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นางสาวมานิตา ลุนพรหม

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

Role of satellite glial cell on electrophysiological properties of trigeminal ganglion neurons

Miss Manita Lunprom

A Thesis Submitted in Partial Fulfillment of the Requirements
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แซทเทลไลท์เกลี่ยเซลล์มีบทบาทและหน้าที่สำคัญหลายอย่าง ซึ่งส่งผลต่อเซลล์ประสาทไตรเจมินาล มีหลายงานวิจัยพบว่า แซทเทลไลท์เกลี่ยเซลล์มีผลให้เซลล์ประสาทไตรเจมินาลหลั่งสารที่ก่อให้เกิดการอักเสบเพิ่มมากขึ้น จุดประสงค์ของงานวิจัยนี้เพื่อศึกษาบทบาทการทำงานของแซทเทลไลท์เกลี่ยเซลล์ต่อสรีรวิทยาไฟฟ้าของเซลล์ประสาทไตรเจมินาลขนาดเล็กถึงปานกลางและขนาดใหญ่ โดยให้การกระตุ้นด้วยแคลซิโทนินยีนรีเลทเปปไทด์และแคปไซซินด้วยโฮลเซลล์แพทช์แคลมป์เรคคอร์ดดิ้ง โดยเปรียบเทียบความไวในการตอบสนองของเซลล์ประสาทเพาะเลี้ยงชนิดปฐมภูมิแบบเพียวเคาน์เจอร์ (มีเฉพาะเซลล์ประสาท) และโคเคาน์เจอร์ (มีทั้งเซลล์ประสาทและแซลเทลไลท์เกลี่ยเซลล์) โดยวัดค่าตัวแปรต่าง ๆ ซึ่งได้จากรูปร่างของศักย์ไฟฟ้าทำงาน ผลการทดลองแสดงให้เห็นว่าเซลล์ขนาดเล็กถึงปานกลางและเซลล์ขนาดใหญ่ในกลุ่มโคเคาน์เจอร์มีค่าเทรชโฮลด์และค่ารีโอเบสที่น้อยกว่ากลุ่มเพียวเคาน์เจอร์อย่างมีนัยสำคัญทางสถิติ เมื่อให้การกระตุ้นด้วยแคลซิโทนินยีนรีเลทเปปไทด์และแคปไซซินก็ส่งผลเช่นเดียวกัน และพบกว่าเซลล์ขนาดเล็กถึงปานกลางในกลุ่มโคเคาน์เจอร์มีจำนวนการเกิดศักย์ไฟฟ้าทำงานมากกว่าในกลุ่มเพียวเคาน์เจอร์อย่างมีนัยสำคัญ จากผลการทดลองจึงแสดงให้เห็นว่าแซทเทลไลท์เกลี่ยเซลล์มีผลต่อการเปลี่ยนแปลงลักษณะสรีรวิทยาไฟฟ้าของเซลล์ประสาทไตรเจมินาลโดยทำให้เซลล์ประสาทมีความไวในการตอบสนองที่เพิ่มขึ้นทั้งในเซลล์ขนาดเล็กถึงปานกลางและเซลล์ขนาดใหญ่

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MANITA LUNPROM: Role of satellite glial cell on electrophysiological properties of trigeminal ganglion neurons. ADVISOR: ASSOC. PROF. SOMPOL SAGUANRUNGSIRIKUL, M.D., CO-ADVISOR: PROF. ANAN SRIKIATKHACHORN, M.D., 74 pp.

Satellite glial cells (SGCs) have an important role of the trigeminal ganglion (TG) neurons. Several studies found that role of SGCs affect the TG response occurring release various inflammatory mediators. This studies purpose role of SGCs on electrical response property of the small-to-medium sized and large sized TG neurons in the presence of capsaicin or CGRP. The experiments divide to primary co-culture TG neurons (TG+SGCs) and primary pure culture neurons (only TG). The result showed that co culture group was significantly lower threshold and lower rheobase than pure culture group in normal condition. The presence of CGRP and capsaicin, the result showed co culture group was significantly lower threshold and lower rheobase than pure culture group. Additionally, co culture group was higher elicited number of spikes than pure culture group in small-to-medium sized TG neurons. Thus, primary culture neurons that without SGCs causing reduce electrical response TG neurons. This study reveal that SGCs can modulate the neuronal excitability and pain sensation in the trigeminovascular nociceptive system deserve further exploration.

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Student's Signature ....
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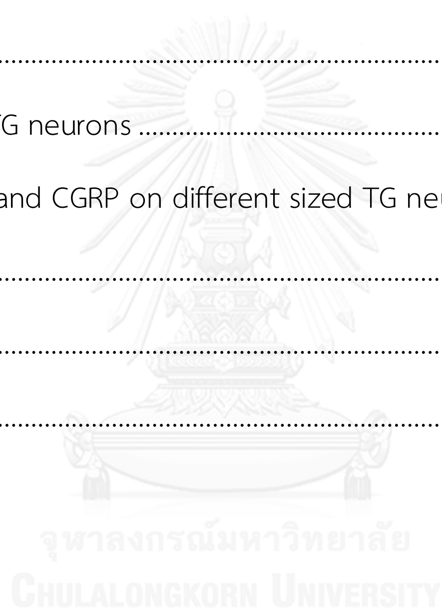
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LIST OF ABBREVIATIONS

°C	Celsius
β	Beta
α	Alpha
µl	microlitre
µm	micromete
AHP	after-hyperpolarization
AP	Action potential
AraC	Cytosine arabinoside
ASICs	Acid-sensing ion channels
CGRP	Calcitonin gene related peptide
CLR	Calcitonin receptor-like receptor
CNS	Central nervous system
CO_2	Carbon dioxide
COX	Cyclooxygenase
DRG	Dorsal root ganglion
ERK	Extracellular signal-regulated kinase
FA	Fast adaptation
GFAP	Glial fibrillary acidic protein
HBSS	Hank's Balance Salt Solution
IL-1β	Interleukin-1β
K	potassium
KCL	Potassium chloride
LCD	Liquid crystal display
LPS	lipopolysaccharide
$MgCl_2$	Magnesium chloride
MF	Multiple firing
ms	millisecond
mV	millivolt

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Na	sodium
NaCl	Sodium chloride
NO	Nitric oxide
NaoH	Sodium hydroxide
pA	picoampere
RAMP1	Receptor activity-modifying protein 1
PDL	Poly-d-lysine
RF	Rapid firing
PGE_2	Prostaglandin
RMP	resting membrane potential
PNS	peripheral nervous system
SEM	standard errors of the mean
SGCs	Satellite glial cells
SS	Single spike
TG	Trigeminal ganglionic
TNC	Trigeminal nucleus caudalis
TRPV1	Transient receptor potential cation channel V1
TTX-R	Tetrodotoxin-resistant sodium channels

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CHAPTER I

INTRODUCTION

Background and Rationale

Satellite glial cells (SGCs) is a supporting cell commonly found encircling neuronal cell bodies in sensory and autonomic ganglia (1). The main functions of these cells is to supply nutrients to the surrounded neurons and to protect the neurons. In the inflammatory condition, SGC also play role in modulating the neuronal excitability by releasing several chemical messengers. These included interleukin1β, interleukin6, prostaglandin, nitric oxide, etc (2-5).

Trigeminal system is a principle system that conveys information regarding craniofacial nociception. The first order neurons of this system reside in the trigeminal ganglion. Trigeminal ganglionic (TG) neurons could be classified based on the size into three groups, namely small, medium and large sized neurons. Small-to-medium cells detect a noxious stimulus while large cells respond to the non-noxious stimuli. Previous studies have shown the neuron-glia interaction in trigeminal ganglion. For instance, induction of inflammation by applied complete Freund's adjuvant leads to the glial cell activation and released of pro-inflammatory cytokines such as interleukin-1β (IL-1β) (2). Activation of TG neurons leads to the release of calcitonin gene related peptide (CGRP) and nitric oxide (NO) within the ganglia(6). It has been shown that CGRP can act on its receptors on SGC and activates IL-1 β secretion. The released IL-1 β and NO further activates glial cells by activating the prostaglandin production (7). These messengers subsequently bind to their receptors on TG neurons, hence modulating its excitability.

In this study, we aimed to investigate the interaction between neuron and glia cells in primary cultured TG model. We compared the responsiveness of TG to CGRP and direct stimulant (capsaicin) in the cultured system comprising either pure neurons or neuron-glia co-cultured. The results of the study will provide better understanding of neuron-glia interaction in the trigeminal ganglion.

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CHAPTER II

LITERATURE REVIEW

Trigeminal system – the principle pathway for craniofacial nociception

The trigeminal system is the most important pathway in the process of craniofacial pain perception. The first order neurons of this system reside in trigeminal ganglion. These cells innervate the pain-sensitive structures via three major branches, namely ophthalmic, maxillary and mandibular. The ophthalmic branch innervates upper face, tip of the nose, upper cornea, nasal cavity, frontal sinuses and parts of the meninges. The maxillary branch innervates middle face (lower lid, side of nose, upper lip and skin under eyelid), upper teeth and nasal cavity. The mandibular branch innervates temporal region to side of head and lower face (anterior of tongue, the lower lip, chin, jaw and the lower teeth and gums) (8).

The nociceptive trigeminal ganglionic neurons send their central axons and terminate by making synapse with second-ordered neuron in caudal part of the trigeminal brainstem complex known as trigeminal nucleus caudalis (TNC). The neurons in TNC then further convey the nociceptive information to ventral posteromedial nucleus of thalamus. The thalamic third-ordered neurons then send axons to somatosensory cortex where nociceptive information is interpreted (9) (Figure 1).

Altered function of trigeminal nociceptive system is believed to play major roles in pathogenesis of various forms of primary headaches. For instance, sensitization of TG and TNC neurons is pivotal mechanism underlying pulsating headache and cutaneous allodynia observed in patients with migraine. Increased

nociceptive activity of from neck and pericranial muscles may lead to pressure liked headache in patients with tension-type headache.

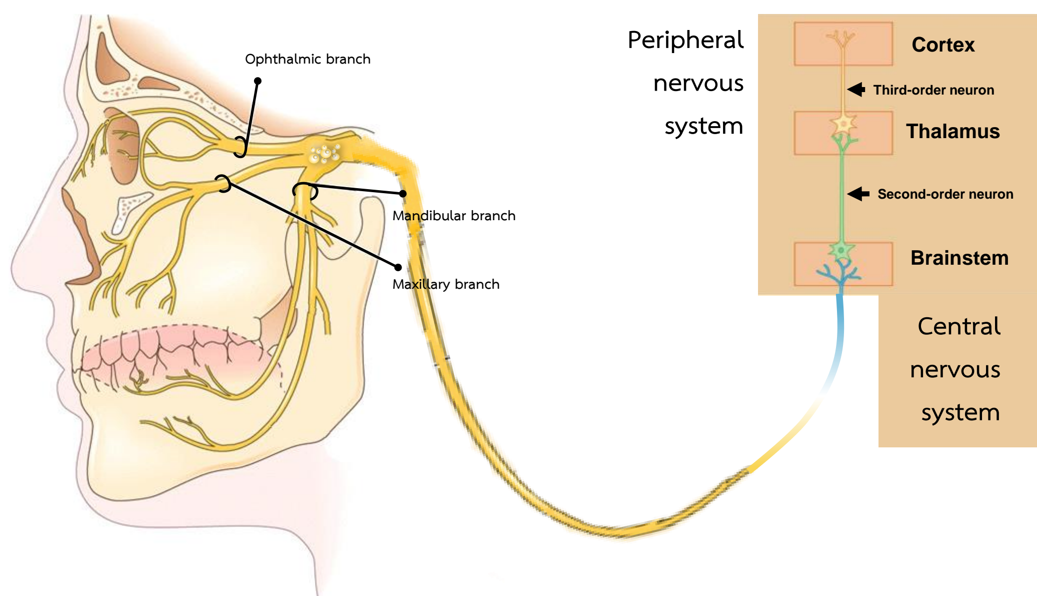

Figure 1. Trigeminal system

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Trigeminal ganglionic neurons – the first-ordered neurons

As previously mentioned, TG cells can be classified into several populations based on their structures and functions. Using morphological criterion, TG cells can be divided into as small-sized (15-24μm), medium-size (30-37μm) and large-sized (>37μm) neurons (10). The neurotransmitters and neuropeptides used in these TG neurons vary according to the type of neuronal cells. The small sized neurons (likely to be nociceptive) contain substance P, CGRP, somatostatin, vasoactive intestinal polypeptide and gelatin while the medium sized neurons contain CGRP and neuropeptide Y. The large sized neurons, mainly non-nociceptive, contain cholecystokinin and neuropeptide Y (11).

<u>**The small sized and medium sized TG neurons**</u> – Small-to-medium sized TG neurons are situated everywhere in the TG. These cells can be divided into light and dark according to the color of their cytoplasm. The cells have a defined nucleus with slightly oval shape situated eccentrically. These cells give rise to the C-fiber and A-delta fiber that detect a noxious stimulus. Therefore, these small-to-medium sized TG cells are deem to be nociceptive (11, 12).

The nociceptive TG cells can be further classified according to their response to noxious stimuli. For example, the neurons which respond to high temperatures or low temperature are defined as ‘thermal nociceptive neurons’ (13). The variation in pattern of response of TG cells is the result of the difference in the expression of specific receptors especially those belong to the transient receptor potential channel (TRP receptor). For instance, cells that express TRPA1 receptor will respond to the extremely low temperatures (below 5 $^{\circ}C$) and generate the sensation of cold pain (14). On the contrary, extremely high temperatures (above 45 $^{\circ}C$) will activate the TRPV1 and TRPV2 receptor and is responsible for the development of burning pain (15, 16).

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In addition to thermal stimuli, small TG neurons also respond to low pH and some chemicals such as capsaicin (a chemical found in chili). Hydrogen ion (or proton) generated in the low pH state can act on specific ionotropic receptor on TG cells. Examples of these pH responsive ionotropic receptors are acid-sensing ion channels (ASICs) (17) and TRPV1. ASICs receptors are activated at the pH of 7.0, while TRPV1 activation requires pH to be lower than 6.0 (18) (19). Via these receptors, local tissue acidosis, as occur in inflammation, can activate TG cells and initiates the process of nociception.

