ผลของการได้รับสารไฟโตเอสโตรเจนขนาดต่ำในระยะยาวต่อการแสดงออกของโปรตีนและยีนแคลซิโทนินรีเลทเปปไทด์และซับแสตนพีในปมประสาทไขสันหลังระดับลัมบาร์และซาครัมในหนูแรทเพศผู้

นางสาวสุชาวดี ทองทา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาสรีรวิทยา (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

EFFECT OF LONG-TERM TREATMENT WITH LOW-DOSE PHYTOESTROGENS ON THE CALCITONIN-GENE RELATED PEPTIDE AND SUBSTANCE P PROTEIN AND GENE EXPRESSION OF LUMBOSACRAL DORSAL ROOT GANGLIA IN MALE RATS

Miss Sushawadee Tongta

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Physiology

(Interdisciplinary Program)

Graduate School

Chulalongkorn University

Academic Year 2011

Thesis Title	EFFECT OF LONG-TERM TREATMENT WITH LOW-DOSE PHYTOESTROGENS ON THE CALCITONIN-GENE RELATED PEPTIDE AND SUBSTANCE P PROTEIN AND GENE EXPRESSION OF LUMBOSACRAL DORSAL ROOT GANGLIA IN MALE RATS
By	Miss Sushawadee Tongta
Field of Study	Physiology
Thesis Advisor	Associate Professor Sutthasinee Poonyachoti
Thesis Co-advisor	Assistant Professor Sarinee Kalandakanond-Thongsong

---

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

…….. Dean of the Graduate School
(Associate Professor Pornpote Piumsomboon,Ph.D.)

THESIS COMMITTEE

…….. Chairman
(Assistant Professor Suwanakiet Sawangkoon, DVM, Ph.D.)

……. Thesis Advisor
(Associate Professor Sutthasinee Poonyachoti, DVM, Ph.D.)

…….. Thesis Co-advisor
(Assistant Professor Sarinee Kalandakanond-Thongsong, DVM, Ph.D.)

…….. Examiner
(Professor Anan Srikiatkhachorn, M.D.)

…….. Examiner
(Professor Suchinda Malaivijitnond, Ph.D.)

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

สุชาวดี ทองทา : ผลของการได้รับไฟโตเอสโตรเจนขนาดต่ำในระยะยาวต่อการแสดงออกของโปรตีนและยีนแคลซิโทนินรีเลทเปปไทด์และซับแสตนพีในปมประสาทไขสันหลังระดับลัมบาร์และซาครัมในหนูแรทเพศผู้. (EFFECT OF LONG-TERM TREATMENT WITH LOW-DOSE PHYTOESTROGENS ON THE CALCITONIN-GENE RELATED PEPTIDE AND SUBSTANCE P PROTEIN AND GENE EXPRESSION OF LUMBOSACRAL DORSAL ROOT GANGLIA IN MALE RATS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.สพญ.ดร.สุทธาสินี ปุญญโชติ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.สพญ.ดร.สฤณี กลันทกานนท์ ทองทรง, 73 หน้า.

ปมประสาทรากบนของไขสันหลังเป็นบริเวณลัมบาร์และซาครัมที่รับความรู้สึกจากช่องท้องและขา ที่ปมประสาทนี้จะมีสารสื่อประสาทแคลซิโทนินยีนรีเลทเปปไทด์และซับแสตนพี โดยการควบคุมการสร้างและหลั่งสารสื่อประสาทเหล่านี้ส่วนหนึ่งมาจากฮอร์โมนเอสโตรเจนซึ่งเป็นฮอร์โมนเพศหญิง ไฟโตเอสโตรเจนเป็นสารที่สร้างจากพืชสามารถจับกับตัวรับของเอสโตรเจนได้และสามารถออกฤทธิ์คล้ายหรือตรงข้ามกับเอสโตรเจน โดยเจนิสเต- อีนและไดดซีนเป็นไฟโตเอสโตรเจนพบได้มากในถั่วเหลือง มีการศึกษาพบว่าการได้รับเจนิสเตอีนและไดดซีนในปริมาณมากจะรบกวนการเจริญเติบโตและการทำงานของอวัยวะเพศชาย ซึ่งในปัจจุบันผู้ชายนิยมบริโภคอาหารที่มาจากถั่วเหลืองซึ่งมีไฟโตเอสโตรเจนขนาดต่ำเป็นจำนวนมาก เช่น น้ำเต้าหู้ จึงเป็นที่น่าสนใจว่าการได้รับเจนิสเตอีนและไดดซีนในปริมาณต่ำในระยะเวลานาน จะมีผลต่อการแสดงออกของสารสื่อประสาทแคลซิโทนินยี นรีเลท เปปไทด์และซับแสตนพีและอวัยวะสืบพันธุ์เพศชายหรือไม่ ในการศึกษานี้ได้ทำการให้เอสโตรเจน (0.1-10 µg/kg BW) เจนิสเตอีนและไดดซีนขนาดต่ำ (250-1000 µg/kg BW) ในหนูแรทเพศผู้ โดยการฉีดใต้ผิวหนังเป็นเวลา 35 วัน จดบันทึกน้ำหนักตัวและน้ำหนักอาหารทุกวัน จากนั้นนำ ปมประสาทบนของไขสันหลังเป็นบริเวณลัมบาร์และซาครัม (L1-S2) มาวิเคราะห์ผลการแสดงออกของยีนด้วยเทคนิค real-time PCR และดูการแสดงออกของโปรตีนด้วยเทคนิค immunohistochemistry และวิเคราะห์ผลต่ออวัยวะสืบพันธุ์โดยการบันทึกน้ำหนักของอวัยวะสืบพันธุ์ จากการศึกษาดังกล่าวพบว่า เจนิสเตอีนและไดดซีนไม่มีผลต่อระบบสืบพันธุ์ในขณะที่เอสโตรเจนมีผลลดน้ำหนักตัวและอวัยวะสืบพันธุ์ อย่างไรก็ตามพบว่าไดดซีนมีผลเพิ่มการแสดงออกของยีนสร้างซับแสตนพีที่ไขสันหลังเป็นบริเวณลัมบาร์และซาครัม 3-4 เท่า ในขณะที่เจนิสเตอีนลดการแสดงออกของสารสื่อประสาทแคลซิโทนินยีนรีเลทเปปไทด์และซับแสตนพีที่ปมประสาทไขสันหลังบริเวณซาครัม อย่างไรก็ตามพบว่าเอสโตรเจนมีผลเพิ่มสารสื่อประสาทเหล่านี้ จากผลดังกล่าวแสดงให้เห็นว่าเจนิสเตอีนและไดดซีนขนาดต่ำมีผลลดสารสื่อประสาทแคลซิโทนินยีนรีเลทเปปไทด์และซับแสตนพีที่ปมประสาทไขสันหลังบริเวณซาครัมโดยมีผลตรงข้ามกับเอสโตรเจน ในด้านการทำงานเกี่ยวกับการรับความเจ็บปวดและความรู้สึกโดยไม่รบกวนระบบสืบพันธุ์ ดังนั้นการได้รับไฟโตเอสโตรเจนจากถั่วเหลืองขนาดต่ำเป็นประจำน่าจะช่วยลดการรับความรู้สึกเจ็บปวดและการอักเสบที่เกี่ยวกับระบบประสาท

สาขาวิชา .....สรีรวิทยา.....ลายมือชื่อนิสิต .....

ปีการศึกษา.....2554.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

##5187332220 : MAJOR PHYSIOLOGY
KEYWORDS : CALCITONIN-GENE RELATED PEPTIDE / SUBSTANCE P / DORSAL ROOT GANGLIA / PHYTOESTROGEN

SUSHAWADEE TONGTA : EFFECT OF LONG-TERM TREATMENT WITH LOW-DOSE PHYTOESTROGENS ON THE CALCITONIN-GENE RELATED PEPTIDE AND SUBSTANCE P PROTEIN AND GENE EXPRESSION OF LUMBOSACRAL DORSAL ROOT GANGLIA IN MALE RATS. ADVISOR: ASSOC. PROF. SUTTHASINEE POONYACHOTI, D.V.M., Ph.D., CO-ADVISOR: ASST. PROF. SARINEE KALANDAKANOND-THONGSONG, D.V.M., Ph.D., 73 pp.

Lumbosacral dorsal root ganglion (DRG) regulates pain and sexual behaviors through sensory neuropeptides calcitonin-gene related peptides (CGRP) and substance P (SP). These neuropeptide syntheses regulated by female sex steroids has been suggested. Soybean phytoestrogens, genistein or daidzein, revealed both estrogenic and anti-estrogenic effects in many organs. High-dose genistein or daidzein supplementation interrupted male reproductive organs growth and function but low doses of phytoestrogens contaminated in daily consuming products such as soymilk have never been investigated for their neuromodulatory effect in adult males. Therefore, this study aimed to examine genomic effects of low-doses of genistein and daidzein on CGRP- and SP- mRNA and protein expressions in DRG neurons of adult male rats using real time reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical analysis (IHC), respectively. Adult male Wistar rats were daily subcutaneously injected with vehicle, 17β-estradiol (E_2 0.1-10 µg/kg BW), genistein or daidzein (250-1000 µg/kg BW) for consecutive 35 days. Body weight and food intake were daily recorded. DRGs (L1-S2) were collected and prepared for real-time RT-PCR or the IHC. The weight of testes and accessory sex organs were evaluated for the disrupted effects of chemical treatment. In the present study, genistein or daidzein did not exhibit any effects on reproductive organs in male rats, whereas E_2 reduced body weight gain and accessory organ weight weight. However, daidzein but E_2 or genistein induced PPT-A gene encoding substance P expression in both lumbar and sacral DRG by 3-4 folds. In contrast to mRNA expression, E_2 increased sacral neuropeptides CGRP- and SP-expressed neuron subpopulations. However, genistein but daidzein significantly decreased CGRP- and SP-expressed neurons (>80%) in sacral DRG. Therefore, long-term exposure with low-dose genistein or daidzein but E_2 has negatively regulatory effects on the expression of CGRP and SP neuropeptides that mediated pain and sensation without the male reproductive organs disruption. These evidences may provide the new insights for daily consuming of soybean phytoestrogens as an anti-nociceptive and anti-inflammatory compound in neuropathic pain

Field of Study : Physiology ..... Student's Signature .....
Academic Year : 2011 ..... Advisor's Signature .....
Co-advisor's Signature .....

ACKNOWLEDGMENTS

This research project would not have been possible without the support of many people. I wish to express my gratitude to my supervisor, Associate Professor Dr. Sutthasinee Poonyachoti, and my co-advisor, Assistant Professor Dr. Sarinee Kalandakanond-Thongsong who were abundantly helpful and offered invaluable assistance, support and guidance.

I would like to express my sincere thanks to the chairman, Assistant Professor Suwanakiet Sawangkoon and my thesis committee, Professor Anan Srikiatkhachorn Professor Dr. Suchinda Malaivijitnond and Professor Dr. Pansiri Phansuwan, for their valuable comments, suggestions and correction of this thesis.

I wish to express my sincere thanks to all my teachers and staffs in the Interdisciplinary program of Physiology, Graduate School, Chulalongkorn University for all their helps during the time I was studying, Mrs. Jutarat Jirasupphachok for her help in molecular laboratory instrument, Mr. Supradit Wangnaitham for his help on immunohistological technique and great taking care of my immunohistochemical work. This work was supported by “Chulalongkorn University Centernary Academic Development Project” for real time PCR equipment.

My appreciations are also devoted to my dear family and all of my friends, Miss Suwaporn Daendee, Miss Massupha Wiyaporn, and Miss Sureerat Suthongsa for their love, kindness and support throughout this study.

Finally, many thanks go to the financial support from the Office of the National Research Council of Thailand.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α-CGRP	Alpha-Calcitonin gene related peptide
β-CGRP	Beta-Calcitonin gene related peptide
AIB1	Amplified in breast cancer 1
BSA	Bovine serum albumin
BW	Body weight
CBP/p300	Cyclic AMP response element binding protein
cDNA	Complementary deoxyribonucleic acid
Con	Control
CGRP	Calcitonin gene related peptide
CGRP-ir	Calcitonin gene related peptide immunoreactivity
CLR	Calcitonin–like receptor
cm	centimeter
Dai	daidzein
DFI	Daily food intake
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
E_2	17β-estradiol
ER	Estrogen receptor
ER-α	Estrogen receptor alpha
ER-β	Estrogen receptor beta
Gen	genistein
GRIP-1	Glucocorticoid receptor-interacting protein-1
hr	Hour
kDa	Kilodalton

kg	Kilogram
μg	Microgram
M	Molar
MAPK	Mitogen/extracellular activated protein kinase
mg	Milligram
min	Minute
ml	Milliliter
mRNA	Messenger ribonucleic acid
NK1	Neurokinin 1
nm	Nanometer
OVX	Ovariectomized
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
PE	Phytoestrogen
peak	Melt curve
PGC-1	Peroxisome proliferators activated receptor gamma coactivator 1α
PGP 9.5	Protein gene product 9.5
PGP-ir	Protein gene product 9.5 immunoreactivity
PI3K	phosphatidylinositol 3-OH kinase
PKA	Protein kinase A
PKC	Protein kinase C
PPT-A	Preprotachykinin-A
RAMP	Receptor activity-modifying protein
S.E.M.	Standard error of mean
SP	Substance P
SP-ir	Substance P immunoreactivity
SRC-1	Steroid receptor coactivitor-1
UCH-L1	Ubiquitin carboxy-terminal hydrolase L1

CHAPTER I

INTRODUCTION

Spinal sensory ganglion or dorsal root ganglion (DRG) is important in neural pathway to convey sensory information into the central nervous system via spinal cord. Lumbosacral cord and lumbosacral dorsal root ganglia, in particular, it is an area involved in reproductive functions such as sexual reflexes. These segments receive touch and tactile stimulations from hindquarter integument and pelvic. Calcitonin gene-related peptide (CGRP) and substance P (SP) are common neuropeptides in DRG and released in the spinal dorsal horn (de Groat, 1987; Malykhina, 2007). Both neuropeptides predominantly play neuromodulatory role in the relaying of somatic and visceral pain involved in tactile stimulation and inflammatory response (Brain et al., 1985; McCulloch et al., 1986; Cao et al., 1998; Harrison and Geppetti, 2001)

The number of CGRP neurons and nerve fibers are suggested to be under male sex hormonal control as in male the numbers of CGRP-expressed neuron in DRG were greater than in female (Yang, 1996; 1998). However, CGRP and SP levels in plasma and expression in uterine cervix, DRG and spinal cord were increased in corresponded with high estrogen in order to regulate the relaxation of uterine artery in late pregnant rat (Mowa et al., 2003a; b). Estrogen treatment also increased CGRP and SP expressions in both primary cell culture DRG and in ovariectomized rat (Gangula et al., 2000; Mowa et al., 2003a; b). There are many reports of estrogen receptors mRNA and protein expression in DRG (Mowa, 2003a; b; Taleghany et al.; 1999; Yang et al., 1998) which co-localized with CGRP or SP in neuron (Yang et al., 1998; Bennett et al., 2000). Testosterone can be converted to estrogen by aromatase enzyme to act on DRG via estrogen receptor. Therefore, lumbosacral DRG has been predicted to be regulated by estrogen, such as the synthesis and release of CGRP and SP in response to stimuli in both male and female.

Phytoestrogens, plant-derived compounds, can bind and activate estrogen receptors to produce genomic and non-genomic action in many organs. Many data supported that the phytoestrogens could act in nervous system as estrogen (Belcher and Zsarnovszky, 2001; Lephart et al., 2002; Shir et al., 2002; Marotta et al., 2006). Genistein and daidzein are phytoestrogens those are mostly found in soybean. Even though consuming soy and soy-based products has been claimed as an antioxidant or anti-cancer agents, it could disrupt the hypothalamic-pituitary-gonadal axis in both male and female including male reproductive system (Murkies et al.; 1998; Weber et al., 2001; Yildiz, 2006; Chavarro et al., 2008; Perez-Rivero et al., 2008). Humans and animals are exposed simultaneously to various environment and food contaminated with isoflavones at the low level during the puberty/adult period but life-long or neonatal stage could not clearly show the effect on male reproductive tract and fertility (Roberts et al., 2000). Moreover, the exposure to low-dose phytoestrogen (genistein 1 mg/kg/day) has been correlated to the induction of cluster genes belong to neuroactive-ligand receptor interaction family (Eustache et al., 2009) suggesting that phytoestrogen can affect nervous system. Results from the studies in rodent model additionally suggested roles of phytoestrogen on neural plasticity and modulation of many behaviors such as anxiety, learning and memory (Lephart et al., 2002).

Of interest, long-term exposure to low-dose phytoestrogens (i.e genistein or daidzein) during adult may affect CGRP and SP expression in DRG by altering the gene and protein expression of CGRP and SP. **The present study will focus on the effects of phytoestrogen and estrogen at the lumbosacral DRG since this region relay both pain, tactile and proprioceptive signals from hindquarter and reproductive organs which may have sexually dimorphic and may be affected by exogenous sex steroids in male rats.** The results of CGRP and SP expression in lumbosacral DRG affected by sex steroid or soybean estrogen may provide the either benefit or caution of low dose soybean consumption in male.

Research Question

Does estrogen, daidzein or genistein at the low dose has long-term effect on reproductive organs and their relevant sensory nervous system i.e. CGRP and SP protein and mRNA expression in DRG at lumbar and sacral levels in male rats?

Hypothesis

long-term of low-dose estrogen, daidzein and genistein can effect on CGRP and SP protein and mRNA expression in DRG at lumbar and sacral levels but does not effect on reproductive organs in intact male rats

Research Objective

1. To examine the long-term effect of low-dose estrogen, daidzein and genistein on reproductive organs in intact male rats

2. To examine the long-term effect of low-dose estrogen, daidzein and genistein on CGRP and SP protein and mRNA expression in DRG at lumbar and sacral levels in male rats

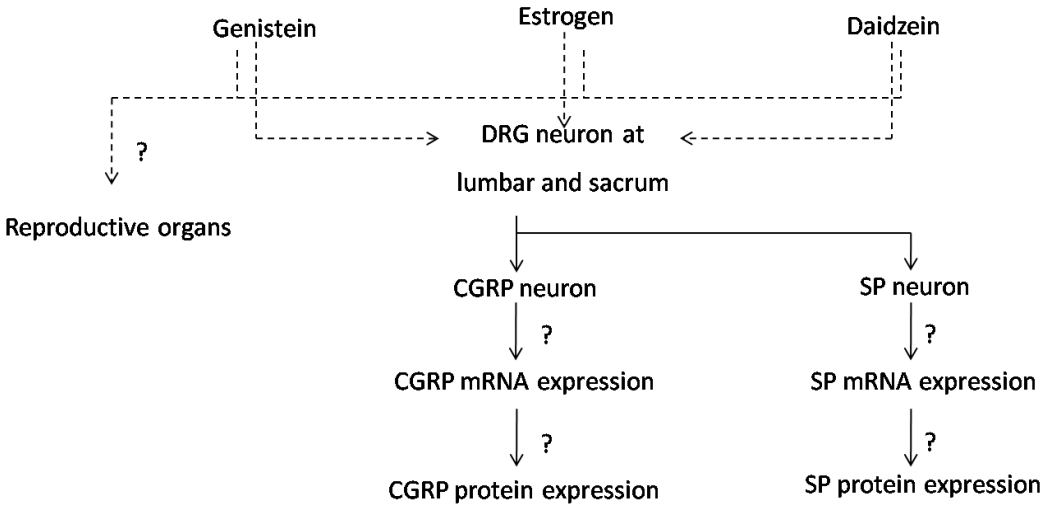

Figure 1-1 Conceptual Framework

CHAPTER II

LITERATURE REVIEWS

Spinal sensory ganglion or dorsal root ganglion

Spinal sensory ganglion or DRG is a nodule on a dorsal root that contains cell bodies of neurons in afferent spinal nerves. The neurons in DRG are pseudo-unipolar neurons and have a bifurcated axon which was divided to central spinal cord and peripheral tissue. The peripheral branch innervates the skin, muscle, or other tissues and associates with specialized receptors in terminal branch. DRG picks up sensory signals from skin, muscle, joints of the limbs and trunk or internal organs and send the signal through the nerve to spinal cord and brain for integration. This signaling pathway is called ascending sensory system (pathway) (Kandel et al., 2000; Patestas and Gartner, 2006).

Anatomically, the ascending sensory systems are composed of three main pathways: the anterolateral system (ALS), the dorsal column-medial lemniscal (DCML) pathway, and the sensory pathway to the cerebellum (Patestas and Gartner, 2006).

1) The anterolateral systems consist of the spinothalamic, spinomesencephalic, and spinotectal tract. They relayed pain, temperature, nondiscriminative (crude) touch, pressure, and some proprioceptive sensation (Patestas and Gartner, 2006).

2) The dorsal column-medial lemniscal pathways consist of the fasiculus gracilis, fasiculus cuneatus, and medial lemniscus. They relay discriminative (fine) touch, vibratory sense, and position sense (Patestas and Gartner, 2006).

3) The sensory pathways to the cerebellum consist of the anterior, posterior, and rostral spinocerebellar, including cuneocerebellar tracts. They relay primarily proprioceptive information and also some pain and pressure information (Patestas and Gartner, 2006).

These ascending sensory pathways had the first order neuron cell bodies reside in DRG. DRG lied along the vertebral column by spinal cord and can be divided into

cervical, thoracic, lumbar and sacral segments. In dermatomes of skin, cervical DRGs innervate head, neck and upper appendages, thoracic DRGs innervate trunk, lumbar and sacral DRGs innervate lower back and lower appendages (Patestas and Gartner, 2006).

Moreover, DRG is a part of local reflex circuits by directing signal sending from sensory neuron to target organs. The brain could not receive sensory signal from that nerve when the DRGs were cut-off, meaning that the patient could loss sensation from that area (numbness, blindness, deafness, etc) (Kandel et al., 2000; Patestas and Gartner, 2006).

The neuron has three parts:

1. Dendrite is a fiber that receives the signal and relays to the soma.

2. Soma is the cell body of the neuron and contains the nucleus. It is the metabolic center of the cell including protein synthesis.

3. Axon sends information from the soma to the main conducting unit to signal other neurons at the synapse. (Kandel et al., 2000).

