การควบคุมพฤติกรรมการฟักไข่และการเลี้ยงลูกโดยระบบประสาทและระบบต่อมไร้ท่อในไก่พื้นเมืองไทยเพศเมีย: บทบาทของมีโซโทซิน

นางสาวปานประดับ สินปรุ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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NEUROENDOCRINE REGULATION OF INCUBATION AND REARING BEHAVIORS IN THE FEMALE NATIVE THAI CHICKEN: ROLE OF MESOTOCIN

Panpradub Sinpru

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ปานประดับ สินปรุ : การควบคุมพฤติกรรมการฟักไข่และการเลี้ยงลูกโดยระบบประสาทและระบบต่อมไร้ท่อในไก่พื้นเมืองไทยเพศเมีย: บทบาทของมีโซโทซิน (NEUROENDOCRINE REGULATION OF INCUBATION AND REARING BEHAVIORS IN THE FEMALE NATIVE THAI CHICKEN: ROLE OF MESOTOCIN) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.ยุพาพร ไชยสีหา, 190 หน้า.

ไก่พื้นเมืองไทย (แกลลัสโดเมสติกัส) มีการแสดงออกของพฤติกรรมความเป็นแม่ ได้แก่ พฤติกรรมการฟักไข่และการเลี้ยงลูกอย่างโดดเด่น การแสดงออกของพฤติกรรมดังกล่าวนับว่าเป็นปัญหาสำคัญที่ส่งผลทำให้ศักยภาพในด้านการผลิตไข่ลดลงเป็นอย่างมาก ความสัมพันธ์ของมีโซโทซิน (โปรตีนในสัตว์ปีกที่มีโครงสร้างคล้ายออกซิโทซิน) กับพฤติกรรมการฟักไข่และความสัมพันธ์ของมีโซโทซินร่วมกับบทบาทของโปรแลคตินและโดปามีนในระหว่างการเปลี่ยนจากพฤติกรรมการฟักไข่ไปสู่พฤติกรรมการเลี้ยงลูกถูกตรวจสอบในแม่ไก่พื้นเมืองไทย เทคนิคอิมมูโนฮิสโตเคมิสทรีถูกใช้เพื่อเปรียบเทียบจำนวนเซลล์ประสาทที่ผลิตมีโซโทซินในสมองของแม่ไก่ที่ปล่อยให้ฟักไข่ตามปกติและแม่ไก่ที่ถูกพรากออกจากรัง และในระหว่างที่มีการเปลี่ยนจากพฤติกรรมการฟักไข่ไปสู่พฤติกรรมการเลี้ยงลูก จำนวนเซลล์ประสาทที่ผลิตมีโซโทซินและเซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซีเลส (ตัวบ่งชี้ถึงเซลล์ประสาทที่ผลิตโดปามีน) ถูกเปรียบเทียบระหว่างแม่ไก่ที่ปล่อยให้ฟักไข่ตามปกติและแม่ไก่ที่ถูกแทนที่ไข่ด้วยลูกไก่ ระดับโปรแลคตินในพลาสมาของแม่ไก่ที่ปล่อยให้ฟักไข่ตามปกติและแม่ไก่ที่ถูกแทนที่ไข่ด้วยลูกไก่ถูกวัดโดยใช้เทคนิคเอนไซม์ลิงค์อิมมูโนซอร์เบนท์แอสเสย์ ผลการศึกษาพบว่าเซลล์ประสาทที่ผลิตมีโซโทซินบริเวณนิวเคลียสซุพราออพติคัสพาร์สเวนทราลิส นิวเคลียสพรีออพติคัสมีดิเอียลิส และนิวเคลียสพาราเวนทริคูลาลิสแมกโนเซลลูลาริสของแม่ไก่ที่ปล่อยให้ฟักไข่ตามปกติมีจำนวนมากตลอดช่วงการฟักไข่ และลดลงอย่างมีนัยสำคัญเมื่อแม่ไก่ถูกพรากออกจากรัง การแทนที่ไข่ด้วยลูกไก่ในแม่ไก่ที่ฟักไข่ทำให้จำนวนเซลล์ประสาทที่ผลิตมีโซโทซินบริเวณนิวเคลียสซุพราออพติคัสพาร์สเวนทราลิส นิวเคลียสพรีออพติคัสมีดิเอียลิสและนิวเคลียสพาราเวนทริคูลาลิสแมกโนเซลลูลาริสเพิ่มขึ้นในแม่ไก่ที่ถูกแทนที่ไข่ด้วยลูกไก่เมื่อเปรียบเทียบกับแม่ไก่กลุ่มที่ปล่อยให้ฟักไข่ตามปกติ ในทางตรงกันข้ามพบว่าจำนวนเซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซีเลสในบริเวณนิวเคลียสอินทราเมดิเอลิสลดลงในแม่ไก่กลุ่มที่ถูกแทนที่ไข่ด้วยลูกไก่ในวันที่ 13 และ 17 (แทนที่ไข่ด้วยลูกไก่ในวันที่ 10 และ 14 ของการฟักไข่และชักนำให้เกิดพฤติกรรมการเลี้ยงลูกเป็นระยะเวลา 3 วัน) และจำนวนเซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซีเลสในบริเวณนิวเคลียสแมมิลลาริสแลเทอราลิสลดลงเฉพาะในแม่ไก่กลุ่มที่ถูกแทนที่ไข่ด้วยลูกไก่ในวันที่ 13 เมื่อเปรียบเทียบกับกลุ่มแม่ไก่ที่ปล่อยให้ฟักไข่ตามปกติ การลดลงของ

จำนวนเซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซีเลสในบริเวณนิวเคลียสอินทราเมดิเอลิสและนิวเคลียสแมมิลลาริสแลเทอราลิสมีความสัมพันธ์กับการลดลงของระดับโปรแลคตินในพลาสมา ผลการศึกษานี้บ่งชี้ว่าเซลล์ประสาทมีโซโทซินบริเวณนิวเคลียสซุพราออพติคัสพาร์ส์เวนทราลิส นิวเคลียสพรีออพติคัสมีดิเอียลิส และนิวเคลียสพาราเวนทริคูลาลิสแมกโนเซลลูลาริสไม่เพียงแต่ถูกควบคุมโดยพฤติกรรมการเลี้ยงลูกแต่อาจจะเกี่ยวข้องกับการเริ่มต้นและการคงอยู่ของพฤติกรรมการฟักไข่ด้วย อีกทั้งการปรากฏของไข่หรือลูกไก่ยังเป็นปัจจัยสำคัญในการควบคุมระบบเซลล์ประสาทมีโซโทซินในบริเวณดังกล่าว นอกจากนี้ระบบเซลล์ประสาทโดปามีนในบริเวณนิวเคลียสอินทราเมดิเอลิสและนิวเคลียสแมมิลลาริสแลเทอราลิสยังควบคุมการหลั่งโปรแลคตินในระหว่างการเปลี่ยนจากการฟักไข่ไปสู่ช่วงเวลาของการเลี้ยงลูกในไก่พื้นเมืองไทย ผลจากการศึกษายังแสดงให้เห็นอีกว่าระบบเซลล์ประสาทมีโซโทซินและระบบโดปามีน/โปรแลคตินมีบทบาทสำคัญต่อพฤติกรรมการฟักไข่และการเลี้ยงลูกในไก่พื้นเมืองไทย

สาขาวิชาชีววิทยา
ปีการศึกษา 2560

ลายมือชื่อนักศึกษา ฟานประดับ สินปร
ลายมือชื่ออาจารย์ที่ปรึกษา _____
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PANPRADUB SINPRU : NEUROENDOCRINE REGULATION OF INCUBATION AND REARING BEHAVIORS IN THE FEMALE NATIVE THAI CHICKEN: ROLE OF MESOTOCIN. THESIS ADVISOR : PROF. YUPAPORN CHAISEHA, Ph.D. 190 PP.

DOPAMINE/INCUBATION BEHAVIOR/MESOTOCIN/NATIVE THAI CHICKEN/PROLACTIN/REARING BEHAVIOR

Native Thai chicken (*Gallus domesticus*) exhibits strong maternal behaviors including incubation and brooding or rearing behaviors. The expression of such behaviors is a costly problem, resulting in substantial loss of potential egg production. The association of mesotocin (MT; the avian homolog of oxytocin) with incubation behavior and MT in conjunction with the roles of prolactin (PRL) and dopamine (DA) during the transition from incubation to rearing behavior were investigated in native Thai hens. Using an immunohistochemistry technique, the numbers of MT-immunoreactive (-ir) neurons were compared in the brain of incubating (INC) and nest-deprived hens. During the transition from incubation to rearing behavior, the numbers of MT-ir and tyrosine hydroxylase-ir (TH-ir; as a marker for DA neurons) neurons were compared between the INC and replaced-eggs-with-chicks (REC) hens. Plasma PRL levels of the INC and REC hens were determined by an enzyme-linked immunosorbent assay. The results revealed that the numbers of MT-ir neurons within the nucleus supraopticus, pars ventralis (SOv), nucleus preopticus medialis (POM), and nucleus paraventricularis magnocellularis (PVN) of the INC hens remained high throughout the incubation period and significantly decreased when hens were nest-

deprived. Replacement of eggs with chicks in the INC hens increased the numbers of MT-ir neurons within the SOv, POM, and PVN in the REC hens when compared with those of the INC hens. On the other hand, the number of TH-ir neurons in the nucleus intramedialis (nI) decreased in the REC13 and REC17 (replaced eggs with chicks at days 10 and 14 of incubation and induced rearing behavior for 3 days), and the number of TH-ir neurons in the nucleus mamillaris lateralis (ML) only decreased in the REC13 hens when compared with the INC hens. The decrease in the numbers of TH-ir neurons within the nI and ML is associated with the decrease in the levels of plasma PRL. Taken together, the present findings indicate that the MTergic neurons within the SOv, POM, and PVN are not only regulated by rearing behavior but also might have a role in the initiation and maintenance of incubation behavior and the presence of either eggs or chicks is the key factor regulating the MTergic system within these nuclei. Moreover, the DAergic system within the nI and ML controls the release of PRL during the transition from incubation to rearing behavior in native Thai chickens. The results further suggest that the MTergic and DA/PRL systems play pivotal roles in incubating and rearing behavior in the native Thai chickens.

School of Biology

Academic Year 2017

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Panpradub Sinpru

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LIST OF ABBREVIATIONS

AADC	=	1-aromatic amino acid decarboxylase
AM	=	Nucleus anterior medialis hypothalami
AVP	=	Arginine vasopressin
AVT	=	Arginine vasotocin
BNST	=	Bed nucleus of the stria terminalis
BSTmd	=	Bed nucleus of the stria terminalis, dorsomedial subdivision
CA	=	Catecholamines
Cb	=	Cerebellum
CNS	=	Central nervous system
DA	=	Dopamine
DA-MEL	=	Dopamine-melatonin
DBH	=	Dopamine beta-hydroxylase
E	=	Epinephrine
ELISA	=	Enzyme-linked immunosorbent assay
FSH	=	Follicle stimulating hormone
GH	=	Growth hormone
GnRH	=	Gonadotropin releasing hormone
HPG	=	Hypothalamo-pituitary-gonadal axis
ICV	=	Intracerebroventricular
IH	=	Nucleus inferioris hypothalami

LIST OF ABBREVIATIONS (Continued)

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
LS	=	Lateral septum
ME	=	Eminentia mediana (median eminence)
ML	=	Nucleus mamillaris lateralis
MPOA	=	Area praeoptica medialis
MT	=	Mesotocin
nCPa	=	Nucleus commissurae pallii
ND	=	Nest-deprived hen
NE	=	Norepinephrine
nI	=	Nucleus intramedialis
OT	=	Oxytocin
PBS	=	Phosphate buffered saline
Pit-1	=	Pituitary-specific transcription factor 1

LIST OF ABBREVIATIONS (Continued)

PL	=	Placental lactogen
PMM	=	Hypothalamic premammillary nucleus
POA	=	Preoptic area
POM	=	Nucleus preopticus medialis
POP	=	Nucleus preopticus periventricularis
PR	=	Progesterone receptor
PRF	=	Prolactin-releasing factor
PRL	=	Prolactin
PRLR	=	Prolactin receptor
PVN	=	Nucleus paraventricularis magnocellularis
PVO	=	Organum paraventriculare
REC	=	Replaced-eggs-with-chicks hens
SON	=	Nucleus supraopticus
SOv	=	Nucleus supraopticus, pars ventralis
Stat	=	Signal transducers and activators of transcription
TH	=	Tyrosine hydroxylase
TR	=	Tubular region
V III	=	Ventriculus tertius (third ventricle)
VIP	=	Vasoactive intestinal peptide
VLT	=	Nucleus ventrolateralis thalami
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 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 (incubating 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 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 proteins 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 recognizing, and its popularity is rapidly growing, providing the good opportunity for producing them in industrial scale. Furthermore, Thai government policies 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. Based on this concept, the farmers focus on mixed farming, which is the strategy for helping rural farmers to increase self-sufficiency. One of the natural resources that needs 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, which are heritable traits such as incubating and rearing behaviors. The onset of incubation and rearing behaviors affects the number of eggs produced because it terminates egg laying and the hen spends much time rearing chicks.

Generally, the native Thai hen lays eggs 3-4 times per year and 4-17 eggs per clutch, producing about 30-40 chicks per year, which is significantly lower than that of the imported hen, which produces eggs all year long (240-270 eggs per year). At present, market demands of the native Thai chickens cannot be met by suppliers, mainly because of their low egg laying performance. They tend to lay eggs in clutches rather than evenly distributed over the year, leading to production of chicks irregularly. In addition, growth rate of the native Thai chickens is significantly slower than that of the imported ones. Thus, improving the efficiency of the native Thai chicken production would benefit the poultry industry in Thailand. However, in order to increase the production of the native Thai chicken, it is deemed important to understand the basic neuroendocrinology influencing its reproductive activities. Presently, there are only a limited number of researchers studying the neuroendocrine regulation of reproduction of the native Thai chicken. The reproductive cycle of the native Thai chicken is divided into four reproductive stages; non-egg laying, egg laying, incubating eggs, and rearing chicks. It is very well documented that avian

reproduction is regulated by the integration of the hypothalamus, the pituitary, and the gonads (testis and ovary). There are two major neuroendocrine systems that play pivotal roles in avian reproduction. One system involves 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). Both systems are governed by dopaminergic (DAergic) neurotransmission. The GnRH/FSH-LH system regulates the period of egg laying. On the other hand, the VIP/PRL system initiates and maintains maternal behaviors and may influence the onset of gonadal regression. Recently, mesotocin (MT) has been implicated to be associated with the reproductive cycle and rearing behavior in the native Thai chicken.

It is well established that hormones, neurohormones, neuromodulators, and neurotransmitters play a significant role in maternal behaviors of avian species. PRL, an anterior pituitary hormone, has been indicated to be associated with the reproductive cycle in several avian species such as canvasback ducks, cockatiels, emperor penguins, geese, king penguins, mallards, tropical seabirds, Japanese quails, bantams, ring doves, pigeons, turkeys, and native Thai chickens. PRL has been implicated as a causative factor in the onset and maintenance of maternal behaviors, playing a significant role in incubation behavior, crop milk production and secretion, feeding of the young, and nest defense. Elevated plasma PRL level is involved in the transition from sexual to parental activities. High circulating PRL levels are well known to be associated with incubation and rearing behaviors in birds. In the native Thai chickens, plasma PRL levels of the incubating and rearing hens are higher than

those of the non-rearing ones. It is well established that PRL is under stimulatory control by hypothalamic VIP, the avian PRL-releasing factor, and DAergic influences are involved in both stimulating and inhibiting avian PRL secretion.

DA, a neurotransmitter/neuromodulator, is found in both central and peripheral nervous systems of many species and has several important physiological functions involved in a wide variety of behaviors and reproduction. 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 in mammalian species. Removal of this inhibition results in an increased PRL release and hyperprolactinemia. This is not the case in birds, in which removal of this hypothalamic input results in the complete cessation of PRL secretion. DA has been measured and visualized in many avian species including domestic fowls, Japanese quails, pigeons, zebra finches, chickens, budgerigars, collared doves, turkeys, canaries, and native Thai chickens. Unlike mammals, it has been established that DAergic influences are involved in both stimulating and inhibiting avian PRL secretion depending on multiple DA receptor subtypes. It is also 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. 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. In the native Thai chickens, changes in the number of tyrosine hydroxylase-immunoreactive (TH-ir) neurons (a marker for DAergic neurons) in the nucleus intramedialis (nI) are observed across the reproductive cycle and mirrored directly with circulating PRL levels. The

number of TH-ir neurons is low in non-egg laying hens and markedly increases in incubating hens. Disruption of incubation behavior by nest deprivation decreases the numbers of TH-ir neurons within the nI and nucleus mamillaris lateralis, suggesting that the expression of incubation behavior is regulated by the DAergic system which, in turn, stimulates VIP and subsequent PRL release. In addition, disruption of rearing behavior by removing chicks from hens decreases the number of TH-ir neurons in the nI, and this is accompanied by declined plasma PRL levels, implicating that the activities of the DA/PRL system play a significant role in rearing behavior as well.

MT, a nonapeptide neurohypophysial hormone in birds, is a homolog of oxytocin in mammals. MT neurons are found in several brain areas. However, little is known regarding the physiological function(s) of MT in birds. MT facilitates uterine contractions in hens, acts as a vasodepressor in cockerels, decreases food intake in chicks, promotes social behaviors in zebra finches and emberizid sparrows, and may be involved with nest building in zebra finches. MT is essential to the onset and maintenance of maternal activities in birds. In turkeys, the numbers of MT-ir neurons within the nucleus paraventricularis magnocellularis (PVN) and nucleus supraopticus, pars ventralis (SOv) increase in incubating hens when compared with laying hens. In the native Thai chickens, the MTergic system is associated with the reproductive cycle, and the numbers of MT-ir neurons within the SOv, nucleus preopticus medialis, and PVN increase in rearing hens when compared with non-rearing hens.

For successful reproduction, the survival of the offspring until reproduction has marked effects upon population growth, and it is more sensitive to environmental changes than the survival of the adult. Thus, parental care behavior is an important key to promote and maintain the survival and well-being of the offspring. Parental

behavior is defined as the behavior of the parents that contributes to the survival of its 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 offspring to independence. Maternal care behaviors are limited to incubation and brooding or rearing behaviors. Paternal care behavior refers to behaviors performed by the mature male, which have a positive influence on development, growth, well-being, and survival of the offspring. Therefore, reproductive efforts in birds are extended past fertilization with a variety of parental behaviors. These phenomenons involving parental behaviors may occur due to a complex neuronal/hormonal interaction of many hormones, neurohormones, neuromodulators, and neurotransmitters. Recently, behavioral endocrine studies in the galliform birds have focused on the roles of several neurotransmitters/neuromodulators/neurohormones/hormones that mediate maternal care behaviors.

The expression of maternal behaviors including incubation and rearing behaviors is a costly problem, resulting in substantial loss of potential egg production. Some evidences suggest that plasma PRL levels also play a role in terminating egg laying and regulating clutch size in species that lay clutches of more than two eggs. The reproductive efficiency of native Thai hens is low in comparison to those of the imported ones. Thus, in order to increase the production of the native Thai chickens in Thailand, it is very important to understand the neuroendocrine regulation of the

incubation and rearing behaviors. To date, an association of the neuronal interactions between PRL, GnRHergic, VIPergic, and DAergic systems in the regulation of incubation and rearing behaviors has been reported. Recently, the MTergic system in the regulation of rearing behavior has also been reported. However, the role of the MTergic system on the neuroendocrine regulation of incubation behavior has never been studied. Thus, the objectives of this dissertation were carried out to elucidate the neuroendocrine regulation of maternal behaviors in the native Thai chickens. This dissertation focuses on the role of MT associated with incubation behavior and the role of MT in conjunction with the roles of PRL and DA in rearing behavior in the female native Thai chickens. The findings gained from this study will provide an insight into the neuroendocrine mechanism(s) underlying the regulation of the incubation and rearing behaviors in the native Thai chickens, which has limited number of studies. The knowledge gained can be then applied commercially in the poultry industry to increase egg production of the native Thai chicken in Thailand.

1.2 Research Objectives

1.2.1 To study the localization and differential expression of MT neurons that might be associated with the neuroendocrine regulation of incubation behavior in the female native Thai chickens.

1.2.2 To study the role of PRL and the differential expression of MT and DA neurons that might be associated during the transition from incubation to rearing behavior by replacement of eggs with chicks in the female native Thai chickens.

CHAPTER II

LITERATURE REVIEW

2.1 Native Thai Chicken

Historically, 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. It has been accepted that all domesticated chickens descend from a single ancestor, the red jungle fowl (*Gallus gallus*), originating in 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 by village people approximately 3,000 years ago. The native Thai chickens still express strong maternal behaviors (incubation and rearing or brooding behaviors), which are inherited from its ancestor (Beissinger et al., 1998). The main objectives of raising the native Thai chicken are for consumption, sport competition, and recreation. It is not only a main protein food source for families, but it can be also sold for supplemental income. Generally, they are easy to raise, resistant to diseases, acclimatized to the local environments, and tolerate a large variety of available local feed. The native Thai chicken has a slower growth rate than those of the imported commercial broilers when raised under the same conditions. However, it can be raised commercially with lower production costs by raising it as free range or under the farming system in a backyard using organic local feed. It also has been reported that when fed with local feeds, high performance breeds lose their advantages more than the native Thai chickens in terms

of weight gains (Leotarakul and Pimkamlia, 1999; Haitook et al., 2003). In addition, the native Thai chicken is well adapted to the poor conditions of small farms or simple rural environments. It has been reported that the native Thai chickens can adapt to high temperature better than imported broilers (Aengwanich, 2008), resulting in high potential for raising the native Thai chickens commercially in the urban areas (Kajaroen et al., 1989).

To date, in Thailand, there are about 89 million native Thai chickens or 21 % of total chicken production, which are 62 % broilers, 13 % layers, 3 % commercial broiler breeders, and 1 % commercial layer breeders (Department of Livestock Development, 2017). The meat of native Thai chicken is very popular among Thai consumers because of its unique taste and texture, which is regarded as a greater delicacy than those of commercial broilers (Wattanachant et al., 2005; Puttaraksa et al., 2012). It also provides higher meat quality with less fat and low cholesterol contents than those of the imported commercial broilers (Wattanachant et al., 2004; Jaturasitha et al., 2008; Teltathum and Mekchay, 2009). The textural characteristics of the native Thai chicken's meat are similar to the spent hen meat, but they are much different from the imported broiler's meat (Wattanachant et al., 2004; Chuaynukool et al., 2007). 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; Puttaraksa et al., 2012). In addition, the shear values of raw and cooked native Thai chicken muscles are higher than those of the broiler muscles (Wattanachant et al., 2004; 2005; Jaturasitha et al., 2008). The imported breeds (Bresse and Rhode Island Red) are heavier at slaughter and have higher contents of fat and cholesterol than those of the indigenous strains (black-boned and native Thai chicken; Jaturasitha et al., 2008). In addition, it has been

suggested that Pradu Hang Daum breed is suitable to be developed as a meat type chicken because its lower genetic distance to broiler strains (Dorji et al., 2011). However, the meat quality is determined by several factors such as genotype, rearing system, feed, age, muscle pH, chemical composition, microstructure of muscle, postmortem aging, and processing methods (Chotesangasa and Gongruttananun, 1999; Jaturasitha et al., 2002; Wattanachant et al., 2005; Wattanachant, 2008). It has been reported that the domestic market for native Thai chickens has increased substantially and there is also strong potential for sales in overseas markets (Huo and Na-Lampang, 2012), and these advantages of its meat lead to a higher price of about 2-3 times higher than those of the imported commercial broilers in Thailand, Hong Kong, China, and Japan (Chotesangasa and Gongruttananun, 1999; Jaturasitha et al., 2008).

The egg production of native Thai chickens is much lower than those of the crossbred and hybrid breeds, which is necessary for sufficient number of chicks for fattening (Chotesangasa et al., 1994). Therefore, the native Thai chickens are suited for the small farmers raising system. However, there are needed to be developments in improving the supply of chicks for fattening (Haitook et al., 2003). The limited number of eggs per hen causes the problem of chick production in the hatchery. Generally, the native Thai hen lays eggs 3-4 times/year and 4-17 eggs/clutch rather than laying eggs continuously all year long. In addition, the hen-day egg production of the native Thai hen is lower than that of the commercial laying hen. The peak production for native Thai hens is 38.0 %, while commercial laying hens is 75.5 %. The amount of eggs per hen of native Thai hen is about 30-92 eggs/year, which is significantly lower than that of the imported commercial hen (243 eggs/hen/year; Chotesangasa et al., 1994). Thus, with a hatching rate of 80-85 %, a typical native Thai hen produces 25-40 chicks/year

(Klinhom et al., 2005). This low potential in egg production of the native Thai chickens causes a problem in order to produce them commercially in the poultry industry in Thailand. The main cause of low egg production and the short egg laying period is the expression of maternal behaviors (incubation and rearing behaviors). These behaviors are highly expressed during egg laying, nesting, and rearing periods, which are certainly not desired for commercial scale production (Choprakarn and Wongpichet, 2007). 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.