Activation of nociceptive TG neurons leads to several subsequent processes. One of the most important changes is the release to pro-inflammatory peptides such

as substance P and CGRP. These peptides, as well as other chemical substances found during inflammation such as bradykinin, histamine, etc., can decrease the threshold of nociceptors. Therefore, the nociceptors which normally are high threshold sensory receptors become more responsive and can respond to low intensity stimulus. The process is called 'peripheral sensitization'(6, 7, 20).

In addition to the patterns of response, small size and medium sized TG cells can be also classified based on their electrophysiological properties. At least four patterns of firing have been reported in TG cells. These include a single spike (SS), multiple firing (MF), fast adaptation (FA) and rapid firing (RF) (21). These cells exhibit different response to stimuli. For instance, the TG neurons with MF pattern can be activated by capsaicin but are irresponsive to ATP (21, 22).

The large-sized neurons – In TG, the large-sized neurons are situated near the places where the three branches of the trigeminal nerve exit. They are typical pseudounipolar neurons with light cytoplasm and big hypochromic oval nucleus. The peripheral axons of these large neurons are A-beta fibers which respond to the non-noxious stimulation such as touch and proprioception (11, 12). Although these cells are considered to be non-nociceptive, several lines of evidences imply that they also play roles in process of nociception. For instance, these cells express CGRP receptor and, therefore, can interact with CGRP released during the nociceptive activation and inflammation (23, 24).

Satellite glial cell

Satellite glial cells (SGCs) are the principal glial cells found in the peripheral nervous system, specifically in sensory and autonomic ganglia. Satellite glial cell are supporting cells that encircle neuronal cell bodies and initial portions of axons. The role of these cells resemble the roles of astrocytes in the central nervous system

(CNS) (1). The primary functions of SGC are to control the microenvironment of ganglia and to support the functions of neurons by providing nutrients and protection.

SGCs plays an importance role in inflammation and release several mediators for modulate neuronal activity. Inflammation and nerve injury initiate many changes in glial cells. These include the activation of extracellular signal-regulated kinase (ERK) in astrocytes and microglia (25). Injection with capsaicin leads to glial cell proliferation and increased expression of calcium-binding protein B (S100B), a protein of the S-100 protein family, which is involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation (5). Administration of lipopolysaccharide (LPS) activates cyclooxygenase (COX) enzymes and enhances expression of COX-mRNA, TNF-α-mRNA and microglial marker (26). The expression of P38 mitogen-activated protein kinases (P38), an enzyme involving in the stress response is increased after being exposed to nitric oxide (NO) and TNF-α (5).

In addition to the supporting functions, accumulating evidences show that SGC also play active roles in modifying neuronal responses. These cells express a variety of receptors that allow for a range of interactions with neuroactive chemicals (27). Several of these receptors and other ion channels have recently been implicated in health issues including chronic pain (28). Previous studies have shown the importance of satellite glial cells to modify the function of sensory transmission including nociception (2, 6, 7, 29).

Concerning trigeminal system, SGC in TG also involves in the trigeminal nociceptive process. Increased expression of glial fibrillary acidic protein (GFAP), a marker for activated glial cells, in TG has been observed following chemically induced inflammation in tooth pulp (30). Activated glial cells can release proinflammatory cytokines such as interleukin-1β (IL-1β) (2). IL-1β is known to play

important roles in inflammatory process and can alter function of TG neurons and SGCs (2, 4, 6, 7). Increased GFAP expression is also seen after injection with CGRP into temporomandibular joints. This inflammatory induced glial cell activation persists for at least 24 hours (3).

Neuronal and satellite glial cell interaction

Satellite glial cells are actively participate in the modulation of trigeminal neuronal activity. They can augment and sustain inflammatory processes within the ganglia. After noxious stimulation, TG neurons will release CGRP and NO within the ganglia. The released CGRP will activate SGC by binding with its receptors on SGC, causing the release IL1β. Increased mount of NO will activate cyclooxygenase enzyme in SGC and increase the production of prostaglandin (PGE_2) (4, 6). These pronociceptive chemicals will enhance the activity of TG neurons by the process of sensitization (Figure 2).

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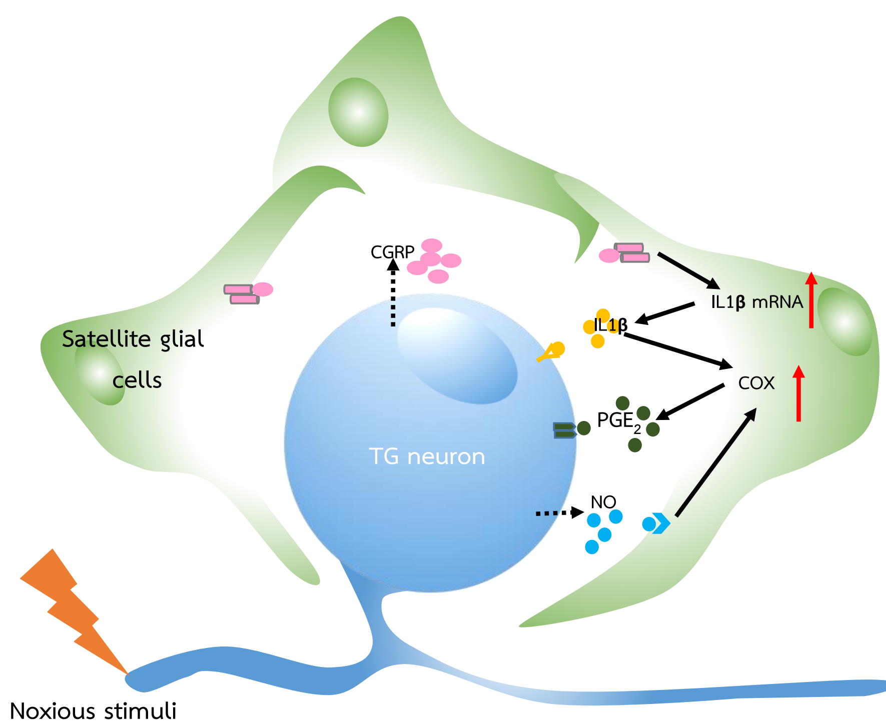

Figure 2. Trigeminal ganglion (TG) neurons interact with satellite glial cells

Calcitonin gene related peptide (CGRP)

CGRP is a 37-amino acid neuropeptide in a family of peptides include in calcitonin, adrenomedullin, amylin and the recently cloned calcitonin receptor-stimulating peptides. Two isoform of CGRP are known, namely α-CGRP and β-CGRP (31). The α-CGRP is formed from the alternative splicing located on chromosome 11 of the CGRP gene (32). The less-studied β-CGRP diverge in three amino acids (in humans) and is encoded in the same chromosome in a separate gene (33). Both isoforms of the peptide are structurally similar, with the β-CGRP only differing by 3 amino acid residues in humans and 1 amino acid in rodents. Indeed, both isoforms present comparable biological activity and primarily differ in their tissue distribution. α-CGRP is the most commonly found within peripheral sensory neurons, whereas β-CGRP seems to be more restricted to nerves of the enteric plexues.

CGRP is expressed in both central and peripheral nervous systems, and is especially enhance in dorsal root ganglion (DRG), trigeminal ganglion neurons and their respective fibers (34). Anatomical studies showed that small-to-medium sized are abundant CGRP, while large sized TG neurons are less CGRP. Correspondingly, CGRP is secreted from unmyelinated fibers (c fibers) and thinly myelinated fibers (Aδ fibers) originating from DRG and TG neurons (23, 24).

The effect of CGRP on sensitizing nociceptors is supported by several studies. For instance, study using experimental osteoarthritis pain model showed that administration of CGRP could increase mechanical sensitivity of joint nociceptors. Applying of CGRP into the temporomandibular joint capsule enhanced expression of several downstream transduction proteins such as active forms of the MAP kinases p38 and ERK, and PKA in trigeminal ganglia, implying an augmentation of nociceptive transduction (3, 35, 36). However, some studies showed the opposing results. Levy et al showed that topical and systemic CGRP administration, in the dose that produced substantial vasodilation, neither activated nor sensitized the mechanosensitive meningeal nociceptors (37, 38). More recently, Capuano et al showed that CGRP can activate trigeminal neurons only after these neurons have been sensitized (22). In addition, the study of electrophysiology showed that CGRP could enhanced the peak amplitude of membrane currents induce by α, β-me ATP (35), enhanced TTX-resistant sodium currents (39) and enhanced the inward currents evoked by capsaicin (40), as result indicate CGRP could enhanced excitation of TG neurons. In the study of trigeminal sensory neurons in brainstem slices showed that CGRP increase the excitability that increased the firing of action potential and reduced threshold of neurons via CGRP receptors because the effect was negated by an antagonist (CGRP8-37) (41).

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Although the effect of CGRP in the process of peripheral sensitization of TG cells is well recognized, the mechanisms by which this peptide affects trigeminal nociceptive process are still unclear. Ample evidences indicate that its direct effects upon trigeminal ganglionic neurons are rather unlikely. Anatomical studies revealed that small trigeminal ganglionic neurons do not express any CGRP receptor components namely calcitonin receptor-like receptor (CLR) and the receptor activity-modifying protein 1 (RAMP1). On the contrary, both components are expressed in large ganglionic neurons and SGCs. These findings imply that CGRP does not directly affect small trigeminal fibers. Therefore, its effect on the peripheral nociceptor is more likely to be indirect. The findings also indicate the role of large non-nociceptive fibers and SGCs in the process of trigeminal sensitization.

Electrophysiological properties

Electrophysiology is the study of the electrical properties of biological cells and tissues. We used the whole-cell patch clamp technique for this study. The whole-cell patch clamp technique is the standard laboratory for determine electrophysiological properties of neurons that is action potential. Action potentials (AP) occur excitable cells such as neurons, muscle cell and endocrine cell. In neurons, they play a central role in cell-to-cell communication. Action potential elicited by square pulse depolarizing current and parameter for described the electrophysiological properties such as threshold (mV), rheobase (pA), AP rising time (ms), AP height (mV), AP falling time (ms), After hyperpolarization (AHP) and duration time (ms) (42, 43). Characterizing of action potential relationship between the stimulus and individual neuronal responses.

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Conclusion

Based on the above evidences, it is clear that SGCs can interact with TG neurons. However, the precise interaction between these two cells are not well understood, especially their responses to CGRP. The proposed study aims at investigating the influence of SGCs on the electrical properties of the TG neurons in response to direct stimulant (capsaicin) and CGRP. The information obtained from this study will provide better understanding of the interaction between SGCs and TG cells.

Research questions

What is the effect of SGC on electrical response of TG neurons in the presence of direct stimulant (capsaicin) or CGRP?

Objective

- To compare the electrical response of TG neurons provoked by capsaicin in co-culture (SGC and neuron) and pure culture of trigeminal ganglion neurons
- To compare the electrical response of TG neurons in the presence of CGRP in co-culture (SGC and neuron) and pure culture of trigeminal ganglion neurons

Hypothesis

Satellite glial cell activation increases the neuronal excitability in co-culture trigeminal ganglion neurons, CGRP increases the neuronal excitability in co-culture trigeminal ganglion neurons and capsaicin increases the neuronal excitability in small-to-medium sized of trigeminal ganglion neurons.

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Conceptual framework

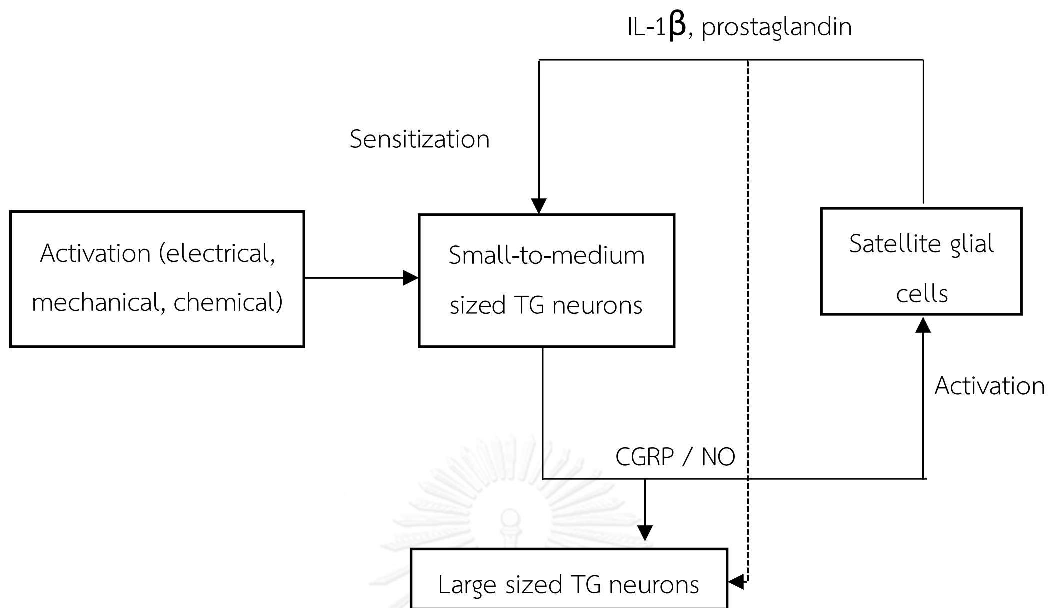

Figure 3. Conceptual framework

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CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Animals

Adult male wistar rats (National Laboratoey Animal Center, Mahidol University, Nakorn-Pathom, Thailand)

2. Materials

Medical supplies (needles, syringes, gloves)

Capillary glass (for electrode recording) (Sutter Instruments, Navoto, CA, USA)

1.5 ml microcentrifuge tube (Biologix)

50 ml centrifuge tube (Corning, NY, USA)

Stripette 25 ml. (Corning NY, USA)

Syringe filter 25 mm. 0.2 µm SFCA (Corning NY, USA)

Cell strainer 40 µm (BD falcon)

60 x 15 mm. TC dish (Corning NY, USA)

3. Instrumental device

Bravia LCD TV BX300 (Sony Thai Co., Ltd, Thailand)

BX51WI fixed-stage upright microscope (Olympus Corporation, Japan)

Cellculture Co_2 incubator (Esco, Global, Singapore)

Digitdata 1440 series interface (Axon Instruments, Foster City, CA, USA)

Flaming/Brown micropipette puller (P-97) (Sutter Instruments, Navoto, CA, USA)

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Fiske® 210 Micro-osmometer (Advanced Instruments, Inc., MA, USA)

High output vacuum/pressure pump (EMD Milipore Corporation, MA, USA)

MP-385-2 Micromanipulator (Sutter Instruments, Navoto, CA, USA)

