NEUROENDOCRINE REGULATION OF REPRODUCTIVE SYSTEM IN THE MALE NATIVE THAI CHICKEN

Boonyarit Kamkrathok

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เป็นที่ทราบกันดีว่า ระบบประสาทและต่อมไร้ท่อควบคุมวงจรการสืบพันธุ์และพฤติกรรมความเป็นแม่ในไก่พื้นเมืองไทยเพศเมียโดยเกี่ยวข้องกับโกนาโดโทรปินรีลิสซิงฮอร์โมน วาโซแอคทีฟอินเทสทินอลเปปไทด์ โดปามีน และมีโซโทซิน ไก่พื้นเมืองไทยเพศผู้มีการแสดงออกของพฤติกรรมการเลี้ยงลูกเช่นเดียวกันกับไก่เพศเมีย แต่ยังไม่มีข้อมูลมากพอที่อธิบายเกี่ยวกับการควบคุมโดยระบบประสาทและต่อมไร้ท่อในเพศผู้ วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาการกระจายตัวของเซลล์ประสาทที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์ ไทโรซีนไฮดรอกซีเลส (ตัวบ่งชี้ถึงเซลล์ประสาทที่ผลิตโดปามีน) และมีโซโทซินในสมองของไก่พื้นเมืองไทยเพศผู้ การกระจายตัวของเซลล์ประสาทและไฟเบอร์ที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์ ไทโรซีนไฮดรอกซีเลส และมีโซโทซินถูกศึกษาโดยใช้เทคนิคอิมมูโนฮิสโตเคมิสทรี ระดับของฮอร์โมนโปรแลคตินและเทสทอสเทอโรนในพลาสมาถูกวัดโดยใช้เทคนิคเอนไซม์ลิงค์อิมมูโนซอร์เบนท์แอสเสย์ ผลการศึกษาพบการกระจายตัวของเซลล์ประสาทที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์เฉพาะในบริเวณนิวเคลียสอินเฟอริโอริสไฮโปทาลาไมและนิวเคลียสอินฟันดิบูไลไฮโปทาลาไม พบการเปลี่ยนแปลงของจำนวนเซลล์ประสาทที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์ ระดับของฮอร์โมนโปรแลคตินและเทสทอสเทอโรนมีระดับต่ำในไก่ก่อนวัยเจริญพันธุ์และในไก่หลังวัยเจริญพันธุ์และมีระดับสูงขึ้นอย่างชัดเจนในไก่วัยเจริญพันธุ์ การกระจายตัวของเซลล์ประสาทที่ผลิตมีโซโทซินและไฟเบอร์ของมีโซโทซินพบการกระจายตัวทั่วไปในบริเวณสมองโดยพบมากที่สุดในบริเวณไดเอนเซฟาลอน เซลล์ประสาทที่ผลิตมีโซโทซินและไฟเบอร์ของมีโซโทซินพบมากที่สุดในบริเวณนิวเคลียสซุพราออพติคัสพาร์สเวนทราลิส นิวเคลียสพรีออพติคัสมีดิเอียลิส นิวเคลียสเวนโทรลาเทอราลิชทาลาไม นิวเคลียสพาราเวนทริคูลาลิสแมกโนเซลลูลาริส และรีจีโอเลเทอราลิสไฮโปทาลาไม นอกจากนี้ยังพบว่าจำนวนเซลล์ประสาทที่ผลิตมีโซโทซินในบริเวณนิวเคลียสซุพราออพติคัสพาร์สเวนทราลิส และนิวเคลียสพรีออพติคัสมีดิเอียลิสมีจำนวนมากกว่าเมื่อเปรียบเทียบกับบริเวณนิวเคลียสเวนโทรลาเทอราลิชทาลาไม นิวเคลียสพาราเวนทริคูลาลิสแมกโนเซลลูลาริส และรีจีโอเลเทอราลิสไฮโปทาลาไม การกระจายตัวของเซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซีเลสและไฟเบอร์ของไทโรซีนไฮดรอกซีเลสพบกระจายตัวทั่วไปในบริเวณสมองโดยพบมากที่สุดในบริเวณไดเอนเซฟาลอนและมีเซนเซปฟาลอน โดยพบการกระจายตัวของเซลล์ประสาทที่ผลิตไทโร

ซีนไฮดรอกซีเลสและไฟเบอร์ของไทโรซีนไฮดรอกซีเลสมากที่สุดในบริเวณนิวเคลียสอินทราเมดิเอลิส และนิวเคลียสแมมิลลาริสแลเทอราลิส เมื่อเปรียบเทียบจำนวนของเซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซีเลสในบริเวณนิวเคลียสแอนทีเรียมีเดียลิสไฮโปทาลาไม นิวเคลียสพาราเวนทริคูลาลิสแมกโนเซลลูลาริส นิวเคลียสอินทราเมดิเอลิส และนิวเคลียสแมมิลลาริสแลเทอราลิส พบว่าเซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซีเลสในบริเวณนิวเคลียสอินทราเมดิเอลิส และนิวเคลียสแมมิลลาริสแลเทอราลิสมีจำนวนมากกว่าเมื่อเปรียบเทียบกับบริเวณแอนทีเรียมีเดียลิสไฮโปทาลาไม และนิวเคลียสพาราเวนทริคูลาลิสแมกโนเซลลูลาริส ผลการศึกษานี้แสดงให้เห็นว่าการทำงานเซลล์ประสาทที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์ มีโซโทซิน และโดปามีนมีส่วนเกี่ยวข้องกับระบบสืบพันธุ์ในไก่พื้นเมืองไทยเพศผู้

สาขาวิชาชีววิทยา
ปีการศึกษา 2562

ลายมือชื่อนักศึกษา ____________
ลายมือชื่ออาจารย์ที่ปรึกษา ____________
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม ____________

BOONYARIT KAMKRATHOK : NEUROENDOCRINE REGULATION OF REPRODUCTIVE SYSTEM IN THE MALE NATIVE THAI CHICKEN. THESIS ADVISOR : PROF. YUPAPORN CHAISEHA, Ph.D. 215 PP.

BRAIN/DOPAMINE/HYPOTHALAMUS/MALE/MESOTOCIN/NATIVE THAI CHICKEN/PROLACTIN/VASOACTIVE INTESTINAL PEPTIDE

It is well established that the neuroendocrine regulation of the reproductive cycle and maternal behaviors in the female native Thai chickens is associated with gonadotropin releasing hormone, vasoactive intestinal peptide (VIP), dopamine (DA), and mesotocin (MT). Like females, male native Thai chickens also exhibit parental care behaviors. However, there are no data describing the neuroendocrine regulation of paternal behaviors in males. The aims of this study were to elucidate the distributions of VIP-, tyrosine hydroxylase- (TH; as a DA marker), and MT-immunoreactive (-ir) neurons in the brain of male native Thai chickens. The distributions of VIP-ir, TH-ir, and MT-ir neurons and fibers were studied utilizing the immunohistochemistry technique. Plasma prolactin (PRL) levels and testosterone (T) were determined by an enzyme-linked immunosorbent assay. The results revealed that the highest accumulations of VIP-ir neurons were concentrated only within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN). Numbers of VIP-ir neurons and PRL and T levels were low in premature and postmature males and markedly increased in mature males. MT-ir neurons and fibers were distributed throughout the brain and extensively in the diencephalon. MT-ir neurons and fibers were predominantly located within the nucleus supraopticus, pars

ventralis (SOv), nucleus preopticus medialis (POM), nucleus ventrolateralis thalami (VLT), nucleus paraventricularis magnocellularis (PVN), and regio lateralis hypothalami (LHy). In addition, the numbers of MT-ir neurons within the SOv and POM were significantly higher than those of the VLT, PVN, and LHy. TH-ir neurons and fibers were located throughout the brain and extensively in the diencephalon and mesencephalon. The highest density of TH-ir neurons and fibers was found within the nucleus intramedialis (nI) and nucleus mamillaris lateralis (ML). The numbers of TH-ir neurons within the nucleus anterior medialis hypothalami (AM), PVN, nI, and ML were then compared and revealed that the numbers of TH-ir neurons within the nI and ML were significantly higher than those of the AM and PVN. These findings indicate that the VIPergic, MTergic, and DAergic systems might be associated with the reproductive activities in the male of this species.

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LIST OF ABBREVIATIONS

AADC	=	1-aromatic amino acid decarboxylase
AM	=	Nucleus anterior medialis hypothalami
AVP	=	Arginine vasopressin
AVT	=	Arginine vasotocin
BCA	=	Brachium conjunctivum ascendens
BCD	=	Brachium conjunctivum descendens
BST	=	Bed nucleus of the stria terminalis
CA	=	Catecholamines
cAMP	=	3'-5'-cyclic adenosine monophosphate
Cb	=	Cerebellum
DA	=	Dopamine
DBH	=	Dopamine beta-hydroxylase
DLAmc	=	Nucleus dorsolateralis anterior thalami, pars magnocellularis
E	=	Epinephrine
ELISA	=	Enzyme-linked immunosorbent assay
FSH	=	Follicle stimulating hormone
GCt	=	Substantia grisea centralis
GH	=	Growth hormone
GnRH	=	Gonadotropin releasing hormone
HL	=	Nucleus habenularis lateralis

LIST OF ABBREVIATIONS (Continued)

HPG	=	Hypothalamo-pituitary-gonadal axis
ICV	=	Intracerebroventricular
IF	=	Tractus infundibularis
IH	=	Nucleus inferioris hypothalami
IHC	=	Immunohistochemistry
IN	=	Nucleus infundibuli hypothalami
INC	=	Incubating hen
INF	=	Infundibular nuclear complex
-ir	=	-Immunoreactive
Jak2	=	Janus kinase 2
kDa	=	Kilodalton
L-DOPA	=	3,4-dihydroxyphenylalanine
LH	=	Luteinizing hormone
LHy	=	Regio lateralis hypothalami
LoC	=	locus ceruleus
ME	=	Eminentia mediana (median eminence)
ML	=	Nucleus mamillaris lateralis
MM	=	Nucleus mamillaris medialis
MPOA	=	Area praeoptica medialis
MT	=	Mesotocin
nCPa	=	Nucleus commissurae pallii
NE	=	Norepinephrine

LIST OF ABBREVIATIONS (Continued)

nI	=	Nucleus intramedialis
NR	=	Non-rearing hens
nVm	=	Nucleus mesencephalicus nervi trigemini
OM	=	Tractus occipitomesencephalicus
OT	=	Oxytocin
OVLT	=	Organum vasculosum lamina terminalis
PACAP	=	Adenylate cyclase activating peptide
PBS	=	Phosphate buffered saline
Pit-1	=	Pituitary-specific transcription factor 1
PL	=	Placental lactogen
PMM	=	Nucleus premamillaris
POA	=	Preoptic area
POM	=	Nucleus preopticus medialis
POP	=	Nucleus preopticus periventricularis
PRF	=	Prolactin-releasing factor
PRL	=	Prolactin
PRLR	=	Prolactin receptor
PHI	=	Peptide histidine isoleucine
PHM	=	Peptide histidine methionine
PHN	=	Nucleus periventricularis hypothalami
PVN	=	Nucleus paraventricularis magnocellularis
PVO	=	Organum paraventriculare

LIST OF ABBREVIATIONS (Continued)

QF	=	Tractus quintofrontalis
R	=	Rearing hens
REC	=	Replaced-eggs-with-chicks
ROT	=	Nucleus rotundus
SCNm	=	Nucleus suprachiasmaticus
SL	=	Nucleus septalis lateralis
SM	=	Nucleus septalis medialis
SON	=	Nucleus supraopticus
SOv	=	Nucleus supraopticus, pars ventralis
SSO	=	Organum subseptale (supseptal organ)
Stat	=	Signal transducers and activators of transcription
T	=	Testosterone
TH	=	Tyrosine hydroxylase
TPc	=	nucleus tegmenti pedunculo-pontinus, pars compacta
TSM	=	Tractus septomesencephalicus
V III	=	Ventriculus tertius (third ventricle)
VIP	=	Vasoactive intestinal peptide
VLT	=	Nucleus ventrolateralis thalami
VMN	=	Nucleus ventromedialis hypothalami
VP	=	Vasopressin
VT	=	Vasotocin

CHAPTER I

INTRODUCTION

1.1 Rational of the Study

Native Thai chicken (*Gallus domesticus*) belongs to genus Gallus of the family Phasianidae. It originated from the wild jungle fowl, which is still found wildly distributed throughout in Southeast Asia and was domesticated by village people approximately 3,000 years ago. Some inherited characteristics from the wild jungle fowl such as maternal behaviors (incubation and rearing (brooding) behaviors) of the native Thai chicken are still highly expressed. In Thailand, historically, the native Thai chickens have long been in the countryside, and the main objectives of raising them are for consumption, sport competition, and recreation. Indeed, it is not only a main protein food source for families, but it can be sold for supplemental income as well. To date in Thailand, there are about 89 million native Thai chickens, which are raised by 2.4 million farmers, gaining income of about 2.2 million baht per year. They are easy to raise, resistant to diseases, and acclimatized to the local environments. It can be raised with lower production costs by raising them as free range using organic local feed. Its meat is firm in texture and contains high protein as well as low fat and cholesterol contents, resulting in high demand by consumers who prefer low fat white meat. Thus, the high price of its meat has been recognized, and its popularity is rapidly growing, providing a good opportunity for producing them in industrial scale. Moreover, Thai government policies are to encourage the development and the use of

natural resources in supporting of His Majesty the King Bhumibol Adulyadej's concept for self-sufficiency in agriculture. Regarding to this concept, the farmers focus on mixed farming which is the strategy for helping rural farmers to increase self-sufficiency. One of the important natural resources that need to be developed is the native Thai chicken. However, the native Thai chickens suffer from their low productivity. One of the main causes of this low reproductive performance is the incidence of maternal behaviors such as incubation and rearing behaviors, which are heritable traits. The onset of incubation and rearing behaviors affect the number of eggs produced because it terminates egg laying and the hen spends much time rearing chicks.

In the female native Thai chickens, the reproductive cycle is divided into four reproductive stages; non-egg laying, egg laying, incubating eggs, and rearing chicks. Generally, the native Thai hen lays eggs 3-4 times per year and 4-17 eggs per clutch, producing about 25-40 chicks per year, which is significantly lower than that of the imported hen which produces eggs all year long (240-270 eggs per year). Presently, market demands of the native Thai chickens cannot be met by suppliers, mainly because of their low egg laying performance as they tend to lay eggs in clutches rather than evenly distributed over the year, leading to production of chicks irregularly. These cause a problem for producing them commercially in the poultry industry in Thailand.

The reproductive stage of male native Thai chickens is divided into three stages: immature (6 months of age), mature (12 months of age), and aging (36 months of age). The male has higher body weight when compared with the female, which is a good reason to raising them for consumption in industrial scale. Therefore, improving

the efficiency of the male native Thai chicken production would benefit to the poultry industry in Thailand. However, in order to increase their production, it is deemed important to understand the basic neuroendocrinology influencing their reproductive activities. At present, there are only a limited number of researchers studying the neuroendocrine regulation of reproduction of the male native Thai chicken.

It is very well documented that avian reproductive cycle is regulated by the integration of the hypothalamus, the pituitary, and the gonads (testis and ovary). This system is referred to as the hypothalamo-pituitary-gonadal axis (HPG). There are two major neuroendocrine systems play an important role in the avian reproduction. These systems include the gonadotropin releasing hormone (GnRH) and the subsequent secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH; GnRH/FSH-LH system), and another system involves vasoactive intestinal peptide (VIP) and the subsequent secretion of prolactin (PRL; VIP/PRL system). The GnRH/FSH-LH system regulates the egg laying period and parental care behaviors. GnRH is synthesized from the hypothalamus and released to stimulate the synthesis and secretion of the gonadotropins (FSH and LH). FSH and LH are then transported to the gonads, causing the release of testosterone (T) from the testis or estrogens from the ovary. The VIP/PRL system initiates and maintains parental behaviors and influences the onset of gonadal regression. Both systems are governed by the dopaminergic (DA) system. Recently, mesotocin (MT) has been implicated to associate with the reproductive stages and maternal care behaviors in the female native Thai chickens.

It is well documented that hormones, neurohormones, neuromodulators, and neurotransmitters play a significant role in the maternal behaviors of avian species. In

addition, male birds also exhibit parental behaviors such as nest building, brooding, and feeding of the young in many species. PRL, an anterior pituitary hormone, has been shown to be associated with the reproductive cycle in several avian species such as turkeys, quails, bantams, ring doves, pigeons, mallard ducks, Florida scrub-jays, zebra finches, Eurasian hoopoes, red-eyed vireos, and native Thai chickens. PRL is best known for its role in the onset and maintenance of maternal behaviors in birds. PRL plays an important role in incubation behavior, crop milk production and secretion, feeding of the young, and nest defense. Circulating PRL levels are low in non-laying hens, and then increase gradually when the hens enter the laying period. Prior to incubation period, PRL levels increase markedly and are maintained at high levels during incubation. The levels of plasma PRL then decline sharply to the same level of non-laying immediately after hatching in several gallinaceous birds. PRL has been well known to play a significant role in parental behaviors in mammalian and avian species. In addition, PRL, an incubation promoting hormone, has also been implicated to be involved as a crucial factor to the onset and maintenance of the rearing behavior in birds. Elevated PRL level is associated with the transition from sexual to parental activities. Circulating PRL levels increase in both male and female breeders during various stages of nest building, egg laying, incubating, and feeding of the young. PRL reduces courtship behaviors and facilitates male care of eggs and it is necessary for maintenance of the brooding pouch and normal embryo development. In the native Thai hens, rearing behavior is maintained by high levels of PRL and upon hatching. It is very well documented that PRL is under stimulatory control by hypothalamic VIP, the avian PRL-releasing factor (PRF) and DA plays an intermediary role in PRL secretion.

Vasoactive intestinal peptide, an octacosapeptide, is extensively distributed in the central and peripheral nervous systems, with high concentrations found in the hypothalamus and functions as a neurotransmitter and neuroendocrine substance. VIP neurons in the hypothalamus whose axons project to the eminentia mediana (median eminence; ME) and a high concentration of VIP in the hypophysial portal blood, leading to the involvement of VIP in the regulation of anterior pituitary functions. In mammals, the physiological functions of VIP have been reported such as vasodilation, smooth muscle relaxation, reproduction, immunomodulation, PRL secretion, and increased of gastric motility. In birds, VIP acts directly on the anterior pituitary gland to stimulate PRL synthesis and secretion during the reproductive cycle. The distributions of VIP neurons and fibers have been mapped in many avian species such as Pekin ducks, bantams, pigeons, ring doves, dark-eyed juncos, chickens, turkeys, Japanese quails, collared doves, starlings, zebra finches, blue tits, and native Thai chickens. In the female native Thai chickens, VIP-immunoreactive (-ir) neurons and fibers are extensively distributed throughout the brain and are predominantly expressed in the diencephalon, where VIP-ir neurons are highly concentrated within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) areas. Changes in the number of VIP-ir neurons within the IH-IN are directly correlated to plasma PRL levels across the reproductive cycle, and the number of VIP-ir neurons within the IH-IN decreases concurrently with circulating PRL levels in nest-deprived incubating and disruption of rearing behavior hens. Moreover, the number of VIP-ir neurons in the IH-IN is markedly decreased in the replaced-egg-with chicks hens when compared with the incubating hens. These findings suggest that the VIPergic system in the IH-IN plays a regulatory role in year-round

reproductive activities and indicate its importance in the regulation of maternal behaviors in this equatorial species.

Dopamine is a neurotransmitter/neuromodulator found in both central and peripheral nervous systems in many species and has several important physiological functions involved in a wide variety of behaviors and reproduction. In mammals, the regulation of PRL secretion is under the inhibitory control of hypothalamic tuberoinfundibular DAergic neurons, which release DA that acts directly upon D_2 DA receptors located on pituitary lactotrophs. Removal of this inhibition results in an increased PRL release and hyperprolactinemia. However, this is not the case in birds, in which removal of this DAergic input results in the complete cessation of PRL secretion. As aforementioned, PRL secretion is tonically stimulated by VIP; the avian PRF. It is very well established that DA plays an intermediary role in PRL secretion, requiring an intact VIPergic system in order to cause the release of PRL. Indeed, dynorphin, serotonin, DA, and VIP all appear to stimulate avian PRL secretion along a common pathway expressing κ opioid, serotonergic, DAergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator. It has been established that D_1 DA receptors stimulate PRL secretion at the hypothalamic level by operating through VIP. DA also inhibits PRL secretion at the pituitary level by activating via D_2 DA receptors. To date, there are numerous studies regarding the physiological roles of DA and its receptors in birds such as food intake, food reward, cognitive performance, feather pecking, singing behavior, song learning, social activity, aggressive behavior, mate competition, courtship motivation, egg production, incubation, reproductive cycle, and male sexual behavior. Moreover, DA is involved in the regulation of the

reproductive cycle and maternal behaviors (incubating and rearing behaviors) in the female native Thai chicken, a non-seasonally breeding species. Numerous studies have been reported that DA neurons and fibers are distributed in several regions of the brain in both birds and mammals. The anatomical distributions of DA-containing neurons and fibers have been mapped in many avian species such as domestic fowls, Japanese quails, pigeons, zebra finches, chickens, budgerigars, collared doves, turkeys, canaries, and female native Thai chickens. In the female native Thai chickens, tyrosine hydroxylase (TH; a DA marker)-ir neurons and fibers are widely distributed throughout the brain, especially in the diencephalic and mesencephalic regions with the highest densities are found within the nucleus intramedialis (nI) and nucleus mamillaris lateralis (ML). Changes in the number of TH-ir neurons in the nI are associated with the reproductive stages of the female native Thai chickens. The number of TH-ir neurons in the nI is lowest in non-egg laying stage, then markedly increases in the egg laying, and reaches the highest density in incubating eggs, and decreases in the rearing chicks hens. Disruption of incubation behavior by nest-deprivation decreases the numbers of TH-ir neurons within the nI and ML. In addition, the number of TH-ir neurons in the nI decreases in non-rearing hens when compared with rearing hens. Recently, it has reported that the presence of eggs or chicks is associated with the decreased numbers of TH-ir neurons within the nI and ML, and the increased numbers of MT-ir neurons within the nucleus supraopticus; pars ventralis (SOv), nucleus preopticus medialis (POM), and nucleus paraventricularis magnocellularis (PVN) during the transition from incubating to rearing behavior. These findings suggest that the initiation and maintenance of

incubation and rearing behaviors is regulated by the DAergic system in the nI which, in turn, stimulate VIP and subsequent PRL release in this species.

Mesotocin, a homolog of oxytocin (OT) in mammals, is a nonapeptide neurohypophysial hormone that is synthesized in specific neuronal groups within the hypothalamus and from nerve terminals of the posterior pituitary gland or into the hypophysial portal blood via the ME in amphibian, reptilian, and avian species. MT in birds and OT in mammals are different at amino acid position 8; MT is isoleucine, but OT is leucine. MT is also distributed within several regions of the avian brain and several tissues of the reproductive tract including the corpus luteum, follicle, and uterus. There are a limited number of studies regarding the role(s) of MT in birds. It has been reported that MT facilitates uterine contractions in hens, acts as a vasodepressor in cockerels, inhibits feeding behavior in chicks, and promotes sociality of female zebra finches and field sparrows. The role of MT in avian brooding behavior was first documented in the turkeys. MT has then reported to be involved in the regulation of the reproductive cycle and maternal behaviors in the female native Thai chickens. The distributions of MT-containing neurons and fibers have been mapped in many avian species such as chickens, domestic mallards, Japanese quails, domestic fowls, White Leghorn cockerels, turkeys, and female native Thai chickens. In the female native Thai chickens, MT-ir neurons and fibers are found distributed throughout the brain. The highest accumulations of MT-ir neurons and fibers are concentrated within the SOv, POM, nucleus ventrolateralis thalami, regio lateralis hypothalami, and PVN. Changes in the numbers of MT-ir neurons within the SOv, POM, and PVN are associated with the reproductive stages of the female native Thai chickens, with the highest density observed in the incubating and rearing hens.

Disruption of incubation behavior by nest deprivation causes the numbers of MT-ir neurons within the SOv, POM, and PVN to decrease. In addition, the numbers of MT-ir neurons within these nuclei of rearing hens are higher than those of non-rearing ones. These findings indicate that the MTergic system in these nuclei plays a regulatory role in reproductive activities and the neuroendocrine reorganization to establish and maintain maternal behaviors in the native Thai chickens.

Testosterone, a steroid hormone, is synthesized and secreted from the testis. It regulated spermatogenesis, spermiogenesis, and also plays a pivotal role in the development of secondary sexual characteristics in both mammalian and avian species. T has been indicated to be associated with the reproductive activities in several avian species such as pied flycatchers, white-crowned sparrows, song sparrows, red-winged blackbirds, yellow-headed blackbirds, dark-eyed juncos, European starlings, house finches, and spotted sandpiper. Hypothalamic GnRH neurons synthesize and release GnRH via the ME to stimulate the synthesis and secretion of FSH and LH from the anterior pituitary gland which, in turn, stimulate the testis to synthesize and release T. Numerous studies have been reported the involvements of T and PRL in paternal cares in many species. The HPG axis leads to elevation of T during breeding in seasonal breeding species. In males, during breeding season, this elevation is important for seasonal development of some secondary sexual characteristics, spermatogenesis, sexual behavior, mate attraction, and territory defense. T secretion follows a seasonal pattern, reaching the lowest levels during non-breeding period and increasing during breeding period for sperm production and reproduction. Moreover, high levels of T are important in inhibiting and maintaining parental behaviors. Therefore, T secretion is thought to be tightly regulated during

breeding period, increasing when aggressive behavior is important for reproduction. Despite these general patterns, the role(s) of T in aggression behavior and parental care varies greatly between species and some species may be behaviorally insensitive to this hormone. Thus, the physiological function(s) of T involving behaviors are needed to further elucidated in birds.

For successful reproduction, the survival of the young until reproduction has marked effects on population growth, and it is more sensitive to environmental changes than the survival of adult. Parental care is an important key to promote the survival and well-being of the offspring. Parental behavior is defined as the behavior of the parents that contributes to the survival of the offspring. Maternal behavior is defined as the collection of behaviors by the mother that can increase offspring survival. Nurturing behaviors analogous to maternal behaviors are called paternal behaviors by fathers/male mating partners and alloparental behavior by older conspecifics. In birds, parental behaviors include nest preparation, egg laying into a preferred site such as a nest, egg incubation, and post hatch care of the young to independence. Maternal behaviors are limited to incubation and brooding or rearing behaviors. Paternal care refers to behaviors performed by the mature male, which have a positive influence on development, growth, well-being, and survival of the offspring. An association of the neuronal interactions between the GnRHergic, VIPergic, DAergic, and MTergic systems in the regulation of parental behaviors has been extensively studied, particularly in females. In many species, male birds display parental behaviors such as nest building, brooding, and feeding of the young. These parental behaviors may happen due to a complex neuronal/hormonal interaction of many hormones, neurohormones, neuromodulators, and neurotransmitters. To date, it has been

reported that the neuronal interactions between PRL, VIP, DA, GnRH, and MT are involved in the regulation of reproductive cycle and maternal behaviors in the female native Thai chickens. However, the roles of T, PRL, VIP, DA, and MT in the regulation of reproductive system and paternal behaviors of the male native Thai chicken have never been studied. Thus, the objectives of this dissertation were carried out to elucidate the neuronal association/interaction of T, PRL, VIP, DA, and MT in the neuroendocrine regulation of reproductive system and paternal behaviors in the male native Thai chickens. The findings gained from this study will help to understand the basic neuroendocrine regulation of the reproductive system and parental behaviors in birds. In addition, the findings of the distributions of VIP, DA, and MT neurons and fibers in the brain might be related to the regulation of reproductive activities and paternal behaviors in the male native Thai chickens. The knowledge gained can be then applied commercially in the poultry industry to increase the production of the native Thai chickens in Thailand.

1.2 Research Objectives

1.2.1 To study the association of T, PRL, and VIP with the reproductive stages of the male native Thai chickens that might be related to the regulation of reproductive activities and paternal behaviors.

1.2.2 To study the localization and differential expression of VIP, DA, and MT neurons that might be associated with the neuroendocrine regulation of reproductive activities and paternal behaviors in the male native Thai chickens.

CHAPTER II

LITERATURE REVIEW

2.1 Native Thai Chicken

Native Thai chickens or Thai indigenous chicken (*Gallus domesticus*) have long been in the countryside of Thailand. The native Thai chicken belongs to genus Gallus of the family Phasianidae. Its ancestor might be the red jungle fowl (*Gallus gallus*), which is widely distributed throughout Southeast Asia (Austic and Nesheim, 1990; Fumihito et al., 1994; Hillel et al., 2003; Sawai et al., 2010; Peters et al., 2016). It was domesticated approximately 3,000 years ago by village people. Historically, the native Thai chickens have been in the countryside of Thailand for many generations. Some inherited characteristics from the wild jungle fowl, such as maternal behaviors (incubation and rearing (brooding)) are still highly expressed in the native Thai chicken (Beissinger et al., 1998; Chaiseha and El Halawani, 2015; Namken et al., 2017; Sinpru et al., 2017; 2018). Raising of the native Thai chickens is widespread throughout Thailand and Southeast Asia, because they are easy to raise, resistant to diseases, and acclimatized to the local environments, especially high temperature. In addition, they can be raised with lower production costs by raising them as free range using organic local feed (Leotarakul and Pimkamlia, 1999). The main objectives of raising them are for consumption, sport competition, and recreation. In Thailand, the native Thai chicken has become a new economic domestic animal with presently

growing demand and relatively high price. The market price of the native Thai chickens is 2-3 times higher than those of the imported broilers. Indeed, it is not only a main protein food source, but it can be also sold for supplemental income for Thai smallholder farmers (Na-Lampang, 2012). Nowadays, there are about 89 million native Thai chickens or 21 % of total chicken production, which consists of broilers (62 %), layers (13 %), commercial broiler breeders (3 %), and commercial layer breeders (1 %). They are raised by 2.4 million farmers in Thailand (Department of Livestock Development, 2017). This exported goods gains income of about 2.2 million baht per year (Department of Livestock Development, 2015). The domestic markets of the native Thai chickens have increased substantially as well as external markets (Huo and Na-Lampang, 2012). The meat of the native Thai chicken is very popular among Thai consumers, because its unique taste and texture resulting in a greater delicacy than that of commercial broilers (Wattanachant et al., 2004, 2005; Puttaraksa et al., 2012). Moreover, its meat is firm in texture and contains high protein as well as low fat and cholesterol contents, resulting in high demand by consumers who prefer low fat, chewy texture, good taste, and white meat (Wattanachant et al., 2004; 2005; Jaturasitha et al., 2008; Wattanachant, 2008). Therefore, the high price of native Thai chickens' meat has been recognized and their popularity is rapidly growing (Teltathum and Mekchay, 2009). The native Thai chicken muscles contain higher protein and collagen contents but lower fat content than those of the broiler muscles (Wattanachant et al., 2004; Wattanachant, 2008). This provides a good opportunity for producing them in industrial scales. Moreover, recent Thai government policies are to encourage the development and the use of natural resources in supporting of His Majesty the King Bhumibol Adulyadej's concept for

self-sufficiency in agriculture. According to this concept, farmers tend to focus on mixed farming that is the strategy for helping rural farmers to increase self-sufficiency. One of the important natural resources that need to be developed is the native Thai chicken. However, the native Thai chickens have low productivity. One of the main causes of this low reproductive performance is the incidence of maternal behaviors such as incubation and rearing behaviors, which are heritable traits (Beissinger et al., 1998). The onset of incubation and rearing behaviors affects the number of eggs produced, because it terminates egg laying and the hen spends much time for rearing chicks (Kosonsiriluk et al., 2008). These behaviors are highly expressed which are certainly not desired for commercial scale production (Choprakarn and Wongpichet, 2007).