2.2 Neuroendocrine Regulation of the Avian Reproductive Cycle

The control of avian reproduction involves the interaction of external stimuli with neuroendocrine mechanisms and is integratively regulated by the hypothalamus, the pituitary, and the gonads, namely the hypothalamo-pituitary-gonadal (HPG) axis. It is well established that neurotransmitters, neuromodulators, neurohormones, and hormones of this HPG axis play a significant role in avian reproduction. This axis involves two major neuroendocrine systems including the gonadotropin releasing hormone/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH) and vasoactive intestinal peptide/prolactin (VIP/PRL) systems. The GnRH/FSH-LH system regulates the period of sexual maturity and egg laying. GnRH stimulates the secretion

of FSH and LH, which in turn are responsible for ovarian follicular growth and ovulation. The VIP/PRL system initiates and maintains maternal behaviors and may influence the onset of gonadal regression. Both systems are governed by the dopaminergic (DAergic) system (Chaiseha and El Halawani, 2005; 2015).

In birds, FSH, LH, and PRL are associated with the reproductive cycle in several species such as canvasback ducks (Bluhm et al., 1983b), cockatiels (Myers et al., 1989), emperor penguins (Lormee et al., 1999), geese (Huang et al., 2008), king penguins (Mauget et al., 1994), mallards (Bluhm et al., 1983a; Boos et al., 2007), tropical seabirds (Lormee et al., 2000), chickens (Hu and Zadworny, 2017), turkeys (Mashaly et al., 1976; El Halawani et al., 1984b; 2001; Wong et al., 1992b), and native Thai chickens (Chaiseha and El Halawani, 2015). Egg laying period is associated with high plasma levels of FSH, LH, and gonadal steroids (estrogens and progesterone; El Halawani et al., 1988b; Kosonsiriluk et al., 2008). The onset of incubation behavior is associated with declining plasma levels of LH and gonadal steroids and increasing plasma levels of PRL (Lea et al., 1981; Kosonsiriluk et al., 2008; Angelier et al., 2016). PRL is a causative factor for the declined plasma FSH and LH levels and subsequently ovarian regression, when birds make the transition from egg laying to incubation period in bantam hens (Lea et al., 1981), canaries (Goldsmith et al., 1984), cockatiels (Myers et al., 1989), cowbirds (Hohn, 1959), domestic chickens (Sharp et al., 1977; 1979; Bedrak et al., 1981), Japanese quails (Goldsmith and Hall, 1980), mallard ducks (Bluhm et al., 1983a), pheasants (Breitenbach and Meyer, 1959), pied flycatchers (Silverin and Goldsmith, 1983), pigeons (Riddle et al., 1935), ring doves (Goldsmith et al., 1981), snow geese (Campbell et al., 1978), spotted sandpipers (Oring et al., 1986), turkeys (Cogger et al., 1979; Burke and Dennison, 1980; El Halawani et al., 1997), white-

crowned sparrows (Wingfield and Farner, 1978; Hiatt et al., 1987), European wide starlings (Dawson and Goldsmith, 1982), zebra finches (Vleck and Priedkalns, 1985), and native Thai chickens (Chaiseha and El Halawani, 2015). Indeed, PRL is involved in many aspects of reproductive physiology and behaviors. It plays a pivotal role in parental behaviors by mediating increases in incubation, crop milk production/secretion, feeding of young, and nest defense (Buntin et al., 1991; Buntin, 2010). It has been indicated that PRL acts centrally to reduce LH levels by reducing hypothalamic GnRH concentrations (Rozenboim et al., 1993). The abundance of LH-β subunit and PRL mRNAs expression shows an inverse relationship in photostimulated/laying and incubating turkey hens (Wong et al., 1992b). Administration of PRL to laying turkeys suppresses ovariectomy-induced increases in LH release, delays the onset of egg laying, and induces incubation behavior (El Halawani et al., 1991). In addition, immunoneutralization against PRL slows down ovarian follicular development in large white follicles into small yellow follicles and reduces egg laying performance (Li et al., 2011). PRL modulates steroidogenesis in chicken ovarian follicles, and its effects are different depending on the concentration, type of gonadotropin (FSH or LH), and stage of follicle development (Hu and Zadworny, 2017; Hu et al., 2017). Changes in LH and PRL circulating levels during the reproductive cycle are well established in birds. In reproductively quiescent birds, plasma PRL and LH levels are low, while the levels are increased in reproductively active laying hens. Plasma PRL levels are then dramatically increased during the incubating stage, while plasma LH levels are gradually suppressed. Thus, it is this rising PRL levels that have been implicated as the cause of cessation of ovulation, ovarian regression, and induction and maintenance of incubation behavior. Subsequently, PRL levels decrease, while LH levels begin to increase when incubation

behavior is terminated, and as soon as molting is ended (El Halawani et al., 1997; 2001; Angelier et al., 2016). Although avian species exhibit elevated circulating PRL levels during incubation behavior, PRL levels drop at the time of hatching in many precocial species and remain elevated during the rearing period (Chaiseha and El Halawani, 2015; Angelier et al., 2016).

2.2.1 Gonadotropin Releasing Hormone/Follicle Stimulating Hormone-Luteinizing Hormone System

The synthesis and secretion of the pituitary gonadotropins (FSH and LH) are regulated by the central nervous system (CNS) at the hypothalamic level. The hypothalamus synthesizes GnRH, which in turn stimulates the synthesis and release of these gonadotropins (Ulloa-Aguirre and Timossi, 2000; Shalev and Leung, 2003). In both mammals and birds, environmental stimuli transduced by specific receptors govern the synthesis and secretion of GnRH, for which secretion occurs episodically from the hypothalamus. The amplitude and frequency of pulsatile GnRH release influence the pattern of gonadotropins secretion (Levine and Ramirez, 1982; Moenter et al., 1992). In birds, GnRH is synthesized by hypothalamic neurons, secreted from the median eminence (ME), and reaches target cells within the anterior pituitary gland via the hypophysial portal vessels, which in turn stimulates the synthesis and secretion of gonadotropins. In birds, three subtypes of GnRH and two subtypes of GnRH receptors have been identified (Sun et al., 2001; Shimizu and Bedecarrats, 2006; McFarlane et al., 2011). Two distinct forms of GnRH have been isolated in chicken; cGnRH-I and cGnRH-II (Miyamoto et al., 1984; Sherwood et al., 1988). GnRH-III was first characterized in lamprey and is also found in the brain of songbirds (Bentley et al.,

2004). However, GnRH-I is the only form that is known to be directly involved in regulating reproduction in birds (Sharp et al., 1990).

GnRH neurons and fibers are found extensively distributed throughout the avian brain including chickens (Mikami et al., 1988; Kuenzel and Blahser, 1991), ducks (McNeill et al., 1976; Bons et al., 1978), white-crowned sparrows (Blahser et al., 1986; 1989), Japanese quails (Mikami et al., 1988; van Gils et al., 1993; Teruyama and Beck, 2000), European starlings (Dawson et al., 1985; Goldsmith et al., 1989), garden warblers (Bluhm et al., 1991), great tits and ring doves (Silver et al., 1992), turkeys (Millam et al., 1993), dark-eyed juncos (Saldanha et al., 1994), house sparrows (Hahn and Ball, 1995), cockerels (Sun et al., 2001), canaries (Bentley et al., 2004), and native Thai chickens (Chaiseha and El Halawani, 2015). GnRH increases pituitary LH and FSH secretion both *in vitro* and *in vivo* (Millar et al., 1986; Peczely, 1989). Injection of cGnRH-I or cGnRH-II stimulates an increase in plasma LH levels in the domestic hens (Guemene and Williams, 1999; Proudman et al., 2006). Incubation of turkey anterior pituitary cells with GnRH results in an increase in LH-β-subunit mRNA expression and also stimulates LH secretion (You et al., 1995). In chickens, GnRH inhibits FSH-stimulated steroidogenesis, but it enhances LH-stimulated progesterone production (Hertelendy et al., 1982). In seasonal breeders, GnRH neuronal activity is regulated by photoperiod (Sharp and Blache, 2003), and photostimulatory inputs to hypothalamic GnRH neurons increase GnRH mRNA transcription and translation (Dunn and Sharp, 1999), and increase the sensitivity of pituitary cells to GnRH (Davies and Follett, 1975). In many avian species, GnRH peptide contents increase during long day stimulation and decrease during photorefractoriness. Changes in GnRH contents are observed during the avian reproductive cycle (Hahn and Ball, 1995; Millam et al., 1995; Dunn et

al., 1996; Kang et al., 2006; Kuenzel and Golden, 2006). In temperate zone birds, the turkeys, GnRH-I mRNA is abundance within the nucleus commissurae pallii (nCPa), organum vasculosum lamina terminalis, and nucleus septalis lateralis, is greater in the laying hens than those of the non-photostimulated and incubating hens, while lower GnRH-I mRNA expression is observed in the photorefractory hens (Kang et al., 2006). In non-temperate zone birds, the native Thai chickens, GnRH-I-immunoreactive (-ir) neurons are distributed in a discrete region lying close to the third ventricle from the preoptic area (POA) through the anterior hypothalamus, with the greatest abundance found within the nCPa. The number of GnRH-I-ir neurons in the nCPa is highest in the laying hens when compared with those in the other reproductive stages. Nest deprivation causes an increase in the number of GnRH-I-ir neurons in the nCPa of nest-deprived hens when compared with those of the incubating hens. High numbers of GnRH-I-ir neurons are found in the nCPa of non-rearing hens, whereas fewer GnRH-I-ir neurons are observed in the nCPa of rearing hens. These results indicate an association of the GnRH system with maternal behaviors in this non-photoperiodic, continuously breeding avian species. The expression of incubation and brooding behaviors of the native Thai chickens might be regulated, in part, by the differential expression of GnRH-I neurons in the nCPa (Sartsoongnoen et al., 2012; Chaiyachet et al., 2013a). Indeed, VIP, DA, gonadotropin inhibitory hormone, and gonadal steroids are considered to be involved in the regulation of GnRH secretion (Sharp et al., 1984; Tsutsui et al., 2000). In addition, active immunoneutralization against VIP increases pituitary LH-β and FSH-β mRNA expression, subsequently a decrease in PRL mRNA expression and increase the number of small yellow follicles, presumably due to

elevated FSH (Ahn et al., 2001). Taken together, it can be concluded that GnRH plays a significant role in the neuroendocrine regulation of avian reproduction.

2.2.2 Vasoactive Intestinal Peptide/Prolactin System

The regulation of PRL synthesis and secretion in birds involves the interaction of external stimuli with neuroendocrine mechanisms, which includes photoperiod, ambient temperature, and the presence of eggs and young. The external stimuli and internal stimuli are important for initiation and maintenance of PRL secretion, although their relative importances vary with the reproductive stages (Curlewis, 1992). It is very well documented that avian PRL secretion and gene expression are under stimulatory control by hypothalamic VIP, the avian PRL-releasing factor (PRF; El Halawani et al., 1997; 2001; Chaiseha and El Halawani, 2005; 2015). Indeed, it has been established for a long time that avian PRL secretion is controlled by the tonic stimulation of the hypothalamus (Kragt and Meites, 1965; Bern and Nicoll, 1968), and that the principal PRF is VIP, which is secreted from neurons located in the infundibular nuclear complex (INF) of the caudo-medial hypothalamus (Sharp et al., 1989; El Halawani et al., 1997; 2001; Chaiseha and El Halawani, 2015). To date, VIP is very well accepted as the avian PRF, because it meets the classical criteria for defining it as the hypophysiotrophic PRF in birds (Chaiseha and El Halawani, 2015).

Hypothalamic VIP-ir neurons and its mRNA content and hypophyseal portal blood VIP levels are correlated with changes in circulating PRL levels throughout the reproductive cycle in turkeys (Mauro et al., 1989; Youngren et al., 1996a; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999). These observed variations in PRL are, in part, regulated by changes in VIP receptors at the pituitary level (Chaiseha et al., 2004). Unlike mammals, DAergic system influences are involved in both stimulating and

inhibiting PRL secretion in birds, depending upon multiple subtypes of DA receptors (Youngren et al., 1995; 1996b; Chaiseha et al., 1997; 2003a; 2003b; Lv et al., 2018). DA also plays an intermediary role in PRL secretion, requiring an intact VIPergic system to cause PRL secretion (Youngren et al., 1996b). Dynorphin, serotonin, DA, and VIP all appear to stimulate PRL secretion via a pathway expressing κ opioid, serotoninergic, 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).

In birds, VIP neurons and fibers are extensively distributed throughout the hypothalamus (Yamada et al., 1982; Peczely and Kiss, 1988; Hof et al., 1991; Kosonsiriluk et al., 2008; Chaiyachet et al., 2013b; Montagnese et al., 2015; Kamkrathok et al., 2016), especially within 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). During the avian reproductive cycle, VIP acts on the anterior pituitary gland directly to stimulate PRL synthesis and secretion (El Halawani et al., 1997). 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 turkeys (Mauro et al., 1989), pigeons (Peczely and Kiss, 1988), ring doves (Cloues et al., 1990), and native Thai chickens (Kosonsiriluk et al., 2008; Chaiyachet et al., 2013b). Changes in pituitary VIP receptor mRNA expression are observed during the avian reproductive cycle. Increased pituitary VIP receptor mRNA expression is observed in 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 non-temperate zone breeding species, the native Thai chickens, 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 in the IH-IN are associated with the reproductive cycle and directly mirrored with changes in plasma PRL levels. These results suggest that VIP expression in the IH-IN plays a regulatory role in year-round reproductive activity in this equatorial species (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 (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). It also has been reported that the numbers of VIP-ir neurons in the IH-IN areas are high in the rearing hens, whereas the numbers of VIP-ir neurons decrease in the non-rearing hens, and these changes are correlated with plasma PRL levels (Chaiyachet et al., 2013b). Recently, replacement of eggs with chicks in incubating hens decreases the numbers of VIP-ir neurons in the IH-IN when compared with hens that incubate their eggs naturally (Namken et al., 2017). These results indicate that the VIP/PRL system plays an important role in neuroendocrine reorganization to establish the maternal behaviors in this equatorial precocial species.

Thus, the VIP/PRL system is not only a key well established regulator of the incubation behavior, but it is involved in the regulation of rearing behavior as well. It is possible that VIP and the decline in the numbers 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.

2.3 Prolactin: Structure, Functions, and Regulation of Secretion

2.3.1 The Structure of Prolactin

PRL was discovered (Riddle et al., 1932; 1935), and its name is coined on the findings that an extract of bovine pituitary gland causes 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 the lactotrophs of the anterior pituitary gland (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). Variant forms of PRL have been characterized in several mammalian species, and its variants result of alternative splicing of the primary transcript, proteolytic cleavage, phosphorylation, glycosylation, and other posttranslational modifications, thereby altering its physiological functions (Sinha, 1995). The PRL gene in mammals encodes into 277 amino acids of the PRL prehormone (Maria, 2016). Depending on species, the mature hormone consists of 194-199 amino acids (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 belongs to the families of growth hormone (GH) and placental lactogen (PL), and its amino acid sequence is similar to those of GH and PL, sharing genomic, structure, and biological features (Boulay and Paul, 1992; Horseman and Yu-Lee, 1994; Maria, 2016). Their encoding genes are evolved from a common ancestral gene by gene duplication about 500 million years ago (Niall et al., 1971). In birds, it has been suggested that the mechanisms that regulate its gene expression may be extensively conserved (Kansaku et al., 2005; Hiyama et al., 2009b). It has also been reported that PRL is also synthesized by a number of extra-pituitary cells or tissues in both mammals (Ben-Jonathan et al., 1996; Freeman et al., 2000; Soares, 2004) and birds (Berghman et al., 1992; Ramesh et al., 2000; Chaiseha et al., 2012), but its physiological function(s) is far from understood and needed to be further elucidated.

PRL is synthesized and secreted by 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, over 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 omnipotent hormone. Furthermore, it has been suggested that the physiological functions and biological activities of PRL are, at least in part, regulated additionally by posttranslational modifications such as phosphorylation in the various physiological stages (Hiyama et al., 2009a).

PRL receptor (PRLR) is a member of the Class I cytokine receptor superfamily. The family includes receptors of GH, leptin, erythropoietin, and interleukins (Bazan, 1989; 1990; Kelly et al., 1991). PRL binds not only to its cognate receptor (PRLR), but 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 involved in this receptor as well. Activation of mitogen-activated protein kinases 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, pigeons (Chen and Horseman, 1994), and turkeys (Zhou et al., 1996; Pitts et al., 2000). Tissue distributions of the PRLR mRNA have been reported in rats (Nagano and Kelly, 1994; Bakowska and Morrell, 1997), turkeys (Zhou et al., 1996; Pitts et al., 2000), and chickens (Ohkubo et al., 1998). In mammals, PRLR is found in the CNS and a variety of cells, tissues, and organs such as the pituitary gland, heart, lung, thymus, spleen, liver, pancreas, kidney, adrenal gland, uterus, skeletal muscle, prostate gland, epididymis, epithelial cells, bone, and skin (Nagano and Kelly, 1994; Nevalainen et al., 1997; Bole-Feysot et al., 1998; Clement-Lacroix et al., 1999; Pratt et al., 2015). 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).

2.3.2 The Physiological Functions of Prolactin in Birds

As aforementioned, PRL has been known as the parental hormone involved in many physiological functions and behaviors in avian species (Riddle, 1963).

Circulating PRL levels increase in 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). It has been well established that PRL is associated with the avian reproductive cycle in canvasback ducks (Bluhm et al., 1983b), cockatiels (Myers et al., 1989), emperor penguins (Lormee et al., 2000), geese (Huang et al., 2008), king penguins (Mauget et al., 1994), mallards (Bluhm et al., 1983a; Boos et al., 2007), tropical seabirds (Lormee et al., 1999), Japanese quails (Camper and Burk, 1977), bantams (Sharp et al., 1979), ring doves (Lea et al., 1986), zebra finches (Smiley and Adkins-Regan, 2016), turkeys (El Halawani et al., 1984a; 1997), and native Thai chickens (Chaiseha and El Halawani, 2015). Plasma PRL levels are low during reproductively quiescent stages (non-egg laying and rearing stages) in many birds (Buntin, 1996; Sockman et al., 2006; Kosonsiriluk et al., 2008; Angelier et al., 2016). During the period of egg laying, circulating PRL levels increase, and then the levels increase dramatically during the incubation period (El Halawani et al., 1984b; Kosonsiriluk et al., 2008). It is this rising PRL level that causes the cessation of ovulation, ovarian regression, and induction and maintenance of incubation behavior. Thus, the onset of incubation behavior is associated with increasing PRL concentrations (Chaiseha and El Halawani, 2015; Angelier et al., 2016). Numerous studies have suggested that the rising plasma PRL levels during incubating period may suppress FSH and LH secretion (Zadworny and Etches, 1987; Sharp et al., 1988; 1998; El Halawani et al., 1991; 1993; Porter et al., 1991). It has been suggested that PRL acts centrally to reduce circulating levels of LH by reducing hypothalamic GnRH levels (Rozenboim et al., 1993). Suppression of FSH and LH secretion during the incubating period involves a mechanism independent of increased PRL secretion (Sharp et al., 1988; 1989; Lea

and Sharp, 1989; Lea et al., 1996). PRL may also directly inhibit ovarian steroidogenesis (Rozenboim et al., 1993) and lead to involution of the ovary with reduced ovarian steroidogenesis and regression of the oviduct (Porter et al., 1991). Taken together, PRL has been well implicated as a causative factor for the reduced circulating gonadotropins and subsequent ovarian regression, when birds make the transition from egg laying to incubation period in bantam hens (Sharp et al., 1988), cockerels (Sharp et al., 1977), canaries (Goldsmith et al., 1984), Japanese quails (Goldsmith and Hall, 1980), mallard ducks (Goldsmith and Williams, 1980; Bluhm et al., 1983a), pheasants (Breitenbach and Meyer, 1959), pigeons (Riddle et al., 1935), ring doves (Goldsmith et al., 1981), spotted sandpipers (Oring et al., 1986), turkeys (Burke and Dennison, 1980; El Halawani et al., 1984a; 1988a; 1997; Youngren et al., 1991), white-crowned sparrows (Hiatt et al., 1987), wild starlings (Dawson and Goldsmith, 1982), and native Thai chickens (Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). PRL circulating levels increase gradually at the onset of incubation behavior and high levels are maintained during incubation period (Saeki and Tanabe, 1955; Proudman and Opel, 1988). Terminating incubation decreases PRL to the same levels of reproductively quiescent stages (El Halawani et al., 1980; Wentworth et al., 1983; Chaiseha and El Halawani, 2015; Angelier et al., 2016). In addition, changes in PRL gene expression are also highly correlated with the reproductive cycle (Wong et al., 1991; Tong et al., 1997).

2.3.3 The Regulation of Prolactin Secretion in Birds

In birds, the neuroendocrine regulation of PRL secretion involves the interaction of external stimuli with neuroendocrine mechanisms. Critical environmental stimuli include sensory information concerning photoperiod, ambient

temperature, and the presence of eggs and offspring. These external stimuli and steroid hormones such as estrogen and progesterone are important in initiating and maintaining PRL secretion, although their relative importance varies with the stages of the reproductive cycle (Curlewis, 1992). It has long been established that the hypothalamic control of PRL secretion in birds involves a stimulatory mechanism rather than the inhibitory DAergic system found in mammals (Kragt and Meites, 1965; Bern and Nicoll, 1968; El Halawani et al., 1984a; Hall et al., 1986). It is well documented that PRL secretion and its gene expression in birds are controlled by hypothalamic VIP, the avian PRF (Chaiseha and El Halawani, 2015). VIP is secreted from neurons located in the 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, 1999; 2005; 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 et al., 1997; 2003a), and DA plays an intermediary role in PRL secretion, requiring an intact VIPergic system in order to cause the release of PRL (Youngren et al., 1996b). There are evidences indicating that dynorphin, serotonin, DA, and VIP all appear to stimulate avian PRL secretion along a common pathway expressing κ opioid, serotoninergic, 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). Tactile stimuli from the nests and eggs maintain the elevated circulating levels of PRL and up-regulate VIP gene expression in the incubating hens (Silver et al., 1988; Buntin et al., 1991; Massaro et al., 2007). In addition, several

hypothalamic neurotransmitters, neurohormones, and neuropeptides have been studied during the past six decades for their physiological effects on PRL such as thyrotropin releasing hormone, angiotensin II, oxytocin (OT), vasopressin (VP), pituitary adenylate cyclase-activating polypeptide, and peptide histidine isoleucine, but only VIP is thought to be a physiologically significant PRF in birds (Chaiseha and El Halawani, 2015).

2.4 Dopamine: Structure, Functions, and Regulation of Secretion

2.4.1 The Structure of Dopamine

DA is found in both CNS and peripheral nervous system of many vertebrate and invertebrate species (Carlsson and Hillarp, 1956; Benes, 2001). Its chemical name 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. It communicates between neurons and acts synaptically within the 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 molecule that are decarboxylated derivatives of amino acids. CA have distinctive structures, a nucleus of catechol, benzene group with two adjacent hydroxyl groups, an ethylamine side chain, and an amine group (Wood-Gush and Gilbert, 1973).