Natishige MF-830 microforge (Narishige, Japan)

Patch clamp amplifier: Axopatch 200B (Axon Instruments, Foster City, CA, USA)

Peristaltic pump: Minipuls 3 (Gilson, S.A.S., Villiers le Bel, France)

Rotina 420 R (Hettich Instruments, Bach, Switzerland)

Software for data acquisition: Clampex 10.2 (Axon Instruments, Foster City, CA, USA)

Software for data analysis: pClampfit 10.2 (Axon Instruments, Foster City, CA, USA)

Super HAD CCD II camera (Sony corporation, Japan)

Vibration isolation platform (Newport Corporation, CA, USA)

4. Drugs

Alpha calcitonin gene-related peptide (CGRP) (Sigma-Aldrich, MO, USA)

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Capsaicin (Sigma-Aldrich, MO, USA)

Sodium pentobatbital (Ceva Sante Animale, Liboune, France)

5. Chemical

Sodium Chloride (Sigma-Aldrich, MO, USA)

Potassium Chloride (Sigma-Aldrich, MO, USA)

Calcium Chloride (Sigma-Aldrich, MO, USA)

D-glucose (Sigma-Aldrich, MO, USA)

EGTA (Sigma-Aldrich, MO, USA)

Magnesium Chloride (Sigma-Aldrich, MO, USA)

Potassium gluconate (Sigma-Aldrich, MO, USA)

Potassium Chloride (Merck, Germany)

Sodium Hydroxide (Merck, Germany)

Laminin (Sigma-Aldrinch, MO, USA)

Poly-d-lysine (PDL) hydrobromide (Sigma-Aldrich, MO, USA)

HEPES free acid (Sigma-Aldrich, MO, USA)

Leibovitz's L-15 medium (Gibco, Grand island, NY, USA)

Fetal bovine serum (Gibco, Grand island, NY, USA)

Hank's balanced salt solution (HBSS) (Gibco, Grand island, NY, USA)

L-Glutamine (Gibco, Grand island, NY, USA)

Ham's F-12 (Gibco, Grand island, NY, USA)

Cytosine Arabinoside (Sigma-Aldrinch, MO, USA)

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

Dispase II (Invitrogen, Carlsbad, CA, USA)

Glutamax (Gibco, Grand island, NY, USA)

Papain (Sigma-Aldrinch, MO, USA)

Penicillin (10000 U/ml) / Streptomycin (10000 U/ml) (Gibco, Grand island, NY, USA)

B27 (Sigma-Aldrinch, MO, USA)

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Experimental design

The study is *in vitro* study using primary cultured TG neurons without SGCs and with SGCs.

Research Methodology

Experiment I

To investigated the electrical response of TG neurons provoked by capsaicin in co-culture (SGCs and neurons) and pure culture (TG neurons only).

The effect of capsaicin in TG neurons was studied by using whole-cell patch-clamp. The TG neurons divided into 2 groups from co-culture and pure culture; small-to-medium sized and large sized. Both groups of neurons were administration **1 μM capsaicin** with perfusion (22). The response of TG neurons between co-culture and pure culture will be compared. The diagram showing experimental design is as follow (Figure 4):

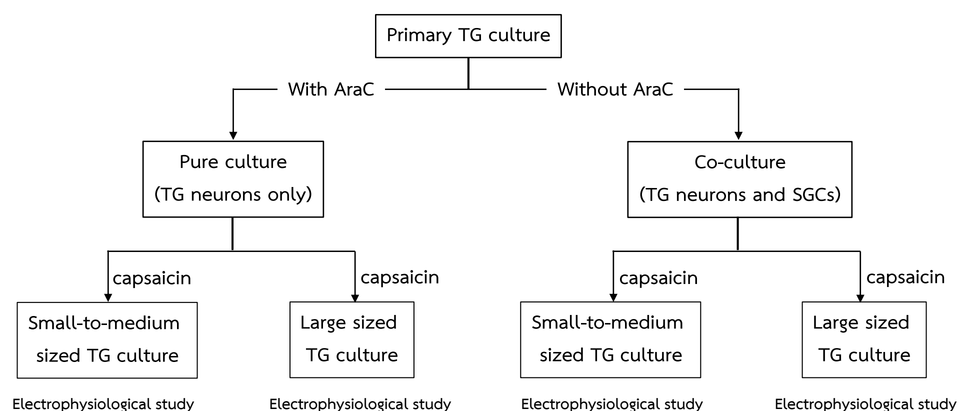

Figure 4. Diagram of experiment design I

Experiment II

To investigated the electrical response of TG neurons provoked by CGRP in co-culture (SGCs and neurons) and pure culture (TG neurons only).

The effect of CGRP in TG neurons was studied by using whole-cell patch-clamp. The TG neurons are to be divided into 2 groups from co-culture and pure culture; small-to-medium sized and large sized. Both groups of neurons incubated with **1μM CGRP** for 1 hour at 37°C before recording (35). The response of TG neurons between co-culture and pure culture will be compared. The diagram showing experimental design is as follow (figure 5):

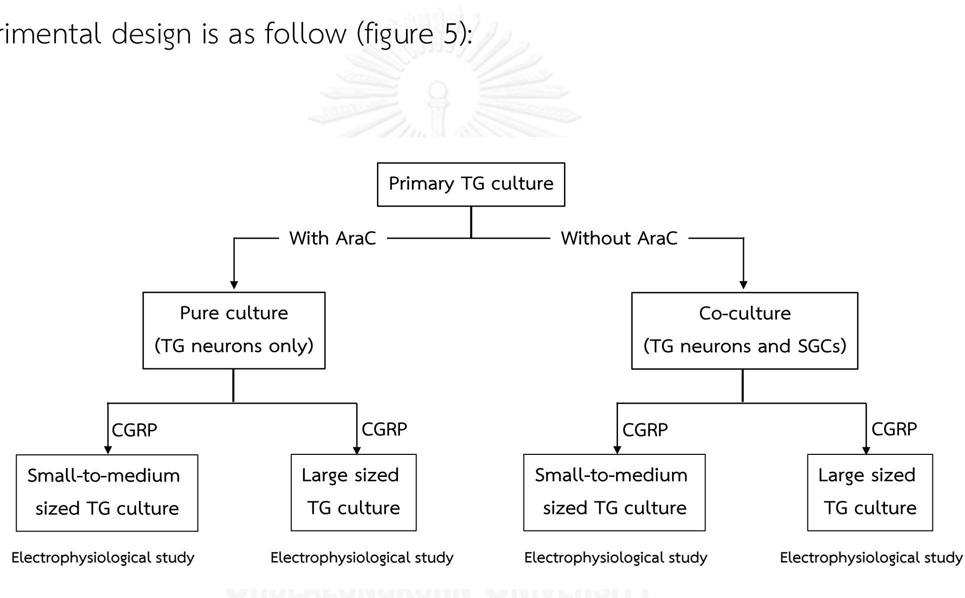

Figure 5. Diagram of experiment design II

Methods

Animal

Adult male wistar rats was used in all experiments. The rats purchased from the National Laboratory Animal Center, Mahidol University, Nakorn-Pathom, Thailand. Rats was housed in stainless cages in the ventilation room under a 12-hour dark-light cycle and allowed food and water ad libitum. All of the protocols are approved by the Animal Care and Use Committee of Faculty of Medicine, Chulalongkorn University, Thailand.

Primary cell culture trigeminal ganglion neurons

1. **Co-culture**

This experiment used wistar rats 4 to 8 week old. Animal were anesthetized by intraperitoneal injection of overdose sodium pentobarbitol before decapitation. Trigeminal ganglia were rapidly removed and immersed in 35 mm culture dish of ice-cold Hank's Balance Salt Solution (HBSS) with penicillin/ streptomycin, and excised in 1 ml of HBSS. Next, collagenase and dispersed was added with a 0.22 μm filter into sample, incubated at 37 °C for 20 min. Papain was added with a 0.22 μm filter, incubated at 37 ° C for 20 min. Next, sample was centrifuged 2 min at RCF 400 g, and supernatant was pumped out excepting precipitate to grind tissue in L-15 complete medium by using glass pipette 3 times. Sample was centrifuged 8 min at RCF 400 g, and supernatant was pumped out excepting precipitate, and immediately washed by neurobasal medium 2 times. Next step, sample was added 400 μl of neurobasal medium and placed on 35 mm Laminin/PDL dish, then maintained in an incubator (37 ° C, 5% CO_2 for 3 h) and washed by neurobasal medium in 2 times.

2. **Pure culture**

Primary culture of TG neurons prepared using similar method except for the addition of pre-plating 3 h and then add cytosine arabinoside (final concentration 10 µM; Sigma Aldrich) to inhibit the proliferation of satellite glial cells. Culture dish incubated at 37°C with 5% CO_2 and fed with neurobasal medium + B27 medium. The purification processes were observed and recorded under phase contrast microscope (29) (figure 6).

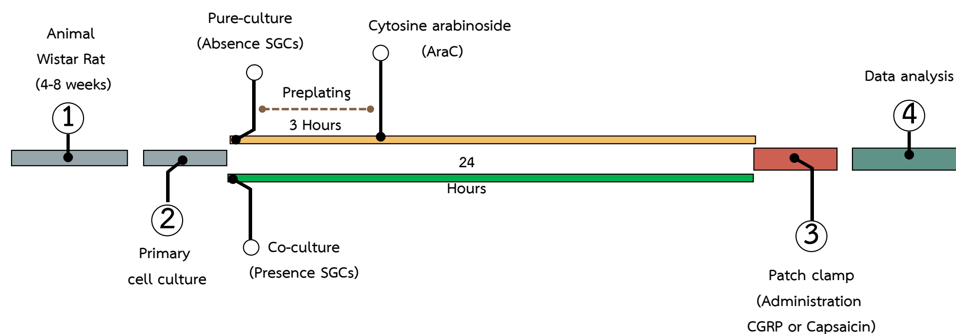

Figure 6. Timeline of experiment

Whole-cell patch-clamp recording

Whole-cell patch-clamp recording techniques were used to valuation the electrical properties of TG neurons that was dissociated and maintained in culture for 22-24 hours after isolation by using an Axopatch 200B amplifier (Axon instruments, Foster City, CA). Plastic chamber's primary TG neurons are placed on the sample stand of microscopy (Olympus BX51WI microscope, Olympus, USA); after that, superfusion of extracellular solution into plastic chamber occurs (composition in mM: 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-Glucose and 10 mM HEPES; adjusted pH value to 7.40 with 1 M NaoH, Osmolatity 320 mOsm/kg with glucose) start at flow rate 1 ml/min in room temperature. Glass pipettes are pulled

by a microelectrode puller Sutter Instrument) and heat polished on a Narashige microforge with a resistance of 3-5 MΩ. Glass pipettes are filled with intracellular solution (composition: K-gluconase pH7.3, 2 mM $MgCl_2$, 10 mM HEPES, 0.3 mM Na-GTP, and 10 mM Mg-ATP), and then inserted to the headstage of an Axopatch amplifier showed in figure 7 and figure 8. This experiment assessed the neuronal excitability, we used current-clamp mode and performed the following two protocols: (I) Applying 1 ms depolarizing current pulses to assessed the action potential shape (Figure 10,11); (II) Applying 500 ms depolarizing current pulses to assessed the characterized of firing pattern (Figure 12,13) (22). The protocols applied step current pulses from -15 pA to 85 pA with increase 5 pA per step (44). Moreover, in this experiment selected small-to-medium sized (15-37µm) (Figure 9A) and large sized TG neurons (>37 µm) (Figure 9B) for whole cell patch clamp recording. Morphologies of primary culture TG neurons was sphere, cleary nucleus, double cell membrane and clearly granule (Figure 9). Data obtained from short stimulation were used for action potential shape analysis.

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Figure 7. Whole cell patch clamp set up recording; A: Peristatic pump, B: Culture dish, C: Vacuum pump, D: Up light microscope, E: Anti-vibration isolation table, F: Manipulator, G: LCD monitor, H: Axopatch 200B amplifier, I: pClamp10.2

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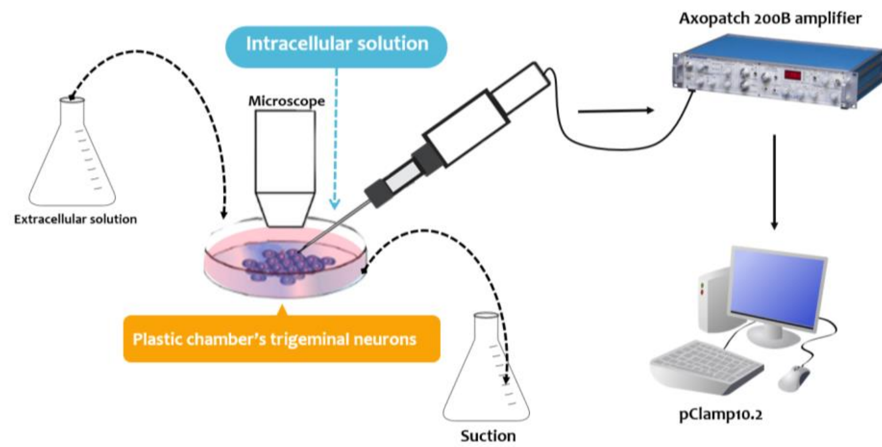

Figure 8. Extracellular circuit

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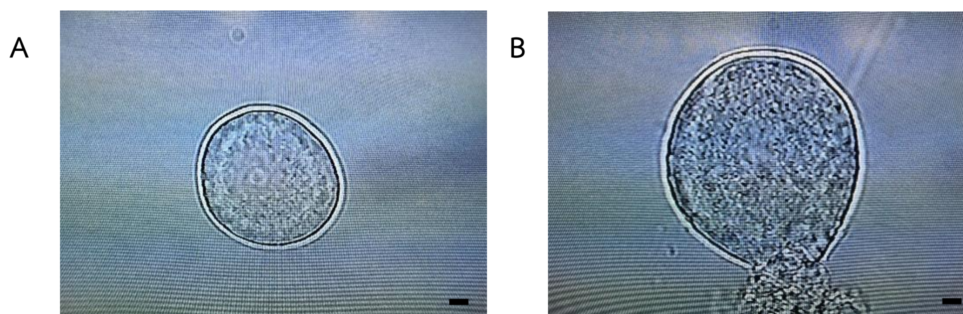

Figure 9. Representative morphology of TG neuron primary cell culture. A: The small-to-medium sized TG neuron was diameter 26.3 µm, B: The large sized TG neuron was diameter 43.5 µm, Scale bar = 5 µm. Using a 40x objective lens for measured these neurons.