In female native Thai chickens, the reproductive cycle is divided into 4 reproductive stages: non-egg laying, egg laying, incubating eggs, and rearing chicks. Generally, the native Thai hen lays eggs 3-4 times per year and 4-17 eggs per clutch, rather than laying eggs continuously all year long. Egg production of the native Thai hen is lower than that of the imported laying hen, with the peak production for native Thai hens and commercial laying hens being 38.0 % and 75.5 %, respectively. The total number of eggs per hen of native Thai hen is between 30-92 eggs/year, which is significantly lower than that of 240-270 eggs per hen per year of the imported hen (Chotesangasa et al., 1994). In general, the native Thai chicken spends about 10-15 weeks for each reproductive cycle; 2 weeks for laying, 3 weeks for hatching, and 6-10 weeks for taking care of the chicks (Katawatin et al., 1997; Choprakarn et al., 1998). Moreover, growth rate of the native Thai chicken is significantly slower than those of the imported breeds. It takes about 4-5 months to reach marketable size with an 80-85 %

carcass (Choprakarn and Wongpichet, 2007). Thus, improving the efficiency of native Thai chicken production would benefit the poultry industry in Thailand.

Like females, male native Thai chickens also exhibit parental care behaviors for their chicks and such behaviors might effect their reproductive activities. Thus, improving the efficiency of production in both male and female native Thai chickens would benefit the poultry industry in Thailand. The reproductive stage of male native Thai chickens is divided into 3 stages: immature (6 months of age), mature (12 months of age), and aging (36 months of age). Normally, the male native Thai chickens are fighting cock. The main objective of raising them is for sport competition, not only for food materials (Phianmongkhol et al., 2012). Moreover, the male has higher body weight when compared with the female (Sawasdee et al., 2015), which is a good reason for raising them for consumption in industrial scale. Therefore, improving the efficiency of the male native Thai chicken production would benefit the poultry industry in Thailand. However, in order to increase their production, it is deemed important to understand the basic neuroendocrinology influencing their reproductive activities.

2.2 Neuroendocrine Regulation of the Avian Reproductive Cycle

The regulation of the avian reproductive system involves the interaction of external stimuli with neuroendocrine mechanisms and is integratively regulated by the hypothalamus, the pituitary, and the gonads (testis and ovary), namely the hypothalamo-pituitary-gonadal (HPG) axis. It is very well documented that neurotransmitters, neuromodulators, neurohormones, and hormones of this HPG axis play a pivotal role in avian reproduction. There are two major neuroendocrine systems

that play important roles in avian reproduction. These systems include the gonadotropin releasing hormone (GnRH) and the subsequent secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH; GnRH/FSH-LH system) and another system that involves vasoactive intestinal peptide (VIP) and the subsequent secretion of prolactin (PRL; VIP/PRL system). The GnRH/FSH-LH system regulates the egg laying period and parental care behaviors. GnRH is synthesized in the hypothalamus and released to stimulate the synthesis and secretion of the gonadotropins (FSH and LH). Gonadotropins are then transported to the gonads, causing the release of testosterone (T) from the testis or estrogens from the ovary and expression of reproductive behaviors (Murton and Westwood, 1977; Deviche et al., 2010). The VIP/PRL system initiates and maintains parental behaviors and influences the onset of gonadal regression. VIP stimulates pituitary lactotrophs to synthesize and secrete PRL. Both systems are governed by the dopaminergic (DAergic) system (Chaiseha and El Halawani, 2015).

2.3 Prolactin: Structure, Functions, and Regulation of Secretion

2.3.1 The Structure of Prolactin

Historically, PRL was discovered (Riddle et al., 1932; 1935), and its name is coined on the findings that an extract of bovine pituitary gland caused the proliferation and growth of crop sac, stimulates the elaboration of crop milk in pigeons, or promotes lactation in rabbits (Riddle et al., 1933; Bern and Nicoll, 1968). PRL is synthesized in and secreted from specialized cells of the anterior pituitary gland, the lactotrophs (Bern and Nicoll, 1968; Velkeniers et al., 1988; Freeman et al., 2000). The molecular weight of the major form of PRL found in the pituitary gland is

about 23 kilodaltons (kDa), encoded by a gene consisting of 5 exons and 4 introns (Maria, 2016). The mature hormone consists of 194-199 amino acids, depending on species (Sinha, 1995), and its structure is stabilized by 3 intramolecular disulfide bonds. The primary structure of PRL was first reported in the ovine (Li et al., 1970), and subsequently the complete amino acid sequences of PRLs of more than 25 species have been documented (Sinha, 1995). A comparison of the amino acid sequence from different species shows varying degrees of sequence homology, reflecting to a great extent order of the phylogenetic relationships. However, some 32 amino acids seem to be conserved among different species (Watahiki et al., 1989). The homology of sequences of PRLs among different species and their primary structures are depicted in Figures 2.1 and 2.2, respectively.

	Human	Baboon	Monkey	Ovine	Bovine	Porcine	Equine	Camel	Elephant	Fin whale	Rat	Mouse	Hamster	Chicken	Turkey	Crocodile	Alligator	Sea turtle	Bullfrog	Lungfish	Sturgeon	Catfish	Carp	Chum salmon	Chinook salmon	Rainbow trout	Tilapia-188	Tilapia-177
Human		97	97	76	76	81	82	81	67	82	64	61	62	72	70	72	73	75	65	58	36	35	36	35	35	35	34	31
Baboon			99	73	73	79	80	80	66	78	61	58	62	70	68	69	70	71	64	54	36	34	34	35	35	35	33	31
Monkey				74	73	79	78	80	66	77	61	56	60	70	67	70	70	72	64	53	37	35	34	35	35	34	34	31
Ovine					99	83	79	80	74	84	61	56	58	69	70	71	71	71	59	53	34	34	35	34	34	34	33	30
Bovine						84	80	80	73	85	62	56	59	70	70	72	71	72	60	54	35	34	35	34	34	34	33	30
Porcine							93	96	76	96	65	61	64	79	79	81	81	80	67	61	35	34	35	34	34	34	33	30
Equine								93	73	91	64	61	63	79	79	81	82	80	69	61	35	35	36	35	35	35	34	30
Camel									72	93	63	61	63	80	78	83	84	84	69	59	37	34	34	35	35	34	33	29
Elephant										76	57	54	57	67	67	66	66	69	57	55	37	36	37	36	36	36	37	31
Fin whale											64	60	61	79	79	80	82	80	66	61	36	35	36	35	35	35	34	31
Rat												85	82	59	60	60	61	60	53	52	30	31	33	31	31	31	31	30
Mouse													72	55	56	56	56	56	48	47	35	32	35	31	31	31	33	31
Hamster														58	58	62	61	60	53	47	36	29	29	29	29	30	28	28
Chicken															93	90	91	89	72	65	31	36	38	35	35	35	35	31
Turkey																89	90	85	71	64	35	35	35	35	35	35	35	30
Crocodile																	99	85	73	66	35	35	35	33	33	33	32	29
Alligator																		86	72	65	34	34	34	34	34	34	31	28
Sea turtle																			74	66	37	36	38	35	35	35	34	31
Bullfrog																				64	40	35	35	35	35	35	34	31
Lungfish																					40	35	37	37	37	37	33	31
Sturgeon																						46	45	46	47	46	43	36
Catfish																							79	68	67	68	64	53
Carp																								73	71	73	65	52
Chum salmon																									97	99	69	56
Chinook salmon																										98	68	56
Rainbow trout																											69	56
Tilapia-188																												69
Tilapia-177																												

Figure 2.1 The percentage of homology sequence of PRLs among different species (Sinha, 1995).

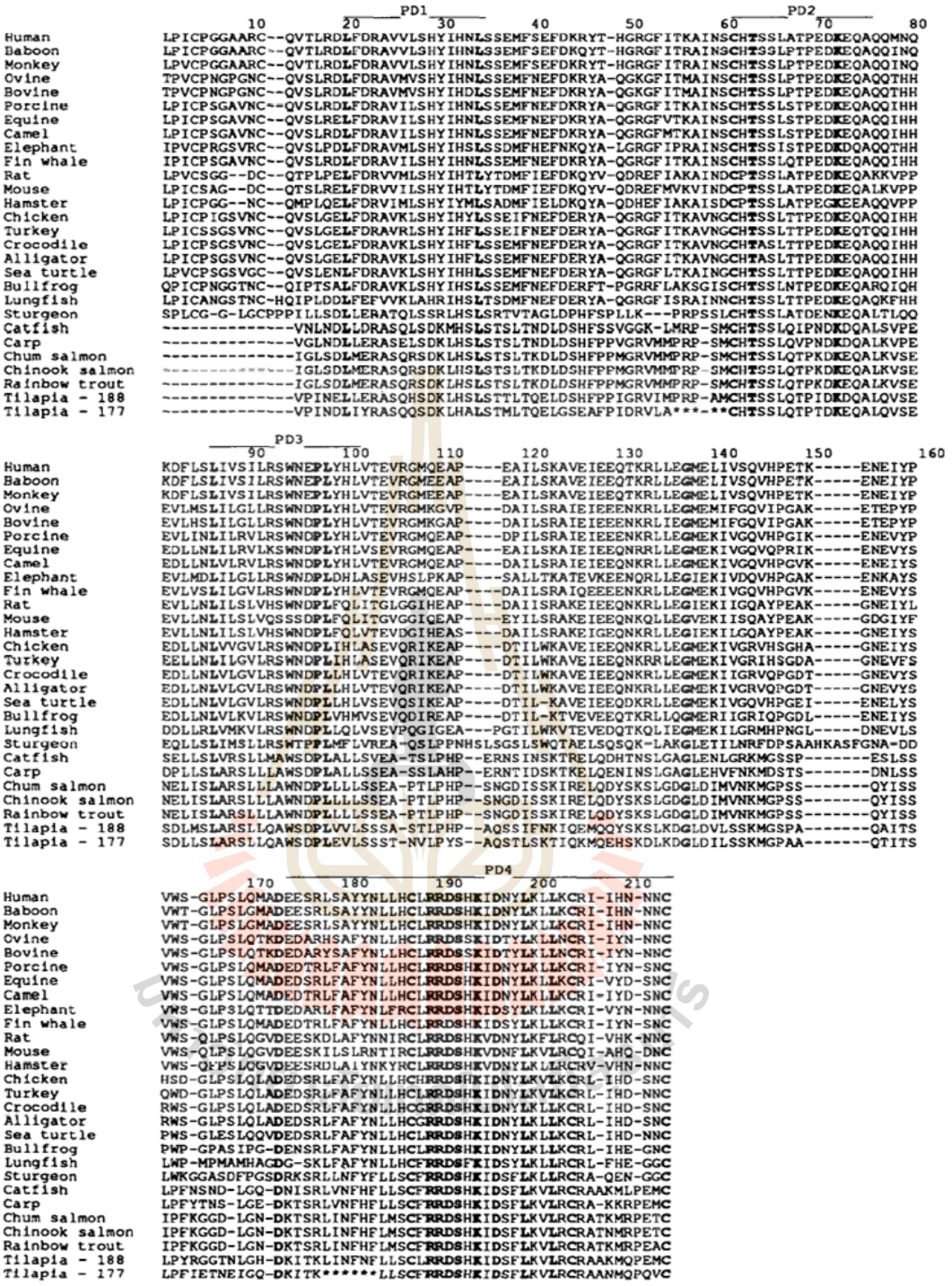

Figure 2.2 Primary structures of PRLs of different species. (-) indicates the positions left blank to optimize alignment of amino acid sequences. (*) indicates the absence of residues from a genetic variant of tilapia PRL. PD is PRL domain. PD1-PD4 indicates the four highly conserved domains of the PRLs (Sinha, 1995).

PRL is synthesized and secreted by a variety of cells, tissues, and organs including the immune cells, mammary epithelium, placenta, deciduas of the pregnant uterus, lacrimal gland, adrenal gland, corpus luteum, prostate gland, testis, pancreas, and brain (Ben-Jonathan et al., 1996; Freeman et al., 2000). To date, more than 500 different physiological functions of PRL have been reported (Houdebine, 1983; Bole-Feysot et al., 1998; Harris et al., 2004) in such areas as reproduction, osmoregulation, growth and development, brain and behavior, endocrinology and metabolism, and immunoregulation as well as behaviors such as migration and the nurturing of the young in different vertebrate species, highlighting the significant role of this pluripotent hormone. It has been further suggested that the physiological functions and biological activities of PRL are, at least in part, regulated by additional posttranslational modifications such as phosphorylation in the various physiological stages (Hiyama et al., 2009).

PRL receptor (PRLR) is a membrane-bound receptor that is part of the class 1 cytokine receptor superfamily that includes the receptors of growth hormone (GH), leptin, erythropoietin, and interleukins (Bazan, 1989; 1990; Kelly et al., 1991). Not only PRL binds to its cognate receptor (PRLR), but placental lactogen (PL) and GH also bind the PRLR. PRL and GH receptors share several structural and functional features despite their low (30 %) sequence homology (Goffin and Kelly, 1996). The PRLR is activated by the binding of a single ligand to the receptor to dimerize two identical receptors, resulting in activation of the Janus kinase 2 (Jak2) associated with the cytoplasmic domain, which then activates a number of signalling pathways through which PRL exerts its physiological effects (Bole-Feysot et al., 1998; Freeman et al., 2000). Subsequently, Jak2 phosphorylates tyrosine residues on different target

proteins, the best identified is named signal transducers and activators of transcription (Stat). The Jak2-Stat cascade is the major signalling pathway of the PRLR, but other signalling pathways are also associated with this receptor as well. Activation of mitogen-activated protein kinase signalling pathway has also been reported in different cellular systems under PRL stimulation (Bole-Feysot et al., 1998). Activation of the nucleotide exchange protein, Vav, has been reported as well (Clevenger et al., 1995).

Various PRLR isoforms have been identified in different cells or tissues in both mammals and birds (Davis and Linzer, 1989; Ali et al., 1991; Lesueur et al., 1991; Pitts et al., 2000). Alternative splicing of the PRLR gene results in the multiple isoforms, which differ in the length and composition of their cytoplasmic tails. These isoforms are referred to the short (291 amino acids; Boutin et al., 1988) and long (591 amino acids; Shirota et al., 1990) PRLR isoforms (Harris et al., 2004). PRLR and its mRNA are found in the mammary gland and ovary, the best characterized sites of PRL physiological actions in mammals (Nagano and Kelly, 1994). cDNAs encoding the PRLR gene have been cloned in chickens (Tanaka et al., 1992), doves (Chen and Horseman, 1994), pigeons (Chen and Horseman, 1994), and turkeys (Zhou et al., 1996; Pitts et al., 2000). A few studies of PRLR distribution have been reported in the avian brain. The distribution of PRLRs is limited to five avian species: pigeons (Muccioli et al., 1988), ring doves (Fechner and Buntin, 1989; Hnasko and Buntin, 1993), Wilson's phalaropes (Buntin et al., 1998), dark-eyed juncos (Deviche and Buntin, 1992), and zebra finches (Smiley, 2019). In birds, PRLR is found in the crop sac (Tanaka et al., 1992; Chen and Horseman, 1994; Zhou et al., 1996), brood patch (Ohkubo et al., 1998), thyroid gland (Ohkubo et al., 1998), liver (Tanaka et al., 1992;

Pitts et al., 2000), kidney (Tanaka et al., 1992; Ohkubo et al., 1998; Pitts et al., 2000; Wang et al., 2009; Xing et al., 2011), leg skin (Ohkubo et al., 1998), large and small intestines (Tanaka et al., 1992; Zhou et al., 1996; Pitts et al., 2000; Xing et al., 2011), adipose tissue (Ohkubo et al., 1998), muscle (Ohkubo et al., 1998), adrenal gland (Ohkubo et al., 1998), thymus (Ohkubo et al., 1998), spleen (Ohkubo et al., 1998; Xing et al., 2011), gizzard (Zhou et al., 1996), brain (Zhou et al., 1996; Ohkubo et al., 1998), pineal gland (Pitts et al., 2000), ovary (Ohkubo et al., 1998; Wang et al., 2009; Xing et al., 2011), testis (Pitts et al., 2000; Wang et al., 2009; Xing et al., 2011), seminal duct (Xing et al., 2011), and oviduct (Tanaka et al., 1992; Pitts et al., 2000; Xing et al., 2011). This wide distribution of PRLR expression reflects the many functional roles proposed for PRL.

2.3.2 The Physiological Functions of Prolactin in Birds

As aforementioned, numerous studies have shown that PRL is an important hormone associated with parental care behaviors and increased reproductive success in avian species (Chastel et al., 2005; Miller et al., 2009; Ouyang et al., 2011; Riechert et al., 2014; Smiley and Adkins-Regan, 2016). Circulating PRL levels increase during incubation period, crop milk secretion, feeding of young, and nest defense in birds (Silver, 1984; Janik and Buntin, 1985; Lea et al., 1986; Buntin et al., 1991; Angelier et al., 2016). The role of PRL in parental care in males has been investigated in several species. PRL has been found to be associated with paternal care behaviors in human (Storey et al., 2000; Fleming et al., 2002), primates (Ziegler et al., 2000; Schradin et al., 2003; Almond et al., 2006; da Silva Mota et al., 2006), rodents (Schradin and Pillay, 2004; Carlson et al., 2006; Schradin, 2008), fish (Kindler et al.,1991; Whittington and Wilson, 2013), and birds (Buntin, 1996; Garcia

et al., 1996; Schoech et al., 1996; 1998; Ziegler, 2000; Van Roo et al., 2003; Vleck and Vleck, 2011). In birds, it has been well documented that PRL is associated with parental behaviors in ring doves (Buntin, 1996; Wang and Buntin, 1999), dark-eyed juncos (Schoech et al., 1998), King penguins (Garcia et al., 1996), scrub-jays (Schoech et al., 1996), songbirds (Van Roo et al., 2003), house finches (Badyaev and Duckworth, 2005), Adelie penguins (Cottin et al., 2014), and zebra finches (Smiley and Adkins-Regan, 2018a, 2018b). Plasma PRL levels are high in breeding birds, and they have been positively correlated with increased parental care behaviors and increased reproductive success (Vleck et al., 1991; Schoech et al., 1996; Khan et al., 2001; Chastel et al., 2005; Miller et al., 2009; Ouyang et al., 2011, Riechert et al., 2014; Smiley and Adkins-Regan, 2016). In contast, low plasma PRL levels are related to poor body condition, nest abandonment, low rates of parental care, and offspring mortality (Angelier and Chastel, 2009; Angelier et al., 2016; Smiley and Adkins-Regan, 2016). Among species in which parental care is provided by the female only, such as pied flycatchers, circulating PRL levels are more elevated in females than in males, whereas in species in which parental care is provided only by the male, such as Wilson's phalaropes and red-necked phalaropes, circulating PRL levels are higher in males than in females during the parental phase of the breeding cycle (Buntin, 1996; 2010; Schradin and Anzenberger, 1999). In addition, a positive correlation between the intensity/quality of parental care and PRL levels has been observed in many parental birds and cooperative breeders. In the cooperatively breeding species, Florida scrub-jays and Harris' hawks, PRL levels in helpers are positively correlated with the rate of nestling provisioning (Dawson and Mannan, 1991; Schoech et al., 1996; Angelier and Chastel, 2009). In male ring doves, rising and lowering PRL levels have

demonstrated that PRL plays a direct role in stimulating incubation behavior, the formation of crop milk, and the frequency of chick feeding behavior (Lea and Sharp, 1989; Koch et al., 2004). PRL levels elevate in both male and female breeders during various stages of nest building, egg laying, incubating, and feeding of young birds (Ziegler, 2000).

2.3.3 The Regulation of Prolactin Secretion in Birds

The control of PRL secretion involves the interaction of external stimuli with neuroendocrine mechanisms (Curlewis, 1992). It is very well established that the regulation of avian PRL secretion as well as its gene expression is influenced by hypothalamic VIP, the avian PRL-releasing factor (PRF; Chaiseha and El Halawani, 2015). VIP is released from neurons in the infundibular nuclear complex (INF) of the caudo-medial hypothalamus (Sharp et al., 1989; Talbot et al., 1991; El Halawani et al., 1997; 2001; Chaiseha et al., 1998; Chaiseha and El Halawani, 2015). VIP meets the classical criteria for defining it as the hypophysiotrophic PRF in birds (El Halawani et al., 1997). In contrast with mammals, it has been established that DAergic influences are involved in both stimulating and inhibiting avian PRL secretion depending on multiple subtypes of DA receptors (Youngren et al., 1995; 1996b; Chaiseha and El Halawani, 2015). It is very well documented that DA plays an intermediary role in PRL secretion, requiring an intact VIPergic system in order to cause the release of PRL. Dynorphin, serotonin, DA, and VIP all appear to stimulate avian PRL secretion along a pathway expressing κ opioid, serotonergic, DAergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator (El Halawani et al., 2001).

2.4 Testosterone: Structure, Functions, and Regulation of Secretion

2.4.1 The Structure of Testosterone

In 1849, Arnold Berthold conducted a series of experiments on roosters to link the physiological and behavioral changes of castration to a substance secreted by the testes (Berthold, 1849). Berthold was curator of the local zoo and concluded that the castrated roosters lose male aggressive behavior and lose interest in hens following castration. More importantly, by ablation and replacement experiments, he also found that these castration-induced changes could be reversed by administration of a crude testicular extract (or prevented by transplantation of the testes; Figure 2.3). Berthold concluded that the testes must affect behavioral and sexual characteristics by secreting a substance into the bloodstream. Thus, almost a century before gonadal hormones were isolated, it was shown that substances secreted by the testes 'androgens' are responsible for the activation of male sexual behaviors.

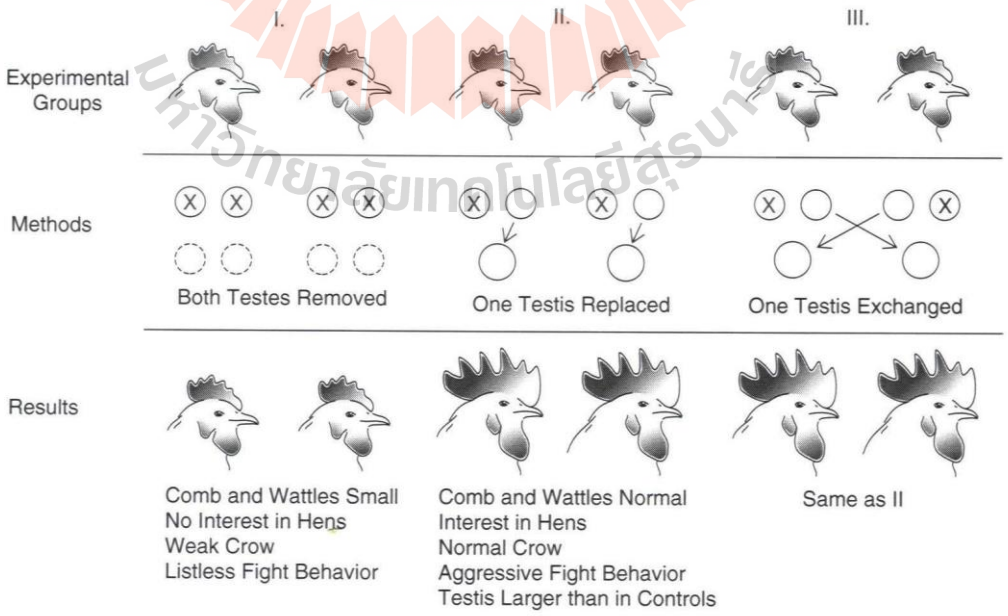

Figure 2.3 The first endocrine experiment by Berthold (1849).

Eventually, testosterone, the hormone of the testes, was isolated in 1935. The paper was published about the male hormone from testicles, and its name reflects its source and structure (testo = testes, ster = sterol, one = ketone; David et al., 1935). In 1940, there was convincing evidence that male sexual behavior in birds is facilitated by androgens secreted by the testes (Beach, 1948; Collias, 1950). It has been reported that the correlation between testicular cycles and seasonal variations in courtship behavior, and androgen dependence of distinctive male behavior had been demonstrated in Galliformes, Columbiformes, Charadriiformes, Ciconiformes, Anseriformes, and Passeriformes (Beach, 1948; Collias, 1950). T is synthesized and secreted by Leydig cells in the testis (Freeman et al., 2001; Fusani, 2008). A series of enzymes convert the side chain at C-17 of the cholesterol precursor to a hydroxyl group, transfer a double bond from C-6 to C-4 and oxidize the hydroxyl group at C-3 to a carbonyl group (Figure 2.4). Secretion is controlled by a negative feedback mechanism involving LH and FSH, tropic hormones synthesized by the anterior pituitary gland. T stimulates the synthesis of specific proteins by crossing the cell membrane and binding with a receptor in the nucleus, activating particular genes (Freeman et al., 2001).

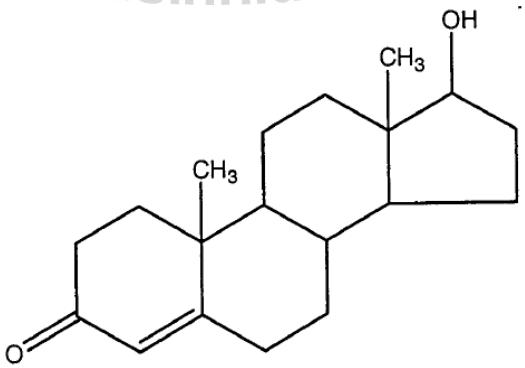

Figure 2.4 Molecular structure of testosterone, steroid hormone, includes lipid soluble cholesterol nucleus and polar hydroxyl side chain (Freeman et al., 2001).

2.4.2 The Physiological Functions of Testosterone in Birds

Testosterone is an important regulator of male sexual differentiation, reproductive and nonreproductive behaviors, development of secondary sex characteristics, and spermatogenesis. T influences many aspects of physiology and behavior in birds. Physiological effects include metabolic rate (Hannsler and Prinzinger, 1979; Feuerbacher and Prinzinger, 1981), lipid storage (Wingfield, 1984), and timing of molt (Runfeldt and Wingfield, 1985; Schleussner et al., 1985). Behavioral effects include aggression, reproductive and parental care behaviors, ornamentation, and fitness measured in the wild (Greives et al., 2006; McGlothlin et al., 2007; 2008; 2010; Vleck and Vleck, 2011). In birds, it has been well documented that T is associated with the parental behaviors and reproductive activities in pied flycatchers (Silverin, 1980), house sparrows (Hegner and Wingfield, 1987), reed warblers (Dittami et al., 1991), dark-eyed juncos (Ketterson et al., 1992; Schoech et al., 1998), yellow-headed blackbirds (Beletsky et al., 1995), barn swallows (Saino and Moller, 1995), spotless starlings (Moreno et al., 1999), European starlings (De Ridder et al., 2000), house finches (Stoehr and Hill, 2000), blue headed vireos (Van Roo, 2004), snow buntings (Lynn et al., 2005), yellow-legged gulls (Alonso-Alvarez, 2001), rufous whistlers (McDonald et al., 2001), and spotted sandpiper (Oring et al., 1989). T follows similar seasonal patterns, T levels reaching lowest during non-breeding periods and increasing during breeding for sperm production and reproduction success (Hau, 2007). Similarly, T can inhibit parental care behaviors such that individuals that maintain high levels of T throughout offspring rearing often reduce their parental care investment, which could subsequently lead to reduced reproductive success (Silverin, 1980; Lynn et al., 2009; Horton et al., 2010; Rosvall,

2013). Implant studies have clearly shown that elevated T increases expression of behaviors associated with reproduction in male birds. Males with T implants had significantly higher spontaneous song rates in a variety of species, including dark-eyed juncos (Ketterson et al., 1992; Chandler et al., 1994; Titus, 1998), pied flycatchers (Silverin, 1980), house sparrows (Hegner and Wingfield, 1987), reed warblers (Dittami et al., 1991), European starlings (De Ridder et al., 2000), house finches (Stoehr and Hill, 2000), great tits (Van Duyse et al., 2000), Lapland longpspurs (Hunt et al., 1997), chestnut-collared longspurs (Lynn et al., 2002), and snow buntings (Lynn et al., 2005). Moreover, the elevation of T may increase intensity and persistence of male aggression in appropriate contexts. For example, administration of T implants at various stages of breeding increased agonistic behavior in free-living male pied flycatchers (Silverin, 1980), white-crowned sparrows (Moore, 1984), song sparrows (Wingfield, 1984), red-winged blackbirds (Beletsky et al., 1990), and yellow-headed blackbirds (Beletsky et al., 1995). Whereas the effects of T to enhance sexual and territorial behavior may impart males with reproductive benefits, extension of the early season peak in T has also been shown to alter behavior such that a male's reproductive success may be compromised. For example, sustained high concentration of plasma T suppressed feeding of young in pied flycatchers (Silverin, 1980), house sparrows (Hegner and Wingfield, 1987), reed warblers (Dittami et al., 1991), dark-eyed juncos (Ketterson et al., 1992; Schoech et al., 1998), yellow-headed blackbirds (Beletsky et al., 1995), barn swallows (Saino and Moller, 1995), spotless starlings (Moreno et al., 1999), European starlings (De Ridder et al., 2000), house finches (Stoehr and Hill, 2000), blue headed vireos (Vireo solitarius, Van Roo, 2004), and snow buntings (Lynn et al., 2005). In addition, high

concentration of plasma T disrupted incubation of eggs by males that normally assist females in incubation in European starlings (De Ridder et al., 2000), yellow-legged gulls (Alonso-Alvarez, 2001), rufous whistlers (McDonald et al., 2001), blue headed vireos (Van Roo, 2004) and by males that typically incubate alone in spotted sandpiper (Oring et al., 1989). T levels usually decrease dramatically when eggs are laid and the males enter the parental phase of reproduction (Lynn et al., 2002). In Adelie penguins, males take the first long bout of incubation on the eggs, and, as incubation begins, T decreases to less than 2 % of the level in the courtship phase (Vleck et al., 1999). In yellow-eyed penguins, this drop can even be stimulated by an artificial egg in the nest (Massaro et al., 2007). In many avian and mammalian species, elevated androgen levels have often been found to interfere with the exhibition of paternal behaviors, and androgen levels tend to be lower during the paternal care period (Wingfield et al., 1990; Logan and Wingfield, 1995; Hirschenhauser et al., 2003; Goymann et al., 2007; McGlothlin et al., 2007). In song sparrows and white-crowned sparrows, T concentrations increase during the breeding season, but decrease while the males care for the young. The levels of T increase again as the males begin to mate and guard for the second clutch (Wingfield and Farner, 1978; Wingfield, 1984). Thus, T implant studies have demonstrated a relatively common pattern when the early season peak in T is experimentally extended, males often show marked increases in sexual and aggressive behavior, but tend to also significantly decrease paternal investment.

2.4.3 The Regulation of Testosterone Secretion in Birds

It is very well established that seasonal activation of the HPG axis leads to elevations in T in anticipation of breeding in male birds (Lynn, 2016). Hypothalamic

GnRH neurons synthesize and release GnRH via the eminentia mediana (median eminence; ME) to stimulate the synthesis and secretion of FSH and LH from the anterior pituitary gland which, in turn, stimulate the testis to synthesize and release T (Jawor et al., 2006; Deviche et al., 2010). Reproduction is regulated by the activity of the HPG axis, beginning with the release of GnRH from the hypothalamus (Sharp, 2005; Clarke, 2011). This neuropeptide stimulates the secretion of FSH and LH from the anterior pituitary gland (Kuenzel, 2000; Clarke, 2011). In males, FSH and LH stimulates gonadal growth, gametogenesis, and the synthesis and secretion of T (Murton and Westwood, 1977). T regulates a variety of male reproductive characteristics, such as the development of primary (Kempenaers et al., 2008) and secondary sexual characteristics (Buchanan et al., 2003), courtship, and aggression towards conspecific males (Hegner and Wingfield, 1987; Landys et al., 2010). Individual male birds, vary consistently in the amount of T produced in response to an experimental injection with GnRH (Jawor et al., 2006), and these repeatable individual differences in T production correlate with immune function, aggression, parental care, ornamentation, and fitness measured in the wild (Greives et al., 2006; McGlothlin et al., 2007; 2008; 2010).

