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NEUROENDOCRINE REGULATION OF REARING BEHAVIOR IN THE NATIVE THAI HEN: ROLES OF DOPAMINE AND MESOTOCIN

Duangsuda Chokchaloemwong

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NEUROENDOCRINE REGULATION OF REARING BEHAVIOR IN THE NATIVE THAI HEN: ROLES OF DOPAMINE AND MESOTOCIN

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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_____

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ไก่พื้นเมืองไทย (สายพันธุ์กาลลัส โดเมสติคัส) เป็นไก่ที่มีระบบการสืบพันธุ์ที่ให้ผลผลิตไข่ตลอดทั้งปี ซึ่งพบในเขตศูนย์สูตร ลักษณะเด่นของไก่พื้นเมืองไทยคือมีการแสดงออกของพฤติกรรมความเป็นแม่สูงอยู่เสมอ พฤติกรรมความเป็นแม่นี้เกี่ยวข้องกับฮอร์โมนโดยเริ่มต้นจากการเกิดพฤติกรรมการฟักไข่และต่อเนื่องไปจนถึงระยะที่ลูกไก่ถูกเลี้ยงดูโดยพ่อแม่ (พฤติกรรมการเลี้ยงลูก) การแสดงออกของพฤติกรรมดังกล่าวนับว่าเป็นปัญหาสำคัญที่ส่งผลทำให้ศักยภาพในด้านการผลิตไข่ลดลงเป็นอย่างมาก การวิจัยนี้ได้ศึกษาความสัมพันธ์ของโดปามีน มีโซโทซิน (โปรตีนในสัตว์ปีกที่มีโครงสร้างคล้ายออกซิโทซิน) และโปรแลคตินกับการควบคุมพฤติกรรมการเลี้ยงลูกโดยระบบประสาทและระบบต่อมไร้ท่อในไก่พื้นเมืองไทยโดยได้เปรียบเทียบการเปลี่ยนแปลงของจำนวนเซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซีเลส (ตัวบ่งชี้ถึงเซลล์ประสาทที่ผลิตโดปามีน) และจำนวนเซลล์ประสาทที่ผลิตมีโซโทซินของไก่พื้นเมืองไทย โดยใช้เทคนิคอิมมูโนฮิสโตเคมิสทรี ระดับโปรแลคตินในพลาสมาถูกวัดโดยใช้เทคนิคเอนไซม์ลิงค์อิมมูโนซอร์เบนท์แอสเสย์ ผลการศึกษาพบว่าโปรแลคตินมีระดับสูงในวันที่ลูกไก่ถูกฟักออกจากไข่และลดลงอย่างรวดเร็วภายใน 4 วัน หลังจากที่ลูกไก่ฟักออกจากไข่ และคงอยู่ในระดับต่ำตลอดช่วงระยะเวลาการเลี้ยงลูก 28 วัน ระดับของโปรแลคตินของแม่ไก่ที่ให้เลี้ยงลูกสูงกว่าแม่ไก่ที่ถูกพรากลูกตลอดช่วงเวลาของการทดลอง เซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซีเลสพบทั่วไปในบริเวณสมองส่วนไฮโปทาลามัสของแม่ไก่ที่เลี้ยงลูกและแม่ไก่ที่ถูกพรากลูก และพบมากที่บริเวณนิวเคลียสอินทราเมดิเอลิสและนิวเคลียสแมมิลลาริสแลเทอราลิส จำนวนเซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซีเลสบริเวณนิวเคลียสอินทราเมดิเอลิสลดลงอย่างมีนัยสำคัญทางสถิติในแม่ไก่กลุ่มที่ถูกพรากลูกเมื่อเปรียบเทียบกับแม่ไก่กลุ่มที่เลี้ยงลูกตั้งแต่วันที่ลูกไก่ฟักออกจากไข่จนถึงระยะเวลา 14 วันของการศึกษา แต่ไม่พบความแตกต่างระหว่างแม่ไก่กลุ่มที่ให้เลี้ยงลูกและแม่ไก่กลุ่มที่ถูกพรากลูกที่บริเวณนิวเคลียสแมมิลลาริสแลเทอราลิส ส่วนเซลล์ประสาทที่ผลิตมีโซโทซินพบว่ากระจายตัวทั่วไปในบริเวณสมองส่วนหน้าของไฮโปทาลามัสใกล้กับบริเวณที่ติดกับโพรงสมองที่สาม โดยพบมากที่สุดบริเวณนิวเคลียสซุพราออพติคัสพาร์เวนทราลิส นิวเคลียสพรีออพติคัสมีดิเอียลิส และนิวเคลียสพาราเวนทริคูลาลิสแมกโนเซลลูลาริส จำนวนเซลล์ประสาทที่ผลิตมีโซโทซินที่บริเวณ

นิวเคลียสซุพราออพติคัสพาร์สเวนทราลิส นิวเคลียสพรีออพติคัสมีดิเอียลิส และนิวเคลียสพาราเวนทริคูลาลิสแมกโนเซลลูลาริส มีระดับต่ำในไก่ที่ไม่ออกไข่ เพิ่มสูงขึ้นทีละน้อยเมื่อแม่ไก่เข้าสู่ช่วงของการออกไข่ และเพิ่มสูงสุดในช่วงที่แม่ไก่ฟักไข่และเลี้ยงลูก เมื่อเปรียบเทียบจำนวนเซลล์ประสาทที่ผลิตมีโซโทซินที่บริเวณนิวเคลียสซุพราออพติคัสพาร์สเวนทราลิส นิวเคลียสพรีออพติคัสมีดิเอียลิส และนิวเคลียสพาราเวนทริคูลาลิสแมกโนเซลลูลาริสระหว่างแม่ไก่กลุ่มที่เลี้ยงลูกและแม่ไก่กลุ่มที่ถูกพรากลูก พบว่าจำนวนของเซลล์ประสาทที่ผลิตมีโซโทซินในบริเวณเหล่านี้มีจำนวนมากในแม่ไก่กลุ่มที่ให้เลี้ยงลูกและลดลงอย่างมีนัยสำคัญทางสถิติในแม่ไก่กลุ่มที่ถูกพรากลูก ผลการศึกษานี้แสดงให้ประจักษ์เป็นครั้งแรกว่าเซลล์ประสาทที่ผลิตโดปามีนและมีโซโทซินมีบทบาทในการควบคุมระบบประสาทและระบบต่อมไร้ท่อเพื่อให้พฤติกรรมความเป็นแม่เกิดขึ้นและดำรงอยู่ในไก่พื้นเมืองไทย โดยอาจเป็นไปได้ว่าการทำงานของเซลล์ประสาทที่ผลิตโดปามีนและมีโซโทซินรวมถึงระดับของโปรแลคตินในระหว่างการขัดขวางพฤติกรรมการเลี้ยงลูกอาจมีส่วนเชื่อมโยงกับการมีส่วนร่วมของพฤติกรรมการเลี้ยงลูกในสัตว์ปีกที่ลูกอาศัยอยู่ในไข่จนโตเต็มที่แล้วจึงฟักออกจากไข่ที่อาศัยอยู่ในแถบเส้นศูนย์สูตรชนิดนี้

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

DUANGSUDA CHOKCHALOEMWONG : NEUROENDOCRINE REGULATION OF REARING BEHAVIOR IN THE NATIVE THAI HEN: ROLES OF DOPAMINE AND MESOTOCIN. THESIS ADVISOR : ASSOC. PROF. YUPAPORN CHAISEHA, Ph.D. 318 PP.

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

Native Thai chicken (*Gallus domesticus)* is a continuously breeding species found in the equatorial zone that produces eggs all year. It always expresses high maternal behaviors. Maternal behaviors are hormonal dependent and initiated with the onset of incubation behavior and continue through the period when the parents are taking care of the young (broody/rearing behavior). The expression of such behaviors is a costly problem, resulting in substantial loss of potential egg production. The association of dopamine (DA), mesotocin (MT; the avian homolog of oxytocin), and prolactin (PRL) with the neuroendocrine regulation of rearing behavior were investigated in the native Thai chickens. Changes in the numbers of tyrosine hydroxylase-immunoreactive (TH-ir; as a marker for DA neurons) and MT-immunoreactive (MT-ir) neurons in the brain of the native Thai chicken were studied using immunohistochemistry. Plasma PRL levels were determined by enzyme-linked immunosorbent assay. The plasma PRL levels remained at high levels on the day of chicks' hatched and then rapidly decreased within 4 days after they hatched and remained at low levels through 28 days. Plasma PRL levels in the rearing hens (R)

were higher than those of the non-rearing hens (NR) throughout the observation periods. TH-ir neurons and fibers were extensively distributed throughout the hypothalamic areas of R and NR native Thai hens and were highly expressed in the nucleus intramedialis (nI) and nucleus mammillaris lateralis (ML). Significant decreases in the number of TH-ir neurons of the NR hens when compared to those of the R hens were observed in the nI after the day of hatch until 14 days of the observation periods, but there are no significantly differences between R and NR hens in the ML. MT-ir neurons and fibers were found in discrete regions located closely to the third ventricle from the levels of preoptic area through the anterior hypothalamus with the greatest abundance observed in the nucleus supraopticus; pars ventralis (SOv), nucleus preopticus medialis (POM), and nucleus paraventricularis magnocellularis (PVN). The numbers of MT-ir neurons in the SOv, POM, and PVN were low in non-laying hens, gradually increased when hens entered the laying stage, and peaked in the incubating and rearing hens. Comparisons of MT-ir neurons in the SOv, POM, and PVN between the R and NR hens were elucidated. The numbers of MT-ir neurons in these nuclei were high in the R hens which significantly decreased in the NR hens. These results indicate, for the first time, that DAergic and MTergic systems play a role in the neuroendocrine reorganization to establish and maintain maternal behaviors in the native Thai chickens. It is possible that DAergic and MTergic activities as well as PRL levels during disrupting the rearing behavior might be related to the contribution of rearing behavior in this equatorial precocial species.