Data collection

Data analyzed using Clampfit 10.2 software (Molecular Devices). After whole-cell establish, passive membrane recorded in membrane test program with clampex 10.2 software (Molecular Devices). In voltage-clamp recording (holding potential at -65 mV), measured in terms of peak amplitude, time of desensitization. Action potential (AP) variables analyzed the procedure of data analysis by Grigaliunas (45).

Electrophysiology variables to be measured comprise:

- Amplitude of action potentials (AP height) (mV) is measured from resting membrane to peak potential.
- Threshold (mV) is the minimum current that can stimulated neurons by elicited depolarization. Low threshold indicate neurons is increased excitability. Threshold of excitation is determined from injecting series of hyperpolarizing and depolarizing current step.
- Rheobase (pA) is the minimum current that will produce an action potential, for measure of membrane excitability. Lower rheobase indicate neurons is increased excitability.
- AP rising time (ms) is the duration of a depolarization, that is the time between threshold and peak amplitude of an AP. Longer AP rising time indicate enhancing neuronal excitability.
- Half duration of AP (ms) is measured from spike duration at half amplitude.
- AP height (mV) is measured as the enhancement of an AP from holding potential to peak amplitude of the AP. Higher AP height indicates enhancing neuronal excitability that Na+ equilibrium potential of neuron is raised.

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- Decay time of AP (ms) is measured from duration of the falling phase of spike amplitude. AP falling time (ms) is the duration of the positive phase decreased in cell membrane that is the time between peak amplitude and returning point to holding potential. AP falling time point to the function of K channel, shorter AP falling time indicate K channel is open for long periods as a result the decreasing of K^+ efflux. Thus shorter AP falling time indicates enhancing neuronal excitability.
- After hyperpolarization (AHP) amplitude (AHP depth) is measured from the level of RMP to the lowest point and AHP duration is defined as recovery time from the lowest point to the level of RMP describes the hyperpolarizing phase of a neuron action potential where the cell membrane potential falls below the normal resting potential.
- AHP duration time (ms) is the duration time from the lowest point of AHP to 50% of the recovery of the holding potential. Longer AHP duration indicates K channel is open for long periods.
- Total spikes are the frequency of AP. Higher spikes indicate enhancing neuronal excitability.

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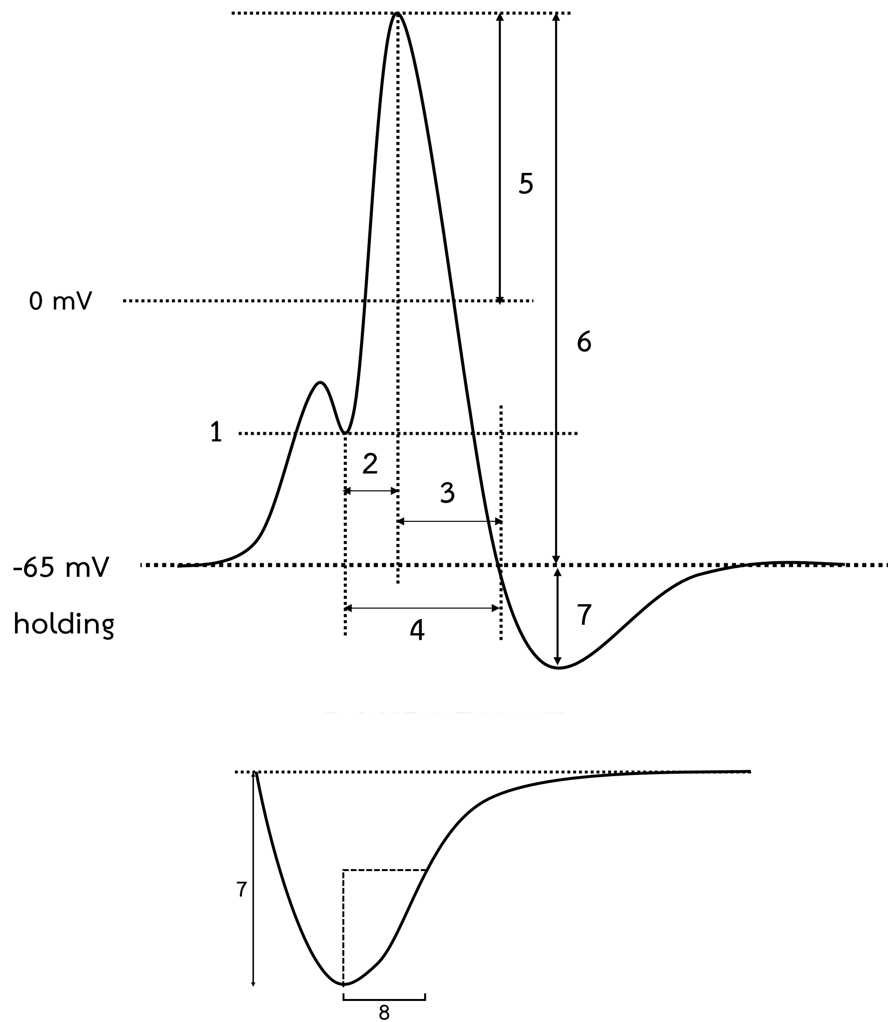

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Figure 10. Represent the assessment of AP properties by 1 ms depolarizing current pulses (5 pA /step) to assessed the action potential shape. These parameters were membrane potential: (1) Threshold, (2) AP rising time, (3) AP falling time, (4) AP duration, (5) AP overshoot, (6) AP height, (7) AHP depth, (8) 50% of hyperpolarization (AHP) duration.

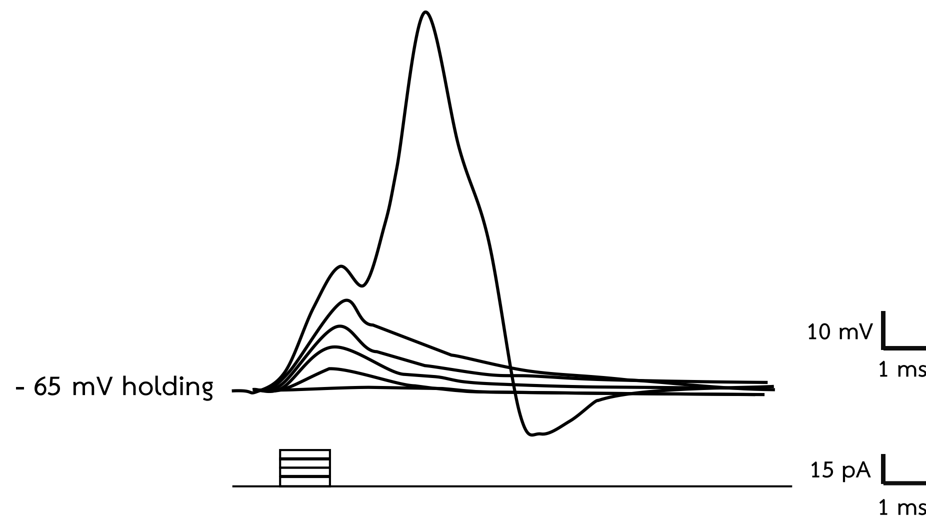

Figure 11. Sample of action potential obtained in response to 1 ms current pules. The rheobase (pA) was the minimum current that generated an AP. The depolarization phase of an AP generated in 6 step. This experiment applied current 5 pA /step thus rheobase of this AP was 30 pA.

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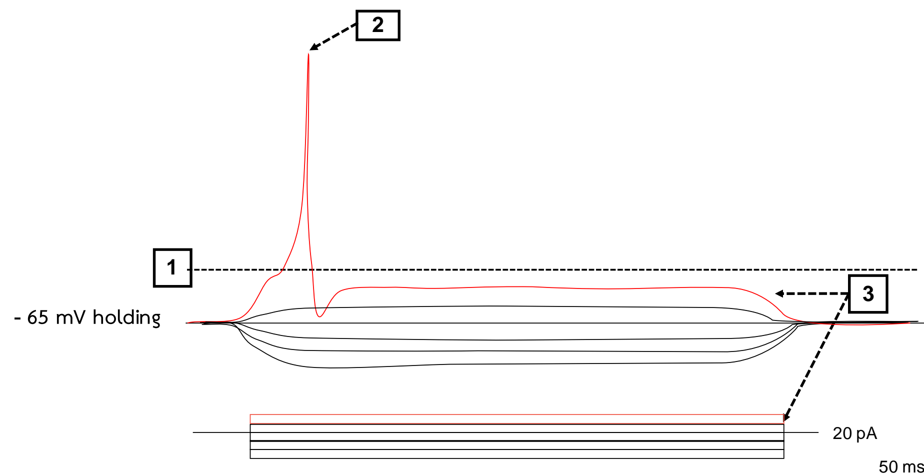

Figure 12. Represent the assessment of AP properties by 500 ms current pulses (5 pA /step). 1: Threshold, 2: Total spikes (1 total spikes), 3: Rheobase (10 pA) is measured from holding membrane potential (-65 mV) until occur the first AP. The depolarization phase of an AP produce in 2 step. This experiment applied current 5 pA per step thus rheobase of this AP is 10 pA.

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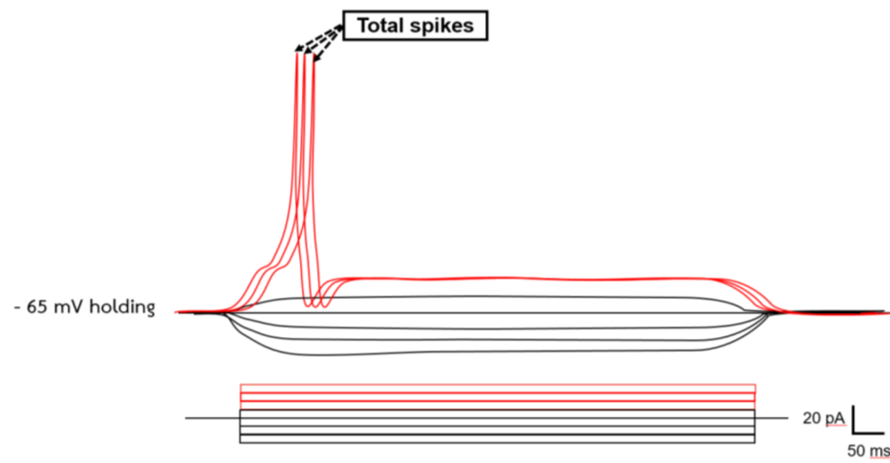

Figure 13. Showed the total spikes were measured frequency of AP. This AP have 3 total spikes.

Statistical analysis

All data showed as means ± SEM. Statistical analysis were done using ANOVA and post hoc test. A probability value (p value) of < 0.05 was accepted as indicative of a statistically significant difference.

CHAPTER IV

RESULT

Effect of SGCs on electrical property of small-to-medium sized TG neurons and effect of CGRP and capsaicin

This experiment tested the role of SGCs on the excitability of small-to-medium sized TG neuron. Primary TG neurons culture with and without SGCs showed in figure 14 and 15 on phase contrast microscope. The morphology of small-to-medium TG neurons measured using a 40x objective lens up light microscope (Figure 16). The stimulation started by applying 1 ms current to determine the change in the action potential property in presence and absence of SGCs (Figure 17). The result showed no significant difference in RMP between two conditions (Table 1) (Figure 18). Despite the absence of change during the resting stage, we found that TG neurons were more readily to generate action potential in the presence of SGCs. The threshold for action potential generation was more negative in the presence of SGCs compared to the pure culture group (-28.91 ±0.82 and -26.85 ±1.07, p= 0.0262 respectively). In alignment with the more negative threshold, the rheobase measured from the co-cultured group was significantly lower than the pure cultured group. (43.65 ± 1.41 and 54.07 ± 3.49 pA, for the presence and absence of SGCs respectively, p= 0.0006).

Exposure to CGRP led to an increase in excitability of TG neurons without affecting the resting membrane potential. The threshold for action potential was more negative in all tested groups compared with the controls. The rheobase of the CGRP groups were significantly lower than the respective controls (42.64 ± 2.51 and 54.07 ± 3.49 pA, respectively, p<0.0001) (Figure 19, 20).

Similar pattern was observed in the TG groups exposed to capsaicin. Capsaicin did not alter the resting membrane potential. The threshold for action potential generation in the pure neuronal cultured and co-cultured group with capsaicin exposure were -32.34 ±0.84 and -37.10 ±0.75 mV, respectively (p=0.0269). The rheobase from the co-cultured group was lower than the pure culture group. Our results also showed that the change in the repolarizing phase of action potential. The AP falling time, AP duration and AHP duration were shorter in the presence of SGCs. Interestingly, in the presence of CGRP or capsaicin, the AP falling time and AP duration of the co-cultured group was longer than those of the pure neuronal group. These findings were different from the data obtained from the control condition of which AP falling time and AP duration was shorter in the presence of SGCs. The AHP duration was shorter in the presence of SGCs but the difference in the capsaicin did not reach statistical significance.

To determine the pattern of action potential by applying 500 ms depolarizing current pulse of increasing intensity to TG neurons in presence and absence of SGCs (Figure 21,23). The result showed similar pattern for those obtained from short stimulation study. Comparing between pure neuronal and co-cultured group, the RMP was not different but the threshold was more negative in the presence of SGCs (Table 2) (Figure 24). The thresholds for action potential generation were -23.49 ± 1.20 and -32.98 ±1.26 mV, for pure neuronal and co-cultured group respectively ($p<0.0001$). The rheobase was significantly lower in the co-cultured group. Higher numbers of spikes were demonstrated in the co-culture group. These findings suggest that TG neurons are more sensitive in the presence of SGCs. The relationship of current-voltage (I-V) showed in figure22.

The effect of SGCs in enhancing excitability of TG neurons was more pronounced when TG neurons were exposed to CGRP or capsaicin. Compared with the pure neuronal cultured condition, the threshold was more negative, rheobase

was lower and total spikes were higher in the presence of SGCs. The rheobase of the CGRP groups were significantly lower than the respective controls (23.28 ± 2.05 and 33.09 ± 2.28 pA, respectively, $p<0.0001$. The numbers of spikes observed in the CGRP groups were higher than the respective controls in both pure and co-cultured conditions) (Figure25, 26). In addition, capsaicin did not alter the resting membrane potential but shifted the threshold towards more negative. The threshold for action potential generation in the pure neuronal cultured and co-cultured group with capsaicin exposure were -41.94 ±0.78 and -45.71 ± 0.69 mV, respectively ($p=0.0068$).