2.5 Vasoactive Intestinal Peptide: Structure, Functions, and Regulation of Secretion

2.5.1 The Structure of Vasoactive Intestinal Peptide

Vasoactive intestinal peptide, an octacosapeptide, consists of 28 amino acids. It was first isolated from porcine duodenum (Said and Mutt, 1970; Mutt and Said, 1974). Subsequently, it has been found to be widely distributed in the central and

peripheral nervous systems (Larsson et al., 1976; Said and Rosenberg, 1976; Giachetti et al., 1977; Rosselin et al., 1982), with high concentrations found in the hypothalamus (Emson et al., 1979; Samson et al., 1979; Ceccatelli et al., 1991) and is considered to function as a neurotransmitter and neuroendocrine substance (Larsson et al., 1976; Marley and Emson, 1982). The discovery of a large population of VIP-immunoreactive (-ir) neurons in the hypothalamus whose axons project to the ME (Samson et al., 1978; 1979; Polak and Bloom, 1982; Lam, 1991; Dalcik and Phelps, 1993) and a high concentration of VIP in hypophysial portal blood (Said and Porter, 1979; Shimatsu et al., 1981; Brar et al., 1985; Mutt, 1988) led to the hypothesis that VIP participates in the regulation of anterior pituitary functions.

Vasoactive intestinal peptide is a neuropeptide of the VIP/glucagon/secretin superfamily including secretin, glucagon, gastric inhibitory peptide, GH releasing factor (GRF), peptide histidine isoleucine (PHI), and pituitary adenylate cyclase activating peptide (PACAP). VIP exerts its biological effects by binding to its specific receptors that are coupled to the G proteins, whose actions are mediated via the adenylate cyclase and the production of 3'-5'-cyclic adenosine monophosphate (cAMP; Hokfelt et al., 1980; Couvineau et al., 1990; Lutz et al., 1995). The peptides of this family are probably the results of exon duplication coupled to gene duplication. It has been ducumented that the VIP gene contains 7 exons, each exon encoding a different functional domain in the final mRNA and protein. Two adjacent exons in the genome encoding VIP and the related peptide histidine methionine (PHM) or PHI are exon 5 and exon 4, respectively (Bodner et al., 1985; Yamagami et al., 1988; Giladi et al., 1990). It has been reported that chicken VIP is different from mammalian VIP in its amino acid sequence at position 11, 13, 26, and 28, but the number of amino acid

residue is the same (Nilsson, 1975). In addition, chicken and turkey VIP share complete amino acid homology and are 98 % homologus at the neucleotide level. VIP mRNA may exist with or without PHI. Both mRNA forms are found in the chicken digestive tract and hypothalamus. In contrast, the short form is found only in the turkey hypothalamus and comprises 4-6 % of all VIP transcripts (You et al., 1995b). The amino acid sequence of VIP and the members in the VIP/glucagon/secretin family are shown in Figure 2.5.

Figure 2.5 The amino acid sequences of VIP, PACAP, secretin, GRF, peptide having an histidine residue in N-terminal position and an isoleucine residue in C-terminal position (PHI and its human homolog PHM), helodermin, glucagon, gastric inhibitory polypeptide (GIP), glucagon-likepeptide 1 and 2 (GLP-1andGLP-2; Couvineau et al., 2012).

Vasoactive intestinal peptide receptors are the members of the G protein-coupled family, whose biological actions are mediated via interaction with specific receptors that are coupled to adenylate cyclase and the production of cAMP (Gourdji et al., 1979; Bjoro et al., 1987; Couvineau et al., 1990; Lutz et al., 1995). There have

been reports that VIP receptor is present in several organs including lung, liver, small intestine, and many regions of the brain such as cerebral cortex and hippocampus (Besson et al., 1986; Martin et al., 1987; Csillag et al., 1993; Usdin et al., 1994; Sherward et al., 1995). Avian VIP receptors have been cloned and characterized in chickens (Kansaku et al., 2001) and turkeys (You et al., 2001). It has been reported that the circulating PRL variations observed across the turkey reproductive cycle are, in part, regulated by changes in VIP receptors at the pituitary level (Chaiseha et al., 2004). In birds, VIP receptors are present on the surface membranes of the anterior pituitary cells (Rozenboim et al., 1993b; Gonzales et al., 1994a; 1994b), hypothalamus (Gonzales et al., 1995), small intestine, and granulosa cells (Kawashima et al., 1995).

2.5.2 The Localization of Vasoactive Intestinal Peptide in the Avian Brain

Vasoactive intestinal peptide has been found to be distributed in the central and peripheral nervous systems (Larsson et al., 1976; Said and Rosenberg, 1976; Giachetti et al., 1977; Hokfelt et al., 1982; Rosselin et al., 1982). Significant concentrations of VIP are detected in the gastrointestinal tract, heart, lung, thyroid gland, kidney, immune system, urinary bladder, and genital organs. The distributions of VIP-containing neurons have been mapped in many avian species such as Pekin ducks (Korf and Fahrenkrug, 1984), bantams (Macnamee et al., 1986), pigeons (Hof et al., 1991), ring doves (Norgren and Silver, 1990), dark-eyed juncos (Saldanha et al., 1994), chicks (Kuenzel et al., 1997), turkeys (Chaiseha and El Halawani, 1999), Japanese quails (Teruyama and Beck, 2001), collared doves (Den Boer-Visser and Dubbeldam, 2002), starlings (Dawson et al., 2002), zebra finches (Kingsbury et al., 2015), blue tits (Montagnese et al., 2015), and native Thai chickens (Kosonsiriluk et

al., 2008). VIP neurons are extensively distributed throughout the hypothalamus (Chaiseha and El Halawani, 2015), especially in the areas of the medial preoptic area (MPOA), medial hypothalamic region, regio lateralis hypothalami (LHy), nucleus anterior medialis hypothalami (AM), and INF (den Boer-Visser and Dubbeldam, 2002). In general, three types of VIP-ir neurons and fibers are described. The first consists of a large number of spindle or bipolar neurons that connected the third ventricle to the external layer of the ME. A second set of VIP-ir fibers extends from the infundibular nucleus to the ME. The third type of VIP-ir neurons terminates upon small capillaries within the hypothalamus. It has been suggested that VIP in the ME is derived from neurons located within the INF (Macnamee et al., 1986; Mauro et al., 1989; Chaiseha and El Halawani, 1999; Youngren et al., 2002). VIP terminals are observed in the external portion of the ME and the majority of VIP-containing cell bodies are located in the INF. The number of the VIP-ir neurons in the INF increases following a gonadal stimulatory photoperiod. The hypothalami of incubating turkey hens contain more VIP-ir neurons than those of non-photostimulated hens. Depriving incubating birds from their nests was found to reduce circulating PRL levels and hypothalamic VIP immunoreactivity (Mauro et al., 1989). Fluctuations in hypothalamic VIP immunoreactivity and expression within the INF parallel fluctuations in circulating PRL concentrations (Chaiseha and El Halawani, 1999). The number, area, and density of hypothalamic VIP-ir neurons are greater in incubating than in laying hens (Sharp et al., 1989). In addition, in the domesticated pigeons, increases in the number and size of VIP-ir neurons within this region following the periods of elevated circulating PRL have been reported (Peczely and Kiss, 1988; Cloues et al., 1990). These VIP neurons project to the ME, where VIP is transported

through the hypothalamic-pituitary portal vessels to the anterior pituitary gland (Yamada et al., 1982; Macnamee et al., 1986; Mauro et al., 1989). Moreover, lesions in the INF can prevent the PRL increase after photostimulation (Youngren et al., 1989).

All these data indicate that the VIP neurons in the INF are an important factor in the stimulation of PRL secretion. Moreover, it has been established that VIP axon terminals have been found in close apposition to GnRH neurons in the lateral septal organ and POA (Teruyama and Beck, 2001), and an inverse relationship between VIP in the INF and GnRH in the POA has been reported (Deviche et al., 2000). A subset of VIP-ir neurons within the medial basal hypothalamus and septal region of the dove brain has been proposed to be encephalic photoreceptors (Silver et al., 1988; Norgren and Silver, 1990).

2.5.3 The Physiological Functions of Vasoactive Intestinal Peptide in Birds

Several hypothalamic neurotransmitters and neuropeptides have been studied during the past six decades for their effects upon PRL such as thyrotropin-releasing hormone, angiotensin II, oxytocin (OT), vasopressin (VP), PACAP, and PHI. Only VIP is thought to be a physiologically significant PRF in birds. VIP is a potent releaser of PRL *in vivo* (Lea and Vowles, 1986; Macnamee et al., 1986; Opel and Proudman, 1988; El Halawani et al., 1990a; Pitts et al., 1994) and *in vitro* (Macnamee et al., 1986; Proudman and Opel, 1988; El Halawani et al., 1990b; Xu et al., 1996). Thus, VIP plays a pivotal role in the regulation of PRL secretion in birds. VIP regulates PRL gene expression by enhancing the transcription rate of PRL and up-regulating PRL mRNA stability (Tong et al., 1998). The abundance of pituitary lactotrophs increases during incubation behavior (Lopez et al., 1996), and VIP was

shown to be able to mimick this increase *in vitro* (Porter et al., 2006). It is very well documented that variations in hypothalamic VIP immunoreactivity, VIP contents, VIP mRNA steady-state levels, VIP mRNA expression in the INF, VIP receptor mRNA in the pituitary cells, and VIP concentrations in hypophysial portal blood are correlated with the changes in circulating PRL levels in many avian species such as turkeys (Mauro et al., 1989; Youngren et al., 1996a; Chaiseha et al., 1998; 2004; Chaiseha and El Halawani, 1999), chickens (Sharp et al., 1989), doves (Cloues et al., 1990), and native Thai chickens (Kosonsiriluk et al., 2008). In male birds, VIP expression and VIP-Fos coexpression are associated with nesting behavior in male zebra finches (Kingsbury et al., 2015).

During the reproductive cycle in birds, VIP acts on the anterior pituitary gland directly to stimulate PRL synthesis and release (Lea and Vowles, 1986; Macnamee et al., 1986; Proudman and Opel, 1988; El Halawani et al., 1990b; 1997; Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011; Chaiyachet et al., 2012). From immunohistochemistry (IHC) studies, hypothalamic VIP-ir neurons in the INF and VIP-ir fibers in the ME correspond to the enhanced plasma PRL levels in the turkeys and native Thai chickens (Mauro et al., 1989; Kosonsiriluk et al., 2008; Chaiyachet et al., 2012). Other studies have also demonstrated the increases in the number and cell size of VIP-ir neurons within this region in the pigeons and ring doves during the periods of hyperprolactinaemia (Peczely and Kiss, 1988; Cloues et al., 1990). Changes in pituitary VIP receptor mRNA expression are observed during the reproductive cycle of the turkey. Increased pituitary VIP receptor mRNA expression is observed in the turkey hens with normal (laying) or high PRL levels (incubating), while lower VIP receptor mRNA expression is observed in the hypoprolactinemic non-photostimulated

and photorefractory turkey hens. This suggests that the VIP receptors located in the INF are involved in PRL secretion and indicates that PRL secretion is principally governed by VIP receptors at the pituitary level (Chaiseha et al., 2004).

In the native Thai chicken, VIP-ir neurons and fibers are extensively distributed throughout the brain and are predominantly expressed in the diencephalon, where VIP-ir neurons are concentrated within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) areas. Changes in the number of VIP-ir neurons within the IH-IN are directly correlated with changes in plasma PRL levels throughout the reproductive cycle. These results suggest that VIP expression in the IH-IN plays a regulatory role in year-round reproductive activity in this equatorial bird (Kosonsiriluk et al., 2008). Further studies indicate that an increase in the numbers of VIP-ir neurons in the IH-IN during incubation behavior is associated with an increase in DAergic neurons within the nucleus intramedialis (nI) and nucleus mamillaris lateralis (ML), and an increase in plasma PRL levels parallels these systems. These results suggest that nesting activity stimulates PRL secretion through activation of the DAergic system, which in turn stimulates the VIPergic system (Prakobsaeng et al., 2011). Recently, it has been reported that the numbers of VIP-ir neurons in the IN-IH areas are high in rearing (R) hens, whereas the numbers of VIP-ir neurons decrease in non-rearing (NR) hens, and these changes correlate with plasma PRL levels. These results indicate that the VIP/PRL system plays an important role in neuroendocrine reorganization to establish the rearing behavior in this non-seasonal breeding, equatorial, precocial species. The VIP/PRL system is not only a key, well established, regulator of incubation behavior, but it is involved in the

regulation of rearing behavior as well. It is possible that VIP and the decline in the number of VIP-ir neurons in the IH-IN and in turn VIPergic activity and the decrease in PRL levels are related to their contributions to rearing behavior of the native Thai chickens.

2.5.4 The Regulation of Vasoactive Intestinal Peptide Secretion in Birds

It is well established that avian pituitary PRL secretion is tonically stimulated by VIP, which is secreted from neurons located in the INF of the caudo-medial hypothalamus (El Halawani et al., 1997). The findings that immunoneutralization of endogenous VIP reduces levels of circulating PRL and pituitary PRL mRNA and totally blocks the PRL release affected by electrical stimulation of the MPOA (El Halawani et al., 1990b; Youngren et al., 1994) as well as blocks the hormonal and behavioral characteristics of incubating (INC) hens (El Halawani et al., 1995). In birds, the regulation of PRL synthesis and secretion involves the interaction of external stimuli with neuroendocrine mechanisms, and these critical stimuli include photoperiod, ambient temperature, and the presence of eggs and offspring. These external stimuli and internal stimuli such as estrogen and progesterone are important in initiating and maintaining PRL secretion. However, their relative importances vary with stages of the reproductive cycle (Curlewis, 1992). It is very well documented that the regulation of avian PRL secretion and its gene expression are governed by hypothalamic VIP, the avian PRF (Chaiseha and El Halawani, 2015). VIP acts directly on the lactotrophs to stimulate PRL secretion (El Halawani et al., 1997). There are increases in the number and cell size of VIP neurons reported in the pigeons and ring doves when circulating concentrations of PRL are elevated (Peczely and Kiss, 1988; Cloues et al., 1990). Increased pituitary VIP receptor expression is also

found in laying or incubating turkey hens supporting PRL secretion being principally regulated by VIP at the pituitary level (Chaiseha et al., 2004). Tactile stimuli from the nests and eggs maintain elevated circulating concentrations of PRL and up-regulate VIP gene expression in the INC hens (Silver et al., 1988; Buntin et al., 1991; Massaro et al., 2007). It has been suggested that VIP mediates the effects of photoperiod on PRL secretion in the turkey (El Halawani et al., 1996), and quantification of hypothalamic VIP revealed increased VIP content following photostimulation (Mauro et al., 1992). It also has been demonstrated that VIP content in the ME, hypothalamic cytoplasmic VIP mRNA steady-state levels, and hypothalamic nascent VIP mRNA levels are all increased and correlated with increased PRL secretion following photostimulation (Chaiseha et al., 1998). These results lend support to a hypothetical scheme for photoperiodic regulation of PRL in which VIP serves as the PRF that is intimately linked to photoperiodic mechanisms. Furthermore, the results also imply that VIP transcription is coupled to the photoperiodic state of the birds. However, it remains to be clarified how photoperiodic information is transduced to VIP-ir neurons located in the INF region of the hypothalamus (Mauro et al., 1989). Whether photoperiodic cues directly influence VIP remains an open question. Silver et al. (1988) found that VIP was colocalized with an opsin-like pigment in the INF area. This area is thought to contain extra-retinal hypothalamic photoreceptors which are important for the induction of seasonal reproductive function in birds (Oksche and Farner, 1974; Oliver and Bayle, 1976; Oliver et al., 1977). Alternatively, photoperiod may modulate VIP expression by acting upon unknown neuronal circuits that influence VIP transcription. Recently, turkey melanopsin was found in DA-melatonin co-localized neurons in the nucleus premamillaris (PMM) and was implicated as an

important component of the photoreceptive system regulating reproductive activity in temperate zone birds (Kang et al., 2010). It has also been reported that VIP is also inhibited by high concentration of circulating PRL. Intracerebroventricular (ICV) PRL injections into incubating ring doves reduce the number of infundibular VIP-like neurons, which indicates the existence of a hypothalamic negative feedback loop for PRL (Saldanha and Silver, 1995). Intracranial and systemic administrations of ovine PRL into laying turkey hens reduce circulating PRL concentrations (Youngren et al., 1991; Rozenboim et al., 1993b). In addition, systemic PRL administration also reduces hypothalamic VIP content and the number of anterior pituitary VIP binding sites (Rozenboim et al., 1993a), suggesting that PRL may act directly at the pituitary level. Moreover, PRL binding sites have been found within the avian hypothalamus (Buntin and Ruzycki, 1987; Buntin and Walsh, 1988) and PRL receptor mRNA is also detected in the brain of chicken (Tanaka et al., 1992) and the hypothalamus of turkey (Zhou et al., 1996; Pitts et al., 2000). Furthermore, PRL may cross the blood-brain barrier at the choroid plexus (Buntin and Walsh, 1988) and binds to PRL receptors lining the third ventricle, thereby decreasing the number of hypothalamic VIP-containing neurons (Saldanha and Silver, 1995).

2.6 Dopamine: Structure, Functions, and Regulation of Secretion

2.6.1 The Structure of Dopamine

DA is found in both the central and the peripheral nervous systems of several vertebrate and invertebrate species (Carlsson and Hillarp, 1956; Benes, 2001). The chemical name of DA is 4-(2-aminoethyl) benzene-1,2-diol, and the formula is $C_6H_3(OH)_2$-CH_2-CH_2-NH_2. It is a neurotransmitter/neuromodulator belonging to a

group of catecholamines (CA) and functions as a classical neurotransmitter in the brain. Thus, it communicates between neurons and acts synaptically within anatomically confined neuronal networks. DA is a precursor of norepinephrine (NE) and epinephrine (E) in the biosynthetic pathway. CA and indolamines are referred to as monoamines, water soluble molecules that are decarboxylated derivatives of amino acids. CA have distinctive structures, which are the single amine group, a nucleus of catechol; a benzene ring with two adjacent hydroxyl groups, and a side chain of ethylamine or one of its derivatives (Wood-Gush and Gilbert, 1973).

Tyrosine is the precursor of DA synthesis, which the majority of this circulating amino acid is from diets, and small amounts are derived from hydroxylation of phenylalanine by phenylalanine hydroxylase in the liver (Missale et al., 1998). Tyrosine is taken up by the neurons and then converted to DA by two enzymes, which are tyrosine hydroxylase (TH) and 1-aromatic amino acid decarboxylase (AADC). These enzymes are named dihydroxyphenylalanine decarboxylase. TH is the rate-limiting enzyme in this biosynthetic pathway. TH converts tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA), and L-DOPA is then catalyzed to DA by AADC. DA is then processed to NE by DA beta-hydroxylase (DBH), and NE is then converted into E by phenylethanolamine N-methyl transferase. The CA biosynthetic pathway is illustrated in Figure 2.6. TH is the most critical enzyme that regulates DA synthesis. In human, TH gene is localized at chromosome 11p and encodes a single form of TH that can be alternatively spliced (Powell et al., 1984). The mature TH is composed of 4 subunits of approximately 60 kDa each. Each monomer consists of an inhibitory regulatory domain at the N terminus and a catalytic domain at the C terminus. The catalytic domain contains a protein-binding region and

a putative leucine zipper at the C terminus involved in intersubunit binding (Kumer and Vrana, 1996).

The DA receptors belong to the G protein coupled receptors family. There are five subtypes (D_1-D_5) in vertebrate species that are divided in two families according their structures and biological responses. The D_1-like DA subfamily includes D_1 and D_5 DA receptors, while D_2-like DA subfamily consists of D_2, D_3, and D_4 DA receptors (Rangel-Barajas et al., 2015). cDNA characterization of these receptor subtypes shows that the D_1 and D_5 DA receptors share high homology in their transmembrane sequences, and the transmembrane sequences of D_2, D_3, and D_4 DA receptors are highly conserved among these three receptor subtypes (Missale et al., 1998). The D_1 DA and D5 receptor subtype has been classified as being stimulatory, and the D_2 DA receptor subtype has been classified as being inhibitory (Porter et al., 1994). Activation of the D_1-like DA receptors increases adenylate cyclase activity via the $G_{s\alpha}$ subunit. Activation of the D_2-like DA receptors inhibits adenylate cyclase activity via the $G_{i\alpha}$ subunit. However, the G_o and G_q proteins associated with ion channels and the phosphoinositide cascade are also involved (Bentivoglio and Morelli, 2005).

In birds, more than seven D_1-like (D_{1A}, D_{1B}, D_{1C}, and D_{1E}) and D_2-like (D_2, D_3, and D_4) DA receptors have been characterized (Haug-Baltzell et al., 2015; Yamamoto et al., 2015). Cloning of cDNAs encoding the D_1 and D_2 DA receptors has been reported in turkeys (Schnell et al., 1999a; 1999b). The nucleotide sequence of the avian D_2 DA receptor reveals 75 % homology to the mammalian D_2 DA receptor.

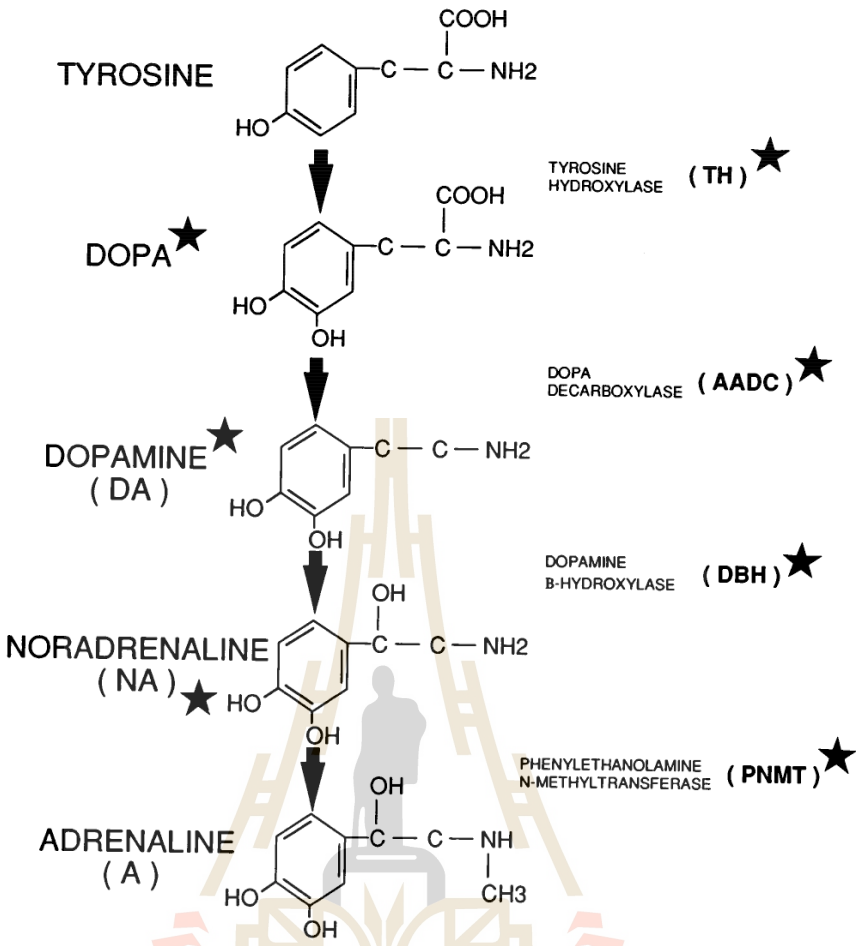

Figure 2.6 Catecholamines biosynthetic pathway and available antibodies as indicated by asterisks (Smeets and Gonzalez, 2000).

The D_1-like DA receptor has been found in the brain of pigeons (Richfield et al., 1987; Dietl and Palacios, 1988), European starlings (Casto and Ball, 1994), Japanese quails (Ball et al., 1995), chickens (Schnabel et al., 1997; Sun and Reiner, 2000; Kubikova et al., 2010), zebra finches (Kubikova et al., 2010), and turkeys (Schnell et al., 1999a; Chaiseha et al., 2003a). The D_2-like DA receptor has been found in the brain of pigeons (Richfield et al., 1987), Japanese quails (Levens et al., 2000), and turkeys (Schnell et al., 1999a; 1999b; Chaiseha et al., 2003a). The distributions of D_2

DA receptor mRNA have been found widespread throughout the brain, pineal gland, cortex, cerebellum (Cb), and also in the pituitary gland of the turkeys and chickens (Chaiseha et al., 2003a; Lv et al., 2018).

2.6.2 The Localization of Dopamine in the Avian Brain

The DAergic system of birds is similar to that of mammals in terms of anatomy (Durstewitz et al., 1999). The anatomical distributions of DA have been determined and visualized in several avian species including Japanese quails (Ottinger et al., 1986; Bailhache and Balthazart, 1993; Balthazart et al., 1998; Absil et al., 2001), pigeons (Kiss and Peczely, 1987; Berk, 1991; Divac et al., 1994; Durstewitz et al., 1998), zebra finches (Barclay and Harding, 1990; Bottjer, 1993; Mello et al., 1998), chickens (Contijoch et al., 1992; Moons et al., 1994; 1995), budgerigars (Roberts et al., 2001), turkeys (Al-Zailaie and El Halawani, 2000; Thayananuphat et al., 2011), collared doves (den Boer-Visser and Dubbeldam, 2002), canaries (Appeltants et al., 2001), and female native Thai chickens (Sartsoongnoen et al., 2008; Prakobsaeng et al., 2011; Chokchaloemwong et al., 2015; Sinpru et al., 2018). The majority of DA neurons and fibers is distributed in the diencephalon and mesencephalon. The distributions of DA neurons and fibers are located in a discrete region lying close to the ventriculus tertius (third ventricle) from the level of POA. The anatomical distributions of DA neurons and fibers are found in several regions of the avian brain such as the POA, nucleus preopticus medialis (POM), nucleus ventrolateralis thalami (VLT), suprachiasmatic nucleus, AM, LHy, nucleus paraventricularis magnocellularis (PVN), nucleus ventromedialis hypothalami, nucleus dorsomedialis hypothalami, nI, PMM, nucleus mamillaris medialis, ML, area ventralis, nucleus tegmenti pedunculo-pontinus, pars compacta (substantia nigra),

locus ceruleus, brachium conjunctivum ascendens, brachium conjunctivum descendens, Cb, lateral septum, pons, and medulla oblongata (Kiss and Peczely, 1987; Bailhache and Balthazart, 1993; Moons et al., 1994; Reiner et al., 1994; Al-Zailaie and El Halawani, 2000; den Boer-Visser and Dubbeldam, 2002; Acerbo et al., 2003). DA fibers are extensively distributed within the external layer of the ME (Bailhache and Balthazart, 1993; Kiss and Peczely, 1987; Moons et al., 1994; Al-Zailaie et al., 2006). Similarly, in the female native Thai chickens, TH-ir neurons and fibers are extensively distributed throughout the brain, especially in the diencephalic (Sartsoongnoen et al., 2008; Prakobsaeng et al., 2011; Chokchaloemwong et al., 2015; Sinpru et al., 2018) and mesencepharic regions (Sartsoongnoen et al., 2008). DA neurons are found throughout the hypothalamus (Kiss and Peczely, 1987; Reiner et al., 1994; Al-Zailaie and El Halawani, 2000) and have been shown to be immunoreactived for VIP and its mRNA (Mauro et al., 1989; 1992; Hof et al., 1991; Kuenzel et al., 1997; Chaiseha and El Halawani, 1999). Given their widespread distributions, DA neurons and their fibers are found intermingled with VIP neurons in the INF, GnRH neurons in the POA, and with both VIP and GnRH terminals in the external layer of the ME (Contijoch et al., 1992; Fraley and Kuenzel, 1993). Thus, it is reasonable to consider whether any regional specificity exists in DA neurons that are neuroendocrine in nature such as controlling the release and expression of VIP/PRL and GnRH/FSH-LH systems.

2.6.3 The Physiological Functions of Dopamine in Birds

There are numerous reports regarding the physiological roles of DA and its receptors in birds such as food intake (Khodadadi et al., 2017), food reward (Moe et al., 2014), cognitive performance (Taufique and Kumar, 2016), feather pecking (Kops

et al., 2017), singing behavior (Sasaki et al., 2006; Heimovics and Riters, 2008; Riters et al., 2014; Merullo et al., 2018), song learning (Budzillo et al., 2017), social activity (Heimovics et al., 2009), aggressive behavior (Komiyama et al., 2014), mate competition (Kabelik et al., 2009), courtship motivation (Goodson et al., 2009), egg production (Xu et al., 2010a), incubation (Xu et al., 2010b), reproductive cycle (Lea et al., 2001), and male sexual behavior (Balthazart et al., 1997). DA influences are involved in both stimulating and inhibiting PRL secretion in birds. ICV infusion of DA in the laying turkey hens can stimulate or inhibit PRL secretion depending on the concentrations used (Youngren et al., 1995). Therefore, both stimulatory and inhibitory effects of DA on avian PRL secretion are dependent upon multiple subtypes of DA receptors (Youngren et al., 1996b). These actions are confirmed by the presence of both D_1 and D_2 DA receptor mRNAs in the brain and the pituitary cells of turkeys (Schnell et al., 1999a; 1999b; Chaiseha et al., 2003a). These findings suggest that the stimulatory effect of DA on PRL synthesis and secretion is regulated via the D_1 DA receptors residing in the INF, where the VIP neurons are located. DA inhibits PRL synthesis and secretion at the pituitary level via the D_2 DA receptors by blocking the effect of VIP (Youngren et al., 1996b; 1998; 2002; Chaiseha et al., 1997; 2003a; Al Kahtane et al., 2003). It has also been reported that DA activates hypothalamic VIP gene expression in the INF (Bhatt et al., 2003). Additionally, it has been suggested that the signalling mechanism(s) underlying the interaction between VIP and DA in the regulation of PRL secretion involves protein kinase A (Kansaku et al., 1998), calcium ion (Ca^{2+}; Hall et al., 1985; Al Kahtane et al., 2003; 2005), and protein kinase C (Sun and El Halawani, 1995) signalling pathways.

There is some evidence suggesting an inhibitory role of DA on GnRH synthesis and release in both mammals and birds (Ramirez et al., 1984; Sharp et al., 1984). In chickens, there is evidence suggesting the involvement of DA in GnRH regulation, due to the dense concentration of TH- and GnRH-containing processes located in the lateral and mediobasal portion of the external layer of the ME. This finding provides an opportunity for synaptic interaction between DA and GnRH. DA inhibits GnRH release via presynaptic inputs at the ME in the chickens (Contijoch et al., 1992; Fraley and Kuenzel, 1993). Activation of the DA neurons in the ML is associated with the activation of GnRH-I and VIP neurons and the subsequent release of LH and PRL (Al-Zailaie et al., 2006). The relationship of the DAergic system in the PMM and the GnRH-I system in the nucleus commissurae pallii during the photo-induction of reproductive activity has been reported, demonstrated by c-fos mRNA expression within the PMM that is differentially activated by light and corresponding with a rhythm of photosensitivity (Thayananuphat et al., 2007a; 2007b). It is further suggested that DA in the PMM proposed to be the DA A11 group controls the reproductive seasonality in the temperate zone birds.