School of Biology

Academic Year 2012

Student's Signature _____

Advisor's Signature _____

Co-advisor's Signature _____

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Duangsuda Chokchaloemwong

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CHAPTER I

INTRODUCTION

1.1 Rational of the Study

The native Thai chicken (*Gallus domesticus*) belongs to genus Gallus of the family Phasianidae. It is probably originated from the wild jungle fowl in Southeast Asia and was domesticated approximately 3,000 years ago by village people. In Thailand, they have been raised in the countryside for many generations. Traditionally, Thai people raised chickens mostly for consumption, competition, and recreation. It is not only a main protein food source, but it can be sold for supplemental income for families in the urban areas as well. Raising the native Thai chickens is found widespread throughout Thailand because it is easy to raise, resistant to diseases, and acclimatized to the local environments, especially high temperature. Beside its meat has a unique taste, it is firm in texture and contains high content of proteins as well as low fat and cholesterol contents, resulting in high demand by consumers who prefer low fat white meat. In general, native Thai and imported broiler chickens are consumed at approximately the same commercial live weight. However, the native Thai chickens have slower growth rate than those of the imported ones, which may contribute to differences in properties of their meats. There are some evidences reported that the muscles of native Thai chicken possess firmer texture, particularly after cooking than those of the commercial broilers, and this might be

related to the differences in total and soluble collagen contents between their muscles. The high price of native Thai chicken has been recognizing, because the consumers have acquired a taste of native Thai chicken and its popularity is rapidly growing. This situation provides a good opportunity for producing the native Thai chicken in industrial scale. Furthermore, recent Thai government policies are to encourage the development and the use of natural resources in supporting of His Majesty the King's concept for self-sufficiency in agriculture. Regarding to this concept, the farmers tend to focus on mixed-farming that is the strategy for helping rural farmers to increase self-sufficiency. One of the significant natural resources of Thailand that needs to be developed is the native Thai chicken. Recently, the native Thai chicken has become a new economic domestic animal of Thailand with presently growing demand and relatively high price. The market price of the native Thai chickens is 2-3 times higher than those of the imported broilers. Up to date, there are about 76 million native Thai chickens in Thailand, which are raised by 2.7 million farmers. This exported goods gains income about 2.2 million baht per year.

Although, the native Thai chickens can be raised under poor environmental conditions such as in the backyard with local feed, minimum care, and management, but they have low productivity. The reproductive performance of the native Thai chicken is much lower than those of the cross breeds and hybrids, especially egg laying performance. One of the main causes of low reproductive performance in the native Thai chickens is the incidence of maternal behaviors; incubating, and brooding or rearing behaviors, which are heritable traits. The onset of incubation and rearing behaviors affects the number of egg production, because it terminates egg laying and the native Thai hen spends much time for rearing chicks, respectively. Generally, the

native Thai hen lays eggs 3-4 times per year, 4-17 eggs per clutch. Thus, it produces about 30-40 chicks per year, which is significantly lower than that of the imported hen, which produces eggs all year long (240-270 egg per year). At present, market demands of the native Thai chickens cannot be met by supplies, mainly because of their low egg laying performance, and they tend to lay eggs in clutches rather than evenly distributed over the year, leading to production of chicks irregularly. These cause a problem for producing the native Thai chickens commercially in the poultry industry in Thailand. Indeed, since it was domesticated, the native Thai chicken is never been genetically selected and always expresses high maternal behaviors, the heritable traits from their ancestors.

Well establishedly, neurotransmitters, neurohormones, neuromodulators, and hormones of the hypothalamo-pituitary-gonadal (HPG) axis play an important role in the reproductive cycle of avian species. This HPG axis involves two major neuroendocrine systems controlling avian reproduction. These systems include the gonadotropin releasing hormone/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH), and vasoactive intestinal peptide/prolactin (VIP/PRL) systems and both systems are influenced by dopamine (DA).

PRL, an anterior pituitary hormone, has been indicated to be associated with the reproductive cycle in several avian species such as turkeys, quails, bantams, ring doves, pigeons, mallard ducks, and native Thai chickens. PRL has been implicated as a causative factor in the onset and maintenance of maternal behaviors, especially play a significant role in incubation behavior, crop milk production and secretion, feeding of the young, and nest defense. Rearing behavior is maintained by high levels of PRL and upon hatching. It is very well documented that PRL is under stimulatory control

by VIP, the avian PRL-releasing factor (PRF). In many avian species, the onset and maintenance of maternal behaviors is correlated with low levels of GnRH, FSH, LH, and high levels of circulating PRL.

DA 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 releases DA that acts directly upon D_2 DA receptors located on pituitary lactotrophs in mammalian species. Removal of this DAergic inhibition results in an increased PRL release and hyperprolactinemia. This is not the case in birds, while removal of hypothalamic inputs results in the completed cessation of PRL secretion. In birds, it has been documented that PRL secretion is tonically stimulated by the VIP; the avian PRF. At present, unlike the mammalian DAergic strategy for PRL control, the role of DA in the regulation of avian PRL secretion is poorly understood. DA neurons are found throughout the hypothalamus and have been shown to be immunoreactive (ir) for VIP. DA has been measured and visualized in various avian species including domestic fowls, quails, pigeons, zebra finchs, 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 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.

Recently, the presence of hypothalamic VIP-ir, tyrosine hydroxylase (TH)-ir (as a marker for DA), and GnRH-I-ir neurons at different reproductive stage and throughout the incubation period have been reported in the native Thai chickens. Changes in the number of VIP-ir, TH-ir, and GnRH-I-ir neurons in the native Thai chickens are observed across the reproductive stages and during incubation and nest deprivation period, which is mirrored directly with variations in plasma PRL levels. VIP-ir neurons and fibers are extensively distributed throughout the brain of the native Thai chickens, and are predominantly expressed in the diencephalon, where VIP-ir neurons are highly concentrated within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) areas. Changes in numbers of VIP-ir neurons within the IH-IN are directly correlated with changing of plasma PRL levels throughout the reproductive cycle, suggesting that VIP expression in the IH-IN plays a regulatory role in year-round reproductive activity of the native Thai chickens. Further studies reveal that an association exists between DA neurons in the nucleus intramedialis (nI) and the regulation of the reproductive system in the native Thai chickens, indicating that the differential expression of DA neurons in the nI might play a role in the control of VIP secretion and subsequent PRL release in the native Thai chickens. Moreover, it has been further demonstrated that changes in the number of VIP-ir neurons in the IH-IN are associated with DAergic neurons within the nI and nucleus mamillaris areas, resulting in PRL release to induce and maintain incubation behavior in the native Thai hens. It is then further suggested that nesting activity stimulates PRL secretion by the activation of the DAergic system, which in turn

stimulates the VIPergic system. These elevated PRL levels increase nesting activity and maintain incubation behavior. In several avian species, PRL secretion has been shown to be stimulated by the presence of chicks. This hormone is indicated to be associated with parental care, and is known to decline steadily immediately after hatching in precocial bird species or drop at the end of the rearing period in altricial bird species. Moreover, in the native Thai hens, disruption of rearing behavior by removing the chicks from the hens markedly decreases plasma PRL levels, a parallel decline in the number of VIP-ir neurons in the IH-IN, and an accompanying increase in the number of GnRH-I-ir neurons in the nucleus commissurae pallii (nCPa), suggesting that the VIPergic system in the IH-IN and the GnRH system in the nCPa may be involved in the regulation of the reproductive neuroendocrine system and the initiation and maintenance of rearing behavior in the native Thai chickens.

It is well established that oxytocin (OT) and arginine vasopressin (AVP), neurohypophysial hormones, play an important role mainly in parturition and lactation in mammals and regulate a variety of important physiological functions. In mammals, AVP regulates fluid and electrolyte balance, blood pressure, and plays a role in the stress response, whereas OT regulates various reproductive behaviors and functions including uterine contraction and milk ejection. The physiological actions of OT range from the modulation of the neuroendocrine reflexes to the establishment of complex social and bonding behaviors related to the reproduction and care of the offspring. Furthermore, it has been suggested that the expression pattern and high homology of OT receptors may stimulate myometrial contraction, and therefore play a critical role in oviposition in birds. Both OT and AVP have central effects on sexual, maternal, and social behaviors as well as on memory and learning. The avian

neurohypophysial hormones have been then characterized. The avian antidiuretic hormone is arginine vasotocin (AVT) and the oxytotic principle is mesotocin (MT).