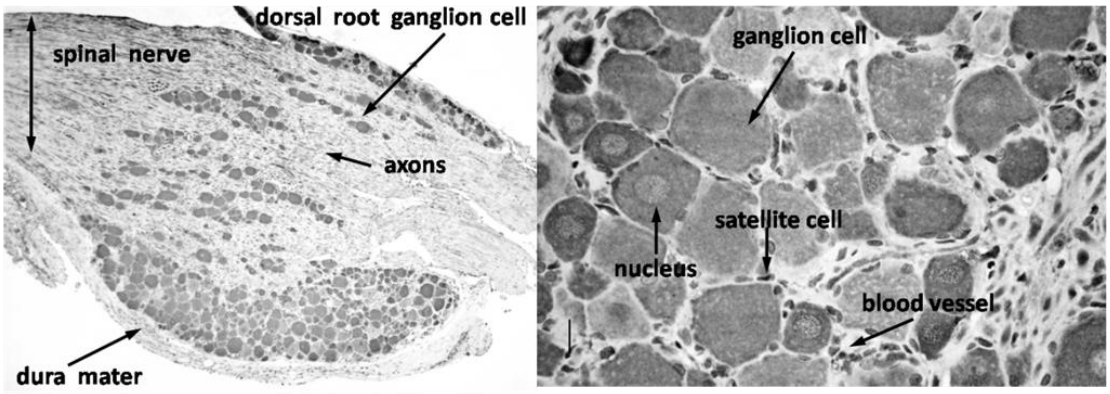

Figure 2-1 Histology of dorsal root ganglion

Base on the histology, ganglion cells, their perikaryon are a very large with light nucleus, and typically larger than other cells in the ganglia. Some ganglion cells are surrounding with one flattened layer of satellite cells. In addition, ganglion cells are

contact with other parts of the nervous system and with the peripheral tissues which they innervate. Nerve fibers were found close to or within the ganglion.

Ganglion cells of the DRGs were also neuroanatomically classified into three types (A, B and C) on the basis of their size and the distribution of their organelles, type A and type B are main types in DRG. The **type A cells** were **light** and **large neurons** (50-75 μm in diameter), the **type B cells** were dark and **smaller neurons** (20-50 μm in diameter), and the **type C** cells were **the smallest** of the DRG neurons, i.e. with a diameter of less than 20 μm (Rambourg, et al, 1982; Averill et al., 1995). **The type A cells** were about 40% of lumbar DRG cells. However, they were non-peptidergic and did not bind the lectin IB4 which were usually used as sensory marker. The "light" DRG cells had myelinated axon Aβ fibers in particular, that innervate mechanoreceptor. However, some of type A cells appear to had Aδ-size axons which could be nociceptors. Many of axon of the type A belonged to cutaneous receptors and projected to laminae III and IV of dorsal horn, but others were large muscle afferent that project to the intermediate nucleus and ventral horn. **The type B cells**, medium-sized cells were about 30% of DRG. They immunoreactive to LA4 antibody that reacted with an oligosaccharide located on the surface of the neurons. These types of DRG neurons had unmyelinated axon (Averill et al., 1995). Similar to type A neurons, they did not contain neuropeptides (Guo et al., 1999) but expressed P2X3 purinoreceptors and their synaptic ending form a narrow band in the inner part of substantia gelatinosa, lamina II_{inner}. The evidence suggests its significance of type B neurons in relaying mechanoceptive and nociceptive input (Guo et al., 1999). **The type C cells** were group of "small dark" DRG cells which contained many neuropeptides (Rambourg, et al, 1982; Averill et al., 1995).

There were many neuropeptides in DRG neurons such as CGRP, substance P, cholecytokinin, somatostatin, or galanin. Normally, CGRP was the neuropeptide that was mostly found in DRG neurons of all animals including rodents. The rank order of number of neuropeptides in lumbar ganglion cells, approximately was CGRP>substance P>somatostatin>galanin. CGRP commonly co-expressed with most of these other

peptides, the basis of content of one or more other peptides but mostly co-expressed in substance P containing neurons (Ju et al., 1987). Even though DRG contained many neuropeptides, mostly are CGRP and SP (de Groat, 1987; Malykhina, 2007). CGRP and SP usually co-released from the DRG neurons. Moreover, CGRP potentiated the release of SP from the primary afferent terminal and promoted the transmission of nociceptive information induced by mechanical noxious stimuli (Maggi, 1995).

The most exciting lined of neuroscience research was the suggestion of the roles of primary sensory neurons which conducted not only sensory modalities but they also released biologically active substances mostly CGRP and SP, at the peripheral terminals of their processes to affected target organ function blood vessels and immune cells in particular (Maggi, 1995). In addition, the sensory nerve fibers provided synaptic collateral to the neurons in autonomic ganglia to form the axon reflexes that permit sensory modulation of autonomic neurons activity (Suzuki et al., 1989). This suggest that CGRP and SP neuropeptidergic transmission in DRG may be the significant pathway to link between immune system and central nervous system.

Calcitonin gene-related peptide (CGRP)

Calcitonin gene-related peptide (CGRP) is a 37 amino acid neuropeptide (Rosenfeld et al., 1983). CGRP was widely distributed in various peripheral tissues as well as in the central and peripheral nervous systems, including sensory neurons (Gibson et al., 1984; Ju et al., 1987; Ghatta and Nimmagadda, 2004). CGRP could be divided into two isoforms: α-CGRP and β-CGRP with three amino acid difference in rat and one amino acid difference in human. Both isoforms were transcribed from different gene which only α-CGRP was synthesized by the alternative splicing of the primary calcitonin gene (Ghatta and Nimmagadda, 2004; Russo and Dickerson, 2006). Normally, α-CGRP was mainly expressed in sensory neurons, whereas β-CGRP was mainly expressed in motor neurons (Russo and Dickerson, 2006). However, both subtypes had similar biological effects (Ghatta and Nimmagadda, 2004).

About 65-80% of DRG neurons expressed CGRP. It was co-expressed in almost all DRG neurons that expressed SP and co-expressed with other pain-related peptides

in primary sensory neurons suggesting that CGRP predominantly played role in nociception (Hay and Dickerson, 2010). Basically, CGRP was known as the potent vasodilator peptides and the neuro-transmission of pain (Brain et al., 1985; McCulloch et al., 1986). Injection of CGRP induced a transient pain response in tail flick test (Cridland and Henry, 1988; Ma and Quirion, 2006) while injection of CGRP antagonist, CGRP 8-37, induced antinociception (Yu et al., 1994). Moreover, injection of CGRP in rats induced hyperalgesia in the paw withdrawal test and it was reduced by CGRP 8-37 and inhibitors of protein kinase A (PKA) or protein kinase C (PKC) (Sun et al., 2004). These data indicate that CGRP and PKC or PKA signaling have a direct nociceptive effect in the spinal dorsal horn and CGRP is a pain neurotransmitter in pain transmission pathway (Hay and Dickerson, 2009).

In addition, CGRP indirectly co-operated sensory signal including pain and tactile by feeding signals to produce many behaviors by enhancing the release of excitatory neurotransmitters such as glutamate, aspartate and SP in the spinal cord. For an example, CGRP enhanced SP neural activity to induce pain behaviors and inflammatory response. Moreover, CGRP played significance roles in regulating SP induced pain responses by facilitation of the release or inhibition of the degradation of SP. These data indicate that CGRP does not only show a direct effect as a pain transmitter at the first synapse of pain signaling pathway, but it also showed a pain modulator by interactions with excitatory neurotransmitters (Hay and Dickerson, 2009).

Moreover, CGRP was related with neurogenic inflammation. CGRP was released at peripheral nerve endings when the neurons are activated by noxious stimuli. CGRP led to vasodilation at target tissue, inflammatory cell leak to tissue and was induced to secrete proinflammatory and pain-inducing mediators, including chemokines, cytokines and histamine by CGRP (Hay and Dickerson, 2009).

CGRP mediated its effect through receptor, called calcitonin-like receptor (CLR) and receptor activity-modifying protein (RAMP), which were G protein-coupled receptors (Poyner et al., 2002). CGRP function was suggested to relate with migraine, pain, motor activity, depression- and anxiety-like behaviors and mating behavior

(Herbison et al., 1995; Schorscher-Petcu et al., 2009). However, CGRP had been indicated to play a critical role in reducing gastrointestinal mucosal injury through an increase the level of prostacyclin. Interestingly, the production of CGRP sensory neurons was different between female and male. Estrogen seemed to enhance nerve growth factor-mediated CGRP production in sensory neurons of female compared to those of male (Shimozawa et al., 2006). However, in male, CGRP was an essential substance for reproductive system by increasing blood flow in blood vessel including penile artery leading to erectile response (Morales, 1998). CGRP-immunoreactive afferent nerves that localized in the carvernosal smooth muscle of penis have been suggested to depend on androgens including its metabolite, dihydrotestosterone (DHT). Castrated rat which had the low levesl of testosterone and DHT, the proportion of CGRP-positive afferent nerve fiber in penis was decreased suggesting role of male sex steroid on the CGRP expression (Shen et al., 2005). In addition, the sensory nucleus of genitofemoral nerve (L1-L2 DRG) was sexually dimorphic and had a role in testicular descent. The intrauterine flutamide antiandrogen treatment on day 16 to 19 of pregnancy decreased the number of CGRP neurons of the male newborn rats similar to those of female newborn rats (Hrabovszky et al., 2000). The evidence suggests the regulatory roles of sex steroid on the production of CGRP and its function in both sexes.

Substance P (SP)

Substance P (SP) an 11 amino acid neurotransmitter, a part of the tachykinins family, synthesized from the preprotachykinin-A (*PPT-A*) gene. Substance P was originally discovered and isolated from brain and intestinal tissue in 1931 by von Euler and Gaddum.

SP widely expressed not only in the nervous system, central nervous system and peripheral nervous system i.e. enteric nervous system (Keast et al., 1987) but also expressed in immune cells. It was released from both central and peripheral ending of primary afferent neurons and function as neurotransmitter (Harrison and Geppetti, 2001). SP and its receptor, the NK1 receptor were commonly distributed in the brain and found in many brain regions that regulate emotion such as amygdala,

periaqueductal gray and hypothalamus (Mantyh et al., 1984; Arai and Emson, 1986). The demonstration of SP immunoreactivity (SP-ir) in the cell bodies of dorsal root ganglia, in sensory nerve fibers, and in the dorsal horn of the spinal cord also suggested the main function of substance P on pain perception pathway (Ljungdahl et al, 1978). Basically, the major source of SP production was the sensory ganglion cells but transports to the interneurons resided in spinal cord and nerve ending of the peripheral branches during transmission of sensory signal (Harrison and Geppetti, 2001; Maggi, 1995). Moreover, SP accumulates distal to a ligature of a dorsal root suggesting that it was transported to the spinal cord from DRG (Harrison and Geppetti, 2001). Stimulation of primary afferent fibers with capsaicin could induce not only release SP but it also released the other neurotransmitters produced in the DRG neurons including CGRP in particular indicating the significance of co-releasing of sensory neuropeptides

Besides the relating pain perception, many behavioral and electrophysiological studies suggested that SP releasing from nociceptive primary afferent fibers could affect neurogenic inflammation and vasodilation, and cause depression, nausea or vomiting (Harrison and Geppetti, 2001). Activation or damage of neurons changed biosynthesis of neuropeptides by induction of neuropeptide gene expression (Hokfelt et al., 1994). The modification of neuropeptide expression in DRG neurons was found in models of acute and chronic inflammation (Noguchi et al., 1988). Peripheral inflammation led to increased SP-ir within the superficial spinal cord and release of SP (Schaible et al., 1990; Marlier et al., 1991). Moreover, the expression of preprotachykinin (*PPT*) mRNA was up-regulated in the periphery during noxious stimulation or neurogenic inflammation (Noguchi et al., 1988). In addition, transgenic mice with a deletion of the preprotachykinin A (PPT-A) gene had remain responses to mildly noxious stimuli but slow in responses to moderate or intense noxious stimuli. Interestingly, they were complete absence of neurogenic inflammation (Cao et al., 1998).

The biological actions of SP was mediated by tachykinin (neurokinin: NK) receptors, which were G protein-coupled receptors. There are three types of tachykinin receptors: NK1, NK2 and NK3 which SP could act on all subtypes but commonly act on

NK1 (Harrison and Geppetti, 2001). In the periphery, during noxious stimulation or neurogenic inflammation, the expression of NK1 receptor mRNA is upregulated (Harrison and Geppetti, 2001).

In male, chemical lesion using SP-saporin on SP-neural population of lumbar spinothalamic cells abolishes ejaculatory behavior explicitly indicating the significance of substance P expressed neurons in reproductive behavior (Young et al., 2009). However, a little report of sex-related difference in SP production.

Protein gene product 9.5 (PGP 9.5)

Protein gene product 9 (PGP 9.5), also known as ubiquitin carboxyl-terminal hydrolase-1 (UCH-L1), is a 27-kDa protein that was first isolated from whole brain extracts and found in total soluble brain proteins about 1–5% (Jackson and Thompson, 1981). This protein is found in cytoplasm and highly specifically express in neuron and neuroendocrine cell in vertebrates. However, PGP 9.5 can be found in other cells such as spermatogonia, Leydig cells, oocytes, prostatic secretory epithelium, ejaculatory duct cells, epididymis, mammary epithelial cells, kidney and carcinoma cell (D'Andrea et al, 1997; Martin et al., 2000; Aumuller et al, 1999; Day and Thompson, 2010).

The ubiquitin carboxyl terminal hydrolases are a group of proteases used for removing attached ubiquitin from substrate. Ubiquitin is a small protein that regulates proteins by targeting for degradation. This regulation is important in many cellular pathways such as cell cycle regulation, cellular response to stress, and DNA repair (Ciechanover et al., 2000; Day and Thompson, 2010).

PGP 9.5 is expressed about 50-fold concentration in the brain more than in other tissues (Jackson and Thompson, 1981). **At present, many reports in databases used PGP 9.5 as an immunochemical pan-neuronal marker** (reviewed by Day and Thompson, 2010)

Estrogen

Estrogen (17β-estradiol or E_2) is a steroid hormone that is converted from testosterone by aromatase enzyme and acts on estrogen receptor (ER). ER is not only found in reproductive organs but it is also identified in nervous system. It plays role on the regulation of many brain functions including behavioral function, such as feeding behavior, memory, emotion and sexual behavior (Simpson and Davis, 2000). Estrogen mediates its effect via two types of nuclear receptors: ER alpha (ER-α) and ER beta (ER-β) which they are difference in the C-terminal ligand-binding domain and in the terminal trans-activation domain. When estrogen binds to ERs, ERs form dimers and regulate transcription by binding to estrogen response element (ERE) in promoter region of target genes and requires recruitment of other transcription factors and multiple co-activators such as SRC-1 (steroid receptor coactivitor-1), GRIP-1 (glucocorticoid receptor-interacting protein-1), AIB1 (amplified in breast cancer 1), CBP/p300 (cAMP response element binding protein), PGC-1 (peroxisome proliferators activated receptor gamma coactivator 1α) and p68 RNA helicase. Estrogen not only acts on receptors in intracellular, but it also acts on cell surface receptor (membrane estrogen receptor) which is characterized by a rapid onset of their effects, such as signaling pathway in neuronal cells. Membrane estrogen receptor is G protein coupled receptor or GPR30. GPR30 can effect on cell through various protein kinase cascades in intracellular signaling pathway i.e. PKA, PKC, mitogen/extracellular activated protein kinase (MAPK) or/and phosphatidylinositol 3-OH kinase (PI3K). These proteins may regulate a rapid effect as modulate ion influx or transcription of target gene (Yildiz, 2006; Zhang and Trudeau, 2006).

Estrogen treatment was found to affect tactile sensitivity in rats in which overly sensitive cutaneous sensation may lead to sexual pain, while inadequate cutaneous sensation may lead to impair desire and/or arousal functioning (Bereiter et al., 1980; Frohlich and Meston, 2005). On the other hand, in menopausal women, estrogen deficiency, increased tactile sensitivity such as hot flashes, sweating and a sensation of heat was occurred (Freedman, 2001). Moreover, estrogen also reduces inflammation by

decrease cytokines (Nilsson, 2007). These evidences suggest that estrogen may affect tactile sensitivity, pain and inflammatory responses through CGRP and SP.

Effect of estrogen on CGRP and substance P

Besides the suggestion of the sexual dimorphism of CGRP- or SP-expression and function between female and male in nervous system, there are more evidences of estrogenic effect on these neuropeptides expression. For an example, in late pregnant rat (high level of estrogen) showed high level of CGRP and SP neuron in DRG (Mowa et al., 2003a; b) as in estrogen treated ovariectomized (OVX) rat (Gangula et al., 2000; Mowa et al., 2003a; b) and DRG cell culture treated with estrogen (Gangula et al., 2000). Moreover, CGRP and SP neurons in DRG co-localized with ER (Mowa et al., 2003a; b). Estrogen treatment in OVX rat with the ER blocker (ICI 182,780) decreased CGRP and SP expression suggesting these estrogenic effects mediated by ER (Gangula et al., 2000; Mowa et al., 2003a; b). In contrast, Yang and co-workers (1998) found that CGRP immunoreactivity in the peripheral nervous system of adult rats is sexually dimorphic and regulated by sex steroid. The numbers of CGRP-immunoreactive neurons in DRG at the cervical, lumbar and sacral levels in the female rat are significantly lower than those of male rats. Ovariectomy increases CGRP neurons in consistent with the increase in ER but it is decreased in estrogen treated-OVX rats (Yang et al., 1998). The CGRP expression perhaps is down-regulated by estrogen through ERs. **These results indicate that DRG containing neuropeptides are under regulation of sex steroid. Typically in the lumbar and sacral level of the spinal cord, this evidence suggests that the gender differences are subjected in the peripheral nerves projecting to sex organs or the skin that may be related to the different sexual function and sexual behavior of female and male rats.**

Phytoestrogens

Phytoestrogens (PEs) are plant-derived compounds with their molecular structures similar to those of estrogens and therefore they can bind and activate ERs. Phytoestrogens are basically classified into isoflavones, coumestans and lignans. They can be found in many types of foods such as fruits, vegetables, grain products, soybean, alcoholic beverages, cow's milk and meat. Soybean, a typical ingredient in many kind of food, is rich in isoflavones, mainly consist of genistein and daidzein (Yildiz, 2006).

In plants and foods, isoflavones are present in multiple chemical forms. Isoflavones are conjugated with glucose called glycosides. These forms are non-absorbable forms which are indicated as inactive forms. In soybean, the major glycosides (conjugated with glucose) are genistin and daidzin and are converted to absorbable forms as aglycone by intestinal microflora. Genistein and daidzein are present as aglycone forms or unconjugated forms which are active forms (Murkies et al., 1998; Yildiz, 2006).

In general, phytoestrogens are weak estrogenic compound compared with estrogen. Therefore, they are required higher concentration than estrogen to generate similar response. The rank order of estrogenic potency of phytoestrogens can be estimated that genistein = coumestrol > zearalenone > daidzein > biochanin A = apigenin = kaempferol = naringenin > phloretin = quercetin = ipriflavone = formononetin = chrysin for ERβ (Kuiper et al., 1998). Coumestans are the most potent phytoestrogens, however they are mainly found in animal feeding plants, clover and alfalfa which are rarely found in human diet. At the same time, isoflavones are major component in human diet as soybean. Phytoestrogens can bind with both ERβ and ERα , however, they have higher binding affinity for ER-β than the ER-α (Kuiper et al., 1998; Yildiz, 2006).

In many previous studies, the administration of phytoestrogens in adult intact primates or rats had no sex interrupted effects in males (Mitchell et al., 2001; Faqi, et al., 2004; Perry et al., 2007). For an example in human study, 18-35 years old males

consumed 40 mg of isoflavones which is similar to the amounts consumed in many eastern countries for 2 months had not affected the gonadotrophin or sex hormone levels, or semen quality (Mitchell et al., 2001). In adult intact male rats, consumption of 200 or 2000 mg/kg soy diet for 12 months had no effect on sperm count, sperm production, or sperm morphology (Faqi et al., 2004). In adult male macaques, consumption of 6 or 12 mg/kg/d isoflavonoid diet for 3 years had no effect on estrogen or testosterone level in serum and sperm count (Perry et al., 2007). However, **some reports shown that high phytoestrogen treatment reduced testosterone level in serum, reproductive organ weight or sperm quality** (Weber et al., 2001; Chavarro et al., 2008; Perez-Rivero et al., 2008). In adult male Sprague-Dawley rats (70 days old), consumption of 600 μg/g phytoestrogen diet for 35 days decreased plasma testosterone and androstenedione levels but it did not affect the plasma estrogen and LH levels (Weber et al., 2001). In male, consumption of soy food containing genistein or daidzein more than 2 mg/day for 3 months decreased sperm concentration (Chavarro et al., 2008). In dog (2-4 years old), consumption of coumestrol 300 μg once a week for 4 weeks reduced tubular spermatogenic epithelium and lumen opening. In addition, the number of abnormal spermatozoa was increased and decreased sperm concentration (Perez-Rivero et al., 2008). Moreover, Pan and co-worker (2008) had found that daidzein negatively affected erectile function.

In addition, many data supported that phytoestrogens could affect both behavior and neuronal system such as sexual behavior (lordosis), anxiety, stress, memory and learning (Lephart et al., 2002; Moore et al., 2004; Patisaul, 2005). The relevant mechanism had been suggested by the up-regulation of oxytocin receptor by phytoestrogen in the rat paraventricular nucleus of the hypothalamus which was a nucleus critical for the generation of the lordosis response in female (Patisaul, 2005). In addition, phytoestrogen could increase open arm time and number of entries into open arm in the anxiety model suggesting their anxiolytic effects (Lephart et al., 2002). Furthermore, phytoestrogens could reduce neuropathic pain in male rats and proinflammatory response in central nervous system of OVX rat (Shir et al., 2002;

Marotta et al., 2006). These data support that phytoestrogens can affect nervous system.