Tyrosine is the precursor of DA synthesis. 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), namely dihydroxyphenylalanine decarboxylase. TH is the rate limiting step for CA biosynthesis by converting 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 2.3. 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) of the 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. The D_1 DA receptor subtype has been classified as being stimulatory, and the D_2 DA receptor subtype has been classified as being inhibitory. Activation of the D1-like DA receptors

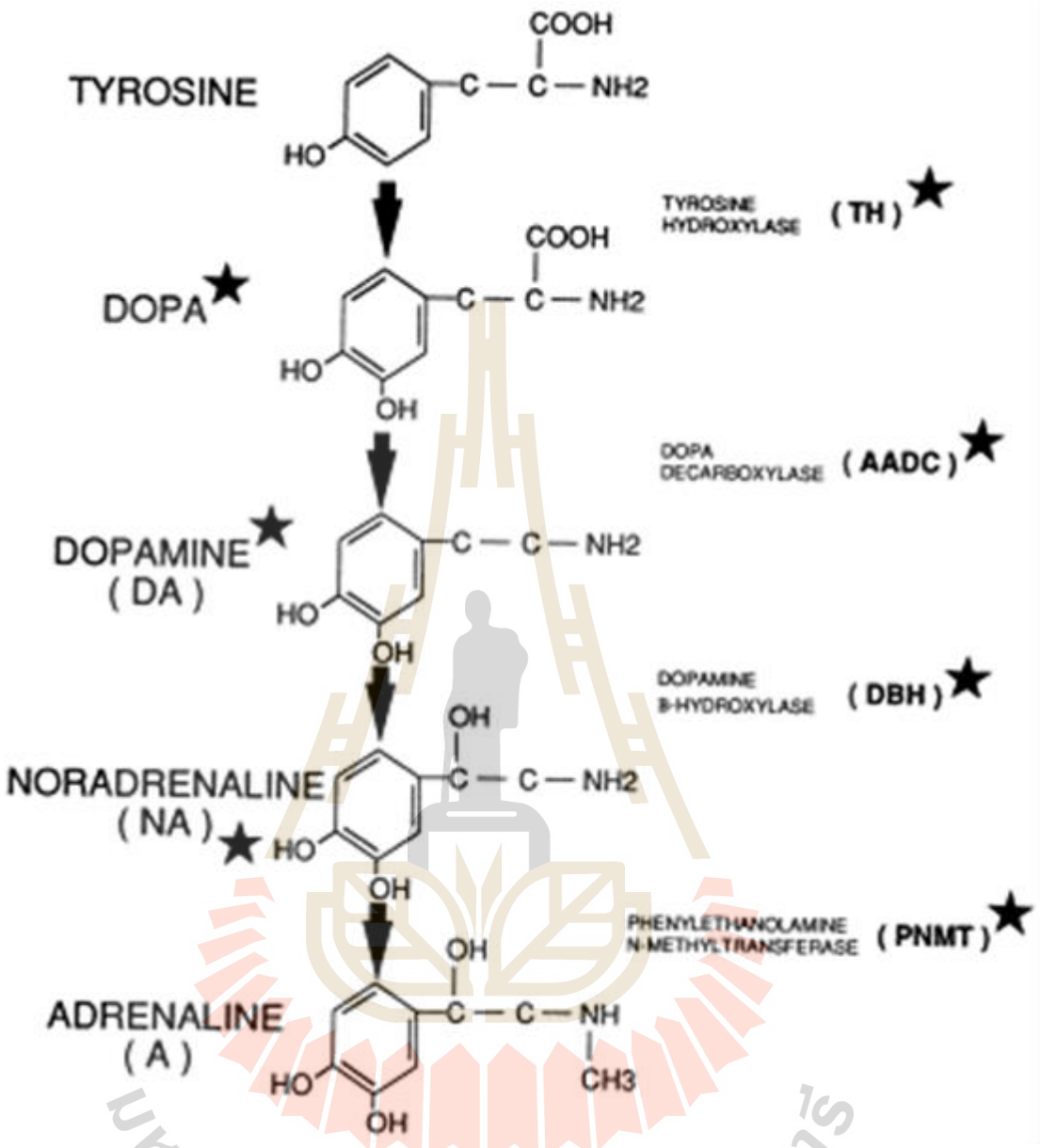

Figure 2.3 Catecholamines biosynthetic pathway and available antibodies as indicated by asterisks (Smeets and Gonzalez, 2000).

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 mammals, the distributions of DA receptor subtypes have been well elucidated. They have distinct localization within the brain and are expressed in a tissue-specific manner in the peripheral tissues (Sunahara et al., 1993; Contreras et al., 2002). In birds, unlike in mammals, 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. 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).

2.4.2 The Dopaminergic System in the Avian Brain

The DAergic system of birds is similar to that of mammals in terms of anatomy (Durstewitz et al., 1999). DA has been determined and visualized in several avian species including domestic fowls (Knigge and Piekut, 1985), Japanese quails (Ottinger et al., 1986; Balthazart et al., 1992; 1998; Bailhache and Balthazart, 1993; 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), collared doves (den Boer-Visser and Dubbeldam, 2002), turkeys (Al-Zailaie and El Halawani, 2000), canaries (Appeltants et al., 2001), and native Thai chickens (Sartsoongnoen et al., 2008; Prakobsaeng et al., 2011; Chokchaloemwong et al., 2015). DA neurons are found throughout the hypothalamus (Kiss and Peczely, 1987; Reiner et al., 1994; Al-Zailaie and El Halawani, 2000) and have been illustrated 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). The localizations of DA-ir neurons in the chicken hypothalamus (Smeets and Gonzalez, 1990) and hindbrain (Kuenzel et al., 1992) have also been reported. Several DA neuronal groups have been found in the preoptic hypothalamic areas of the turkeys including the nucleus preopticus medialis (POM), AM, suprachiasmatic nucleus, nucleus ventrolateralis thalami (VLT), nucleus paraventricularis magnocellularis (PVN), LHy, nucleus ventromedialis hypothalami, nucleus dorsomedialis hypothalami, nucleus mamillaris medialis, and hypothalamic premammillary nucleus (PMM; Al-Zailaie and El Halawani, 2000; Al-Zailaie et al., 2006). The distributions of hypothalamic TH-ir positive and DBH-ir negative cells are found in the turkeys and other avian species (Kiss and Peczely, 1987; Bailhache and Balthazart, 1993; Moons et al., 1994; Reiner et al., 1994; den Boer-Visser and Dubbeldam, 2002). Indeed, TH-ir neurons are predominantly located within the diencephalon and mesencephalon. The presence of DAergic fibers in the ME has been reported in Japanese quails (Bailhache and Balthazart, 1993), chickens (Moons et al., 1994), turkeys (Al-Zailaie et al., 2006), and native Thai chickens (Sartsoongnoen et al., 2008). 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. The existence of DA-melatonin (DA-MEL) neurons in the PMM is demonstrated, where DA and MEL are synthesized and co-localized, suggesting that the pattern of serotonin/CA neuronal distributions and their variable interactions with PMM DA-MEL neurons during different reproductive stages may involve the control of avian reproductive seasonality in seasonally breeding temperate zone birds (Al-Zailaie et al., 2006; Kang et al., 2007; 2009; 2010; Thayananuphat et al., 2007a; 2007b; 2011; El Halawani et al., 2009). Moreover, in non-seasonally breeding species, native Thai chickens, the highest numbers of TH-ir neurons in the nI or VIP-ir neurons in the IH-IN is found in incubating hens when compared with the other reproductive stages, where circulating PRL levels are the greatest (Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). Disruption of incubation behavior by nest deprivation decreases the numbers of TH-ir neurons within the nI and ML and VIP-ir neurons in the IH-IN, mirrored directly by plasma PRL levels (Prakobsaeng et al., 2011). It has been demonstrated that the numbers of TH-ir neurons in the nI and VIP-ir neurons in the IH-IN decrease in parallel with plasma PRL levels when chicks are removed from hens (Chokchaloemwong et al., 2015), but the number of GnRH-ir neurons in the nCPa increases (Chaiyachet et al., 2013a). These also suggest that DA controls the VIP/PRL and GnRH/FSH-LH systems during the reproductive cycle in this species.

2.4.3 The Physiological Functions of Dopamine in Birds

DA and its receptors have been suggested to be involved with several physiological functions in birds such as song learning and production (Budzillo et al., 2017), feather pecking (Kops et al., 2017), food reward (Moe et al., 2014), aggressiveness (Komiyama et al., 2014), egg production (Xu et al., 2010), food intake (Khodadadi et al., 2017), and neurogenesis and neuronal recovery (Lukacova et al., 2016). It has been well established that DA influences are involved in both stimulating and inhibiting PRL secretion in birds. Intracerebroventricular (ICV) infusion of DA in the laying turkey hens can stimulate or inhibit PRL secretion depending on the used concentrations (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 the turkeys (Schnell et al., 1999a; 1999b; Chaiseha et al., 2003a). It has been suggested 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 also activates hypothalamic VIP gene expression in the INF. This increased VIP mRNA in the INF is correlated with increased levels of circulating PRL and LH-β mRNAs in the anterior pituitary gland (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 with 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).

The role of DA as a critical inhibitor of GnRH synthesis and secretion has been reported in both mammals and birds (Ramirez et al., 1984; Sharp et al., 1984). In chickens, the involvement of DA in correlating with GnRH is derived from a dense concentration of TH- and GnRH-containing processes located in the lateral and mediobasal portion of the external layer of the ME, and DA inhibits GnRH release via presynaptic inputs at the ME (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 nCPa during the photo-induction reproductive activity has been reported, demonstrating by c-fos mRNA expressions within the PMM are differentially activated by light and corresponded 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 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 affects 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 the 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).

DAergic activity and DA receptors mRNA expression are changed according the different physiological behaviors and reproduction. DAergic activity in the anterior hypothalamus of bantam hens markedly increases in incubating hens when compared with laying or nest-deprived hens (Macnamee and Sharp, 1989). Moreover, the increasing of stimulatory D_1 DA receptors mRNA expression has been found in hypothalamus of hyperprolactinemic incubating and pituitary of laying hens. However, the inhibitory D_2 DA receptors mRNA expression is increased in the pituitary of hypoprolactinemic photorefractory hens (Schnell et al., 1999a; 1999b; Chaiseha et al., 2003a). 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; 1984a; Mauro et al., 1989; Wong et al., 1991; Chaiseha et al., 2003a). In native Thai chickens, changes in the number of TH-ir neurons in the nI are observed across the reproductive cycle and correlated directly with variations in plasma PRL levels. The number of TH-ir neurons in the nI increases significantly during the egg incubation period (Sartsoongnoen et al., 2008). Disruption of incubation behavior by nest deprivation decreases the numbers of

TH-ir neurons within the nI and ML, and these findings are interpreted that the expression of incubation behavior in birds is regulated by the DAergic system which, in turn, stimulates VIP and subsequent PRL release (Prakobsaeng et al., 2011). Moreover, it has been demonstrated that the number of TH-ir neurons in the nI along with plasma PRL levels are significantly higher in rearing hens as compared to non-rearing hens, suggesting that the DA/PRL system is involved with rearing behavior in this species (Chokchaloemwong et al., 2015).

DA also plays a role in many aspects of sexual activities and reproduction 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). Administration of the D_1 DA agonist increases the sexual behavior in Japanese quails (Balthazart et al., 1997). It is hypothesized that DA neuronal groups within the posterior hypothalamus, particularly from the nI, may play a role in the onset of puberty (Fraley and Kuenzel, 1993). 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 A11 DA neurons of the caudal hypothalamus are involved in courtship singing in songbirds such as zebra finches (Bharati and Goodson, 2006). DA is also involved in learning, reward-seeking, motivated behaviors, and social behavior in birds (Arias-Carrion and Poppel, 2007; Riters, 2012).

2.4.4 The Regulation of Dopamine Secretion in Birds

Originally, DA neurons are 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 mammals, DA concentrations in

hypophysial portal blood are maintained at physiologically active levels (Ben-Jonathan et al., 1980). The studies have demonstrated the involvement of the pituitary-specific transcription factor 1 (Pit-1; growth hormone factor 1) in the hormonal regulation of PRL transcriptional activity including the inhibitory response to DA (Iverson et al., 1990; Elsholtz et al., 1991; Yan et al., 1991). In birds, it has been suggested that the inhibitory effects of DA on VIP-induced PRL gene transcription may result from DA suppression of Pit-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., 1992a; 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 temporal interaction maintains the reproductive system in a stimulated condition and prevents reproductive regression, but does not prevent the onset of scotosensitivity in Japanese quails. It is 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). 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).

2.5 Mesotocin: Structure, Functions, and Regulation of Secretion

2.5.1 The Structure of Mesotocin (Oxytocin-Like Peptide)

The best known neurohypophysial nonapeptides are OT and arginine vasopressin (AVP) that are found in most mammalian species. These neurohypophysial hormones and other related peptides are referred to as the VP/OT family. In addition, there are at least 14 additional neurohypophysial hormones that are found in non-mammalian vertebrates. In birds, the two hormones are arginine vasotocin (AVT) and mesotocin (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 2.4).

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

MT 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, so far, 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.

In birds, the distributions of MT neurons and fibers are found in the supraopticus, pars ventralis (SOv), tractus septomesencephalicus, POM, nucleus preopticus periventricularis, VLT, LHy, bed nucleus of the stria terminalis (BNST), PVN, nucleus

habenularis lateralis, IH, Cb, lateral septum (LS), optic lobe, tuberomammillary nucleus, pons, and medulla oblongata (Goossens et al., 1977; Bons, 1980; Robinzon et al., 1988; Barth et al., 1997; Thayananuphat et al., 2011; Chokchaloemwong et al., 2013; Kamkrathok et al., 2017). MT fibers are found within the internal and external layers of the ME (Goossens et al., 1977; Bons, 1980; Chokchaloemwong et al., 2013; Kamkrathok et al., 2017).

2.5.2 The Physiological Functions of Mesotocin in Birds

MT, the avian neurohypophysial hormone, 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 nucleus supraopticus (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). Oviposition is the expulsion of an egg and requires contraction of the myometrium of the shell gland and simultaneous relaxation of abdominal muscles and the sphincter between the shell gland and vagina (Baeyens and Cornett, 2006). 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 excepting 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 cause 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 induces 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, 2013a; 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 incubating hens when compared with laying hens. In addition, c-fos mRNA is induced in the MT-ir neurons within these areas in the incubating 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, with the highest density observed in the incubating and rearing hens. Comparing the numbers of MT-ir neurons within the SOv, POM, and PVN of hens rearing chicks with that of non-rearing chicks, the number of MT-ir neurons is high in the rearing hens, but low in the non-rearing hens in these nuclei (Chokchaloemwong et al., 2013). Blocking MT receptors decreases nesting behavior and time that female zebra finches spent on their nests (Klatt and Goodson, 2013b).

2.5.3 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, replacing eggs with chicks in incubating turkeys, MT-ir neurons are mainly found within the SOv and PVN (Thayananuphat et al., 2011). Taken together, these findings suggest that the presence of either eggs or chicks may regulate the MTergic system during maternal behaviors in birds.

2.6 Maternal Behaviors in Birds

Maternal care behaviors in birds include incubation and brooding or rearing behaviors. Incubation refers to birds incubating their eggs, and rearing is those caring for their chicks after hatching (El Halawani et al., 1988a; Nelson, 2000). Incubation behavior in birds is defined by sitting continually on their eggs until they hatch, whereas rearing behavior is related to the care of young (Richard-Yris et al., 1983; El Halawani et al., 1988a; Ruscio and Adkins-Regan, 2004; Sharp, 2009; Chaiseha and El Halawani, 2015). Generally, the hens develop maternal behaviors gradually in four stages: brooding, titbitting, clucking, and normal broody behavior (Ramsay, 1953). The maternal behaviors concur with a pause in laying and a significant long term fall in the plasma levels of ovarian steroids (Richard-Yris et al., 1983; 1988a). In chickens, incubation behavior is usually associated with increased body temperature, reduced feed and water intake, frequent nest occupancy, turning and retrieval of eggs, aggressive or defensive behaviors, and characteristic clucking (Romanov et al., 2002; Chaiseha and El Halawani, 2015). Rearing behavior consists of sheltering chicks under the wings, leading the chicks to food or away from danger, and calling to the young in some species (Cain et al., 1978). Birds express rearing behavior by allowing the chicks to access and remain underneath their wings, whereas birds that do not exhibit rearing behavior ignore the chicks (Opel and Proudman, 1989; Ruscio and Adkins-Regan, 2004; Edgar et al., 2011).

2.6.1 Incubation Behavior in Birds

Incubation is a complex behavioral and physiological event. In turkeys, the behavioral patterns include nesting activity, nest protection, and anorexia (El Halawani et al., 1988a). Incubating hens typically have increased body temperature, reduced

intake of food and water, frequent nest occupancy, turning and retrieval of eggs, aggressive or defensive behavior, characteristic clucking, and cessation of egg laying (Romanov et al., 2002). The initiation of incubation behavior is related to nesting frequency and egg laying. In bantam hens, nesting frequency increases and progressively extends to occupy the nest most of the day when the hens stop laying, at which point nesting behavior has transformed to full incubation behavior (Lea et al., 1981). During incubation behavior, the hens sit on their clutches, persistently turn their eggs, rearrange them to ensure they are well covered. The development of this behavior is associated with the cessation of egg laying, clucking, and loss of feathers from the breast to form a brood patch. The incubation behavior and the cessation of egg laying begin after the hens accumulate a full clutch of their eggs. In the bantam hens, they have accumulated about 10-20 eggs per clutch. In the native Thai hens, they have accumulated about 10-17 eggs per clutch. However, the turkey hens may incubate their eggs although not stop egg laying (Lea and Sharp, 1982). The same number of eggs is laid whether or not eggs are removed from the nests, while the birds are still laying in some birds (Moss and Watson, 1982).

Most birds that exhibit incubation behavior develop a defeathered, edematous, and hyperemic area of skin including most of the caudal ventral thoracic and portion of the cranial ventral abdominal regions, the so called brood patch. It has been suggested that the brood patch formation begins about 5 days before the onset of incubation (Lea et al., 1981). It is well supplied with blood vessels for the hen to transfer heat to their eggs and facilitates transmission of tactile stimuli from the eggs to the hen (El Halawani et al., 1988a; Deeming, 2008; D'Alba et al., 2009). During incubation behavior, birds eat and drink very little and lose weight, and this weight loss has been reported in

turkeys (Zadworny et al., 1985), bantams (Savory, 1979), geese (Akesson and Raveling, 1981), mallard ducks (Gatti, 1983), and native Thai chickens (Kosonsiriluk, 2008). Generally, incubation behavior is terminated when the chicks are hatched, but it may persist for a prolonged period if the nest still contains unhatched eggs. Several wild birds continue incubating their infertile eggs for about 50 % longer than the time normally required for hatching them (Skutch, 1962). During a prolonged incubation period, the bantam hens show more ingestive behaviors such as feeding and drinking than searching behaviors such as foraging or random walking, and these behaviors are reversed when the duration of incubation increases (Sharp, 1997).

2.6.2 Neuroendocrine Regulation of Incubation Behavior

The onset of incubation behavior is associated with declining levels of gonadotropins and gonadal steroids and increasing levels of PRL (Lea et al., 1981; El Halawani et al., 1988a; El Halawani and Rozenboim, 1993; Chaiseha and El Halawani, 2015). These increasing PRL levels suppress the activity of the GnRH-I/FSH/LH system, resulting in reduced ovarian steroid secretion, a cessation of ovulation, and ovarian regression (Youngren et al., 1991; Rozenboim et al., 1993). Then, birds transfer from egg laying activity to incubation behavior. Subsequently, PRL level decreases, whereas LH level begins to elevate when incubation behavior terminates (El Halawani et al., 1988a; Knapp et al., 1988; Chaiseha and El Halawani, 2015), and the molting is ended (Bluhm et al., 1983a; 1983b; Mauget et al., 1994). LH level begins to increase at the onset of hatching (Sharp et al., 1979; Goldsmith and Williams, 1980; Hall, 1987; Zadworny et al., 1988; Kuwayama et al., 1992) or when there is the presence of chicks (Richard-Yris et al., 1987a; 1987b; 1995; Sharp et al., 1988; Leboucher et al., 1990; 1993).

It is well established that the increase in PRL concentrations maintains incubation behavior (Sharp et al., 1988). Incubation behavior is facilitated by the combined physiological actions of estrogen, progesterone, and PRL in turkeys, (El Halawani et al., 1986). In addition, the stimulus of nesting maintains high circulating PRL levels in the incubating hens. Disruption of incubation behavior by nest deprivation in turkeys and native Thai hens results in a dramatic decrease in plasma PRL levels (El Halawani et al., 1980; Proudman and Opel, 1981; Prakobsaeng et al., 2011). The degree of incubation behavior and the plasma levels of PRL and LH depend on rearing conditions (Bedecarrats et al., 1997). Additionally, the peripheral nervous inputs act on the onset of incubation behavior as well (Book et al., 1991). The nucleus tuberis, POM, nucleus ovoidalis, and paleostriatum primitivum areas are indicated to be involved in the incubation behavior (Georgiou et al., 1995).

2.6.3 Rearing Behavior

Maternal experiences, neurotransmitters, neurohormones, neuromodulators, hormones, and stimuli from the young interact in complex events to promote maternal responsiveness in both mammals and birds. The presence of chicks results in the expression of rearing behavior, inducing the emergence of specific maternal behaviors and produces maternal vocalizations such as clucking and food calling. The hens display physical contact with the chicks by brooding the chicks for longer durations after hatching, while clucking and food calling are regular behaviors exhibited in hens rearing chicks (Chaiseha and El Halawani, 2015). In galliform birds, precocial newly hatched chicks can walk, feed, see, and hear after hatching, but they cannot effectively thermoregulate during the first two weeks after hatching. Therefore, brooding by the hens helps them to survive (Mills et al., 1997).

Rearing behavior consists of the hens allowing the chicks to nestle underneath its slightly raised wings, while assuming a distinct crouching posture (Hess et al., 1976). Stimuli from the chicks are clearly involved in the establishment, appearance, and maintenance of this behavior (Richard-Yris and Leboucher, 1987; Opel and Proudman, 1989). Rearing behavior can be induced in chickens (Richard-Yris et al., 1983; Richard-Yris and Leboucher, 1986; 1987; Leboucher et al., 1990; 1991; 1993), turkeys (Opel and Proudman, 1988), Japanese quails (Ruscio and Adkins-Regan, 2004), and native Thai chickens (Namken et al., 2017) by introducing the newly hatched chicks to them, which the hens present immediate maternal care responses. The behavior is induced by physical contact between the hen and chicks, alone or in combination with visual and/or auditory stimuli from the chicks (Maier, 1963; Richard-Yris and Leboucher, 1987; Richard-Yris et al., 1998b). A bond is formed between the broody hen and chicks, and the chicks learn to respond to the maternal food calling, distress call, and to the hens purring sound (Wauters and Richard-Yris, 2002; 2003; Edgar et al., 2011). These maternal-offspring bonds are strengthened by repeated exposure of the chicks to the hen, accompanied by food, guidance, and protection (Wauters and Richard-Yris, 2001), which is important for the development of post-hatch species-specific maternal call recognition (Gottleib, 1976; Jain et al., 2004). In precocial species, chicks are self-sufficient after hatching, but parents still serve an important protective function, while also teaching the chicks about food avoidance and food preference (Nicol and Pope, 1996; Nicol, 2004). It has been suggested that changes in PRL concentrations may be related to the large changes in intermediary and water metabolism that occur during rearing behavior (Zadworny et al., 1985). Filial imprinting on parents always occurs during the first few days after hatching in precocial species (Rodgers, 1995; Mills et

al., 1997). The relationships between the mothers and the precocial young have been investigated during their first days of life; the characteristics of mothers influence the emotional and social behavioral development of their young (Bertin and Richard-Yris, 2005; Richard-Yris et al., 2005).

2.6.4 Neuroendocrine Regulation of Rearing Behavior

PRL has been well established role as an incubation promoting hormone in birds. However, it has also been implicated as a crucial factor involved in the onset and maintenance of rearing behavior (Vleck, 1998; Sharp, 2009; Buntin, 2010). High circulating concentrations of PRL are associated with rearing behavior in chickens (Sharp et al., 1988; Hoshino and Wakita, 1989), turkeys (Proudman and Opel, 1981), mallard ducks (Goldsmith and Williams, 1980), Australian black swans (Goldsmith, 1982), ring doves (Buntin, 1996), and native Thai chickens (Chaiseha and El Halawani, 2015).