Anatomically, it has been suggested that AVT and MT may play similar hypophysiotropic functions in non-mammalian vertebrates. MT neurons are found in the areas of hypothalamic paraventricular nucleus (PVN), supraoptic nucleus (SON), and tuberomammillary area in chickens, domestic mallards, and Japanese quails. MT fibers are found at both internal and external layer of the median eminence. MT is also detected in areas outside the hypothalamus such as in the cerebellum, lateral septum, optic lobe, pons, and medulla oblongata. There are several lines of evidence, using domestic chicken as the model, demonstrating that AVT, and not MT, is a key regulator of oviposition in birds. This finding is somewhat surprising since structurally MT is mostly like OT. It has been demonstrated that plasma AVT levels transiently increase during oviposition in birds, and this increase at the time of oviposition has been correlated with the increase in uterine contractility. Moreover, AVT, but not MT, has been shown to stimulate contraction of shell gland strips *in vitro*. Taken together, in avian species, the physiological functions of AVT have been well established, but the role(s) of MT is far from understood.

For successful reproduction, not only sexual activity is important, but also successful care of the young. Maternal behavior is crucial to the survival of fertilized eggs or offspring. The offspring need one or both parents to provide food, heat, or protection from any harm. This behavior must be performed immediately after birth or hatching of offspring. Maternal behaviors in mammals are composed of nest building, pup retrieval, crouching, exploration and sniffing of pups, licking and grooming, and placentophagia. Brooding/rearing behavior in precocial birds consists

of vocal signalling, protection from the environment, and food searching, while in artricial birds, the young require substantial attention for feeding and protection. Maternal care in birds is included incubation and brooding/rearing behaviors. The term incubation refers to the maternal care of unhatched eggs, and brooding is the maternal care of chicks after hatching. Incubation behavior in birds is qualified by sitting continually on their eggs until they are hatched, while brooding or rearing behavior is directed to the care of newly hatched chicks. Generally, the hens develop maternal behaviors gradually in four stages; brooding, titbitting, clucking, and normal broody behavior. The incidence of maternal behaviors concurs with a pause in laying and a decrease in plasma gonadal steroid levels. It has been reported that birds that exhibit brooding behavior allow chicks to access and remain underneath their wings and then provide post-hatching care, whereas birds that do not show brooding behavior actively avoided the chicks. Maternal behaviors are hormonal dependent, initiated with the onset of incubation behavior, and continued through the period of broody behavior.

In mammals, the mechanisms underlying the regulation of maternal behaviors may be derived from the processes involving in the gestation, parturition, or the regulation of lactation including changes in circulating levels of progesterone, estrogen, OT, and PRL. These hormonal activities increase in the medial preoptic area (MPOA) during the expression of maternal behaviors. Apparently, some neuropeptides, neurohormones, and hormones, most notably OT and PRL that play a key role in the onset of maternal behaviors, are prominent in the reorganization of the neuronal systems controlling energy balance, stress response, anxiety, and aggression in postpartum females as well. Functional neuroanatomical evidence indicates that the

appetitive aspects of maternal behaviors are regulated through the MPOA interactions with the mesolimbic DA system. Moreover, the interactions between the MPOA and the mesolimbic DA system not only regulate the onset of maternal behaviors, but also control its continuance during the postpartum period in rodents. OT seems to be involved during the onset of maternal behaviors. In mammals, hypothalamic neurons of the PVN and SON are capable of releasing OT into diverse neural sites at the time of birth. Importantly, evidence has shown that OT physiological actions at the level of MPOA, ventral tegmental area, or nucleus accumbens can stimulate the onset of maternal behaviors.

In birds, MT neurons are found in several brain areas. However, little is known regarding the physiological function(s) of MT in birds. It does not appear to be involved in aggression, partner preference, cardiovascular function as well as plasma osmolarity. The first evidence reported the role of MT in avian brooding behavior has only been investigated in the turkeys. The numbers of MT-ir neurons in the PVN and nucleus supraopticus, pars ventralis increase in incubating hens when compared with laying hens. In addition, the induction of c-fos mRNA in MT-ir neurons within these brain nuclei in incubating hens stimulated with poults, and preventing poult brooding from taking place by blocking MT receptors suggest that MT is essential to the onset of maternal activities in the turkeys.

As aforementioned, the expression of maternal behaviors including brooding and rearing behaviors is a costly problem, resulting in substantial loss of potential egg production. Some evidence suggests 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. Cessation of egg laying is associated with increased PRL

concentrations in the turkeys, domestic fowls, and native Thai chickens. Obviously, the reproductive efficiency of native Thai chickens is low in comparison to those of the imported breeds. Thus, in order to increase the production of native Thai chickens in Thailand, it is very important to understand the neuroendocrine regulation of the maternal behaviors. To date, it has been well established that incubation behavior in this species is regulated by the VIP/PRL, GnRH/FSH-LH, and DAergic systems. However, no data are available that describe the interrelationship and the functional aspects of the changes in DAergic and MTergic system during rearing/brooding behaviors in this species. Thus, the objectives of this dissertation were carried out to elucidate the neuroendocrine regulation of maternal behaviors in the native Thai chicken. This dissertation focuses on the roles of DA, PRL, and MT that associated with maternal care for their chicks in the native Thai chickens. The results gained from this study will provide an insight into the neuroendocrine mechanism(s) underlying the regulation of maternal behaviors in the native Thai chickens. The knowledge gained from this study can be then applied commercially in poultry industry to increase egg production of the native Thai chickens in Thailand.

1.2 Research Objectives

1.2.1 To study the changes in plasma PRL levels in the regulation of rearing behavior in the female native Thai chickens.

1.2.2 To study the differential expression of TH (a marker for DA) that is associated with the neuroendocrine regulation of rearing behavior in the female native Thai chickens.

1.2.3 To study the differential expression of MT across the reproductive cycle of the native Thai chickens

1.2.4 To study the differential expression of MT that is associated with the neuroendocrine regulation of rearing behavior 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 suggested that the red jungle fowl (*Gallus gallus*) might be the ancestor of all domestic chickens, which is found wildly distributed throughout Southeast Asia (Austic and Nesheim, 1990; Fumuhito et al., 1994; Hillel et al., 2003; Sawai et al., 2010), and it was domesticated by village people approximately 3,000 years ago. Some inherited characteristics from the wild jungle fowl such as maternal behaviors (incubation and rearing or brooding behaviors) of the native Thai chickens are still strongly expressed (Beissinger et al., 1998). Traditionally, the main objectives of raising native Thai chickens are for consumption, sport competition, recreation, and also for sale as an additional income for families. In general, the native Thai chickens are easy to raise, resistance 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 high performance breeds lost their advantages over the native Thai chickens in terms of weight gain when fed with local feed (Leotarakul and Pimkamlia, 1999). In addition, the native Thai chicken is well adapted to the poor conditions of small farms or simple rural environments. Its resistance to diseases and tolerance to high temperature (heat stress) are considerably higher than those of high performance hybrid breeds. It has been reported that the native Thai chickens can adapt to high temperature, and imported broilers are less tolerant to the high temperature than those of Thai indigenous chickens crossbred and Thai indigenous chickens (Aengwanich, 2008). These result in high potential for raising the native Thai chickens commercially in the urban areas (Kajaroen et al., 1989).

Up to date, in Thailand, there are about 76 million native Thai chickens or 24 % of total chicken production, which are broilers 55 %, layers 15 %, commercial broiler breeders 5 %, and commercial layer breeders 1 % (Department of Livestock Development, 2011). Native Thai chicken's meat has a unique taste and texture regarding to a delicacy and quite popular among consumers (Wattanachant et al., 2005). It also provides higher meat quality with less fat and low cholesterol contents than those of the imported commercial broilers, resulting in high demand by consumers (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 there 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). In addition, the shear values of native Thai chicken muscles both raw and cooked 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). Therefore, there are several factors such as genotype, rearing system, feed, age, muscle pH, chemical composition, microstructure of muscle, postmortem aging, and processing methods contribute to the quality of meat (Chotesangasa and Gongruttananun, 1999; Jaturasitha et al., 2002; Wattanachant et al., 2005; Wattanachant, 2008). The firm texture and low fat and cholesterol meat, free of drug residues such as antibiotics, makes consumers prefer this meat type (Choprakarn et al., 2000), and these advantages of its meat leads to a higher price 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). Furthermore, it has been suggested that the native Thai chickens, especially Pradu Hang Daum breed, is suitable to be developed as a meat type chicken regrading to its lower genetic distance to broiler strains (Dorji et al., 2011).