Since their structures are similar to estrogen, phytoestrogen effects have been revealed as estrogenic or anti-estrogenic effects (Yildiz, 2006). Human and animals are exposed simultaneously to various environment and food contaminated with isoflavones at the low level during the puberty/adult period but life-long or neonatal stage. Even though, the previous studies clearly show non-disrupted effects of phytoestrogens on male reproductive tract and fertility their effects on neural system related to reproductive behaviors is disguised (Roberts et al., 2000). Therefore, it is of interest that phytoestrogens such as genistein and daidzein may affect CGRP and SP expression in DRG which may relevant to the altering of the threshold of nerve stimulation and behavior.

CHAPTER III

MATERIALS AND METHODS

A. Experimental animals

Adult male Wistar rats aged 11-12 weeks at beginning of the experiment were obtained from National Laboratory Animal Center, Mahidol University (NLAC-MU), Thailand. They were housed in 2-3 animals per cage under 12h light/dark cycle (lights on at 06:00 a.m.) and maintained at 25±2°C. The rats were supplied with standard rat chow (CP 082, perfect companion group co. Ltd, Thailand) and water *ad libitum*. All procedures in this study were approved by the Chulalongkorn University Animal Care and Use Committee, Faculty of Veterinary Science, Chulalongkorn University (Animal Use Protocol No: 1031031).

B. Chemicals

17β-estradiol (1,3,5 [10]-estratriene-3, 17 β-diol), E-8875, FW 272.4, lot no.120H0126, Sigma-Aldrich, St. Louis, MO, USA), genistein (98% purity, FW 270.24, lot no.034K0852, Sigma-Aldrich) and daidzein (98% purity, FW 254.20, lot no.02K4102, Sigma-Aldrich) were dissolved in 10% dimethylsulfoxide (DMSO) to give a stock solution with a final concentration of 100 mg/ml. Isoflurane was purchased from Minrad, Inc. (Bethlehem, PA, USA)

Rabbit polyclonal anti-rat PGP 9.5 was purchased from Thermo Fisher Scientific, Inc., (Waltham, MA, USA; lot no. LL1376833). Rabbit polyclonal anti-rat CGRP was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-rat SP was purchased from Phoenix Pharmaceuticals, Inc (Burlingame, CA, USA; lot no.00992). Histostain®-SP Kits was purchased from Invitrogen Corporation (Camarillo, CA, USA). Secondary antibody detection system (Envision®) was purchased from DAKO (Produktionsvej 42, Glostrup, Denmark).

RNAlater was purchased from QIAGEN, Inc. (Valencia, CA, USA). Trizol reagent was purchased from Gibco BRL: Life Technologies, Inc. (Gaithersburg, MD, USA). iScript™ Select cDNA Synthesis Kit was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Real-time PCR Master Mix E4 was purchased from GeneOn GmbH (Ludwigshafen am Rhein, Germany).

C. Experimental protocol

To examine the effect of estrogen or phytoestrogens i.e. genistein and daidzein on CGRP, SP and PGP 9.5 expression in dorsal root ganglia related with multifunction physiology, fifty intact Wistar male rats were divided into 10 groups (5 rats/group): control (vehicle); genistein – treated groups at a dose of 250, 500 or 1000 µg/kg BW; daidzein – treated groups at a dose of 250, 500 or 1000 µg/kg BW; estrogen – treated groups at a dose of 0.1, 1 or 10 µg/kg BW (Fig 3-1). These doses of genistein and daidzein were chosen since their concentration was about containing in soy diet. In addition, they have shown effect behavior or nervous system in our preliminary study.

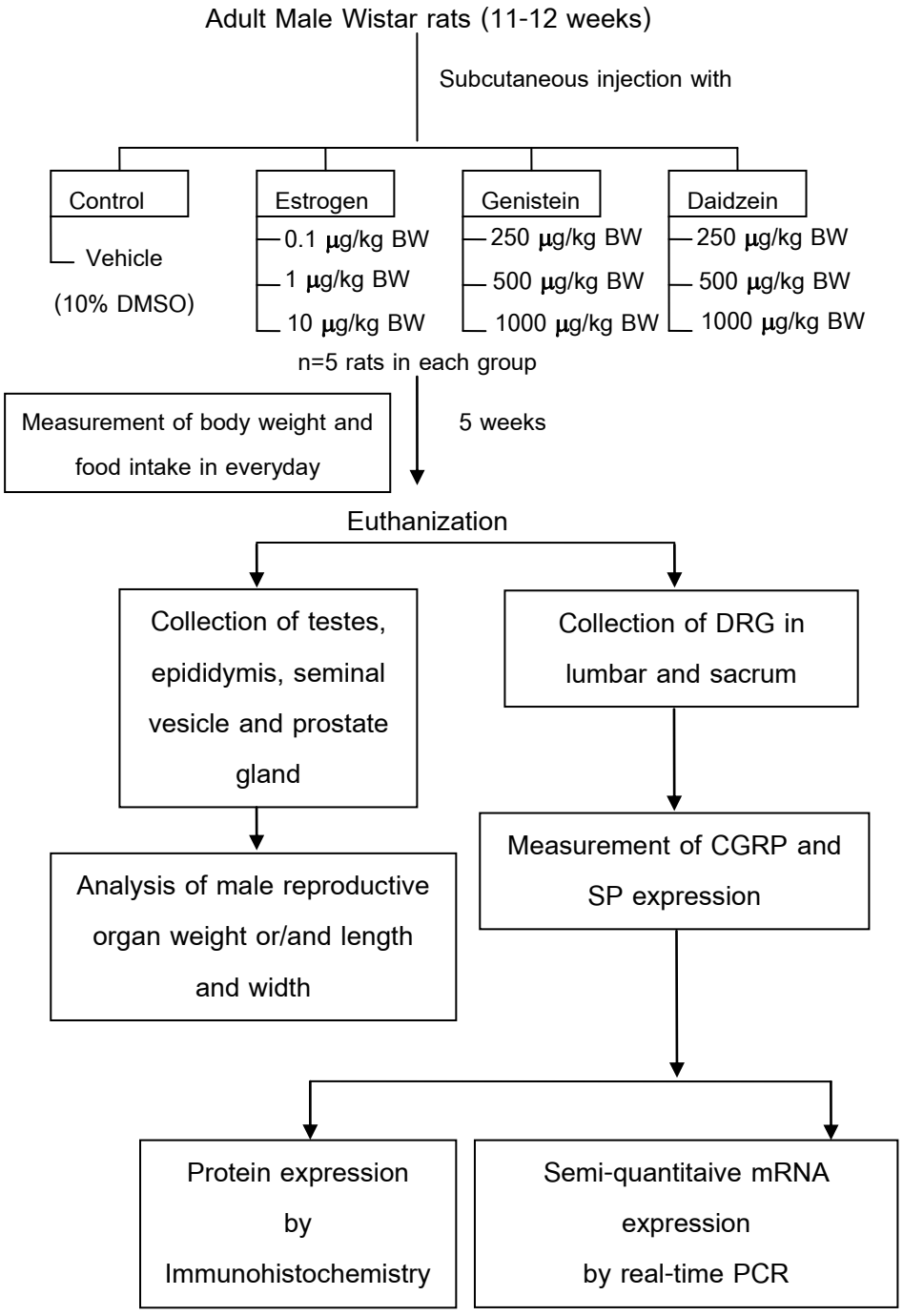

Figure 3-1 Diagram of the experimental protocol

D. Methods

1. Estrogen, genistein and daidzein supplementation

All rats were daily subcutaneously injected with vehicle (10%DMSO in propylene glycol), 17β-estradiol (E_2; 0.1, 1, 10 µg/kg BW), genistein (Gen; 250, 500, 1000 µg/kg BW) or daidzein (Dai; 250, 500, 1000 µg/kg BW) subcutaneously into the dorsal region of the neck for 5 weeks. This time period was selected as it had been shown to affect nervous system in male rats (Lephart et al., 2000).

2. Measurement of body weight, food intake and reproductive organ weight

Body weight of animal were recorded daily. Male reproductive organs i.e. testis, epididymis, prostate gland and seminal vesicle, which are the indicators of androgen responses, were determined immediately by being weighed or measured after sacrifice by inhalation with isoflurane.

The percentage of body weight change (%BWC), the daily food intake (DFI), the percentage of reproductive organ weight-to-body weight ratio (%ROW/BW), and testis width and length were calculated using the following equations.

$$\%\text{BWC} = \left(\frac{[\text{End BW (g)} - \text{Start BW (g)}]}{\text{start BW (g)}} \right) \text{x}100$$

,

$$\% \frac{\text{ROW}}{\text{BW}} = \frac{\text{ROW (g)}}{\text{BW (g)}} \text{x } 100$$

$$\text{Testis width (cm)} = \frac{\text{left testis width (cm)} + \text{right testis width (cm)}}{2}$$

$$\text{Testis length (cm)} = \frac{\text{left testis length (cm)} + \text{right testis length(cm)}}{2}$$

3. Sample collection

After euthanasia and reproductive organs removal, the DRGs from lumbar and sacral segments at the specified levels from L1-L6 and S_1-S_2 were collected. The L_4 and $S_{2\text{ on the left side}}$ were immediately immersed in 4% paraformaldehyde, and kept at 4°C for immunohistochemistry study. The rest of DRGs including L_1-L_6 and S_1-$S_{2\text{ from both sides}}$ were kept in separated vial labelled as L or S segments containg RNAlater and stored at -20°C for real time polymerase chain reaction (RT-PCR).

4. Immunohistochemistry (IHC)

For immunohistochemistry, the sections of DRG tissue were fixed in 4% paraformaldehyde and embedded in paraffin blocks. Transverse sections were cut at the thickness of 4-6 μm and mounted on coated glass slides yielding approximately 20 sections/DRG. The slides positioned at the middle of serial sections (section number 9-11) of each tissue were selected. Then the selected sections were deparaffinized with xylene and rehydrated gradually with graded ethanol (100%, 95%, 80%, and 70% alcohol The IHC were started with antigen retrieval process by treating with 10 mM sodium citrate buffer, pH 6.0, at 95 °C for 5 min. The sections were then treated with 3%

hydrogen peroxide in methanol for 10 min at room temperature to eliminate the endogenous peroxidase. Nonspecific binding sites in tissue sections were blocked by incubating with 4% bovine serum albumin (BSA) for 30 min at room temperature. Then, it was incubated overnight (4°C) with a primary antibody, rabbit polyclonal anti-rat PGP 9.5 (pan-neuronal marker), rabbit polyclonal anti-rat CGRP or rabbit polyclonal anti-rat SP at the dilution of 1:2000, 1:800 or 1:2000, respectively. Excess antibody was removed by washing with phosphate buffer saline (PBS) three times 5 min each, and incubated with a secondary antibody detection system (1:400; Envision®) for 45 min at room temperature. The excess secondary antibody was removed by washing with PBS three times, 5 min each. The sections were then incubated with 3,3'- diaminobenzidine (DAB) (DAKO) for 2 min. Then the reaction was stopped by washing with distilled water. The sections were counterstained with hematoxylin for 30 sec and washed with running tap water. Finally, the sections were dehydrated and mounted with mounting media (DAKO).

The immunostaining slide was visualized under the light microscope at 40x magnification and the images were captured with Hamamatsu digital camera (C4742-95, Nikon, Japan). For each section, a total number of cells within the same section area of each antibody were manually counted (1 section/antibody/rat). The positive cells as indicated by the dark-brown cytoplasmic stain were counted and presented as the percentage of the total cell count. The percentage of immunoreactive cells of all animals in each experiment was calculated as following;

$$\%\ \text{immunoreactive cell (\% ir cells)} = \frac{\text{No. of PGP 9.5, CGRP or SP ir cells}}{\text{numbers of observed cells}}\ \text{X } 100$$

In addition, subpopulations of CGRP and SP in DRG were determined as following;

$$\%\ \text{CGRP or SP subpopulation neurons} = \frac{\%\ \text{CGRP or SP ir cells}}{\%\text{PGP ir cells}}\ \text{X } 100$$

5. Semi-quantitative of mRNA expression

5.1 Total RNA isolation

RNA was isolated from DRG using Trizol reagent (Gibco BRL, USA) in accordance with the manufacturer's instruction. Briefly, isolated DRGs stored in RNAlater were removed and washed with 0.1 M steriled PBS (pH 7.4) for 5 min 2 times, DRGs were then transferred into 400 µl of Trizol reagent, immediately disrupted, homogenized and incubated for 5 min. 80 µl of chloroform was added, mixed and incubated for 3 min. The lysate was centrifuged at 12,000x g (Andreas Hettich universal 32R, Tuttlingen, Germany) for 15 min at 4°C, then the supernatant (aqueous phase) containing the RNAs, was immediately transferred to 1.5 ml microcentrifuge tube, and 200 µl isopropyl alcohol was added, mixed and incubated for 10 min. The sample was centrifuged at 12,000x g for 10 min at 4°C and the supernatant was discarded. The pellet was then washed with 70% ethanol, vortexed and centrifuged at 12,000x g for 10 min. Finally, the pellet containing the total RNA was diluted with diethylpyrocarbonated (DEPC)-treated water. The RNA concentration for each sample was measured and and stored at -20 °C.

5.2 cDNA synthesis

Total RNA samples were reverse transcribed into complementary DNA (cDNA) using oligo-dT primer. This reverse transcription step was done using iScript™ select cDNA Synthesis kit (Bio-Rad). A 20 µl of reverse transcription reaction mixture, containing 2 µl (10 mM) oligo-dT primer, 13 µl (1 µg) total RNA and nuclease-free water, 4 µl of 5x iScript selected reaction mix (containing dNTPs, magnesium chloride and stabilizers) and 1 µl of iScript reverse transcriptase enzyme were incubated at 42 °C for 90 min and 85 °C for 5 min, respectively. The synthesized cDNA was measured and stored at -20°C for later analysis. Qualification of total RNA sample integrity was assessed by both 260 and 280 nm absorbance ratio between 1.8-2.0.

OD_{260}: UV absorbance of total RNA/cDNA was measured at a wavelength of 260 nm.
OD_{280}: UV absorbance of protein was measured at a wavelength of 280 nm

5.3 Calculation of total RNA or cDNA concentrations

The quantity of isolated RNA or cDNA sample was measured with spectrophotometer (Model U-2000, Hitachi Instrument Inc, Tokyo, Japan) under the wavelength of 260 nm and 280 nm. The calculation of RNA or cDNA was calculated by using the equation (Birren et al., 1997) as shown below.

Total RNA concentration (μg /μl) = [40 x OD_{260} x dilution factor]
Total cDNA concentration (μg /μl) = [50 x OD_{260} x dilution factor]

The dilution factor was used 1 μl total RNA in 50 μl water (1/50).
OD_{260}: UV absorbance of total RNA/cDNA was measured at a wavelength of 260 nm.

5.4 Real-Time Polymerase Chain Reaction

Relative quantitative real-time PCR amplification for candidate responsive genes (target genes) was performed with ABI 7300 Real time PCR system (Applied Biosystems, Foster City, CA, USA) using the power Eva® Green master Mix in accordance with the manufacturer's protocol. The specific primer sequences for β-actin, α-CGRP (expression mainly at sensory neuron) and SP are shown in Table 3-1. For each 20 μl PCR reaction, it was composed of 5 μl (1 μg) of template, 10 μl of Real-time PCR Master Mix E4, 1.5 μl of each of the forward and reverse primers (10 μM) and 2 μl of nuclease free water. The PCR was performed under the following conditions: 95°C for 10 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing and extension at 58°C for 1 min. A dissociation step, consisting of 95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec was performed at the end of each PCR to confirm product specificity. Fluorescent signals were detected at the end of extension step of each

cycle. The threshold of fluorescence detection was set at the number of the threshold cycles (Ct) corresponding to the inflection point of the fluorescence curve from the baseline to the exponential phase. Using the $2^{-\Delta\Delta Ct}$ comparative relative method of threshold cycle values using specific primers of CGRP or SP of each sample were normalized to β-actin (endogenous control gene). The fold changes in mRNA expression target of chemical treatments were analyzed from the normalized number of control vehicle group using the following formula:

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Where:

$$\Delta Ct = Ct^{\text{target gene}} - \text{avg.}Ct^{\text{endogenous control gene}}$$

$$\Delta\Delta Ct = [\Delta Ct]_{\text{treatment group}} - \text{avg.}[\Delta Ct]_{\text{control group}}$$

The PCR amplification products were separated on a 2% high resolution agarose (Bio-Rad) gel electrophoresis and visualized under the UV transluminator (Pharmacia Biotech, Uppsala, Sweden) to confirm primer specificity. The specific PCR product for CGRP, SP and β-actin were considered when a single sharp peak was seen.

Table 3-1 Details of Genbank accession numbers, product sizes and nucleotide sequence of primers for RT-PCR analysis of mRNA expression of rat *α–CGRP,* substance P (*PPT-A*) and β-*actin*

Gene	Sequence 5'>3'	Accession No.	Product size (bp)	References
α-CGRP	F: GCTCCAAGTCATCGCTCACC R: CCAAGTTGTCCTTCACCACACC	NM_001033956.1	357	Schorscher-Petcu et al., 2009
Substance P (*PPT-A*)	F: TGGCGGTCTTTTTTCTCGTT R: GCATTGCCTCC TTGATTTGG	NM_001124770.1	114	Yu et al., 2010
β-*actin*	F: AGGGAAATCGTGCGTGAC R: CGCTCATTGCCGATAGTG	NM_031144.2	146	Wang et al., 2011

8. Data analysis

All data were presented as mean and standard error of mean (S.E.M). To study the effects of genistein, daidzein, or estrogen, the comparison was done between treatment and vehicle control groups using orthogonal contrast (Statistical Analysis Software System, 2002). $P<0.05$ was set as a statistical significance.

CHAPTER IV

RESULTS

In this study, the effects of phytoestrogens (i.e. genistein and daidzein) and estrogen on body weight, food intake, reproductive organs, and expressions of CGRP and SP in DRG of intact male rats were studied by treatment with genistein (250, 500 or 1000 µg/kg BW), daidzein (250, 500 or 1000 µg/kg BW) or estrogen (0.1, 1 or 10 µg/kg BW) for 35 days.

1. Effects of genistein, daidzein or 17β-estradiol (E_2) supplementation on body weight and food intake

At the beginning of the experiment, body weights of the animals were not significantly different among groups. As shown in the table 4-1 percentage change of body weight (%BWC) of genistein-, daidzein-, E_2- or vehicle- treated rats, the %BWC of genistein-,daidzein- or E_2-treated intact male rats were not significant different from the vehicle-control rats. However, in male rats treated with E_2 at a dose of10 µg/kg BW had a significant lower %BWC than the control rats [$P < 0.01$] (Figure 4-1).

Table 4-1 Data represents mean±S.E.M. of percentage changes of body weights (%BWC), relative weights of reproductive organs and testis width and length of intact male rats treated with vehicle, genistein, daidzein, or 17β-estradiol for 35 days.

	Vehicle	Genistein			Daidzein			17β-estradiol		
		250 µg	500 µg	1000 µg	250 µg	500 µg	1000 µg	0.1 µg	1 µg	10 µg
%BWC	26.14±2.87	29.79±4.56	28.54±3.31	24.86±5.51	24.47±4.47	20.62±3.92	28.67±5.05	27.81±5.54	17.56±4.51	5.65±3.48**
Testis weight (%)	0.431±0.011	0.428±0.022	0.456±0.009	0.424±0.025	0.435±0.013	0.472±0.025	0.459±0.025	0.406±0.007	0.433±0.007	0.348±0.034**
Testis width	1.316±0.047	1.273±0.034	1.331±0.023	1.302±0.025	1.284±0.011	1.306±0.029	1.338±0.040	1.290±0.042	1.306±0.028	1.161±0.048**
Testis length	2.186±0.027	2.193±0.062	2.181±0.033	2.123±0.034	2.153±0.014	2.180±0.049	2.205±0.032	2.131±0.036	2.109±0.050	1.965±0.065**
Epididymis weight (%)	0.140±0.006	0.132±0.010	0.141±0.005	0.128±0.010	0.142±0.005	0.150±0.009	0.137±0.008	0.127±0.004	0.130±0.006	0.084±0.009**
Seminal vesicle weight (%)	0.359±0.047	0.301±0.035	0.272±0.041	0.303±0.038	0.374±0.031	0.348±0.026	0.356±0.021	0.277±0.016	0.217±0.043**	0.050±0.004**
Prostate gland weight (%)	0.207±0.015	0.207±0.009	0.173±0.014	0.174±0.018	0.196±0.009	0.195±0.016	0.185±0.027	0.145±0.008**	0.126±0.010**	0.067±0.005**

* $P < 0.05$, ** $P < 0.01$ were significant difference from the vehicle control rats using orthogonal contrast. Number of animals = 5 rats per group.

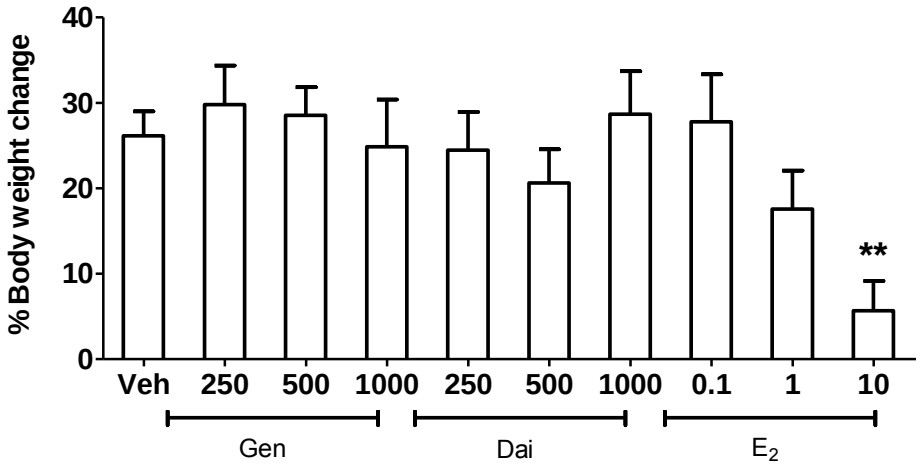

Figure 4-1 Histograms illustrate mean±S.E.M. of % change of body weight of vehicle (Veh)-, genistein (Gen)- (250, 500 or 1000 μg/kg BW), daidzein (Dai)- (250, 500 or 1000 μg/kg BW) and estrogen (E_2)- (0.1,1 or 10 μg/kg BW) treated rats. * $P < 0.05$, ** $P < 0.01$, significant difference from the vehicle treated rats assessed with orthogonal contrast. Number of animals = 5 rats per group.

