form synapses with thalamic neurons that are third order neurons. Finally, thalamic neurons spread the radiation fibers to form synapses with neurons in the cerebral cortex that processes all information of pain perception(13)

## Electrophysiological properties

In the study of excitable and sensitivity cells in neurons, the patch clamp technique is a standard laboratory technique used to determine electrophysiological properties of neurons. In the patch clamp technique, action potentials (AP) are elicited by square pulse depolarizing current. Patch clamp are described for estimating electrophysiological properties of neurons; such as threshold, rheobase, AP height, AP rising time, AP falling time, AP duration, after-hyperpolarization (AHP) depth and AHP duration. The threshold of AP (mV) is characterized by the minimum current for eliciting a single spike Lower threshold indicates higher neuronal excitability. The rheobase of AP is defined as the minimum current injected to elicit a single AP. Lower rheobase (pA) indicates higher neuronal excitability. The AP height (mV) is measured as the elevation of an AP from holding potential to peak amplitude of the AP. Higher AP height indicates higher neuronal excitability that $Na^+$ equilibrium potential of neuron is raised. The AP rising time (ms) is measured as the duration of the rapid depolarization phase that is calculated from time between threshold and peak amplitude of an AP. Longer AP rising time indicates higher neuronal excitability that Na channel open time is extended that results in increase of Na influx. The AP falling time (ms) is measured as the duration of the less positive phase

that is calculated from time between peak amplitude of an AP and returning point to holding potential. Shorter AP falling time indicates higher neuronal excitability that K channel open time is shortened that results in decrease of K efflux. The AHP depth (mV) is the de-escalation of an AHP measured from the holding potential to the negative peak of an AHP. Deeper AHP depth indicates massive flux of K to deeper K+ equilibrium potential of neuron. The AHP duration (ms) is the duration time from the negative peak of an AHP to 50% of the recovery of the holding potential. Longer AHP duration indicates longer K channel open time.

# Conceptual framework

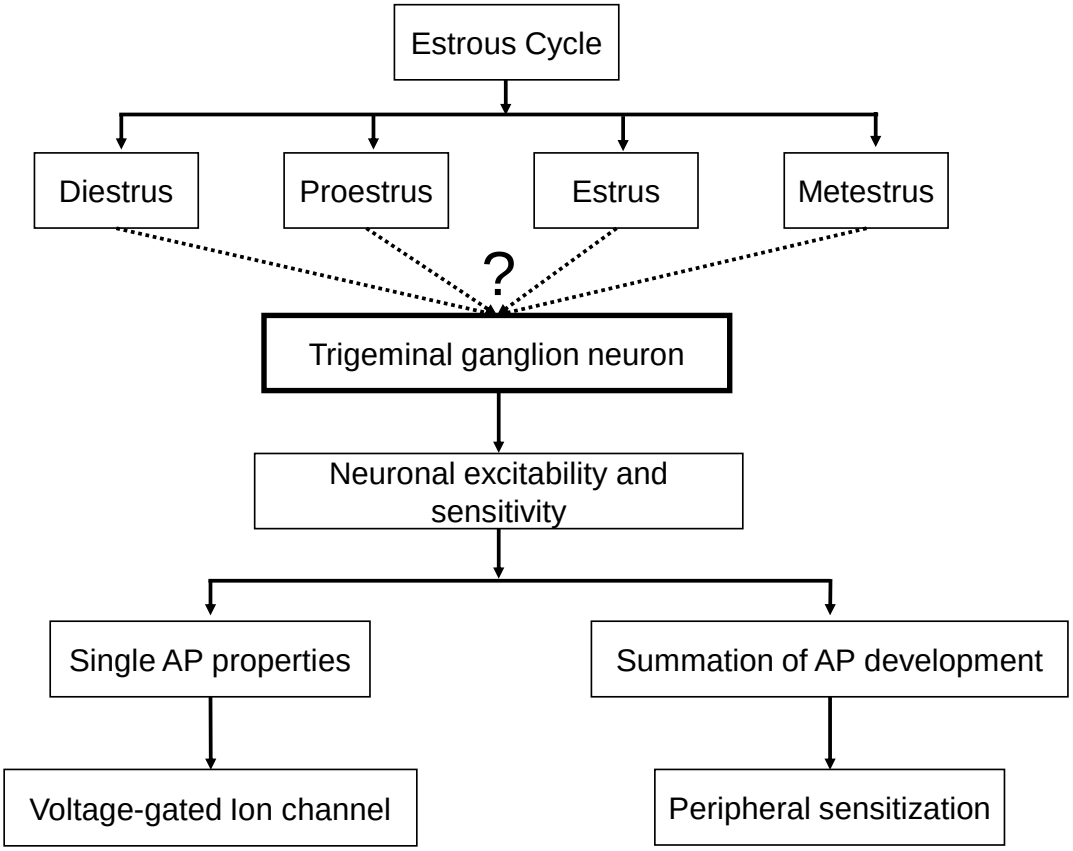


**Figure 4** Conceptual framework

# CHAPTER III

# MATERIALS AND METHODS

## Materials

### 1. Chemicals

Adenosine 5'-triphosphate
Calcium chloride ($CaCl_2$) (Merck, Germany)
Ethylenglycol-bis-(2-aminoethylethyl)-tetraacetic acid (EDTA) (Sigma-Aldrich, MO, USA)
Glucose (Sigma-Aldrich, MO, USA)
HEPES free acid (Sigma-Aldrich, MO, USA)
Laminin (Sigma-Aldrich, MO, USA)
Magnesium chloride ($MgCl_2$) (Sigma-Aldrich, MO, USA)
Poly-d-lysine (PDL) hydrobromide (Sigma-Aldrich, MO, USA)
Potassium chloride (KCL) (Sigma-Aldrich, MO, USA)
Potassium gluconate (K-gluconate) (Sigma-Aldrich, MO, USA)
Potassium hydroxide (KOH) (Merck, Germany)
Sodium chloride (NaCl) (Sigma-Aldrich, MO, USA)
Sodium hydroxide (NaOH) (Merck, Germany)

### 2. Drugs

Sodium pentobarbital (Ceva Sante Animale, France)

### 3. Media

Collagenase type IV (Invitrogen, Carlsbad, CA, USA)