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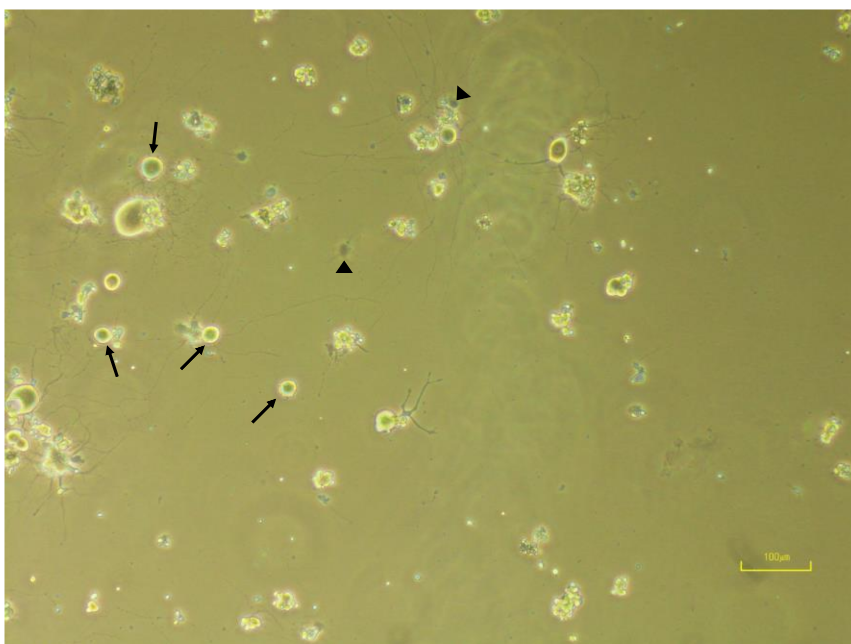

Figure 14. Phase contrast images of TG neurons in primary pure culture at 24 h after pre-plating and adding cytosine arabinoside (AraC), arrow show TG neurons, arrow head show SGCs. Scale bar 100 μm. These cells were measured using a 10x objective lens.

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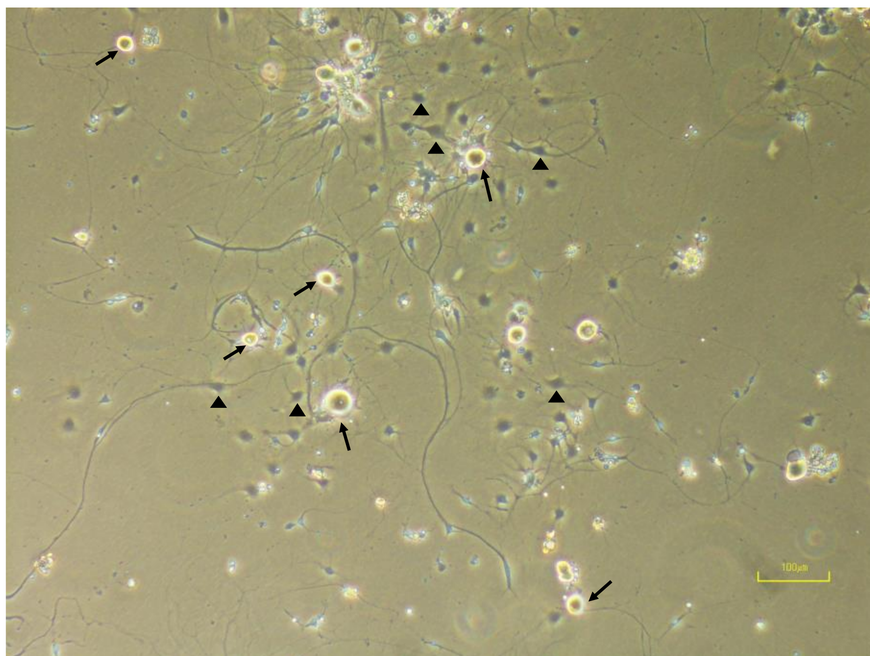

Figure 15. Phase contrast images of TG neurons in primary co-culture at 24 h without cytosine arabinoside (AraC), arrow show TG neurons, arrow head show SGCs. Scale bar 100 µm. These cells were measured using a 10x objective lens.

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Figure 16. Representative morphology of small-to-medium sized TG neuron primary cell culture. The diameter was 21.3 μm, scale bar = 5 μm. Using a 40x objective lens from up light microscope.

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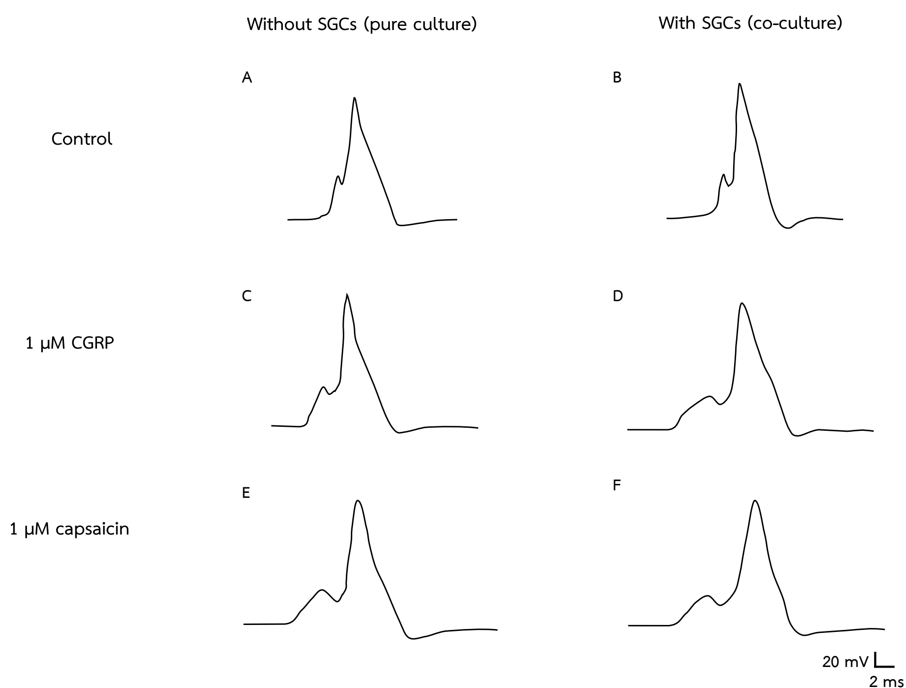

Figure 17. The sample of action potential property small-to-medium sized primary culture TG neurons by 1 ms current. A: Pure culture control (without SGCs) group, B: Co-culture control (with SGCs), C: Pure culture in the presence of 1 μM CGRP, D: Co-culture in the presence of 1 μM CGRP, E: Pure culture in the presence of 1 μM capsaicin, F: Co-culture in the presence of 1 μM capsaicin

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Parameter	Control		With CGRP		With capsaicin	
	Without SGCs (n = 35)	With SGCs (n = 50)	Without SGCs (n = 64)	With SGCs (n = 54)	Without SGCs (n = 43)	With SGCs (n = 55)
RMP (mV)	-50.52 ± 1.10	-48.77 ± 0.97	-50.05 ± 0.79	-49.77 ± 0.97	-49.72 ± 1.64	-48.51 ±1.12
Rheobase (pA)	54.07 ± 3.49	43.65 ± 1.41**	42.64 ± 2.51	35.90 ± 1.37*	34.38 ± 1.23	29.74 ± 0.76
Threshold(mV)	-26.85 ± 1.07	-28.91 ± 0.82*	-32.84 ± 0.62	-37.38 ± 1.02**	-32.34 ± 0.84	-37.10 ± 0.75**
AP Height (mV)	120.2 ± 3.47	119.1 ± 3.36	117.7 ± 2.59	113.1 ± 2.42	117.8 ± 3.02	117.4 ± 2.95
AP Overshoots (mV)	56.24 ± 3.46	58.04 ± 3.29	59.25 ± 1.86	58.78 ± 2.24	55.98 ± 2.73	60.67 ± 1.90
AP Rising Time (ms)	1.20 ± 0.10	1.13 ± 0.13	1.18 ± 0.07	1.15 ± 0.09	1.11 ± 0.10	1.13 ± 0.14
AP Falling Time (ms)	5.35 ± 1.00	2.20 ± 0.43*	3.43 ± 4.22	5.30 ± 0.71*	5.08 ± 0.89	7.09 ± 1.46*
AP Duration (ms)	5.91 ± 1.02	3.99 ± 0.40*	4.67 ± 0.45	6.40 ±0.72*	5.56 ± 0.79	7.81 ± 1.47*
AHP Depth (mV)	-5.41 ± 0.67	-6.76 ± 0.48	-7.54 ± 0..47	-6.58 ± 0.63	-7.40 ± 0.69	-6.90 ± 0.68
AHP Duration (ms)	5.89 ± 0.87	3.77 ± 0.53**	3.034 ± 043	1.55 ± 0.27*	0.96 ± 0.21	0.50 ± 0.17

Table 1. Effect of SGCs on electrical property of small-to-medium sized TG neurons and effect of CGRP and capsaicin by 1 ms current. (Values are mean ± SEM, *p<0.05, **p<0.01 compared with pure culture group in each condition.)

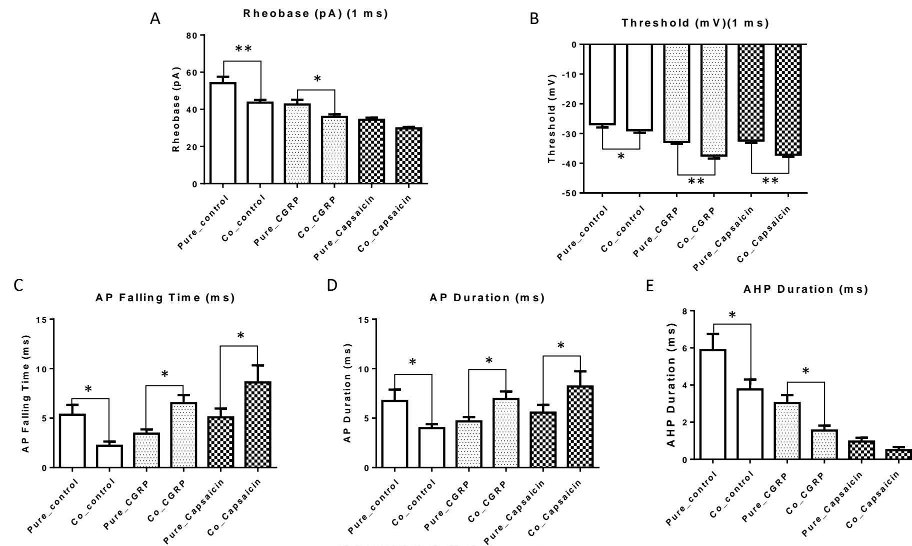

Figure 18. Comparing the electrical properties of small-to-medium TG neurons stimulated by 1 ms current. A: Rheobase (pA), B: Threshold (mV), C: AP falling time (ms), D: AP duration (ms), E: AHP duration (ms). (*p<0.05, **p<0.01 co-culture compared with pure culture group in each condition.)

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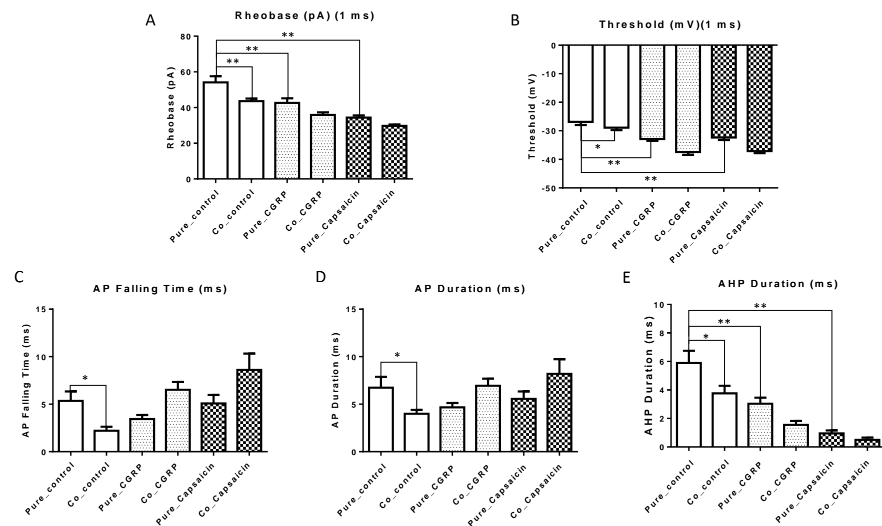

Figure 19. Comparing pure culture group (without SGCs) between each condition on electrical properties of small-to-medium sized TG neurons stimulated by 1 ms current. A: Rheobase (pA), B: Threshold (mV), C: AP falling time (ms), D: AP duration (ms), E: AHP duration (ms). (*p<0.05, **p<0.01 compared with pure culture group in each condition.)

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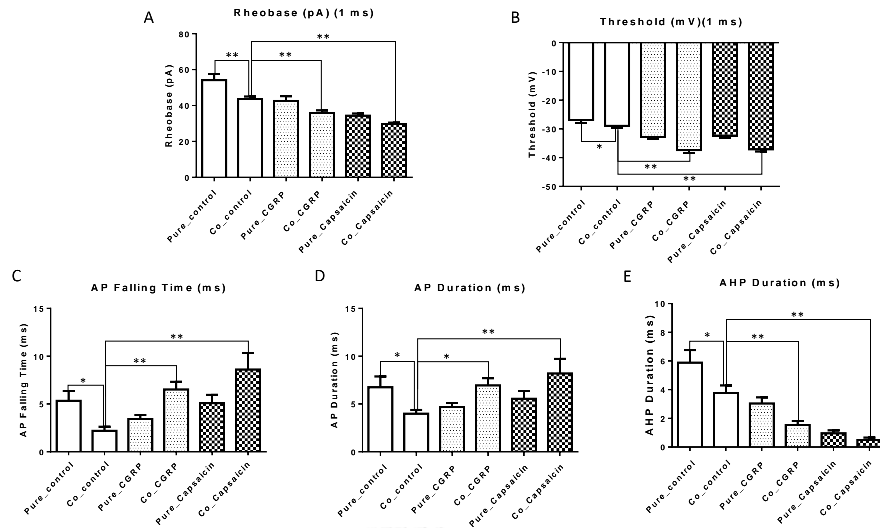

Figure 20. Comparing co-culture group (with SGCs) between each condition on electrical properties of small-to-medium TG neurons stimulated by 1 ms current. A: Rheobase (pA), B: Threshold (mV), C: AP falling time (ms), D: AP duration (ms), E: AHP duration (ms). (*p<0.05, **p<0.01 compared with co-culture group in each condition.)