It is well established that DA plays an intermediary role in PRL secretion in birds and that the stimulatory effect of DA requires the presence of VIP in order to stimulate PRL secretion (Youngren et al., 1996b). Intracranial infusions of DA are ineffective in PRL release in turkeys actively immunized against VIP, suggesting that DA affects PRL secretion by stimulating the release of VIP. This finding is supported with several studies. The infusion of VIP into the turkey pituitary results in a rapid and substantial increase in plasma PRL, and this increase is completely suppressed when DA is infused in conjunction with VIP (Youngren et al., 1998). Co-expression

of D_2 DA receptor mRNA seen in VIP expressing neurons within the LHy and INF has been reported (Chaiseha et al., 2003b). In addition, it has been found that D_2 DA receptor agonist inhibits VIP-stimulated PRL secretion as well as PRL mRNA levels when incubated with turkey anterior pituitary cells (Xu et al., 1996). These results support that DA appears to block VIP-stimulated PRL release by activating D_2 DA receptors. To date, it is concluded that dynorphin, serotonin, DA, and VIP all appear to stimulate avian PRL secretion with the VIPergic system as the final mediator (El Halawani et al., 2001).

Dopamineergic activity and DA receptors mRNA expression are changed according the different physiological behaviors and reproduction. The DAergic expression during the turkey reproductive cycle parallels the changes in plasma PRL levels and VIP immunoreactivity, VIP peptide content, and VIP mRNA expression within the INF (El Halawani et al., 1980; 1984; Mauro et al., 1989; Wong et al., 1991; Chaiseha et al., 2003a). In the native Thai chickens, changes in the number of TH-ir neurons in the nI are associated with the reproductive stages. The number of TH-ir neurons in the nI is lowest in non-egg laying stage, then markedly increases in the egg laying, and reaches the highest density in INC hens, and decreases in the R hens (Sartsoongnoen et al., 2008). Disruption of incubation behavior by nest-deprivation decreases the numbers of TH-ir neurons within the nI and ML (Prakobsaeng et al., 2011). In addition, the number of TH-ir neurons in the nI and plasma PRL levels are significantly higher in R hens when compared with NR hens (Chokchaloemwong et al., 2015). Recently, it has reported that the presence of eggs or chicks is associated with decreased numbers of TH-ir neurons within the nI and ML and increased numbers of mesotocin (MT)-ir neurons within the nucleus supraopticus; pars ventralis

(SOv), POM, and PVN during the transition from incubating to rearing behavior (Sinpru et al., 2017; 2018).

Dopamine also plays a role in many aspects of sexual activities and reproduction in birds. Administration of the D_1 DA agonist increases the sexual behavior in Japanese quails (Balthazart et al., 1997). It is possible that DA neurons located within the PVN and ML might influence gonadal maturation (Kuenzel, 2000). It has been suggested that the rostral DA A11 neurons of the caudal hypothalamus are involved in courtship singing in songbirds such as zebra finches (Bharati and Goodson, 2006). In male Japanese quails, both D_1 and D_2 DA receptors in the POM facilitate sexually-motivated behaviors (Kleitz-Nelson et al., 2010a). D_1 DA in the medial POA is involved in sexual behaviors in male European starlings (Spool et al., 2019). There is evidence suggesting that DA and T play a role in parental behaviors in birds. It has been reported that DA in the medial POM facilitates male sexual behaviors (Hull et al., 1995; Dominguez and Hull, 2005; Bharati and Goodson, 2006; Will et al., 2014; Matheson and Sakata, 2015). DA and T in the POM can stimulate sexually-motivated male behavior. In male starlings, the stimulation of DA in the POM increases sexually-motivated vocal production (Riters et al., 2014). The density of D_1-like receptor in the POM is negatively correlated with sexually-motivated song, when T is high, but correlates positively with non-sexually motivated song, when T is low in male European starlings (Heimovics et al., 2009). DA is also involved in learning, reward-seeking, motivated behaviors, and social behavior in birds (Arias-Carrion and Poppel, 2007; Riters, 2012).

2.6.4 The Regulation of Dopamine Secretion in Birds

Originally, DA neurons were implicated in the regulation of pituitary hormone secretion based on the results of early receptor binding and pharmacological studies illustrating that DA receptors are located in hypophysiotropic regions of the hypothalamus and pituitary gland (Moore, 1987). In birds, it has been suggested that the inhibitory effects of DA on VIP-induced PRL gene transcription may result from DA suppression of the pituitary-specific transcription factor 1 (Pit-1; growth hormone factor 1; Al Kahtane et al., 2003). A conserved consensus Pit-1-biding site has been proposed in the avian and teleost PRL/GH gene family (Ohkubo et al., 1998). Pit-1 cDNA has been cloned in the turkeys (Wong et al., 1992; Kurima et al., 1998) and chickens (Tanaka et al., 1991). In avian species, the studies of the regulation of DA synthesis and secretion are limited. In photorefractory birds, it has been suggested that photosensitivity or photorefractoriness affect the regulatory role of DA and their temporal interactions in the regulation of the neuroendocrine-gonadal axis. It has been suggested that administration of L-5-hydroxytryptophan and L-DOPA at 12 hours intervals maintains the reproductive system in a stimulated condition and prevents reproductive regression, but does not prevent the onset of scotosensitivity in Japanese quails. It was further concluded that the 12 hours temporal relationship of circadian serotoninergic and DAergic oscillations not only eliminates photorefractoriness, but may also reestablish photosensitivity in relative photorefractory Japanese quails (Chaturvedi et al., 2006). Moreover, transcription factors, Phox2 and dHAND, are directly interacted with and transactivate the promoter of the gene encoding the NEergic biosynthetic enzyme, DBH, and are involved in the biosynthesis, transport, and secretion of NE (Rychlik et al., 2005).

2.7 Mesotocin: Structure, Functions, and Regulation of Secretion

2.7.1 The Structure of Mesotocin (Oxytocin-Like Peptide)

It is very well documented that OT and arginine vasopressin (AVP) are neurohypophysial nonapeptides that have been extensively reported to have systemic and physiological roles and known as reproductive hormone in mammals. OT is found within several regions of the brain (Dogterom et al., 1978; Hashimoto et al., 1985; Landgraf and Neumann, 2004) and peripheral tissues (Fields et al., 1983; Ang and Jenkins, 1984; Gimpl and Fahrenholz, 2001). These neurohypophysial hormones and other related peptides are referred to as the VP/OT family. Moreover, there are at least 14 additional neurohypophysial hormones that are found in non-mammalian vertebrates (Hoyle, 1998). In birds, the two hormones are arginine vasotocin (AVT) and MT, respectively the avian homologs of AVP and OT (Acher et al., 1970; 1997; Hoyle, 1998). The avian neurohypophysial hormones have been characterized. The avian antidiuretic hormone is AVT (Munsick et al., 1960) and the OT principle is MT (Acher et al., 1970). The structure of AVT (8-arginine oxytocin) is Cys-Tyr-Ile-Glu(NH_2)-Asp(NH_2)-Cys-Pro-Arg-Gly(NH_2), whereas MT (8-isoleucine oxytocin) is Cys-Tyr-Ile-Glu(NH_2)-Asp(NH_2)-Cys-Pro-Ile-Gly(NH_2). It differs from the mammalian homolog OT, by the substitution of isoleucine for leucine (position 8; Figure 2.7).

Mesotocin is the OT-like hormone found in most terrestrial vertebrates including marsupials, amphibians, reptiles, lungfish, and birds. Only two South American marsupials express OT exclusively, whereas all other marsupials have MT. OT is found together with MT in the Northern brown bandicoots (Rouille et al., 1988) and the North American opossums (Chauvet et al., 1985). Taken together, MT has the

largest distribution in vertebrates after vasotocin (VT) in all non-mammalian vertebrates, and isotocin is identified in bony fish. Despite this invariability, the physiological role(s) of these peptides have not been ascribed. It is still unknown whether the marsupials that are endowed with both OT and MT have two distinct receptors.

	1	2	3	4	5	6	7	8	9	
Oxytocin	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Leu	Gly(NH_2)	Placentals, some marsupials, ratfish (*Hydrolagus colliei*)
Mesotocin	*	*	*	*	*	*	*	Ile	*	Marsupials, nonmammalian tetrapods, lungfishes
Isotocin	*	*	*	Ser	*	*	*	Ile	*	Osteichthyes
Glumitocin	*	*	*	Ser	*	*	*	Gln	*	Skates (Chondrichthyes)
Valitocin	*	*	*	*	*	*	*	Val	*	Sharks (Chondrichthyes)
Aspargtocin	*	*	*	Asn	*	*	*	*	*	Sharks (Chondrichthyes)
Asvatocin	*	*	*	Asn	*	*	*	Val	*	Sharks (Chondrichthyes)
Phasvatocin	*	*	Phe	Asn	*	*	*	Val	*	Sharks (Chondrichthyes)
Cephalotocin	*	*	Phe	Arg	*	*	*	Ile	*	*Octopus vulgaris* (Molluscs)
Annetocin	*	Phe	Val	Arg	*	*	*	Thr	*	*Eisenia foetida* (Annelids)
Vasotocin	*	*	*	*	*	*	*	Arg	*	Nonmammalian vertebrates, cyclostomes
Vasopressin	*	*	Phe	*	*	*	*	Arg	*	Mammals
Lysipressin	*	*	Phe	*	*	*	*	Lys	*	Pig, some marsupials
Phenypressin	*	Phe	Phe	*	*	*	*	Arg	*	Macropodids (Marsupials)
Locupressin	*	Leu	*	Thr	*	*	*	Arg	*	*Locusta migratoria* (Insects)
Arg-conopressin	*	Ile	*	Arg	*	*	*	Arg	*	*Conus geographicus* (Molluscs)
Lys-conopressin	*	Phe	*	Arg	*	*	*	Lys	*	*Lymnaea stagnalis* (Molluscs)

Figure 2.7 Oxytocin and its related peptides, asterisks indicate amino acid residues that are identical to the corresponding residues in the OT sequence (Gimpl and Fahrenholz, 2001).

2.7.2 The Localization of Mesotocin in the Avian Brain

Mesotocin has been determined and visualized in several avian species including chickens (Barth et al., 1997), domestic mallards (Goossens et al., 1977; Bons, 1980), Japanese quails (Goossens et al., 1977; Bons, 1980), domestic fowls (Tennyson et al., 1985), White Leghorn cockerels (Robinzon et al., 1988a), turkeys (Thayananuphat et al., 2011), and female native Thai chickens (Chokchaloemwong et

al., 2013). MT is synthesized in specific neuronal groups within the hypothalamus and released from the posterior pituitary gland into the hypophysial portal blood via the ME in amphibians, reptiles, and birds (Bentrly, 1997). MT is also distributed within several regions of the avian brain (Robinzon et al., 1988a; Thayananuphat et al., 2011; Chokchaloemwong et al., 2013) and several tissues of the reproductive tract including the corpus luteum, follicle, and uterus (Robinzon et al., 1988b). Numerous studies have been reported that MT neurons and fibers are distributed in several regions of the brain such as the SOv; ventral part of the nucleus supraopticus (SON), tractus septomesencephalicus, nucleus preopticus periventricularis, bed nucleus of the stria terminalis, PVN, nucleus habenularis lateralis, IH, Cb, lateral septum, optic lobe, tuberomammillary nucleus, pons, and medulla oblongata (Bons, 1980; Robinzon et al., 1988a; Barth et al., 1997; Thayananuphat et al., 2011). MT fibers are extensively distributed within internal and external layers of the ME (Bons, 1980; Goossens et al., 1977). Similarly to the previous findings in female native Thai chickens, the highest accumulations of MT-ir neurons and fibers are concentrated within the SOv, POM, VLT, LHy, and PVN (Chokchaloemwong et al., 2013; Sinpru et al., 2017; 2018).

2.7.3 The Physiological Functions of Mesotocin in Birds

To date, there are a limited number of studies regarding the role(s) of MT in birds. It has been reported that MT facilitates uterine contractions in hens (Takahashi et al., 1995; Takahashi and Kawashima, 2008), acts as vasodepressors in cockerels (Robinzon et al., 1994), inhibits feeding behavior in chicks (Masunari et al., 2013; 2016), and promotes sociality of female zebra finches and field sparrows (Goodson et al., 2009; 2012a). The role of MT in avian brooding behavior was first documented in the turkey (Thayananuphat et al., 2011). Recently, it has been reported that MT is

involved in the regulation of the reproductive cycle and maternal behaviors in female native Thai chickens (Chokchaloemwong et al., 2013; Sinpru et al., 2017; 2018).

Mesotcin is among 16 naturally occurring neuropeptides found in vertebrates and has been reported to be synthesized by neurons of the anterior hypothalamus, specifically magnocellular neurons of the SON and PVN (Acher et al., 1970; 1997). MT is an important physiological regulator of the anterior pituitary gland. Anatomical and physiological data suggest that AVT and, to a lesser extent, MT may play similar hypophysiotropic functions in non-mammalian vertebrates (Mikami and Yamada, 1984; Tennyson et al., 1985; Tonon et al., 1986; Castro et al., 1988; Moons et al., 1988; Robinzon et al., 1988; Romero et al., 1998; Romero and Wingfield, 2001; Jurkevich et al., 2008). Several lines of evidence, using the domestic chicken as the model, demonstrate that AVT, and not MT, is a key regulator of oviposition in birds (Jurkevich and Grossmann, 2003). This finding is somewhat surprising since structurally MT is most like OT, the neurohypophysial hormone in mammals that stimulates uterine contractility during parturition (Fuchs et al., 1982; Landgraf et al., 1983). Indeed, AVT, but not MT, has been shown to stimulate contraction of shell gland strips *in vitro* (Koike et al., 1988).

Utilizing radioligand binding, studies have established MT-like binding sites in the kidney of the hens, suggesting that MT may function in regulating urine volume (Takahashi et al., 1995; 1996; 1997). However, a definitive MT receptor has not yet been identified in any avian tissues except for the changes in the binding affinity and capacity of MT receptor of the uterus, which may be related to oviposition in hens (Takahashi and Kawashima, 2008). Plasma MT levels are correlated with renal perfusion during hemorrhaging, suggesting that MT may be involved in renal blood

flow regulation in the domestic fowls (Bottje et al., 1989). However, water deprivation that causes dehydration does not affect plasma MT levels in the white leghorn cockerels (Robinzon et al., 1990). In behavioral studies, ICV injections of MT and AVT induce anorexia and wing-flapping in chicks, suggesting that these peptides may bind the same receptor to exert their effects (Masunari et al., 2013; 2016). Moreover, MT modulates numerous social behaviors including pair-bonding (partner preference), stress coping, and affiliative (grouping/flocking) behavior in zebra finches (Goodson et al., 2009; Pedersen and Tomaszycki, 2012; Klatt and Goodson, 2013; Kelly and Goodson, 2014) and emberizid sparrows (Wilson et al., 2016).

Most non-mammalian vertebrates express VT and an OT-like peptide such as isotocin, found in ray-finned fish, or MT, which is ubiquitously expressed in non-mammalian tetrapods (Acher, 1974; Hoyle, 1999). All jawed vertebrates express their two nanopeptides in both magnocellular and parvocellular neurons of the POA and hypothalamus, which in amniotes are located primarily within the SON and PVN (Moore and Lowry, 1998; Goodson, 2008). Thus, given the strong similarities of MT and OT systems, it is likely the case that extra-hypothalamic MT projections in birds are exclusively or almost exclusively derived from the PVN (Goodson and Kingsbury, 2011).

The role of MT in avian maternal behaviors has been reported. In turkeys, the numbers of MT-ir neurons within the PVN and SOv increase in INC hens when compared with laying hens. In addition, c-fos mRNA is induced in the MT-ir neurons within these areas in INC hens stimulated with poults, and blocking MT receptors prevents poult brooding from taking place (Thayananuphat et al., 2011). In native Thai chickens, changes in the numbers of MT-ir neurons within the SOv, POM, and

PVN are associated with the reproductive stages of the native Thai chickens, with the highest density observed in the INC and R hens. In addition, the numbers of MT-ir neurons within the SOv, POM, and PVN of R hens are higher than those of NR hens in these nuclei. These findings indicate that MT-ir neurons play a regulatory role in reproductive activities and neuroendocrine reorganization to establish and maintain rearing behavior in this species (Chokchaloemwong et al., 2013). The numbers of MT-ir neurons within the SOv, POM, and PVN decreased when incubation behavior was disrupted by nest-deprivation (Sinpru et al., 2017). Moreover, the numbers of MT-ir neurons within the SOv, POM, and PVN increase when hens make the transition from incubating to rearing behavior (Sinpru et al., 2018).

2.7.4 The Regulation of Mesotocin Secretion

To date, there are limited data available to elucidate the regulation of MT secretion in avian species. In native Thai chickens, the numbers of MT-ir neurons increase within the SOv, POM, and PVN during rearing behavior and decrease when chicks are removed from hens (Chokchaloemwong et al., 2013). Moreover, when replacing eggs with chicks in incubating turkeys, MT-ir neurons are mainly found within the SOv and PVN (Thayananuphat et al., 2011). Recently, it has been reported that the presence of eggs or chicks is associated with increased numbers of MT-ir neurons within the SOv, POM, and PVN during the transition from incubating to rearing behavior (Sinpru et al., 2017; 2018). Taken together, these findings suggest that the presence of either eggs or chicks may regulate the MTergic system during maternal behaviors in birds.

2.8 Parental Behaviors

2.8.1 Parental Behavior in Birds

For successful reproduction, the survival of the young until reproduction has marked effects on population growth and is more sensitive to environmental changes than adult survival (Stearns, 1992; Clark and Martin, 2007). Parental care behavior is an important key to promote the survival and well-being of the offspring. Parental behavior is defined as the behavior of the parents that contributes to the survival of their offspring (Numan and Insel, 2003). The term maternal refers to the mother and paternal refers to the father. Maternal behavior is defined as the collection of behaviors by the mother that can increase offspring survival (Kinsley, 1990). Nurturing behaviors analogous to maternal behaviors are called paternal behavior by fathers/male mating partners and alloparental behavior by older conspecifics (Kuroda et al., 2011). Furthermore, the factors that may influence male parental behaviors and hormonal changes are stimuli from the pregnant female and stimuli from the newborn, whereas maternal behaviors are influenced by the maternal hormones of the female and stimuli from the offspring (Ziegler, 2000).

In birds, parental behavior includes nest preparation, egg laying into a preferred site such as a nest, egg incubation, and post-hatch care of the young to independence (Rosenblatt, 2003; Ruscio and Adkins-Regan, 2004). One or both parents must incubate their eggs until hatching and then provide post-hatching care. The extent of parental care for their eggs and chicks is depended upon the developmental maturity of the hatchling such as precocial and altricial chicks. Care of the young ranges from guarding and guiding in the most precocial species such as Anseriformes and Galliformes to provisioning of all food and intensive brooding for

thermoregulation in altricial species such as Passeriformes and Psittaciformes (Vleck, 1998). However, some avian species express the most precocial chicks which require no post-hatchcing care. In megapodes such as Australian brush-turkeys that show no parental care, they lay eggs in underground nests and these eggs are incubated by external heat sources and then the chicks dig out of their nests by themselves and live independently of their parents and their siblings (Goth, 2002; Goth and Vogel, 2003). Moreover, in the common cuckoos and the brown headed cowbirds, the parasitic species, they lay their eggs in the nests of other species and let them to raise their young (Winfree, 1999; Kruger, 2007). Likewise, in some duck species such as goldeneye ducks that show intraspecific brood parasitism, the females lay their eggs in the nest of other females (Andersson and Eriksson, 1982).

2.8.2 Paternal Behavior

Paternal care refers to behaviors performed by the mature male, which has a positive influence on development, growth, well-being, and survival of the offspring (Fernandez-Duque et al., 2009). Among the major vertebrate taxa, behaviors in species in the Class Aves are the most extensively studied as birds exhibit an incomparable balance of tractability, diversity, and cognitive complexity (Rosenblatt, 2003; Kentner et al., 2010; Goodson et al., 2012b). Reproductive efforts in birds are extended past fertilization with a variety of parental behaviors. Patterns of parental care range from brood parasitism, in which eggs are laid in the nests of a host species and display no parental behavior to intensively prolonged egg incubation and care of offspring by parents. More than 99 % of the 9,000 species of birds exhibit parental behaviors (Silver et al., 1985). Unlike other vertebrate species, more than 90 % avian species exhibit biparental care (Kendeigh, 1952; Lack, 1968) in various degrees

between species (Schradin and Anzenberger, 1999; Vleck and Vleck, 2011). More than 70 % rear altricial young (Silver et al., 1985). These are poorly developed and helpless at hatch. They depend on their parents to provide food, heat, and protection for an extended period (Buntin, 1996). The remaining 30 % species of birds rear precocial young (Buntin, 2010). These are well developed and able to leave the nest, follow their parents, and forage for food on their own after hatching. In precocial species, parental care after hatching consists of brooding the chicks to keep them warm, defense of the chicks, and leading them to food sources and shelter (Buntin, 2010).

Paternal care can be divided into two patterns: direct and indirect. Direct paternal care includes all activities that fathers do for their young that exert an immediate physical influence on them and are thought to increase their survival rate, such as feeding, warning, and playing. Indirect paternal care includes the activities that fathers do for the young independently of the presence of the young but that have an advantage for them. For example, fathers defend a territory to maintain critical resources (Schradin and Auzenberger, 1999). The neuronal and hormonal control of paternal behavior has been extensively investigated, and there are many hormones associated with paternal care such as PRL, sex steroids, glucocorticoids, OT, and VP (Moore, 1992; Schradin and Anzenberger, 1999; Ziegler, 2000; Bales et al., 2004; Wynne-Edwards and Timonin, 2007).

2.9 References

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CHAPTER III

DISTRIBUTION OF HYPOTHALAMIC VASOACTIVE INTESTINAL PEPTIDE IMMUNOREACTIVE NEURONS IN THE MALE NATIVE THAI CHICKEN

3.1 Abstract

Avian prolactin (PRL) secretion is under stimulatory control by the PRL-releasing factor (PRF), vasoactive intestinal peptide (VIP). The neuroendocrine regulation of the avian reproductive system has been extensively studied in females. However, there are limited data in males. The aim of this study was to elucidate the VIPergic system and its relationship to PRL and testosterone (T) in the male native Thai chicken. The distributions of VIP-immunoreactive (-ir) neurons and fibers were determined by immunohistochemistry. Changes in VIP-ir neurons within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) areas were compared across the reproductive stages. Plasma levels of PRL and T were determined by enzyme-linked immunosorbent assay and then compared across the reproductive stages. The results revealed that the highest accumulations of VIP-ir neurons were concentrated only within the IH-IN, and VIP-ir neurons were not detected within other hypothalamic nuclei. Within the IH-IN, VIP-ir neurons were low in premature and aging males and markedly increased in mature males. Changes in VIP-ir neurons within the IH-IN were directly mirrored with changes in PRL and T

levels across the reproductive stages. These results suggested that VIP neurons in the IH-IN play a regulatory role in year-round reproductive activity in males. The present study also provides additional evidence that VIP is the PRF in non-seasonal, continuously breeding equatorial species.

3.2 Introduction

Avian prolactin (PRL) secretion and its gene expression are tonically stimulated (Kragt and Meites, 1965; Bern and Nicoll, 1968) by vasoactive intestinal peptide (VIP), the avian PRL releasing factor (PRF), which is secreted from neurons located in the infundibular nuclear complex (INF) of the hypothalamus (Chaiseha and El Halawani, 2015). In female birds, VIP acts directly on the pituitary gland to stimulate PRL synthesis and secretion during the reproductive cycle (El Halawani et al., 1997). Immunocytochemical studies have reported that VIP-immunoreactive (-ir) neurons within the INF and VIP-ir fibers in the median eminence (ME) correspond to the enhanced circulating PRL levels in turkeys and native Thai chickens (Mauro et al., 1989; Kosonsiriluk et al., 2008). Increases in the number and size of VIP-ir neurons within this hypothalamic region have also reported in domesticated pigeons and ring doves during periods of hyper-prolactinemia (Peczely and Kiss, 1988; Cloues et al., 1990).

The distributions of VIP-containing neurons and fibers have been mapped in many avian species such as Pekin ducks (Korf and Fahrenkrug, 1984), bantams (Macnamee et al., 1986), pigeons (Hof et al., 1991), ring doves (Norgren and Silver, 1990), dark-eyed juncos (Saldanha et al., 1994), chicks (Kuenzel et al., 1997), turkeys (Chaiseha and El Halawani, 1999), Japanese quails (Teruyama and Beck,

2001), collared doves (Den Boer-Visser and Dubbeldam, 2002), starlings (Dawson et al., 2002), zebra finches (Kingsbury et al., 2015), blue tits (Montagnese et al., 2015), and native Thai chickens (Kosonsiriluk et al., 2008). In the native Thai chicken, VIP-ir neurons and fibers are extensively distributed throughout the brain and are predominantly expressed in the diencephalon, where VIP-ir neurons are concentrated within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) areas. Changes in the number of VIP-ir neurons within the IH-IN are directly correlated to plasma PRL levels across the reproductive cycle (Kosonsiriluk et al., 2008), and the number of VIP-ir neurons decreases concurrently with circulating PRL levels in nest-deprived incubating and disruption of rearing behavior hens (Prakobsaeng et al., 2011; Chaiyachet et al., 2013b). These findings suggest that VIP expression in the IH-IN plays a regulatory role in year-round reproductive activity and indicate its importance in the regulation of reproductive activity in this species (Kosonsiriluk et al., 2008).

The native Thai chicken, an equatorial, tropical, non-seasonally breeding species, has been domesticated without genetic selection. It expresses strong maternal behaviors, which are inherited from the ancestor, the wild jungle fowl (Sawai et al., 2010). It is well established that the neuroendocrine regulation of maternal behaviors (incubation and rearing behaviors) in the female native Thai chickens are associated with the gonadotropin releasing hormoneergic (GnRHergic), VIPergic, dopaminergic (DAergic), and mesotocinergic (MTergic) systems (Prakobsaeng et al., 2011; Sartsoongnoen et al., 2012; Chaiyachet et al., 2013a; 2013b; Chockchaloemwong et al., 2013; 2015). Recently, behavioral endocrine studies in galliform birds have focused on the roles of several neurotransmitters, neurohormones, and hormones that

function in maternal care behaviors. As indicated, the neuroendocrine regulation of rearing behavior has been extensively studied, particularly in females. However, there are limited data regarding the neuroendocrine regulation of parental behaviors in males. Indeed, in many species, male birds show parental care behaviors such as nest building, brooding, and feeding of the young (Chaiseha and El Halawani, 2015; Lynn et al., 2015). These phenomenons involving parental behaviors may occur due to a complex neuronal/hormonal interaction of many hormones, neurohormones, neuromodulators, and neurotransmitters.

To date, there has been no report on the VIPergic system of the male native Thai chicken. The aim of this study was to delineate the hypothalamic VIPergic system in the male native Thai chicken and investigate its relationship to PRL and testosterone (T). The findings of the differential distribution of hypothalamic VIP neurons and fibers and their associated circulating levels of PRL and gonadal steroids may provide an insight into the role(s) of the VIP/PRL system in the neuroendocrine regulation of reproductive activities in male galliform birds.

3.3 Materials and Methods

3.3.1 Experimental Animals

Male native Thai chickens (*Gallus domesticus*), ranging between 5 and 36 months old and mature females, ranging between 20 and 24 weeks old, were used. They were reared and housed (8-10 females: 1 male) in floor pens equipped with basket nests under natural light (approximately 12 h of light and 12 h of darkness; 12L:12D). Feed and water were given *ad libitum*. The animal protocols used adhered

to the guidelines approved by the Suranaree University of Technology Animal Care and Use Committee.

3.3.2 Experimental Procedures

3.3.2.1 Experiment 1: Distribution and Localization of VIP-ir Neurons and Fibers in the Hypothalamus of the Male Native Thai Chicken

To determine the distributions of VIP-ir neurons and fibers in the hypothalamus of the male native Thai chicken, 6 mature males (about 12 months old) were used. The brains were fixed by pressure perfusion with 4 % paraformaldehyde prior to sectioning in a cryostat and further processing by immunohistochemistry (IHC).

3.3.2.2 Experiment 2: Changes in Number of VIP-ir Neurons in the IH-IN Area and Plasma Levels of PRL and T across the Reproductive Stages of the Male Native Thai Chicken

To compare changes in the number of VIP-ir neurons within the IH-IN and plasma PRL and T levels, 21 male native Thai chickens, 5-36 months old, were used. The males were divided into 3 groups (7 birds/group) according to their reproductive stages: Group 1, premature male, 6months old; Group 2, mature male, 12 months old; and Group 3, aging male, 36 months old. Birds were sacrificed according to their reproductive stages. Blood samples were collected from the brachial vein of each bird. The blood samples were then fractionated by centrifugation, and the plasma was stored at -20 °C until assayed for PRL and T. The brains were fixed by pressure perfusion with 4 % paraformaldehyde, sectioned with a cryostat, and processed by IHC. A postmortem examination of each male was performed to confirm its reproductive stage.

3.3.3 Measurement of Plasma PRL Levels

Plasma PRL levels were measured using an enzyme-linked immunosorbent assay (ELISA) according to a previously described method (Kosonsiriluk et al., 2008). The plasma PRL levels determined by this assay in native Thai chickens were validated using the parallelism test (Kosonsiriluk et al., 2008). All samples were determined in duplicate within a single assay. The intra-assay coefficient of variation was 9.2 % and the sensitivity of the assay was 3.9 ng/ml.

3.3.4 Measurement of Plasma T Levels

Plasma T levels were determined utilizing an ELISA according to a previously described method (Mobarkey et al., 2010). Dilutions of primary antibody and tracer were 1:250,000 and 1:250 for T. The assay of plasma T levels in native Thai chickens was validated as follows. Pooled plasma samples of native Thai chickens produced a dose-response curve that paralleled a chicken T standard curve. Plasma samples were determined in duplicate within a single assay. The intra-assay coefficient of variation was 5.7 % and the sensitivity of the assay was 0.78 pg/ml.

3.3.5 Tissue Preparation and Immunohistochemistry

To determine the distributions of VIP-ir neurons and fibers in the hypothalamus of the male native Thai chicken, brain tissue sections were prepared. Briefly, after collecting a blood sample, each bird was intravenously injected with 3 ml of heparin (Baxter Healthcare Corporation, Deerfield, IL, USA; 1000 unit/ml) and then euthanized with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France; 2 ml/kg). The head was removed and immediately fixed by pressure-perfusion via the carotid arteries. Reagents included 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 3-5 min, followed by freshly prepared 4 %

paraformaldehyde (pH 7.4) for 30 min according to a previously described method (Sartsoongnoen et al., 2008). The brain with the pituitary gland attached was then removed from the skull and for cryo-protection was placed in 20 % sucrose in PBS at 4 °C for 48 h or until saturated. The brain was then frozen in powdered dry ice for 1 h, and stored at -35 °C until sectioned. Frozen brains were sectioned in the coronal plane at a thick-ness of 16 µm using a cryostat (Leica CM1850, Leica Instruments GMbH, Nussioch, Germany). Sections were mounted onto gelatin-subbed slides with 2 sections per slide and stored desiccated at -20 °C until further processed for IHC. IHC was performed according to a previously described method (Chaiyachet et al., 2013b). The primary and secondary antibodies used for detecting VIP immunoreactivity were VIP primary antibody (polyclonal anti-chicken VIP antiserum (VIP4-DYC8); generously provided by Dr. M.E. El Halawani, University of Minnesota, USA) and Cy^{TM}3-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), respectively. VIP immunoreactivity using these antibodies has been previously described (Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011; Chaiyachet et al., 2013b).