Dispase II (Invitrogen, Carlsbad, CA, USA)
Fetal bovine serum (FBS) (Gibco, Grand Island. NY, USA)
Glutamax (Gibco, Grand Island. NY, USA)
Ham's F-12 (Gibco, Grand Island. NY, USA)

Hank’s balanced salt solution (HBSS) (Gibco, Grand Island. NY, USA)
Leibovitz’s L-15 medium (L-15) (Gibco, Grand Island. NY, USA)
Papain (Sigma-Aldrich, MO, USA)
Penicillin (1000U/ml)/Streptomycin (10000ug/ml) (Gibco, Grand Island. NY, USA)

## 4. Materials

Borosilicate glass pipettes (1.5 mm OD x 0.86 mm ID) (Sutter Instruments, Navato, CA, USA)
Calibrate graticule (Pyser, Kent, UK)
Glass Pasteur pipette (BRAND, Wertheim, Germany)
35 m x 10 mm dish (Corning, NY, USA)

## 5. Instrumental devises

Bravia LCD TV BX300 (Sony Thai Co., Ltd, Thailand)
BX51WI fixed-stage upright microscope (Olympus Corporation, Japan)
Class II biological safety cabinet model AC2 (Esco Global, Singapore)
Digitdata 1440 series interface (Axon instrument, Foster City, CA, USA)
Flaming/Brown micropipette puller (P-97) (Sutter Instruments, Navato, CA, USA)
Fiske®210 micro-osmometer (Advanced Instruments, Inc., MA, USA)
High output vacuum/pressure pump (EMD Millipore Corporation, MA, USA)
MP-385-2 Micromanipulator (Sutter Instruments, Navato, CA, USA)
Narishige MF-830 microforge (Narishige, Japan)
Patch clamp amplifier: Axopatch 200B (Axon instrument, Foster City, CA, USA)
Peristaltic pump: Minipuls 3 (Gilson, S.A.S., Villiers le Bel, France)
Rotina 420 R (Hettich Istruments, Bäch, Swizerland)
Software for data acquisition: Clampex 10.2 (Axon instrument, Foster City, CA, USA)
Software for data analysis: pClampfit 10.2 (Axon instrument, Foster City, CA, USA)

Super HAD CCD II camera (Sony Thai Co., Ltd, Thailand)
Vibration isolation platform (Newport Corporation, CA, USA)

## Experimental designs

We investigated whether different four stages of estrous cycle may involve in neuronal excitability of TG neurons by performing electrophysiological recording. Female rats were separated into four experimental group following four different stages of estrous cycle. We used adult female rats (6-8 weeks), the stage of the estrous cycle for each female rat is identified by cytology of vaginal cells. After decapitation, TG was rapidly removed for performing primary cell culture. After culturing 3 hours, TG primary-cultured neurons were dissociated to perform single cell patch-clamp recording to investigate electrophysiological properties of TG neurons in four different stages of estrous cycle **(Figure 5).**

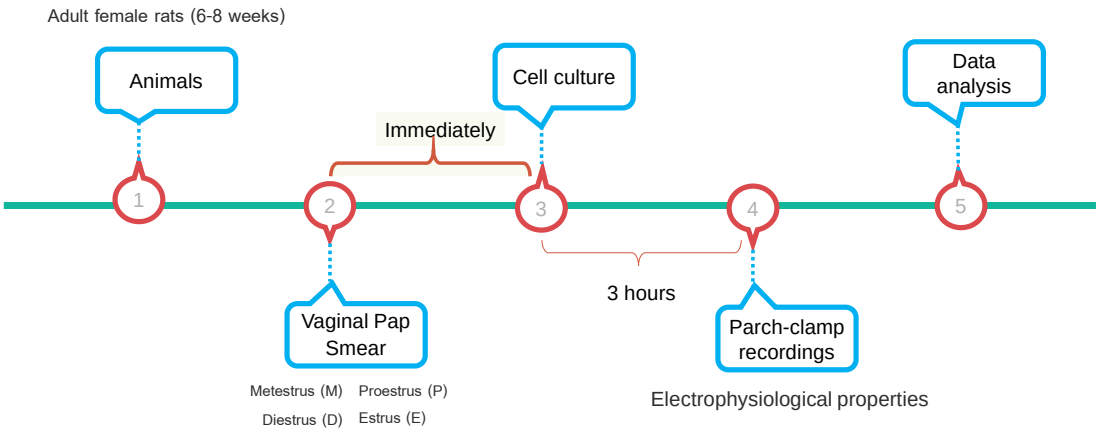


**Figure 5** Timeline of experimental process. Adult female rats (6-8 weeks) were used in all experiments. The stage of the estrous cycle for each female rat was identified by histological of cells in vaginal pap smears. TG was immediately removed to perform primary cell culture after vaginal pap smear. After 3 hour in primary cell culture, we used patch-clamped recording to examine electrophysiological properties of TG neurons in different four stages of estrous cycle. Finally, data analysis was performed.

# Methods

Female Sprague–Dawley rats, 6-8 weeks old, have been defined as female adult rats (35, 36). They had a sufficient estrogen level for observing the estrous cycle. Animals used in all experiments were purchased from the National Laboratory Animal Center, Mahidol University, Nakorn-Pathom, Thailand. Rats were housed in stainless cages in a ventilated room under a 12-hour dark-light cycle and were fed ad libitum. All of the protocols were approved by the Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Thailand (No. 4/58).

## 1. Animal preparation

Rats were divided into four major experimental groups following four different stages of estrous cycle; metestrus (M), diestrus (D), proestrus (P), and estrus (E) stages. The stage of the estrous cycle for each female rat was identified by examining cytology of vaginal cell. These technique of examinations was widely used for detecting stage of the estrous cycle (32, 37). We inserted a plastic dropper filled with 0.9% saline into the vagina. The saline was allowed to fill the vagina, and then we mixed two to three times until injected saline became turbid. The turbid fluid is placed on a glass slide as a sample. A sample was fixed with fire or air dried, and was stained with 1% methylene blue for 5 min and was rinsed in water. Four different stages of the estrous cycle were determined by observing the population of three types of cells in the vaginal smear (i.e., epithelial cells, cornified cells and leukocytes) (6). Immediately after decapitation, the arterial blood was collected approximately 400 – 500 µl from the left cardiac ventricle for storage in a 1.5-ml microcentrifuge tube. The collection tubes were centrifuged at 3,200 rpm for 10 min. Then, serum was collected and stored at -20° C. The serum concentration of estradiol (E2) was measured using the automated Chemiluminescent Microparticle Immunoassay (CMIA)

method. Total 138 neuron cells from 26 female rats were used in the experiment. There were 52 neuron cells from 6 female rats in diestrus stage, 24 neuron cells from 5 female rats in proestrus stage, 35 neuron cells from 9 female rats in estrus stage and 27 neuron cells from 6 female rats in metestrus stage **(Table 1).**