The reproductive performance of native Thai chickens is much lower than those of the cross breeds and hybrid breeds, especially egg laying performance, which is critical to secure a sufficient number of chicks for fattening (Chotesangasa et al., 1994). The production of native Thai chickens is suited for the small farm raising system, but improving the supply of chicks for fattening needs to be developed (Haitook et al., 2003). Hatchability is not the problem for commercially producing the chicks, if the number of eggs per hen is not limited. Generally, the native Thai hen lays eggs 3-4 times/year, 4-17 eggs/clutch rather than laying eggs continuously all

year long. The hen-day egg production of the native Thai hen is lower than that of the commercial laying hen with the peak production for native Thai hens and commercial laying hens are 38.0 % and 75.5 %, respectively (Chotesangasa et al., 1994). The total number of eggs per hen of native Thai hen is between 30-92 eggs/year, which is significantly lower than that of 243 eggs/hen/year of the imported commercial hen (Chotesangasa et al., 1994). Thus, with a hatching rate of 80-85 %, a typical hen produces 25-40 chicks/year (Klinhom et al., 2005). The low potential in egg production of the native Thai chickens causes the problem in order to be produced commercially in poultry industry in Thailand. In the native Thai chickens, the main cause of low egg production and short egg laying period is the expression of the maternal behaviors (incubation and rearing behaviors). These behaviors are highly expressed during egg laying, nesting, and brooding periods, which are certainly not desired for commercial scale production (Choprakarn and Wongpichet, 2007). In general, the native Thai chicken takes about 2 weeks for laying, 3 weeks for hatching, and 6-10 weeks for taking care of the chicks. Therefore, the hen spends about 10-15 weeks for each reproductive cycle (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, taking 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

In birds, the regulation of the reproductive system involves the interaction of external stimuli with neuroendocrine mechanisms. The avian reproductive system is

integratively regulated by the hypothalamus, the pituitary, and the gonads, namely the hypothalamo-pituitary-gonadal (HPG) axis. It is very well established that neurotransmitters, neurohormones, neuromodulators, and hormones of this HPG axis play a pivotal role in reproductive cycle of avian species. This axis involves two major neuroendocrine systems controlling avian reproduction including the gonadotropin releasing hormone/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH), and vasoactive intestinal peptide/prolactin (VIP/PRL) systems and both systems are influenced by dopamine (DA; Bhatt et al., 2003; Chaiseha et al., 2003a; Chaiseha and El Halawani, 2005). Moreover, in temperate zone birds such as turkeys, Canadian geese, Japaneese quails, etc., both neuroendocrine systems depend upon the photoperiod as well as the transduction of photoperiodic information, resulting in either gonad recrudescence and its associated sexual activity or gonad regression and the termination of reproductive activity. The final common pathway regulating these neuroendocrine systems is formed by a system of peptidergic neurons, whose axons terminate around portal capillaries in the external layer of the median eminence (ME; Chaiseha and El Halawani, 2005). GnRH stimulates pituitary gonadotrophs to synthesize and secrete gonadotropins, FSH and LH, which in turn are responsible for ovarian follicular growth and ovulation at the egg laying period. During the egg incubation period, VIP stimulates pituitary lactotrophs to synthesize and secrete PRL, and then regression of the gonads. Indeed, GnRH and VIP can also directly affect the gonads via the gonadal receptors (Asem and Novero, 1993; Johnson, 2000; Sun et al., 2001).

In birds, it has been well documented that FSH, LH, and PRL are associated with the reproductive cycle in several species such as canvasback ducks, cockatiels,

emperor penguins, geese, king penguins, mallards, tropical seabirds, turkeys, and native Thai chickens (Mashaly et al., 1976; Bluhm et al., 1983a; 1983b; El Halawani et al., 1984b; 2001; Myers et al., 1989; Wong et al., 1992b; Mauget et al., 1994; Lormee et al., 1999; 2000; Boos et al., 2007; Huang et al., 2008; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008; Prakobsaeng et al., 2011; Chaiyachet et al., 2012). In the native Thai chickens and turkeys, during reproductively quiescent stages, plasma PRL levels are low (El Halawani et al., 1984b; 1997; Karatzas et al., 1997; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). At the onset of incubation period, plasma progesterone and LH levels start to rise continuously and reach the highest values at about 8 to 2 hours right before ovulation (Mashaly et al., 1976). Plasma FSH levels are low throughout the ovulatory cycle, but a significant decrease occurs just before the preovulatory surge of LH and a significant increase occurs during 3 hours before oviposition as plasma LH levels decrease (Krishnan et al., 1993). Plasma LH levels subsequently continue to decline during the incubating period (Myers et al., 1989). In contrary, during the periods of laying and incubating, plasma PRL levels rise sharply (El Halawani et al., 1984b; Kosonsiriluk et al., 2008).

It is well established that PRL is a causative factor for the reduced circulating FSH and LH levels and subsequently ovarian regression, when birds make the transition from egg laying to incubation period in bantam hens, canaries, domestic chickens, cowbirds, ducks, mallard ducks, native Thai chickens, pheasants, pigeons, ring doves, spotted sandpipers, turkeys, white-crowned sparrows, and wild starlings (Sharp et al., 1977; Burke and Dennison, 1980; Goldsmith and Hall, 1980; Goldsmith et al., 1981; 1984; Dawson and Goldsmith, 1982; Bluhm et al., 1983a; El Halawani et al., 1984b; 1997; Oring et al., 1986; Hiatt et al., 1987; Kosonsiriluk et al., 2008;

Sartsoongnoen et al., 2008). It has been indicated that PRL acts centrally to reduce LH levels by reducing GnRH concentrations at the hypothalamic level (Rozenboim et al., 1993b). In the turkey hens, the abundance of LH-β subunit and PRL mRNAs expression shows an inverse relationship in photostimulated/laying and incubating periods (Wong et al., 1992b). PRL administration suppresses the photo- and ovariectomy-induced increases in LH release, delays the onset of egg laying, and induces incubation behavior in the laying turkey hens (El Halawani et al., 1991). Changes in LH and PRL circulating levels during the reproductive cycle are well established in birds (Follett, 1984; El Halawani et al., 1988b). In reproductively quiescent birds, plasma PRL and LH levels are low, while the levels are increased in reproductively active laying hens. During the incubating stage, plasma PRL levels are dramatically increased (El Halawani et al., 1984b; Sharp et al., 1989; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008), while plasma LH levels are gradually suppressed (Lea et al., 1981; El Halawani and Rozenboim, 1993). As mentioned above, there are abundant evidences indicating that an increased PRL secretion is the causative factor for the reduced circulating gonadotropins' levels and has been observed in many avian species. For example, in galliform birds, the onset of incubation behavior is associated with declining levels of gonadotropins and ovarian steroid hormones (Sharp et al., 1979; Burke and Dennison, 1980; Bedrak et al., 1981; Lea et al., 1981), and a sharp elevate in circulating PRL levels (Goldsmith, 1985; 1991; Lea, 1987; El Halawani et al., 1988b; Sharp et al., 1988). It is this rising PRL levels, which has 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 (El Halawani et al., 1988b; Knapp et al., 1988), and as soon as molting is ended (Bluhm et al., 1983a; 1983b; Mauget et al., 1994). Furthermore, it has been suggested that high levels of PRL inhibit LH release (Zadworny and Etches, 1987; Taira and Beck, 2006). They have reported that breeding season is terminated after PRL concentrations increase above a critical threshold to suppress GnRH neuronal and LH activities (Sharp and Blache, 2003). Thus, it can be concluded that seasonal reproductive activity is inhibited by increasing circulating PRL levels, which in turn suppresses LH release, inhibits follicular development, and finally terminates egg laying (Huang et al., 2008). 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).

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

Pituitary FSH and LH secretion is regulated by the central nervous system (CNS) at the hypothalamic level. The hypothalamus synthesizes GnRH, which in turn stimulates the synthesis and secretion of these gonadotropins (Ulloa-Aguirre and Timossi, 2000; Shalev and Leung, 2003). In both mammals and birds, environmental stimuli transduced by specific receptors influence the synthesis and secretion of hypothalamic GnRH, which its secretion occurs episodically from the hypothalamus. The amplitude and frequency of pulsatile GnRH release determine the pattern of gonadotropins secretion (Levine and Ramirez, 1982; Moenter et al., 1992). In birds, GnRH is synthesized by hypothalamic neurons, released from the ME into the hypophysial portal vessels, and transported to the pituitary gland, which in turn

stimulates the synthesis and secretion of pituitary gonadotropins. Three types of GnRH and two types of GnRH receptor have been found in the avian brain (Sun et al., 2001; Shimizu and Bedecarrats, 2006). Two distinct forms of GnRH have been isolated in chicken; cGnRH-I or GnRH-I and cGnRH-II (King and Millar, 1982; Millar and King, 1984; Miyamoto et al., 1984; Sherwood et al., 1988). To date, GnRH-III which is first characterized in lamprey is also found in the brain of songbirds (Bentley et al., 2004). Of the three forms, GnRH-I is the form that is directly involved in controlling reproduction in birds (Sharp et al., 1990).