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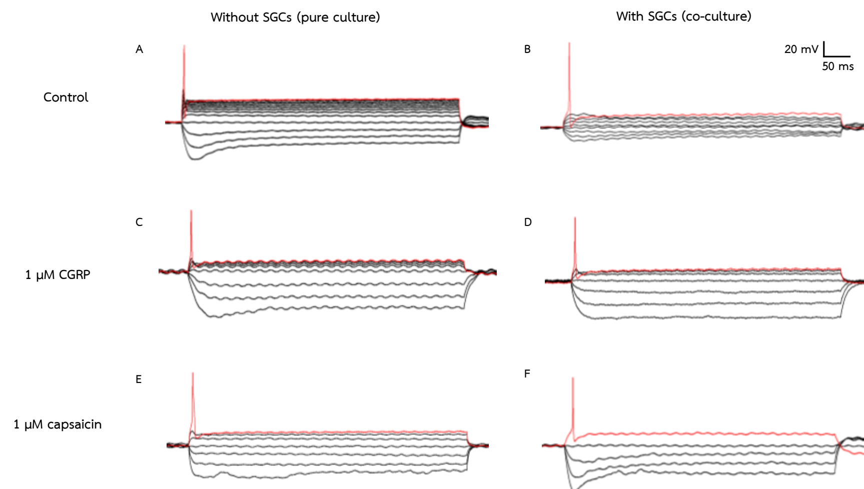

Figure 21. Voltage traces of small-to-medium sized primary culture TG neurons obtained in response to 500 ms current pules (5pA per step, 21 step). The red trace referred to applying current pluses which the first action potential was elicited. A: Pure culture control (without SGCs) group (11step, 35pA), B: Co-culture control (with SGCs) group (9step, 25pA), C: Pure culture in the presence of 1 μM CGRP (8step, 20pA), D: Co culture in the presence of 1 μM CGRP (7step, 15pA), E: Pure culture in the presence of 1 μM capsaicin (7step, 15pA), F: Co-culture in the presence of 1 μM capsaicin (5step, 5 pA)

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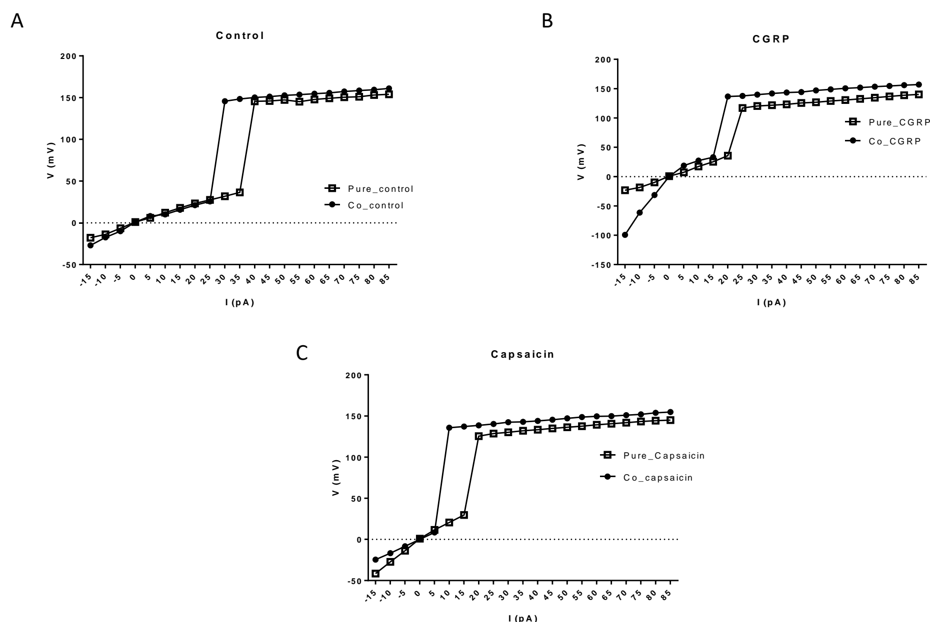

Figure 22. Current-voltage (I-V) curve of small-to-medium TG neurons correlate AP height (mV) and current pulses at -15 to 85 pA (21 step). This curve showed that AP height was no different in each condition, (A): co-culture control group request current pulses lower than pure culture (25pA, 35pA respectively), (B): In CGRP group, the rheobase in co-culture and pure culture were 15pA and 20pA respectively, (C): In capsaicin, the rheobase in co-culture and pure culture were 5pA and 15pA respectively.

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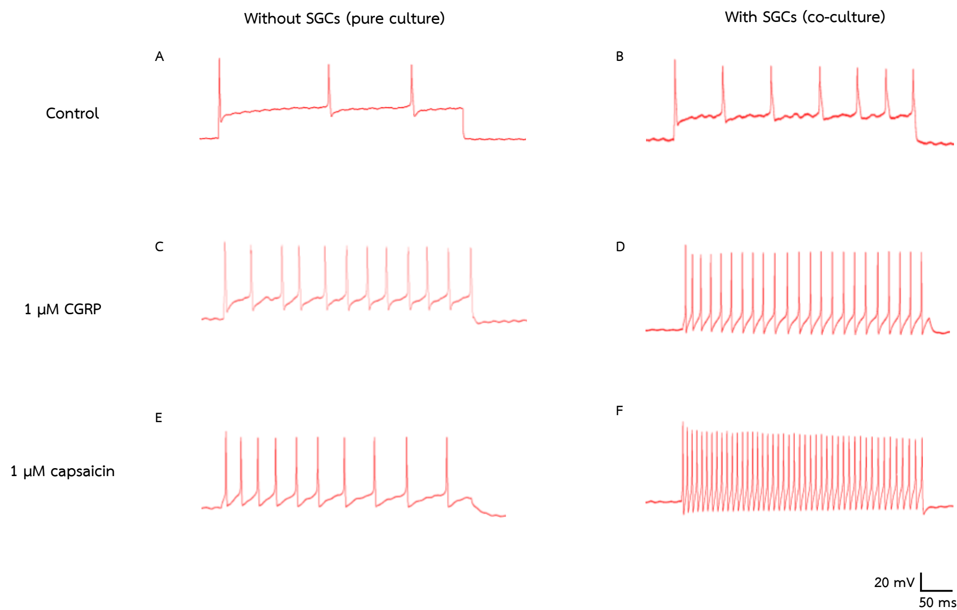

Figure 23. Characteristic of trace action potential small-to-medium sized primary culture TG neurons by 500 ms current (5pA per step, 21 step). The red trace referred the frequency of action potential. A: Pure culture control (without SGCs) group, B: Co-culture control (with SGCs) group, C: Pure culture in the presence of 1 μM CGRP, D: Co-culture in the presence of 1 μM CGRP, E: Pure culture in the presence of 1 μM capsaicin, F: Co-culture in the presence of 1 μM capsaicin.

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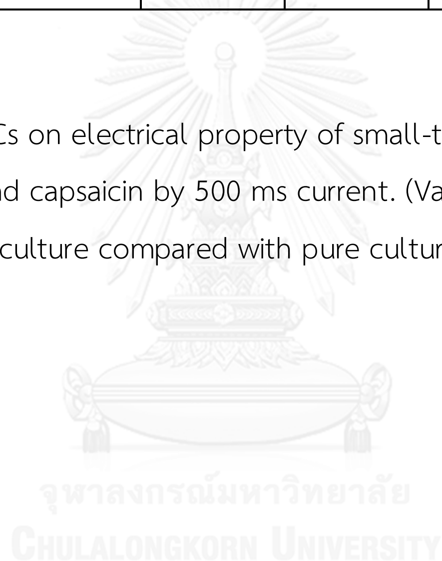
Parameter	Control		With CGRP		With capsaicin	
	Without SGCs (n = 35)	With SGCs (n = 50)	Without SGCs (n = 64)	With SGCs (n = 54)	Without SGCs (n = 43)	With SGCs (n = 55)
RMP (mV)	-50.52 ± 1.10	-48.77 ± 0.97	-50.05 ± 0.79	-49.77 ± 0.97	-49.72 ± 1.64	-48.51 ±1.12
Threshold (mV)	-23.49 ± 1.20	-32.98 ±1.26**	-36.25 ± 0.83	-40.50 ±0.66**	-41.94 ±0.78	-45.71 ± 0.69**
Rheobase (pA)	33.09 ± 2.28	27.06 ± 1.52*	23.28 ± 2.05	17.07 ± 1.30*	17.17 ± 1.29	10.12 ± 0.68*
Total spikes	9.946 ± 1.78	21.65 ± 4.93	33.42 ± 4.95	45.56 ± 9.46	36.42 ± 8.03	69.46 ± 12.65**

Table 2. Effect of SGCs on electrical property of small-to-medium sized TG neurons and effect of CGRP and capsaicin by 500 ms current. (Values are mean ± SEM, *p<0.05, **p<0.01 co-culture compared with pure culture group in each condition.)



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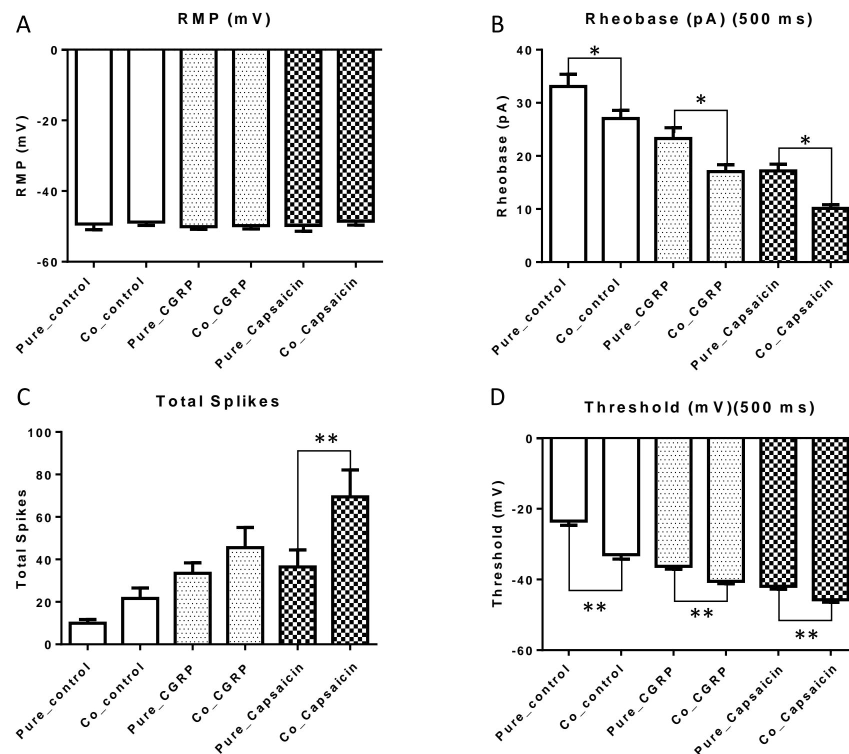

Figure 24. Comparing the electrical properties of small-to-medium sized TG neurons stimulated by 500 ms current. A: Resting membrane potential (RMP) (mV), B: Rheobase (pA), C: Total spikes, D: Threshold (mV). (*p<0.05, **p<0.01 co-culture compared with pure culture group in each condition.)

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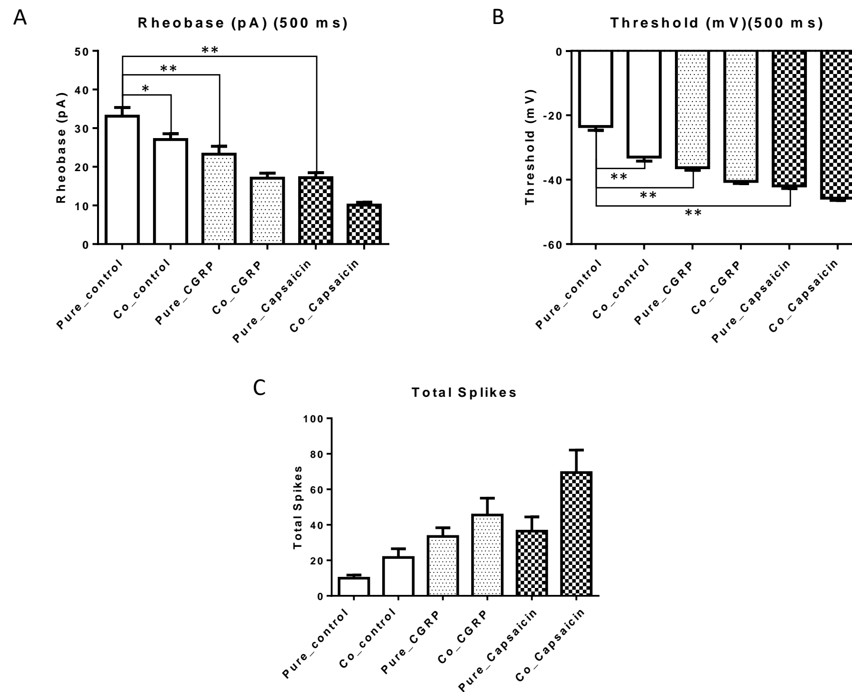

Figure 25. Comparing pure culture group (without SGCs) between each condition on electrical properties of small-to-medium TG neurons stimulated by 500 ms current. A: Rheobase (pA), B: Total spikes, C: Threshold (mV). (*p<0.05, **p<0.01 compared with pure culture group in each condition.)