## 2. Primary cell culture of trigeminal ganglion neurons

The primary cell culture process was modified from dissociated primary sensory neurons protocol (38). Rats were anesthetized with an intra-peritoneal injection overdose of sodium pentobarbital before decapitation. Both trigeminal ganglia were removed and cultured in a 35 mm culture dish of ice-cold Hank's Balance Salt Solution (HBSS) integrated with penicillin/streptomycin and wash twice in HBSS, and were minimized into small pieces with blade in 1 ml of HBSS. Collagenase and dispase were added after filtrated by a 0.22 um filter and immediately incubated at 37 °C for 20 min. Papain was filtrated with a 0.22 um filter and added, and then incubated at 37 ° C for 20 min. After that, the sample was centrifuged at RCF 400 g for 2 min, and supernatant is removed. The precipitate is grinded 3 times in L-15 complete medium by using glass pipette. Moreover, sample is centrifuged at RCF 400 g for 8 min. The precipitate was collected and washed twice by F-12 complete medium. Finally, 400 µl of F-12 completed medium was added into the sample and placed into 35 mm laminin / PDL dish, and incubated in an incubator (37 ° C, 5% $CO_2$) for 3 hours. The sample is washed twice by F-12 for further electrophysiological study.

Electrophysiological study used only TG neuron had diameter approximately 15-38 um (34) **(Figure 6).** TG neuron was observed under a light microscope with 40x objective lens.

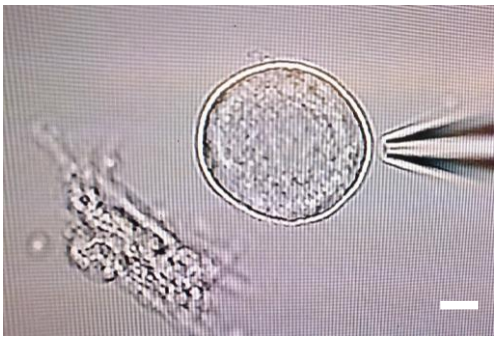

**Figure 6** Representative TG neuron in the experiment. This TG neurons has a typical morphology and diameter (26.29 μm). Scale bar = 5 um.

### 3. Electrophysiological recording

Whole-cell patch clamp recording was used to record the electrophysiological properties of dissociated TG neurons that were maintained in primary culture for 3 hours. Whole-cell parch-clamp recording techniques were operated at room temperature (26.0 ± 0.5°C) using an Axopatch 200B amplifier (Axon instruments, Foster City, CA) and recorded in a computer for data analysis with pClampfit 10.2 software (Axon instruments, Foster City, CA).The output signal was filtered at 2 kHz and sampling rate of 5 kHz. The glass microelectrodes was the outer diameter 1.5 mm and inner diameter 0.86 mm (Sutter Instruments, Navato, CA, USA). The glass microelectrodes was pulled by Flaming/Brown micropipette puller (P-97) (Sutter Instruments, Navato, CA, USA) with resistance of 3-5 MΩ, to a diameter of 1-2 um (39, 40). Microelectrodes were filled by an intracellular solution (composed of 140 mM K-gluconate, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES and 10 mM ATP; osmolality adjusted to 280 ± 5 with glucose).

Plastic chambers containing TG neurons were placed on the microscope sample stand (Olympus BX51WI microscope, Olympus, USA). TG neurons were superfused with extracellular solution flowing into the plastic chamber at a flow rate of 1 ml/min at room temperature. The extracellular solution was composed of 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-Glucose and 10 mM HEPES; the pH value was adjusted to 7.40 with 1 M NaOH, and the osmolarity was

adjusted to 320 mOsm/kg with glucose. Then, microelectrodes were inserted to the headstage of an Axopatch amplifier (Axon, Sunnyvale, CA, USA).

To determine single AP properties; short period current was injected in current-clamp recording mode for evaluating AP properties in response to the different four stages of estrous cycle, the membrane potential was manually held at -60 mV and injected with a current of 10 pA/step with 100 ms duration. The criteria for successful recording was a minimum 10 min recording time, with a stable RMP of more negative than -40 mV; an amplitude of the action potential that was greater than 70 mV; and an input resistance that was higher than 100 mega-ohm. The protocol was adapted from previous reports (41, 42).

To determine summation of AP development; long period current was injected in current-clamp recording mode for evaluating AP properties in response to the different four stages of estrous cycle, the membrane potentials of the TG neurons were manually held at -60 mV and injected with -30 to 70 (10 pA/step) in 11 steps of 500-msec duration. The measurement of electrophysiological properties was performed. Threshold was considered as the depolarizing potential that triggered the first action potential. The rheobase was the lowest injecting current that produced the first action potential. The cell diameter was evaluated as the average of the longest and shortest axis in a BX51WI upright microscope (Olympus, Tokyo, Japan). Only cells with diameter 15-38 μm were analyzed.

**4. Action potential parameter assessment**

Single AP properties were recorded after injection of brief (100 ms-duration) current pulses from a holding potential at -60 mV. The threshold (mV) was the lowest membrane potential that yielded the first depolarization phase of an AP. Rheobase (pA) was the minimal current injection that was able to cause the depolarization phase of an AP. The AP height (mV) was measured as the elevation of an AP measured from the holding potential to peak amplitude of the AP. The AP overshoot (mV) was measured as the elevation of an AP measured from 0 mV to peak amplitude of the AP. The rising time (ms) was measured as the duration of the rapid depolarization phase, which was measured from the threshold to peak amplitude of an AP. The AP falling time (ms) was measured as the duration of the less positive phase, which was measured from a peak amplitude of an AP to the holding potential. The after-hyperpolarization depth (AHP depth mV) is the de-escalation of an AP measured from the holding potential to the negative peak of an AHP. The AHP duration (ms) is the duration time from the negative peak of an AHP to 50% of the recovery of the holding potential is shown in Figure 7.