PRL secretion is stimulated by exposure of the hens to tactile and visual stimuli from the chicks, and in the meantime PRL facilitates and stimulates the expression of maternal behaviors such as incubating, brooding, and feeding (Angelier and Chastel, 2009). An increasing PRL secretion has been implicated in the transition from sexual to parental activity (Sharp et al., 1988; Sockman et al., 2006). Thus, the PRL levels are elevated during the rearing period (Buntin, 1996). This is reflected in changes in circulating PRL levels with different maternal care behaviors in birds. Either sharp declines (bantams (Sharp et al., 1979, 1988; Lea et al., 1981), barheaded geese (Dittami, 1981), common eiders (Criscuolo et al., 2002), domestic ducks (Hall and Goldsmith, 1983; Hall, 1987), Japanese bantams (Zadworny et al., 1988), mallard ducks (Goldsmith and Williams, 1980), Australian black swans (Goldsmith, 1982), turkeys

(Wentworth et al., 1983), and native Thai chickens (Chaiseha and El Halawani, 2015)) or slow decreases (spotted sandpipers (Oring et al., 1986), Wilson's phalarope (Oring et al., 1988), and red-necked phalarope (Gratto-Trevor et al., 1990)) of circulating concentrations of PRL in hens after hatching of the chicks have been reported. PRL facilitates the induction of rearing behavior in galliform birds. Injection of PRL induces the display of the maternal covering stance normally adopted during brooding of chicks (Opel and Proudman, 1980). In precocial species, PRL concentrations generally decline shortly after the chicks are hatched, and the presence of the chicks can modify this rate of declined PRL (Dittami, 1981; Opel and Proudman, 1989). In contrast, in altricial species, PRL secretion increases after hatching while the chicks are being intensively fed and guarded (Buntin, 2010). Among species in which parental care is performed by the females only, PRL levels are higher in females than those of males in parental pied flycatchers (Silverin and Goldsmith, 1984; Angelier and Chastel, 2009). On the other hand, in species in which parental care is performed only by the males, PRL secretion is higher in males than in females such as Wilson's phalaropes and red-necked phalaropes (Buntin, 1996; Buntin et al., 1998). Replacing chicks for eggs or the presence of chicks at hatching in incubating hens is associated with an increase in plasma LH levels and a marked decrease in plasma PRL levels from the high levels presented during the incubation period (Zadworny et al., 1988; Leboucher et al., 1991). Furthermore, exposure to chicks can induce maternal behaviors in the incubating, non-incubating, and ovariectomized hens, which show marked differences in circulating levels of ovarian steroids and patterns of PRL secretion (Richard-Yris et al., 1987a; Leboucher et al., 1991; Lea et al., 1996). In altricial species, the parents jointly care for the young. Pigeons and ring doves, columbiform birds, feed their newly hatched chicks

by regurgitating crop milk, which is produced by epithelial mucosa cells that proliferate in response to PRL and ultimately slough from the crop sac wall (Buntin, 1996; Wang and Buntin, 1999). In addition, the elevated PRL levels during the early post-hatching phase may also promote the display of parental behaviors that are essential for transferring the crop milk to the young squabs (Buntin et al., 1991). After the chicks achieve their thermal independence, PRL circulating levels begin to decrease, and the chicks do not require constant brooding from the mothers (Goldsmith, 1991).

Rearing behavior is associated with low levels of LH and ovarian steroids. The onset of maternal behaviors is accompanied by a significant long-term fall in plasma LH levels. LH secretion may be inhibited by increased plasma PRL levels, indicating that high levels of circulating PRL are involved in the onset or maintenance of brooding behavior and the possibility of an antigonadal role in birds (Bedrak et al., 1981; Sharp et al., 1988; Zadworny et al., 1988; 1989). The relationship between circulating PRL levels and rearing behavior in the parents of precocial young has been reported. The gradual decrease of plasma PRL levels is related to the decline in rearing behavior with the age of chicks (Dittami, 1981; Opel and Proudman, 1989). In shorebirds and red-necked phalaropes, which are facultatively polyandrous and only males care for the eggs and chicks, plasma PRL levels in broody males decrease gradually with increasing age of the broods (Gratto-Trevor et al., 1990). In several avian species, circulating PRL levels decline dramatically at the time of hatch (Goldsmith and Williams, 1980; Dittami, 1981; Goldsmith, 1982; Hall and Goldsmith, 1983; Wentworth et al., 1983). PRL stimulates the growth and development of specialized epithelial cells lining the crop sac, leading to production of crop milk, which is fed to the newly hatched in the pigeons and ring doves. This rise in PRL concentration is associated with the onset or

maintenance of incubation and rearing behaviors in a number of free-living passerine species (Goldsmith, 1991; Buntin, 1996). In turkey hens, endocrinological parameters and production performances are changed during the expression of rearing behavior (Guemene and Williams, 1992). Plasma LH levels decrease progressively, while plasma PRL levels increase while the hens exhibit broodiness. High levels of PRL are then maintained for a long period throughout the rearing behavior and cause the decrease in ovulation rate and egg production. Additionally, an *in vitro* study illustrates that PRL synthesis and secretion are high in the pituitary gland of rearing hens, and these changes are related to rearing behavior (Hoshino and Wakita, 1989).

PRL is involved in maternal behaviors and PRL release is presented throughout the rearing period in galliform birds. PRL secretion in rearing hens is facilitated by the presence of chicks, and those high levels of plasma PRL levels maintain rearing behavior (Sharp et al., 1988). The presence of chicks induces the emergence of specific maternal behaviors in many avian species (Maier, 1963; Richard-Yris et al., 1983; Richard-Yris and Leboucher, 1987; Leboucher et al., 1990; 1993; Wang and Buntin, 1999). Substitution of the eggs by chicks induces maternal behaviors in incubating, non-incubating, and ovariectomized hens (Richard-Yris et al., 1987a; 1995; 1998a; Leboucher et al., 1990; 1993; Lea et al., 1996). In the meantime, PRL levels decline, whereas levels of ovarian steroids remain at low levels (Richard-Yris et al., 1987a; Sharp et al., 1988, Leboucher et al., 1990). Physical contact with newly hatched chicks during brooding bouts also slows down the decrease of PRL release and inhibits LH and estrogen release in maternal hens (Leboucher et al., 1993). Moreover, on the day when chicks are introduced, brooding hens immediately show maternal responses in conjunction with slightly decreased plasma estradiol levels. Thus, it is possible that the

coexistence of newly hatched chicks may suppress LH synthesis and secretion of the hen in the normal/natural breeding cycle (Kuwayama et al., 1992). Rearing behavior progressively declines when the chicks grow older and become fledged, leading to a sharp fall in PRL levels (Richard-Yris et al., 1987a; 1989; Sharp et al., 1988). Thus, plasma PRL levels decrease after the eggs are hatched, the levels remain high for several days, decline gradually as the chicks are brooded, and then reach basal levels by the time the chicks are fledged. The expression of maternal behaviors results from the presence of chicks, and the introduction of chicks induces a drop in plasma PRL levels and a moderate increase in levels of LH and ovarian steroids in incubating domestic chickens (Richard-Yris et al., 1987a; 1995; Sharp et al., 1988; Opel and Proudman, 1989; Leboucher et al., 1990; 1993; Lea et al., 1996) and turkeys (Opel and Proudman, 1988). The number of VIP (the avian PRL-releasing factor) neurons, together with immunoreactivity and cell size, markedly increases during the incubating period and then decreases sharply during the rearing period (Cloues et al., 1990). Disruption of rearing behavior in native Thai chickens markedly decreases PRL secretion with a parallel decline in the number of VIP neurons and an accompanying increase in the number of GnRH-I neurons (Chaiyachet et al., 2013a; 2013b). In addition, the involvement of the DAergic and MTergic systems with rearing behavior also has been demonstrated. The numbers of TH-ir and MT-ir neurons decrease in non-rearing hens when compared with rearing hens, and the decrease in the number of TH-ir neurons is associated with a decline in plasma PRL levels (Chokchaloemwong et al., 2013; 2015). Taken together, these findings suggest that maternal care and particularly physical contact with the young may play a key role in producing these differences (Richard-Yris et al., 1995). It is well established that the presence of chicks inhibits the

hypothalamo-adenohypophysis-ovarian axis in the incubating and non-brooding hens (Richard-Yris et al., 1983; 1987a; 1987b; Sharp et al., 1988). In *vice versa*, physical contact with the chicks induces brooding behavior, an immediate fall in PRL levels, and a gradual rise in LH levels (Richard-Yris et al., 1998b). After hatching the chicks, the circulating levels of LH start to increase gradually, while plasma PRL levels begin to decline (Sharp et al., 1979; Zadworny et al., 1988). It has been stated that PRL is not released at an increased rate while the hens are caring for their young. The bantam hens stop exhibiting broody behavior between 4-10 weeks after the chicks are hatched, and this corresponds to the time when the levels of LH elevate to the levels found in the laying hens (Sharp et al., 1979). In contrast, plasma PRL levels remain at high levels after hatching and then decrease when body mass and structure size of the young are close to those of the hens. The maternal care behavior then decline linearly with brooding behavior as well (Boos et al., 2007). In some avian species, stimuli from the young or from the parent-young interactions may promote or sustain the elevated PRL levels. A definite threshold in circulating PRL levels is necessary to promote and/or maintain post-hatching maternal behaviors in precocial birds. Hens rearing chicks and subsequent hens that are removed from all of their chicks exhibit an abrupt increase in plasma LH levels concurrently with the decrease of plasma PRL levels (Leboucher et al., 1990). Similarly with brooding Gifujidori hens, plasma PRL levels decrease dramatically on the day of hatching, and reach minimum values about 1 week after hatching, while levels of LH and estrogen gradually increase after hatching and reach the maximum values immediately after the removal of chicks (Kuwayama et al., 1992).

IHC studies revealed that the expression of progesterone receptor (PR) immunoreactivity in the tuberal hypothalamic area (TR) decreases in brooding hens as

VIP immunoreactivity increases (Askew et al., 1997; Clark et al., 1999), suggesting that progesterone may act on PR in the TR to inhibit VIP release and subsequently to delay PRL release until incubation behavior has become firmly established (Lea et al., 2001). It is further suggested that PR in the POA mediates the expression of incubation behavior, while PR in the TR is involved in the control of neuroendocrine function(s) (Askew et al., 1997). During the transition from egg laying to the parenting period, PR immunoreactivity decreases in the TR. The numbers of VIP-ir neurons in the IH-IN decrease when hens are forced to make the transition from incubating to rearing behavior (Namken et al., 2017). The DAergic activity and the numbers of MT-ir neurons occurred in specific neuronal regions including the PVN, nucleus dorsomedialis anterior thalami, and SOv, and their numbers are significantly increased in incubating hens when compared with those of laying hens. When the hens make the transition from incubating eggs to brooding of the young, the majority of c-fos mRNA expression by the MT-ir neurons is observed within the PVN and SOv, while the majority of c-fos mRNA expression in the DAergic neurons is observed in the ventral part of the POM (Thayananuphat et al., 2011). So far, in birds, the brain areas that have been implicated in the regulation of parental behaviors are the POA, ventromedial nucleus of the hypothalamus, and PVN (Slawski and Buntin, 1995; Schoech et al., 1998; Lea et al., 2001). Lesion of the POA disrupts PRL-induced parental feeding behavior in ring doves (Slawski and Buntin, 1995). The expression of an immediate early gene protein product, fos-like immunoreactivity, in the brains of brooding ring doves and Japanese quails exposed to their young reveals high density of fos immunoreactivity within the POA, LHy, LS, MPOA, and BNST than those of the parents not allowed to contact with their young (Ruscio and Adkins-Regan, 2004;

Buntin et al., 2006). ICV injections of D_2 DA or OT receptor antagonists into hens brooding poults resulted in over 80 % of those hens failing to brood their poults, and they had lower c-fos mRNA in the dorsal part of POM and the medial part of the BNST areas, indicating that the DAergic, through its D_2 DA receptor and the MTergic systems may play a role in regulating brooding behavior in birds (Thayananuphat et al., 2011). ICV injection of OT also causes a dose-dependent decrease in food intake, feeding time, and pecking frequency. These results suggest that OT might play a unique role in inducing a state of arousal in chickens that resembles fear/anxiety and also in reducing feed intake by acting on MT and/or VT receptors (Jonaidi et al., 2003).

To date, there are limited data regarding the reproductive neuroendocrinology of the native Thai chickens. The role of the MTergic system in the neuroendocrine regulation of incubation behavior has never been studied. Thus, this dissertation was designed to elucidate the role of MT associated with the neuroendocrine regulation of incubation behavior in the female native Thai chickens. In addition, the role of MT in conjunction with the roles of PRL and DA was investigated in rearing behavior as well. The results of this proposed study will provide an insight into the neuroendocrine mechanism(s) underlying the regulation of incubation and rearing behaviors in the native Thai chicken, which has a limited number of studies. The knowledge gained can be then applied commercially in the poultry industry to increase egg production of the native Thai chicken in Thailand.

2.7 References

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CHAPTER III

EFFECTS OF NEST-DEPRIVATION ON HYPOTHALAMIC MESOTOCIN IN INCUBATING NATIVE THAI HENS (*Gallus domesticus*)

3.1 Abbreviations

BSTmd, bed nucleus of the stria terminalis, dorsomedial subdivision; DA, dopamine; GnRH, gonadotropin releasing hormone; IH, nucleus inferioris hypothalami; IHC, immunohistochemistry; IN, nucleus infundibuli hypothalami; INC, incubating hen; -ir, -immunoreactive; ME, eminentia mediana (median eminence); MPOA, area praeoptica medialis; MT, mesotocin; nCPa, nucleus commissurae pallii; ND, nest-deprived hen; nI, nucleus intramedialis; OT, oxytocin; PBS, phosphate buffered saline; POA, area praeoptica; POM, nucleus preopticus medialis; POP, nucleus preopticus periventricularis; PRL, prolactin; PVN, nucleus paraventricularis magnocellularis; SON, nucleus supraopticus; SOv, nucleus supraopticus, pars ventralis; TH, tyrosine hydroxylase; V III, ventriculus tertius (third ventricle); VIP, vasoactive intestinal peptide

3.2 Abstract

Avian mesotocin (MT) is homologous to oxytocin in mammals. Native Thai chickens (*Gallus domesticus*) strongly express maternal behaviors including incubation

and rearing. However, the role of MT during incubation behavior has never been studied. The objective of this study was to determine the physiological function(s) of the MTergic system in incubation behavior in native Thai chickens. The brains were collected from incubating (INC) and nest-deprived (ND) hens at different time points (days 3, 6, 8, 10, 14, 18, and 21; n = 6). Immunohistochemistry technique was used to compare the numbers of MT-immunoreactive (-ir) neurons between the INC and ND hens within the nucleus supraopticus, pars ventralis (SOv), nucleus preopticus medialis (POM), and nucleus paraventricularis magnocellularis (PVN). The results revealed that the numbers of MT-ir neurons within the SOv, POM, and PVN remained high during the incubating stage. The number of MT-ir neurons in the SOv was lower than that of the POM and PVN. Disruption of incubation behavior by nest deprivation caused the numbers of MT-ir neurons within the SOv, POM, and PVN to decrease throughout the observation periods. For the first time, this study demonstrates that the MTergic system within the SOv, POM, and PVN may be involved with incubation behavior. In addition, these results further suggest that the MTergic neurons in these nuclei are not only regulated by rearing behavior but also might have a role in the initiation and maintenance of incubation behavior in this tropical species.

3.3 Introduction

In birds, maternal care behaviors are limited to incubation and brooding or rearing behaviors. Incubation refers to the maternal care of unhatched eggs, and brooding is the maternal care of chicks after hatching (El Halawani et al., 1988; Chokchaloemwong et al., 2013; 2015). Incubation behavior is defined by sitting

continually on eggs until hatching, while brooding is caring for the young after birth (Ruscio and Adkins-Regan, 2004; Chaiseha and El Halawani, 2015). In birds, incubation behavior is composed of frequent nest occupancy, turning and retrieval of eggs, characteristic clucking, and protecting of nests (Romanov et al., 2002; Chaiseha and El Halawani, 2015). In many avian species, a brood patch develops on the ventral abdominal surface, which facilitates the transfer of heat from the hen's body to the eggs, and hens must supply this source of heat at least until the eggs are hatched (Deeming, 2008; D'Alba et al., 2009). These behaviors occur coincidentally with a cessation of egg laying. During the incubating period, plasma levels of luteinizing hormone and follicle-stimulating hormone decline, resulting in ovarian regression and a decline in the plasma levels of ovarian steroids, while prolactin (PRL) secretion is sharply increased (Lea et al., 1981; Myers et al., 1989; Porter et al., 1991; Chaiseha and El Halawani, 2015). In native Thai chickens, (*Gallus domesticus*), plasma PRL levels increase and remain high throughout the incubating stage, whereas the disruption of incubation behavior by nest deprivation is accompanied by a precipitous decline in plasma PRL levels. Moreover, the decline of plasma PRL levels parallels the decrease in the numbers of vasoactive intestinal peptide (the avian PRL releasing factor)-immunoreactive (VIP-ir) neurons in the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) and tyrosine hydroxylase-ir (TH-ir) neurons, a marker of dopaminergic (DAergic) neurons in the nucleus intramedialis (nI) and nucleus mamillaris lateralis (Prakobsaeng et al., 2011; Chaiseha and El Halawani, 2015).

Mesotocin (MT) is a neurohypophyseal hormone in amphibian, reptilian, and avian species, and it is homologous to oxytocin (OT) in mammals. Both neurohypophyseal hormones consist of 9 amino acids, and their sequences differ by only one amino acid, at position 8; OT is leucine, but MT is isoleucine (Acher et al., 1970; Parry et al., 2000). MT neurons are found in the nucleus supraopticus (SON), nucleus paraventricularis magnocellularis (PVN), and tuberomammillary area in chickens, domestic mallards, and Japanese quails, and MT fibers are found at both internal and external layers of the eminentia mediana (ME; Goossens et al., 1977; Bons, 1980). MT is also found in areas outside of the hypothalamus, such as the cerebellum, lateral septum, optic lobe, pons, and medulla oblongata (Robinzon et al., 1988). In turkeys, MT-ir neurons and fibers are found within several hypothalamic areas including the nucleus preopticus periventricularis (POP), nucleus supraopticus, pars ventralis (SOv; ventral part of the SON), the medial part of the bed nucleus of the stria terminalis, PVN, IH, and nucleus habenularis lateralis (Thayananuphat et al., 2011). Recently, in native Thai chickens, the MT-ir neurons and fibers were found in a discrete area lying close to the third ventricle through the anterior hypothalamus, with the greatest density of MT-ir neurons was found in the SOv, nucleus preopticus medialis (POM; largest cell group of the area praeoptica medialis (MPOA) in birds), nucleus ventrolateralis thalami, regio lateralis hypothalami, and PVN. A few MT-ir neurons are also found in the POP, nucleus perventricularis hypothalami, nucleus suprachiasmaticus, pars medialis, tractus septomesencephalicus, and nucleus dorsolateralis anterior thalami, pars magnocellularis. A small group of MT-ir neurons is found at the end of the PVN area. Small groups of MT-ir fibers are found in the

organum vasculosum lamina terminalis, organum subseptale, organum interventriculare, and the external layer of the ME (Chokchaloemwong et al., 2013).

Little is known regarding the physiological function(s) of MT in birds. It is not a regulator of oviposition, cardiovascular function, or plasma osmolarity (Robinzon et al., 1994), but it may participate in regulating urine volume (Takahashi et al., 1997; Takahashi and Kawashima, 2008a; 2008b) and renal blood flow in the kidney (Bottje et al., 1989). MT induces anorexia and wing-flapping in chicks (Masunari et al., 2013; 2016). Studies in zebra finches demonstrate that MT modulates pair-bonding (partner preference; Pedersen and Tomaszycki, 2012; Klatt and Goodson, 2013a; Kelly and Goodson, 2014a), nesting behavior (Klatt and Goodson, 2013b), and stress coping and feeding behavior (Kelly and Goodson, 2014a). Moreover, a function of MT in affiliative (grouping/flocking) behavior has been documented in zebra finches (Goodson et al., 2009) and emberizid sparrows (Wilson et al., 2016). A role for MT in maternal behaviors has been reported in turkeys, in which the numbers of MT-ir neurons in the SOv and PVN increase in incubating hens when compared with laying hens. Mainly, co-localization of MT and Fos (an immediate early gene) mRNA was found within the SOv and PVN after introduction of poults to incubating turkeys (Thayananuphat et al., 2011). These are in accordance with OT in mammals. OT mRNA in the PVN increases in both male and female prairie voles postpartum, and co-localization of OT and Fos also increases with pup exposure relative to a control stimulus (Kelly and Goodson, 2014b). Blocking MT receptors inhibits the expression of brooding behavior in turkeys (Thayananuphat et al., 2011). In native Thai chickens, the highest number of MT-ir neurons is observed in the POM and PVN, when the hens

make the transition from incubating to rearing behaviors, and the numbers of MT-ir neurons in the SOv, POM, and PVN of the rearing hens are significantly higher than those of the non-rearing hens (Chokchaloemwong et al., 2013). This evidence suggests that MTergic activity within the SOv, POM, and PVN is involved with the reproductive cycle and the onset of maternal behaviors in birds (Thayananuphat et al., 2011; Chokchaloemwong et al., 2013).

The native Thai chicken (*G. domesticus*), an equatorial, tropical, non-seasonally breeding species, has been domesticated without genetic selection. It expresses strong maternal behaviors, which are inherited from its ancestor, the wild jungle fowl of Southeast Asia (Austic and Nesheim, 1990; Fumihito et al., 1994; Hillel et al., 2003; Sawai et al., 2010). The neuroendocrine regulation of maternal behaviors (incubation and rearing behaviors) in female native Thai chickens are associated with the VIPergic and DAergic systems (Prakobsaeng et al., 2011; Chaiyachet et al., 2013; Chockchaloemwong et al., 2015).

Recent previous findings indicate that the MTergic system is associated with the reproductive cycle and plays an important role in neuroendocrine reorganization to establish and maintain rearing behavior in native Thai chickens (Chokchaloemwong et al., 2013). However, the role of the MTergic system in incubation behavior has never been studied. Thus, the objective of this study was to elucidate the role of MT associated with the neuroendocrine regulation of incubation behavior in female native Thai chickens. Comparisons were made between the numbers of MT-ir neurons within the SOv, POM, and PVN of incubating and nest-deprived hens. The findings of differential expression of MT-ir neurons in these nuclei may provide insight into their

role in the mechanism(s) underlying the regulation of incubation behavior in native Thai chickens.

3.4 Materials and Methods

3.4.1 Experimental Animals

Female native Thai chickens, Pradoohangdum breed, 18-20 weeks old, were used. They were reared and housed with mature roosters (10-12 females/1 male/pen) in floor pens provided with basket nests under natural day light (approximately 12 h of light and 12 h of darkness, 12L:12D). Food and water were constantly available. At 22 weeks of age, the hens were subjected to the experimental treatments. Each hen was identified by wing band number. The animal protocols used adhered to the guidelines approved by the Suranaree University of Technology Animal Care and Use Committee.

3.4.2 Experimental Design

To study the association of MT-ir neurons and the neuroendocrine regulation of incubation behavior, 84 female chickens and 8 mature roosters were reared and housed in 8 floor pens (10-12 females/1 male/pen). At the first laying cycle, the hens were randomly divided into 2 treatment groups: incubating hens (INC; hens that were allowed to incubate their eggs naturally), and nest-deprived hens (ND; hens that were deprived of their nests and eggs on the third day after the onset of incubation). To compare the time courses of the changes in the numbers of MT-ir neurons within the SOv, POM, and PVN, both INC and ND hens were sacrificed at different time points (days 3, 6, 8, 10, 14, 18, and 21; n = 6) after they started to incubate their eggs or after

nest deprivation. The brain of each hen was fixed by pressure perfusion with 4 % paraformaldehyde (#416780010, Acros Organics, Inc., New Jersey, USA), sectioned with a cryostat, and processed by immunohistochemistry (IHC).

3.4.3 Processing of Tissues for Immunohistochemistry

Prior to perfusion, hens were intravenously injected with 3 ml of heparin (1000 U/ml; Baxter Healthcare Corporation, Deerfield, IL, USA) and then euthanized with pentobarbital sodium (2 ml/kg; Nembutal, Ceva Sante Animale, Libourne, France). The head was removed and immediately fixed by pressure perfusion via the carotid arteries with 100 ml of phosphate buffered saline (PBS, pH 7.4) for 3-5 min, followed by a freshly prepared 4 % paraformaldehyde in 0.1 M of phosphate buffer (pH 7.4, 650 ml) for 30 min according to the previously described method (Chokchaloemwong et al., 2013). The brain was dissected intact from the skull and soaked in 20 % sucrose (#477187, Carlo Erba, Inc., Val-de-Reuil, France) in PBS at 4 °C for 48 h or until saturated for cryoprotection. The brain was then frozen in powdered dry ice for 1 h and stored at -35 °C until sectioned in the coronal plane at a thickness of 16 μm utilizing a cryostat (Microtome cryostat HM525, Microm International GMbH, Walldorf, Germany). The sections were mounted on chrome alum-gelatin-coated glass slides, with 2 sections per slide, and stored under desiccating conditions at -20 °C. Four adjacent sections were processed for IHC to visualize changes in the number of MT-ir neurons within the SOv, POM, and PVN. The stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988) was used to choose the sections containing these nuclei. For each area, the sections were selected starting with the most rostral section that contained each nucleus, and each subsequent section was processed until the nucleus

disappeared from view. The plane that expressed the greatest density of MT-ir neurons was chosen to analyze. These planes of sections have been published in earlier reports (Chokchaloemwong et al., 2013).