2. Effects of genistein, daidzein and estrogen supplementation on reproductive organs of intact male rats

The reproductive organs of the rats treated with vehicle, genistein, daidzein or estrogen are summarized in Table 4-1. The parameters of the reproductive organs of the rats treated with vehicle, genistein, daidzein or E_2 are testis width and length, relative weights of testis, epididymis, seminal vesicle and prostate gland. There were no significantly difference in all measured parameters of genistein (250, 500 or 1000 μg/kg BW) or daidzein (250, 500 or 1000 μg/kg BW) treated rats compared to the vehicle treated group as shown in Figure 4-2 and 4-3. However, rats treated with all three doses of 17β-estradiol had lower testis length, relative weights of epididymis, seminal vesicle and prostate gland than those of vehicle control rats [P <0.05, <0.01, <0.01, <0.01, respectively] (Table 4-2; Figure 4-2, and Figure 4-3). When the results of each dose of estrogen were compared to vehicle control, the dosage of 10 μg/kg BW had significant effects on all parameters [P < 0.01, 0.01, 0.01, 0.01, 0.01 and 0.01 for relative testis weight, testis width, testis length, relative epididymis, seminal vesicle and prostate gland weights, respectively]. In addition, the relative weights of seminal vesicle and prostate gland in the estrogen treated rats at the dosage of 1 μg/kg BW were lower than control [$P < 0.01$ and $P < 0.01$, respectively]. The relative weight of prostate gland in the lowest dose of estrogen (0.1 μg/kg BW) treated group in this study was also lower than control [$P < 0.01$](Figure 4-3C).

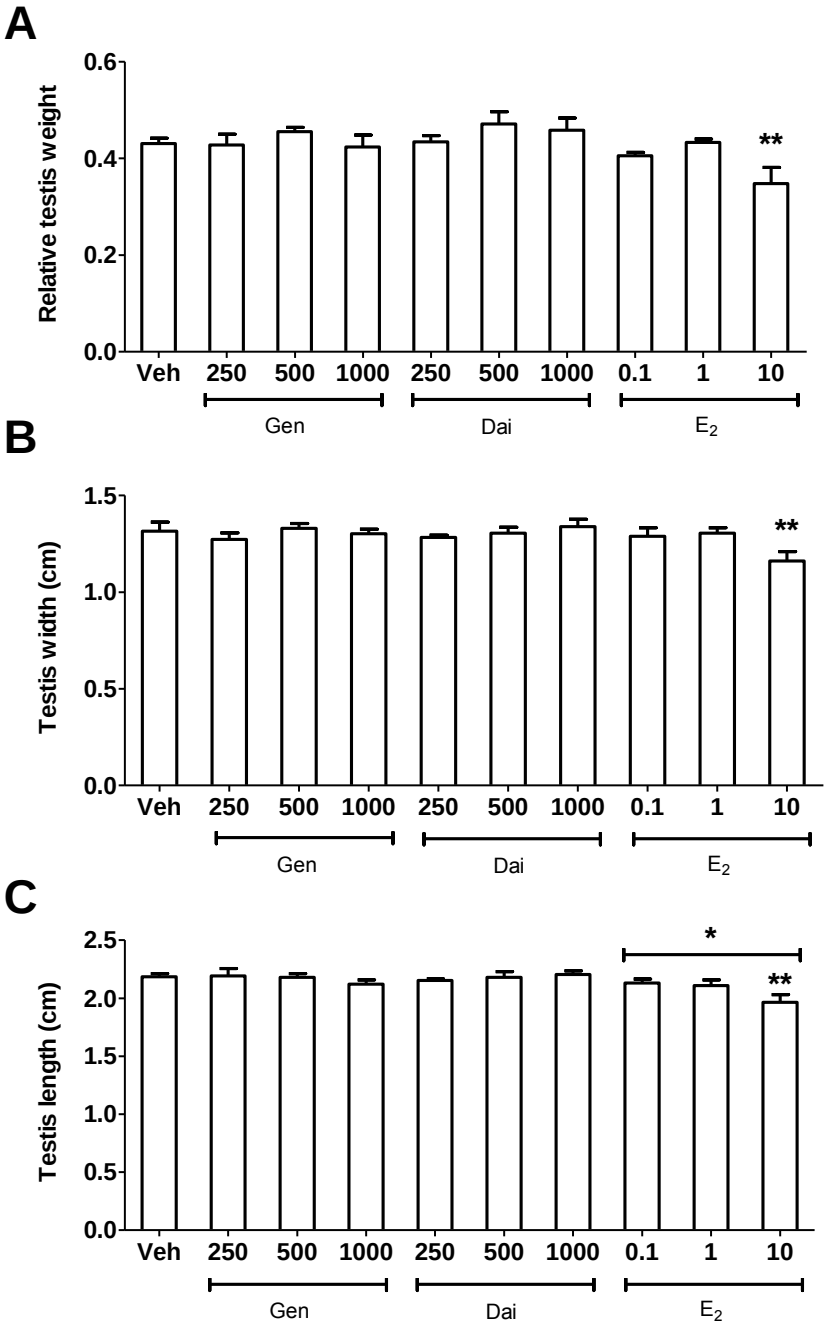

Figure 4-2 Histograms illustrate mean±S.E.M. (A) relative testis weight to body weight (B) testis width and (C) testis length of vehicle (Veh)-, genistein (Gen)- (250, 500 or 1000 μg/kg BW), daidzein (Dai)- (250, 500 or 1000 μg/kg BW) and estrogen (E_2)- (0.1,1 or 10 μg/kg BW) treated rats. * $P < 0.05$, ** $P < 0.01$, significant difference from the vehicle treated rats assessed with orthogonal contrast. Number of animals = 5 rats per group.

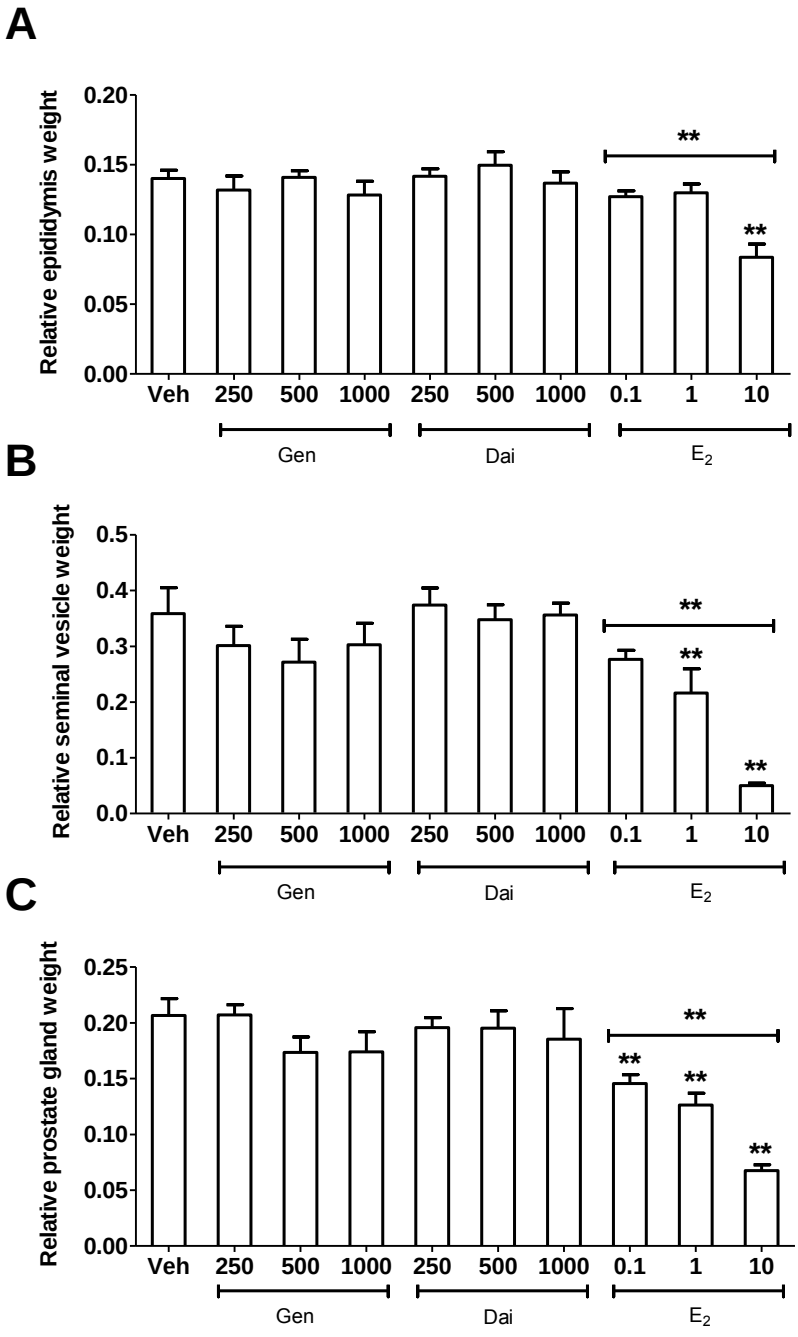

Figure 4-3 Histograms illustrate mean±S.E.M. of relative weights of (A) epididymis (B) seminal vesicle and (C) prostate gland of vehicle (Veh)-, genistein (Gen)- (250, 500 or 1000 μg/kg BW), daidzein (Dai)- (250, 500 or 1000 μg/kg BW) and estrogen (E_2)- (0.1,1 or 10 μg/kg BW) treated rats. * $P < 0.05$, ** $P < 0.01$, significant difference from the vehicle treated rats assessed with orthogonal contrast. Number of animals = 5 rats per group.

3. Effects of genistein, daidzein and estrogen supplementation on gene expression in dorsal root ganglia

Gene transcripts for the *CGRP* and *preprotachykinin-A* (*PPT-A*) gene encoding substance P were detected in the lumbosacral DRG neurons (Figure 4-4). Their levels of expression did not differ significantly from β-actin endogenous control gene expression and also did not differ between whole DRG collected from animals in vehicle control group. DRG contains endothelial and stromal cells not only the neurons. Therefore, the expression of *CGRP* and *PPT-A* which could also be transcribed in those tissues, could not be dismissed. However, the specificity of each primer used in this study was confirmed by performing a high resolution gel electrophoresis and a dissociation curve at the end of PCR. As shown in Figure 4-4 and 4-5, a single band and a single peak were evidenced in an agarose gel electrophoresis and dissociation curves, respectively; indicating primers specificity.

3.1 *CGRP* gene expression

Compared to vehicle-treated rats, *CGRP* mRNA expression was not significantly different in both lumbar and sacral DRGs in male rats treated with genistein (250, 500 or 1000 μg/kg BW), daidzein (250, 500 or 1000 μg/kg BW) or estrogens (0.1, 1.0 or 10 μg/kg BW) as shown in Table 4-2 and Figure 4-5.

3.2 *Preprotachykinin-A* (*PPT-A*) gene expression

Compared to vehicle-treated rats, *PPT-A* mRNA expression which encodes substance P protein was not significantly different in both lumbar and sacral DRGs in male rats treated with genistein (250, 500 or 1000 μg/kg BW), daidzein (250, 500 or 1000 μg/kg BW) or estrogens (0.1, 1.0 or 10 μg/kg BW) as shown in Figure 4-5. However, when each group was compared to vehicle, *PPT-A* mRNA expression in lumbar DRGs was significantly higher only in male treated rats with Dai 250 μg/kg BW [$P < 0.05$]. Similarly, *PPT-A* mRNA expression in sacral DRGs was significantly higher only in male treated rats with Dai 500 μg/kg BW [$P < 0.01$] as shown Table 4-3 in Figure 4-6.

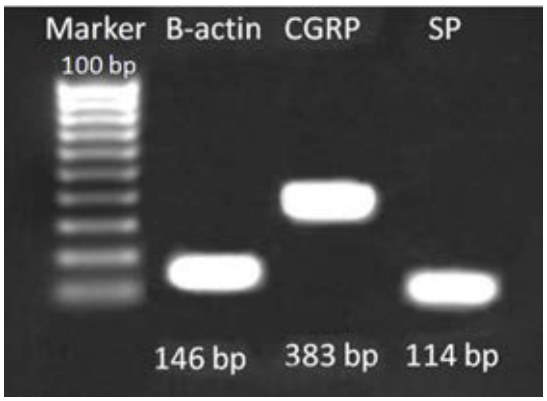

Figure 4-4 Photograph represents agarose gel electrophoresis of PCR amplification products of endogenous control gene *β-actin* (β-actin) and target genes calcitonin gene-related peptides (*CGRP*) and *preprotachykinin-A* (SP) in lumbar dorsal root ganglion neurons. The numbers below each band indicate the size(s) of each PCR product.

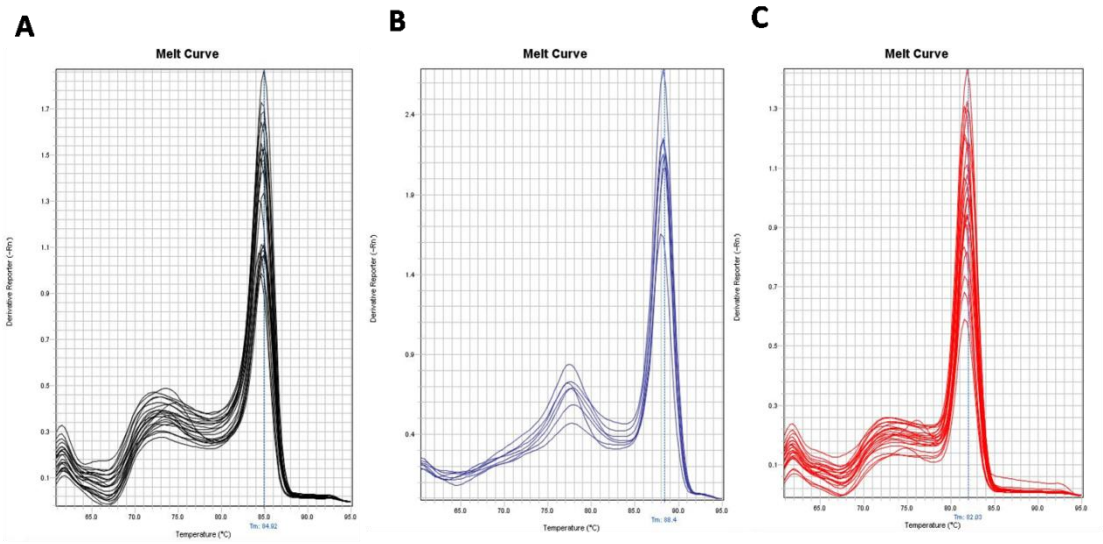

Figure 4-5 Photograph represents the dissociation curves of PCR amplification products of (A) β-actin, (B) calcitonin gene-related peptides and (C) substance P.

Table 4-2 Data represents mean±S.E.M. of ΔCt, ΔΔCt and $2^{-\Delta\Delta_{Ct}}$ (fold change) of *CGRP* mRNA expression in dorsal root ganglion of male rats treated with vehicle (Veh), genistein 250 (250Gen) , 500 (500Gen) or 1000 (1000Gen) μg/kg BW, daidzein 250 (250Dai) , 500 (500 Dai) or 1000 (1000 Dai) μg/kg BW and estrogens 0.1 ($0.1E_2$), 1.0 ($1E_2$) or 10 ($10E_2$) μg/kg BW). Number of animals = 5 rats per group.

Sample	Treatment	ΔCt	ΔΔCt	$2^{-\Delta\Delta_{Ct}}$
Lumbar	Veh	-0.60±0.47	0.00±0.00	1.00±0.00
	250Gen	0.35±0.59	0.96±0.59	0.70±0.26
	500Gen	-0.32±0.65	0.28±0.65	1.08±0.39
	1000Gen	-0.64±0.55	-0.03±0.57	1.32±0.40
	250Dai	-0.80±0.77	-0.19±0.77	1.56±0.53
	500Dai	-1.18±0.26	-0.57±0.26	1.56±0.29
	1000Dai	-0.51±0.88	0.36±0.80	1.22±0.41
	$0.1E_2$	-0.38±0.38	0.23±0.38	0.92±0.26
	$1E_2$	0.14±0.44	0.74±0.44	0.75±0.30
	$10E_2$	-0.55±0.38	-0.08±0.40	1.18±0.30
Sacrum	Veh	-0.45±0.69	0.00±0.00	1.00±0.00
	250Gen	0.35±0.44	0.14±0.44	1.03±0.29
	500Gen	5.14±1.51	4.93±1.51	0.07±0.03
	1000Gen	0.81±0.51	0.61±0.51	0.73±0.20
	250Dai	1.00±0.89	0.79±0.89	0.86±0.54
	500Dai	0.22±0.85	0.01±0.85	1.74±1.12
	1000Dai	0.47±0.03	0.26±0.03	0.83±0.02
	$0.1E_2$	0.49±0.91	0.28±0.91	1.13±0.50
	$1E_2$	0.97±0.36	0.76±0.36	0.64±0.15
	$10E_2$	0.84±0.40	0.64±0.40	0.73±0.22

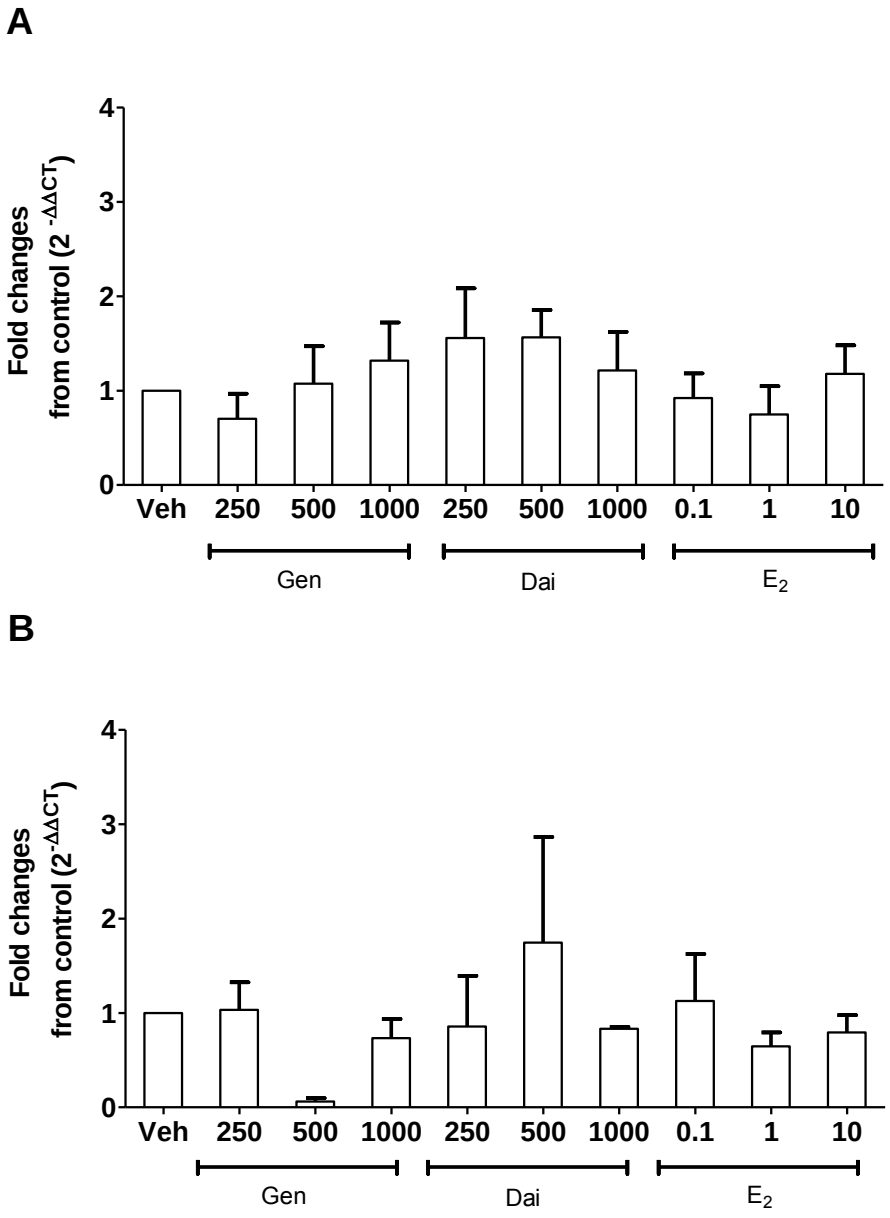

Figure 4-6 Histograms illustrate mean±S.E.M. of the fold change of *CGRP* mRNA expression in (A) lumbar and (B) sacral dorsal root ganglion of vehicle (Veh)-, genistein (Gen)- (250, 500 or 1000 μg/kg BW), daidzein (Dai)- (250, 500 or 1000 μg/kg BW) and estrogen (E_2)- (0.1,1 or 10 μg/kg BW) treated rats. Number of animals = 5 rats per group.

Table 4-3 Data represents mean±S.E.M. of ΔCt, ΔΔCt and $2^{-\Delta\Delta_{Ct}}$ (fold change) of *PPT-A* (substance P) mRNA expression in dorsal root ganglion of male rats treated with vehicle (Veh), genistein 250 (250Gen) , 500 (500Gen) or 1000 (1000Gen) μg/kg BW, daidzein 250 (250Dai) , 500 (500 Dai) or 1000 (1000 Dai) μg/kg BW or estrogens 0.1 ($0.1E_2$), 1.0 ($1E_2$) or 10 ($10E_2$) μg/kg BW). Number of animals = 5 rats per group.