Summation of AP development was recorded by current-clamp recording mode. The membrane potentials of the TG neurons were manually held at -60 mV and injected current 11 steps of 500-msec duration. The measurement of electrophysiological properties are shown in Figure 8. Threshold was considered as the depolarizing potential that triggered the first action potential. Rheobase was the lowest injecting current that produced the first action potential. Total spike was number of peak lead to action potential by injecting step current from 1-11step (10pA per step).

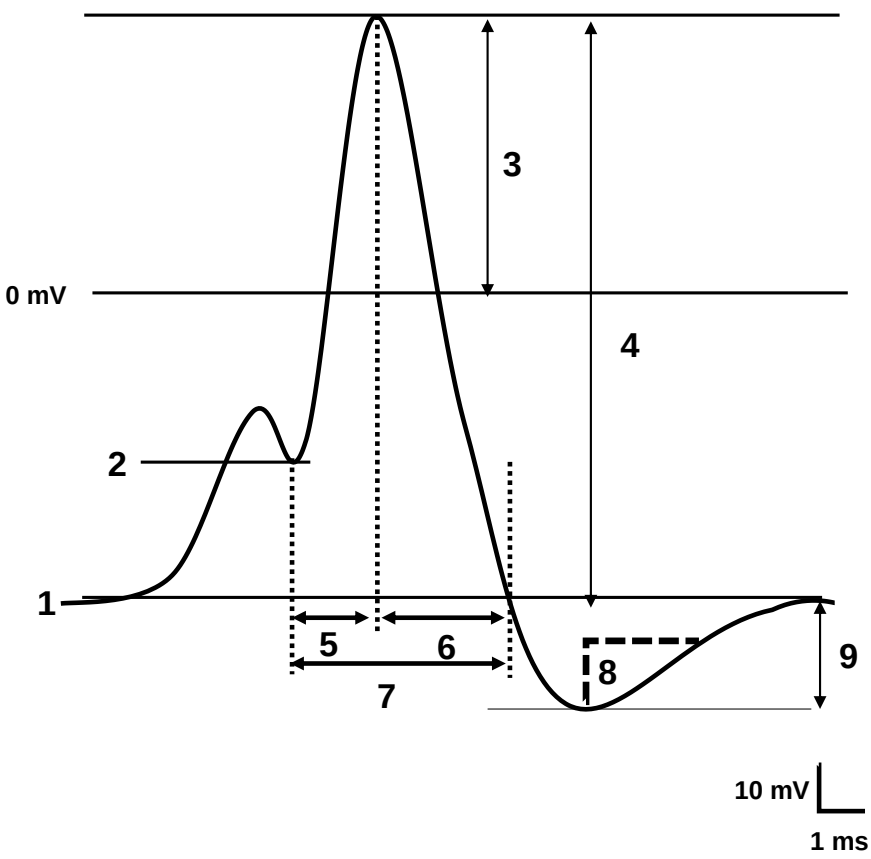


**Figure 7** Assessment of AP properties. 1: Resting membrane potential, 2: Threshold, 3: AP overshoot, 4: AP amplitude, 5: AP rising time, 6: AP falling time, 7: AP duration, 8: 50% of hyperpolarization (AHP) duration, 9: AHP depth

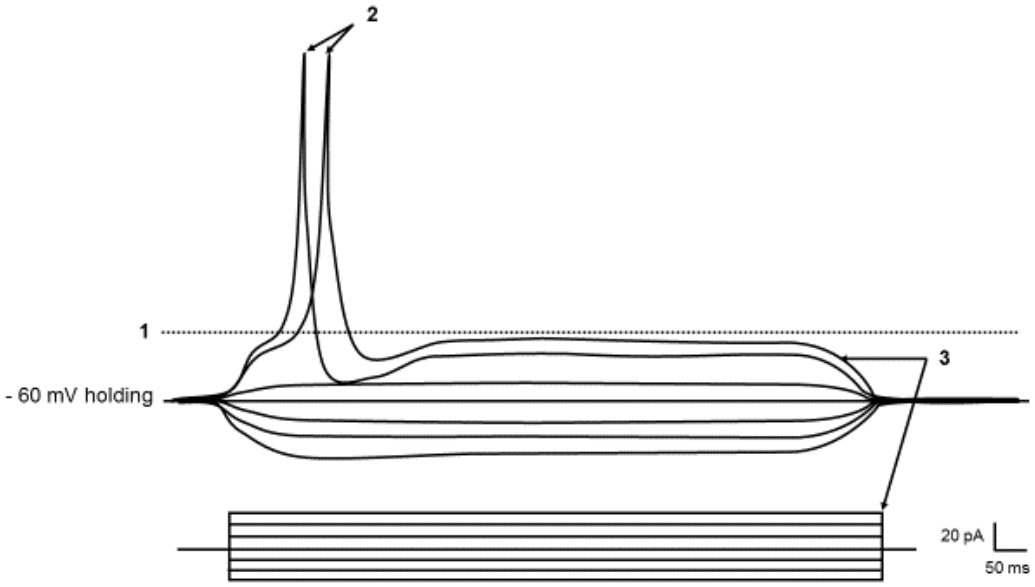


**Figure 8** Electrophysiological parameters; currents in each step are incremented by 10 pA (total 7 currents and 2 spikes). 1: Threshold, 2: Total spikes (2 total spikes), 3: rheobase (30 pA).

## Statistical

All data are presented as mean ± standard errors of the mean (SEM). Statistical analysis was performed using IBM SPSS Statistics data editor. A one-way ANOVA was used to detect the change among groups that are varied due to the stages of estrous cycle. An independent Bonferroni post hoc test was used to determine intergroup differences. A $p < 0.05$ was accepted as statistically significant.

# CHAPTER IV

# RESULTS

## Determination of four different stages in estrous cycle

Morphological studies revealed that the TG neurons in different four stages of the estrous cycle exhibited similar structures (**Figure 9; A-D**). TG neurons had a diameter approximately 15-38 μm, and contained clear nucleus and nucleolus. In contrast, the cytological studies of vaginal smears demonstrated differences in the distributions of vaginal cells. There were many leukocytes and few epithelial nucleated cells in the diestrus stage (**Figure 9 E**), and only clusters of nucleated epithelial cells were observed in the proestrus stage (**Figure 9 F**). The nucleated epithelial cells differentiated to the clusters of cornified epithelial cells in the estrus stage (**Figure 9 G**). The changes in the cell distributions completed with many leukocytes and few cornified epithelial cells in the metestrus stage (**Figure 9 H**). The representative structure of nucleated epithelial cells, cornified epithelial cells and leukocyte are shown in **Figure 9 I, J,** and **K**, respectively.