A pulsatile pattern of GnRH release is observed from the medial basal hypothalamus and the preoptic area (POA) *in vitro* (Li et al., 1994). GnRH neurons and fibers are found extensively distributed throughout the avian brain including chickens (Jozsa and Mess, 1982; Sterling and Sharp, 1982; 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 (Foster et al., 1988; Mikami et al., 1988; Perera and Follett, 1992; van Gils et al., 1993; Teruyama and Beck, 2000), European starlings (Dawson et al., 1985; Foster et al., 1987; 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 (Chaiyachet et al., 2012; Sartsoongnoen et al., 2012). 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., 1995a). In chickens, GnRH inhibits FSH-stimulated steroidogenesis, but it enhances LH-stimulated progesterone production (Hertelendy et al., 1982). In 3 weeks old cockerels, GnRH-I has no effect on circulating FSH levels, but it stimulates LH secretion (Krishnan et al., 1993). Furthermore, it has been indicated that adrenergic stimulation can release hypothalamic GnRH and subsequently increase gonadotropins secretion (Yu et al., 1991).

In avain species, the egg laying period is associated with relatively high concentrations of FSH, LH, and ovarian steroids, and this event is regulated by hypothalamic GnRH (El Halawani et al., 1988b). In fact, GnRH-I is the primary hypophysiotropic factor stimulating the secretion of LH, because active immunoneutralization against GnRH-I decreases plasma LH levels and causes a complete regression of the reproductive system (Sharp et al., 1990). However, seasonal changes in the GnRH-II-immunoreactive (-ir) neurons are observed, suggesting an involvement of GnRH-II in the neuroendocrine regulation of avian reproduction (Maney et al., 1997a; Teruyama and Beck, 2000; Bentley et al., 2004; Stevenson and MacDougall-Shackleton, 2005).

As aforementioned, 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 several 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. (Dawson et al., 1985; Foster et al., 1987; 1988;

Goldsmith et al., 1989; Bluhm et al., 1991; Perera and Follett, 1992; Rozenboim et al., 1993a; Saldanha et al., 1994; Hahn and Ball, 1995; Millam et al., 1995; Dunn et al., 1996; Kang et al., 2006; Kuenzel and Golden, 2006). At the peak level of reproductive activity, birds have more GnRH-ir neurons and fibers than those of the sexually inactive or photorefractory ones (Sharp et al., 1988; 1990; Hahn and Ball, 1995; Parry et al., 1997; Cho et al., 1998; Chaiyachet et al., 2012; Sartsoongnoen et al., 2012). GnRH contents of discrete medial preoptic, infundibulum, and arcuate samples are higher in the laying hens than those of the non-laying hens (Advis et al., 1985). In temperate zone birds, the turkeys, GnRH-I mRNA is abundance within the nucleus commissurae pallii (nCPa), organum vasculosum lamina terminalis (OVLT), and nucleus septalis lateralis (SL), and 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-ir neurons are distributed in a discrete region lying close to the third ventricle from the 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 number of GnRH-I-ir neurons is 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, for the first time, 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 (Chaiyachet et al., 2012; Sartsoongnoen et al., 2012).

Indeed, VIP, DA, gonadotropin inhibitory hormone, and gonadal steroids are considered to be involved in the regulation of GnRH secretion (Ramirez et al., 1984; Sharp et al., 1984; Deviche et al., 2000; Tsutsui et al., 2000; Bentley et al., 2004). In addition, active immunoneutralization against VIP increases LH-β and FSH-β mRNAs expression, and is accompanied by a decrease in PRL mRNA expression (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

In birds, the regulation of PRL synthesis and secretion involves the interaction of external stimuli with neuroendocrine mechanisms, and these critical stimuli include photoperiod, ambient temperature, and the presence of eggs and offspring. These external stimuli and internal stimuli such as estrogen and progesterone are important in initiating and maintaining PRL secretion. However, their relative importances vary with stages of the reproductive cycle (Curlewis, 1992). It is very well documented that the regulation of avian PRL secretion and its gene expression are governed by the hypothalamic VIP, the avian PRL-releasing factor (PRF; El Halawani et al., 1997; 2001; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999; 2005; Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011; Chaiyachet et al., 2012). Indeed, it has been established for a long time that avian PRL secretion is tonically stimulated by the hypothalamus (Kragt and Meites, 1965; Bern and Nicoll, 1968), and that the principal

PRF is VIP (El Halawani et al., 1997; 2001), 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 et al., 1998; Chaiseha and El Halawani, 1999; 2005). 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 (El Halawani et al., 1997; 2001).

Variations in VIP immunoreactivity and VIP mRNA steady-state levels occurring within the hypothalamus, VIP peptide contents in the ME, and plasma VIP levels in hypophysial portal blood are correlated with changes in the circulating PRL levels throughout the turkey reproductive cycle (Mauro et al., 1989; Youngren et al., 1996a; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999), and these observed variations in PRL are, in part, regulated by changes in VIP receptors at the pituitary level (Chaiseha et al., 2004). DA also plays an intermediary role in PRL secretion. Unlike mammals, DAergic system influences are involved in both stimulating and inhibiting avian PRL secretion, depending upon multiple subtypes of DA receptors (Youngren et al., 1995; 1996b; Chaiseha et al., 1997; 2003a; 2003b), requiring an intact VIPergic system to cause the release of PRL (Youngren et al., 1996b). In addition, dynorphin, serotonin (5-HT), DA, and VIP all appear to stimulate PRL secretion via a pathway expressing κ opioid, 5-HTergic, 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 are extensively distributed throughout the hypothalamus (Yamada et al., 1982; Korf and Fahrenkrug, 1984; Mikami and Yamada, 1984; Macnamee et al., 1986; Peczely and Kiss, 1988; Mauro et al., 1989; Cloues et al.,

1990; Norgren and Silver, 1990; Hof et al., 1991; Kuenzel and Blahser, 1994; Kuenzel et al., 1997; Chaiseha and El Halawani, 1999; den Boer-Visser and Dubbeldam, 2002; Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011; Chaiyachet et al., 2012), especially in the areas of the medial preoptic area (MPOA), medial hypothalamic region, anterior hypothalamus, hypothalamus pars lateralis, and INF.

During the reproductive cycle in birds, VIP acts on the anterior pituitary gland directly to stimulate PRL synthesis and release (Lea and Vowles, 1986; Macnamee et al., 1986; Proudman and Opel, 1988; El Halawani et al., 1990b; 1997; Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011; Chaiyachet et al., 2012). From immunohistochemistry (IHC) studies, hypothalamic VIP-ir neurons in the INF and VIP-ir fibers in the ME correspond to the enhanced plasma PRL levels in the turkeys and native Thai chickens (Mauro et al., 1989; Kosonsiriluk et al., 2008; Chaiyachet et al., 2012). Other studies have also demonstrated the increases in the number and cell size of VIP-ir neurons within this region in the pigeons and ring doves during the periods of hyperprolactinaemia (Peczely and Kiss, 1988; Cloues et al., 1990). Changes in pituitary VIP receptor mRNA expression are observed during the reproductive cycle of the turkeys. Increased pituitary VIP receptor mRNA expression is observed in the turkey hens with normal (laying) or high PRL levels (incubating), while lower VIP receptor mRNA expression is observed in the hypoprolactinemic non-photostimulated and photorefractory turkey hens. This suggests that the VIP receptors located in the INF are involved in PRL secretion and indicates that PRL secretion is principally governed by VIP receptors at the pituitary level (Chaiseha et al., 2004).

In birds, VIP neuronal activity is also regulated by photoperiod. In temperate zone birds, VIP/PRL secretion is increased gradually and progressively in response to long day photoperiod, and both their release and gene expression are up-regulated in the turkey hens, a long day seasonal breeders (Mauro et al., 1989; Youngren et al., 1989; Wong et al., 1991; El Halawani et al., 1996; Tong et al., 1997; Chaiseha et al., 1998). During activation of the GnRH/FSH-LH system in the photosensitive female turkeys initiates the reproductive activity, gonadotropins stimulated-estrogen secretion, and induces sexual receptivity, and these events prime the VIP/PRL system to enhance PRL secretion (Wineland and Wentworth, 1975; El Halawani et al., 1983; 1986). Furthermore, it has been established that VIP perikariya axon terminals have been found in close apposition to GnRH neurons in the lateral septal organ (LSO) and POA (Teruyama and Beck, 2001), and an inverse relationship between VIP in the INF and GnRH in the POA has been noted (Deviche et al., 2000). In addition, a subset of VIP-ir neurons within the mediobasal hypothalamus (MBH) and septal region has been proposed to be encephalic photoreceptors in doves (Silver et al., 1988; Norgren and Silver, 1990). Recently, it has been suggested that turkey melanopsin (tOPN4x) in the hypothalamic premammillary nucleus (PMM), DA-melatonin (DA-MEL) neurons acts as an important component of the photoreceptive system regulating reproductive activity in temperate zone birds (Kang et al., 2010).