Sample	Treatment	ΔCt	ΔΔCt	$2^{-\Delta\Delta_{Ct}}$
Lumbar	Veh	-0.40±0.98	0.00±0.00	1.00±0.00
	250Gen	0.94±0.86	1.33±0.86	0.65±0.25
	500Gen	0.32±0.95	0.72±0.95	1.00±0.32
	1000Gen	-1.45±0.61	-1.05±0.61	3.03±1.40
	250Dai	-1.61±0.95	-1.21±0.95	3.86±1.72*
	500Dai	-1.23±0.66	-0.83±0.66	2.76±1.41
	1000Dai	-1.24±0.58	-0.84±0.58	2.27±0.60
	$0.1E_2$	-0.68±1.08	-0.28±1.08	2.48±0.98
	$1E_2$	0.18±0.90	0.57±0.90	1.41±0.81
	$10E_2$	-0.27±0.50	0.13±0.50	1.13±0.47
Sacrum	Veh	1.30±0.44	0.00±0.00	1.00±0.00
	250Gen	2.05±0.52	0.74±0.52	0.71±0.21
	500Gen	10.23±2.33	8.93±2.33	0.02±0.02
	1000Gen	1.53±0.40	0.22±0.40	1.01±0.32
	250Dai	0.77±0.15	-0.54±0.15	1.47±0.15
	500Dai	0.40±0.88	-0.90±0.88	3.55±1.73*
	1000Dai	1.48±0.47	0.17±0.47	1.10±0.36
	$0.1E_2$	1.76±0.43	0.46±0.43	0.79±0.19
	$1E_2$	1.54±0.47	0.23±0.47	0.99±0.32
	$10E_2$	1.15±0.10	-0.16±0.10	1.12±0.08

* $P < 0.05$, ** $P < 0.01$ were significant difference from vehicle control rats using orthogonal contrast).

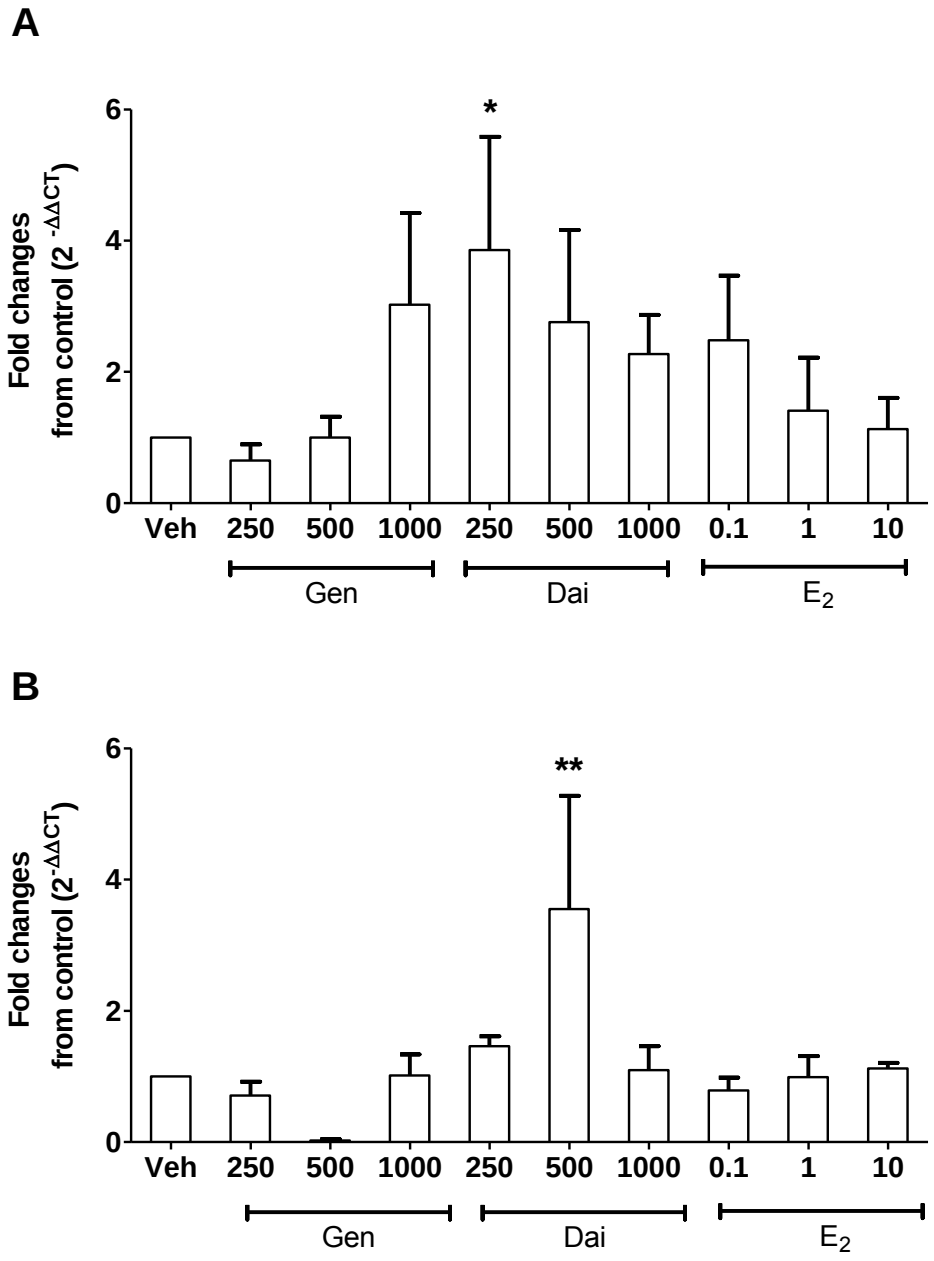

Figure 4-7 Histograms illustrate mean±S.E.M. of the fold change of *PPT-A* (substance P) mRNA expression in (A) lumbar and (B) sacral dorsal root ganglion of vehicle (Veh)-, genistein (Gen)- (250, 500 or 1000 μg/kg BW), daidzein (Dai)- (250, 500 or 1000 μg/kg BW) and estrogen (E_2)- (0.1,1 or 10 μg/kg BW) treated rats. * $P < 0.05$, ** $P < 0.01$, significant difference from vehicle treated rats assessed with orthogonal contrast. Number of animals = 5 rats per group.

4. Effects of genistein, daidzein and estrogen supplementation on protein expression of PGP9.5, CGRP and substance P in dorsal root ganglia neurons

The PGP9.5, CGRP and SP protein expressions were determined by immunohistochemical technique; the immunoreactivity (ir) was defined by reddish brown staining as shown in Figure 4-8 and 4-9. The present study did not reveal types of immunoreactive neurons determined by size of neurons, since tissue of some neurons in the chosen section were not cut in the same level (i.e at the middle of neurons). The specificity of primary antibodies used in this study was confirmed by omitting a primary antibody as seen in Figure 4-8D and 4-9D.

In the present study, the lumbosacral DRG in all rats were observed as encapsulated cells in the condensed connective tissues as seen in Figure 4-8. The neuronal cells or ganglion cells was identified by the immunoreactivity of anti PGP 9.5 antibodies. It seems that most of ganglion cells which have round shape and one nucleus with either large or small perikaryon were positive to.PGP 9.5 antibody (Figure 4-8A and 4-9A). On the other hand, the small spindle-like shape cell surrounding the ganglion cells which could be satellite or endothelial cells were negative to PGP 9.5 antibody (Figure 4-9A). In the PGP 9.5-ir cells, the granules with dark brownish staining of immunoreactivity were mostly localized in the cytoplasm or perikaryon of neurons, however a little of nucleated immunostaining was also indicated (Figure 4-9A). In all DRG, their axons or innervated nerve fibers from spinal cord also showed the light brown immunostaining, however it was not seen in the omitted control. In this study, the morphology and number of PGP 9.5-ir cells did not differ between the control and treatment groups (Table 4-4).

4.1 CGRP expression

For the CGRP-containing neurons, the immunoreactivity of CGRP was also observed in the ganglion cells mostly in the cytoplasm of cells. In contrast to PGP 9.5 immunoreactivity, the small numbers and weak signal of CGRP-immunostaining in ganglion cells was observed suggesting the heterogeneity of DRG neural population. In

lumbar section of DRG neurons of normal control male rats, less than 10% of neurons express CGRP proteins whereas 30% of sacral DRG contained CGRP proteins (Table 4-4 and 4-5). In addition, some CGRP immunoreactivity was detected in satellite cells, endothelial cells and DRG capsules (Figure 4-8B, 4-9B).

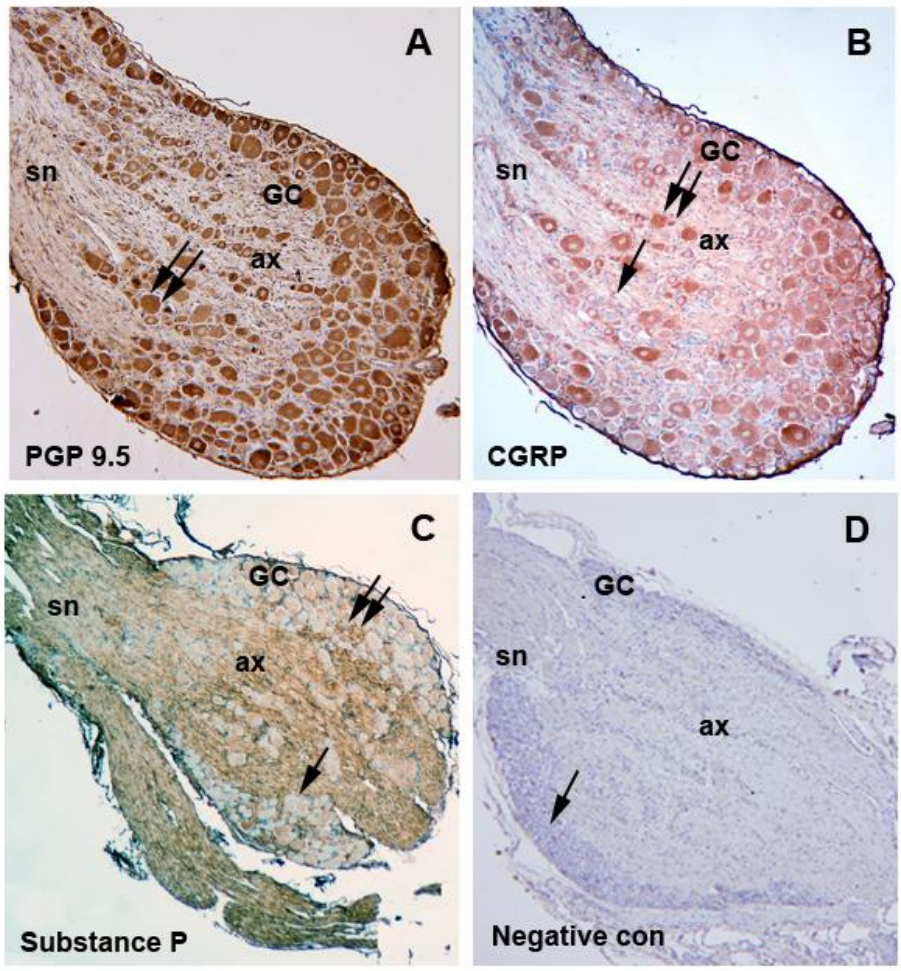

Figure 4-8 Photograph illustrates (A) protein gene product 9.5 (PGP9.5)-, (B) calcitonin gene-related peptides (CGRP)-, (C) substance P (SP)-ir neurons or (D) Negative control (omitted antibody) in lumbar dorsal root ganglion of normal male rats. Single arrow or double arrows indicate non-immunoreactive and imunoreactive cells, respectively. ax=axon, GC= ganglion cell, sn=spinal nerve fibers. (100x magnification).

Treatment with high dose of estrogen (10 μg/kg BW) and all three doses of daidzein did not modulate the numbers of CGRP containing neurons in both lumbar and sacral DRG section (Figure 4-14). However, the subpopulation of ir-cell in male rats treated with genistein were significantly higher in the lumbar DRGs [$P < 0.05$] but it was significantly lower in the sacral DRGs compared to the vehicle treated rats. It is of interest when the comparison between each dosage of genistein was done. If revealed that the lowest dosage (250 μg/kg BW) of genistein increased the CGRP subpopulation in the lumbar [$P < 0.05$] and decreased in the sacral [$P < 0.05$], respectively (Table 4-4 and 4-5; Figure 4-14A). Additionally, in the sacral DRGs, the significant increase in the ir-cells was also found in male rats treated with E_2 at the low dosages of 0.1 and 1 μg/kg BW compared to the vehicle control group [$P < 0.05$ and $P < 0.05$, respectively] as shown in (Table 4-4 and 4-5; Figure 4-14B).

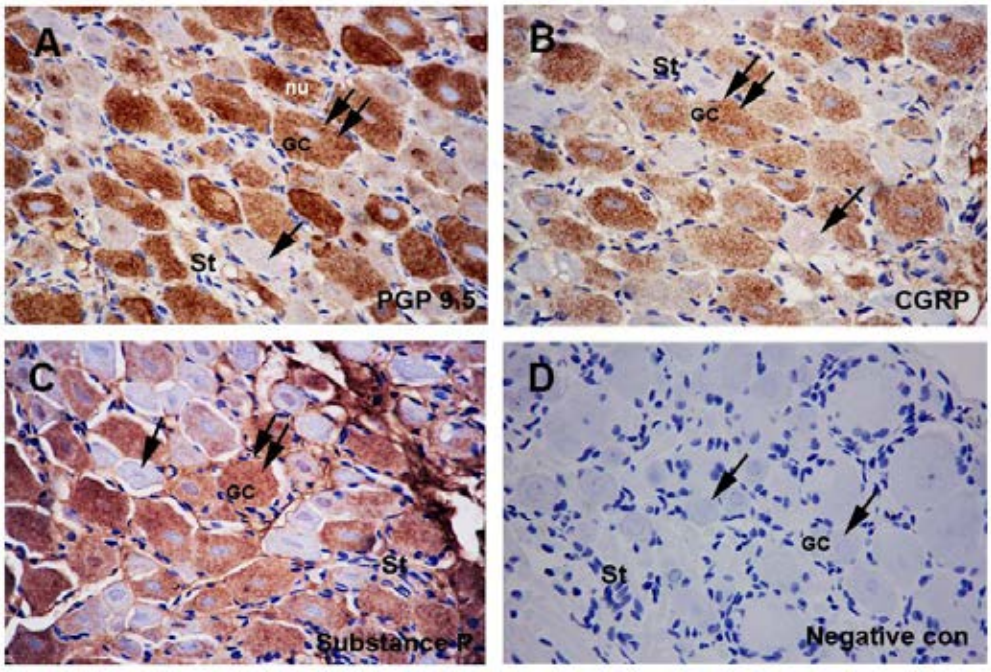

Figure 4-9 Photograph illustrates (A) protein gene product 9.5 (PGP9.5)-, (B) calcitonin gene-related peptides (CGRP)-, (C) substance P (SP)-ir neurons or (D) Negative control (omitted antibody) in lumbar dorsal root ganglion of normal male rats. Single arrow or double arrows indicate non-immunoreactive and imunoreactive cells, respectively GC= ganglion cells, nu=nucleus or St=satellite cells. (400x magnification).

4.2 substance P expression

Similar to the CGRP expression, the immunoreactivity of SP was also revealed in the perikaryon of ganglion cells but more intense signals than those of the CGRP immunostaining (Figure 4-8C or -9C). SP-ir cells were also observed as subpopulation of DRG neurons about 9% and 20% in lumbar and sacrum, respectively (Table 4-4 and 4-5).

For the SP expression, the numbers of ir cell in lumbar DRGs were not significant difference in male rats treated with genistein, daidzein or estrogen. Interestingly, when the comparison was done between each dosage, the numbers of ir cell in the male rats treated with 250 μg/kg BW of genistein was tended to be higher than the vehicle treated rats [$P = 0.0539$] (Figure 4-15A).

For the SP expression in sacral DRGs, the numbers of ir-cell were significant decrease in genistein treated groups [$P < 0.05$] but significant increase in estrogen treated groups [$P < 0.05$] and not significant difference in genistein treated group compared to the vehicle treated rats (Figure 4-15). When the comparison was done between each dosage, genistein at the dosage of 1000 μg/kg BW reduced the numbers of ir-cell compared to the vehicle control rats [$P < 0.05$]. On the contrary, estrogen at the dosage of 0.1 μg/kg BW increased the numbers of ir-cell compared to the vehicle treated rats [$P < 0.05$] as shown in (Table 4-4 and 4-5; Figure 4-15B). .

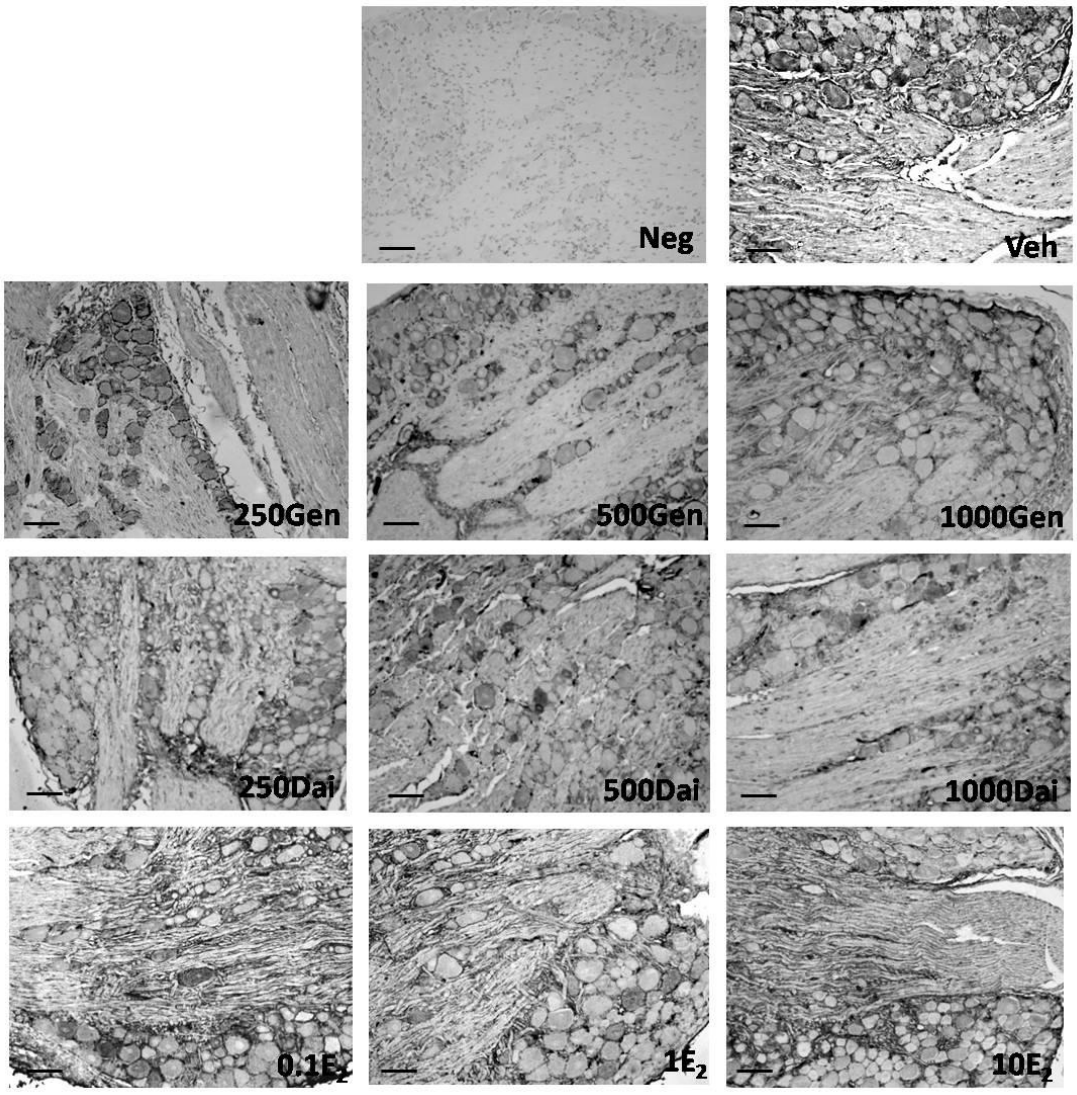

Figure 4-10 Photograph illustrates calcitonin gene-related peptides (CGRP)-ir neurons in lumbar dorsal root ganglion of negative (Neg), vehicle (Veh)-, genistein (Gen)- (250, 500 or 1000 μg/kg BW), daidzein (Dai)- (250, 500 or 1000 μg/kg BW) and estrogen (E_2)- (0.1,1 or 10 μg/kg BW) treated rats. Scale bars = 100 μm. (100x magnification).