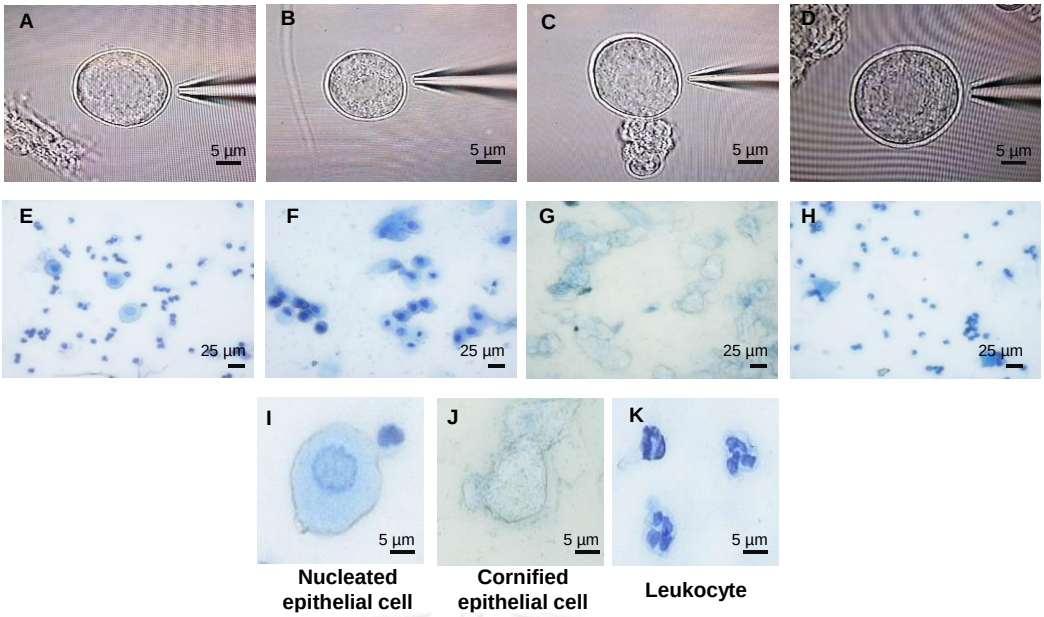


**Figure 9** Evaluation of different four stages of female rat estrous cycle. (A-D) Representative morphologies of primary cultured TG neurons at each stage of the estrous cycle. (A) Diestrus stage, (B) proestrus stage, (C) estrus stage, and (D) metestrus stage. (E-H) Representative vaginal cell types. (E) Diestrus stage, (F) proestrus stage, (G) estrus stage, and (H) metestrus stage. (I-K) Cytology of vaginal cells. (I) nucleated epithelial cells, (J) cornified epithelial cells, and (K) leukocytes.

We confirmed estrogen level in each stage of estrous cycle in serum. Found that, estrogen level was peak in proestrus stage (63.43 ± 2.99 pg/ml, n = 7) when comparing with diestrus; 32.22 ± 1.20 pg/ml, n = 9; $P < 0.05$). In estrus stage was significantly higher of estrogen level than in diestrus stage (44.70 ± 3.74 pg/ml, n = 10; $P < 0.05$), Furthermore, the estrogen level in metestrus stage was significantly lower of estrogen level than in diestrus stage (25.00 ± 4.14 pg/ml, n = 8; $P < 0.05$). The results showed that the overall estrogen level among four different stages of estrous cycle was significantly changed (one-way ANOVA, $p < 0.05$). The concentrations of estrogen level in different four stages are shown in **Figure 10.**

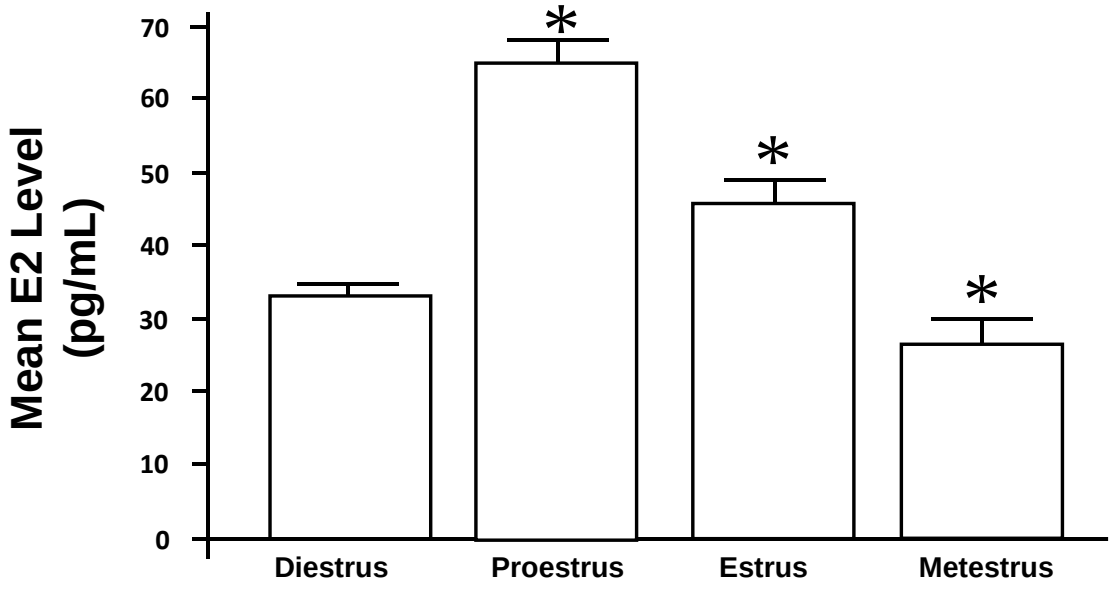


**Figure 10** Concentration of estrogen level at different four stages of estrous cycle. *p < 0.05 compared with diestrus stage (one-way ANOVA followed by post hoc test).