In non-temperate zone breeding species, the native Thai chicken, VIP-ir neurons and fibers are extensively distributed throughout the brain and are predominantly expressed in the diencephalon, where VIP-ir neurons are concentrated within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) areas. Changes in the number of VIP-ir neurons within the IH-IN are directly

correlated with changing in plasma PRL levels throughout the reproductive cycle. These results suggest that VIP expression in the IH-IN plays a regulatory role in year-round reproductive activity in this equatorial bird (Kosonsiriluk, 2007; Kosonsiriluk et al., 2008). Further studies indicate that the differential expression of DA neurons in the nucleus intramedialis (nI) might play a role in the control of VIP secretion and subsequent PRL release in the tropical non-seasonally breeding avian species (Sartsoongnoen et al., 2008). Moreover, it has been demonstrated that changes in the numbers of VIP-ir neurons in the IH-IN are associated with DAergic neurons within the nI and nucleus mamillaris (ML) areas, resulting in PRL release to induce and maintain incubation behavior in the native Thai hens. It is further suggested that nesting activity stimulates PRL secretion through activation of the DAergic system, which in turn stimulates the VIPergic system. The elevated PRL levels increase nesting activity and maintain incubation behavior (Prakobsaeng et al., 2011). Recently, it has been reported that the numbers of VIP-ir neurons in the IN-IH 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. These results indicate that the VIP/PRL system plays an important role in neuroendocrine reorganization to establish the rearing behavior in this non-seasonal breeding, equatorial precocial species. The VIP/PRL system is not only a key well established regulator of 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 number of VIP-ir neurons in the IH-IN and in turn VIPergic activity and the decrease in PRL levels are related to their contributions to rearing behavior of the native Thai chickens.

2.3 Prolactin: Structure, Functions, and Regulation of Secretion

2.3.1 The Structure of Prolactin

PRL, a polypeptide hormone, was discovered (Riddle et al., 1932), and its name is based on the findings that an extract of bovine pituitary gland causes the 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). It is synthesized in and secreted from the lactotrophs of the anterior pituitary gland (Bern and Nicoll, 1968; Velkeniers et al., 1988; Freeman et al., 2000). The molecular weight (MW) of the major form of PRL found in the pituitary gland is about 23 kilodaltons (kDa). It is encoded by a gene consisting of 5 exons and 4 introns (Cooke et al., 1981; Truong et al., 1984). Variant forms of PRL have been characterized in several mammalian species, and its variants can be the results of alternative splicing of the primary transcript, proteolytic cleavage, phosphorylation, glycosylation, and other posttranslational modifications, and thereby altering its physiological functions (Sinha, 1995). PRL is synthesized as a preprohormone (227 amino acids) in most mammals (Miller and Eberhardt, 1983). The mature hormone consists of 194-199 amino acids, depending on species. Hormone structure is stabilized by 3 intramolecular disulfide bonds. The primary structure of PRL is first reported in the ovine (Li et al., 1970). The complete amino acid sequences of PRLs of more than 25 species have been identified (Sinha, 1995). A comparison of the amino acid sequence from different species illustrates 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 shown in Figures 2.1 and 2.2, respectively.

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). 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, which regulate its gene expression may be widely conserved (Kansaku et al., 2005; Hiyama et al., 2009b). However, it has been reported that PRL is also synthesized by a number of extra-pituitary cells/tissues in both mammals (Ben-Jonathan et al., 1996; Freeman et al., 2000; Soares, 2004) and birds (Berghmam et al., 1992; Ramesh et al., 2000; Chaiseha et al., 2012), but its physiological function(s) in these extra-pituitary cells/tissues is far from understood and needed to be further investigated.

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

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

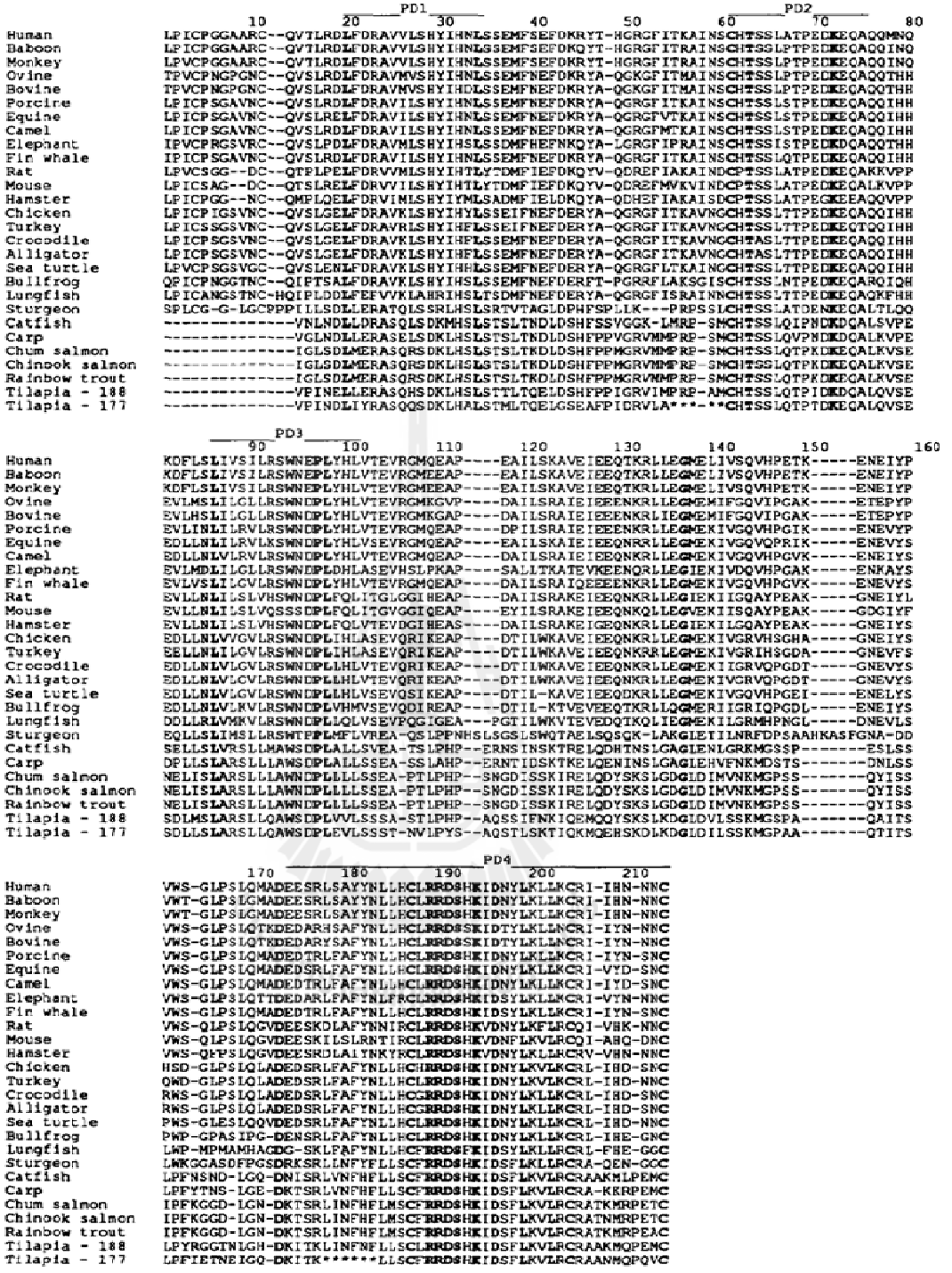

Figure 2.2 Primary structures of PRLs of different species. (-) indicates the positions left blank to optimize alignment of amino acid sequences. (*) indicates the absence of residues from a genetic variant of tilapia PRL. PD is PRL domain. PD1-PD4 indicates the four highly conserved domains of the PRLs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

PRL is synthesized and secreted by variety of cells, tissues, and organs including the immune cells, mammary epithelium, placenta, deciduas of the pregnant uterus, and brain (Ben-Jonathan et al., 1996). Moreover, PRL synthesis is found in the lacrimal gland, adrenal gland, corpus luteum, prostate gland, testis, and pancreas (Ben-Jonathan et al., 1996; Freeman et al., 2000). Up to date, more than 300 different physiological functions of PRL have been documented (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, the nurturing of the young in different vertebrate species, highlighting the significant role of this omnipotent hormone. It has been further suggested that the physiological functions and biological activities of PRL are, at least in part, regulated by additionally 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 that includes the receptors of GH, leptin, erythropoietin, and interleukins (Bazan, 1989; 1990; Kelly et al., 1991). PL and primate GH also bind the PRLR. Despite their low sequence homology (30 %), PRL and GH receptors share some structural and functional features (Goffin and Kelly, 1996). The PRLR is activated by the binding of a single ligand to the receptor to dimerize two identical receptors, resulting to activation of the Janus kinase (Jak2)-kinase 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 termed 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). Moreover, activation of the nucleotide exchange protein, Vav, has been reported (Clevenger et al., 1995).

Various PRLR isoforms have been identified in different cells/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, and are named as 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 the ovary, the best characterized sites of PRL physiological actions in mammals (Nagano and Kelly, 1994). cDNAs encoding the PRLR gene have been cloned in the 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 the rats (Nagano and Kelly, 1994; Bakowska and Morrell, 1997), turkeys (Zhou et al., 1996; Pitts et al., 2000), and chickens (Ohkubo et al., 1998).