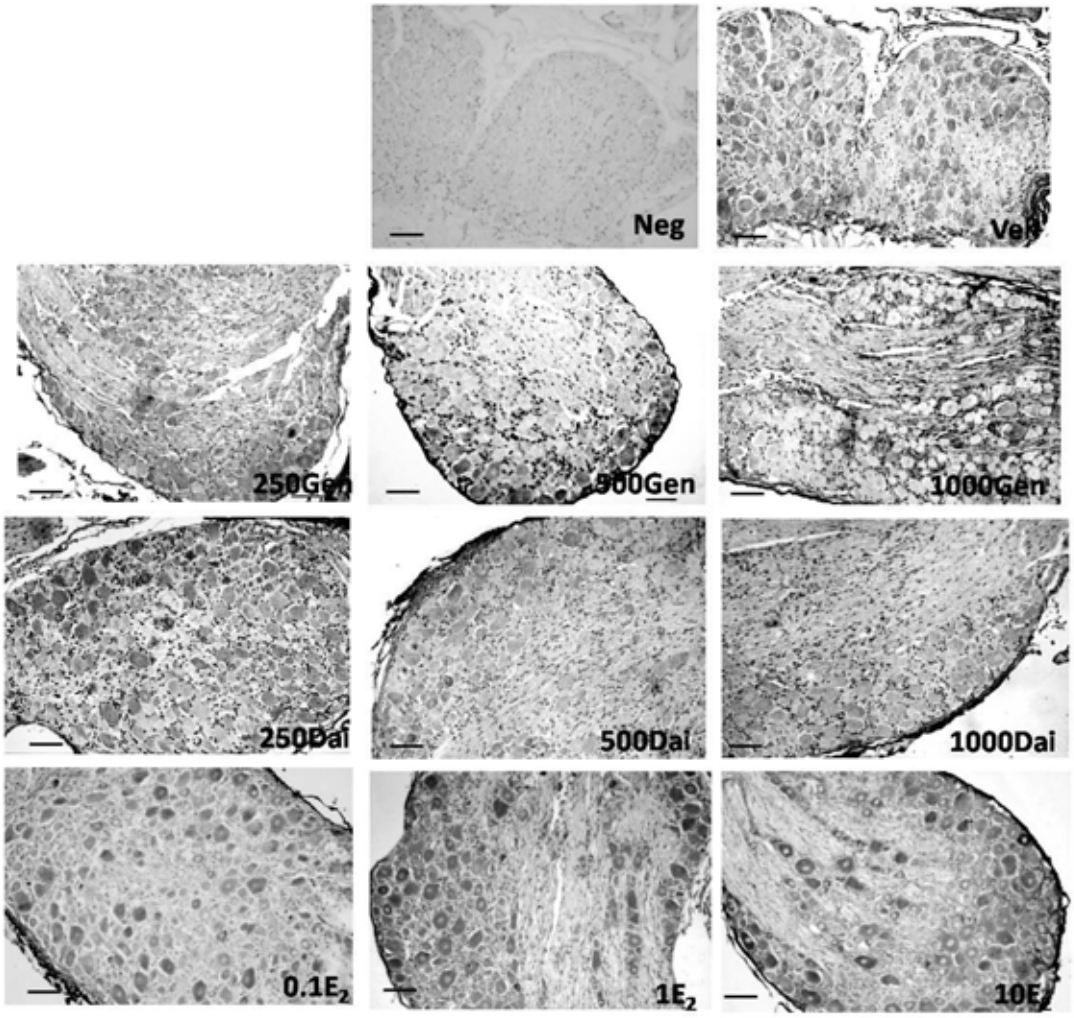

Figure 4-11 Photograph illustrates calcitonin gene-related peptides (CGRP)-ir neurons in sacral dorsal root ganglion of negative (Neg), vehicle (Veh)-, genistein (Gen)- (250, 500 or 1000 μg/kg BW), daidzein (Dai)- (250, 500 or 1000 μg/kg BW) and estrogen (E_2)- (0.1,1 or 10 μg/kg BW) treated rats. Scale bars = 100 μm. (100x magnification).

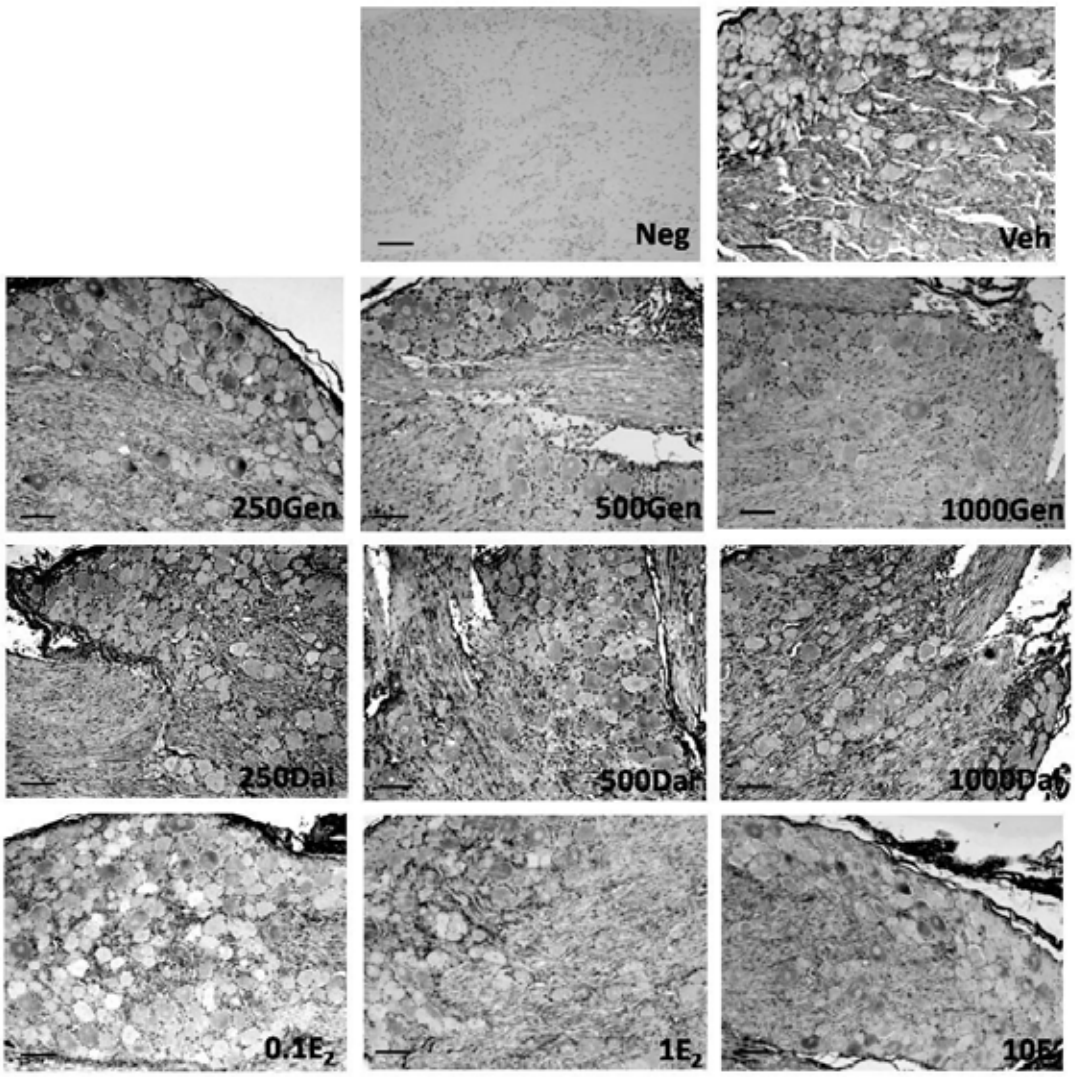

Figure 4-12 Photograph illustrates substance P (SP)-ir neurons in lumbar dorsal root ganglion of negative (Neg), vehicle (Veh)-, genistein (Gen)- (250, 500 or 1000 μg/kg BW), daidzein (Dai)- (250, 500 or 1000 μg/kg BW) and estrogen (E_2)- (0.1,1 or 10 μg/kg BW) treated rats. Scale bars = 100 μm. (100x magnification).

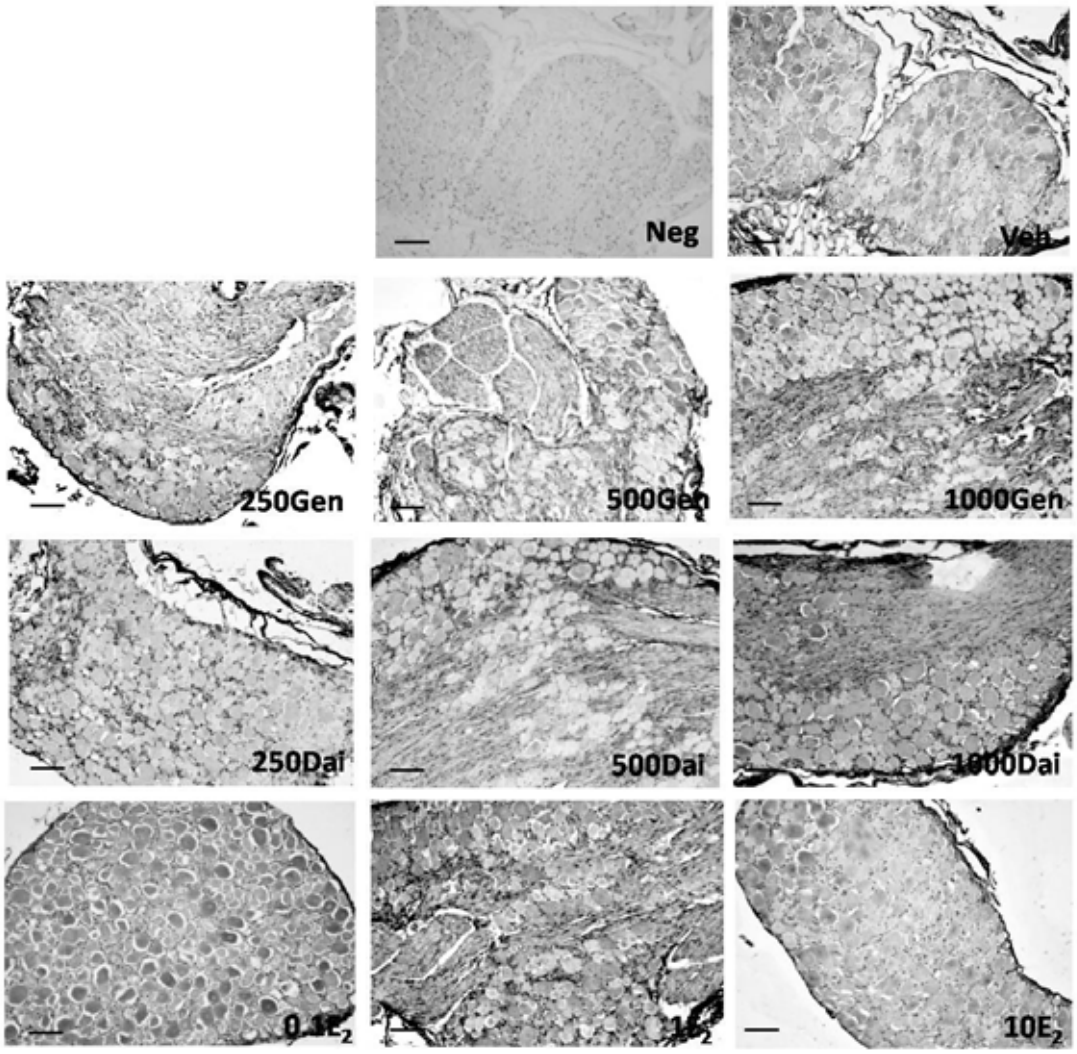

Figure 4-13 Photograph illustrates substance P (SP)-ir neurons in sacral dorsal root ganglion of negative (Neg), vehicle (Veh)-, genistein (Gen)- (250, 500 or 1000 μg/kg BW), daidzein (Dai)- (250, 500 or 1000 μg/kg BW) and estrogen (E_2)- (0.1,1 or 10 μg/kg BW) treated rats. Scale bars = 100 μm. (100x magnification).

Table 4-4 Data presents mean±S.E.M. of percentage of PGP 9.5-, CGRP- and SP-IR-cell in DRG of male rats treated with vehicle (Veh), genistein 250 (250Gen) , 500 (500Gen) or 1000 (1000Gen) μg/kg BW, daidzein 250 (250Dai) , 500 (500 Dai) or 1000 (1000 Dai) μg/kg BW or estrogens 0.1 ($0.1E_2$), 1.0 ($1E_2$) or 10 ($10E_2$) μg/kg BW).

DRGs segment	Treatment	% PGP Positive Cell	% CGRP Positive Cell	% SP positive cell
Lumbar	Veh	83.36±4.20	6.36±2.20	8.19±2.89
	250Gen	91.62±2.18	31.22±11.85	19.94±5.29
	500Gen	95.47±1.13	19.94±4.66	9.64±4.78
	1000Gen	93.53±2.82	25.88±11.87	14.36±6.03
	250Dai	96.82±1.50	24.12±9.97	9.57±2.36
	500Dai	93.82±2.70	3.16±1.10	6.92±0.86
	1000Dai	96.72±1.08	6.17±2.32	9.50±2.61
	$0.1E_2$	89.86±2.73	14.26±2.98	15.94±3.94
	$1E_2$	89.28±2.38	15.04±4.89	12.90±3.51
	$10E_2$	81.24±2.50	10.21±3.49	9.57±4.02
Sacrum	Veh	92.52±1.35	31.59±4.89	19.54±3.94
	250Gen	97.93±1.00	4.07±3.42	7.38±4.53
	500Gen	90.50±2.42	10.75±4.08	7.13±5.14
	1000Gen	93.53±2.23	28.01±9.91	1.31±1.31
	250Dai	95.10±2.26	41.06±11.71	10.60±5.63
	500Dai	83.64±8.42	26.33±8.91	11.79±5.71
	1000Dai	83.97±6.54	12.27±6.69	5.71±2.94
	$0.1E_2$	96.87±1.81	58.88±5.63	43.81±4.71
	$1E_2$	97.21±0.94	62.14±9.94	37.70±10.95
	$10E_2$	90.16±4.46	26.55±6.30	26.79±9.86

Table 4-5 Data presents mean±S.E.M. of CGRP and SP-IR cell sub-population in DRG of male rats treated with vehicle (Veh), genistein 250 (250Gen) , 500 (500Gen) or 1000 (1000Gen) μg/kg BW, daidzein 250 (250Dai) , 500 (500 Dai) or 1000 (1000 Dai) μg/kg BW or estrogens 0.1 (0.1E), 1.0 (1E) or 10 (10E) μg/kg BW).

DRGs segment	Treatment	% CGRP–IR sub-population	% SP–IR sub-population
Lumbar	Veh	7.64±2.63	9.31±3.20
	250Gen	35.18±13.88*	21.55±5.50
	500Gen	20.71±4.64	10.05±4.96
	1000Gen	27.19±11.80	15.48±6.76
	250DAI	24.84±10.10	9.95±2.49
	500Dai	3.29±1.10	7.38±0.89
	1000Dai	6.36±2.41	9.81±2.69
	$0.1E_2$	15.56±2.81	18.12±4.80
	$1E_2$	16.82±5.34	14.53±3.82
	$10E_2$	12.51±4.44	12.04±5.23
Sacrum	Veh	34.27±5.42	21.23±4.42
	250Gen	4.12±3.47*	7.44±4.57
	500Gen	12.11±4.83	7.64±5.43
	1000Gen	29.28±9.87	1.38±1.38*
	250Dai	43.52±12.29	10.96±5.69
	500Dai	32.15±10.90	13.11±5.77
	1000Dai	14.12±7.19	6.80±3.34
	$0.1E_2$	60.73±5.48*	45.31±4.86**
	$1E_2$	63.88±10.19*	38.45±10.94
	$10E_2$	29.45±6.54	31.34±12.25

* $P < 0.05$, ** $P < 0.01$, significant different from vehicle treated rats assessed with orthogonal contrast. Number of animals = 5 per group..

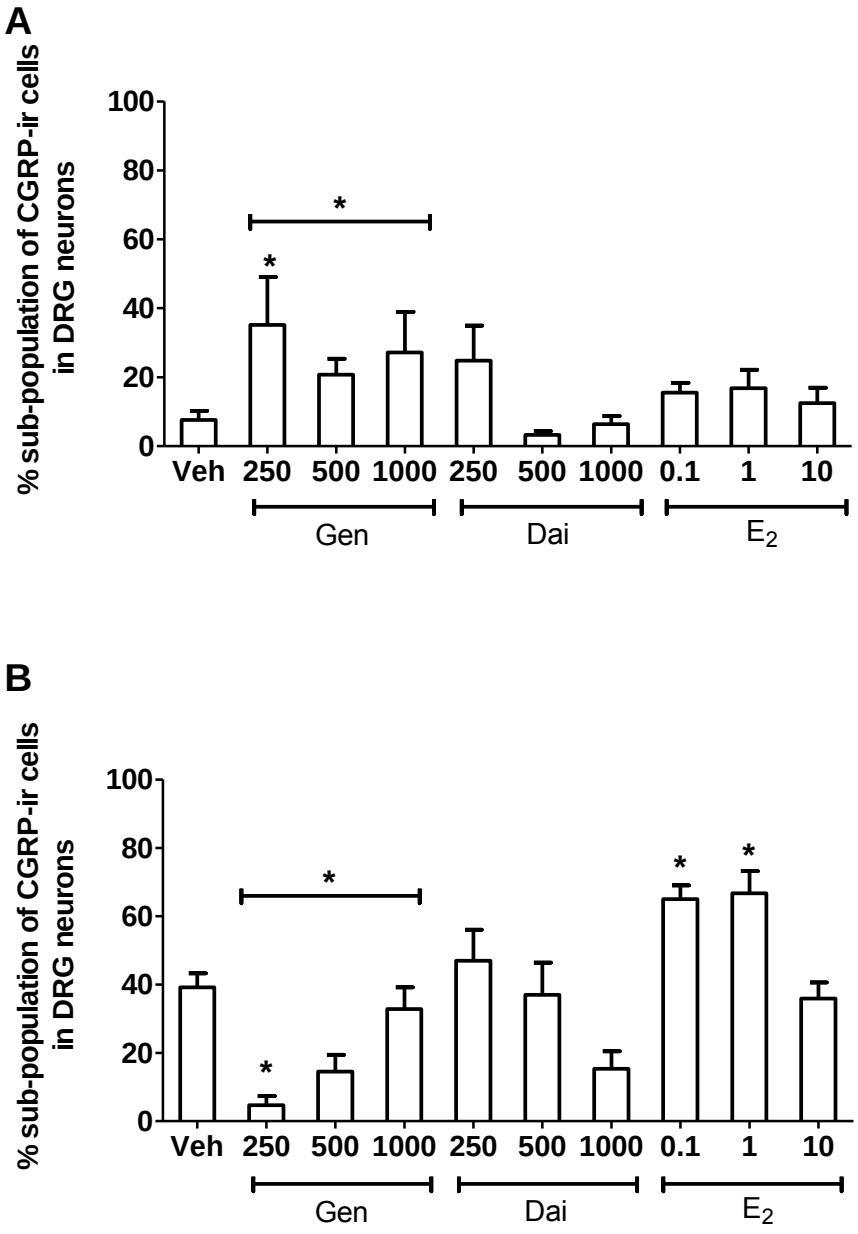

Figure 4-14 Histograms illustrate mean±S.E.M. of sub-population of CGRP-ir cell in (A) lumbar and (B) sacrum of vehicle (Veh)-, genistein (Gen)- (250, 500 or 1000 μg/kg BW), daidzein (Dai)- (250, 500 or 1000 μg/kg BW) and estrogen (E_2)- (0.1,1 or 10 μg/kg BW) treated rats. * $P < 0.05$, significant difference from vehicle treated rats assessed with orthogonal contrast. Number of animals = 5 per group.

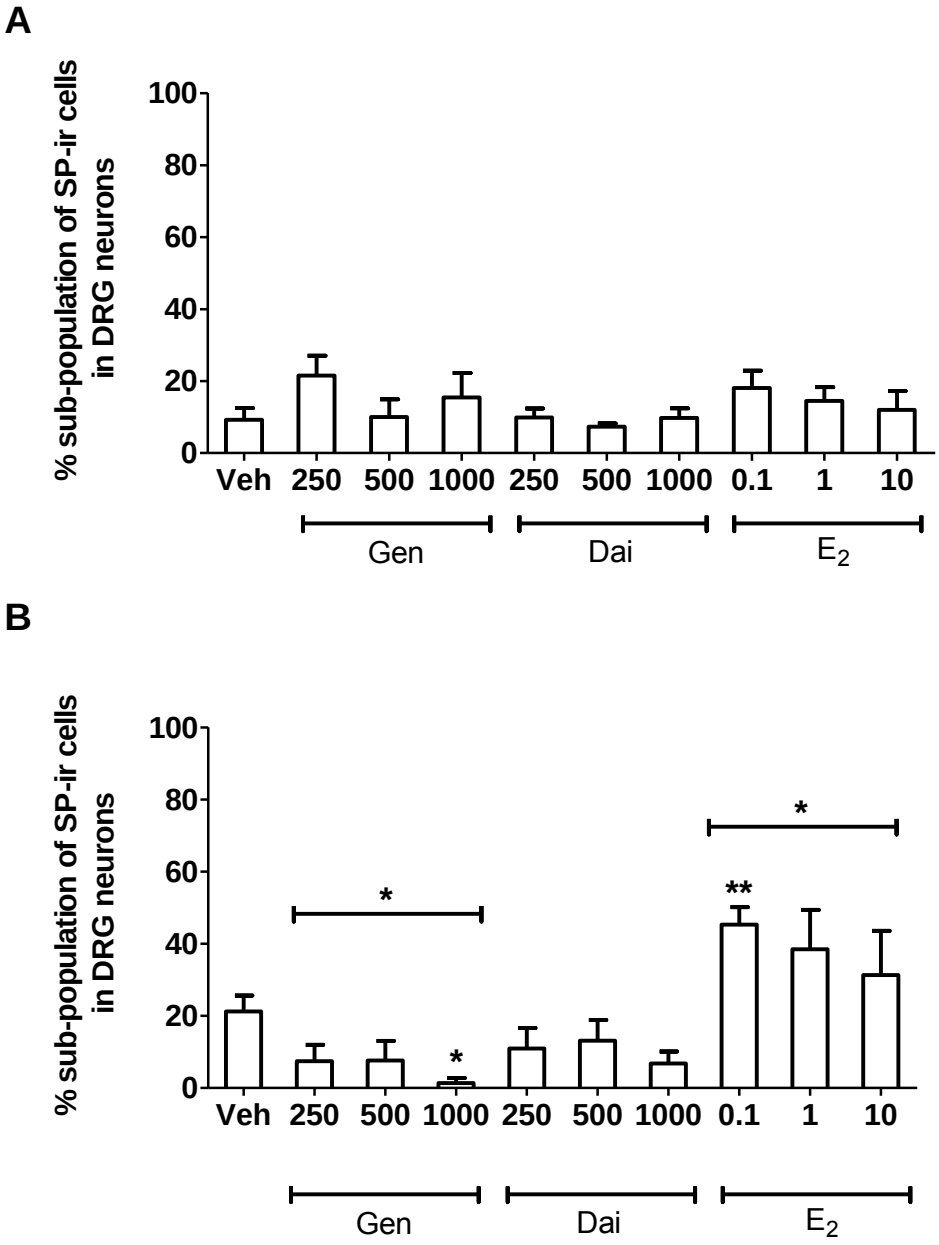

Figure 4-15 Histograms illustrate mean±S.E.M. of sub-population of SP-ir cell in (A) lumbar and (B) sacrum of vehicle (Veh)-, genistein (Gen)- (250, 500 or 1000 μg/kg BW), daidzein (Dai)- (250, 500 or 1000 μg/kg BW) and estrogen (E_2)- (0.1,1 or 10 μg/kg BW) treated rats. * $P < 0.05$, significant difference from vehicle treated rats assessed with orthogonal contrast. Number of animals = 5 per group.