## Single AP properties induced by short-period current injection

The dissociated TG neurons in the primary culture were estimated using a whole-cell patch clamp configuration, of which depolarizing current steps were used to stimulate TG neurons to analyze the AP properties (**Figure 11**). The TG neurons at each stage of the estrous cycle had similar RMP values (Table 1). The threshold at the proestrus (-29.36 ± 0.52 mV, n = 24) and estrus stages (-26.92 ± 2.05 mV, n = 35) was significantly lower than thresholds at the diestrus stage (-19.10 ± 1.67 mV, n = 52; $p < 0.05$, $p < 0.05$, respectively). The rheobase at the proestrus (56.67 ± 2.99 pA, n = 24) and estrus stages (50.69 ± 2.78 pA, n = 35) was also lower than the rheobase at the diestrus stage (74.65 ± 3.70 pA, n = 52; $p < 0.05$, $p < 0.05$, respectively). The AP height and overshoot at the proestrus stage (AP height; 122.65 ± 1.68 mV and AP overshoot; 67.41 ± 1.02 mV, n = 24) were significantly higher compared to the diestrus stage (AP height; 110.09 ± 3.17 mV and AP overshoot; 52.41 ± 2.76 mV, n = 52; $p < 0.05$, $p < 0.05$, respectively). The rising time of the AP was not significant at any stage, while the falling time of the AP at the proestrus (3.22 ± 0.56 ms, n = 24) and estrus stages (2.43 ± 0.31 ms, n = 35) were significantly shorter compared to the diestrus stage (5.21 ± 0.40 ms, n = 52; $p < 0.05$, $p < 0.05$, respectively). Moreover, the duration of the AP at the proestrus (4.48 ± 0.20 ms, n=24) and estrus stage (3.67 ± 0.31 ms, n= 35) were also significantly shorter compared to the diestrus stage (6.46 ± 0.46 ms, n= 52; $p < 0.05$, $p < 0.05$, respectively). The depth of the AHP at the proestrus (-12.15 ± 2.36 mV, n = 24) and estrus stages (-13.56 ± 1.00 mV, n=35) was significantly deeper compared to the diestrus stage (-5.08 ± 0.48 mV, n= 52; $P < 0.05$, $P < 0.05$, respectively), whereas the duration of the AHP was not changed at any stage **(Table 1).**

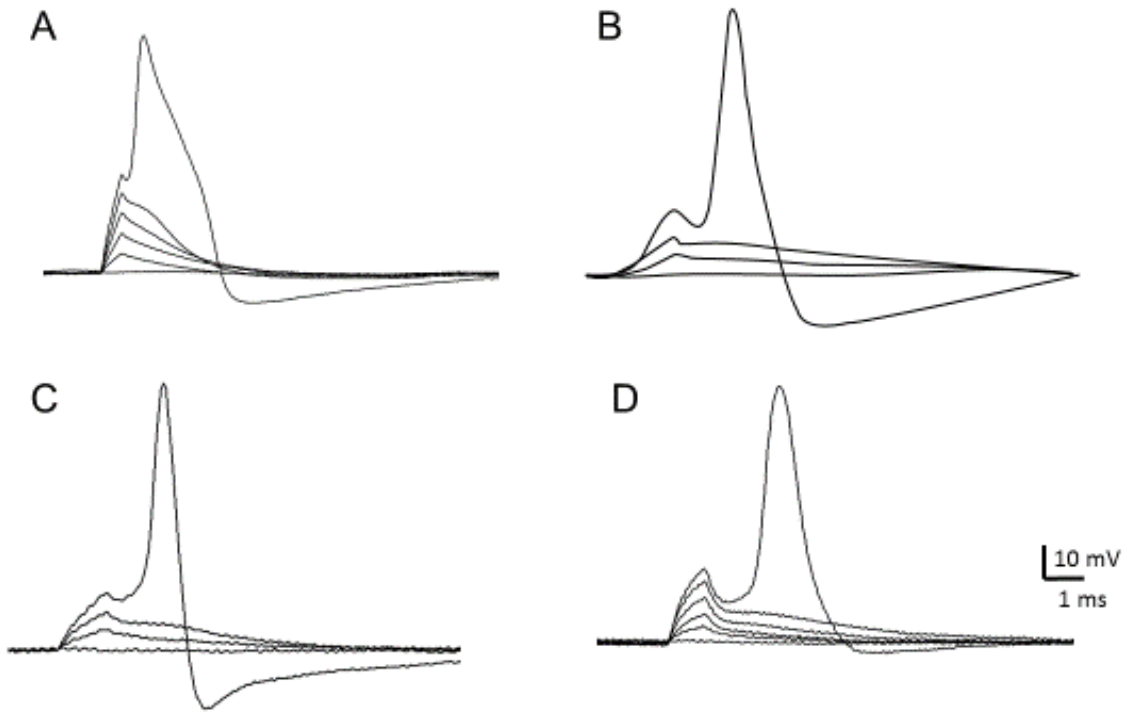


**Figure 11** Representative traces of single AP in TG neurons at different four stages of rat estrous cycle. (A) Diestrus stage, (B) proestrus stage, (C) estrus stage, and (D) metestrus stage.

| | Diestrus (n = 52) | Proestrus (n = 24) | Estrus (n = 35) | Metestrus (n = 27) |
|---|---|---|---|---|
| **RMP (mV)** | -46.85 ± 1.04 | -50.53 ± 2.77 | -45.79 ± 3.26 | -43.85 ± 1.69 |
| **Threshold (mV)** | -19.10 ± 1.67 | -29.36 ± 0.85***** | -26.92 ± 2.05***** | -28.18 ± 2.79 |
| **Rheobase (pA)** | 74.65 ± 3.70 | 56.67 ± 2.99***** | 50.69 ± 2.78***** | 77.22 ± 9.98 |
| **AP height (mV)** | 110.09 ± 3.17 | 122.65 ± 1.68***** | 102.20 ± 4.21 | 100.55 ± 7.60 |
| **AP overshoot (mV)** | 52.41 ± 2.76 | 67.41 ± 1.02 ***** | 51.63 ± 3.84 | 44.43 ± 6.65 |
| **AP Rising time (ms)** | 1.52 ± 0.09 | 1.26 ± 0.13 | 1.24 ± 0.10 | 1.63 ± 0.26 |
| **AP Falling time (ms)** | 5.21 ± 0.40 | 3.22 ± 0.56***** | 2.43 ± 0.31***** | 4.94 ± 0.66 |
| **Duration (ms)** | 6.46 ± 0.46 | 4.48 ± 0.20 ***** | 3.67 ± 0.31 ***** | 6.36 ± 0.82 |
| **AHP depth (mV)** | -5.08 ± 0.48 | -12.15 ± 2.36***** | -13.56 ± 1.00***** | -4.78 ± 1.79 |
| **AHP duration (ms)@half of AHP depth** | 11.75 ± 1.46 | 7.92 ± 1.12 | 5.20 ± 0.68 | 12.48 ± 3.36 |

**Table 1** Single AP properties of the TG neurons in different four stages of rat estrous cycle. The values are presented as the means ± the SEMs. *p < 0.05 compared with the diestrus stage (one-way ANOVA followed by post hoc test).