3.4.4 Immunohistochemistry

Changes in the number of MT-ir neurons of INC and ND hens were determined by IHC according to a previously described method (Chokchaloemwong et al., 2013). The primary and secondary antibodies used for detecting MT-ir neurons were primary rabbit polyclonal antibody directed against OT (ImmunoStar, Inc., Hudson, WI, USA, Catalog No. 20068, Lot No. 642001) and Cy™3-conjugated AffiniPure donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA, Code No. 715-165-150, Lot No. 105237). The specificity of the primary antibody against MT was previously tested (Chokchaloemwong et al., 2013). Briefly, tissue sections were thawed to room temperature before use. They were treated with PBS (pH 7.4) for 30 min at room temperature. After PBS removal, the sections were then incubated with 60 µl of primary antibody at 1:1000 dilution with PBS containing 1 % bovine serum albumin (#268130100, Acros Organics, Inc.) and 0.3 % Triton-X 100 (#215680010, Acros Organics, Inc.) at 4 °C overnight in a moist chamber. The slides were washed three times with PBS (pH 7.4) for 5 min each. After washing, 60 µl of secondary antibody at 1:500 dilution was applied onto the sections under dark conditions. The slides were further incubated in a moist, dark, chamber at room temperature for 1 h, washed with PBS (pH 7.4) 3 times for 5 min each, and then mounted with DPX mountant (#06522, Sigma-Aldrich, Inc., Steinheim, Germany). Microscopic images of brain sections were visualized and further analyzed.

3.4.5 Image Analysis

Microscopic images of the brain sections of the hens 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). The number of MT-ir neurons in four adjacent sections (6 hens per area) was counted manually to determine changes in the number of MT-ir neurons within the SOv, POM, and PVN. The mean values were compared between INC and ND hens. To avoid double-counting neurons with cell bodies that appeared on two adjacent sections, sections were viewed under 400× magnification and only neurons with detectable nuclei were included in the analysis. 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 the MT immunoreactivity.

3.4.6 Statistical Analysis

Significant differences within treatment groups in the number of MT-ir neurons per section (mean ± SEM) in each individual hypothalamic area were compared employing two-way analysis of variance (ANOVA), with the main effects of group and day and the group-by-day interaction. $P < 0.05$ was considered statistically significant. All statistical tests were analyzed using SPSS for Windows software (version 17.0, SPSS Inc., Chicago, IL, USA).

3.5 Results

The numbers of MT-ir neurons were compared between the INC and ND groups across the SOv, POM, and PVN areas (Figures 3.1-3.4). To investigate the association between MT-ir neurons and the neuroendocrine regulation of incubation behavior, changes in the number of MT-ir neurons of the INC and ND hens were compared at different time periods in each area. When compared between the INC and ND groups, the results showed that the number of MT-ir neurons within the SOv of INC group remained high throughout the incubation period and significantly decreased ($P < 0.05$) in the hens deprived of their nests (Figures 3.2 and 3.5A; Table 3.1). The number of MT-ir neurons in the POM (Figures 3.3 and 3.5B) and PVN (Figures 3.4 and 3.5C) of the INC group remained high throughout the incubation period. When the hens were deprived of their nests, the MT-ir neurons were markedly and significantly decreased ($P < 0.05$) in the POM and PVN (Table 3.1). ANOVA revealed that there were no significant differences in the number of MT-ir neurons between the days of observation and interaction effects between the observation time periods and groups in the SOv, POM, and PVN (Table 3.1, $P > 0.05$).

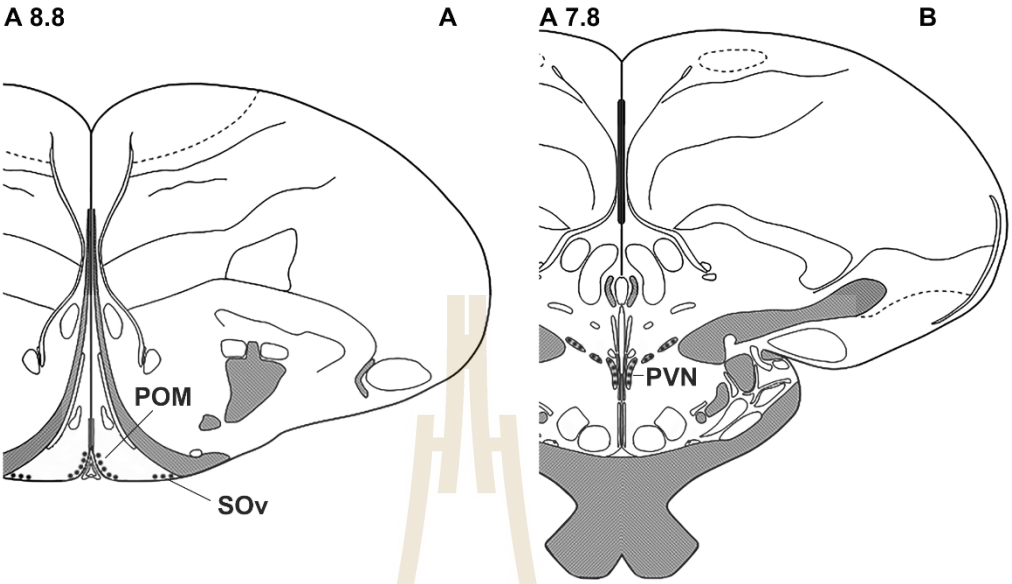

Figure 3.1 Schematic coronal sections showing the sampling regions for counting the numbers of MT-ir neurons in the SOv and POM (A) and in the PVN (B). Coronal illustrations are redrawn, with the given coordinates, from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988).

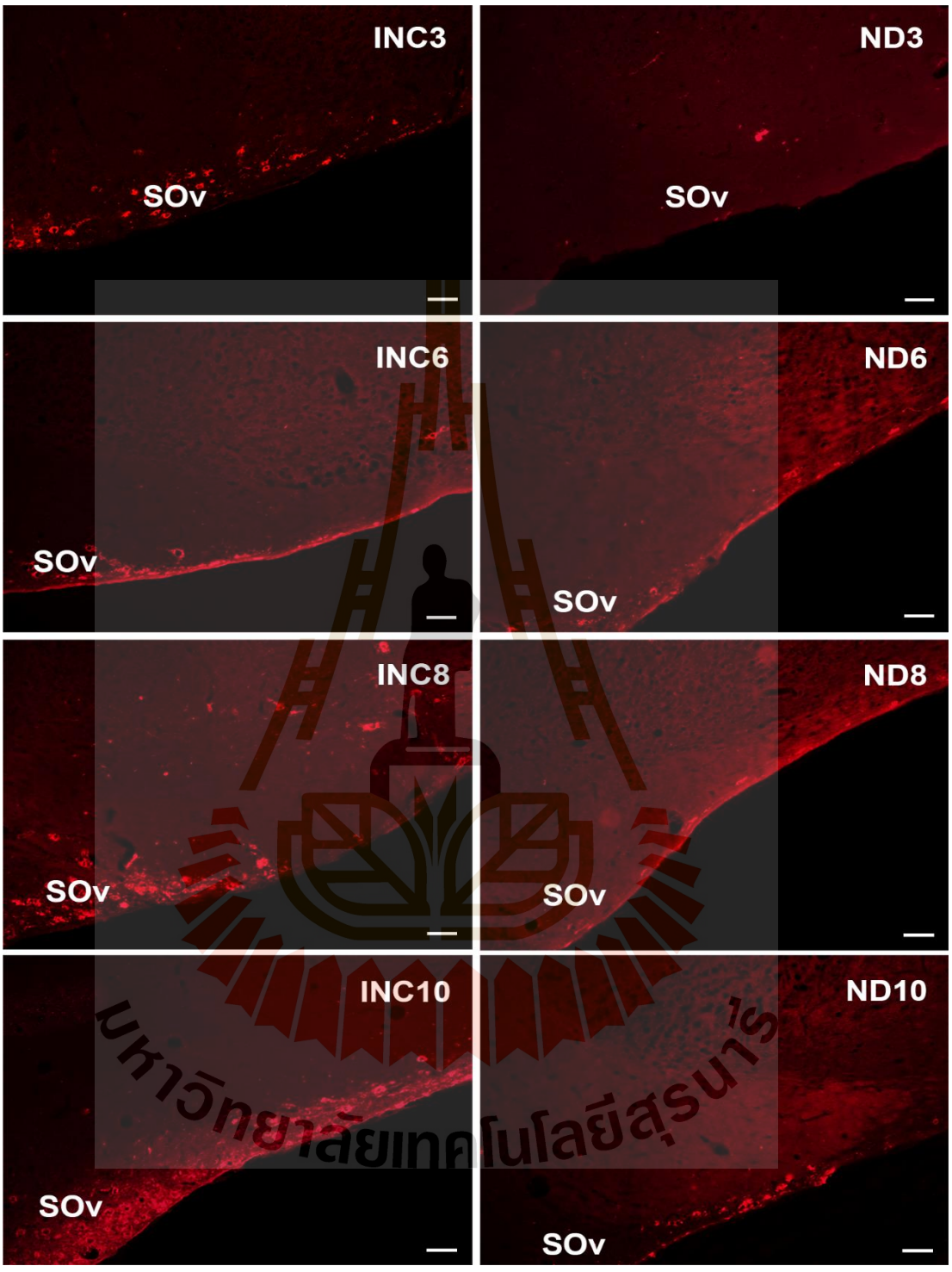

Figure 3.2 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the SOv of incubating (INC) and nest-deprived (ND) native Thai hens during different days of incubating periods. Scale bar = 100 μm.

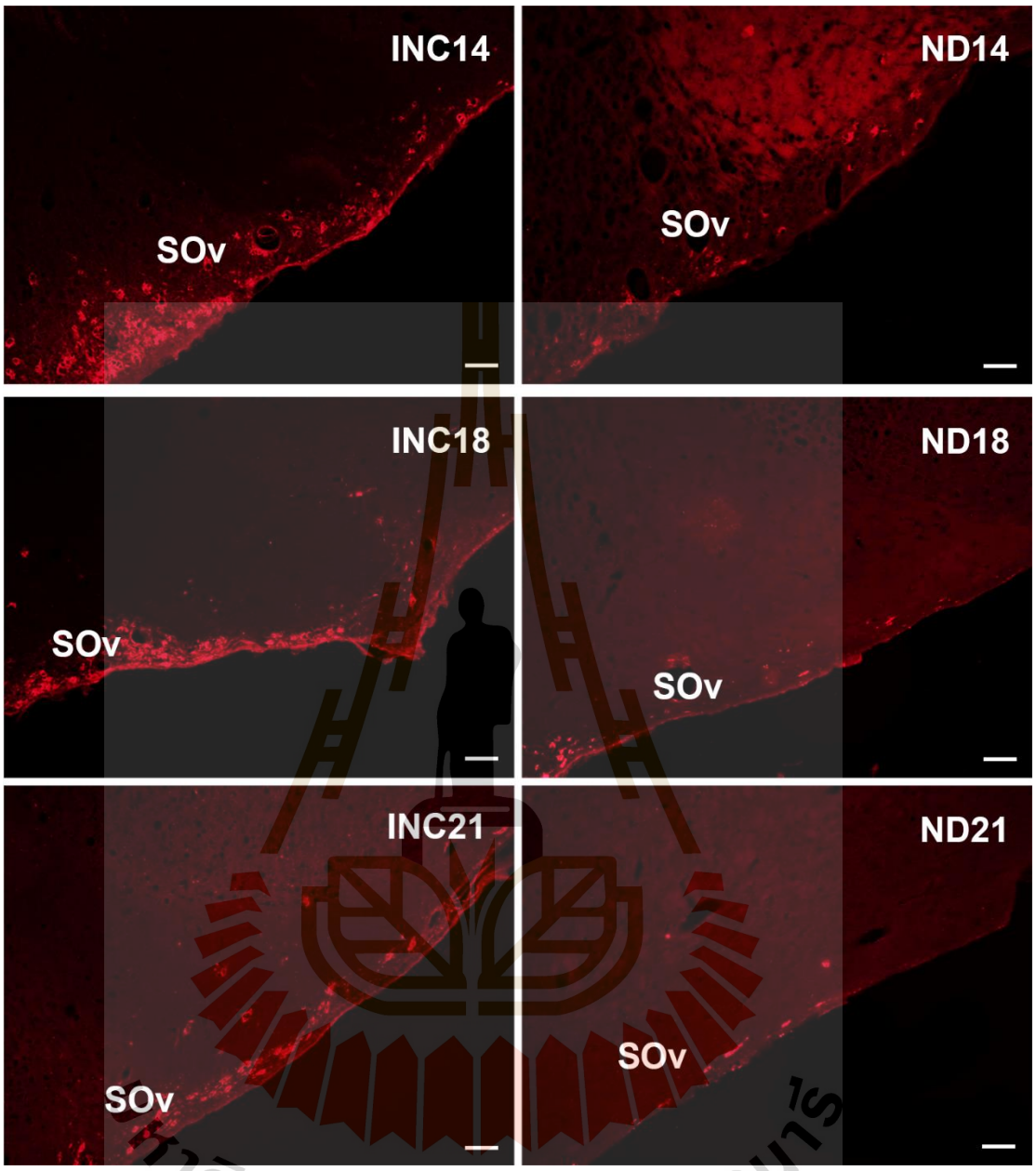

Figure 3.2 (Continued) Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the SOv of incubating (INC) and nest-deprived (ND) native Thai hens during different days of incubating periods. Scale bar = 100 μm.

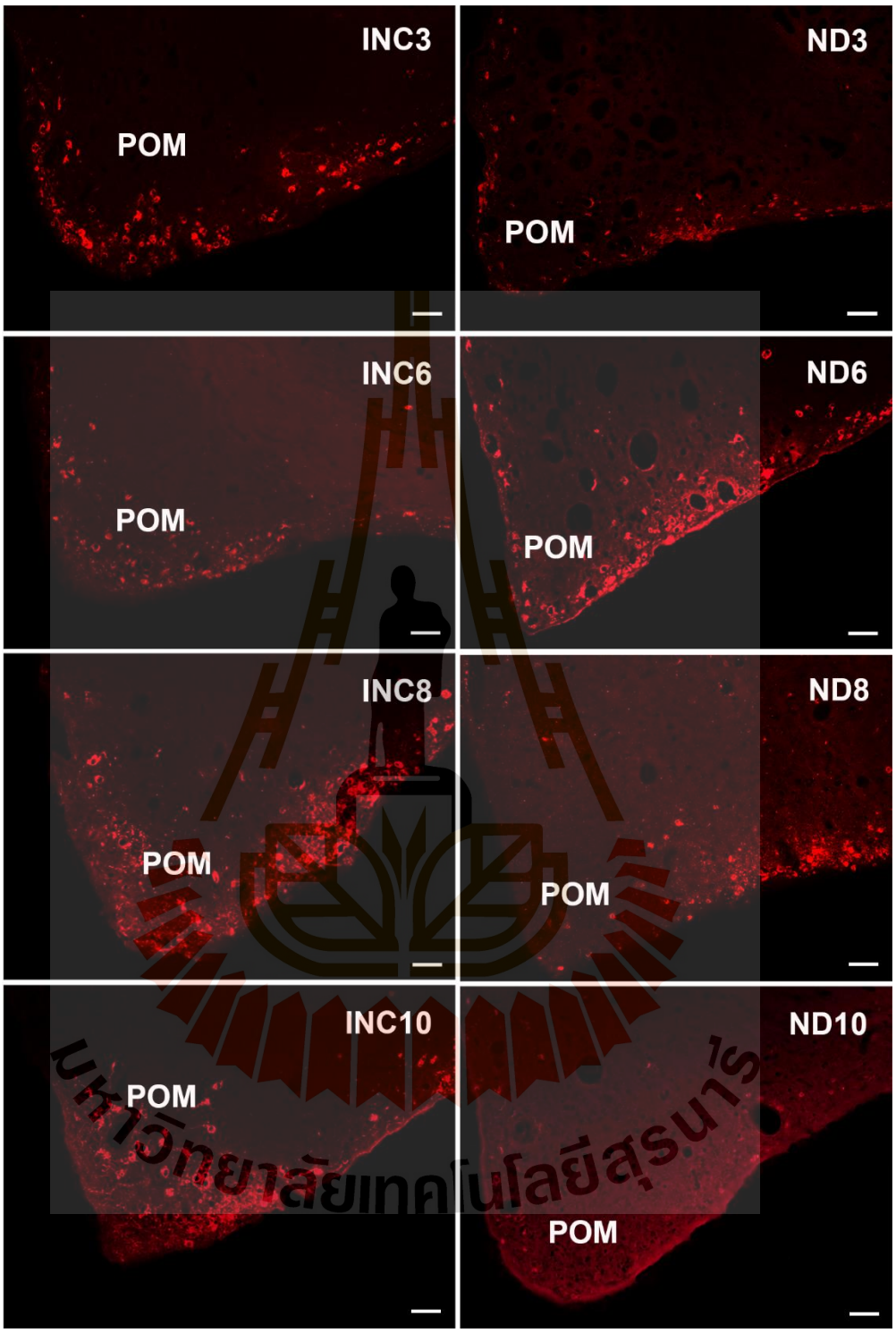

Figure 3.3 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the POM of incubating (INC) and nest-deprived (ND) native Thai hens during different days of incubating periods. Scale bar = 100 μm.

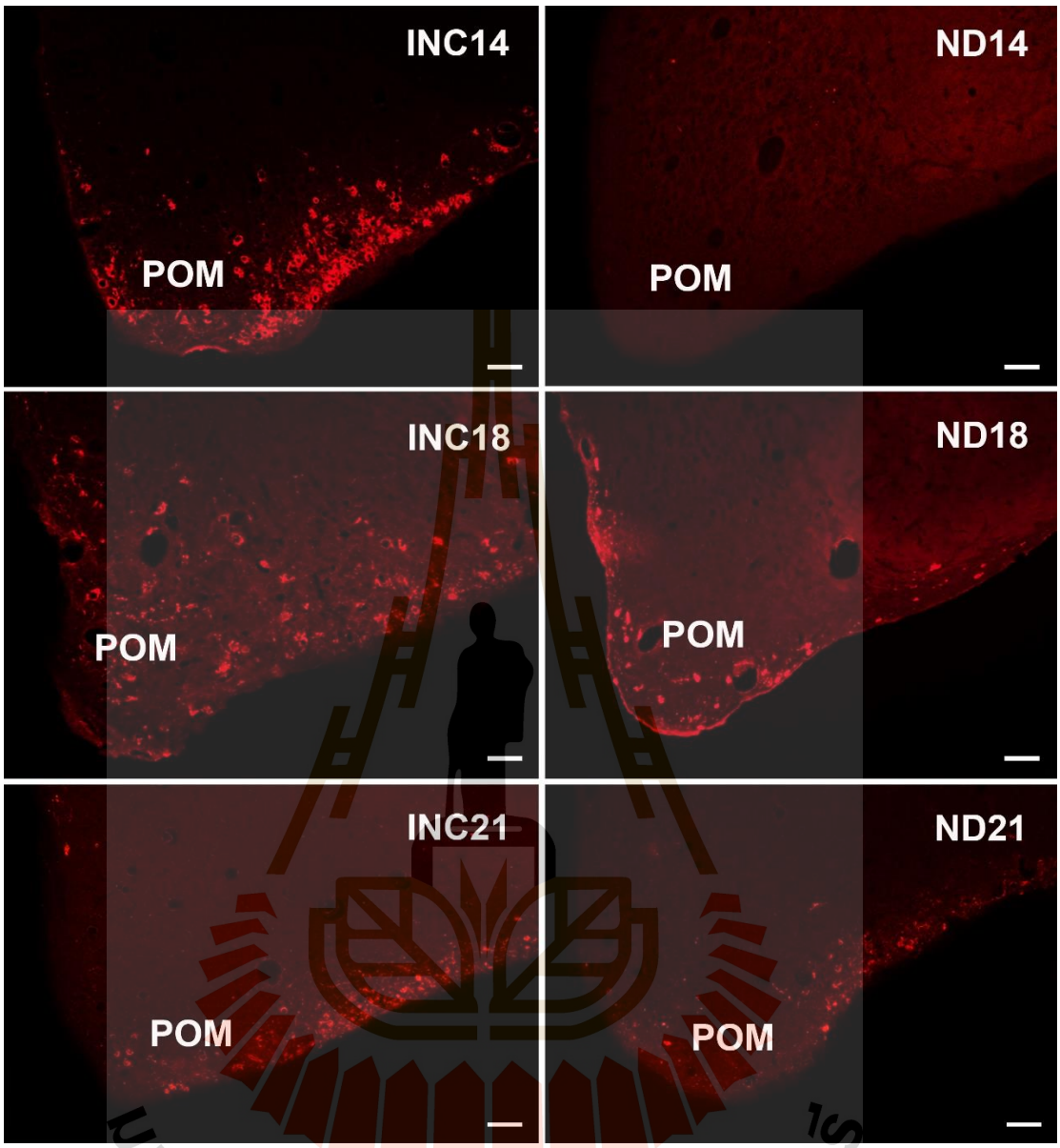

Figure 3.3 (Continued) Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the POM of incubating (INC) and nest-deprived (ND) native Thai hens during different days of incubating periods. Scale bar = 100 μm.

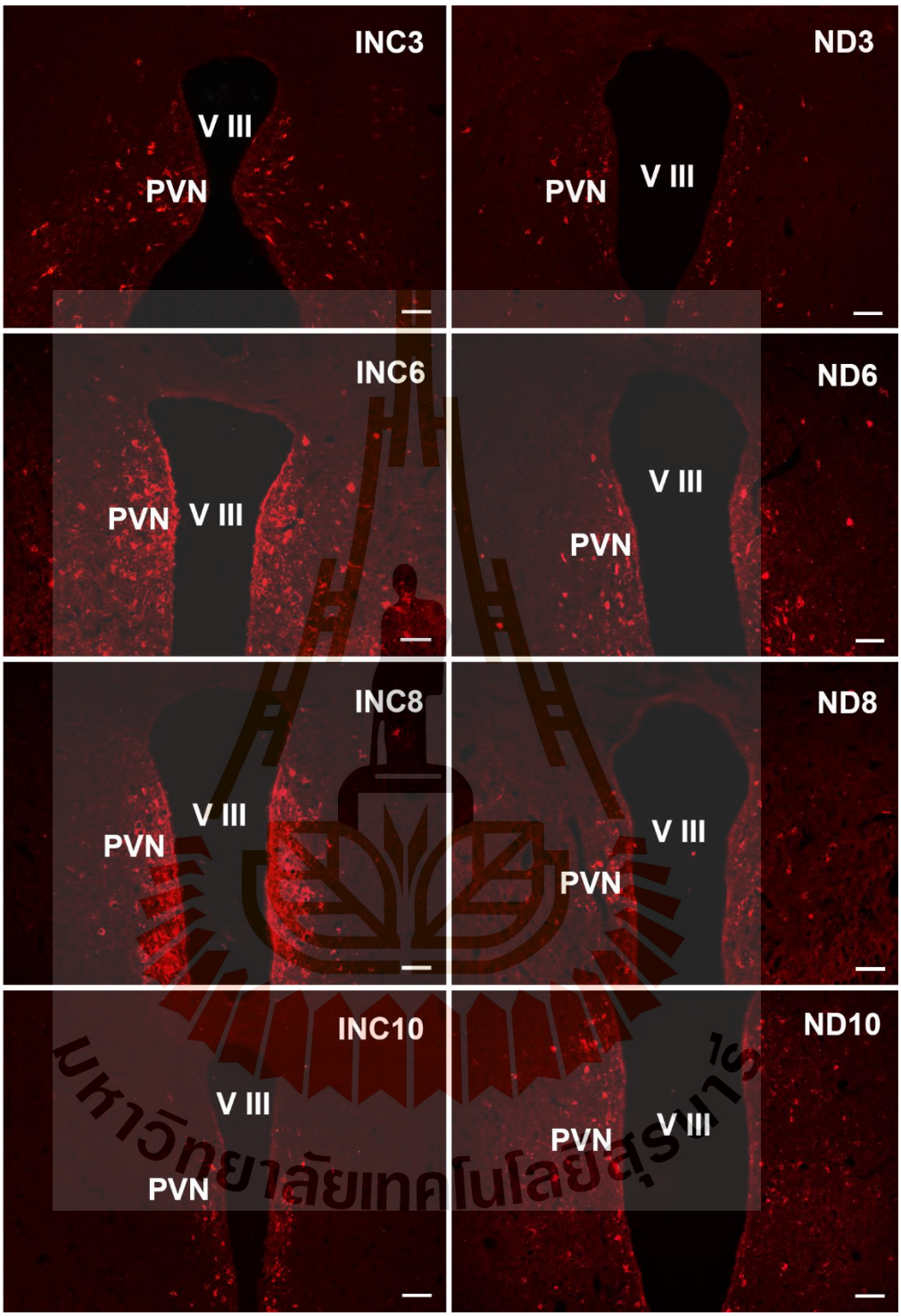

Figure 3.4 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the PVN of incubating (INC) and nest-deprived (ND) native Thai hens during different days of incubating periods. Scale bar = 100 μm.