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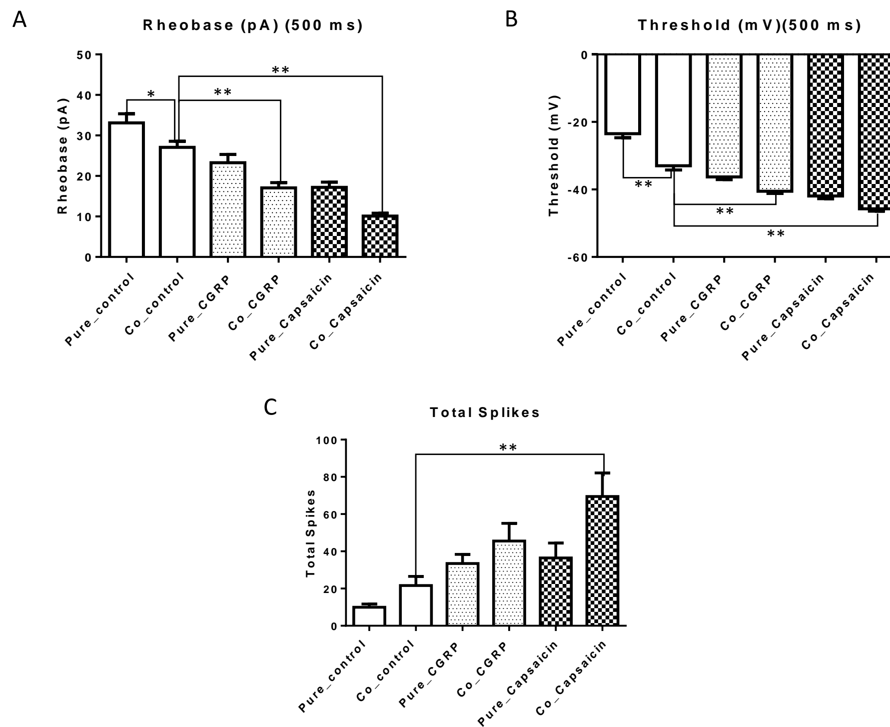

Figure 26. Comparing co-culture group (with SGCs) between each condition on electrical properties of small-to-medium TG neurons stimulated by 500 ms current. A: Rheobase (pA), B: Total spikes, C: Threshold (mV). (*p<0.05, **p<0.01 compared with co-culture group in each condition.)

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Effect of SGCs on electrical property of large sized TG neurons and effect of CGRP and capsaicin

The same stimulation protocol was used to determine the effect of SGCs on the excitability of large sized TG neuron (Figure 27, 28). The overall results showed that the effect of SGCs on large and small TG neurons was not markedly different. Presence of SGCs did not affect the RMP of large TG neurons. Similar to the results obtained in the small-to-medium sized neurons, the threshold of large TG neurons became more negative when co-cultured with SGCS (-20.99 ± 2.19 and -31.58 ± 2.67 mV for pure and co-cultured groups respectively, p= 0.0354). No changes in other action potential parameters between pure and co-cultured groups was observed (Table 3) (Figure 29).

Our results showed that TG neurons were more excitable when being exposed to CGRP. In control condition, presence of SGCs caused large TG neurons to fire at more negative potential. The thresholds for action potential generation measured in the control and CGRP groups were -20.99 ± 2.19 and -34.79 ± 1.33 mV, respectively. Threshold was more negative (-43.72 ±0.64 mV) when neurons were co-cultured with SGCs. In accordance with the change in threshold, the rheobase was significantly lower in the co-cultured group as compared with the pure cultured group (61.67 ± 4.65 and 75.77 ± 6.20 pA for co-cultured and pure cultured group, respectively, p=0.0262).

Similar pattern was observed in the groups exposed to capsaicin. The threshold for action potential generation in the co-cultured group was more negative than the pure cultured condition (-38.22 ± 1.72and -31.20 ± 3.31 mV respectively, p=0.0402). The rheobase was lower than the pure culture group (Figure 30). Our results also showed that the AP falling time, AP duration and AHP duration were no difference. Interestingly, it was noted that the effect of capsaicin on threshold was less pronounced compared to that seen in the CGRP group.

Similar patterns were observed TG neurons were stimulated by 500 ms depolarizing current pulse of increasing intensity (Figure31). Compared between pure neuronal and co-cultured group, the RMP was not different but the threshold was more negative in the presence of SGCs (Table 4) (Figure 33). The thresholds for action potential generation were -25.85 ± 3.14 and -34.21 ± 3.55 mV, for pure neuronal and co-cultured group respectively (p=0.0255). The rheobase also became lower in the co-cultured group but the difference did not reach the statistically significant level. Number of total spikes was not different. The relationship of current-voltage (I-V) showed in figure 32.

Exposure to CGRP or capsaicin enhanced the excitability of large TG neurons. For pure cultured condition, thresholds for action potential were -38.20 ± 2.16, -25.85 ± 3.14 and -37.47 ± 1.13 mV for CGRP, capsaicin-treated and control groups respectively. Compared with the pure neuronal cultured condition, the thresholds of all respective co-cultured groups were significantly more negative. No change in number of spikes was evident (Figure 34).

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Figure 27. Representative morphology of large sized TG neuron primary cell culture was diameter 45.2 µm, scale bar = 5 µm. Using a 40x objective lens from up light microscope.

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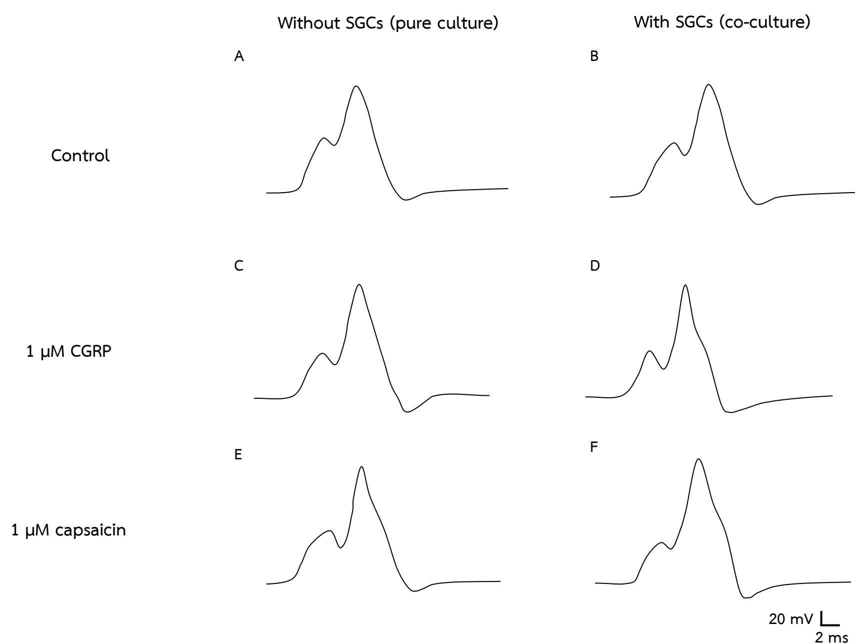

Figure 28. The sample of action potential property large sized primary culture TG neurons by 1 ms current. A: Pure culture control (without SGCs) group, B: Co-culture control (with SGCs), C: Pure culture in the presence of 1 μM CGRP, D: Co-culture in the presence of 1 μM CGRP, E: Pure culture in the presence of 1 μM capsaicin, F: Co-culture in the presence of 1 μM capsaicin

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Parameter	Control		With CGRP		With capsaicin	
	Without SGCs (n = 6)	With SGCs (n = 14)	Without SGCs (n = 16)	With SGCs (n = 13)	Without SGCs (n = 16)	With SGCs (n = 14)
RMP (mV)	-55.58 ± 1.05	-54.92 ± 1.39	-54.79 ± 0.76	-58.19 ± 0.78	-54.19 ± 0.88	-54.11 ± 1.45
Rheobase (pA)	76.43 ± 3.73	70.63 ± 9.33	75.77 ± 6.20	61.67 ± 4.65*	75.25 ± 3.35	61.67 ± 2.70*
Threshold (mV)	-20.99 ± 2.19	-31.58 ± 2.67*	-34.79 ± 1.33	-43.72 ± 0.64*	-31.20 ± 3.31	-38.22 ± 1.72*
AP Height (mV)	119.50 ± 7.45	119.90 ±4.71	118.60 ± 4.26	113.60 ± 3.40	122.70 ± 1.85	120.20 ± 4.70
AP Overshoots (mV)	60.18 ± 6.39	59.61 ± 4.33	57.63 ± 4.43	55.97 ± 5.19	61.24 ± 1.98	57.14 ± 4.49
AP Rising Time (ms)	0.74 ± 0.11	0.80 ± 0.08	0.68 ± 0.08	0.88 ± 0.10	0.68 ± 0.07	0.83 ± 0.10
AP Falling Time (ms)	1.14 ± 0.44	1.19 ± 0.35	1.12 ± 0.27	1.17 ± 0.24	1.25 ± 0.27	1.07 ± 0.08
AP Duration (ms)	1.75 ± 0.36	1.72 ± 0.25	1.77 ± 0.28	1.65 ± 0.18	1.90 ± 0.24	1.83 ± 0.17
AHP Depth (mV)	-14.24 ± 1.37	-8.91 ± 1.44	-10.75 ±1.21	-8.67 ± 1.13	-10.08 ± 1.12	-8.33 ± 1.08
AHP Duration (ms)	1.75 ± 1.02	1.22 ± 0.51	1.87 ± 0.43	1.34 ± 0.57	0.72 ± 0.24	0.47 ± 0.18

Table 3. Effect of SGCs on electrical property of large sized TG neurons and effect of CGRP and capsaicin stimulated by 1 ms current. (Values are mean ± SEM, *$p<0.05$, **$p<0.01$ co-culture compared with pure culture group in each condition.)

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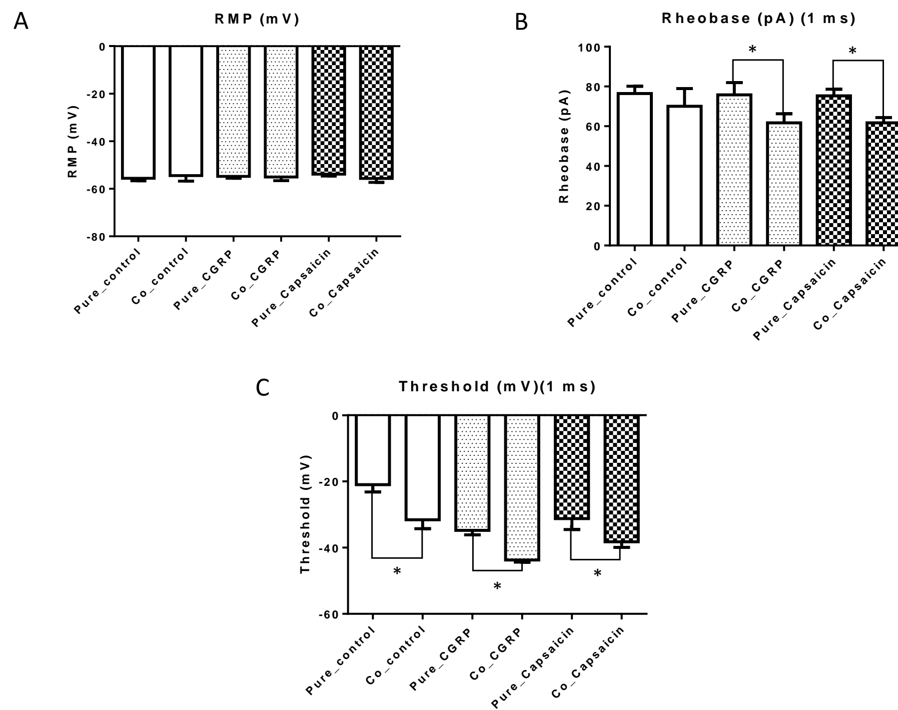

Figure 29. Comparing the electrical properties of large TG neurons stimulated by 1 ms current. A: Resting membrane potential (RMP) (mV), B: Rheobase (pA), C: Threshold (mV). (*p<0.05, **p<0.01 co-culture compared with pure culture group in each condition.)

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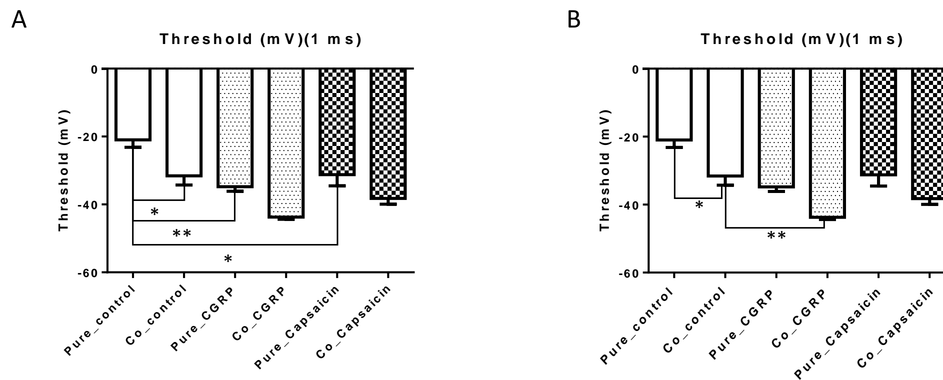

Figure 30. The electrical properties of large TG neurons stimulated by 1 ms current. A: Comparing threshold of pure culture group with each condition, B: Comparing threshold of co-culture group with each condition. (*p<0.05, **p<0.01)

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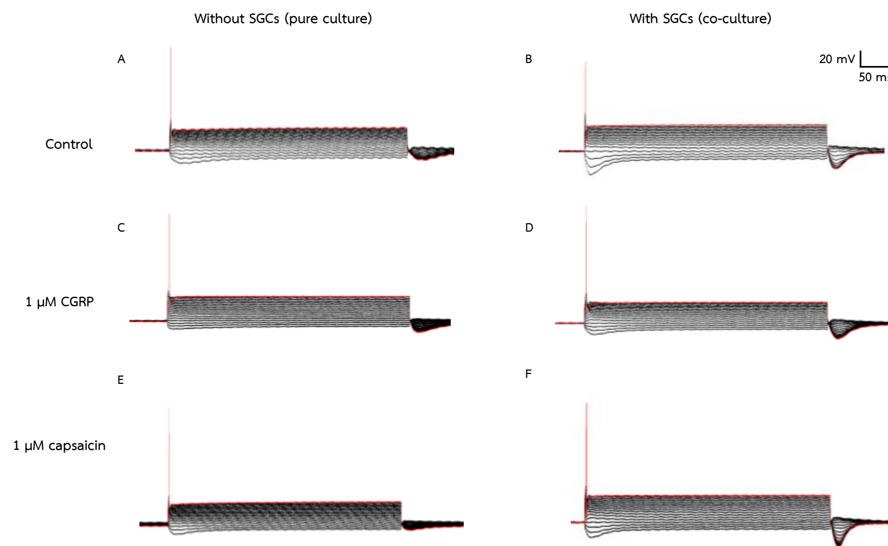