## Summation of AP development induced by long-period current injection

We applied 500-msec step pulses and examined the electrophysiological properties of the TG neurons with whole-cell patch clamp techniques (**Figure 12)**. The TG neurons in each stage of the estrous cycle exhibited similar RMP values that were not significantly different from those of the diestrus stage (**Table 2**). The TG neurons in proestrus (-40.47 ± 0.03 mV, n = 24) and estrus phase (-39.41 ± 1.51 mV, n = 35) had a lower threshold compared with TG neurons in diestrus phase (-31.94 ± 1.07 mV, n = 52), and there were statistical significances ($p < 0.05$, $p < 0.05$, respectively). Similarly, The rheobase of TG neurons at the proestrus (18.75 ± 0.17 pA, n = 24) and estrus stages (23.71 ± 2.46 pA, n = 35) was also lower than the rheobase at the diestrus stage (38.08 ± 4.09 pA, n = 52; $p < 0.05$, $p < 0.05$, respectively). Furthermore, the total spikes of TG neurons in proestrus (107.13 ± 7.58 spikes, n = 24) and estrus phase (84.23± 13.46 spikes, n = 35) were increased when compared with TG neurons of diestrus phase (16.65 ± 4.22 spikes, n = 52), and there were statistical significances ($p < 0.05$, $p < 0.05$, respectively).

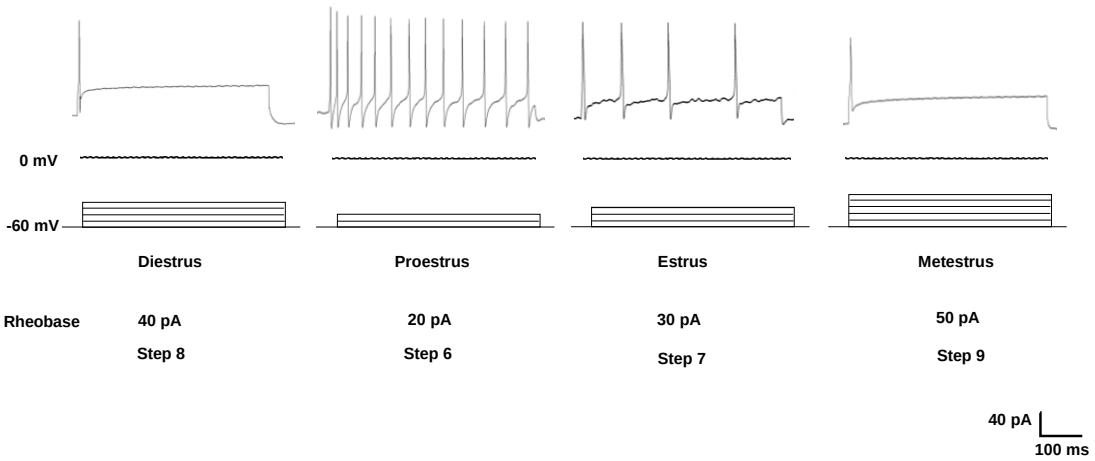


**Figure 12** Summation of AP development occurring in TG neurons at different four stages of rat estrous cycle. A: In the diestrus stage, a rheobase of 40 pA induced the first response. B: In the proestrus stage, a rheobase of 20 pA induced the first response. C: In the estrus stage, a rheobase of 30 pA induced the first response. D: In the metestrus stage, a rheobase of 50 pA induced the first response.

| | Diestrus (n = 52) | Proestrus (n = 24) | Estrus (n = 35) | Metestrus (n = 27) |
|---|---|---|---|---|
| **RMP (mV)** | -46.85 ± 1.04 | -50.53 ± 2.77 | -45.79 ± 3.26 | -43.85 ± 1.69 |
| **Threshold (mV)** | -31.94 ± 1.07 | -40.47 ± 0.03***** | -39.41 ± 1.51***** | -31.12 ± 3.69 |
| **Rheobase (pA)** | 38.08 ± 4.09 | 18.75 ± 0.17***** | 23.71 ± 2.46***** | 42.92 ± 9.83 |
| **Total spikes** | 16.65 ± 4.22 | 107.13 ± 7.58***** | 84.23 ± 13.46***** | 23.67 ± 7.40 |

**Table 2** Summation of AP development properties of the TG neurons in different four stages of the estrous cycle. The values are presented as the means ± the SEMs. *p < 0.05 compared with the diestrus stage (one-way ANOVA followed by post hoc test).

# CHAPTER V

# DISCUSSION

The present study investigated the effects of four different stages of estrous cycle on the alteration of the single AP properties and summation of AP development linked to the trigeminal nociceptive system. We demonstrated that each stage of the estrous cycle was related with differences in the morphology of the vaginal epithelium, which was influenced by estrogen level. Estrogen level was high in proestrus and estrus stages that induce increases of nucleated epithelium cells and cornified epithelium cells without an increase of white blood cells, whereas estrogen was stable at the baseline level in the diestrus and metestrus stages. However, estrogen level had no effect on the morphologies of the TG neurons. Our findings are consistent with Goldman, et al.(38), while single AP properties and summation of AP development were altered according to the fluctuations in the estrogen levels across the estrous cycle. TG neurons exhibited increased excitability in the proestrus and estrus stages which estrogen levels were high. These alterations in TG neuron may be related to neuronal excitability and sensitivity which indicated activation of voltage gated ion channel and peripheral sensitization in the trigeminal nociceptive system.