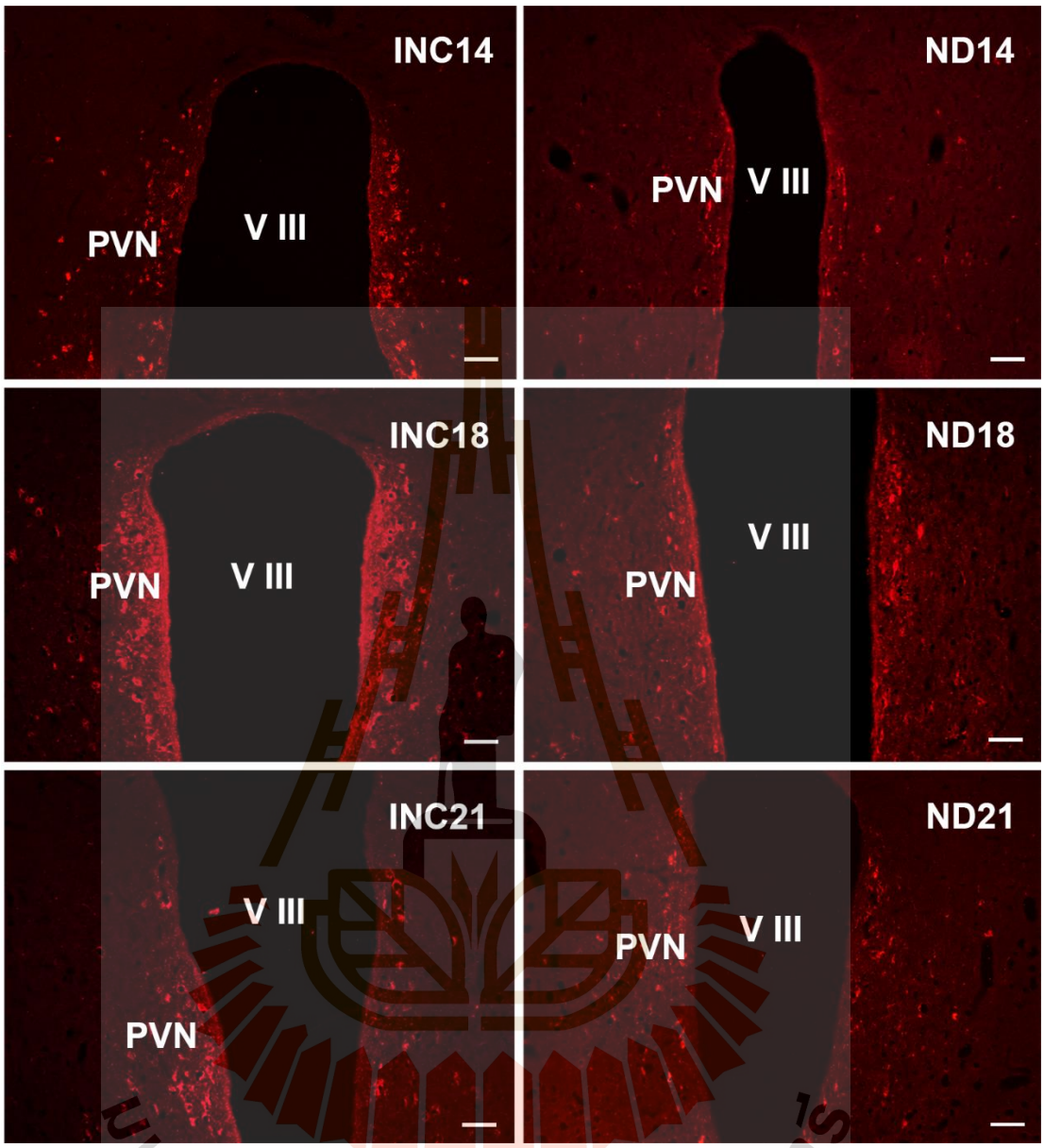

Figure 3.4 (Continued) Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the PVN of incubating (INC) and nest-deprived (ND) native Thai hens during different days of incubating periods. Scale bar = 100 µm.

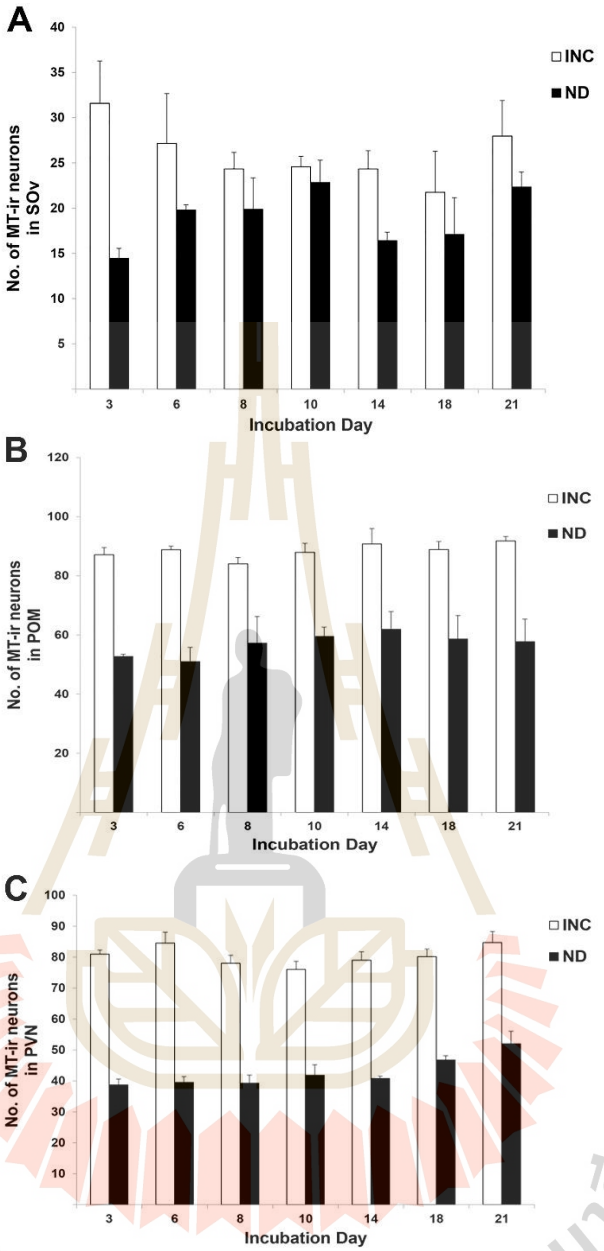

Figure 3.5 Numbers of MT-ir neurons in the SOv (A), POM (B), and PVN (C) of incubating (INC) and nest-deprived (ND) native Thai hens (n = 6). Values are presented as the mean ± SEM. Numbers of MT-ir neurons were greater in the INC hens than in the ND hens ($P < 0.05$) in the SOv, POM, and PVN. However, no differences in MT-ir neurons were found for any region among incubation days or for the group-by-day interactions ($P > 0.05$).

Table 3.1 Results of two-way ANOVA in numbers of MT-ir neurons.

Region	Effect	F	P-value
SOv	Group	17.471	<0.01
	Day	0.813	0.563
	Group x Day	1.234	0.300
POM	Group	147.377	<0.01
	Day	0.554	0.765
	Group x Day	0.333	0.917
PVN	Group	450.397	<0.01
	Day	2.156	0.058
	Group x Day	0.851	0.535

3.6 Discussion

The results of the present study, for the first time, demonstrate a relationship between the MTergic system and incubation behavior in the female native Thai chickens. The results revealed that the numbers of MT-ir neurons in the SOv, POM, and PVN remained high when the hens incubated their eggs and that nest deprivation decreased the numbers of MT-ir neurons in the SOv, POM and PVN significantly. These findings suggest a role for the MTergic system in the initiation and maintenance of incubation behavior through the same nuclei that regulate rearing behavior in this species.

In this study, the distributions of MT-ir neurons and fibers of INC hens were observed throughout the hypothalamus, with an abundance found within the SOv, POM, and PVN, confirming those previously reported in chickens, Japanese quails, domestic mallards, native Thai chickens, and turkeys (Goossens et al., 1977; Bons, 1980; Barth et al., 1997; Thayananuphat et al., 2011; Chokchaloemwong et al., 2013). The greatest density of MT-ir neurons was found in the POM and PVN, and fewer MT-ir neurons were found in the SOv. These findings are consistent with previous results in the rearing native Thai hens, demonstrating that the MT-ir neurons are greater in the POM and PVN than in the SOv (Chokchaloemwong et al., 2013). In addition, MT mRNA is mainly found in the PVN, with little MT mRNA found in the SON of chickens (Barth et al., 1997).

In the present study, the numbers of MT-ir neurons within the SOv, POM, and PVN decreased throughout the observation periods when compared between the INC and ND groups. These results suggest that the MTergic system in the SOv, POM, and

PVN may be involved with the initiation and maintenance of incubation behavior in native Thai chickens.

The number of MT-ir neurons in the POM of the INC group remained high throughout the observation periods. The number of MT-ir neurons in the POM decreased significantly when the hens were deprived of their nests. This result is consistent with other findings from our laboratory showing that the number of MT-ir neurons in the POM increases in the incubating native Thai hens (Chokchaloemwong et al., 2013). It has been suggested that the area praeoptica (POA) is important for the regulation of maternal behaviors in avian species (O'Connell and Hofmann, 2011). Lesions of the POA disrupt PRL-induced parental feeding in ring doves (Slawski and Buntin, 1995). Fos-ir cells in the POA decrease in nonmaternal female Japanese quails and ring doves (Ruscio and Adkins-Regan, 2004; Buntin et al., 2006), while Fos in the POM increases with maternal behavior induction of turkeys (Thayananuphat et al., 2011). In addition, the POA regulates nesting behavior, in which the expression of Fos increases during nesting behavior of zebra finches (Kingsbury et al., 2015). During nest building in male zebra finches, Fos immunoreactivity increases in the POM and bed nucleus of the stria terminalis, dorsomedial subdivision (BSTmd; Hall et al., 2014), and co-localization of MT-ir neurons and Fos increases in the BSTmd (Hall et al., 2015a), suggesting that the MTergic system may be involved with nest building (Hall et al., 2015b). In addition, the MPOA (medial part of the POA in mammals) is involved with initiation and maintenance of maternal behaviors in mammals (Numan and Numan, 1997; Tsuneoka et al., 2013). In rodents, maternal behaviors are mediated by the POA, damage to the MPOA disrupts all aspects of maternal behavior (Terkel et al., 1979;

Jacobson et al., 1980; Miceli et al., 1983; Kalinichev et al., 2000; Oxley and Fleming, 2000). Moreover, infusion of an OT antagonist into the MPOA prevents maternal behaviors in rats (Pedersen et al., 1994). Thus, the present findings provide evidence that incubation behavior in the native Thai chicken may be regulated by the MTergic system in the POM.

In the present study, the number of MT-ir neurons in the PVN remained high throughout the incubation period. The number of MT-ir neurons in the PVN declined when the hens were nest deprived. These results are in good agreement with previous studies in turkeys and native Thai chickens, demonstrating that the numbers of MT-ir neurons increased within the PVN during incubating, prior to the onset of brooding behavior (Thayananuphat et al., 2011; Chokchaloemwong et al., 2013). During incubation, hens show aggressive nest protection activity or defensive behaviors, issues warning vocalization when danger is coming (Chaiseha and El Halawani, 2015). Treatment with an OT receptor antagonist decreases aggression in male and female violet-eared waxbills, and co-localization of MT and Fos increases in the PVN when the males exhibit defense behavior (Goodson et al., 2015). Knockdown of OT synthesis in the PVN increases struggling behavior in finches (Kelly and Goodson, 2014a). In lactating female rats, OT released within the PVN increases during maternal defense, which correlates with amount of aggressive behavior displayed during the maternal defense test (Bosch et al., 2004; 2005). Lesion of OT in the PVN reduces maternal aggression during postpartum in rats (Consiglio and Lucion, 1996), suggesting that OT in the PVN modulates maternal aggression after delivery in rats (Giovenardi et al., 1998). In addition, birds eat and drink very little and lose body weight during

incubation (Chaiseha and El Halawani, 2015). Intracerebroventricular injection of MT decreases food intake in chicks (Masunari et al., 2013), and OT knockdown in the PVN tends to increase food intake in zebra finches (Kelly and Goodson, 2014a). OT acts as a “homeostatic” inhibitor of ingestive behavior, and the PVN mediates eating behavior and energy homeostasis in mammals (Olszewski et al., 2010; Hill, 2012). The present findings are interpreted to suggest that the MTergic system in the PVN might be related to the initiation and maintenance of incubation behavior.

The SON and PVN are the hypothalamic nuclei that regulate maternal behaviors in mammals. The OTergic system in the SON and PVN stimulates the onset of maternal behaviors in rats (Pedersen et al., 1982; Insel and Harbaugh, 1989; Neumann et al., 1996; Bosch et al., 2005). In sheep and rabbits, the numbers of OT-ir neurons and Fos expression in the PVN increased when maternal behaviors were induced (Broad et al., 1993; Caba et al., 2003). OT receptor binding increases in the SON and PVN during pregnancy when compared with ovariectomized rats, suggesting that the OTergic system in the SON and PVN may prepare the systemic release of OT before birth (Bealer et al., 2006). In rats, the OTergic system within the SON is key in the processes of parturition and milk ejection (Moos et al., 1989; Neumann et al., 1996). In the present study, the numbers of MT-ir neurons in the SOv and PVN remained high throughout the incubation period. The numbers of MT-ir neurons in the SOv, and PVN declined when the hens were nest deprived. Taken together, the present findings suggest that activation of the MTergic system in the SOv and PVN during the incubating is important for preparing for maternal care of the young before hatching in this species.

During the incubating stage in native Thai chickens, it has been reported that VIP-ir neurons increase in the IH-IN (Kosonsiriluk et al., 2008), TH-ir neurons increase in the nI (Sartsoongnoen et al., 2008), gonadotropin releasing hormone (GnRH) decreases in the nucleus commissurae pallii (nCPa; Sartsoongnoen et al., 2012), and plasma PRL levels increase, accompanied by a decrease in ovary and oviduct weights (Prakobsaeng et al., 2011). Disruption of incubation behavior revealed a significant increase in the number of GnRH-ir neurons in the nCPa (Sartsoongnoen et al., 2012), a decrease in VIP-ir neurons in the IH-IN, a decrease in TH-ir neurons in the nI, and a decline of plasma PRL levels, associated with an increase in ovary and oviduct weights and initiation of the new laying cycle (Prakobsaeng et al., 2011). Incubation behavior stimulates PRL secretion by the activation of DAergic neurons, which in turn stimulate VIP (Prakobsaeng et al., 2011). The MTergic system in this study is in good agreement with the VIPergic and DAergic systems, in which the numbers of immunoreactivity are low in the nest-deprivation group when compared with the incubation group. Therefore, the MTergic system might play a significant role in incubation behavior in this species as well.

In conclusion, the findings of the present study clearly demonstrate that the MTergic system is associated with incubation behavior in the native Thai chicken. The numbers of MT-ir neurons in the SOv, POM, and PVN remained high during the incubation period and decreased when the hens were nest deprived. This finding suggests that the MTergic system in these nuclei is not only regulating rearing behavior in the native Thai hens, but also likely plays a significant role in the initiation and maintenance of incubation behavior.

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CHAPTER IV

THE EFFECTS OF REPLACING EGGS WITH CHICKS ON MESOTOCIN, DOPAMINE, AND PROLACTIN IN THE NATIVE THAI HEN

4.1 Abbreviations

AM, nucleus anterior medialis hypothalami; DA, dopamine; ELISA, enzyme-linked immunosorbent assay; FSH, follicle stimulating hormone; GnRH, gonadotropin releasing hormone; IHC, immunohistochemistry; INC, incubating hens; -ir, -immunoreactive; LH, luteinizing hormone; ML, nucleus mamillaris lateralis; MT, mesotocin; nI, nucleus intramedialis; OT, oxytocin; PBS, phosphate buffered saline; POM, nucleus preopticus medialis; PRL, prolactin; PVN, nucleus paraventricularis magnocellularis; PVO, organum paraventriculare; REC, replaced-eggs-with-chicks hens; SOv, nucleus supraopticus, pars ventralis; TH, tyrosine hydroxylase; V III, ventriculus tertius (third ventricle); VIP, vasoactive intestinal peptide

4.2 Abstract

The mesotocinergic (MTergic) and dopaminergic (DAergic) systems have been documented to play pivotal roles in maternal behaviors in native Thai chickens. In native Thai chickens, plasma prolactin (PRL) concentrations are associated with maternal behaviors, which are also controlled by the DAergic system. However, the

role of MT in conjunction with the roles of DA and PRL on the neuroendocrine regulation of the transition from incubating to rearing behavior has never been studied. Therefore, the aim of this study was to investigate the association of MT, DA, and PRL during the transition from incubating to rearing behavior in native Thai hens. Using an immunohistochemistry technique, the numbers of MT-immunoreactive (-ir) and tyrosine hydroxylase-ir (TH-ir, a DA marker) neurons were compared between incubating hens (INC; n = 6) and hens for which the incubated eggs were replaced with 3 newborn chicks for 3 days after 6, 10, and 14 days of incubation (REC; n = 6). Plasma PRL levels were determined by enzyme-linked immunosorbent assay. The results revealed that the numbers of MT-ir neurons within the nucleus supraopticus, pars ventralis (SOv), nucleus preopticus medialis (POM), and nucleus paraventricularis magnocellularis (PVN) increased in the REC hens when compared with those of the INC hens at 3 different time points (at days 9, 13, and 17). On the other hand, the number of TH-ir neurons in the nucleus intramedialis (nI) decreased in the REC13 and REC17 hens when compared with those of the INC hens. However, the number of TH-ir neurons in the nucleus mamillaris lateralis (ML) only decreased in the REC13 hens when compared with the INC13 hens. The decrease in the numbers of TH-ir neurons within the nI and ML is associated with the decrease in the levels of plasma PRL. This study suggests that the presence of either eggs or chicks is the key factor regulating the MTergic system within the SOv, POM, and PVN and the DAergic system within the nI and ML during the transition from incubating to rearing behavior in native Thai chickens. The results further indicate that these two systems play pivotal roles in the transition from incubating to rearing behavior in this equatorial species.

4.3 Introduction

Maternal care behaviors in birds include incubation and brooding or rearing behaviors. After egg-laying, birds display incubation behavior by sitting on their eggs, turning and retrieval of eggs, characteristic clucking, and defense of their nests (Romanov et al., 2002; Chaiseha and El Halawani, 2015). After hatching, birds display rearing behavior by emitting a characteristic vocalization when danger is coming, fluffing their feathers, calling the chicks to eat food, and crouching over the chicks to protect and make them warm (Edgar et al., 2011).

In birds, two major neuroendocrine systems controlling the reproductive cycle are the gonadotropin releasing hormone/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH) and vasoactive intestinal peptide/prolactin (VIP/PRL) systems. The first system regulates the period of egg-laying. The latter initiates and maintains maternal behaviors and may influence the onset of gonadal regression. Both systems are influenced by the dopaminergic (DAergic) system (Chaiseha and El Halawani, 2015). In addition, the functions of mesotocin (MT) have been implicated with reproductive stages and maternal activities in birds (Thayananuphat et al., 2011; Chokchaloemwong et al., 2013; Klatt and Goodson, 2013b; Kelly and Goodson, 2014; Hall et al., 2015b; Sinpru et al., 2017).

PRL secretion is associated with the reproductive cycle and plays a pivotal role in the onset and maintenance of incubation behavior in bird (Sharp, 2009; Buntin, 2010; Chaiseha and El Halawani, 2015; Ryan et al., 2015; Angelier et al., 2016; Smiley and Adkins-Regan, 2016b). The elevation of PRL concentrations in incubating hens is maintained by contact stimuli from nests and eggs (Silver et al., 1988; Buntin et al., 1991; Massaro et al., 2007). During the rearing period, the presence of chicks maintains

elevated PRL concentrations (Sharp et al., 1988; Adkins-Regan et al., 2013; Chaiyachet et al., 2013b; Chokchaloemwong et al., 2015). Replacement of eggs with chicks in incubating hens is associated with a decrease in circulating of PRL concentrations, an increase in circulating of LH levels, resulting in disrupted incubation behavior, and then the hens display full brooding activities (Leboucher et al., 1993; Richard-Yris et al., 1998).

In birds, the synthesis and secretion of PRL are controlled by the VIPergic and DAergic systems. DA stimulates PRL secretion at the hypothalamic level via D_1 DA receptors, whereas it inhibits PRL secretion at the pituitary level via D_2 DA receptors (Al Kahtane et al., 2003; Chaiseha et al., 1997; 2003). DAergic activity in the anterior hypothalamus markedly increases during incubation in bantam chickens (Macnamee and Sharp, 1989) and increases in the periventricular regions during parental behaviors in ring doves (Lea et al., 2001). In native Thai chickens, tyrosine hydroxylase-immunoreactive (TH-ir) neurons (a marker for DAergic neurons) and fibers are found throughout the brain and are predominantly located within the nucleus anterior medialis hypothalami (AM), nucleus intramedialis (nI), and nucleus mamillaris lateralis (ML). Changes in the number of TH-ir neurons in the nI mirrored directly circulating PRL concentrations during the reproductive cycle (Sartsoongnoen et al., 2008). Disruption of incubation behavior by nest-deprivation decreases the numbers of TH-ir neurons within the nI and ML, suggesting that the expression of incubation behavior is regulated by the DAergic system which, in turn, stimulates VIP and subsequent PRL release (Prakobsaeng et al., 2011). In addition, the involvement of the DAergic system with rearing behavior also has been demonstrated. The number of TH-ir neurons in the nI and plasma PRL concentrations were significantly higher in rearing

hens when compared with the non-rearing hens, suggesting that the DA/PRL system plays a significant role in rearing behavior as well (Chokchaloemwong et al., 2015).

MT, a nonapeptide hormone from the posterior pituitary gland, is an avian homolog of oxytocin (OT) in mammals. MT-ir neurons and fibers have been mapped in many avian species (Goossens et al., 1977; Bons, 1980; Tennyson et al., 1985; Robinzon et al., 1988; Barth et al., 1997; Thayananuphat et al., 2011) including native Thai chickens (Chokchaloemwong et al., 2013; Kamkrathok et al., 2017). The majority of MT-ir neurons and fibers are found within the nucleus supraopticus, pars ventralis (SOv; ventral part of the nucleus supraopticus), nucleus preopticus medialis (POM), and nucleus paraventricularis magnocellularis (PVN) of the native Thai chicken (Chokchaloemwong et al., 2013). MT is involved with pair-bonding, nesting behavior, affiliative behavior, aggressive behavior, and stress coping and feeding behavior in zebra finches (Goodson et al., 2009; Pedersen and Tomaszycki, 2012; Klatt and Goodson, 2013a; 2013b; Kelly and Goodson, 2014) and grouping behavior in emberizid sparrows (Wilson et al., 2016). In native Thai chickens, the MTergic system is associated with the reproductive cycle. The numbers of MT-ir neurons within the SOv, POM, and PVN increase in incubating and rearing hens when compared with non-incubating and non-rearing hens, respectively (Chokchaloemwong et al., 2013, Sinpru et al., 2017). These findings suggest that MTergic neurons in these nuclei are not only regulated by rearing behavior but also might have a role in the initiation and maintenance of incubation behavior in native Thai chickens.

Presently, it has been reported that the GnRHergic, VIPergic, DAergic, and MTergic systems are associated with maternal behaviors of native Thai chickens (Prakobsaeng et al., 2011; Sartsoongnoen et al., 2012; Chaiyachet et al., 2013a; 2013b;

Chokchaloemwong et al., 2013; 2015; Namken et al., 2017; Sinpru et al., 2017). However, the role of the MTergic system in conjunction with the roles of DA and PRL in the neuroendocrine regulation of incubating and rearing behaviors has never been studied. Native Thai chickens exhibit strong maternal behaviors (Austic and Nesheim, 1990; Fumihito et al., 1994; Hillel et al., 2003; Sawai et al., 2010), and thus it is an excellent animal model for the study of this phenomenon. Therefore, the aim of this study was to investigate the role of PRL and the differential expression of TH-ir and MT-ir neurons that might be associated during the transition from incubating to rearing behavior in native Thai chickens. Comparisons were made in the numbers of TH-ir neurons within the AM, organum paraventriculare (PVO), nI, and ML of incubating and replaced-eggs-with-chicks hens. Also, comparisons were made on the numbers of MT-ir neurons within the SOv, POM, and PVN of incubating and replaced-eggs-with-chicks hens. The findings of differential expression of TH-ir and MT-ir neurons in each hypothalamic area of incubating and replaced-eggs-with-chicks hens and their associated PRL concentrations may provide an insight into the roles of the DAergic and MTergic systems during the transition from incubating to rearing behavior.