3.3.6 Image Analysis

Microscopic images of the brain sections were visualized under a fluorescence microscope (Olympus IX71, Tokyo, Japan) fitted with a cooled digital color camera (Olympus DP70, Tokyo, Japan). The images were captured and stored by DP70-BSW software (Olympus, Tokyo, Japan). VIP-ir neurons in each individual hypothalamic area were visualized. The number of VIP-ir neurons in the IH-IN was quantified as previously described (Chaiyachet et al., 2013b). Briefly, 4 adjacent sections were manually counted to determine changes in the number of VIP-ir

neurons in the IH-IN. The number of VIP-ir neurons was then averaged across the 4 sections for each bird and for each treatment group to determine the number of VIP-ir neurons counted per section in the IH-IN area. The mean values were compared among treatment groups. To aid in the documentation of neuroanatomical results, the nomenclature and schematic diagrams from the stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988) and the chicken hypothalamus (Kuenzel and van Tienhoven, 1982) were used to illustrate VIP immunoreactivity. The specificity of the VIP antibody was previously tested (Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011; Chaiyachet et al., 2013b).

3.3.7 Statistical Analysis

Significant differences among treatment groups in VIP-ir neurons and plasma PRL and T levels (means ± SEM) at different reproductive stages were compared employing one-way analysis of variance. Significant differences between treatment groups were computed utilizing Tukey's HSD Test. $P < 0.05$ was considered statistically significant. All statistical tests were analyzed using SPSS (2007) for Windows software (version 13.0, SPSS, IBM, Chicago, IL, USA).

3.4 Results

3.4.1 Distribution and Localization of VIP-ir Neurons and Fibers in the Hypothalamus of the Male Native Thai Chicken

The highest accumulations of VIP-ir neurons and fibers occurred within the IH-IN, and most of the VIP-ir neurons were found in the caudal portion, whereas there were only a few VIP-ir neurons found in the rostral portion of the IH-IN (Figures 3.1 and 3.2). Dense accumulation of VIP-ir fibers was observed in the

nucleus suprachiasmaticus (SCNm), nucleus anterior medialis hypothalami (AM), regio lateralis hypothalami (LHy; Figures 3.3A, B, and C), nucleus paraventricularis magnocellularis (PVN), nucleus ventromedialis hypothalami (VMN), nucleus periventricularis hypothalami (PHN), and nucleus commissurae pallii (nCPa; Figure 3.4). Very dense accumulations of VIP-ir fibers were found in the external layer of the ME (Figure 3.2B).

3.4.2 Changes in the Number of VIP-ir Neurons in the IH-IN Area and Plasma PRL and T Levels across the Reproductive Stages of the Male Native Thai Chicken

Changes in the number of VIP-ir neurons within the IH-IN (7 birds/group) were observed across the male reproductive cycle (Figures 3.5A and 3.6). VIP-ir neurons were low in premature males (39.57 ± 3.83 cells) and markedly increased (61.21 ± 6.80 cells; $P < 0.05$) in mature males. In aging roosters, the number of VIP-ir neurons was decreased (41.89 ± 2.40 cells), equaling that of premature males.

Plasma PRL levels (6 birds/group) were found to be low in premature males (13.43 ± 1.94 ng/ml), and increased to the highest levels (31.38 ± 8.06 ng/ml; $P < 0.05$) in mature males. When the roosters began aging, circulating PRL levels dropped to a low level (8.64 ± 1.76 ng/ml; Figure 3.5B).

Plasma T levels (5 birds/group) are shown in Figure 3.5C. Pre-mature males had low levels of T (1.61 ± 0.25 ng/ml), and the levels increased (3.43 ± 0.49 ng/ml; $P < 0.05$) in mature males. In aging roosters, circulating T levels declined to a low level (2.85 ± 0.24 ng/ml).

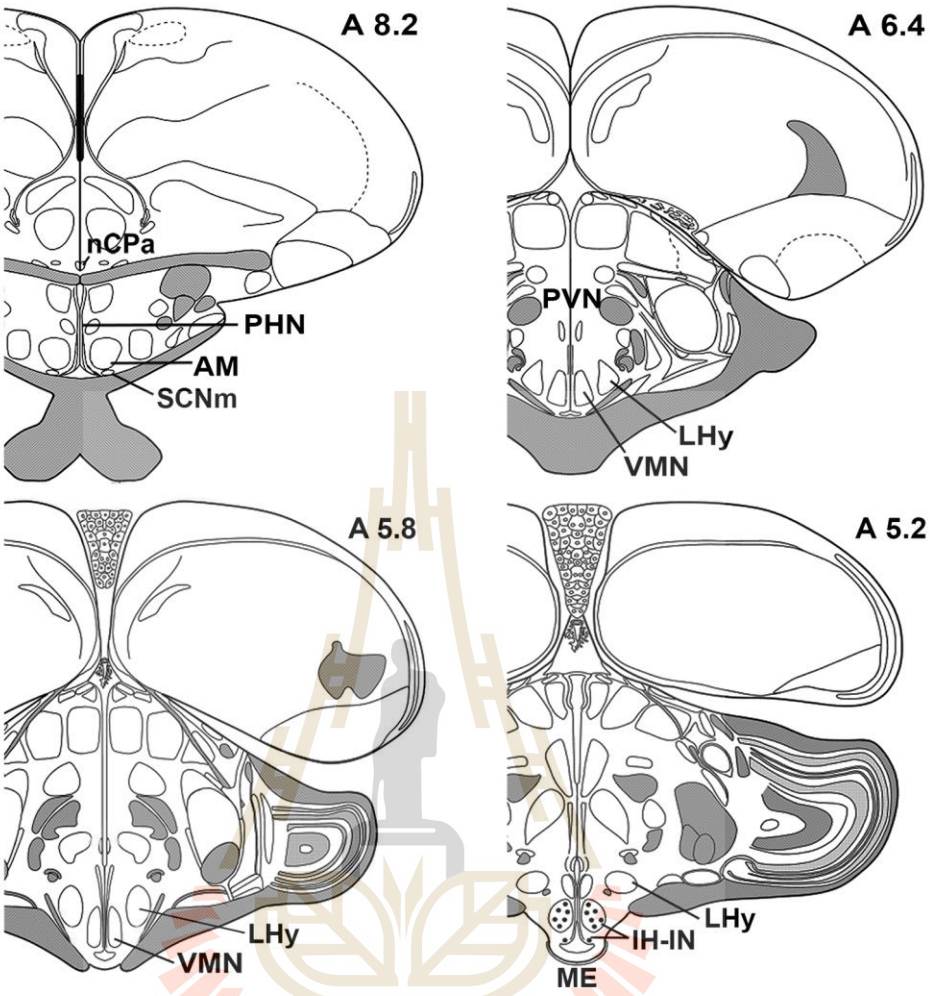

Figure 3.1 Schematic diagrams of coronal sections illustrating the areas of the chick brain showing the distribution of VIP-ir neurons (black dots) and fibers throughout the brain of the male native Thai chicken. Coronal illustrations were redrawn from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988). The following abbreviations are used in the figure legends: AM, nucleus anterior medialis hypothalami; IH, nucleus inferioris hypothalami; IN, nucleus infundibuli hypothalami; LHy, regio lateralis hypothalami; ME, eminentia mediana (median eminence); nCPa, nucleus commissurae pallii; PHN, nucleus periventricularis hypothalami; PVN, nucleus paraventricularis magnocellularis; SCNm, nucleus suprachiasmaticus; VMN, nucleus ventromedialis hypothalami.

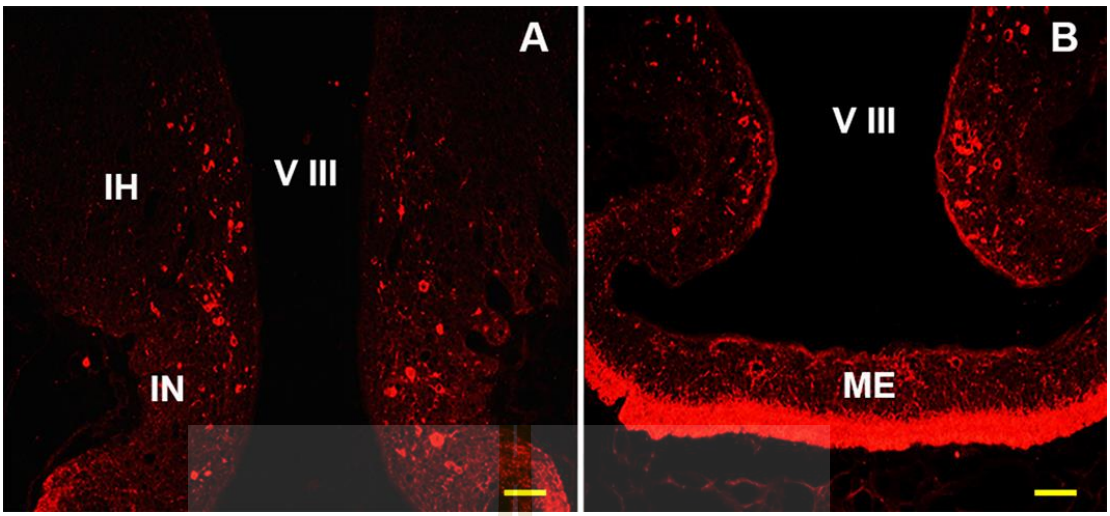

Figure 3.2 Photomicrographs illustrating VIP-ir neurons and fibers in the IH-IN (A and B) and a very dense accumulation of VIP-ir fibers in the ME (B). V III, ventriculus tertius (third ventricle). Scale bar = 100 µm. For abbreviations, see legend of Figure 3.1.

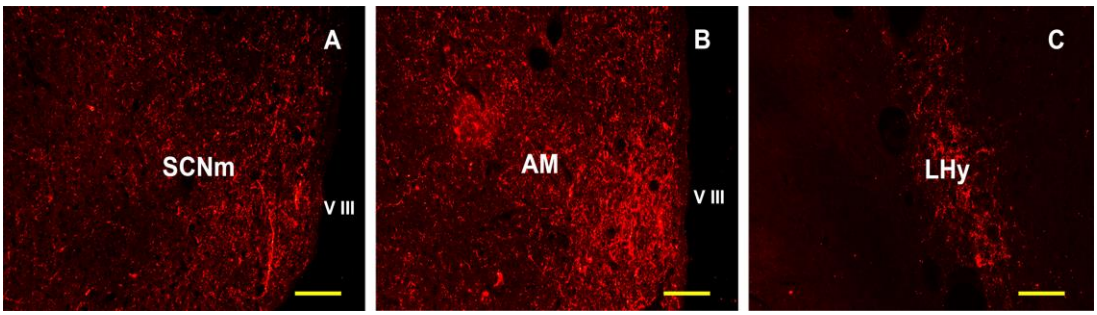

Figure 3.3 Photomicrographs of coronal sections in the hypothalamus demonstrating the dense accumulation of VIP-ir fibers in the SCNm (A), AM (B), LHy (C). V III, ventriculus tertius (third ventricle). Scale bar = 50 µm. For abbreviations, see legend of Figure 3.1.

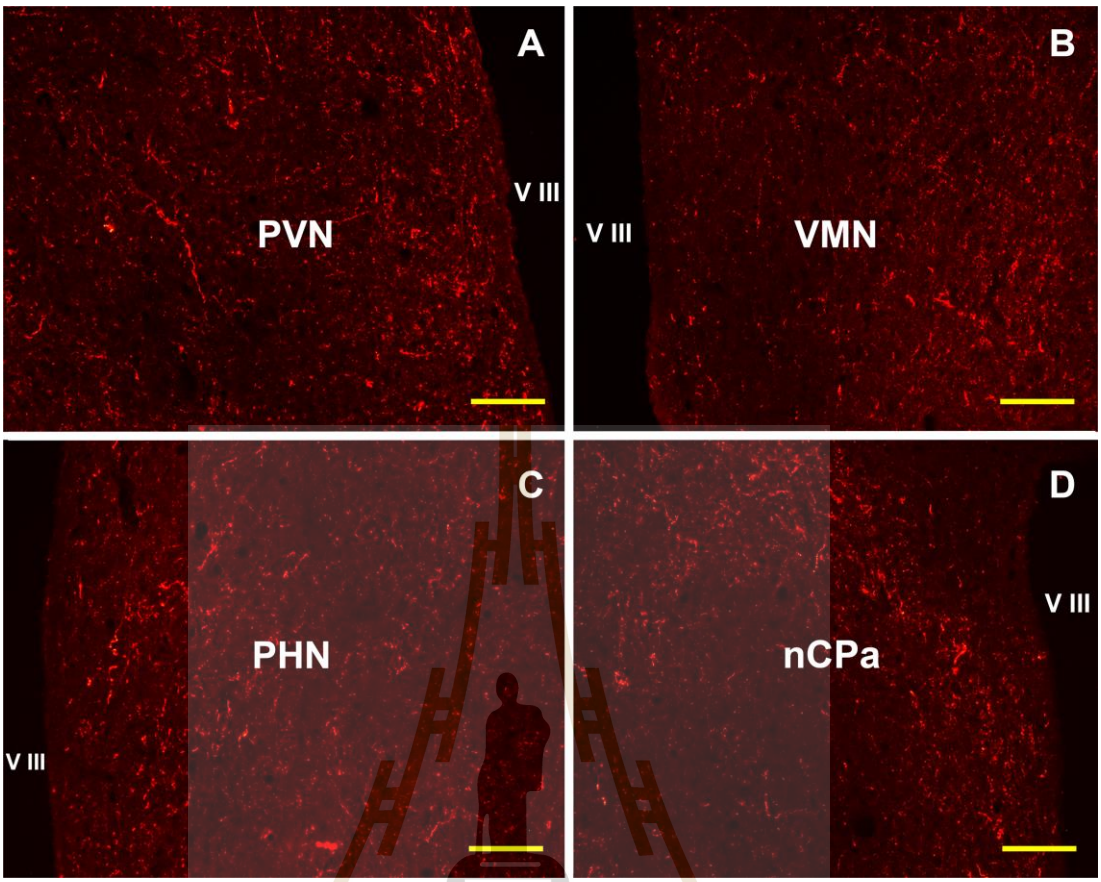

Figure 3.4 Photomicrographs of coronal sections in the hypothalamus showing VIP-ir fibers in the PVN (A), VMN (B), PHN (C), nCPa (D). V III, ventriculus tertius (third ventricle). Scale bar = 50 µm. For abbreviations, see legend of Figure 3.1.

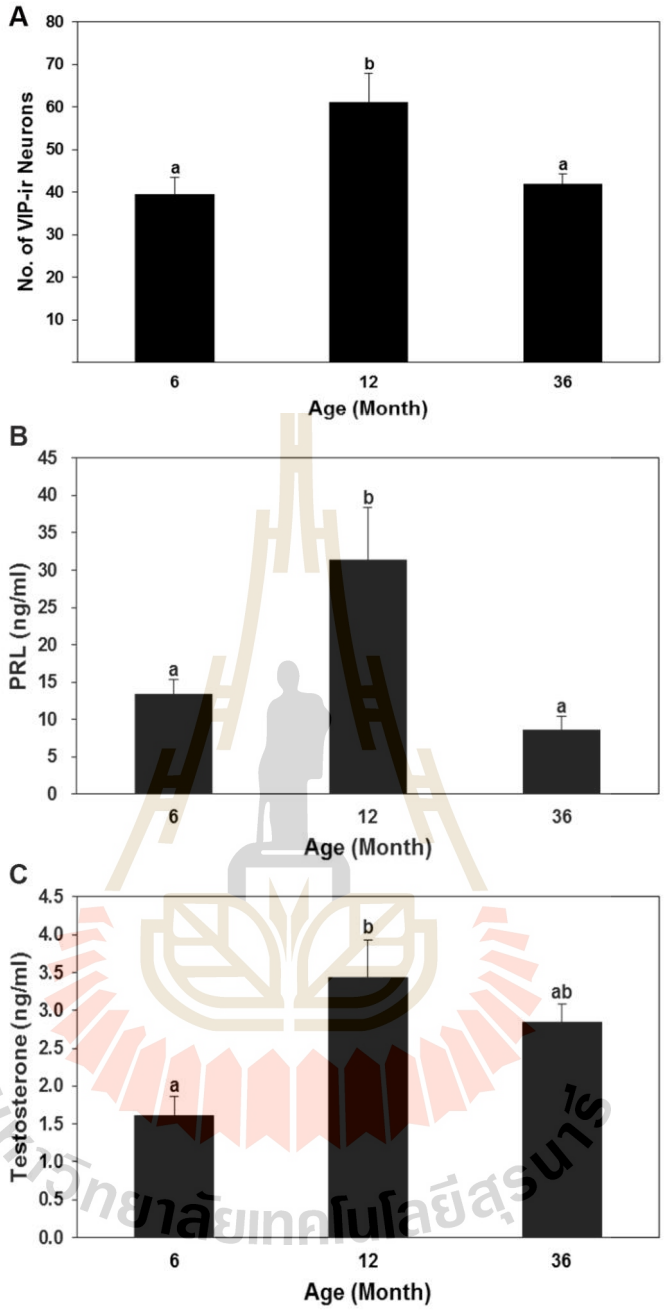

Figure 3.5 (A) Changes in the number of VIP-ir neurons in the IH-IN (7birds/group), (B) plasma PRL levels (6 birds/group), and (C) plasma T levels (5 birds/group) during the reproductive stage of the male native Thai chickens. Values are presented as mean ± SEM. Significant differences between means in each group are denoted by different letters ($P < 0.05$).

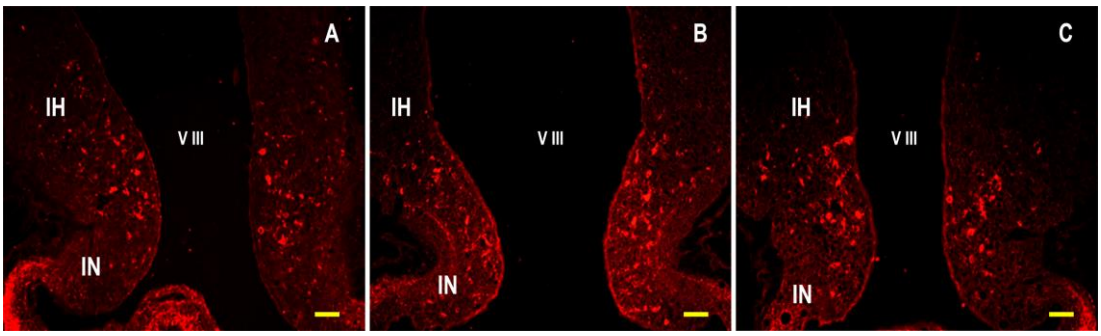

Figure 3.6 Photomicrographs showing the distributions of VIP-ir neurons in the IH-IN during the reproductive stage of the native Thai chickens; premature male (A), mature male (B), aging male (C). V III, ventriculus tertius (third ventricle). Scale bar = 100 µm. For abbreviations, see legend of Figure 3.1.

3.5 Discussion

The results of the present study demonstrate a relationship between the VIPergic system and circulating PRL and T levels during the reproductive cycle of the male native Thai chicken. VIP-ir neurons and fibers were found predominantly concentrated within the IH-IN area. VIP-ir fibers were found distributed throughout the hypothalamus, and a dense accumulation of VIP-ir fibers was found in the external layer of the ME. Changes in VIP-ir neurons within the IH-IN were directly mirrored with changes in circulating PRL and T levels throughout the reproductive cycle of the male native Thai chicken. The present results provide evidence supporting that VIP neurons in the IH-IN of the native Thai chicken play a regulatory role in its year-round reproductive activity in males. This study also provides additional evidence that VIP is the PRF in non-seasonal, continuously breeding equatorial species, as the number of VIPergic neurons paralleled the increase in plasma PRL.

The distributions of VIP neurons and fibers have been mapped in several avian species (El Halawani et al., 1997; Chaiseha and El Halawani, 2015; Kingsbury et al., 2015; Montagnese et al., 2015). The present results of the distributions of hypothalamic VIP neurons and fibers in the male native Thai chicken do not correspond with previous reports in the female native Thai chicken (Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011; Chaiyachet et al., 2013b). In the male native Thai chicken, the highest accumulations of VIP-ir neurons were concentrated within the IH-IN, and VIP-ir neurons were not detected within the SCNm, AM, LHy, PVN, and VMN, in which VIP-ir neurons were found in the female brain (Kosonsiriluk et al., 2008). Thus, it could be that different sexes of birds will show characteristic

differences in the distribution of VIP neurons. In the present study, dense accumulations of VIP-ir fibers were observed within the AM, LHy, VMN, PVN, PHN, and nCPa. A very dense accumulation of VIP-ir fibers was found in the external layer of the ME. It has been suggested that VIP in the ME is derived from neurons located within the IH-IN (Chaiseha and El Halawani, 2015). The present results showing the distribution of VIP-ir neurons was found concentrated only within the IH-IN area and VIP-ir fibers were observed throughout the hypothalamic nuclei and ME in the male native Thai chickens corresponds with previous reports in the male blue tits (Montagnese et al., 2015). However, Kingsbury et al. (2015) reported that VIP neurons were observed within the AM, VMN, and INF in nesting male zebra finches, suggesting that VIP is correlated with nesting behavior.

VIP is well accepted as the avian PRF (Chaiseha and El Halawani, 2015). In this study, circulating PRL and T were at low levels in premature males and then markedly increased to the highest levels in mature roosters. When the roosters began aging, circulating PRL and T levels declined to a lower level not different from those of premature males. These changes in circulating PRL and T levels paralleled the changes in VIP-ir neurons within the IH-IN. In female birds, previous studies have reported that changes in VIP immunoreactivity and gene expression within the native Thai chicken IH-IN and the turkey INF parallel changes in circulating PRL levels (Mauro et al., 1989; Chaiseha and El Halawani, 1999; Kosonsiriluk et al., 2008). In addition, the number of VIP neurons decreased concurrently with circulating PRL levels in nest-deprived incubating and disruption of rearing behavior hens (Prakobsaeng et al., 2011; Chaiyachet et al., 2013b). Additionally, in ring doves, domesticated pigeons, and bantam hens, increases in the number of VIP neurons and

cell size are observed in the ventral infundibulum, anticipating the increase in plasma PRL levels (Peczely and Kiss, 1988; Sharp et al., 1989; Cloues et al., 1990). In male chickens, active immunoneutralization against VIP decreases plasma PRL levels and mRNA expressions of pituitary PRL, luteinizing hormone, follicle stimulating hormone, VIP, and GnRH (Avital-Cohen et al., 2011; 2012). High levels of plasma PRL and high mRNA expressions of VIP and PRL mRNA have been reported in aging roosters (Avital-Cohen et al., 2013).

PRL has long been known to play a significant role in parental care in both birds and mammals. More than 300 different physiological functions of PRL have been documented in areas such as reproduction, osmoregulation, growth and development, brain and behavior, metabolism, and immunoregulation and behaviors such as migration and the nurturing of the young, highlighting the significant role of this pluripotent hormone (Houdebine, 1983; Bole-Feysot et al., 1998; Harris et al., 2004). In female birds, PRL has a well-established role as an incubation-promoting hormone, and it has also been implicated to be involved in the onset and maintenance of rearing behavior (Chaiseha and El Halawani, 2015). In the galliform and columbiform birds, PRL plays a pivotal role in a series of parental behaviors by mediating increases in incubation behavior, crop milk secretion, feeding of young, and nest defense (Schradin and Anzenberger, 1999; Ziegler, 2000; Buntin, 2010; Chaiseha and El Halawani, 2015). Indeed, more than 99 % of the 9000 species of birds exhibit parental behaviors and more than 90 % of avian species exhibit biparental care in various degrees between species (Buntin, 1996; 2010; Schradin and Anzenberger, 1999; Vleck and Vleck, 2011). Among species in which parental care is provided by the female only, pied flycatchers, circulating PRL levels are more

elevated in females than in males, whereas in species in which parental care is provided only by the male, Wilson's phalaropes and red-necked phalaropes, circulating PRL levels are higher in males than in females during the parental phase of the breeding cycle (Buntin, 1996; 2010; Schradin and Anzenberger, 1999). In addition, a positive correlation between the intensity/quality of parental care and PRL levels has been observed in many parental birds and cooperative breeders. In the cooperatively breeding species, Florida scrub-jays and Harris' hawks, PRL levels in helpers are positively correlated with the rate of nestling provisioning (Dawson and Mannan, 1991; Schoech et al., 1996; Angelier and Chastel, 2009).

The role of PRL in parental care in males has been investigated in several vertebrate species (Harris et al., 2004). PRL has found to be associated with paternal care in fish (de Ruiter et al., 1986; Kindler et al., 1991), birds (ring doves; Buntin, 1996; King penguins; Garcia et al., 1996; scrubjays; Schoech et al., 1996; dark-eyed juncos; Schoech et al., 1998; songbirds; Van Roo et al., 2003), rodents (Schradin and Pillay, 2004; Carlson et al., 2006), primates (Ziegler et al., 2000; Schradin et al., 2003; da Silva Mota et al., 2006), and human (Fleming et al., 2002), showing an increase in PRL levels among animals participating prior to and during infant care. It has been suggested that PRL in males is associated with the onset of paternal behaviors rather than maintenance of these behaviors. An association between elevated PRL levels and male parental behaviors has been previously indicated in a number of avian species (Buntin, 2010). In male ring doves, rising and lowering PRL levels have suggested that PRL plays a direct role in stimulating incubation behavior, the formation of crop milk, and the frequency of chick feeding behavior (Lea and Sharp, 1989; Koch et al., 2004). Circulating PRL levels increase in both male and

female breeders during various stages of nest building, egg laying, incubating, and feeding of the young (Ziegler, 2000). The inverse relationship between PRL and gonadal steroids during paternal care behaviors has been reported. In many avian and mammalian species, elevated androgen levels have often been found to interfere with the exhibition of paternal behaviors, and androgen levels tend to be lower during the paternal care period (Wingfield et al., 1990; Logan and Wingfield, 1995; Hirschenhauser et al., 2003; McGlothlin et al., 2007). In song sparrows and white-crowned sparrows, T concentrations increase during the breeding season, but decrease while the males care for the young. T increases again as the males begin to mate and guard for the second clutch (Wingfield and Farner, 1978; Wingfield, 1984).

In summary, the present findings characterize the VIP/PRL system and its relationship to PRL and T in the male native Thai chicken. VIP-ir neurons and fibers were predominantly located within the IH-IN. Changes in VIP-ir neurons within the IH-IN were directly mirrored with changes in circulating PRL and T levels throughout the reproductive stages, suggesting that VIP neurons in the IH-IN also play a regulatory role in its year-round reproductive activity in male native Thai chickens. The present study also provides additional evidence that VIP is the PRF in non-seasonal breeding, continuously breeding equatorial species.

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CHAPTER IV

DISTRIBUTION OF MESOTOCIN-IMMUNOREACTIVE NEURONS IN THE BRAIN OF THE MALE NATIVE THAI CHICKEN

4.1 Abbreviations

AM, nucleus anterior medialis hypothalami; BST, bed nucleus of the stria terminalis; Cb, cerebellum; DA, dopamine; DLAmc, nucleus dorsolateralis anterior thalami, pars magnocellularis; GCt, substantia grisea centralis; HL, nucleus habenularis lateralis; IH, nucleus inferioris hypothalami; IN, nucleus infundibuli hypothalami; -ir, -immunoreactive; LHy, regio lateralis hypothalami; ME, eminentia mediana (median eminence); MPOA, area praeoptica medialis; MT, mesotocin; NR, non-rearing hens; OM, tractus occipitomesencephalicus; OT, oxytocin; OVLT, organum vasculosum lamina terminalis; PHN, nucleus periventricularis hypothalami; POA, area praeoptica; POM, nucleus preopticus medialis; POP, nucleus preopticus periventricularis; PRL, prolactin; PVN, nucleus paraventricularis magnocellularis; PVO, organum paraventriculare; R, rearing hens; ROT, nucleus rotundus; SCNm, nucleus suprachiasmaticus, pars medialis; SL, nucleus septalis lateralis; SON, nucleus supraopticus; SOv, nucleus supraopticus, pars ventralis; SSO, organum subseptale (supseptal organ); TSM, tractus septomesencephalicus; V III, ventriculus tertius (third ventricle); VIP, vasoactive intestinal peptide; VLT, nucleus ventrolateralis thalami

4.2 Abstract

Mesotocin (MT), a homolog of oxytocin (OT) in mammals, is a nonapeptide neurohypophysial hormone that is mainly synthesized in specific neuronal groups within the hypothalamus and released from the posterior pituitary gland in amphibian, reptilian, and avian species. MT is associated with the neuroendocrine regulation of reproductive cycle and maternal behaviors in female native Thai chickens. Male birds exhibit parental behaviors as well. However, there are limited data regarding the role(s) of the MTergic system in males. Thus, the objective of this study was to elucidate the localization of the MT neuronal groups in the brain of male native Thai chickens. The distributions of MT-immunoreactive (-ir) neurons and fibers in the brain were studied utilizing immunohistochemistry technique. The results revealed that MT-ir neurons and fibers were distributed throughout the brain and extensively in the diencephalon. MT-ir neurons and fibers were predominantly located within the nucleus supraopticus, pars ventralis (SOv), nucleus preopticus medialis (POM), nucleus ventrolateralis thalami (VLT), nucleus paraventricularis magnocellularis (PVN), and regio lateralis hypothalami (LHy), suggesting that MT neurons in these nuclei might be involved in the reproductive activities and/or parental behavior in the male chickens. In addition, the numbers of MT-ir neurons within the SOv and POM were significantly higher than those of the VLT, PVN, and LHy. More importantly, the number of MT-ir neurons in the SOv was high in the male brain when compared with the female brain, indicating that the MTergic system in the SOv might play a significant role in male reproductive activities in this equatorial species.

4.3 Introduction

Mesotocin (MT), a homolog of oxytocin (OT) in mammals, is a nonapeptide neurohypophysial hormone that is synthesized in specific neuronal groups within the hypothalamus and released from the posterior pituitary gland into the hypophysial portal blood via the eminentia mediana (median eminence; ME) in amphibian, reptilian, and avian species (Bentley, 1997). MT in birds and OT in mammals are different at amino acid position 8; MT is isoleucine, but OT is leucine (Acher et al., 1970; Parry et al., 2000). MT is also distributed within several regions of the avian brain (Robinzon et al., 1988a; Thayananuphat et al., 2011; Chokchaloemwong et al., 2013) and several tissues of the reproductive tract including the corpus luteum, follicle, and uterus (Robinzon et al., 1988b). To date, there are a limited number of studies regarding the role(s) of MT in birds. It has been reported that MT facilitates uterine contractions in hens (Takahashi et al., 1997; Takahashi and Kawashima, 2008a; 2008b), acts as a vasodepressor in cockerels (Robinzon et al., 1994), inhibits feeding behavior in chicks (Masunari et al., 2013; 2016), and promotes sociality of female zebra finches and field sparrows (Goodson et al., 2009). The role of MT in avian brooding behavior was first documented in turkeys (Thayananuphat et al., 2011). Moreover, it has been reported that MT is involved in the regulation of the reproductive cycle and rearing behavior in the female native Thai chickens (Chokchaloemwong et al., 2013).