CHAPTER V

DISCUSSION AND CONCLUSION

In this study, effect of the potent isoflavones at the concentration found in soybean the most popular Asian daily food products on the sensory neurons were examined in male using the adult testicular intact Wistar rat model. In our preliminary study, anxiety behavior and neurotransmitters underlying the mechanism were modulated by treated with17β-estradiol (E_2; 0.1, 1, 10 μg/kg BW), genistein (Gen; 250, 500, 1000 μg/kg BW) or daidzein (Dai; 250, 500, 1000 μg/kg BW) in 5 weeks. Treatment with 17b-estradiol revealed their effected on both male reproductive organs and brain whereas genistein or daidzein had effects only on behavior. Whether or not treatment of these substances has the same effect on peripheral nervous system involved in reproductive system CGRP and SP expression in lumbosacral dorsal root ganglia.

The estrogenic effects on sensory neural system of adult male but not female were of interest because male has been in naturally low estrogen condition even in the neonatal stage. Indeed, testosterone [or its metabolite form dihydrotestosterone (DHT)] produced and secreted by testes predominantly plays a role in male reproductive organs, such as the existence of epidydymis and penis etc. In addition, many organs development are testosterone dependent including the sympathetic autonomic nervous system (Wright and Smolen, 1983). However, testosterone regulates these organ functions through testosterone receptors and ERs. In order to activate the ERs on organs in male, testosterone is aromatized to estrogen by aromatase enzyme which can be found in many tissues including gonad and brain. Therefore, the testosterone action depends ERα or ERβ. Since neonatal female animal has never been exposed to any sex steroids, the specific organs that express sex steroid receptor are not under sex steroid regulation. Their organs are therefore the feminized pattern. These evidences have been so called sexually dimorphic phenomena (Breedlove, 1992). In addition, the effects of

sex steroids testosterone or estrogen supplementation on some organ functions, the nervous system in particular, of adult male may not be similar to those of adult female. Exposure to compounds with hormonal activity at the high doses has been hypothesized to relevant to abnormalities of animal reproductive system and behaviors of both sexes. Even though long-term exposure to low dose of estrogen-like compound had no dramatically disrupted effects on reproductive organs (Hess, 2003), it may have estrogenic or anti-estrognic effects on other organs which expressed the ERs. Consuming of soybean phytoestrogens containing genistein or daidzein may compete or synergize with testosterone or its conversion form estradiol for binding to its classical receptors. The evidence may cause the alteration of properties and function of body organs. It has to be noted that the soybean phytoestrogens genistein or daidzein which has similar structural characteristics to that of 17β-estradiol could not bind to androgen receptor to produce androgenic effect (Lund et al., 2004). However, consumption of those phytoestrogens could produce the potent isoflavone equol by colonic microbial organism. Equol was indicated as anti-androgenic compound by binding to endogenous DHT, it lead to DHT could not effect on prostate gland proliferation (Lund et al., 2004). Basically, estrogenic effect of exogenous sex steroid is a disrupted effect on male reproductive organs by reducing growth and functions of male gonads and also accessory sex organs, i.e. seminal vesicles through ERα. Therefore, any effect of phytoestrogens that parallel to the 17β-estradiol in the same experimental model could be indicated as estrogenic effects. While the non-effect of potent phytoestrogens, i.e genistein, daidzein or equol should be described as “weak estrogens” which may be because of its receptor-binding activity on the same estrogen receptor subtypes as estrogen. Briefly, the affinity of genistein and daidzein was 20 and 200 times weaker at ERα but was greater at ERβ sites (Shutt and Cox, 1972). In addition, the contrasting effect of genistein or daidzein, so called as anti-estrogenic effect, has been reviewed in the previous studies. The several possible biological mechanisms could be either mediated by 1) the competition of phytoestrogens and estrogens at same site of receptor, 2) the antagonistic action of different cellular pathways mediated by the

different subtype ERβ which is more favorable to genistein and daidzein than estrogen or 3) the non-receptor mediated mechanism by inhibitory action on the signaling system and effector system of estrogen, tyrosine kinase in particular (Barnes, 2010). In addtion, the genomic effects of genistein and daidzein on many genes including of the estrogen receptors has been reported (Casanova et al., 1999). Therefore, distribution or ratio of estrogen receptor subtypes modified by long-term effects of phytoestrogens may be occurred leading to the alteration of organ function responded to endogenous estrogens or even to phytoestrogens

Accidentally contaminated with estrogen-like compound in daily food containing soybean products has been intrigued for men's reproductive health and behaviors. Sexual incompetence and reproductive dysfunction has been reported in males in pharmaceutical industries and exposed with diethystillbestrol (Mattison et al., 1990). Most previous studies concerned about the disrupted effects on growth and development of reproductive organs. Indeed, male sexual behaviors which is concerned as sexually dimorphic and under sex steroids regulation on the sensorimotor nervous system was unaffected by phytoestrogen consumption in adult male, but it was in the neonatal male. These evidences could not be trusted unless the neuromodulatory effect of phytoestrogen was investigated. Currently, effects of estrogen or phytoestrogens supplementation on the characteristic of sensory nervous system i.e CGRP and SP neuropeptides expression which are the major neurotransmitters and target of estrogen were elucidated in male.

1. Effects of genistein, daidzein and estrogen supplementation on body weight change in intact male rats

In this study, the body weight change in intact male rats treated with genistein (250, 500 or 1000 μg/kg BW), daidzein (250, 500 or 1000 μg/kg BW) or 17β-estradiol (0.1 or 1 μg/kg BW) were not different from the control group (vehicle group). In contrast, percent change of body weight were decreased in rats treated with E_2 at the dose of 10 μg/kg BW. Current evidence showed that estrogen had effect on body weight and food intake

in agreement with the previous study where they indicated the roles of ERα subtype on the regulation on central nervous system (Roesch, 2006). In addition, the metabolic diseases were prevalent in sex differences and ovarian hormones appear to be protective against the metabolic syndrome. After menopause, obesity-related metabolic disorders was found more than menopause women (Bjorntorp; 1997). Moreover, adiposity increased in male and female mice that had a targeted deletion in the ERα subunit (αERKO) which was related estrogen could effect on body weight regulation and adipocyte function (reviewed by Shi and Clegg, 2009). Even though, androgen produces sexual dimorphism of body weight (Nance and Gorski, 1975). Marin in 1995 found that an increase in fatty acid turnover in human males treated with testosterone which testosterone inhibited the activity of lipoprotein lipase in adipose tissue.

Moreover, leptin and ghrelin were hormones that play an important role in the regulation of food intake and body weight (Klok et al, 2007). Leptin induces weight loss by suppression of food intake and by stimulation of metabolic rate whereas ghrelin induces hunger, food intake and obesity. Peripheral daily administration of ghrelin induced adiposity in rodents by reducing fat utilization (Klok et al, 2007). Estrogen could up-regulate ghrelin and leptin and level (Tanaka et al, 2001; Yin et al. 2009). Moreover, it was conform that leptin inhibits ghrelin synthesis (Yin et al. 2009). This study shown that E_2 decreased body weight which mechanism may occur by up-regulate leptin and leptin inhibit ghrelin, so food intake and body weight were decrease. However, genistein and daidzein has high affinity to ERβ subtype more than ERα subtype. These suggest that long-term treatment with genistein and daidzein the potent soybean phytoestrogens have no effect on body weight.

2. Effects of genistein, daidzein and estrogen supplementation on reproductive organ weight in intact male rats

Based on the Hershberger bioassay, a method to identify androgen disruption (Owens et al., 2007), the parameters of seminal vesicle and prostate gland responded to 17β-estradiol, genistein and daidzein supplementation were used instead of plasma testosterone evaluation. The testis weight indicated and referred to the estrogen-like compound could not affect to systemic testosterone concentration and function. Only high dose estrogen treated group but not low-dose estrogen, genistein or daidzein treated groups, their reproductive parameters such as weight and size of testis, epididymis, prostate gland and seminal vesicles were reduced. These evidences were consistent to a previous study where they suggested that phytoestrogen supplementation in adults did not interrupt the gonadotropin-testicular axis function or even the sperm quality (Mitchell et al., 2001; Faqi, et al., 2004; Perry et al., 2007). The mechanism of estrogen in disrupting the hypothalamic-gonadotropin-testicular axis in adult male was suggested to mediate by ERα which parallels with the estrogenic effect of estrogen (Scully et al., 1997). However, in male animals given with either low-dose or high dose of any exogenous estrogen and estrogen-like compound i.e phytoestrogens during the reproductive organ development showed the reduction of luteinizing hormone (LH) concentration and plasma testosterone concentration accompanied with permanently failure of male reproductive growth, function and behaviors (Faber and Hughes, 1991). Since genistein and daidzein has lesser affinity of binding at ERα site than those of estrogen, its endocrine disrupted effect on testes growth and function on male accessory sex organs seminal vesicles or prostate gland was not occurred in the current study.

3. Effects of genistein, daidzein and estrogen supplementation on *CGRP* and *PPT-A* (substance P) mRNA and protein expression

Focus on the modulatory effects of estrogen or phytoestrogens treatment, genomic effects of these chemical compounds, treatment for a long term, were examined in this study. In this work, only daidzein supplementation for 5 weeks modulated the gene encoding substance P *PPT-A* gene expression by 3.5-4 fold increase at rat lumbar and sacral DRG. In the present study, estrogen supplementation could not alter mRNA expression of CGRP and substance P which was not in agreement in the previous study in female rat with or without ovariectomy (Gangula et al., 2000). In female, estrogen exposure on OVX rat for four days upregulated substance P and CGRP synthesis in DRG sensory neurons supplying female reproductive organs. This effect was reversed by non-selective ERs blocker ICI 182,780 (Gangula et al., 2000; Mowa et al., 2003). On the other hand, long-term treatment with 17β-estrogen in female rats for 3 months downregulated mRNA expression of substance P suggesting that estrogenic effects of 17β-estradiol may have several mechanisms which mediated by ERs but depending upon physiological condition and exposure time. ERβ is more predominant and co-localized with CGRP and substance P than ERα in dorsal root ganglia especially in the cervical and sacral DRG neurons whereas subpopulations of ERs expressed in L6-S1 is as yet unclear (Taleghany et al, 1999; Mowa et al, 2003a; b). However, during the cyclic changes in female of estrogen in plasma level increase in the ERα correlated with the augmentation of CGRP and substance P synthesis (Mowa et al., 2003a; b). In contrast, the numbers of cervical, lumbar and sacral DRG neurons expressing CGRP were higher in male and ovariectomized rat compared to the intact female rats (Mills and Sengelaub, 1993). In addition, estrogen receptor expression in female DRG is higher than male and OVX-rats. About 80% of CGRP expressed neurons co-expressed with ERβ. Taken together, these evidences led to the conclusion that **estrogen could either down-regulate or up-regulate neuropeptides (i.e CGRP and substance P) expression mediated by ERβ or ERα, respectively in DRG.**

The effects of estrogen on CGRP and substance P expression may be therefore determined by the distribution and ratio of ERα and ERβ in tissues. In the present study of CGRP and SP expression using the immunohistochemistry techniques showed that estrogen supplementation increased CGRP and SP subpopulation of sacral DRG but not at lumbar DRG whereas genistein decreased those subpopulations in sacral DRG neurons. These opposite effects (or anti-estrogenic effect) between estrogen and genistein which its affinity binding to different ERs isoforms imply that S1-S3 DRG neurons containing CGRP and substance P may express both ERα and ERβ subtypes. Therefore the regulation of those two neuropeptides response to exogenous estrogenic compound depended on drug-receptor binding affinity. 17β-estradiol binds to ERα-better than ERβ leading to the up-regulation of CGRP and SP expression consistent to the previous study as in the literatures mentioned earlier. On the other hand, genistein is favorably binding to ERβ which was known to down-regulate CGRP and SP expression therefore decreased subpopulation of these two neuropeptides. However, the other possibility of the anti-estrogenic effects of genistein on CGRP neurons regulation is because of the estrogen receptor subtype adaptation response to long-term phytoestrogen or estrogen treatment. Taleghany and co-worker (1999) found that ERα was downregulated while ERβ was upregulated in DRG in long-term ovariectomized rats supplement with estrogen. These evidences imply that ER subtypes expressed in DRG neurons are under gonadal sex steroid thus estrogen or genistein may has genomic effect to modulate ER ratio and subsequently response to estrogen and other estrogenic compound through the adapted ER subtypes The later suggestion may be suitable to explain the reason why daidzein which also high affinity to ERβ could not demonstrate down-regulatory effects on CGRP or SP neuron subpopulation similar to those of genistein. Perhaps the adaptation of ERs and neuropeptides response to daidzein treatment for 5 weeks in the present study may be delaying or ongoing whereas the genomic effects of genistein and estrogen was completed. This suggestion is conceivable since those two compound modulated CGRP and SP protein expression without changes of its mRNA expression. Delaying effect of daidzein may be caused by

the different pharmacokinetics beweet genistein and daidzein. Setchell and co-workers (2003) indicated that serum genistein reached the maximum higher, faster and more prolong than serum daidzein studied in premenopausal women exposed with soy diet, even though a few literatures have been reported in male animals. Therefore, it needs to be further investigated or performed the study using the longer daidzein treatment.

In addition, none of estrogenic effects were seen in the lumbar (L1-L6) DRG after long-term treatment with estrogen but genistein. Even though genistein revealed the modulatory effects on CGRP subpopulation neurons, its effect was positively enhanced the numbers of lumbar CGRP neurons. As in the previous study explained earlier, up-regulation of CGRP expression response to estrogen-like compound is theoretically mediated by ERα which should be stimulated with estrogen. Since estrogen could not show any effects on CGRP or substance P expression in lumbar, up-regulation of lumbar CGRP neuron subpopulation may not be mediated by ERs or even the same mechanism as seen in sacral DRG. It is unclear if another cellular target or mechanism such as other neuropeptides, growth factor proteins or signaling protein responded to genistein through non-estrogen pathway.

The present study suggested the modulatory effects of estrogen and genistein on neuropeptide expression in DRG neurons by studying of mRNA in parallel with immunohistochemistry but the not by Western blot analysis. The current study showed the unrelevant results of those two techniques. It may be intrigued that estrogen and genistein or daidzein affected to the number of DRG neural population leading to the relative changes of CGRP and SP subpopulation in DRG. Indeed, gonadal sex steroids and soybean estrogens has been reported to have mitogenic effect and neuroprotective effect in CNS leading to the up-regulation of neuronal cell numbers through estrogen receptors (Toran-Allerand, 2004). In order to exclude this possibility, anti PGP 9.5 antibody was used as an immunochemical pan-neuronal marker (Day and Thompson, 2010). In PGP expression, the numbers of PGP 9.5-ir were not significantly difference in lumbar and sacrum DRGs all groups of treated rat compared to the vehicle group. This showed that estrogen and phytoestrogens had no effect on neuronal population.

However, only the numbers of CGRP- and SP-ir were changes after long-term treatment of drugs suggesting that the neuropeptides synthesized in the neurons resided in DRG was changes in response to drugs.

There are dimorphic in CGRP expression. In intact rat, male rat had CGRP expressed neurons higher than female rat in cervical, lumbar and sacral DRG. There was CGRP neurons about 35% in lumbosacral DRGs in male rat. To compare with intact female rat, CGRP neurons were increased in lumbar DRG (L_3) in OVX rat. ER was found in DRG neuron and express in female more than male. To compare with intact female rat, ER in lumbar DRG (L_3) was increased in OVX rat. Moreover, ER co-expression with CGRP neuron was no difference between male or female rat but this co-expression was increased in OVX rat (Yang et al., 1998). However, CGRP and SP mRNA and protein expression level are related to the level of estrogen during parturition (Mowa et al., 2003). Therefore, CGRP and ER expression was modified by estrogen.

ERα was more expression in L6-S1 than L4-L5 DRG in male rat but ERβ was not different between these areas (Taleghany et al,1999). This indicates that ERα/ERβ ratio and expresion of both ERs in sacram was more than in lumbar DRG. These suggest that estrogen may be effect on CGRP and SP in lumbar DRG more than sacrum DRG.

In previous study, ERα have shown to be lowered in OVX rat than those in OVX treated estrogen for a long-term 21-28 days, but ERβ was increased in L4-S1 DRG However, both ERα and ERβ were increased during proestrus compared to metestrus (short-term) in L4-S1 DRG (Taleghany et al, 1999). These suggest that estrogen regulate ER expression in DRG depend on time exposure of estrogen level.

Moreover, phytoestrogen could increase ERα rather than ERβ in cancer cell. This suggests that phytoestrogen could modulate ER subtypes expression leadiing to the difference effects between daidzein and genistein.

CONCLUSION

Overall results of the present study suggest that exogenous estrogen treatment not only up-regulate CGRP and SP subpopulation neurons but also produce endocrine disruption on male reproductive organs suggesting its neuromodulatory and anti-androgenic effect. Since CGRP and SP neuropeptides plays significant roles in pain and inflammation, long-term exposure to estrogen may cause the neuropathic pain and allodynia. In contrast, long-term treatment with the soybean phytoestrogens had no interrupted effects in male reproductive organs, however genistein or daidzein showed the negative regulatory effects on CGRP and SP expression in DRG. These evidences may provide the new insights for daily consuming of soybean phytoestrogens as an anti-nociceptive and anti-inflammatory compound in neuropathic pain. However, daily consumption of genistein or daidzein should be aware for the alteration of sensual behavior mediated by CGRP and SP in healthy male.

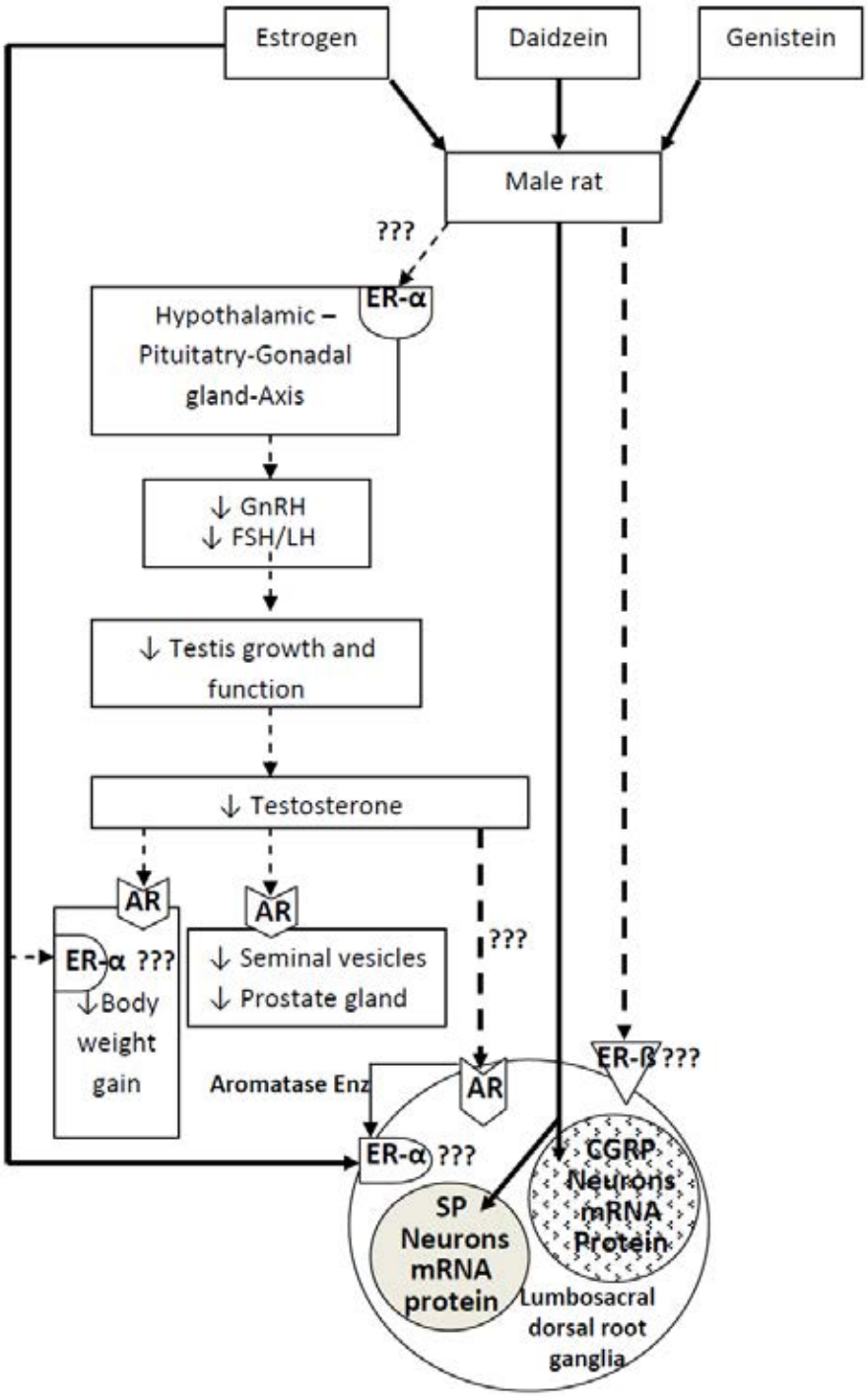

Figure 5-1 Schematic of effects of estrogen and phytoestrogens on body weight changes, male reproductive organs and CGRP or substance P-expression in lumbosacral dorsal root. Bold line represents the increased effect while dotted line represents the reduced effect.

REFERENCES

D'Andrea, V., and others. The immunolocalization of PGP 9.5 in normal human kidney and renal cell carcinoma. G Chir 18 (1997): 521–524.

Arai, H., and Emson, P.C. Regional distribution of neuropeptide K and other tachykinins (neurokinin A, neurokinin B and substance P) in rat central nervous system. Brain Res 399 (1986): 240–249.

Aumuller, G., Renneberg, H., Leonhardt, M., Lilja, H., and Abrahamsson, P.A. Localization of protein gene product 9.5 immunoreactivity in derivatives of the human Wolffian duct and in prostate cancer. Prostate 38 (1999): 261–267.

Averill, S., McMahon, S.B., Clary, D.O., Reichardt, L.F., and Priestley, J.V. Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. Eur J Neurosci 7 (1995):1484-1494.