4.4 Materials and Methods

4.4.1 Experimental Animals

Female and male native Thai chickens (*Gallus domesticus*), 20-22 weeks old, were used. They were reared and housed (9 females/1 rooster/pen) in floor pens equipped with nest baskets under natural light (12 h of light and 12 h of darkness; 12L: 12D). Food and water were provided *ad libitum*. At 24 weeks of age, the hens were subjected to the experimental treatments. The animal handling and maintenance used

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

4.4.2 Experimental Design

To study the role of PRL and the differential expression of TH-ir and MT-ir neurons that might be associated with the transition from incubating to rearing behavior, 36 females and 4 mature roosters, were used. After the egg-laying period, the hens were randomly divided into 2 treatment groups: Group 1; incubating hens (INC; hens that were allowed to incubate their eggs naturally), and Group 2; replaced-eggs-with-chicks hens (REC; hens that were allowed to incubate their eggs for 6, 10, and 14 days, and then they were subjected to rearing period by replacing their eggs with 3 newly hatched chicks for 3 days). After replacement of eggs with chicks, maternal behaviors of the hens were observed for 3 days (4 times/day) for classification of whether the hens adopted the chicks, using indicators of maternal behaviors (Opel and Proudman, 1989; Edgar et al., 2011; Thayananuphat et al., 2011; Chokchaloemwong et al., 2013). During rearing behavior, hens did not peck aggressively at chicks, fluffed their feathers, crouched over the chicks, clucked, and food called. In hens that rejected the chicks, the hens pecked at chicks, and ignored the chicks or left the nest. The REC hens (n = 6) were sacrificed after the chicks were introduced to the hen after 3 days (at days 9, 13, and 17). The INC hens (n = 6) were sacrificed while they were exhibiting incubating behavior on days 9, 13, and 17 after they started to incubate their eggs as the control group. Blood samples were collected from each hen prior to sacrifice and were fractionated by centrifugation, and plasma samples were stored at -20 °C until they were used for assaying PRL concentrations by enzyme-linked immunosorbent assay (ELISA). The brains were fixed by pressure perfusion, sectioned with a cryostat,

and processed for immunohistochemistry (IHC). In each hen, a postmortem examination was conducted to confirm its reproductive status.

4.4.3 Experimental Procedures

4.4.3.1 PRL Assay Plasma PRL concentrations were determined utilizing an ELISA according to a previously described method (Kosonsiriluk et al., 2008). In brief, ELISA plates (Nunc-Maxisorb, Nunc, Roskilde, Denmark) were coated with 0.1 ml per well of goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted 1:2000 in 0.05 M potassium phosphate buffer (pH 7.4) and incubated overnight at 4 °C. In each well, surface was blocked by adding 0.1 ml of 0.4 % casein in 0.15 M phosphate buffered saline (PBS, pH 7.2) containing 1.0 mM EDTA and 0.02 % thimerosal. After incubation, plates were washed three times in 0.03 M PBS containing 0.05 % Tween 20. The assay buffer was 0.15 M PBS (pH 7.2) containing 0.1 % casein, 1.0 mM EDTA, and 0.02 % thimerosal. Fifty microliter samples, either 10 µl of plasma diluted in 40 µl of assay buffer, or serially diluted chicken PRL standard (Dr. Albert F. Parlow, National Hormone and Peptide Program, USA) were added. Twenty five microliters each of biotinylated PRL (1:50,000 dilution) and rabbit anti-chicken PRL (Dr. John Proudman, USDA, USA) at 1:20,000 dilution were then added and incubated overnight at 4 °C. There after incubation, plates were washed and 0.1 ml of streptavidin horseraddish peroxidase (1:5000 dilution) were added. After 2 h at room temperature, plates were washed and 0.1 ml ABTS reagent (0.04 % 2,2'-azino-bis-3-ethylbenzthizoline-6-sulfonic acid and 0.015 % H_2O_2 in 0.1 M citrate phosphate buffer, pH 4) were added. After 1 h incubation at room temperature, the color reaction was measured at 405 nm in a Tecan Sunrise ELISA reader (Tecan Group Ltd., Mannedorf, Switzerland). The assay of plasma PRL

concentrations in native Thai chickens was validated using the parallelism test (Kosonsiriluk et al., 2008). Pooled plasma samples of native Thai chickens produced a dose-response curve that paralleled a chicken PRL standard curve. All samples were performed in duplicate within a single assay. The intra-assay coefficient of variation was 4.46 %, and the sensitivity of the assay was 3.9 ng/ml.

4.4.3.2 Tissue Preparation and Immunohistochemistry To evaluate the distributions of TH-ir and MT-ir neurons and fibers in the brain of the INC and REC hens, brain tissue sections were prepared, and IHC was performed as previously described (Chokchaloemwong et al., 2013; 2015). Briefly, the brains of hens were fixed by pressure perfusion with 4 % paraformaldehyde (pH 7.4). Tissue sectioning was performed in the coronal plane at a thickness of 16 μm utilizing a cryostat (Microtome cryostat HM525, Microm International GMbH, Walldorf, Germany). Sections were mounted on chrome alum-gelatin-coated glass slides with 2 sections per slide and stored desiccated at -20 °C until further processing for IHC. For detecting TH immunoreactivity, primary mouse monoclonal antibody directed against TH (ImmunoStar, Inc., Hudson, WI, USA) and Cy™3-conjugated AffiniPure donkey anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) were used. The primary and secondary antibodies used for detecting MT immunoreactivity were primary rabbit polyclonal antibody directed against OT (ImmunoStar, Inc.) and Cy™3-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.), respectively. Four adjacent sections from INC and REC hens at different time points, in the individual hypothalamic areas, were thawed to room temperature prior to use. The sections were rehydrated in PBS for 30 min at room temperature. After PBS removal, the sections were then incubated with 60 μl of

primary antibody diluted 1:1000 with PBS (pH 7.4) containing 1 % bovine serum albumin and 0.3 % Triton X-100 at 4 °C overnight in a moist chamber. The slides were then washed three times with PBS (pH 7.4) for 5 min each. After washing, 60 µl of secondary antibody (diluted 1:500) was applied onto the sections under dark conditions. The slides were further incubated in a moist, dark, chamber at room temperature for 1 h, washed with PBS (pH 7.4) 3 times for 5 min each, and mounted with DPX mountant (Sigma-Aldrich, Inc., Steinheim, Germany). Microscopic images of brain sections were visualized and further analyzed. Using these antibodies, TH and MT immunoreactivities have been previously described (MT-antiserum; Chokchaloemwong et al., 2013; Kamkrathok et al., 2017; Sinpru et al., 2017, TH-antiserum; Sartsoongnoen et al., 2008; Prakobsaeng et al., 2011; Chokchaloemwong et al., 2015).

4.4.4 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). The numbers of TH-ir and MT-ir neurons of 4 adjacent sections were manually counted to determine the changes in the numbers of TH-ir neurons within the AM, PVO, nI, and ML and MT-ir neurons within the SOv, POM, and PVN. The numbers of TH-ir and MT-ir neurons counted from 4 adjacent sections for each hen (6 hens per area) and for each treatment group were averaged to determine the numbers of TH-ir and MT-ir neurons counted per section in each area. The mean values were compared between the INC and REC hens. 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 the TH and MT immunoreactivities.

4.4.5 Statistical Analysis

All values are expressed as the mean ± SEM. Significant differences in plasma PRL concentrations and the numbers of TH-ir and MT-ir neurons between treatment groups (INC and REC hens) were analyzed using Student's t-test at each time point. For a comparison between means at different time points, one-way analysis of variance (ANOVA) was utilized with Tukey's HSD multiple comparison tests. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS software for Windows (SPSS Windows Software, version 17.0, SPSS Inc., Chicago, IL, USA).

4.5 Results

4.5.1 Effect of Transition from Incubating Eggs to Rearing Chicks on Plasma PRL Concentrations

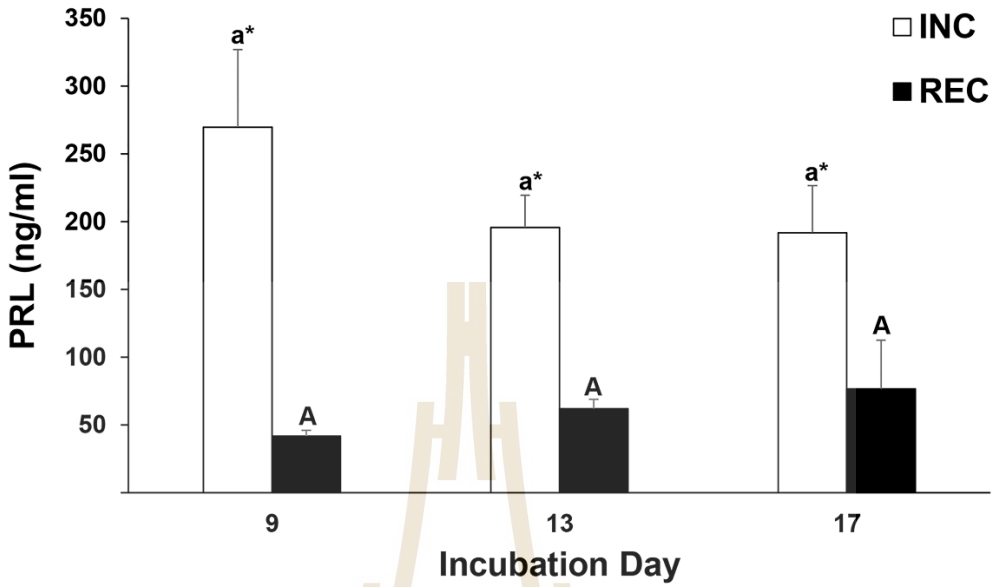
Plasma PRL concentrations in the INC and REC hens are shown in Figure 4.1. The levels remained high in the INC hens and significantly decreased ($P < 0.05$) when eggs were replaced by chicks. However, changes in PRL concentrations over time in both the INC and REC groups were not observed ($P > 0.05$).

4.5.2 Effects of Replacement of Eggs with Chicks on the Numbers of TH-ir Neurons within the AM, PVO, nI, and ML and MT-ir Neurons within the SOv, POM, and PVN

Schematic representations of the distributions of TH-ir and MT-ir neurons throughout the brain are illustrated in Figure 4.2. The numbers of TH-ir neurons within

the AM, PVO, nI, and ML were compared between the INC and REC hens (Table 4.1). In the nI, the number of TH-ir neurons significantly decreased ($P < 0.05$) in the REC hens, in which the eggs were replaced by the chicks at day 10 (REC13) and day 14 (REC17) of incubation for 3 days (Figure 4.3, $P < 0.05$). In addition, changes in the number of TH-ir neurons were markedly decreased in the ML of hens when the eggs were replaced with chicks at day 10 (REC13; Figure 4.4, $P < 0.05$). However, changes in the number of TH-ir neurons between the INC and REC hens were not observed within the AM and PVO.

The distributions of MT-ir neurons and fibers within the SOv, POM, and PVN of the INC and REC hens are illustrated in Figures 4.5, 4.6, and 4.7, respectively. In the INC hens, the numbers of MT-ir neurons within the SOv, POM, and PVN remained high. In the REC hens, the number of MT-ir neurons in the SOv significantly increased ($P < 0.05$) in the REC13 and REC17 hens when compared with the REC9 hens (Figure 4.8A). Replacement of eggs with chicks significantly increased the numbers of MT-ir neurons within the SOv, POM, and PVN (Figures 4.8A, 4.8B, and 4.8C, respectively, $P < 0.05$) when compared with those of the INC hens at 3 different time points (at days 9, 13, and 17).



Figgure 4.1 Plasma PRL concentrations (mean ± SEM) of incubating hens (INC) and hens for which eggs were replaced with chicks (REC) at days 9, 13, and 17. Significant differences between means in each group at different time points are denoted by different letters ($P < 0.05$) and * $P < 0.05$ for comparison between group at a given time point.

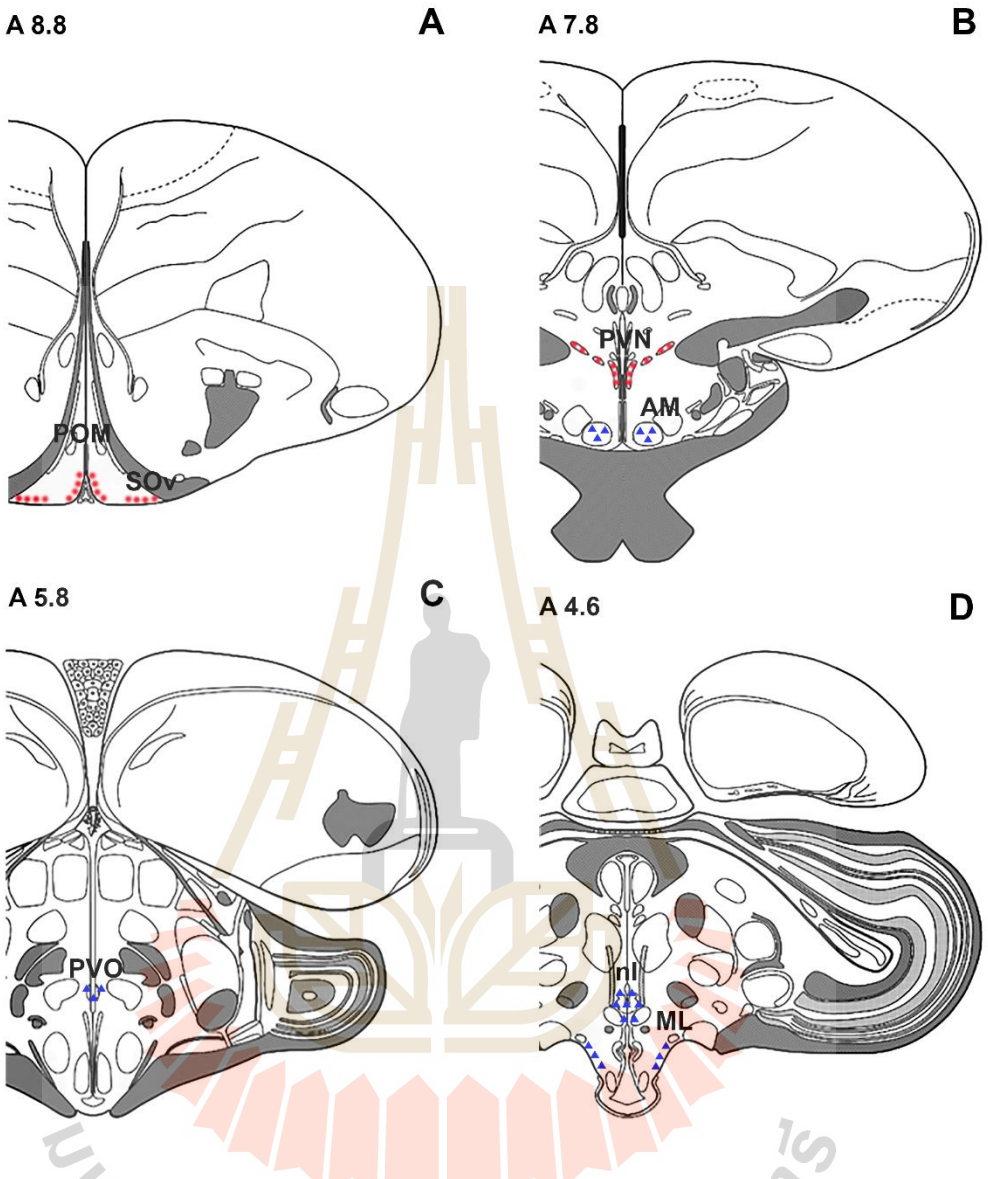


Figure 4.2 Schematic diagrams demonstrated the distributions of TH-ir neurons (blue triangles) and MT-ir neurons (red dots). Sections are presented in a rostral to caudal order from A to D. The illustrations of sections are redrawn with the designated coordinates from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988). The following abbreviations are used in the figure legends: AM, nucleus anterior medialis hypothalami; ML, nucleus mamillaris lateralis; nI, nucleus intramedialis; POM, nucleus preopticus medialis; PVN, nucleus paraventricularis magnocellularis; PVO, organum paraventriculare; SOv, nucleus supraopticus, pars ventralis.

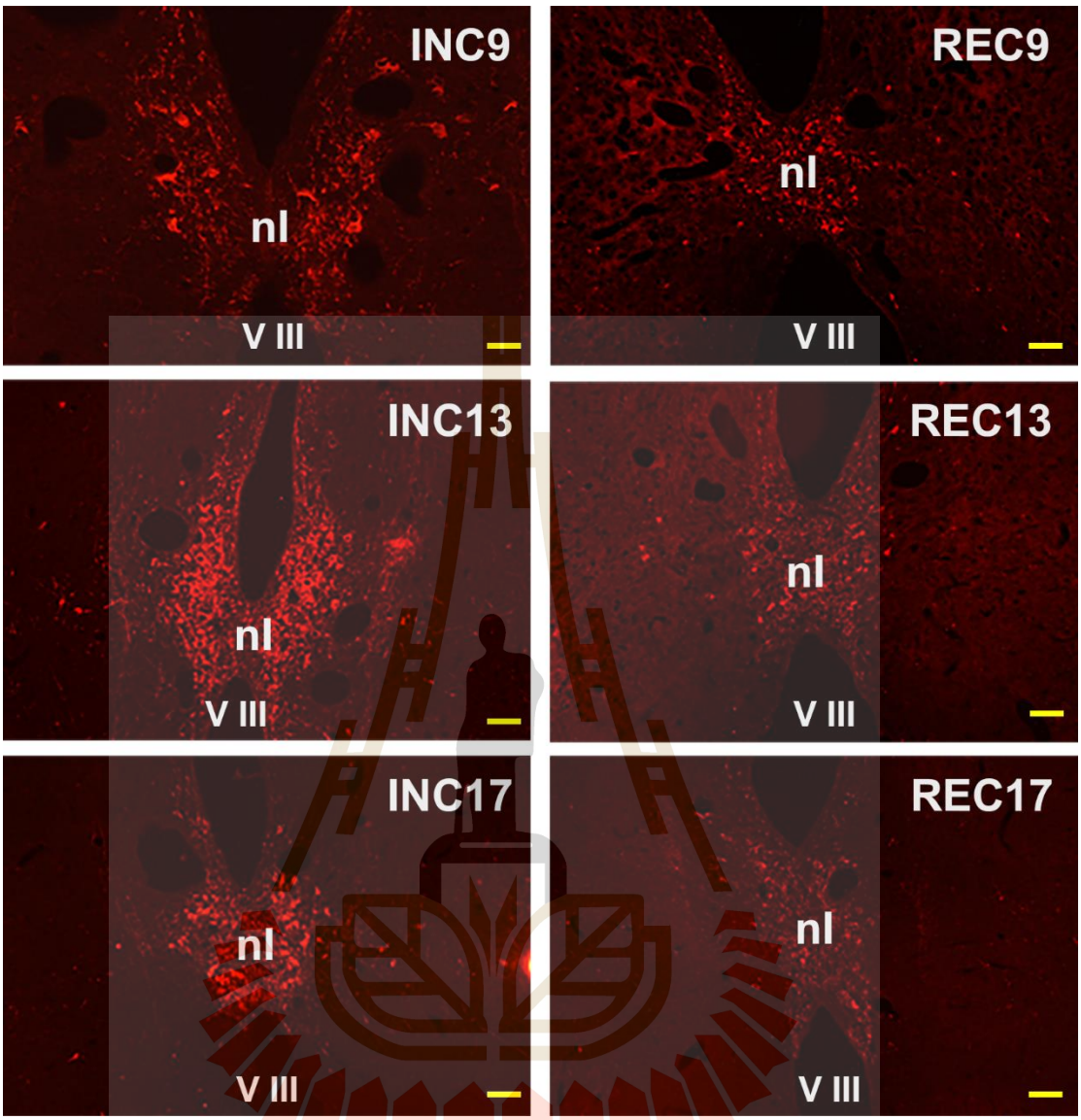


Figure 4.3 The distributions of TH-ir neurons and fibers in the nI of incubating (INC) and replaced-eggs-with-chicks (REC) hens at days 9, 13, and 17. V III, ventriculus tertius (third ventricle). Scale bar = 100 μm. See Figure 4.2 for a description of abbreviations.

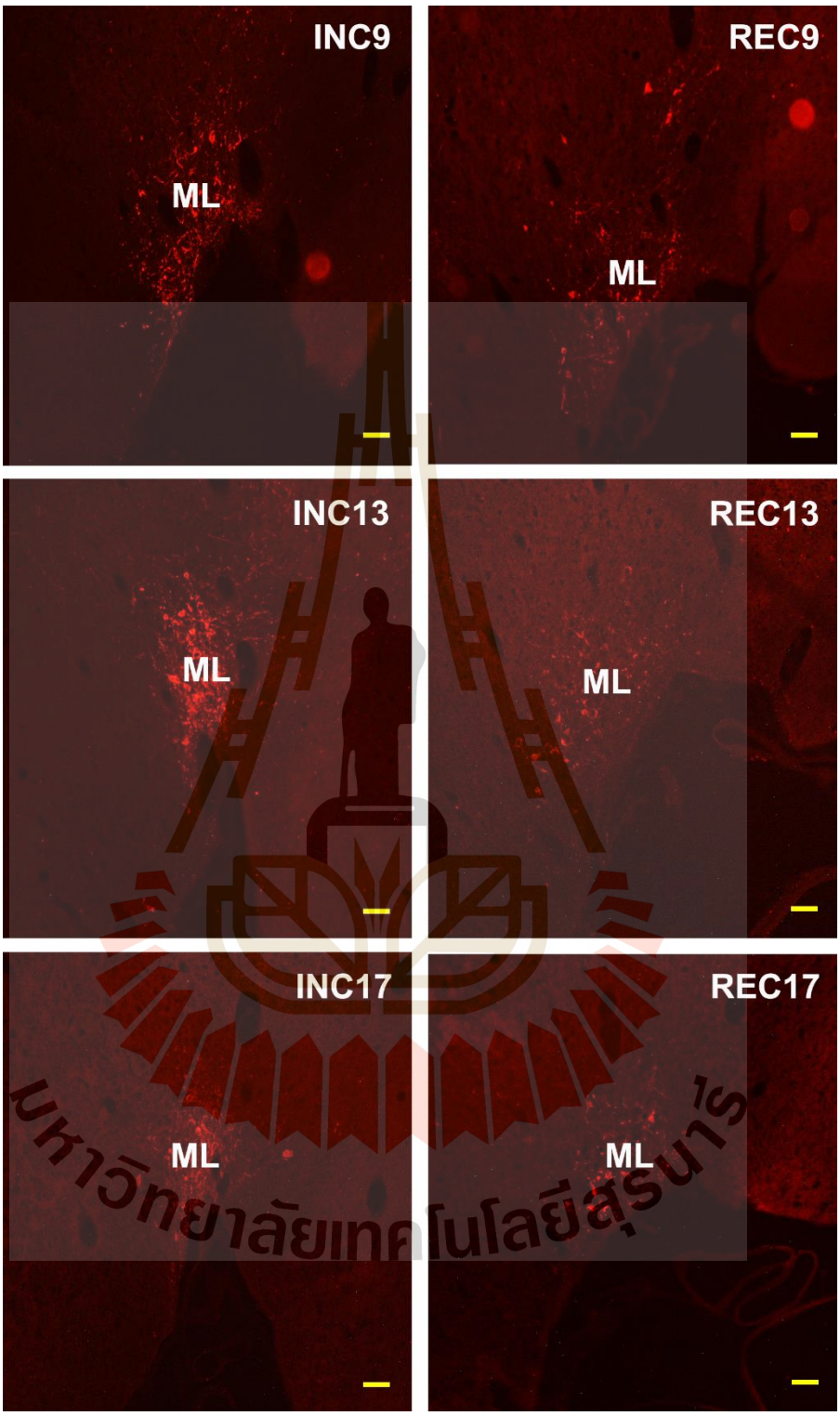


Figure 4.4 The distributions of TH-ir neurons and fibers in the ML of incubating (INC) and replaced-eggs-with-chicks (REC) hens at days 9, 13, and 17. Scale bar = 100 μm. See Figure 4.2 for a description of abbreviations.