In birds, numerous studies have been reported that MT neurons and fibers are distributed in several regions of the brain such as the nucleus supraopticus, pars ventralis (SOv); ventral part of the nucleus supraopticus (SON), tractus septomesencephalicus (TSM), nucleus preopticus periventricularis (POP), bed

nucleus of the stria terminalis (BST), nucleus paraventricularis magnocellularis (PVN), nucleus habenularis lateralis (HL), nucleus inferioris hypothalami (IH), cerebellum (Cb), lateral septum, optic lobe, tuberomammillary nucleus, pons, and medulla oblongata (Goossens et al., 1977; Bons, 1980; Robinzon et al., 1988a; Barth et al., 1997; Thayananuphat et al., 2011). MT fibers are extensively distributed within internal and external layers of the ME (Goossens et al., 1977; Bons, 1980). Similarly to the previous findings, in female native Thai chickens, the highest accumulations of MT-immunoreactive (-ir) neurons and fibers are concentrated within the SOv, nucleus preopticus medialis (POM), nucleus ventrolateralis thalami (VLT), regio lateralis hypothalami (LHy), and PVN. Changes in the numbers of MT-ir neurons within the SOv, POM, and PVN are associated with the reproductive stages of the native Thai chickens, with the highest density observed in the incubating and rearing hens. In addition, the numbers of MT-ir neurons within the SOv, POM, and PVN of rearing hens (R) are higher than those of non-rearing (NR) hens in these nuclei. These findings indicate that MT-ir neurons play a regulatory role in reproductive activities and the neuroendocrine reorganization to establish and maintain rearing behavior in this species (Chokchaloemwong et al., 2013).

Native Thai chicken (*Gallus domesticus*), an equatorial, tropical, nonseasonally breeding species, is originated from the wild jungle fowl in Southeast Asia. It has been domesticated without genetic selection. It expresses strong maternal behaviors which are inherited from the ancestor, the wild jungle fowl (Austic and Nesheim, 1990; Fumihito et al., 1994; Hillel et al., 2003; Sawai et al., 2010). It is well established that the neuroendocrine regulation of reproductive cycle and maternal behaviors in the female native Thai chickens is associated with gonadotropin

releasing hormone, vasoactive intestinal peptide (VIP), dopamine (DA), prolactin (PRL), and MT (Prakobsaeng et al., 2011; Sartsoongnoen et al., 2012; Chaiyachet et al., 2013a; 2013b; Chokchaloemwong et al., 2013; 2015). As aforementioned, the neuroendocrine regulation of reproductive behaviors has been extensively studied, particularly in females. However, there are limited data regarding the neuroendocrine regulation of reproductive activities in males. Recently, it has been reported that VIP neurons within the IH and nucleus infundibuli hypothalami (IN) and its relationship to PRL and testosterone play a pivotal role in its year-round reproductive activity in the male native Thai chickens (Kamkrathok et al., 2016). In addition, male birds exhibit parental behaviors such as nest building, brooding, and feeding of the young in many species (Chaiseha and El Halawani, 2015; Lynn et al., 2015). To date, there has been no report of the MTergic system in the male native Thai chicken. Thus, the objective of this study was to investigate the localization of the MT neuronal groups in the brain of the male native Thai chickens, enabling further studies of neuroendocrinology related to behavior. The findings of the distributions of MT-ir neurons and fibers might be related to the regulation of reproductive activities and paternal behaviors in the male native Thai chickens.

4.4 Materials and Methods

4.4.1 Experimental Animals

Male native Thai chickens, 12 months of age, were used. They were reared and housed together with mature females (1 male: 8 females) in floor pens equipped with basket nests under natural light (approximately 12 h of light and 12 h of darkness; 12L:12D) with free access to feed and water. The animal protocols used

adhered to the guidelines approved by the Suranaree University of Technology Animal Care and Use Committee.

4.4.2 Experimental Design

To determine the distributions of MT-ir neurons and fibers in the brain of the male native Thai chicken, 6 mature males (12 months of age) were used. The brains of mature roosters were fixed by pressure perfusion with 4 % paraformaldehyde (#416780010, Acros Organics, Inc., New Jersey, USA). Tissue sectioning was performed in the coronal plane at a thickness of 16 µm utilizing a cryostat and further processing by immunohistochemistry according to a previously described method (Chokchaloemwong et al., 2013). In this study, the primary and secondary antibodies used for detecting MT immunoreactivity were primary rabbit polyclonal antibody directed against OT (ImmunoStar, Inc., Hudson, WI, USA, Catalog No. 20068, Lot No. 642001), OT antibody diluted 1:1000 with PBS (pH 7.4), containing 1 % bovine serum albumin (#268130100, Acros Organics, Inc.) and 0.3 % Triton-X 100 (#215680010, Acros Organics, Inc.) and Cy™3-conjugated AffiniPure donkey anti-rabbit IgG at 1:500 dilution with PBS (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA, Code No. 715-165-150, Lot No. 105237), respectively. The specificity of primary antibody against MT was previously tested. The specificity of the antibody used was tested by pre-absorption of the antibody with a synthetic peptide based on the chicken MT sequence. Briefly, tissue sections were incubated overnight with normal rabbit serum, OT antibody diluted 1:1000 with PBS (pH 7.4) and 1 % BSA, and OT antibody pre-absorbed with 10 µg/ml of MT (Bachem, Torrance, CA, USA) or vasotocin (VT; Bachem) diluted 1:1000 with PBS (pH 7.4) and 1 % BSA. Pre-absorption of OT antibody with MT completely abolished the

staining of neuronal cells compared to tissues stained with non-preabsorbed OT antibody or the antibody preabsorbed with VT. Immunostaining with normal rabbit antisera also abolished the staining (Chokchaloemwong et al., 2013).

4.4.3 Image Analysis

Microscopic images of the brain sections were visualized under a fluorescence microscope (Nikon ECLIPSE 80i, Tokyo, Japan) fitted with a cooled digital color camera (Nikon DS-Fi1, Tokyo, Japan). The images were captured and stored by NIS-Elements Documentation software (Nikon, Tokyo, Japan). MT-ir neurons and fibers in each individual area of the brain was visualized and analyzed. The numbers of MT-ir neurons in six adjacent sections were counted manually to determine changes in the number of MT-ir neurons in the individual hypothalamic areas (SOv, POM, VLT, PVN, and LHy). The counted MT-ir neurons from the six adjacent sections for each rooster (6 roosters per area) were averaged to determine the numbers of MT-ir neurons counted per section in each hypothalamic area. The mean values were compared across the hypothalamic areas (Chokchaloemwong et al., 2013). Atlas of the chick brain (Kuenzel and Masson, 1988) and the chicken hypothalamus (Kuenzel and van Tienhoven, 1982) were used for identification of specific brain regions of MT-ir neurons and fibers.

4.4.4 Statistical Analysis

Significant differences among the numbers of MT-ir neurons within the SOv, POM, VLT, PVN, and LHy (mean ± SEM) were compared employing one-way analysis of variance (ANOVA). Significant differences between each individual hypothalamic area were computed utilizing Tukey's HSD Test. $P < 0.05$ was

considered statistically significant. All statistical tests were analyzed using SPSS for Windows software (version 17.0, SPSS Inc., Chicago, IL, USA).

4.5 Results

The results of this study revealed that the distributions of MT-ir neurons and fibers were located throughout the telencephalon, diencephalon, and rhombencephalon. Predominantly, MT-ir neurons and fibers were located on sequential sets of diagrams from the rostral throughout the caudal part of diencephalon. MT-ir neurons were extensively distributed from the preoptic region of the hypothalamus, anterior hypothalamus, and toward the end of the septal region. MT-ir fibers were largely located along the ventriculus tertius (third ventricle; V III), also distributed in a discrete region lying close to the V III from the level of area praeoptica (POA) through the anterior hypothalamus, and very dense fibers were observed in the internal and external layers of the ME (Figure 4.1).

Within the diencephalon, the majority of MT-ir neurons and fibers was distributed in a discrete region lying close to the V III through the anterior hypothalamus (Figures 4.2, 4.3, and 4.4). The greatest density of MT-ir neurons and fibers was located within the SOv (Figures 4.2A and B), POM (Figures 4.2A and C), VLT (Figure 4.2D), PVN (Figure 4.2E), and LHy (Figure 4.2F). Moreover, the numbers of MT-ir neurons in five hypothalamic areas including the SOv, POM, VLT, PVN, and LHy were compared (Figure 4.5). The number of MT-ir neurons is significantly higher ($P < 0.05$, $F_{4,25} = 24.532$) within the SOv (57.17 ± 1.49 cells) and POM (54.83 ± 3.20 cells) when compared with the VLT (34.83 ± 3.57 cells), PVN

(39.54 ± 1.94 cells), and LHy (30.64 ± 0.64 cells). Higher magnification of MT-ir neurons within the SOv and POM was illustrated (Figure 4.3).

A modest density of MT-ir neurons and fibers was observed in the TSM (Figure 4.4A), POP (Figure 4.4B), nucleus suprachiasmaticus, pars medialis (SCNm; Figure 4.4C), nucleus anterior medialis hypothalami (AM; Figure 4.4D), nucleus periventricularis hypothalami (PHN; Figure 4.4E), nucleus dorsolateralis anterior thalami, pars magnocellularis (DLAmc; Figure 4.4F), and tractus occipitomesencephalicus (OM; Figure 4.4G). Moreover, MT-ir neurons were also found lining the cortex layer of the Cb (Figure 4.4H).

In the telencephalon, MT-ir fibers were found in abundance within the nucleus septalis lateralis (SL; Figure 4.6A). However, the greatest density of MT-ir fibers was predominantly located in the diencephalon. These fibers were large and found closely to the midline on both sides of the V III. MT-ir fibers were distributed extensively within the OM (Figure 4.6B), nucleus rotundus (ROT; Figure 4.6C), and substantia grisea centralis (GCt; Figure 4.6D). A few of MT-ir fibers were found in the organum vasculosum lamina terminalis (OVLT; Figure 4.2A), organum subseptale, substantia grisea centralis (SSO; Figure 4.7A), organum paraventriculare (PVO; Figure 4.7B), IH (Figure 4.7C), and very dense fibers were observed in the internal and external layers of ME (Figure 4.7D).

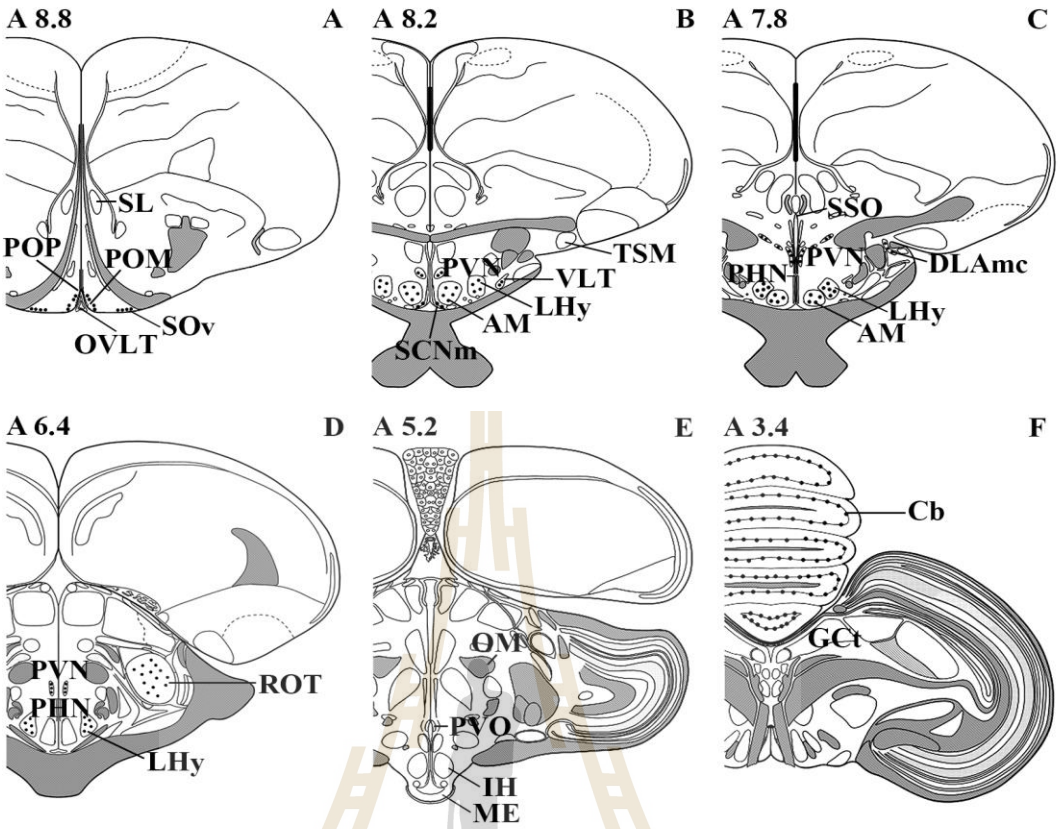

Figure 4.1 Schematic diagrams of coronal sections illustrating the areas of the chick brain showing the distributions of MT-ir neurons (black dots) and fibers throughout the brain of the male native Thai chicken. Coronal illustrations were redrawn from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988). The following abbreviations are used in the figure legends: AM, nucleus anterior medialis hypothalami; Cb, cerebellum; DLAmc, nucleus dorsolateralis anterior thalami, pars lateralis; GCt, substantia grisea centrlis; IH, nucleus inferioris hypothalami; LHy, regio lateralis hypothalami (lateral hypothalamic area); ME, eminentia mediana (median eminence); OM, tractus occipitomesencephalicus; OVLT, organum vaculosum lamina teminalis; PHN, nucleus periventricularis hypothalami; POM, nucleus preopticus medialis; POP, nucleus preopticus periventricularis; PVN, nucleus paraventricularis magnocellularis; PVO, organum paraventriculare; ROT, nucleus

rotundus; SCNm, nucleus suprachiasmaticus, pars medialis; SL, nucleus septalis lateralis; SOv, nucleus supraopticus, pars ventralis; SSO, organum subseptale (supseptal organ); TSM, tractus septomesencephalicus; VLT, nucleus ventrolateralis thalami.

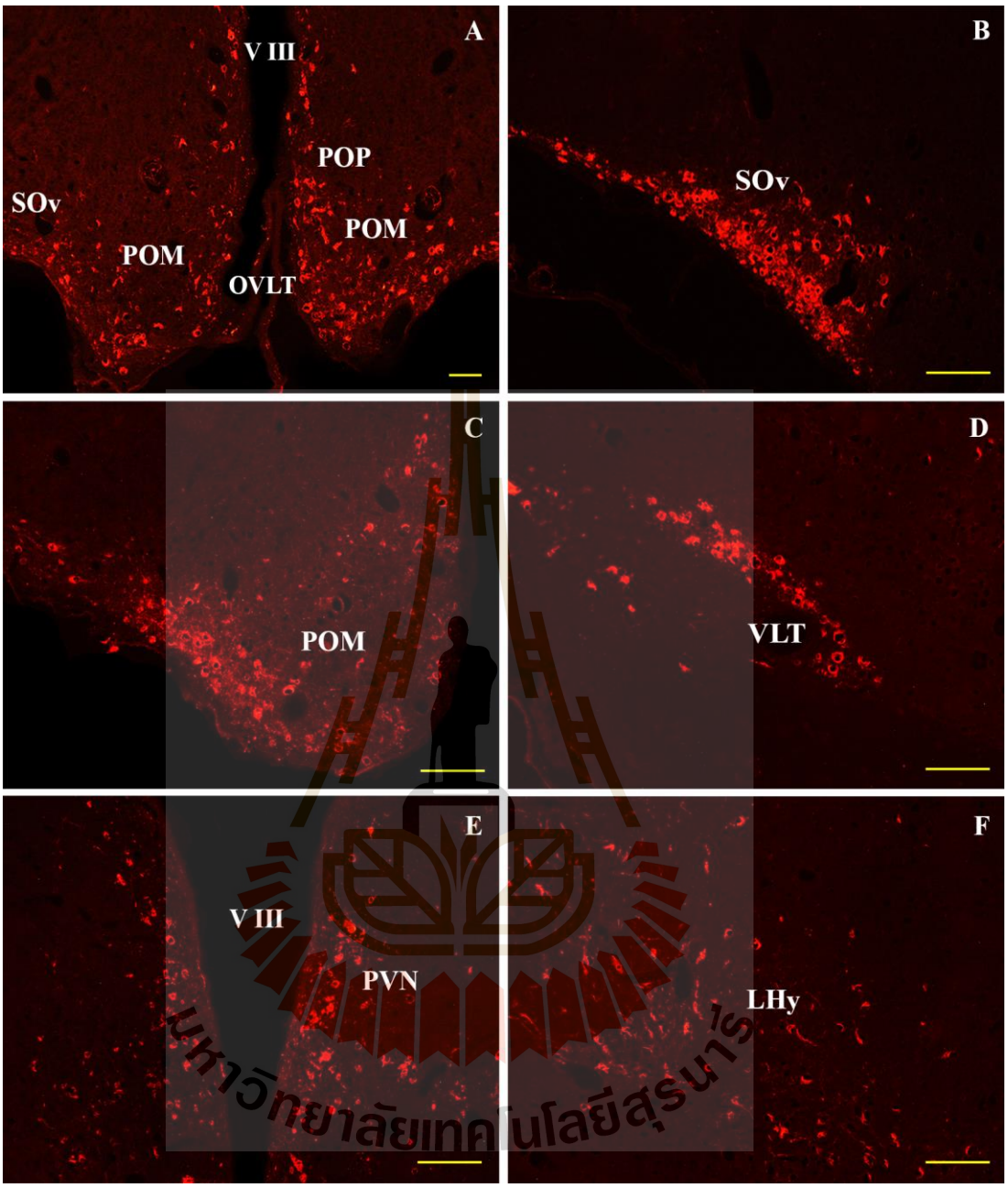

Figure 4.2 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the hypothalamic regions of the male native Thai chicken. MT-ir neurons were found within (A) the OVLT, POM, POP, SOv, (B) POM, (C) SOv, (D) VLT, (E) PVN, and (F) LHy. V III, ventriculus tertius (third ventricle). A, scale bar = 200 µm; B-F, scale bar = 100 µm. See Figure 4.1 for a description of the abbreviations.

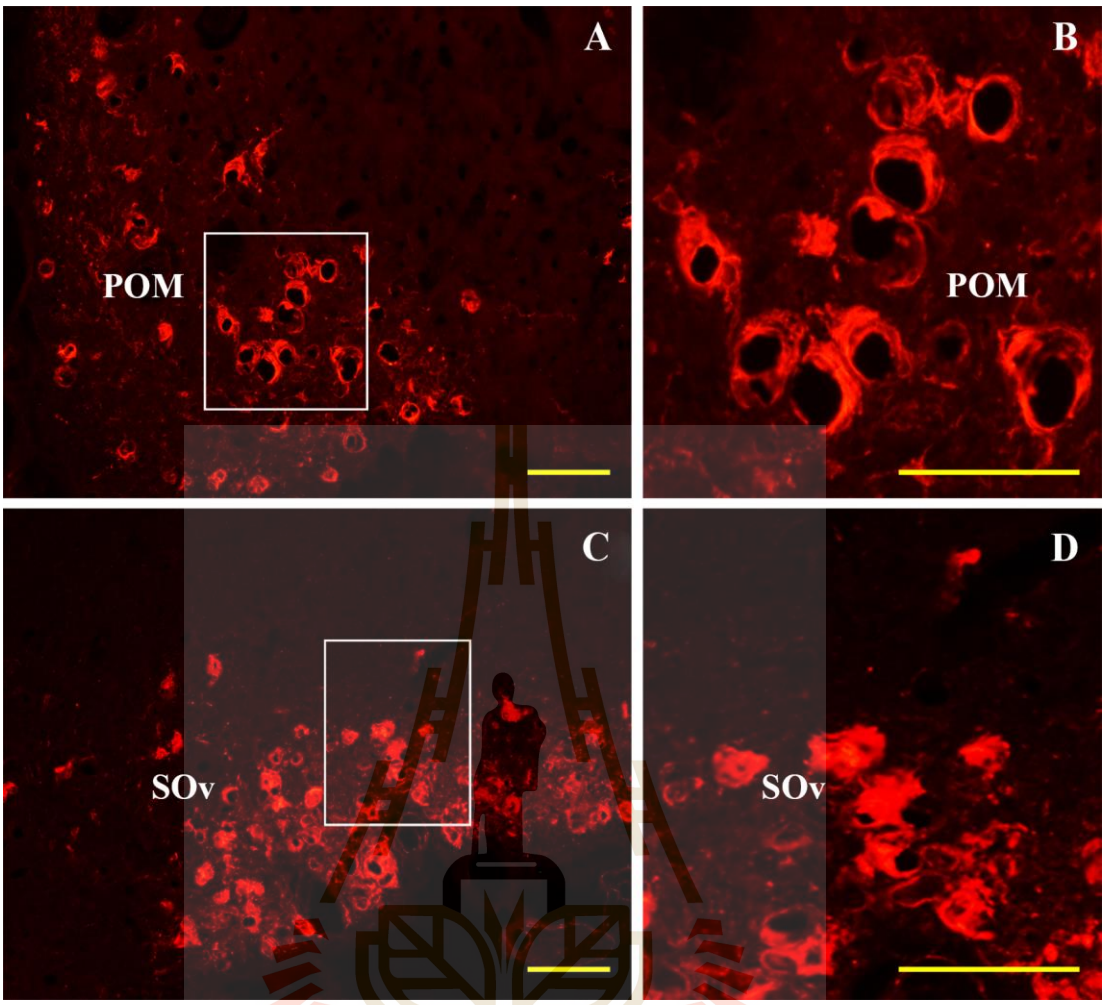

Figure 4.3 The distributions of MT-ir neurons and fibers within the (A) POM and (C) SOv of the male native Thai chicken. Rectangles indicate areas from which higher magnification photomicrographs were taken in the (B) POM and (D) SOv. Scale bar = 50 µm. See Figure 4.1 for a description of the abbreviations.

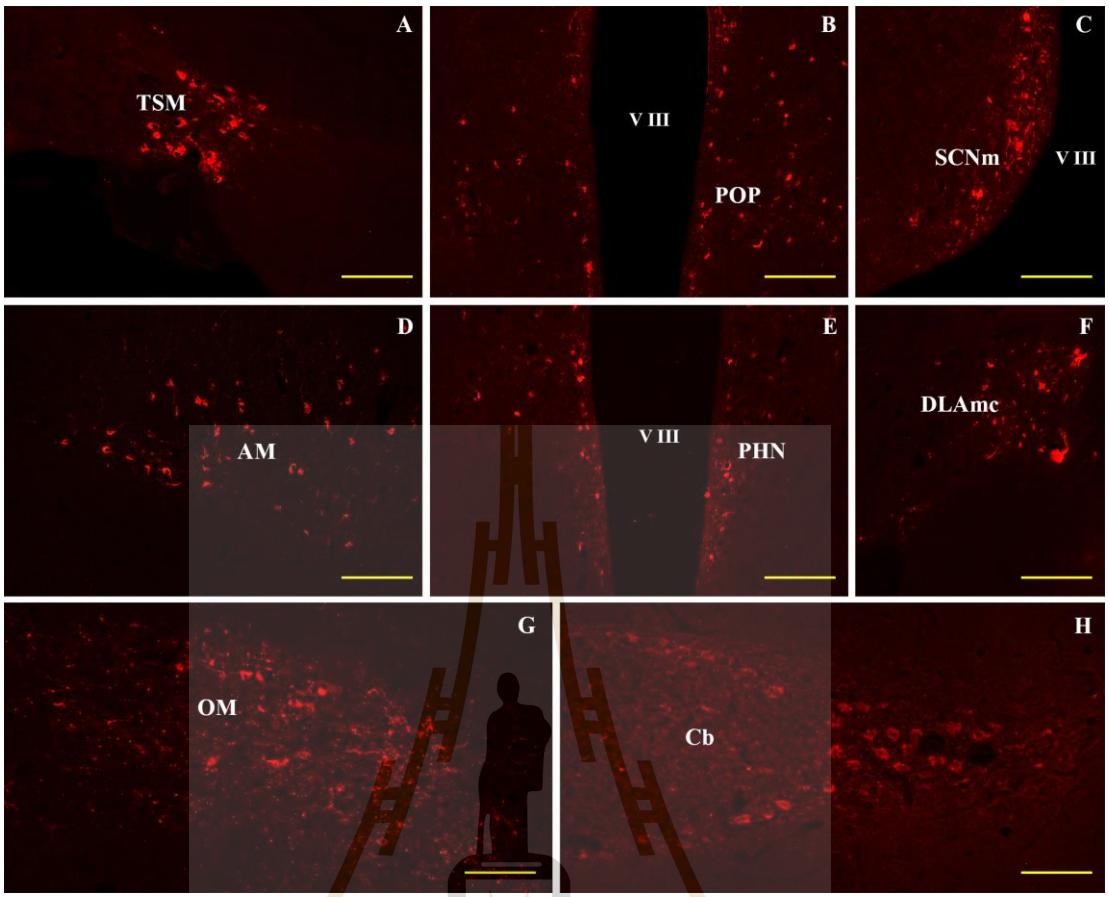

Figure 4.4 Photomicrographs illustrating the distributions of MT-ir neurons and fibers within the diencephalon and rhombencephalon of the male native Thai chicken. Small numbers of MT-ir neurons and fibers were showed within the (A) TSM, (B) POP, (C) SCNm, (D) AM, (E) PHN, (F) DLAmc, (G) OM, and (H) Cb. V III, ventriculus tertius (third ventricle). Scale bar = 100 µm. See Figure 4.1 for a description of the abbreviations.

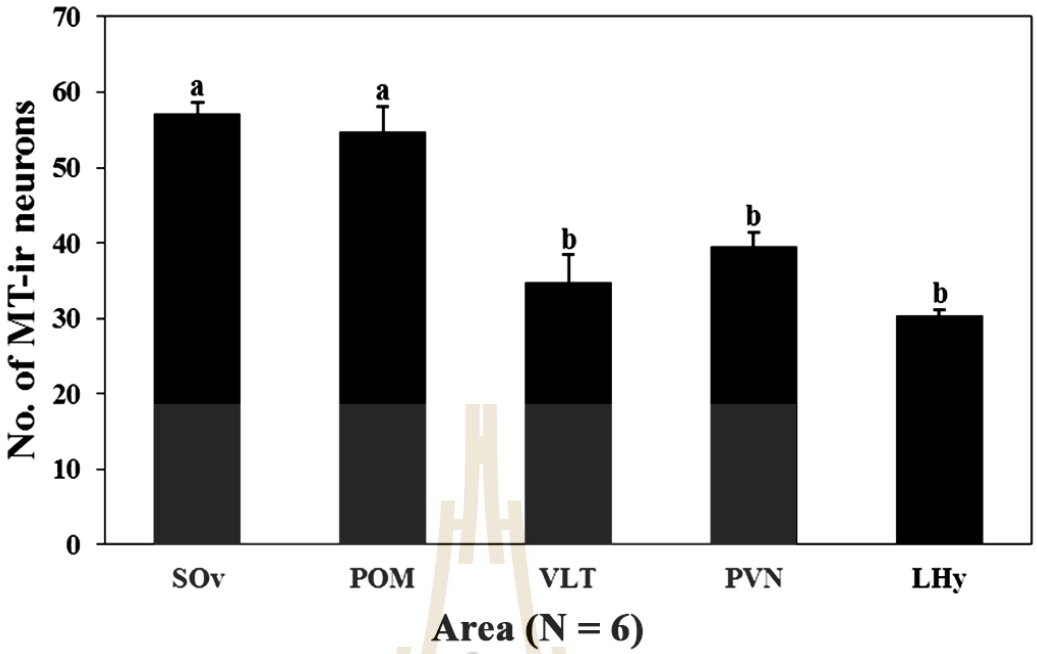

Figure 4.5 The numbers of MT-ir neurons within the SOv, POM, VLT, PVN, and LHy of the male native Thai chicken (6 sections/subject, N = 5). Significant differences between values (mean ± SEM) in each treatment group of different hypothalamic nuclei are indicated by different letters ($P < 0.05$).

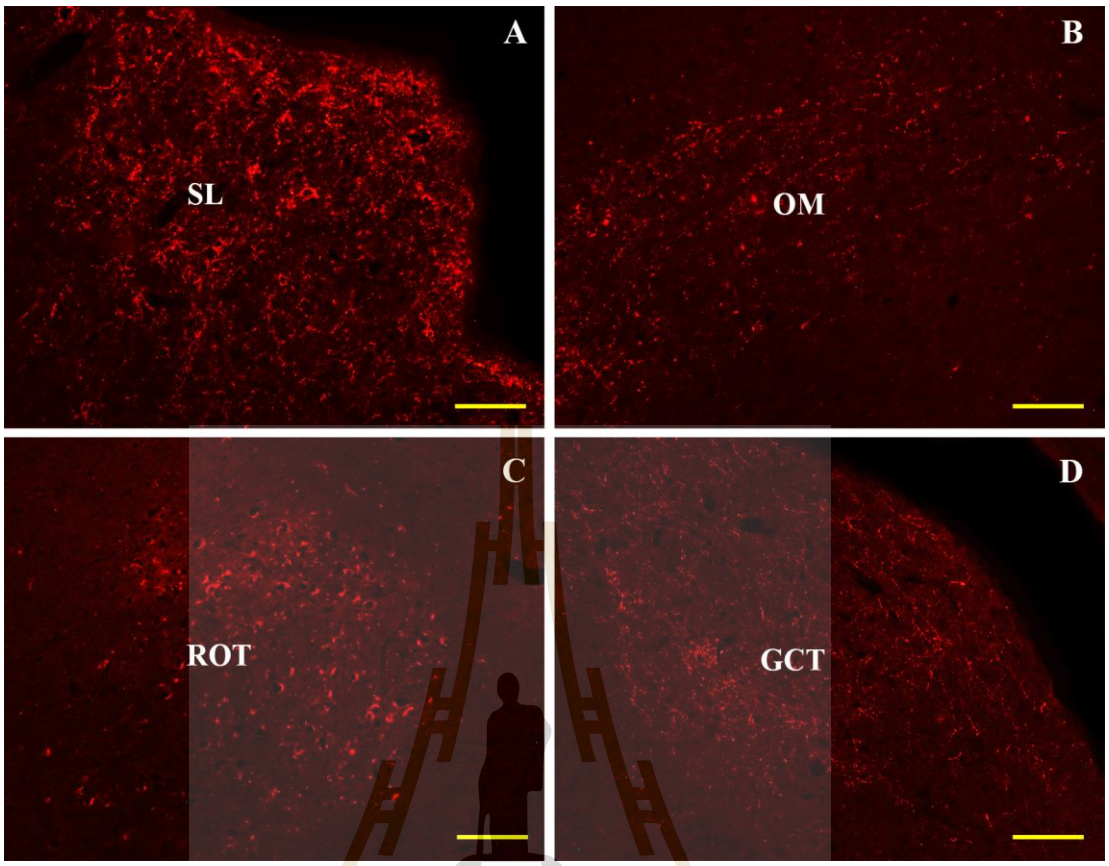

Figure 4.6 Photomicrographs of coronal sections in the telencephalon and diencephalon of the male native Thai chicken. The dense accumulation of MT-ir fibers within the (A) SL, (B) OM, (C) ROT, and (D) GCt. Scale bar = 100 µm. See Figure 4.1 for a description of the abbreviations.