Barnes, S. The biochemistry, chemistry and physiology of the isoflavones in soybeans and their food products. Lymphat Res Biol 8 (2010): 89-98.

Belcher, S.M., and Zsarnovszky, A. Estrogenic actions in the brain: estrogen, phytoestrogens, and rapid intracellular signaling mechanisms. J Pharmacol Exp Ther 299 (2001): 408-414.

Bennett, H.L., Gustafsson, J., and Keast, J.R. Estrogen receptor expression in lumbosacral dorsal root ganglion cells innervating the female rat urinary bladder. Auton Neurosci 105 (2000): 90-100.

Bereiter, D.A., Stanford, L.R., and Barker, D.J. Hormone-induced enlargement of receptive fields in trigeminal mechanoreceptive neurons. II. Possible mechanisms. Brain Res 184 (1980): 411–423.

Birren, B., Green, E.D., Klapholz, S., Myers, R. M., and Roskams, J. A laboratory manual Vol 1. Quantitation of DNA Analyzing DNA. USA: Cold Spring Harbor Laboratory Press. 1997.

Bjorntorp, P. Body fat distribution, insulin reistance, and metabolic diseases. Nutrition 13 (1997): 795–803.

Brain, S.D., Williams, T.J., Tippins, J.R., Morris, and H.R., MacIntyre, I. Calcitonin gene-related peptide is a potent vasodilator. Nature 313 (1985): 54–56.

Breedlove, S.M. Sexual dimorphism in vertebrate nervous system. J Neurosci 12 (1992): 4133-4142.

Cao, Y.Q., Mantyh, P.W., Carlson, E.J., Gillespie, A.M., Epstein, C.J., and Basbaum, A.I. Primary afferent tachykinins are required to experience moderate to intense pain. Nature 392 (1998): 390-394.

Casanova, M., You, L., Gaido, K.W., Archibeque-Engle, S., Janszen, D.B., and Heck, H.A. Developmental effects of dietary phytoestrogens in Sprague-Dawley rats and interactions of genistein and daidzein with rat estrogen receptors alpha and beta in vitro. Toxicol Sci 51 (1999): 236-244.

Chavarro, J.E., Toth, T.L., Sadio, S.M., and Hauser, R. Soy food and isoflavone intake in relation to semen quality parameters among men from an infertility clinic. Hum Reprod 23 (2008): 2584-2590.

Ciechanover, A., Orian, A., and Schwartz, A.L. The ubiquity inmediated proteolytic pathway: mode of action and clinical implications. J Cell Biochem 77 (2000): 40–51.

Cridland, R.A., and Henry, J.L. Effects of intrathecal administration of neuropeptides on a spinal nociceptive reflex in the rat: VIP, galanin, CGRP, TRH, somatostatin and angiotensin II. Neuropeptides 11 (1988): 23–32.

McCulloch, J., Uddman, R., Kingman, T.A., and Edvinsson, L. Calcitonin gene-related peptide: Functional role in cerebrovascular regulation. Proc Natl Acad Sci USA 83 (1986): 5731–5735.

Day, I.N., and Thompson, R.J. UCHL1 (PGP 9.5): neuronal biomarker and ubiquitin system protein. Prog Neurobiol 90 (2010): 327-362.

von Euler, U. S.; and Gaddum, J. H. An unidentified depressor substance in certain tissue extracts. J Physiol 72 (1931): 74–87.

Eustache, F., and others. Chronic dietary exposure to low-dose mixture of genistein and vinclozolin modifies the reproductive axis testis transcriptome, and fertility. Environ Health Perspect 117 (2009): 1272-1279.

Faber, K.A., and Hughes, C.L. The effect of neonatal exposure to diethylstilbestrol, genistein, and zearalenone on pituitary responsiveness and sexually dimorphic nucleus volume in the castrated adult rat. J Biol Reprod 45 (1991): 649–653.

Faqi, A.S., Johnson, W.D., Morrissey, R.L., and McCormick, D.L. Reproductive toxicity assessment of chronic dietary exposure to soy isoflavones in male rats. Reprod Toxicol 18 (2004): 605-611.

Freedman, R.R. Physiology of hot flashes. Am J Hum Biol 13 (2001): 453–464.

Frohlich, P.F., and Meston, C.M. Tactile sensitivity in women with sexual arousal disorder. Arch Sex Behav 34 (2005): 207–217.

Gangula, P.R.R., Lanlua, P., Wimalawansa, S., Supowit, S., DiPette, D., and Yallampalli, C. Regulation of calcitonin gene-related peptide expression in dorsal root ganglia of rats by female sex steroid hormones. Biol Reprod 62 (2000): 1033–1039.

Ghatta, S., and Nimmagadda, D. Calcitonin gene-related peptide: Understanding its role. Indian J Phamacol 36 (2004): 227-283.

Gibson, S.J., and others. Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and of eight other species. J Neurosci 4 (1984): 3101-3111.

de Groat, W.C. Neuropeptides in pelvic afferent pathway. Experientia 43 (1987): 801-813.

Guo, A., Vulchanova, L., Wang, J., Li, X., and Elde, R. Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. Eur J Neurosci 11 (1999): 946-958.

Harrison, S., and Geppetti, P. Substance P. Int J Biochem Cell Biol 33 (2001): 555-576.

Hay, D.L. and Dickerson, I.M. (eds.) The Calcitonin Gene-related Peptide Family: Form, Function and Future Perspectives. New York: Springer-Verlag, 2009.

Herbison, A.E., King, I.S., Tan, K.C., and Dye, S. Increased fos expression in preoptic calcitonin gene-related peptide (CGRP) neurones following mating but not the luteinizing hormone surge in female rats. J Neuroendocrinol 7 (1995): 377-385.

Hess, R.A. Estrogen in the adult male reproductive tract: A review. Reprod Biol Endocrinol 1 (2003): 52.

Hirata, S., Shoda, T., Kato, J., and Hoshi, K. Isoform/variant mRNAs for sex steroid hormone receptors in humans. Trends Endocrinol Metab (2003) 14: 124–129.

Hökfelt, T., Zhang, X., and Wiesenfeld-Hallin, Z. Messenger plasticity in primary sensory neurons following axotomy and its functional implications. Trends Neurosci 17 (1994): 22–30.

Hrabovszky, Z., Farmer, P.J., and Hutson, J.M. Does the sensory nucleus of the genitofemoral nerve have role in testicular descent? J Pediatr Surg 35 (2000): 96-100.

Jackson, P., and Thompson, R.J. The demonstration of new human brain-specific proteins by high-resolution two-dimensional polyacrylamide gel electrophoresis. J Neurol Sci 49 (1981): 429-38.

Ju, G., and others. Primary sensory neurons of the rat showing calcitonin gene-related peptide immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide- and cholecystokinin-immunoreactire ganglion cells. Cell Tissue Res 247 (1987): 417-431.

Kalra, S.P., and Kalra, P.S. Temporal interrelationships among circulating levels of estradiol, progesterone and LH during the rat estrous cycle: effects of exogenous progesterone. Endocrinology 95 (1974): 1711-178.

Kandel, E.R., Schwartz, J.H., and Jessell, T.M. Principles of Neural Science. New York: McGraw-Hill Professional Publishing. 2000.

Keast, J.R., Furness, J.B., and Costa, M. Distribution of peptide-containing neurons and endocrine cells in the rabbit gastrointestinal tract, with particular reference to the mucosa. Cell Tissue Res 248 (1987): 565-577.

Kishida, T., and others. Dietary soy isoflavone-aglycone lowers food intake in female rats with and without ovariectomy. Obesity (Silver Spring) 16 (2008): 290-297.

Klok, M.D., Jakobsdottir, S., and Drent, M.L. The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. Obes Rev (2007) 8: 21–34.

Kuiper, G.G., and others. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139 (1998): 4252-4263.

Lephart, E.D., Thompson, J.M., Setchell, K.D.R., Adlercreutz, H., and Weber, K.S. Phytoestrogens decrease brain calcium-binding proteins but do not alter hypothalamic androgen metabolizing enzymes in adult male rats. Brain Res 859 (2000): 123-131.

Lephart, E.D., and others. Neurobehavioral effects of dietary soy phytoestrogens. Neurotoxic Teratol 24 (2002): 5-16.

Ljungdahl, A., Hokfelt, T., and Nilsson, G. Distribution of substance P-like immunoreactivity in the central nervous system of the rat. I. Cell bodies and nerve terminals. Neuroscience (1978): 861–943.

Lund, T.D., Munson, D.J., Haldy, M.E., Setchell, K.D., Lephart, E.D., and Handa, R.J. Equol is a novel anti-androgen that inhibits prostate growth and hormone feedback. Biol Reprod 70 (2004): 1188–1195.

Ma, W., and Quirion, R. Increased calcitonin gene-related peptide in neuroma and invading macrophages is involved in the up-regulation of interleukin-6 and nerve injury associated thermal hyperalgesia in a rat model of mononeuropathy. J Neurochem 98 (2006): 180–192.

Maggi, C.A. Tachykinin and calcitonin gene related peptide (CGRP) as co-transmitters released from peripheral endings of sensory nerves. Prog Neurobiol 45 (1995): 1-98.

Malykhina, A.P. Neural mechanisms of pelvic organ cross-sensitization. Neuroscience 149 (2007): 660-672.

Mantyh, P.W., Hunt, S.P., and Maggio, J.E. Substance P receptors: localization by light microscopic autoradiography in rat brain using [3H]SP as the radioligand. Brain Res 307 (1984):147–165.

Marin, P. Testosterone and regional fat distribution. Obes Res (1995) 3: 609S-612S.

Marlier, L., Poulat, P., Rajaofetra, N., and Privat, A. Modifications of serotonin-, substance P- and calcitonin gene-related peptide-like immunoreactivities in the dorsal horn of the spinal cord of arthritic rats: a quantitative immunocytochemical study. Exp Brain Res 85 (1991): 482-490.

Marotta, F., and others. Anti-inflammatory and neuroprotective effect of a phytoestrogen compound on rat microglia. Ann N Y Acad Sci 1089 (2006): 276-281.

Martin, R., and others. Immunohistochemical localization of protein gene product 9.5, ubiquitin, and neuropeptide Y immunoreactivities in epithelial and neuroendocrine cells from normal and hyperplastic human prostate. J Histochem Cytochem 48 (2000):1121–1130.

Mattison, D.R., Plowchalk, D.R., Meadows, M.J., al-Juburi, A.Z., Gandy, J., and Malek, A. Reproductive toxicity: male and female reproductive systems as targets for chemical injury. Med Clin North Am 74 (1990): 391-411.

Mills, A.C., and Sengelaub, D.R. Sexually dimorphic neuron number in lumbosacral dorsal root ganglia of the rat: development and steroid regulation. J Neurobiol (1993): 1543-1553.

Mitchell, J.H., Cawood, E., Kinniburgh, D., Provan, A., Collins, A.R., and Irvine DS. Effect of a phytoestrogen food supplement on reproductive health in normal males. Clin Sci 100 (2001): 613-618.

Moore, T.O., Karom, M., and O'Farrell, L. The neurobehavioral effects of phytoestrogens in male Syrian hamsters. Brain Res 1016 (2004): 102-110.

Morales, A. Erectile Dysfunction: Issues in Current Pharmacotherapy. London: Martin Dunitz Publishers Ltd, 1998.

Mowa, C.N., Usip, S., Collins, J., Storey-Workley, M., Hargreaves, K.M., and Papka, R.E. Substance P in uterine cervix, dorsal root ganglion and spinal cord during pregnancy and the effect of estrogen on SP synthesis. Peptides 24 (2003a): 761-771.

Mowa, C.N., Usip, S., Collins, J., Storey-Workley, M., Hargreaves, K.M., and Papka, R.E. The effects of pregnancy and estrogen on the expression of calcitonin gene-related peptide (CGRP) in the uterine cervix, dorsal root ganglia and spinal cord. Peptides 24 (2003b): 1163–1174.

Murkies, A.L., Wilcox, G., and Davis, S.R. Clinical review 92: Phytoestrogens. J Clin Endocrinol Metab 83 (1998): 297-303.

Nance, D.M., and Gorski, R.A. Neurohormonal determinants of sex differences in the hypothalamic regulation of feeding behavior and body weight in the rat. Pharmacol Biochem Behav 3 (1975): 155-162.

Nilsson, B.O. Modulation of the inflammatory response by estrogens with focus on the endothelium and its interactions with leukocytes. Inflamm Res 56 (2007): 269-273.

Noguchi, K., Morita, Y., Kiyama, H., Ono, K., and Tohyama, M. A noxious stimulus induces the preprotachykinin-A gene expression in the rat dorsal root ganglion: a quantitative study using in situ hybridization histochemistry. Brain Res 464 (1988): 31–35.

Owens, W. The OECD program to validate the rat Hershberger bioassay to screen compounds for in vivo androgen and antiandrogen responses: phase 2 dose-response studies. Environ Health Perspect 115 (2007): 671-678.

Pan, L., Xia, X., Feng, Y., Jiang, C., Cui, Y., and Huang, Y. Exposure of juvenile rats to the phytoestrogen daidzein impairs erectile function in a dose-related manner in adulthood. J Androl 29 (2008): 55–62.

Patestas, M.A. and Gartner, L.P. A Textbook of Neuroanatomy. Malden, MA: Blackwell Publishing, 2006.

Patisaul, H.B. Phytoestrogen action in the adult and developing brain. J Neuroendocrinol 17 (2005): 57-64.

Perez-Rivero, J.J., and others. Phytoestrogen treatment induces testis alterations in dogs. Potential use in population control. Vet Res Commun 33 (2008): 87-95.

Perry, D.L., Spedick, J.M., McCoy, T.P. Adams, M.R., Franke, A.A., and Cline, J.M. Dietary soy protein containing isoflavonoids does not adversely affect the reproductive tract of male cynomolgus macaques (Macaca fascicularis). J Nutr 137 (2007): 1390-1394.

Poyner, D.R., and others. International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. Pharmacol Rev 54 (2002): 233–246.

Rambourg, A., Clermont, Y., and Beaudet, A. Ultrastructural features of six types of neurons in rat dorsal root ganglia. J Neurocytol 12 (1982): 47-66.

Roberts, D., Veeramachaneni, D.N., Sclaff, W.D., and Awoniyi, C.A. Effects of chronic dietary exposure to genistein, a phytoestrogen, during various stages of development on reproductive hormones and spermatogenesis in rats. Endocrine 13 (2000): 281-186.

Roesch, D.M. Effects of selective estrogen receptor agonists on food intake and body weight gain in rats. Physiol Behav 87 (2006): 39-44.

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

Russo, A.F., and Dickerson, I.M. CGRP: A Multifunctional Neuropeptide Handbook of Neurochemistry and Molecular Neurobiology. New York: Plenum Press, 2006.

Schaible, H.G., Jarrott, B., Hope, P.J., and Duggan, A.W. Release of immunoreactive substance P in the spinal cord during development of acute arthritis in the knee joint of the cat: a study with antibody microprobes. Brain Res 529 (1990): 214–223.

Schorscher-Petcu, A., Austin, J.S., Mogil, J.S., and Quirion, R. Role of central calcitonin gene-related peptide (CGRP) in locomotor and anxiety- and depression-like behaviors in two mouse strains exhibiting a CGRP-dependent difference in thermal pain sensitivity. J Mol Neurosci 39 (2009): 125-136.

Scully, K.M., Gleiberman, A.S., Lindzey, J., Lubahn, D.B., Korach, K.S., and Rosenfeld, M.G. Role of estrogen receptor-alpha in the anterior pituitary gland. Mol Endocrinol 11 (1997): 674-681.

Setchell, K.D., and others. Comparing the pharmacokinetics of daidzein and genistein with the use of 13C-labeled tracers in premenopausal women. Am J Clin Nutr. 77 (2003): 411-419.

Shen, Z.J., and others. Preliminary study on androgen dependence of calcitonin gene-related peptide in rat penis. Asian J Androl 7 (2005): 55-59.

Shi, H., and Clegg, D.J. Sex differences in the regulation of bodyweight. Physiol Behav 97 (2009): 199-204.

Shimozawa, N., and others. Contribution of sensory neurons to sex difference in the development of stress-induced gastric mucosal injury in mice. Gastroenterology 131 (2006): 1826-1834.

Shir, Y., Campbell, J.N., Raja, S.N., and Seltzer, Z. The correlation between dietary soy phytoestrogens and neuropathic pain behavior in rats after partial denervation. Anesth Analg 94 (2002): 421-426.

Shutt, D.A., and Cox, R.I. Steroid and phytoestrogen binding to sheep uterine receptor in vitro. Endocrinology 52 (1972): 299-310.

Simpson, E., and Davis, S.R. Another role highlighted for estrogen in the male: sexual behavior. Proc Natl Acad Sci USA 97 (2000): 14737-14741.

Sun, R.Q., and others. Calcitonin gene-related peptide receptor activation produces PKA- and PKC-dependent mechanical hyperalgesia and central sensitization. J Neurophysiol 92 (2004): 2859–2866.

Suzuki, N., Hardebo, J.E., and Owman, C. Trigeminal fibre collaterals storing substance P and calcitonin gene-related peptide associate with ganglion cells containing choline acetyltransferase and vasoactive intestinal polypeptide in the sphenopalatine ganglion of the rat. An axon reflex modulating parasympathetic ganglionic activity? Neuroscience 30 (1989): 595-604.

Taleghany , N., Sarajari, S., DonCarlos, L.L., Gollapudi, L., and Oblinger, M.M. Differential expression of estrogen receptor alpha and beta in rat dorsal root ganglion neurons. J Neurosci Res 57 (1999): 603-615.

Tanaka, M., and others. Effects of estrogen on serum leptin levels and leptin mRNA expression in adipose tissue in rats. Horm Res 56 (2001): 98–104.

Toran-Allerand, C.D. Estrogen and the brain: beyond ER-alpha and ER-beta. Exp Gerontol 39 (2004):1579-1586.

Tou, J.C., Chen, J., and Thompson, L.U. Dose, timing, and duration of flaxseed exposure affect reproductive indices and sex hormone levels in rats. J Toxicol Environ Health A (1999): 555-570.

Wang, K., Li, P., Dong, Y., Cai, X., Hou, D., Guo, J., and others. A microarray-based approach identifies ADP ribosylation factor-like protein 2 as a target of microRNA-16. J Biol Chem 286 (2001): 9468-9476.

Weber, K.S., Setchell, K.D., Stocco, D.M., Lephart, E.D. Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5alpha-reductase or testicular steroidogenic acute regulatory peptide levels in adult male Sprague-Dawley rats. J Endocrinol 170 (2001): 591-599.

Wright, L.L., and Smolen, A.J. Neonatal testosterone treatment increases neuron and synapse numbers in male rat superior cervical ganglion. Brain Res 284 (1983): 145-153.

Xuefeng Yin1, Yin Li1, Geyang Xu1, Wenjiao An1, and Weizhen Zhang1,2 Ghrelin fluctuation, what determines its production? Acta Biochim Biophys Sin (2009): 188–197

Yang, Y., Ozawa, H., Lu, H., and Kawata, M. The effects of sex hormones on CGRP-immunoreactive neurons in dorsal root ganglion. Neurosci Res 25 (1996): 78-78.

Yang, Y., and others. Immunocytochemical analysis of sex differences in calcitonin gene-related peptide in the rat dorsal root ganglion, with special reference to estrogen and its receptor. Brain Res 791 (1998): 35–42.

Yildiz, F. Phytoestrogens in Functional Foods. Boca Raton: CRC Press, 2006.

Yin, X., Li, Y., Xu, G., An, W., and Zhang, W. Ghrelin fluctuation, what determines its production? Acta Biochim Biophys Sin 41 (2009): 188–197.

Young, B., Coolen, L., and McKenna, K. Neural regulation of ejaculation. J Sex Med 6 (2009): 229-233.

Yu, L.C., Hansson, P., and Lundeberg, T. The calcitonin gene-related peptide antagonist CGRP8-37 increases the latency to withdrawal responses in rats. Brain Res 653 (1994): 223–230.

Yu, T.M., Yao, G., Man, Y.H., and Mao, X.J. Quantification of substance P mRNA expression in the midbrain of ovariectomized migraine rats with SYBR green I real-time polymerase chain reaction. Afr J Biotechnol 9 (2010): 5481-5487.

Zhang, D., and Trudeau, V.L. Comparative biochemistry and physiology integration of membrane and nuclear estrogen receptor signaling. Comp Biochem Physiol A Mol Integr Physiol 144 (2006): 306–315.

BIOGRAPHY

NAME	Miss Sushawadee Tongta
DATE OF BIRTH	12 December 1985
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTION ATTENDED	Chulalongkorn University 2004-2008
	Bachelor of Science (Biology)
RESEARCH GRANT	National Research Council of Thailand

PUBLICATIONS

1. **Sushawadee Tongta**, Chatsri deachapunya, Sarinee Kalandakanond-Thongsong, Sutthasinee Poonyachoti. 2011. Modulation of Neuropeptide mRNA Expression in Male Rat Sensory Neurons of Dorsal Root Ganglia by Chronic Treatment of Soybean Phytoestrogen. การประชุมวิชาการ ประจำปี 2554 คณะแพทยศาสตร์ มศว เรื่อง "Quality of Care and Patient Safety" วันที่ 10-11 พฤศจิกายน 2554 ณ ตึกคณะแพทยศาสตร์ องครักษ์ จังหวัดนครนายก.

2. **Sushawadee Tongta**, Chatsri deachapunya, Sarinee Kalandakanond-Thongsong, Sutthasinee Poonyachoti. 2012. Effects of estrogen and low-dose phytoestrogen treatment on the calcitonin gene-related peptide and substance P-expressed neurons in the dorsal root ganglia of male rats. In proceeding of The 1st ASEAN Plus Three Graduate Research Congress (AGRC 2012) - March 1-2, 2012 at The Empress Convention Center, The Empress Hotel Chiangmai,Chiang Mai, Thailand.

AWARD

Excellent Poster Presentation at The 1st ASEAN Plus Three Graduate Research Congress (AGRC 2012) - March 1-2, 2012 at The Empress Convention Center, The Empress Hotel Chiangmai,Chiang Mai, Thailand.