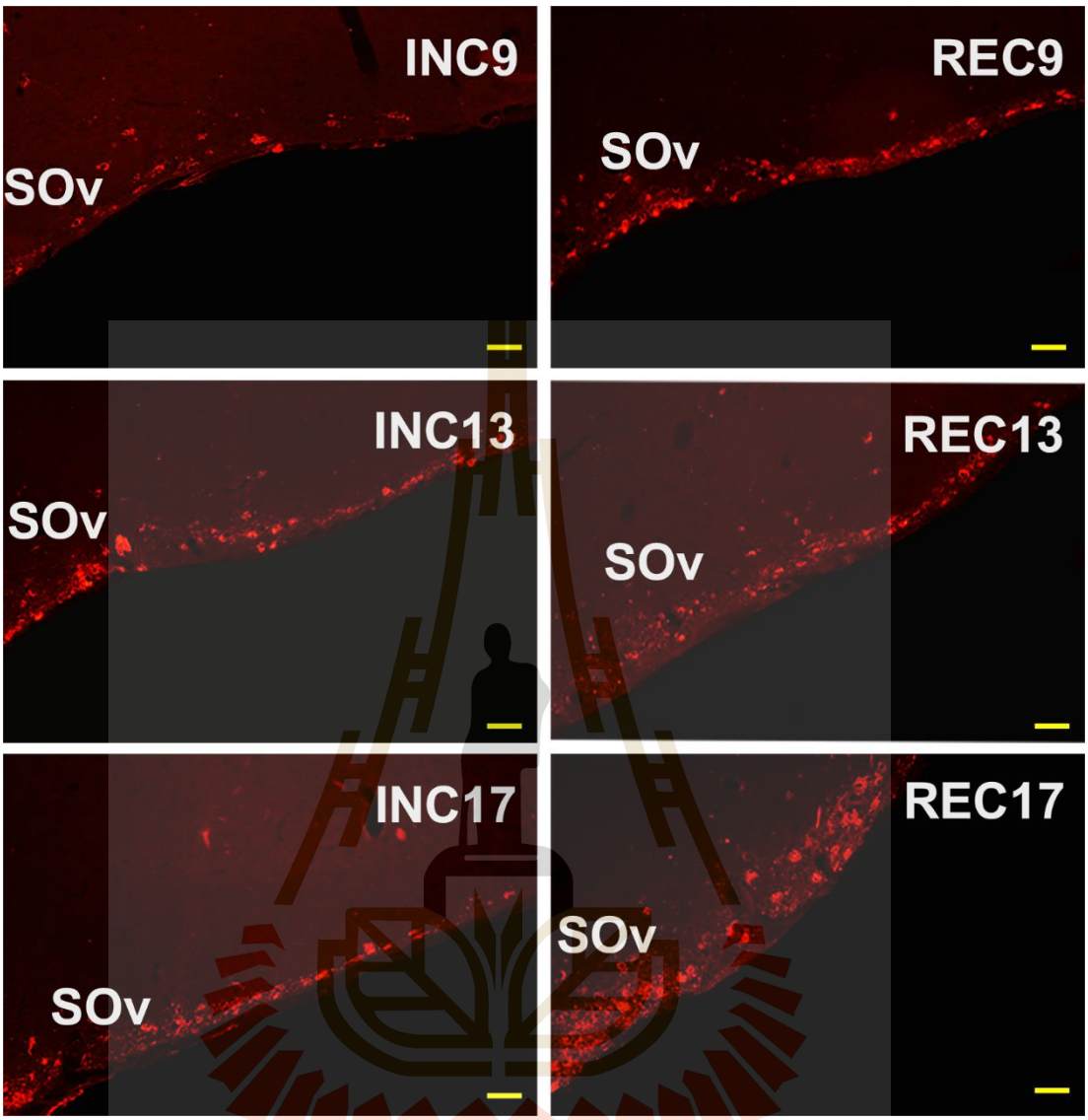


Figure 4.5 The distributions of MT-ir neurons and fibers in the SOv of incubating (INC) and replaced-eggs-with-chicks (REC) hens at days 9, 13, and 17. Scale bar = 100 μm. See Figure 4.2 for a description of abbreviations.

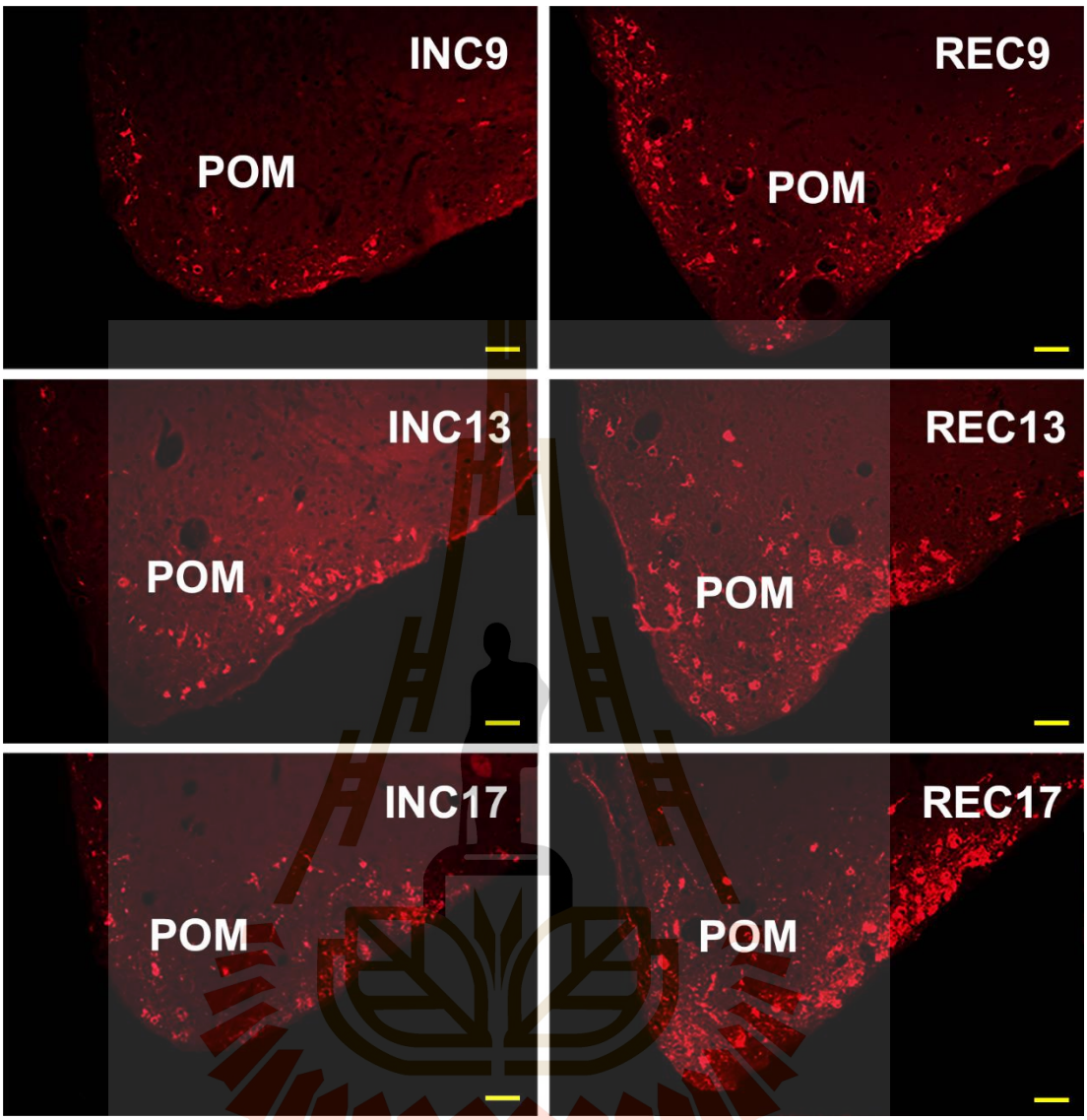


Figure 4.6 The distributions of MT-ir neurons and fibers in the POM of incubating (INC) and replaced-eggs-with-chicks (REC) hens at days 9, 13, and 17. Scale bar = 100 μm. See Figure 4.2 for description of the abbreviations.

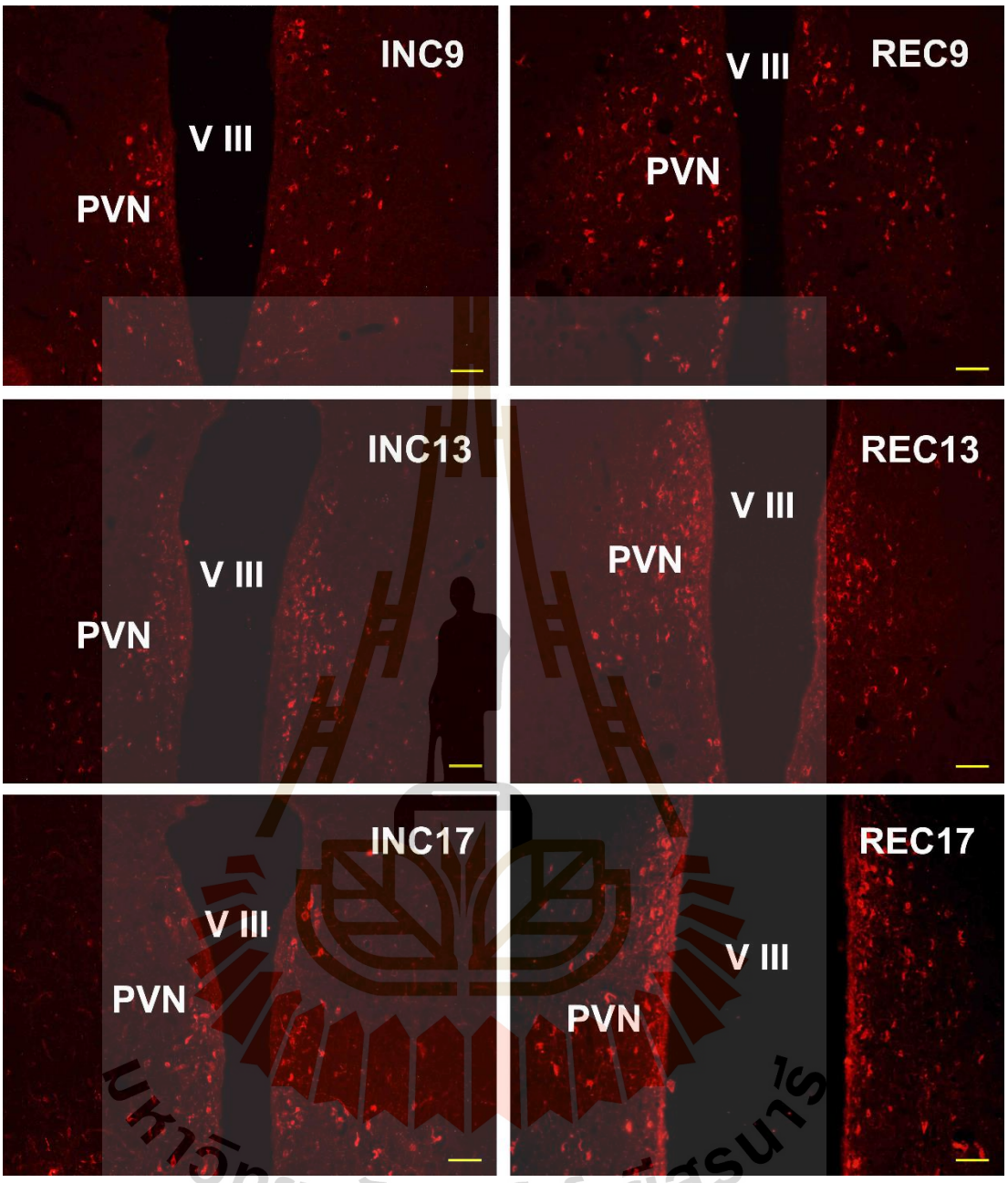


Figure 4.7 The distributions of MT-ir neurons and fibers in the PVN of incubating (INC) and replaced-eggs-with-chicks (REC) hens at days 9, 13, and 17. V III, ventriculus tertius (third ventricle). Scale bar = 100 μm. See Figure 4.2 for description of the abbreviations.

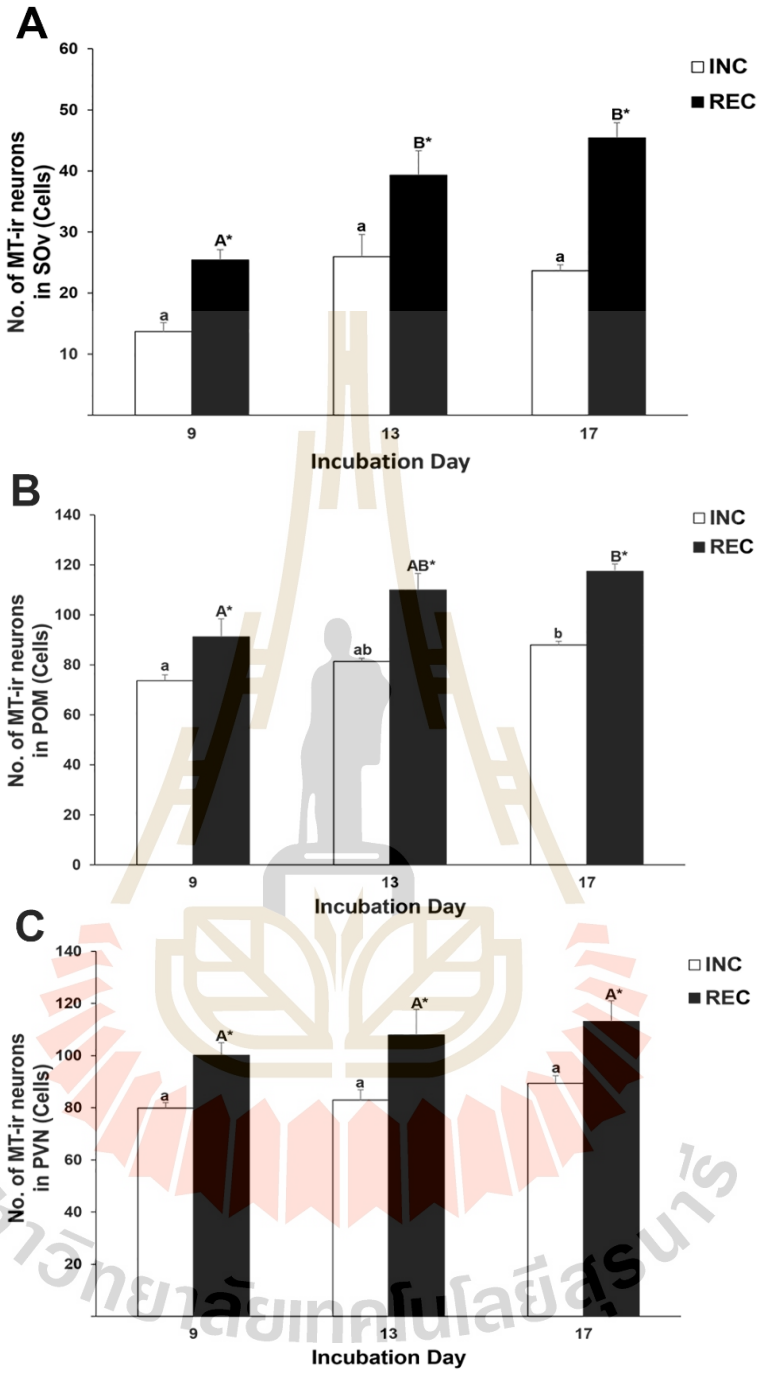


Figure 4.8 The numbers of MT-ir neurons within the SOv (A), POM (B), and PVN (C) of incubating (INC) and replaced-eggs-with-chicks (REC) hens. Significant differences between values (mean ± SEM) in each treatment group of different hypothalamic nuclei are indicated by different letters ($P < 0.05$) and * $P < 0.05$ for the comparison between the treatment groups in each hypothalamic nucleus.

Table 4.1 The numbers of TH-ir neurons within the AM, PVO, nI, and ML of incubating (INC) and replaced-eggs-with-chicks (REC) native Thai hens at different time points.

Areas	Groups	Incubation or Replaced-eggs-with-chicks Days		
		9	13	17
AM	INC	13.08 ± 0.68^{a}	16.67 ± 2.16^{a}	17.00 ± 2.14^{a}
	REC	14.33 ± 0.67^{A}	19.13 ± 1.04^{B}	21.46 ± 0.79^{B}
PVO	INC	12.63 ± 0.65^{a}	13.88 ± 1.53^{a}	14.04 ± 1.19^{a}
	REC	12.75 ± 0.55^{A}	13.92 ± 1.32^{A}	14.21 ± 1.38^{A}
nI	INC	26.67 ± 1.23^{a}	$31.63 \pm 1.41^{b*}$	$27.67 \pm 1.13^{ab*}$
	REC	23.71 ± 1.53^{A}	21.29 ± 1.24^{A}	21.83 ± 1.31^{A}
ML	INC	16.04 ± 1.12^{a}	$18.50 \pm 0.61^{a*}$	18.04 ± 1.45^{a}
	REC	14.42 ± 0.31^{A}	15.67 ± 0.84^{A}	16.92 ± 1.19^{A}

Values represent the means ± SEM (cells; n = 6). Different superscripted letters indicate significant difference of means within the same rows (Tukey's HSD, $P < 0.05$) and * $P < 0.05$ for a comparison between treatment group, based on the Student's t-test.

4.6 Discussion

The data presented herein clearly implicate an involvement of the MTergic system in conjunction with the DAergic system and PRL during the transition from incubating to rearing behavior in native Thai chickens. The results revealed that the substitution of newly hatched chicks for eggs increased the numbers of MT-ir neurons within the SOv, POM, and PVN of the REC hens. In contrast, the number of TH-ir neurons within the nI and ML decreased in the REC hens when compared with those of the INC hens. These neuroendocrine changes were associated with changes in plasma PRL concentrations. These findings further indicate that the presence of eggs and chicks is important for controlling the neuroendocrine system during maternal behaviors by affecting the MTergic system within the SOv, POM, and PVN and the DAergic system within the nI and ML in the initiation and maintenance of rearing behavior in native Thai chickens.

The present study revealed that the distributions of MT-ir neurons and fibers of the INC and REC hens were predominantly found within the SOv, POM, and PVN. The greatest density of MT-ir neurons was found within the POM and PVN, and a few MT-ir neurons were found in the SOv. The numbers of MT-ir neurons within the SOv, POM, and PVN remained high during the incubation period. When rearing behavior of incubating hens was induced by replacement of eggs with chicks, the numbers of MT-ir neurons were significantly increased within the SOv, POM, and PVN, and incubation behavior was disturbed. This study is supported by previous studies in turkeys and native Thai chickens. In turkey hens, the numbers of MT-ir neurons within the SOv and PVN increase in incubating turkey hens when compared with laying turkey hens. Co-expression of MT-ir neurons and Fos mRNA was also found within the SOv and

PVN when incubating turkeys had their eggs substituted by chicks and incubation behavior was terminated and hens exhibited full brooding behavior (Thayananuphat et al., 2011). In native Thai hens, the numbers of MT-ir neurons increased within the SOv, POM, and PVN in the rearing hens when compared with those of the non-rearing ones (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). This evidence suggests that MTergic activity within the SOv, POM, and PVN is involved with the reproductive cycle and the onset of maternal behaviors in birds (Thayananuphat et al., 2011; Chokchaloemwong et al., 2013; Sinpru et al., 2017). In addition, in male zebra finches, stimulation of the MTergic system during nest-building has been demonstrated. The co-localization of MT-ir neurons and Fos increased in stria terminalis, dorsal subdivision during nesting (Hall et al., 2015a). Peripheral administration of OT receptor antagonist decreased nesting behavior and time that female zebra finches spent on their nests (Klatt and Goodson, 2013b). Taken together, these findings clearly suggest that the MTergic system is involved with maternal behaviors in birds.

In the present study, TH-ir neurons and fibers were found within the AM, PVO, nI, and ML of the INC and REC hens, with the highest density of TH-ir neurons found in the nI. The numbers of TH-ir neurons within the nI and ML remained high during the incubation period. When incubation behavior was disturbed by replacing eggs with chicks for 3 days, the number of TH-ir neurons in the nI decreased in the REC13 and REC17 hens when compared with those of the INC13 and INC17 hens, respectively. However, in the ML, the number of TH-ir neurons only decreased in the REC13 hens when compared with that of the INC13 hens. No differences were seen in the numbers

of TH-ir neurons within the nI and ML during early-incubation between the INC and REC hens. Moreover, the numbers of TH-ir neurons within the AM and PVO were not changed. These results are consistent with previous findings from our laboratory showing that the number of TH-ir neurons is high during the incubation period (Sartsoongnoen et al., 2008; Prakobsaeng et al., 2011). Disruption of incubation behavior by removing hens from their nests and eggs decreased the number of TH-ir neurons in the nI at mid- and late-incubation, whereas the number of TH-ir neurons in the ML decreased at early-incubation (days 6 and 8; Prakobseang et al., 2011). In contrast, the number of TH-ir neurons was higher in the rearing hens when compared with the non-rearing hens. Disruption of rearing behavior by removing the hens from their chicks decreased the number of TH-ir neurons in the nI (Chokchaloemwong et al., 2015). This evidence suggests that the DAergic system within the nI and ML affects maternal behaviors in native Thai chickens (Prakobsaeng et al., 2011; Chokchaloemwong et al., 2015). Indeed, the association of the DAergic system and maternal behaviors has been well documented in birds. DAergic activity in the anterior hypothalamus markedly increases in incubating bantam hens when compared with laying or nest-deprived hens (Macnamee and Sharp, 1989). TH-ir neurons co-expressing Fos mRNA are found in the ML when turkey hens are forced to make the transition from incubating to rearing behavior by replacing their eggs with chicks (Thayananuphat et al., 2011). In parenting ring doves, the number of TH-ir neurons in the periventricular regions is higher in brooding than in non-brooding birds (Lea et al., 2001). Furthermore, Fos immunoreactivity in the area tegmentalis ventralis (DAergic reward nucleus) increases with the number of times nest material is picked up by nest-building male zebra finches (Hall et al., 2014). TH-ir neurons are co-localized with Fos

in the central grey and increase in relation to the length of time a male zebra finch spent with his mate in the nest cup (Hall et al., 2015a). These reports support the present findings, suggesting that the DAergic system within the nI and ML regulates the transition from incubating to rearing behavior in this species.

In this present study, plasma PRL concentrations were high throughout the incubation period. The presence of chicks caused plasma PRL concentrations to decrease and decreased the number of TH-ir neurons within the nI and ML. These results are in good agreement with previous reports demonstrating that plasma PRL concentrations decline when chicks are introduced to incubating hens and incubation behavior is subsequently disrupted (Leboucher et al., 1993; Richard-Yris et al., 1998). Disruption of incubation behavior in native Thai chickens by nest-deprivation decreases circulating PRL concentrations, which is associated with decreased numbers of TH-ir neurons within the nI and ML (Prakobsaeng et al., 2011). Removing chicks from hens decreased the number of TH-ir neurons in the nI and plasma PRL concentrations in native Thai chickens (Chokchaloemwong et al., 2015). Substitution of chicks for eggs during early-, mid-, and late-incubation decreased the numbers of VIP (the avian PRL-releasing factor)-ir neurons within the nucleus inferioris hypothalami and nucleus infundibuli hypothalami when compared with incubating hens, suggesting that the presence of eggs and chicks affects the VIPergic system in native Thai chickens (Namken et al., 2017). A role for an increase in plasma PRL concentrations in inducing incubation activities in birds is well established (Burke and Dennison, 1980; El Halawani et al., 1984; Buntin and Tesch, 1985; Youngren et al., 1991; Rozenboim et al., 2004; Williams, 2012 Ryan et al., 2014). Termination of incubation behavior or the presence of young after hatching decreases the levels of

plasma PRL in bantams (Lea et al., 1981), chickens (Sharp et al., 1979; Zadworny et al., 1988; Leboucher et al., 1990; Kuwayama et al., 1992), and native Thai chickens (Prakobsaeng et al., 2011). PRL is positively associated with parental behavior, number of chicks hatched, and chick survival (Smiley and Adkins-Regan, 2016a). Thus, these reports strongly support neuroendocrine interactions between the DAergic system and PRL secretion on maternal behaviors in birds.

The involvement of MT and DA in maternal behaviors in birds has been reported. In native Thai chickens, the numbers of MT-ir neurons increased within the SOv, POM, and PVN and TH-ir neurons increased in the nI in rearing hens when compared with non-rearing hens (Chokchaloemwong et al., 2013; 2015). Intracerebroventricular injection of D_1 or D_2 DA or OT receptor antagonists demonstrated an involvement of DA in initiating brooding behavior via the D_2 DA receptor subtype (Thayanuphat et al., 2011). In zebra finches, MTergic and DAergic neurons were higher in nest-building birds than in control birds (Hall et al., 2015a). Therefore, the interaction of the MTergic and DAergic systems may be associated with maternal behaviors in birds.

In conclusion, the present findings indicate that the numbers of MT-ir neurons within the SOv, POM, and PVN increase when hens make the transition from incubating to rearing behavior. In contrast, the numbers of TH-ir neurons within the nI and ML decreased in parallel with plasma PRL concentrations when the hens were forced to make the transition from incubating to rearing behavior. These findings are interpreted to suggest that the presence of eggs and chicks affects the MTergic system within the SOv, POM, and PVN and the DAergic system within the nI and ML, and

these two systems, in turn, have a significant role in the transition from incubating to rearing behavior in native Thai chickens.

4.7 Acknowledgements

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APPENDIX

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