Several previous studies described that estrogen modulates pain response via neurotransmitter processes and other modulatory systems (42, 43). For AP induction in the trigeminal system, voltage-gated Na channels have a key role in response to depolarization, eliciting an AP in TG neurons, which leads to pain perception. Voltage-gated Na channels Nav1.1 to 1.9 are expressed in TG neurons, including Nav1.7 (a tetrodotoxin-sensitive Na channel; TTX-S), Nav1.8 (a tetrodotoxin-resistant Na channel; TTX-R), and Nav1.9 (TTX-R), which can be stimulated to induce an AP (44, 45). Our results demonstrated that AP threshold, rheobase and AP height were altered in the condition of high estrogen level at proestrus and estrus stages. These findings are consistent

with a previous study demonstrating that high estrogen level increases the specific expression of Nav1.8 and Nav 1.9, as well as the excitability of TG neurons by reducing the AP threshold and rheobase and that estrogen also increases the height of the AP (46). Nav1.7 and Nav1.9 activates at subthreshold resulting to shift of membrane potential closing to AP threshold. After membrane potential reaches AP threshold, opening of Nav1.8 produces AP upstroke in rapid depolarization phase (45, 47). In addition, estrogen has been shown to increase the expression of ERK (36), which phosphorylates voltage-gated Na channels in TG neurons (48). Thus, high estrogen level during the proestrus and estrus stages may increase the expression of Nav1.8, Nav1.9, and ERK, which enhances the excitability of TG neurons resulting in lower threshold, lower rheobase and higher AP height of AP development.

Additionally, voltage-gated K channels play a key role during the falling phase, undershoot phase and repolarization phases of AP induction. Our findings demonstrated that AP falling time were reduced while AHP depth were increased in the condition that estrogen is elevated at proestrus and estrus stages. Previous research has demonstrated that estrogen alters the duration of an AP through the calcium-activated K channel (BKCa) and changes the depth of AHP through the calcium-activated K channel (SKCa) in the hippocampal pyramidal neurons (49). Moreover, estrogen also activates L-type Ca channels to allow $Ca^{2+}$ influx, which increases intracellular $Ca^{2+}$ and activates voltage-gated K channels (45, 48). Consequently, a high level of estrogen at the proestrus and estrus stages may potentiate the K efflux during AP induction in TG neurons, which presents as shorter AP falling time and deeper AHP depth.

Following exposure to estrogen, cells are activated by the process of protein expression. Estrogen binds to the ER-alpha and ER-beta receptors, which function via both genomic and non-genomic mechanisms (43). Systemic high estrogen levels activate the ER receptors in vaginal cells, which results in the proliferation of nucleated epithelium cells and cornified epithelium cells. Estrogen receptors are also expressed in TG neurons (50). Our results revealed that high estrogen levels in

proestrus and estrus stages reduced the thresholds and rheobases of stimulation required to evoke APs of TG neurons. The thresholds and rheobases reflect the neuronal excitability of TG neurons, which are modulated by various neurotransmitters and neuropeptides. It has been demonstrated that the administration of estrogen decreases trigeminal pain thresholds in female rats and also increases the excitability of TG neurons (8). Estrogen increases peripheral sensitization in the trigeminal system by enhancing bradykinin signaling in TG neurons (51, 52). Estrogen also augments endothelial NOS (eNOS) levels, and these levels directly modify peripheral sensitization (53). In addition, Estradiol is a form of estrogen that increases calcitonin gene-related peptide (CGRP) and serotonin (5-HT) in TG neurons, but estradiol does not affect the expressions of the CGRP and 5-HT genes in TG neurons. Because CGRP and 5-HT are known to have important roles in pain perception in the trigeminal system, estrogen is thought to be a modulatory factor in menstrual migraine (54, 55). Other studies have reported enhanced effects of estrogen in CGRP synthesis due to nerve growth factor (NGF)-mediated mechanisms and activation of the transient receptor potential cation channel V1 (TRPV1) in dorsal root ganglia (56). The chronic administration of estrogen increases CGRP levels in dorsal root ganglia (57). Estrogen also modulates nociceptive responses through its effects on other neuropeptides, such as galanin and neuropeptide Y (15). Our results also revealed that the total numbers of spikes during the proestrus and estrus stages were increased. These findings indicate that estrogen may modulate the activation of several voltage-gated ion channels. Estrogen has been found to increase the activity of $Ca^{2+}$-dependent $K^{+}$ channels and induce augmented depolarization in dorsal root ganglion cells (58, 59). Estrogen increases the activation of mitogen-activated protein (MAP) kinase and extracellular signal-regulated kinase (ERK), which result in the phosphorylation of voltage-gated sodium channels and voltage-gated potassium channels (36). These effects are involved in the regulation of peripheral sensitization (60). Overall, the fluctuations of in the estrogen levels during the estrous cycle are increases neurotransmission in the trigeminal system which results in the peripheral sensitization that occurs during menstrual migraine.

In summary, our results indicated that increase of TG neuronal excitability and sensitivity in the condition of high estrogen level during proestrus and estrus stages may involve migraine attacks during menstrual cycle. It has been suggested that activation of voltage-gated ion channels surrounding TG nociceptors induces AP development which increases TG neurons excitability and causes headache pain perception over the migraine attack (61). Upregulation of voltage-gated ion channels following high level of estrogen may facilitate AP development in TG neurons likely reflects a raise in excitability of the trigeminal system to generate migraine attacks in menstrual migraine, as TG neurons increase their excitability before headache starts. Importantly, the influence of estrogen fluctuation on modulation of single AP properties changes in TG neuronal excitability and summation of AP development in peripheral sensitization may help explain the profound headache observed in menstrual migraine as well as suggest a novel target for the treatment of this migraine condition.

# CHAPTER VI

# CONCLUSION

Our study demonstrated that funding support to estrous cycle modulates single AP properties and summation of AP development in TG neurons in intact female rats. Interestingly, modification of those electrophysiological properties of TG neurons at four different stages of the estrous cycle may be induced by the cyclical fluctuation of estrogen levels, which may relate to modulation of voltage-gated ion channels and induction of peripheral sensitization. The results of this study reveal that the neuronal excitability and sensitivity of the trigeminal system alters during ovarian cycle, which may be the fundamental mechanism underlying menstrual migraine.

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# APPENDIX

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