The PRLR is also found in the CNS and a vareity of cells, tissues, and organs including the pituitary gland, heart, lung, thymus, spleen, liver, pancreas, kidney, adrenal gland, uterus, skeletal muscle, prostate gland, epithelial cells, bone, and skin in mammals (Nagano and Kelly, 1994; Nevalainen et al., 1997; Bole-Feysot et al.,

1998; Clement-Lacroix et al., 1999). PRLR mRNA expression is found in the CNS, choroid plexus, bed nucleus of the stria terminalis (BNST) , amygdala, central gray of the midbrain, thalamus, hypothalamus, cerebral cortex, and olfactory bulb in the rats. The PRLR is extensively expressed by the immune cells, and some types of lymphocytes synthesize and secrete PRL, suggesting that PRL may act as an autocrine or paracrine modulator of the immune system (Freemark et al., 1995; 1996). In avain species, PRLR is found in the crop sac, brood patch, thyroid gland, liver, kidney, leg, skin, large and small intestines, adipose tissue, adrenal gland, thymus, lymphoid tissue, spleen, heart, brain, pineal gland, ovary, testis, seminal duct, and oviduct (Tanaka et al., 1992; Chen and Horseman, 1994; Zhou et al., 1996; Ohkubo et al., 1998; Pitts et al., 2000; Kang et al., 2007; Wang et al., 2009; Xing et al., 2011).

2.3.2 The Physiological Functions of Prolactin in Birds

It has been well established that PRL is associated with the avian reproductive cycle (canvasback ducks, cockatiels, emperor penguins, geese, king penguins, mallards, native Thai chickens, tropical seabirds, and turkeys: Mashaly et al., 1976; Bluhm et al., 1983a; 1983b; El Halawani et al., 1984a; 1997; Myers et al., 1989; Wong et al., 1992b; Mauget et al., 1994; Lormee et al., 1999; Lormee et al., 2000; Boos et al., 2007; Huang et al., 2008; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). During reproductively quiescent stages (non-egg laying and rearing stages) of the turkeys (El Halawani et al., 1984b; 1997) and the native Thai chickens (Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008), plasma PRL levels are very low. During the laying and incubating periods, plasma PRL levels increase sharply (El Halawani et al., 1984b; Kosonsiriluk et al., 2008). It is confirmed that this rising PRL

level causes the cessation of ovulation, ovarian regression, and induction and maintenance of incubation behavior. Changes in PRL gene expression are also highly correlated with the reproductive cycle (Knapp et al., 1988; El Halawani et al., 1990a; Talbot et al., 1991; Wong et al., 1991; You et al., 1995b; Tong et al., 1997). The onset of incubation behavior is associated with decreasing plasma levels of LH and ovarian steroids and increasing plasma PRL levels (Cogger et al., 1979; Burke and Dennison, 1980; Lea et al., 1981; Rozenboim et al., 1993a). PRL has been well implicated as a causative factor for the reduced circulating gonadotropins and ovarian regression, when birds make the transition from egg laying to incubation period in bantam hens, canaries, chickens, cowbirds, ducks, mallard ducks, native Thai chickens, pheasants, pigeons, ring doves, spotted sandpipers, turkeys, white-crowned sparrows, and wild starlings (Riddle et al., 1935; Breitenbach and Meyer, 1959; Hohn, 1959; Sharp et al., 1977; 1988; Burke and Dennison, 1980; Goldsmith and Hall, 1980; Goldsmith and Williams, 1980; Goldsmith et al., 1981; 1984; Dawson and Goldsmith, 1982; Bluhm et al., 1983a; El Halawani et al., 1984a; 1988a; 1997; Oring et al., 1986; Hiatt et al., 1987; Youngren et al., 1991; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). PRL circulating levels increase gradually at the onset of incubation behavior and are maintained at high levels during incubation period (Saeki and Tanabe, 1955; Proudman and Opel, 1988), and then decrease to the same levels of reproductively quiescent stages, when incubation behavior is terminated (El Halawani et al., 1980; Wentworth et al., 1983).

PRL is involved in many aspects of reproductive physiology and behaviors. It is thought to play an important role in parental behaviors by mediating increases in incubation, crop milk secretion, feeding of young, and nest defense (Silver, 1984;

Janik and Buntin, 1985; Lea et al., 1986; Buntin et al., 1991). Active immunoneutralization against PRL reduces the incidence, delays the development, or prevents the occurrence of incubation behavior (March et al., 1994), whereas administration of exogenous PRL results to increase parental behaviors (Lea and Vowles, 1986; Macnamee et al., 1986; Pedersen, 1989; Buntin et al., 1991; Youngren et al., 1991).

There are plenty evidences suggesting that PRL plays a role in terminating egg laying, thus it regulates the clutch size in avian species that lay more than 2 eggs/clutch. The cessation of egg laying is associated with an increase plasma PRL levels (Etches et al., 1979; Burke and Dennison, 1980; Lea et al., 1981; Bluhm et al., 1983a; Hall and Goldsmith, 1983; Silverin and Goldsmith, 1983), and numerous studies have been suggested that the rise in plasma PRL levels during incubating period may suppress LH secretion (Zadworny and Etches, 1987; Porter et al., 1991; El Halawani et al., 1993; Sharp et al., 1998). Administration of exogenous PRL suppresses concentrations of FSH and LH in the turkey hens (El Halawani et al., 1991) and domestic fowls (Sharp et al., 1988). It has been suggested that PRL acts centrally to reduce circulating levels of LH by reducing hypothalamic GnRH levels (Rozenboim et al., 1993b). During the incubating period, suppression of FSH and LH secretion involves in a mechanism independent of increased PRL secretion (Sharp et al., 1988; 1989; Lea and Sharp, 1989; Lea et al., 1996). Moreover, PRL may also directly inhibit ovarian steroidogenesis (Rozenboim et al., 1993b), resulting to involution of the ovary with reduced ovarian steroidogenesis and regression of the oviduct (Porter et al., 1991). Additionally, the reproductive activities during long day photoperiod-stimulated PRL secretion are associated with increased pituitary PRL

gene expression, which in turn suppress LH secretion, inhibit follicular development, terminate laying, and induced molting (Huang et al., 2008).

2.3.3 The Regulation of Prolactin Secretion

It has been well established that PRL secretion is regulated by both stimulatory and inhibitory hypothalamic factors, but it is mainly under tonic inhibitory control in mammals (MacLeod and Login, 1976; Neill, 1988; Ben-Jonathan et al., 1989; Lamberts and MacLeod, 1990), and the predominant mammalian PRL-inhibiting factor (PIF) is DA, which is released from a dense neuronal network within the MBH, namely the tuberoinfundibular dopaminergic (TIDA) neurons, and serves as the PIF of PRL secretion (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001). DA acts directly on D_2 DA receptors located on the pituitary lactotrophs (Caron et al., 1978; Civelli et al., 1991), and removal of this DAergic inhibition leads to an increase PRL secretion and hyperprolactinemia (Nicoll and Swearingen, 1970; Nicoll, 1977). DA and its agonists inhibit PRL release and its gene expression and proliferation of the lactotrophs (Birge et al., 1970; Pawlikowski et al., 1978; Maurer, 1981), indicating that the regulation of PRL secretion and its gene expression are under inhibitory control of the TIDA neurons (Pasqualini et al., 1988; Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001). A brief fall of DA levels occurring immediately after physiological stimuli such as suckling (Chiocchio et al., 1979; Selmanoff and Wise, 1981; Demarest et al., 1983) is necessary for the secretion of PRL. Supporting by *in vitro* studies that PRL release in anterior pituitary cells is stimulated after short term exposure of DA (Fagin and Neil1, 1981; Denef et al., 1984). PRL surges occurring during proestrous, pregnancy, lactation, and stress are all associated with the suppression of TIDA neuronal activity and loss of DA inhibition

of PRL secretion (Freeman et al., 2000; Ben-Jonatha and Hnasko, 2001). There are numerous studies confirming the physiological roles of DA as the PIF. However, it has been reported that a lower concentration of DA than those required for inhibition of PRL secretion can stimulate PRL secretion *in vitro* (Shin, 1978; Burris et al., 1992; Porter et al., 1994) and *in vivo* (Arey et al., 1993), suggesting that all lactotrophs have the potential to respond to both inhibitory and stimulatory effects of DA (Kineman et al., 1994). The two opposite effects of DA upon PRL secretion may be mediated via distinct guanine nucleotide binding proteins (G proteins), depending on its specific receptor subtypes (Burris et al., 1992; Niimi et al., 1993; Lew et al., 1994).