Figure 31. Voltage traces of large sized primary culture TG neurons obtained in response to 500 ms current pules (5pA per step, 21step). The red trace referred to applying current pluses which the first action potential was elicited. A: Pure culture control (without SGCs) group (19step, 75pA), B: Co-culture control (with SGCs) group (18step, 70pA), C: Pure culture in the presence of 1 µM CGRP (18step, 70pA), D: Co-culture in the presence of 1 µM CGRP (16step, 60pA), E: Pure culture in the presence of 1 µM capsaicin (18step, 70pA), F: Co-culture in the presence of 1 µM capsaicin (17step, 65 pA)

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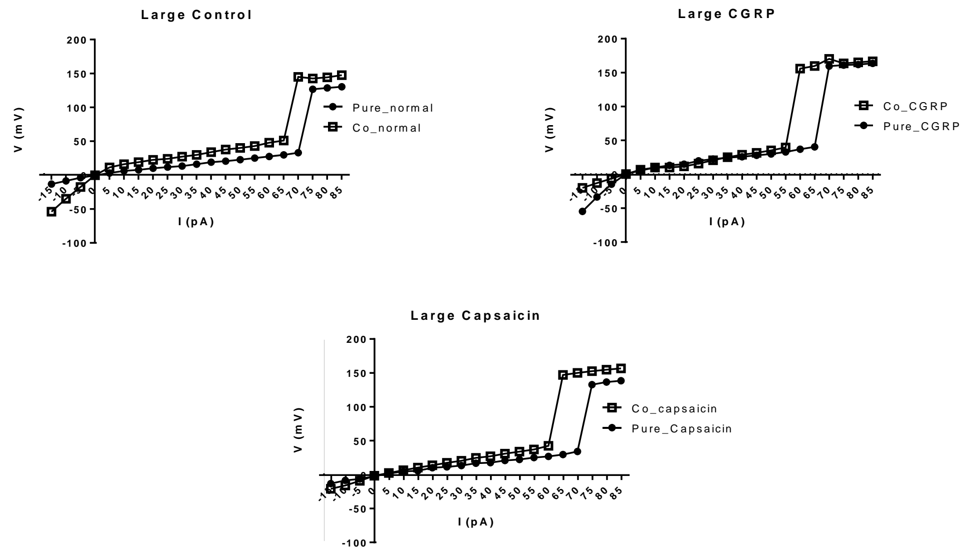

Figure 32. Current-voltage (I-V) curve of large TG neurons correlate AP height (mV) and current pulses at -15 to 85 pA (21 step). This curve showed that co-culture group was increasing excitability in each condition compared with pure culture, (A): co-culture control group request current pulses lower than pure culture (65pA, 70pA respectively), (B): In CGRP group, the rheobase in co-culture and pure culture were 55pA and 65pA respectively, (C): In capsaicin, the rheobase in co-culture and pure culture were 60pA and 70pA respectively

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Parameter	Control		With CGRP		With capsaicin	
	Without SGCs (n = 6)	With SGCs (n = 14)	Without SGCs (n = 16)	With SGCs (n = 13)	Without SGCs (n = 16)	With SGCs (n = 14)
RMP (mV)	-55.58 ± 1.05	-54.43 ± 2.37	-54.79 ± 0.76	-55.01 ± 1.58	-53.84 ± 0.78	-55.66 ±1.66
Rheobase (pA)	-25.85 ± 3.14	-34.21 ± 3.55*	-38.20 ± 2.16	-44.27 ± 1.27*	-37.47 ± 1.13	-39.12 ± 1.85
Threshold (mV)	75.00 ± 4.83	61.00 ± 9.93	70.56 ± 3.58	58.33 ± 5.20	78.00 ± 4.36	63.18 ± 5.45**
Total spikes	8.14 ± 1.41	8.14 ± 1.07	9.27 ± 1.10	9.93 ± 1.62	8.71 ± 1.59	9.20 ± 1.19

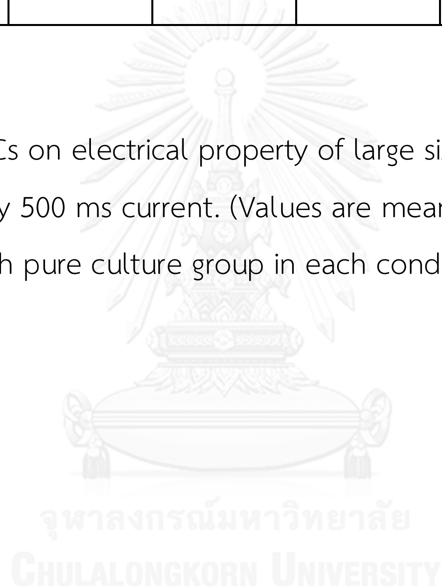

Table 4. Effect of SGCs on electrical property of large sized TG neurons and effect of CGRP and capsaicin by 500 ms current. (Values are mean ± SEM, *p<0.05, **p<0.01 co-culture compared with pure culture group in each condition.)

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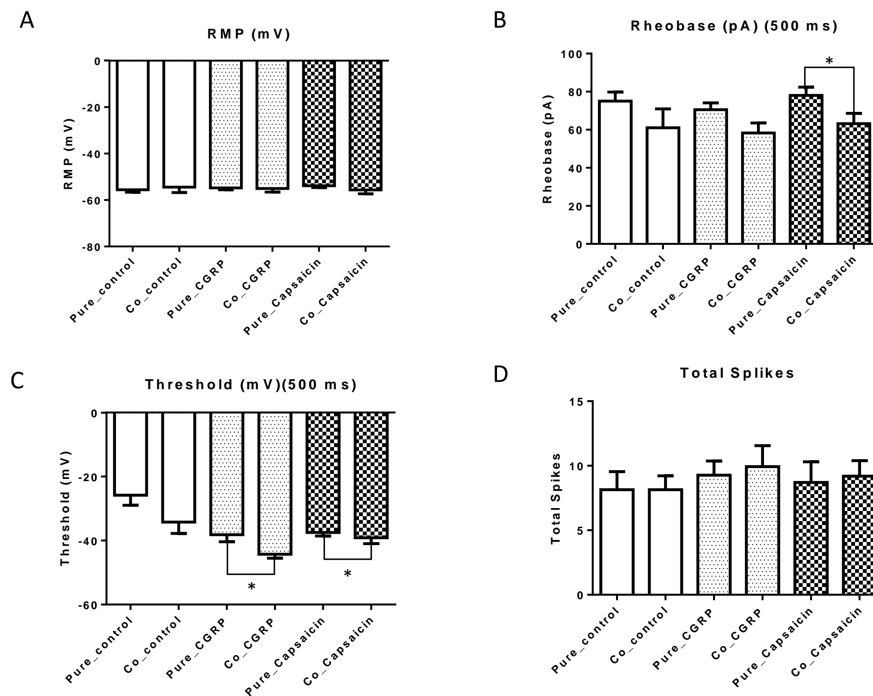

Figure 33. Comparing the electrical properties of large TG neurons stimulated by 500 ms current. A: Resting membrane potential (RMP) (mV), B: Rheobase (pA), C: Threshold (mV), D: Total spikes. (*p<0.05, **p<0.01 co-culture compared with pure culture group in each condition.)

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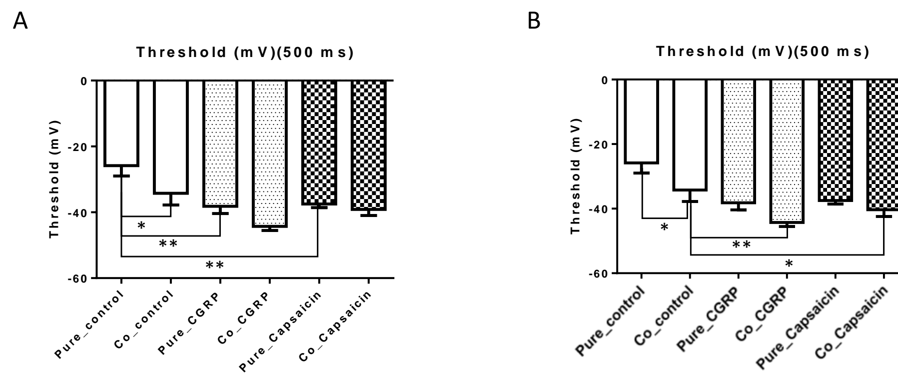

Figure 34. The electrical properties of large TG neurons stimulated by 500 ms current. A: Comparing threshold of pure culture group with each condition, B: Comparing threshold of pure culture group with each condition. (*p<0.05, **p<0.01)

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CHAPTER V

DISSCUSSIONS

The main findings of this study are (1) both small-to-medium sized and large TG neurons were more excitable in the presence of SGC and (2) the change was more pronounced when cells were exposed to CGRP and capsaicin and (3) despite the difference in the expression of membrane ion channels, the electrical response of small-to-medium sized and large TG neurons to the presence of SGCS was not substantially different.

Effect of SGCs on TG neurons

Our study showed that both small-to-medium sized and large TG neurons were more excitable in the presence of SGC and these effects were greater after being exposed to CGRP or capsaicin. These results indicate the role of SGCs in modifying the excitability of TG neurons and may involve in the process of pathogenesis of chronic headache and facial pain.

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It is known that, in addition to its role as supporting system, SGCs can release several chemical messengers involved in the process of nociception. Previous studies showed that SGCs are activated in the inflammatory condition. During the inflammatory process, several pro-nociceptive messengers such as CGRP, substance P, etc. are released from nerve terminals. CGRP can activate SGCs by binding to its receptors on glia cell membrane. These activated glial cells then can release P38, TNF-α, NO as well as several proinflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10) (2, 3, 5). NO can activate cyclooxygenase

enzyme in SGC and increase the production of prostaglandin (4, 6). The inflammatory chemicals will increase the excitability of TG neurons by the process of sensitization.

Opening of voltage-gated Na channel channels play a critical role generating the action potential by initializing the depolarization phase. Concerning nociception, tetrodoxin-resistant (TTX-R) Na_v 1.8 and Na_v 1.9 were expressed in small-diameter dorsal root ganglion, unmyelinated C-fiber axons, weakly myelinated Aδ-fiber axons and nociceptive neurons (46-48). An increase in conductance of these Na channels is the main mechanism underlying the process of peripheral sensitization. For instance, Rush et al showed that prostaglandin-E_2 could enhance the TTX-R Na_v 1.9 in small dorsal root ganglion neurons. This led to the development of action potential (AP) at lower threshold and lower rheobase (48, 49).

Alteration of other ion channels can also involve in the changing the sensitivity of sensory neurons. Opening of voltage-gated K channels play a role in repolarization phase of action potential and the after hyperpolarization (AHP) period. Therefore, changes in these channels may explain our findings of the higher AP falling time and lower AHP duration seen in the CGRP and capsaicin groups (22).

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Effect of capsaicin and CGRP on different sized TG neurons

Our study showed that both small-to-medium sized and large TG neurons were more excitable in the presence of capsaicin or CGRP. It should be noted that small-to-medium sized and large TG neurons express different pattern of ion channel and membrane receptors. TRP channels, the receptors for capsaicin, are expressed in the small-to-medium sized neurons while CGRP receptors are expressed in large TG neurons.

How do the different sized TG neurons exert the similar response? Given that small-to-medium sized neurons are lack of CGRP receptors (23, 50, 51), the effect of CGRP on these neurons cannot be direct. The more likely mechanism is that CGRP binds to its receptors on large TG neurons and SGCs. Upon activation, these cells (both neurons and glia cells), release pro-nociceptive chemicals such as IL-1β, IL-6, IL-10, NO, PGs, etc. These chemical messengers then activate or sensitize the small-to-medium sized nociceptive TG neurons, resulting in the peripheral sensitization. In addition, CGRP can enhance the expression of MAP kinases p38 and ERK, and PKA in trigeminal ganglion cause increasing of nociceptive transduction (3).

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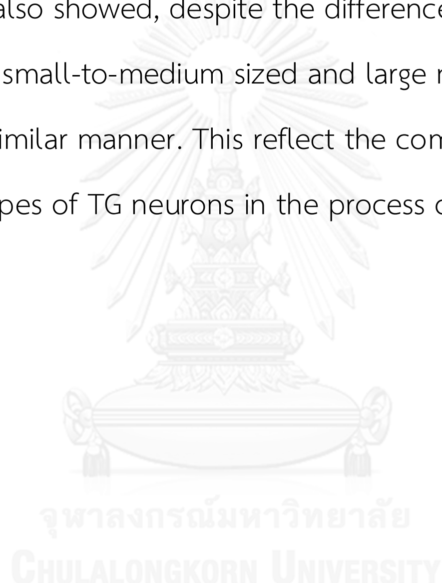
Concerning the response of TG neurons to capsaicin, this chemical messenger exerts its activity by coupling with its intracellular binding site on the transient receptor potential vanilloid type l (TRPV1) receptor. The TRPV1 is a non-selective cation channel which can be activated by noxious temperatures (>43°C), acid (pH <5.9) and capsaicin (52, 53). TRPV1 channels are expressed mainly on the small-to-medium sized TG neurons (54). Activation of TRPV1 channels leads to neuronal phosphorylation and changes in protein expression such as S100B, P38, camKll, protein kinase etc. Coupling between capsaicin and TRPV1 also activates the release of several inflammatory mediators including CGRP (6, 55, 56) cause increasing excitability of TG neurons (13, 21, 22). Previous study found CGRP and TRPV1 are co-

localized in small-to-medium sized TG neurons thus capsaicin has an important role of sensitization (26). In addition to the effect on TG sensitization, CGRP can cause more prolong change in TG neurons. Recent evidence showed that CGRP can enhance TRPV1 expression in trigeminal organ culture model. (40, 57).

CONCLUSION

This study investigating the influence of SGCs on the electrical properties of the TG neurons and TG response to direct stimulant (capsaicin) and CGRP. The result showed the TG neurons co-culture in normal condition was more excitable than TG neurons in pure cultured condition indicating by the lower rheobase, more negative threshold and higher total spikes. This study indicate that SGCs have an important role in modulating the activity of TG neurons both small-to-medium sized and large neurons. The results also showed, despite the difference in ion channel and receptor expression, that both small-to-medium sized and large neurons responded to CGRP and capsaicin in the similar manner. This reflect the complex relationship between SGCs, and different types of TG neurons in the process of nociception.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APPENDIX

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