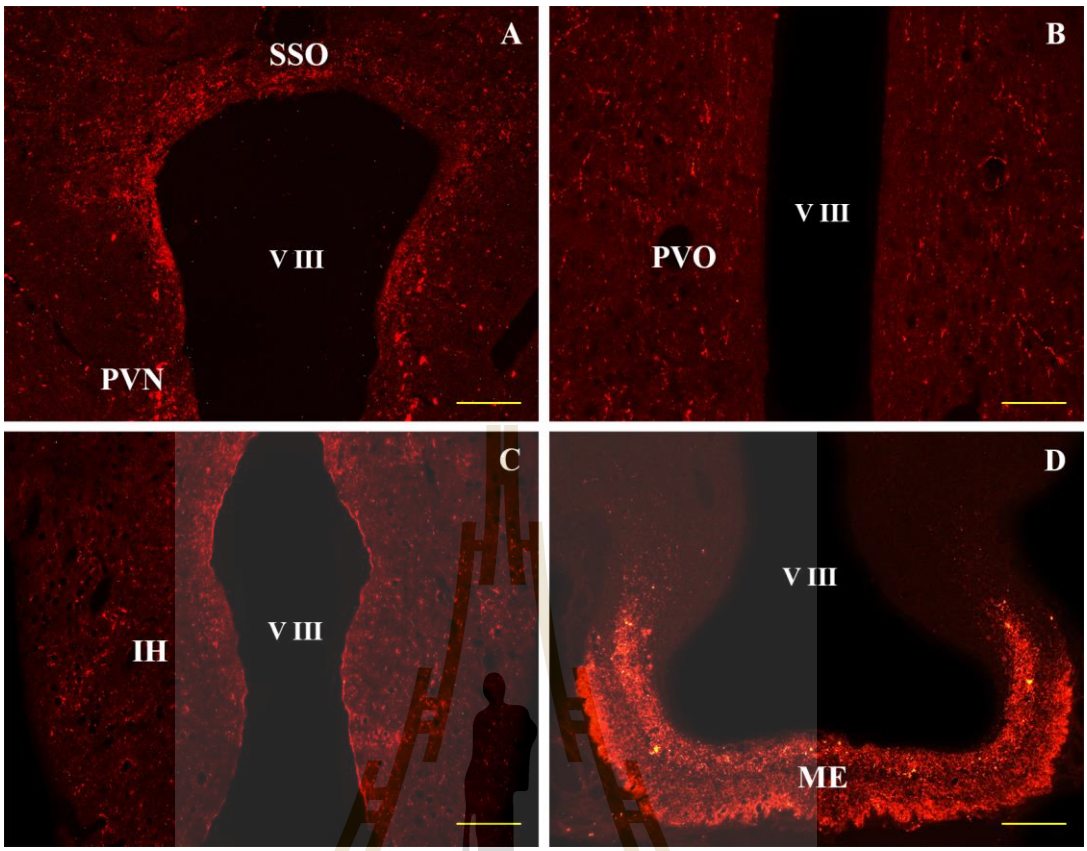

Figure 4.7 Photomicrographs of coronal sections in the hypothalamus showing MT-ir fibers within the (A) SSO, (B) PVO, (C) IH, and (D) ME. V III, ventriculus tertius (third ventricle). Scale bar = 100 μm. See Figure 4.1 for a description of the abbreviations.

4.6 Discussion

The results from the present study demonstrate the distributions of MT-ir neurons and fibers in the brain of male native Thai chickens. MT-ir neurons and fibers were widely located throughout the brain including telencephalic, diencephalic, and rhombencephalic regions, especially in the diencephalic regions. MT-ir neurons and fibers were also located lying close to the midline on both sides of the V III within the POA of the hypothalamus. The greatest density of MT-ir neurons and fibers was found within the SOv, POM, VLT, PVN, and LHy. In addition, the numbers of MT-ir neurons within the SOv and POM were significantly higher than those of the VLT, PVN, and LHy. Dense clusters of MT-ir fibers were innervated within the SL, OM, ROT, GCt, and in the internal and external layers of the ME. Small numbers of MT-ir fibers were also found lying symmetry adjacent to the VIII. These present findings suggest that the MTergic system within the SOv, POM, VLT, PVN, and LHy might be associated with the physiological function(s) of reproductive activities such as nest building, brooding, and feeding of the young in the male native Thai chickens.

The anatomical distributions of MT-ir neurons and fibers in this present study are in accordance with previous studies that indicated the distributions of MT neurons and fibers throughout the avian brain such as chickens (Barth et al., 1997), domestic mallards (Goossens et al., 1977; Bons, 1980), Japanese quails (Goossens et al., 1977; Bons, 1980), domestic fowls (Tennyson et al., 1985), White Leghorn cockerels (Robinzon et al., 1988a), turkeys (Thayananuphat et al., 2011), and female native Thai chickens (Chokchaloemwong et al., 2013). The present findings demonstrated that the majority of MT-ir neurons and/or fibers was distributed in a discrete region lying close to the V III through the diencephalon, especially within the POA, and are

consistent with previous reports in chickens (Barth et al., 1997), domestic mallards (Goossens et al., 1977; Bons, 1980), Japanese quails (Goossens et al., 1977; Bons, 1980), White Leghorn cockerels (Robinzon et al., 1988a), turkeys (Thayananuphat et al., 2011), and female native Thai chickens (Chokchaloemwong et al., 2013).

In this study, the prominent groups of MT immunoreactivity were found abundance within the SOv, POM, VLT, PVN, and LHy. These results are in good agreement with previous reports in female native Thai chickens (Chokchaloemwong et al., 2013). There are no differences in MT-ir neuron distribution between male and female brains. Interestingly, MT-ir neurons within the SOv and POM were highly concentrated when compared with those of the VLT, PVN, and LHy. This finding in the male brain is different from the female brain in which the numbers of MT-ir neurons within the POM and PVN are higher than that of the SOv (Chokchaloemwong et al., 2013). Therefore, it is possible that the number of MT-ir neurons in each area is sexually dimorphic. In male voles, the number of OT immunoreactivity in the SON increases in vole fathers when compared with virgin males (Song et al., 2010). Similarly, OT neurons in these nuclei are involved with tactile stimulation of the penis in rats (Honda et al., 1999). Taken together, these results demonstrate that the MTergic system in the SOv might play an important role in the reproductive activities in the male chickens.

Like in female native Thai chickens, the present results demonstrate the highest density of MT immunoreactivity within the SOv, POM, and PVN. In females, changes in the numbers of MT-ir neurons within the SOv, POM, and PVN are associated with the reproductive cycle. The numbers of MT-ir neurons within the POM and PVN are higher in the R hens when compared with the non-egg laying, egg

laying, and incubating hens. Moreover, the numbers of MT-ir neurons within these nuclei are high in the R hens when compared with the NR hens. In addition, MT-ir neurons increase within the SOv and PVN prior to the onset of brooding behavior in turkeys (Thayananuphat et al., 2011). This evidence indicates that the MTergic system within the SOv, POM, and PVN might play a significant role in the reproductive activities and the neuroendocrine reorganization to establish and maintain rearing behavior in birds. In mammals, it has been well established that the majority of OT is synthesized within the SON and PVN (Ni et al., 2014). OT neurons in the PVN are important for the regulation of male sexual behaviors (Argiolas and Melis, 2004). OT plays an important role in initiating social and reproductive behaviors such as parental care, pair-bond formation, sexual behaviors, mating behavior, emotional-related behavior, and feeding of the young (Insel and Hulihan, 1995; Donaldson and Young, 2008; Ebstein et al., 2009; Stein, 2009; Neumann and Landgraf, 2012). Taken together with the present findings, these previous reports in mammals indicate that functional aspect(s) of the prominent groups of MT-ir neurons and fibers within the SOv, POM, and PVN might be involved in the reproductive activities and/or parental behavior in this species.

In the present study, a modest density of MT-ir neurons and fibers was distributed within the TSM, POP, SCNm, AM, PHN, DLAmc, and OM. In addition, the MT-ir neurons were also found lining the cortex layer of the Cb. These findings correspond with the results of previous studies in females (Chokchaloemwong et al., 2013). Similarly, in other avian species, a few of MT mRNA was found in the TSM in chickens (Tennyson et al., 1985; Barth et al., 1997). In turkey hens, MT-ir neurons

and fibers were detected in the POP (Thayananuphat et al., 2011). However, there are no data regarding the physiological function(s) of these brain areas in birds.

Unlike other avian and mammalian species, MT-ir neurons and fibers within the medial part of BST (BSTM) and HL were not detected in this study, and this is in good agreement with previous report in females (Chokchaloemwong et al., 2013). It has been suggested that the BSTM plays a pivotal role in parental behaviors in both birds and mammals (Cohn and Gerall, 1989; Numan and Numan, 1996; Ruscio and Adkins-Regan, 2004; Thayananuphat et al., 2011; Dumais et al., 2013; 2016; Hall et al., 2014; 2015a; 2015b). Thus, it is possible that the BST is involved with initiation and maintenance of parental behaviors in some avian and mammalian species.

In the present study, MT-ir fibers were largely located along the V III and also distributed in a discrete region lying close to the V III from the level of the POA through the anterior hypothalamus. MT-ir fibers were found abundantly within the SL, OM, ROT, GCt, and internal and external layers of the ME. Moreover, small numbers of MT-ir fibers were found within the OVLT, SSO, PVO, and IH. In females, a few MT-ir fibers were only found within the OVLT, SSO, and external layer of the ME (Chokchaloemwong et al., 2013). Interestingly, MT neurons in the OVLT have been reported in domestic mallards and Japanese quails (Bons, 1980). Thus, it is possible that MT distribution in this area is species-specific. The present results demonstrate that the majority of MT-ir fibers was distributed closely to the V III within the POA area. In avian species, it has been established that the POA is important for the regulation of maternal behaviors (O'Connell and Hofmann, 2011). Lesions of the POA disrupt PRL-induced parental feeding in ring doves (Slawski and Buntin, 1995). In mammals, OT in the area praeoptica medialis (MPOA; medial part

of the POA) facilitates mating in male rats (Gil et al., 2013). OT and PRL act on the MPOA during the exposition of maternal behaviors (Numan et al., 1990; Pedersen et al., 1994; Bakowska and Morrell, 1997; Tsuneoka et al., 2013). In addition, the MPOA is involved with initiation and maintenance of maternal behaviors in mammals (Numan and Numan, 1997; Tsuneoka et al., 2013).

Unlike in turkeys (Thayananuphat et al., 2011), a few MT-ir neurons and fibers were found in the IH in male chickens, but not the MT-ir neurons. In our previous study, VIP-ir neurons were highly accumulated in the IH-IN (Kamkrathok et al., 2016). In mammals, it has been documented that the OTergic neurons, whose axons project to the limbic system, brainstem, and spinal cord, might play a role as neurotransmitter (Raggenbass, 2001). Previous studies report that OT also acts like a neurotransmitter in many physiological and behavioral functions such as cardiovascular system, digestion and metabolism, thermoregulation, pain threshold, memory and learning, sexual activity, and feeding behavior (Lin et al., 1983; Arletti et al., 1990; Richard et al., 1991; Engelmann et al., 1996; Petersson et al., 1996). Therefore, it might be possible that MT-ir fibers located in the IH might synapse with VIP neurons and may be involved with reproductive activities in males. Some evidence suggests that OT neurons interacts with other neurotransmitters such as VIP, DA, and serotonin (Ottesen et al., 1984; Jorgensen et al., 2002; Melis and Argiolas, 2003; 2011; Rosenfeld et al., 2011; Dolen et al., 2013).

In summary, this study illustrates the distribution of MT immunoreactivity throughout the brain of male native Thai chickens. The greatest density of MT-ir neurons and fibers was found within the SOv, POM, VLT, PVN and LHy, suggesting that MT neurons in these nuclei that may be involved with the physiological

function(s) of reproductive activities in the male chickens. More importantly, the number of MT-ir neurons in the SOv was high in the male brain, implicating that the MTergic system in the SOv might play an important role in the male reproductive activities in this equatorial species.

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CHAPTER V

DISTRIBUTION OF DOPAMINE-IMMUNOREACTIVE NEURONS IN THE BRAIN OF THE MALE NATIVE THAI CHICKEN

5.1 Abbreviations

AM, nucleus anterior medialis hypothalami; AVT, area ventralis; BCA, brachium conjunctivum ascendens; BCD, brachium conjunctivum descendens; Cb, cerebellum; DA, dopamine; GCt, substantia grisea centralis; GnRH, gonadotropin releasing hormone; IF, tractus infundibularis; IH, nucleus inferioris hypothalami; IN, nucleus infundibuli hypothalami; INC, incubating; -ir, -immunoreactive; LH, luteinizing hormone; LHy, regio lateralis hypothalami (lateral hypothalamic area); LoC, locus ceruleus; ME, eminentia mediana (median eminence); ML, nucleus mamillaris lateralis; MM, nucleus mamillaris medialis; MT, mesotocin; nI, nucleus intramedialis; NR, non-rearing; nVm, nucleus mesencephalicus nervi trigemini; OM, tractus occipitomesencephalicus; PBS, phosphate buffered saline; PMM, nucleus premamillaris; POA, area praeoptica; POM, nucleus preopticus medialis; POP, nucleus preopticus periventricularis; PRL, prolactin; PVN, nucleus paraventricularis magnocellularis; PVO, organum paraventriculare; QF, tractus quintofrontalis; R, rearing; REC, replaced-eggs-with-chicks; ROT, nucleus rotundus; SCNm, nucleus suprachiasmaticus, pars medialis; SL, nucleus septalis lateralis; SM, nucleus septalis

medialis; SOv, nucleus supraopticus, pars ventralis; TH, tyrosine hydroxylase; TPc, nucleus tegmenti pedunculo-pontinus, pars compacta (substantia nigra); TSM, tractus septomesencephalicus; V III, ventriculus tertius (third ventricle); VIP, vasoactive intestinal peptide

5.2 Abstract

Dopamine (DA) is a neurotransmitter/neuromodulator found in both central and peripheral nervous systems. It plays several physiological functions in some mammalian and avian species. DA has been indicated to be associated with the neuroendocrine regulation of reproductive cycle and maternal behaviors in female native Thai chickens. Male birds express parental behaviors as well. To date, there are no data describing the functional aspects of the DAergic system in the male native Thai chickens. Thus, the objective of this study was to elucidate the localization of tyrosine hydroxylase (TH; a DA marker) neuronal groups in the brain of the male native Thai chickens. The distributions of TH-immunoreactive (-ir) neurons and fibers in the brain were detected utilizing the immunohistochemistry technique. The results revealed that TH-ir neurons and fibers were located throughout the brain and extensively in the diencephalon and mesencephalon. The highest density of TH-ir neurons and fibers was found within the nucleus intramedialis (nI) and nucleus mamillaris lateralis (ML). The numbers of TH-ir neurons within the nucleus anterior medialis hypothalami (AM), nucleus paraventricularis magnocellularis (PVN), nI, and ML were then compared and revealed that the numbers of TH-ir neurons within the nI and ML were significantly higher than those of the AM and PVN. These present findings suggest that the DAergic neurons within the nI and ML might play an

important role in the reproductive activities in the native Thai roosters. Moreover, the highest accumulation of TH-ir neurons in the nI was concentrated in the male brain, suggesting that the DAergic system in the nI might be involved in male reproductive activities and/or parental behaviors in this equatorial species.

5.3 Introduction

Dopamine (DA) is a neurotransmitter/neuromodulator that is found in both central and peripheral nervous systems in many vertebrate and invertebrate species (Benes, 2001; Ben-Jonathan and Hnasko, 2001; Beaulieu and Gainetdinov, 2011). It has several important physiological functions involved in a wide variety of behaviors and reproduction. In mammals, DA is well known to be involved in the onset and maintenance of maternal care and male sexual behaviors (Stolzenberg and Numan, 2011). In birds, it has been well established that DA controls two major neuroendocrine systems that play a pivotal role in avian reproduction; the gonadotropin-releasing hormone/follicle-stimulating hormone-luteinizing hormone (GnRH/FSH-LH) and vasoactive intestinal peptide/prolactin (VIP/PRL) systems (Chaiseha and El Halawani, 2015). Numerous studies have reported that DA neurons are distributed within several regions of the avian brain, especially in the hypothalamus, and have been shown to be immunoreactived for VIP, the avian PRL releasing factor (Al-Zailaie and El Halawani, 2000; Chaiseha and El Halawani, 2003). Activation of the DA neurons is linked to the activation of GnRH-I and VIP neurons and subsequently secretion of LH and PRL (Al-Zailaie et al., 2006). To date, there are numerous reports regarding the physiological roles of DA and its receptors in birds such as food intake (Khodadadi et al., 2017), food reward (Moe et al., 2014),

cognitive performance (Taufique and Kumar, 2016), feather pecking (Kops et al., 2017), singing behavior (Sasaki et al., 2006; Heimovics and Riters, 2008; Riters et al., 2014; Merullo et al., 2018), song learning (Budzillo et al., 2017), social activity (Heimovics et al., 2009), aggressive behavior (Komiyama et al., 2014), mate competition (Kabelik et al., 2009), courtship motivation (Goodson et al., 2009), egg production (Xu et al., 2010a), incubation (Xu et al., 2010b), reproductive cycle (Lea et al., 2001), and male sexual behavior (Balthazart et al., 1997). More importantly, DA is involved in the regulation of the reproductive cycle and maternal behaviors (incubating and rearing behaviors) in the female native Thai chicken, a non-seasonally breeding species (Sartsoongnoen et al., 2008; Prakobsaeng et al., 2011; Chokchaloemwong et al., 2015; Sinpru et al., 2018).

The anatomical distributions of DA neurons and fibers have been mapped in several avian species and there are distributed in several regions of the brain such as the area praeoptica (POA), nucleus preopticus medialis (POM), nucleus ventrolateralis thalami, suprachiasmatic nucleus, nucleus anterior medialis hypothalami (AM), regio lateralis hypothalami (LHy), nucleus paraventricularis magnocellularis (PVN), nucleus ventromedialis hypothalami, nucleus dorsomedialis hypothalami, nucleus intramedialis (nI), nucleus premamillaris (PMM), nucleus mamillaris medialis (MM), nucleus mamillaris lateralis (ML), area ventralis (AVT), nucleus tegmenti pedunculo-pontinus, pars compacta (substantia nigra; TPc), locus ceruleus (LoC), brachium conjunctivum ascendens (BCA), brachium conjunctivum descendens (BCD), cerebellum (Cb), lateral septum, pons, and medulla oblongata (Kiss and Peczely, 1987; Bailhache and Balthazart, 1993; Moons et al., 1994; Reiner et al., 1994; Al-Zailaie and El Halawani, 2000; den Boer-Visser and Dubbeldam,

2002; Acerbo et al., 2003). DA fibers are extensively distributed within external layer of the eminentia mediana (median eminence; ME; Kiss and Peczely, 1987; Bailhache and Balthazart, 1993; Moons et al., 1994). Similarly, in the female native Thai chickens, tyrosine hydroxylase (TH; a DA marker)-immunoreactive (-ir) neurons and fibers are extensively distributed throughout the brain, especially in the diencephalic and mesencephalic regions. Changes in the number of TH-ir neurons in the nI are associated with the reproductive stages. The number of TH-ir neurons in the nI is lowest in non-egg laying stage, then markedly increases in the egg laying, and reaches the highest density in incubating (INC) hens, and decreases in the rearing chicks (R) hens (Sartsoongnoen et al., 2008). Disruption of incubation behavior by nest-deprivation decreases the numbers of TH-ir neurons within the nI and ML (Prakobsaeng et al., 2011). In addition, the number of TH-ir neurons in the nI decreases in non-rearing (NR) hens when compared with R hens (Chokchaloemwong et al., 2015). Recently, it was reported that the presence of eggs or chicks is associated with the decreased numbers of TH-ir neurons within the nI and ML, and the increased numbers of mesotocin (MT)-ir neurons within the nucleus supraopticus; pars ventralis (SOv), POM, and PVN during the transition from incubating to rearing behavior (Sinpru et al., 2017; 2018).

Native Thai chicken (*Gallus domesticus*), an equatorial, tropical, non-seasonally breeding species, has been domesticated without genetic selection. It expresses strong maternal behaviors which are inherited from the ancestor, the wild jungle fowl in Southeast Asia (Austic and Nesheim, 1990; Fumihito et al., 1994; Hillel et al., 2003; Sawai et al., 2010). It is well documented that the neuroendocrine regulation of reproductive cycle and maternal behaviors is associated with GnRH,

VIP, DA, PRL, and MT in the female native Thai chickens (Chaiseha and El Halawani, 2015; Namken et al., 2017; Sinpru et al., 2017; 2018). Presently, the neuroendocrine regulation of reproductive behaviors has been extensively studied in the female native Thai chickens. However, there are limited data regarding the neuroendocrine regulation of reproductive activities in males. It has been reported that changes in the numbers of VIP-ir neurons within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) are observed across the reproductive stages and mirror directly with circulating PRL and testosterone levels in the male native Thai chickens (Kamkrathok et al., 2016). The distributions of MT-ir neurons and fibers has also been reported, suggesting that MT-ir neurons within the SOv and POM might be involved in the reproductive activities and/or parental behaviors in the native Thai roosters (Kamkrathok et al., 2017). Interestingly, it has been documented that male birds exhibit parental behaviors such as nest building, brooding, protection, and feeding of the young in many species (Chaiseha and El Halawani, 2015; Lynn, 2016). To date, there has been no report regarding the physiological role(s) of the DAergic system in the male native Thai chicken. Thus, the objective of this study was to investigate the localization of the DA neuronal groups in the brain of the male native Thai chickens, enabling further studies of neuroendocrinology related to behavior. The findings of the distributions of TH-ir neurons and fibers might be related to the regulation of reproductive activities and/or paternal behaviors in the male native Thai chickens.

5.4 Materials and Methods

5.4.1 Experimental Animals

Male native Thai chickens, 12 months of age, were used. They were reared and housed together with mature females (1 male: 8 females) in floor pens equipped with basket nests under natural light (approximately 12 h of light and 12 h of darkness; 12L:12D) with free access to feed and water. The animal protocols used adhered to the guidelines approved by the Suranaree University of Technology Animal Care and Use Committee.

5.4.2 Experimental Design

To determine the distributions of TH-ir neurons and fibers in the brain of the male native Thai chicken, 6 mature males (12 months of age) were used. The brains of mature roosters were fixed by pressure perfusion with 4 % paraformaldehyde (Acros Organics, Inc., New Jersey, USA). Tissue sectioning was performed in the coronal plane at a thickness of 16 μm utilizing a cryostat and further processing by immunohistochemistry technique according to a previously described method (Chokchaloemwong et al., 2015). In this study, the primary and secondary antibodies used for detecting TH immunoreactivity were primary mouse monoclonal antibody raised directly against TH (ImmunoStar, Inc., Hudson, WI, USA), TH antibody diluted 1:1000 with phosphate buffered saline (PBS; pH 7.4), containing 1 % bovine serum albumin (Acros Organics, Inc.) and 0.3 % Triton-X 100 (Acros Organics, Inc.) and Cy™3-conjugated AffiniPure donkey anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at 1:500 dilution with PBS, respectively. TH immunoreactivity using these antibodies has been previously

described (Prakobsaeng et al., 2011; Sartsoongnoen et al., 2008; Chokchaloemwong et al., 2015; Sinpru et al., 2018).

5.4.3 Image Analysis

Microscopic images of the brain sections were visualized under a fluorescence microscope (Nikon ECLIPSE 80i, Tokyo, Japan) fitted with a cooled digital color camera (Nikon DS-Fi1, Tokyo, Japan). The images were captured and stored by NIS-Elements Documentation software (Nikon, Tokyo, Japan). TH-ir neurons and fibers in each individual area of the brain were visualized and analyzed. The numbers of TH-ir neurons of six adjacent sections for each rooster (6 roosters per area) were counted manually to determine changes in the number of TH-ir neurons in the individual hypothalamic areas (AM, PVN, nI, and ML). The mean values were compared across the hypothalamic areas (Chokchaloemwong et al., 2015). Atlas of the chick brain (Kuenzel and Masson, 1988) and the chicken hypothalamus (Kuenzel and van Tienhoven, 1982) were used for identification of specific brain regions of TH immunoreactivity.

5.4.4 Statistical Analysis

Significant differences among the numbers of TH-ir neurons within the AM, PVN, nI, and ML (mean ± SEM) were compared employing one-way analysis of variance (ANOVA). Significant differences between each individual hypothalamic area were computed utilizing the Tukey's HSD test. $P < 0.05$ was considered statistically significant. All statistical tests were analyzed using SPSS for Windows software (version 17.0, SPSS Inc., Chicago, IL, USA).

5.5 Results

The results of this study revealed that TH-ir neurons and fibers were located throughout the brain including the telencephalon, diencephalon, mesencephalon, and rhombencephalon. The distributions of TH-ir neurons and fibers were predominantly located within the diencephalon and mesencephalon. TH-ir fibers were extensively distributed in the diencephalon, and very dense fibers were observed in the internal and external layers of the ME (Figure 5.1).

The majority of TH-ir neurons and fibers was distributed in the diencephalon and mesencephalon. The densest TH-ir neurons and fibers were distributed in a discrete region lying close to the ventriculus tertius (third ventricle; V III) through the hypothalamus (Figures 5.2, 5.3, 5.4, and 5.5). The greatest density of TH-ir neurons and fibers was located within the nI (Figures 5.2A and B) and ML (Figures 5.2C and D) in the diencephalon. A modest density of TH-ir neurons and fibers was observed within the nucleus septalis lateralis (SL; Figure 5.3A) and nucleus septalis medialis (SM; Figure 5.3B) in the telencephalon. The distributions of TH-ir neurons and fibers were moderately within the AM (Figure 5.3C), LHy (Figure 5.3D), PVN (Figure 5.3E), and organum paraventriculare (PVO; Figure 5.3F) in the diencephalon. Scattered TH-ir neurons and fibers were also found within the AVT, adjacent to the nervus oculomotorius (Figure 5.4A), TPc (Figure 5.4B), LoC (Figures 5.4C and D), BCA (Figures 5.4D and E), and BCD (Figures 5.4D and F) in the mesencephalon. Small numbers of TH-ir neurons and fibers were found within the tractus quintofrontalis (QF; Figure 5.5A), tractus septomesencephalicus (TSM; Figure 5.5B), nucleus preopticus periventricularis (POP; Figure 5.5C), nucleus suprachiasmaticus, pars medialis (SCNm; Figure 5.5D), tractus occipitomesencephalicus (OM; Figure

5.5E), substantia grisea centralis (GCt; Figure 5.5F), tractus infundibularis (IF; Figure 5.5G), and PMM (Figure 5.5H). Moreover, TH-ir neurons were also found lining the cortex layer of the Cb (Figure 5.5I) and nucleus mesencephalicus nervi trigemini (nVm; Figure 5.5J) in the rhombencephalon. The numbers of TH-ir neurons in four hypothalamic areas including the AM, PVN, nI, and ML were then compared (Figure 5.6), revealing that the number of TH-ir neurons was significantly higher ($P < 0.05$) within the nI (35.00 ± 3.75 cells) and ML (28.25 ± 1.56 cells) when compared with the AM (18.83 ± 2.05 cells) and PVN (18.33 ± 2.24 cells).

Dense clusters of TH-ir fibers were innervated in the diencephalon. TH-ir fibers were extensively distributed within the nucleus rotundus (ROT; Figure 5.7A), IH (Figure 5.7B), MM (Figure 5.7C), and very dense fibers were observed in the internal and external layers of ME (Figure 5.7D).

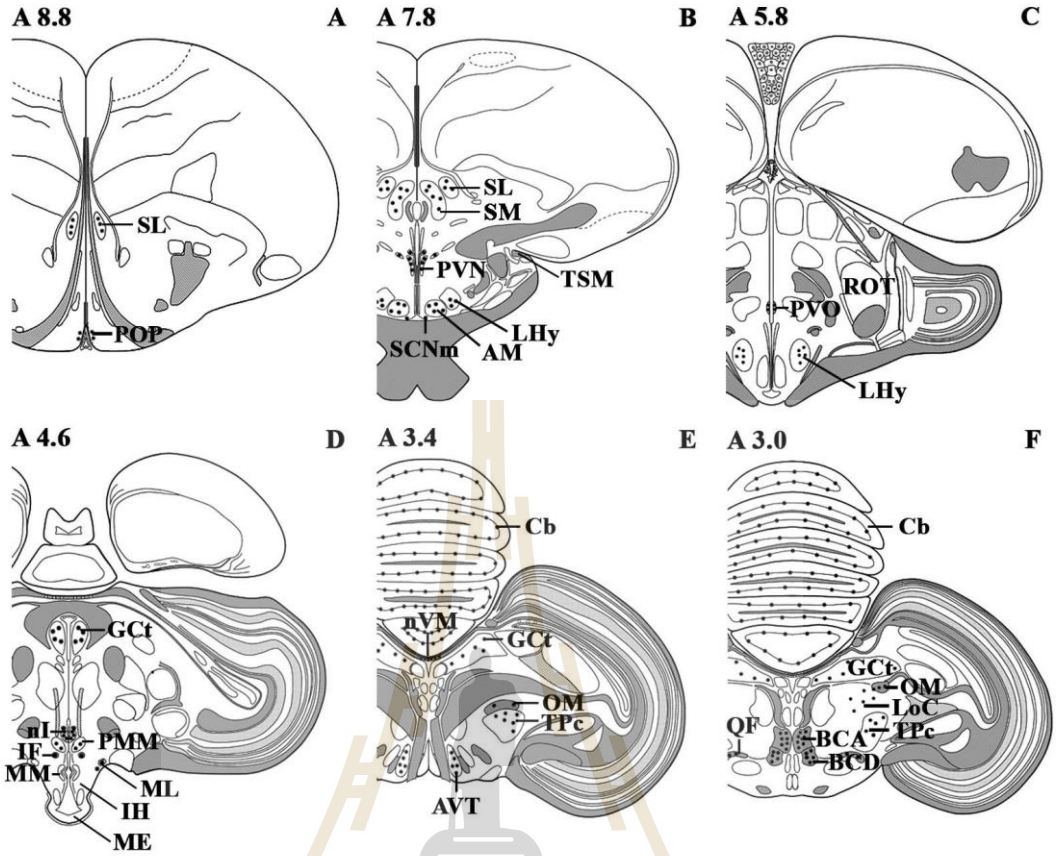

Figure 5.1 Schematic diagrams of coronal sections illustrate the areas of the chick brain showing the distributions of TH-ir neurons (black dots) and fibers throughout the brain of the male native Thai chicken. Coronal illustrations were redrawn from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988). The following abbreviations are used in the figure legends: AM, nucleus anterior medialis hypothalami; AVT, area ventralis; BCA, brachium conjunctivum ascendens; BCD, brachium conjunctivum descendens; Cb, cerebellum; GCt, substantia grisea centrlis; IF, tractus infundibularis; IH, nucleus inferioris hypothalami; LHy, regio lateralis hypothalami (lateral hypothalamic area); LoC, locus ceruleus; ME, eminentia mediana (median eminence); ML, nucleus mamillaris lateralis; MM, nucleus mamillaris medialis; nI; nucleus intramedialis; nVm, nucleus mesencephalicus nervi

trigemini; OM, tractus occipitomesencephalicus; PMM, nucleus premamillaris; POP, nucleus preopticus periventricularis; PVN, nucleus paraventricularis magnocellularis; PVO, organum paraventriculare; QF, tractus quintofrontalis; ROT, nucleus rotundus; SCNm, nucleus suprachiasmaticus, pars medialis; SL, nucleus septalis lateralis; SM, nucleus septalis medialis; TPc, nucleus tegmenti pedunculo-pontinus, pars compacta (substantia nigra); TSM, tractus septomesencephalicus.

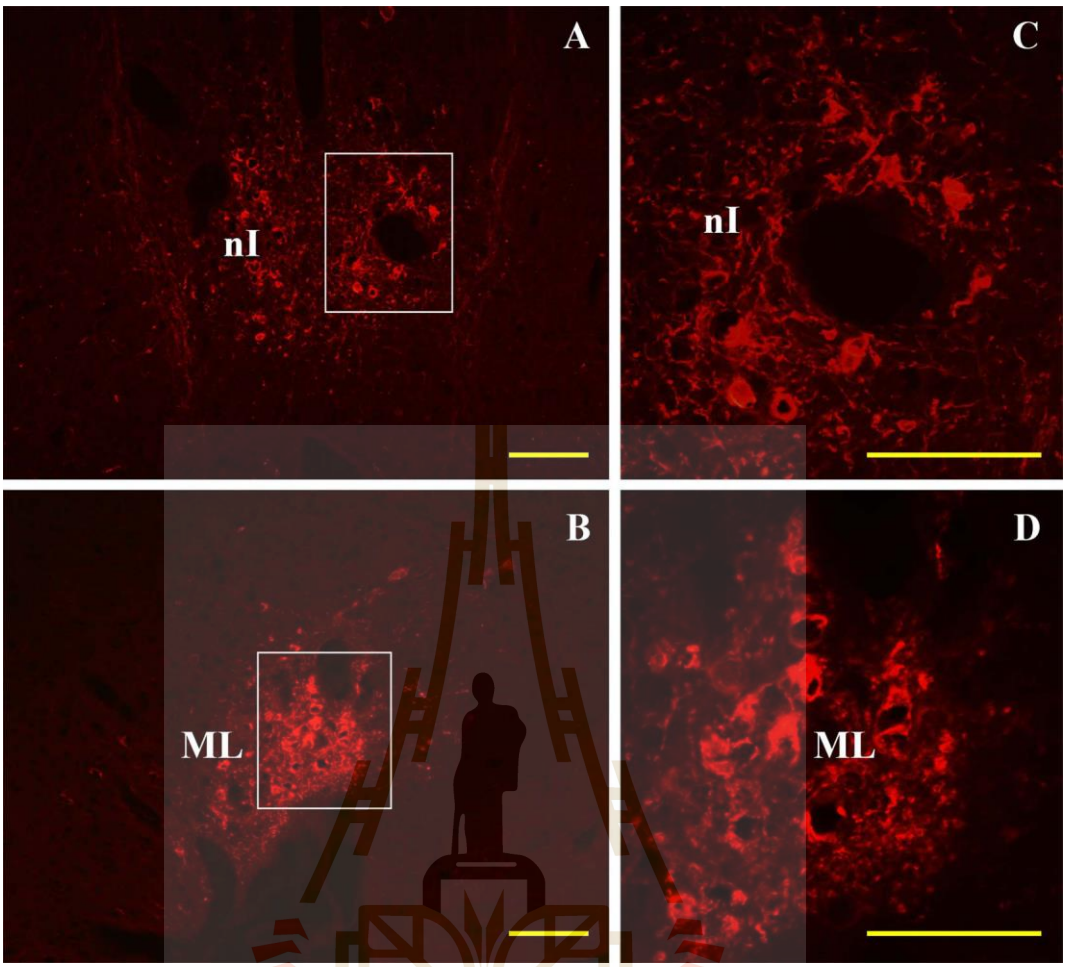

Figure 5.2 Photomicrographs illustrating the greatest density of TH-ir neurons and fibers within the (A and B) nI and (C and D) ML of the male native Thai chicken. Rectangles indicate areas from which higher magnification photomicrographs were taken in the (C) nI and (D) ML. Scale bar = 50 µm. Scale bar = 100 µm. See Figure 5.1 for a description of the abbreviations.

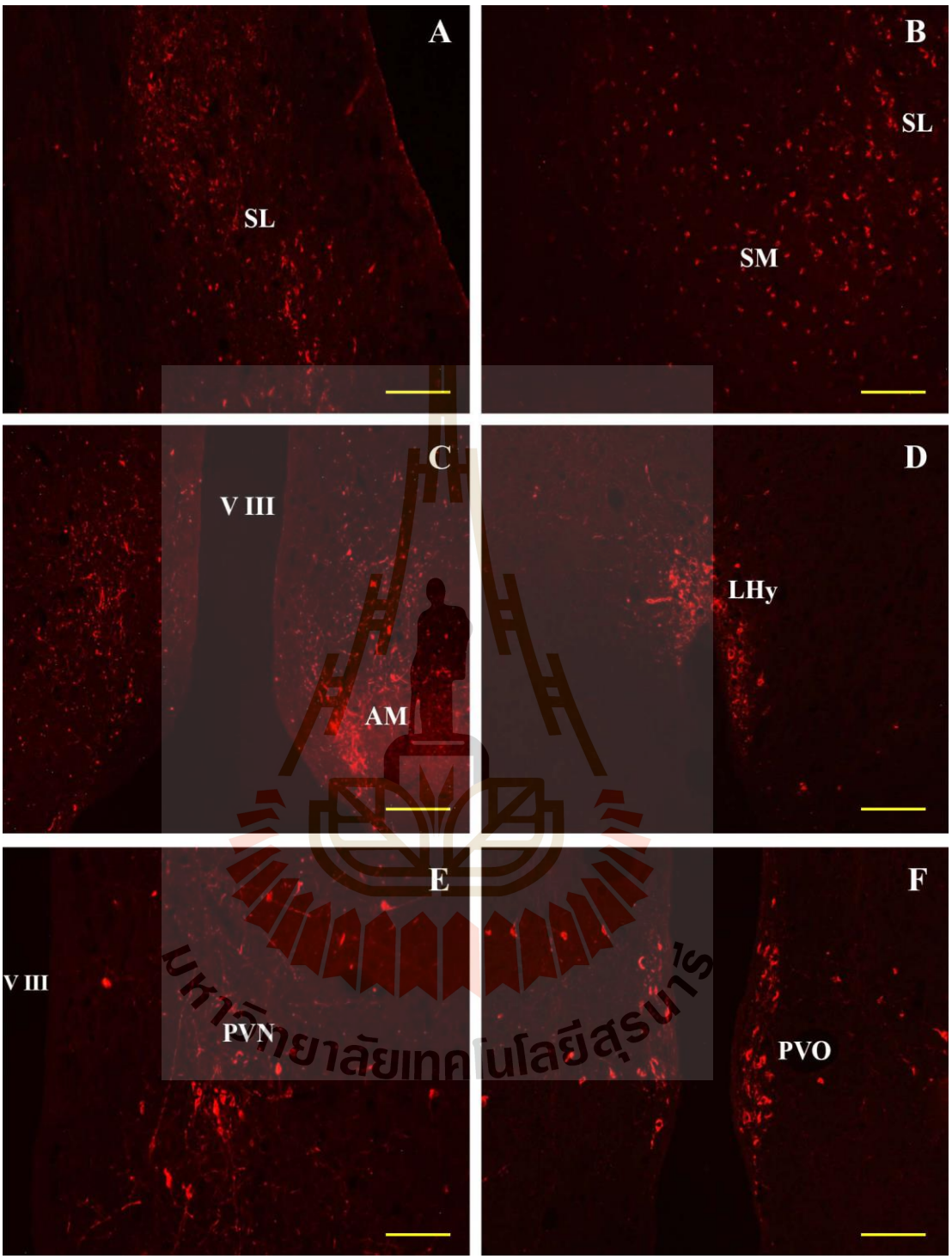

Figure 5.3 Photomicrographs illustrating the distributions of TH-ir neurons and fibers within the (A) SL, (B) SL, SM, (C) AM, (D) LHy, (E) PVN, and (F) PVO. V III, ventriculus tertius (third ventricle). Scale bar = 100 µm. See Figure 5.1 for a description of the abbreviations.

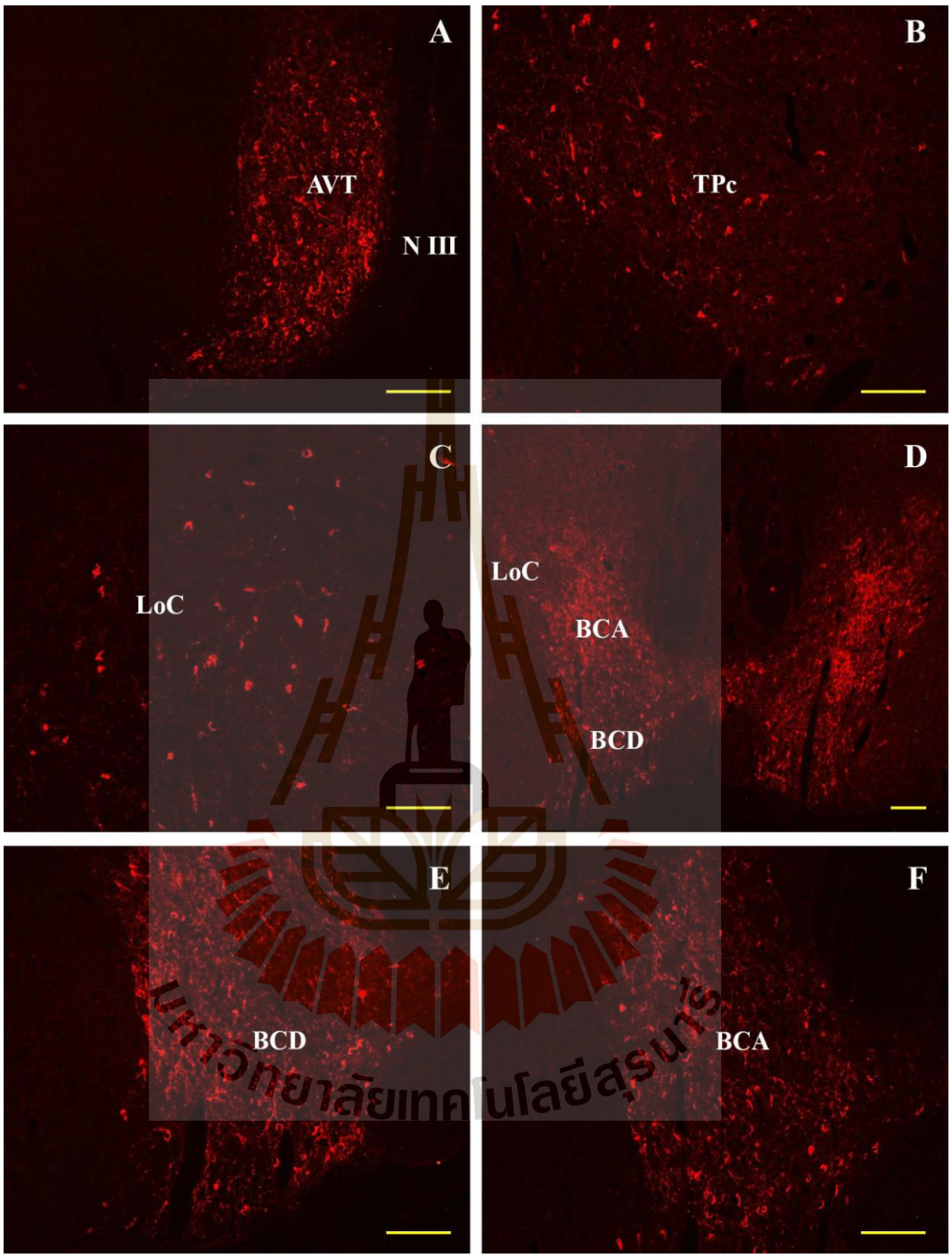

Figure 5.4 Photomicrographs illustrating a modest density of TH-ir neurons and fibers within the (A) AVT, adjacent to the nervus oculomotorius (NIII), (B) TPc, (C) LoC, (D) LoC, BCA, BCD, (E) BCD, and (F) BCA. D, scale bar = 200 µm; A-C, E, F, Scale bar = 100 µm. See Figure 5.1 for a description of the abbreviations.

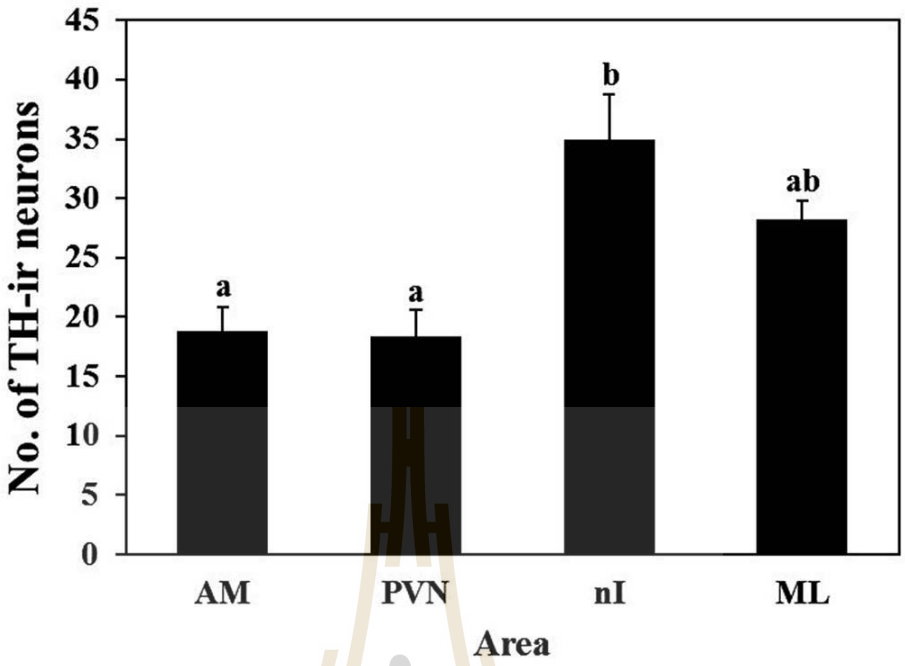

Figure 5.5 The number of TH-ir neurons in the individual hypothalamic areas (AM, PVN, nI, and ML) of the male native Thai chicken. Significant differences between values (mean ± SEM) in each hypothalamic area are indicated by different letters ($P < 0.05$).

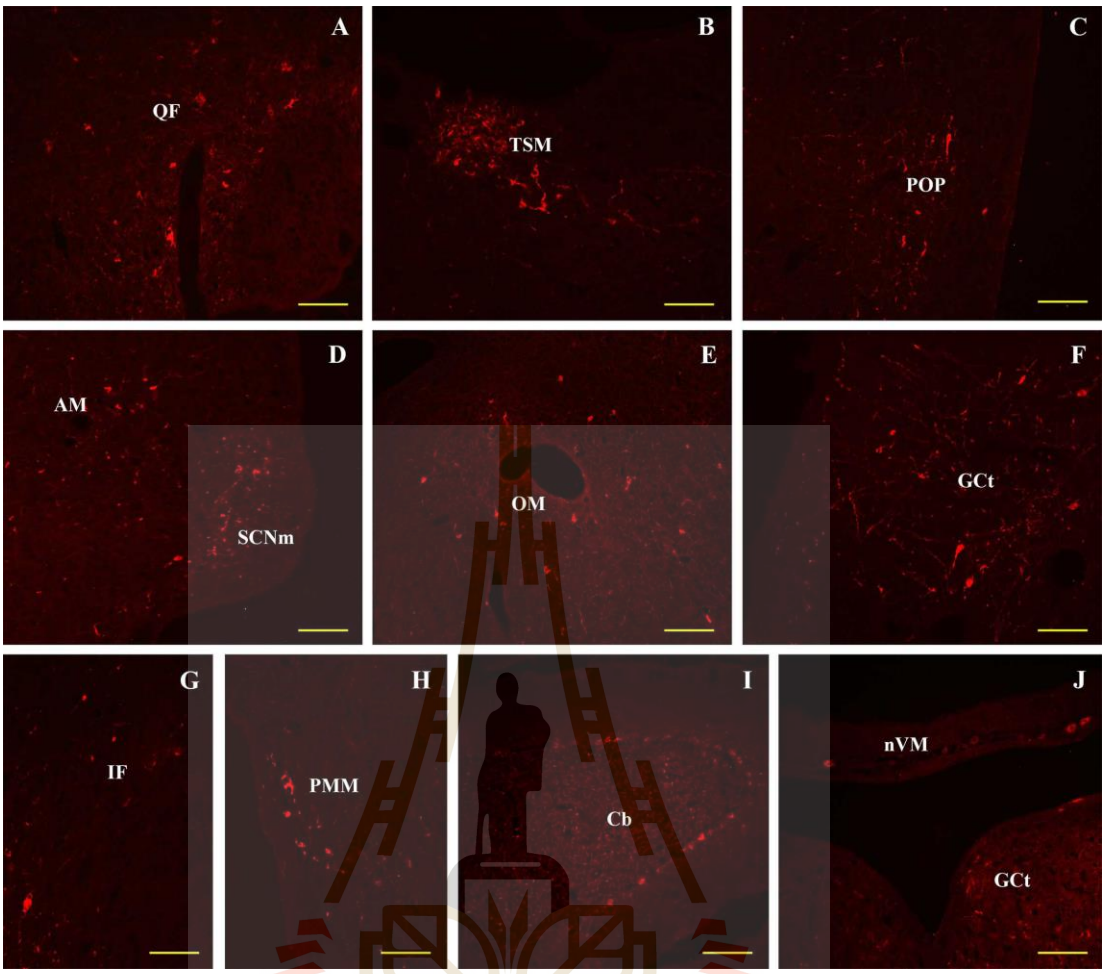

Figure 5.6 Photomicrographs of coronal sections showing small number of TH-ir neurons and fibers within the (A) QF, (B) TSM, (C) POP, (D) AM, SCNm, (E) OM, (F) GCt, (G) IF, (H) PMM, (I) Cb, and (J) nVM. Scale bar = 100 µm. See Figure 5.1 for a description of the abbreviations.

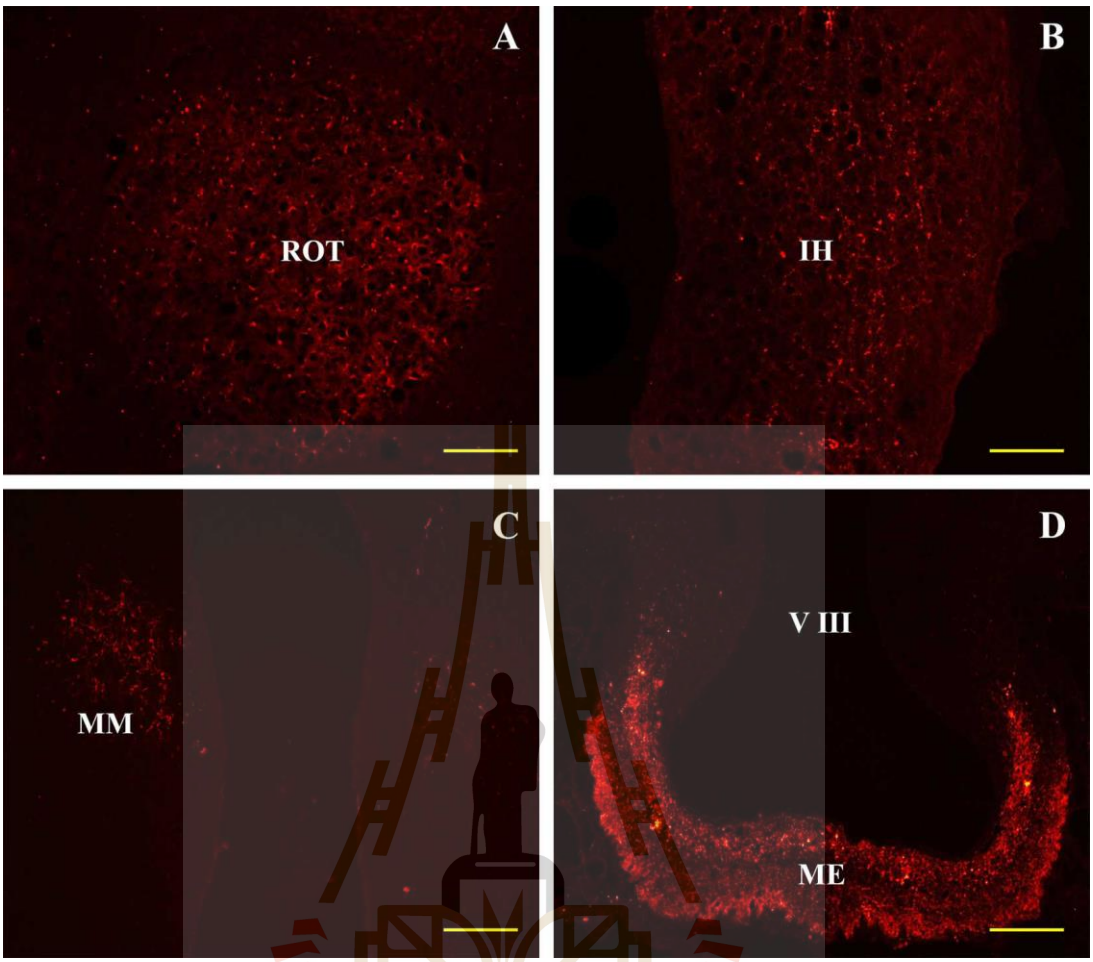

Figure 5.7 Photomicrographs of coronal sections showing TH-ir fibers within the (A) ROT, (B) IH, (C) MM, and (D) ME. V III, ventriculus tertius (third ventricle). Scale bar = 100 μm. See Figure 5.1 for a description of the abbreviations.

5.6 Discussion

The results from the present study demonstrate the distributions of TH-ir neurons and its fibers in the brain of the male native Thai chickens. TH-ir neurons and fibers were extensively located throughout the brain. The majority of TH immunoreactivity was located in the diencephalic and mesencephalic regions. The distributions of TH-ir neurons and fibers were predominantly located within the nI and ML. A modest density of TH-ir neurons and fibers was observed within the SL, SM, AM, LHy, PVN, PVO, AVT, TPc, LoC, BCA, and BCD. The numbers of TH-ir neurons within the nI and ML were significantly higher than those of the AM and PVN. There was a dense accumulation of TH-ir fibers within the ROT, IH, MM, and in the internal and external layers of the ME. The present findings suggest that the DAergic system within the nI and ML might be associated with the physiological function(s) of reproductive activities in the male native Thai chickens.

The anatomical distributions of TH-ir neurons and fibers in this present study are in accordance with previous studies that indicated the distributions of DA neurons and fibers throughout the avian brain including Japanese quails (Ottinger et al., 1986; Bailhache and Balthazart, 1993; Balthazart et al., 1998; Absil et al., 2001), pigeons (Kiss and Peczely, 1987; Berk, 1991; Divac et al., 1994; Durstewitz et al., 1998), zebra finches (Barclay and Harding, 1990; Bottjer, 1993; Mello et al., 1998), chickens (Contijoch et al., 1992; Moons et al., 1994; 1995), budgerigars (Roberts et al., 2001), turkeys (Al-Zailaie and El Halawani, 2000; Thayananuphat et al., 2011), collared doves (den Boer-Visser and Dubbeldam, 2002), canaries (Appeltants et al., 2001), and female native Thai chickens (Sartsoongnoen et al., 2008; Prakobsaeng et al., 2011; Chokchaloemwong et al., 2015; Sinpru et al., 2018). The present findings

demonstrate that the majority of TH-ir neurons and fibers was distributed in the diencephalon and are consistent with previous reports in Japanese quails (Bailhache and Balthazart, 1993; Absil et al., 2001), pigeons (Kiss and Peczely, 1987; Berk, 1991), zebra finches (Bottjer, 1993), and female native Thai chickens (Sartsoongnoen et al., 2008; Prakobsaeng et al., 2011; Chokchaloemwong et al., 2015; Sinpru et al., 2018). In addition, TH-ir neurons and fibers were extensively located within the mesencephalon. These results are in good agreement with previous reports in the female native Thai chickens (Sartsoongnoen et al., 2008). Thus, there are no differences in TH-ir neuron distribution between male and female brains.

The present results illustrate that the distributions of TH-ir neurons and fibers were located in a discrete region lying close to the V III from the level of POA. The distributions of TH-ir neurons and fibers were found in high abundance within the nI and ML. These results are in good agreement with previous reports in females (Sartsoongnoen et al., 2008; Prakobsaeng et al., 2011; Chokchaloemwong et al., 2015; Sinpru et al., 2018). Similarly, in other avian species, the dense immunostaining of TH-ir neurons is extensively distributed along the VIII in canaries and pigeons (Kiss and Peczely, 1987; Appeltants et al., 2001). Moreover, TH-ir neurons and fibers were moderately observed within the SL and SM in the telencephalon and found within the AM, LHy, PVN, and PVO in the diencephalon as in females (Sartsoongnoen et al., 2008; Chokchaloemwong et al., 2015). Indeed, the distributions of TH-ir neurons are scattered within the SL, SM, AM, PVN, and PVO in Japanese quails, collared doves, and canaries (Absil et al., 2001; Appeltants et al., 2001; den Boer-Visser and Dubbeldam, 2002). In this study, like in females, a modest density of TH-ir neurons and fibers was observed within the AVT, TPc, LoC, BCA, and BCD in the

mesencephalon (Sartsoongnoen et al., 2008), and these distributions are also reported in chickens and pigeons (Kiss and Peczely, 1987; Moons et al., 1994). The major groups of TH-ir neurons and some fibers are detected in the AVT (A10), TPc, and LoC in collared doves and canaries as well (Appeltants et al., 2001; den Boer-Visser and Dubbeldam, 2002). In the present study, small numbers of TH-ir neurons and fibers were found within the QF, TSM, POP, SCNm, OM, GCt, IF, and PMM. In addition, TH-ir neurons were also found lining the cortex layer of the Cb and nVm as reported in the females by Sartsoongnoen et al. (2008). Similarly, TH-ir neurons and fibers in this present study are in accordance with previous studies in Japanese quails (Absil et al., 2001), pigeons (Kiss and Peczely, 1987), chickens (Moons et al., 1994), and canaries (Appeltants et al., 2001).

The numbers of TH-ir neurons in four hypothalamic areas reveal that the highest accumulation of TH-ir neurons was observed within the nI and ML when compared with those of the AM and PVN. These results are consistent with previous findings in the female native Thai chickens (Prakobsaeng et al., 2011; Chokchaloemwong et al., 2015; Sinpru et al., 2018). Previous studies in the females reported that the number of TH-ir neurons in the nI is associated with the reproductive stages, with the number of TH-ir neurons in this nucleus and plasma PRL levels increasing significantly in the INC hens, and decreasing in the R hens (Sartsoongnoen et al., 2008). Disruption of incubation behavior by nest-deprivation causes the numbers of VIP-ir neurons in the IH-IN and TH-ir neurons within the nI and ML to decrease (Prakobsaeng et al., 2011). The number of TH-ir neurons in the nI is significantly higher in the R hens when compared with the NR hens while plasma PRL levels are directly mirrored with changes in the number of TH-ir

neurons in the nI, indicating that DA neurons in this nucleus and plasma PRL levels are enhanced to initiate and maintain the rearing behavior than for egg incubation (Chokchaloemwong et al., 2015). Recently, it was shown that the numbers of TH-ir neurons in the nI and ML and plasma PRL levels decrease in the replaced-eggs-with-chicks (REC) hens when compared with the INC hens (Sinpru et al., 2018). Likewise, TH-ir neurons co-expressing Fos mRNA are found in the ML when the hens make the transition from incubating to rearing behavior by REC hens. DA neurons in the nI have been shown to be involved in the regulation of reproductive seasonality in the turkeys (Thayananuphat et al., 2007; 2011). It has been reported that the activation of DA neurons in the ML is associated with the stimulation of GnRH-I and VIP neurons and the subsequent release of LH and PRL (Al-Zailaie et al., 2006). Taken together, these results demonstrate that the DAergic system within the nI and ML might play a regulatory role in year-round reproductive activities and/or parental behaviors in this equatorial species.

In the present study, dense clusters of TH-ir fibers were innervated in the diencephalon. TH-ir fibers were found to be abundant within the ROT, IH, MM, and very dense fibers were observed in the internal and external layers of the ME. These findings correspond with the results of previous studies in the females (Sartsoongnoen et al., 2008; Chokchaloemwong et al., 2015). Studies in chickens suggest that L-DOPA and DA-ir are distributed within the ROT, IH, MM, and ME (Moons et al., 1994). The presence of DAergic fibers in the ME has been reported in the Japanese quails (Bailhache and Balthazart, 1993), pigeons (Kiss and Peczely, 1987), chickens (Moons et al., 1994), and turkeys (Al-Zailaie and El Halawani, 2000). It has been reported that DA inhibits GnRH release via presynaptic inputs at

the ME in the chickens (Contijoch et al., 1992; Fraley and Kuenzel, 1993), and it has been suggested that these areas are involved in the regulation of PRL secretion. PRL secretion is regulated by the inhibitory control of the tuberoinfundibular dopaminergic neurons residing in the infundibular nuclear complex (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001), which release DA that acts directly upon the D_2 DA receptors located on pituitary lactotrophs (Civelli et al., 1991).

In conclusion, this study illustrates the distribution of TH immunoreactivity throughout the brain of male native Thai chickens. The greatest density of TH-ir neurons and fibers was found within the nI and ML, suggesting that the DAergic neurons in these nuclei may be involved with the physiological function(s) of reproductive activities in the male chickens. Moreover, the number of TH-ir neurons in the nI was high in the male brain, implicating that the DAergic system in this nucleus might play an important role in the male reproductive activities and/or parental behavior in this equatorial species.

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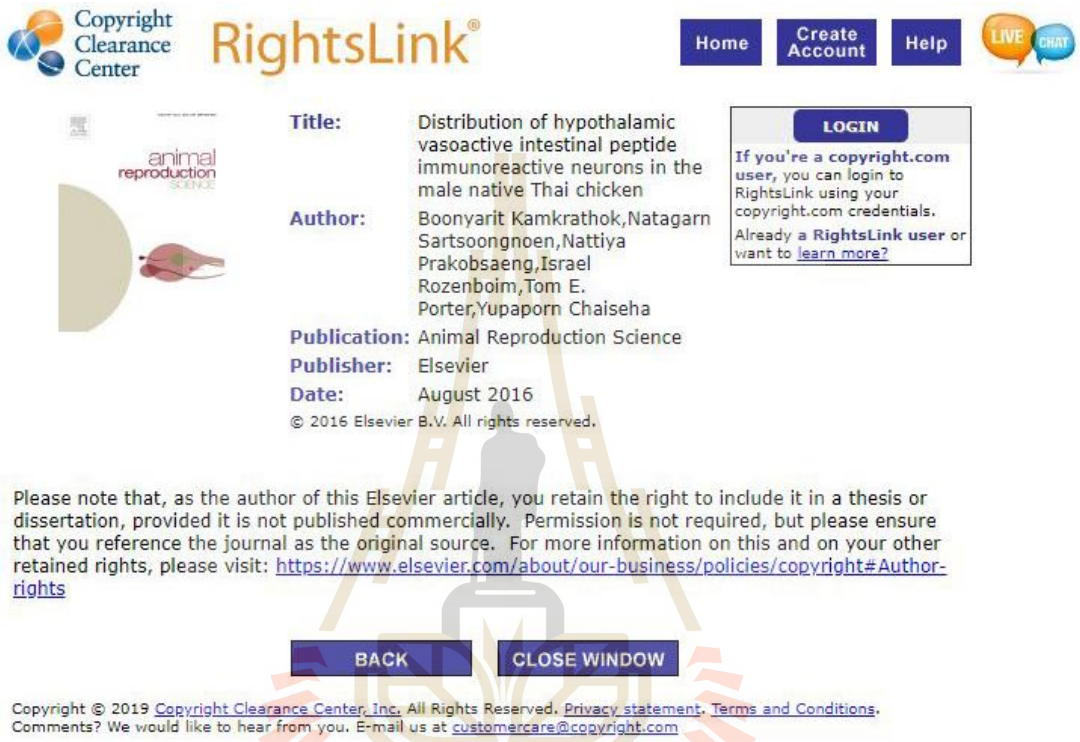
APPENDIX



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Title: Distribution of hypothalamic vasoactive intestinal peptide immunoreactive neurons in the male native Thai chicken

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APPENDIX

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