VIP has been shown to be involved in the regulation of PRL secretion for many decades in mammals (Kato et al., 1978; Rotsztejn et al., 1980; Reichlin, 1988) via a neuroendocrine pathway because of the presence of VIP in the hypothalamic nerve endings, the anterior pituitary gland (Besson et al., 1979), and the hypophysial portal system (Said and Porter, 1979). VIP stimulates PRL release both *in vitro* (Shaar et al., 1979; Enjalbert et al., 1980; Matsushita et al., 1983) and *in vivo* (Kato et al., 1978; Frawley and Neill, 1981). The contents of PRL mRNA and its protein appear to be regulated by VIP (Ben-Jonathan et al., 1989; Maas et al., 1991). VIP fibers are found intermingle with DAergic neurons in the arcuate nucleus (ARC) and periventricular nucleus. This study also demostrates that VIP2 receptors are located on a soma and proximal dendrites of these DA-containing neurons. These results, taken together, suggest that VIP may regulate PRL secretion in mammals by controlling DA delivery to the anterior pituitary gland (Gerhold et al., 2001). DA and 5-HT appear to have a complementary interaction regarding PRL secretion. DA and 5-HT are co-localized within neurons in the hypothlalamus of baboon (Thind et al.,

1987), and synaptic junctions between 5-HTergic nerve endings and TH-containing neurons have been identified in the rat hypothalamus (Kiss and Halasz, 1986). In addition, it has been reported that DA antagonist inhibits an increase in 5-HTergic activity (King et al., 1985), and intraventricular injections of 5-HT reduce DA levels in the hypophysial portal blood in rats (Pilotte and Porter, 1981). As aformentioned, VIP is proposed as a mammalian PRF.

Thyrotropin-releasing hormone (TRH) acts as a hypothalamic PRF in mammals as well. TRH stimulates PRL secretion *in vitro* (Maas et al., 1991) and *in vivo* (Grosvenor and Mena, 1980; Lafuente et al., 1994) and also its gene expression (Laverriere et al., 1988). In addition, the secretion of PRL induced by TRH occurs during a transient depression in DAergic activity (Plotsky and Neill, 1982; Martinez de la Escalera et al., 1988). However, there are several contradictive results that lead to question of its role as the PRF.

Up to date, various PRFs and PIFs have been observed in both birds and mammals such as 5-HT (Chaiseha and El Halawani, 2005; Chaiseha et al., 2010), angiotensin II (Opel and Proudman, 1988; Steele, 1990), oxytocin/vasopressin (Hyde and Ben-Jonathan, 1989), peptide histidine isoleucine (Werner et al., 1983; Chaiseha and El Halawani, 1999; Kulick et al., 2005), and pituitary adenylate cyclase activating polypeptide (Miyata et al., 1989; You et al., 2000).

The regulation of avain PRL also involves the interaction of external stimuli with neuroendocrine mechanisms. These critical environmental stimuli include sensory information (photoperiod, ambient temperature, etc.), 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, and their

relative importances vary with stages of the reproductive cycle (Curlewis, 1992). In the incubating hens, tactile stimuli from the nests and eggs maintain the elevated circulating PRL levels and up-regulate VIP expression (Silver et al., 1988; Buntin et al., 1991; Massaro et al., 2007).

As aformentioned, the regulation of mammalian PRL secretion and its gene expression are under the inhibitory control of the TIDA neurons (Ben-Jonathan and Hnasko, 2001). However, this is not the case in birds, because removal of these hypothalamic inputs results in the complete cessation of PRL secretion (Tixier-Vidal et al., 1966; Hall et al., 1986). Indeed, it has long been well established that the secretion of PRL in birds involves in a tonic stimulatory control by the hypothalamus rather than the inhibitory DAergic system that found in mammals (Kragt and Meites, 1965; Bern and Nicoll, 1968; El Halawani et al., 1984a; Hall et al., 1986). It has been further established that the regulation of avian PRL secretion and its gene expression is influenced by hypothalamic VIP, the avian PRF (El Halawani et al., 1997; 2001; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999; 2005; Kosonsiriluk et al., 2008, Prakobsaeng et al., 2011; Chaiyachet et al., 2012). In the past six decades, several studies confirm the pivotal role of VIP as the only avian PRF. For example, immunoneutralization againt VIP prevents an increase in circulating PRL levels, prevents the induction of incubation behavior, up-regulates content of LH-β- and FSH-β-subunit mRNAs, and extends the duration of egg laying period, but it does not prevent spontaneous gonadal regression and molting (Sharp et al., 1989; El Halawani et al., 1995; 1996; Dawson and Sharp, 1998; Ahn et al., 2001). Changes in VIP immunoreactivity, VIP peptide contents in the INF and ME, and VIP mRNA steady-state levels in the INF are associated with changes in plasma PRL levels throughout

the reproductive cycle in birds (Mauro et al., 1989; Chaiseha et al., 1998; Chaiseha and El Halawani, 1999; Kosonsiriluk et al., 2008).

Unlike mammals, DAergic influences are involved in both stimulating and inhibiting PRL secretion depending on multiple DA receptor subtypes in birds (Youngren et al., 1995; 1996b; Chaiseha et al., 1997; 2003a; Al Kahtane et al., 2003). In the turkeys, stimulatory D_1 DA receptor mRNA expression has been found to increase in the hypothalamus of hyperprolactinemic incubating hens and in the pituitary gland of laying hens, and inhibitory D_2 DA receptor mRNA expression increases in the pituitary gland of hypoprolactinemic photorefractory hens (Schnell et al., 1999a; 1999b; Chaiseha et al., 2003a). This stimulatory effect of DA on PRL secretion is regulated by the D_1 DA receptors residing in the INF, where the VIP neurons are located. In contrast, DA inhibits PRL synthesis and release by blocking the action of VIP via the D_2 DA receptors at the pituitary level (Youngren et al., 1996b; 1998; 2002; Chaiseha et al., 1997; 2003a; Al Kahtane et al., 2003). In the turkeys, changes in the DAergic activity during the reproductive cycle mirror the changes in plasma PRL levels, the number of VIP-ir neurons, VIP peptide contents, and its mRNA expression within the INF (El Halawani et al., 1980; 1984b; Mauro et al., 1989; Wong et al., 1991; Chaiseha et al., 2003a; 2003b). It has been suggested that the variations in PRL secretion observed across the reproductive cycle are, in part, regulated by changes in VIP receptors at the pituitary level (Chaiseha et al., 2004). It is well established that 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). In addition, there are evidences indicating that dynorphin, 5-HT, DA, and VIP all appear to stimulate avian PRL secretion along a common pathway expressing

κ opioid, 5-HTergic, DAergic, and VIPergic receptors at synapses arranged serially in that functional order with the VIPergic system as the final mediator (El Halawani et al., 2001).

2.4 Dopamine: Structure, Functions, and Regulation of Secretion

2.4.1 The Structure of Dopamine

After the discovery, DA is found in both central and peripheral nervous systems of many vertebrate and invertebrate species (Carlsson and Hillarp, 1956; Benes, 2001). Chemical name of DA is 4-(2-aminoethyl) benzene-1,2-diol and the formula is $C_6H_3(OH)_2$-CH_2-CH_2-NH_2. DA is a neurotransmitter/neuromodulator belonging to a group of catecholamines (CA) and functions as a classical neurotransmitter in the brain. Thus, DA communicates between neurons and acts within the anatomically confined neuronal networks of the synapses. Several significant physiological functions have been reported involving in a wide variety of behaviors and reproduction. DA is a precursor of norepinephrine (NE) and epinephrine (E) in the biosynthetic pathway. CA and indolamines such as 5-HT are referred to as monoamine, a water soluble molecule that is decarboxylated derivatives of amino acids. CA has distinctive structures, which are the single amine group, a nucleus of catechol; a benzene ring with two adjacent hydroxyl groups, and a side chain of ethylamine or one of its derivatives (Wood-Gush and Gilbert, 1973).

The precursor for DA synthesis is tyrosine, which the majority of this circulating amino acid is from diets, and small amounts are derived from hydroxylation of phenylalanine by phenylalanine hydroxylase at the liver (Missale et al., 1998). Tyrosine is up taken by the neurons, and then converted to DA by two

enzymes, which are tyrosine hydroxylase (TH) and 1-aromatic amino acid decarboxylase (AADC). These enzymes are named dihydroxyphenylalanine decarboxylase. TH is the rate-limiting enzyme in this biosynthetic pathway. TH converts tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA), and L-DOPA is then catalyzed to DA by AADC. DA is then processed to NE by DA beta-hydroxylase (DBH) in some neurons, and these neurons also contain phenylethanolamine N-methyl transferase (PNMT) that converts NE to E. The CA biosynthetic pathway is shown in Figure 2.3. TH is the most critical enzyme that regulates DA synthesis. TH gene in humans 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 (Kumer and Vrana, 1996). Each monomer is consisted of an inhibitory regulatory domain at the N terminus and a catalytic domain at the C terminus. The catalytic domain contains protein binding region and a putative leucine zipper at the C terminus that involves in intersubunit binding.

DA exerts its physiological actions by binding to its specific receptors, which belongs to the G protein-coupled receptors (GPCR) family. Five distinct subtypes of DA receptors (D_1-D_5) are found prominently in the CNS of the vertebrate species (Contreras et al., 2002). They have been isolated, characterized, and classified into 2 families based upon the basis of their stimulatory or inhibitory activities on adenylate cyclase (Kebabian and Calne, 1979). The D_1-like DA subfamily comprises of D_1 and D_5 DA receptors and named the D_{1A} and D_{1B} DA receptors by some researchers (Monsma et al., 1990; Sibley, 1991). The D_2-like DA subfamily includes D_2, D_3, and

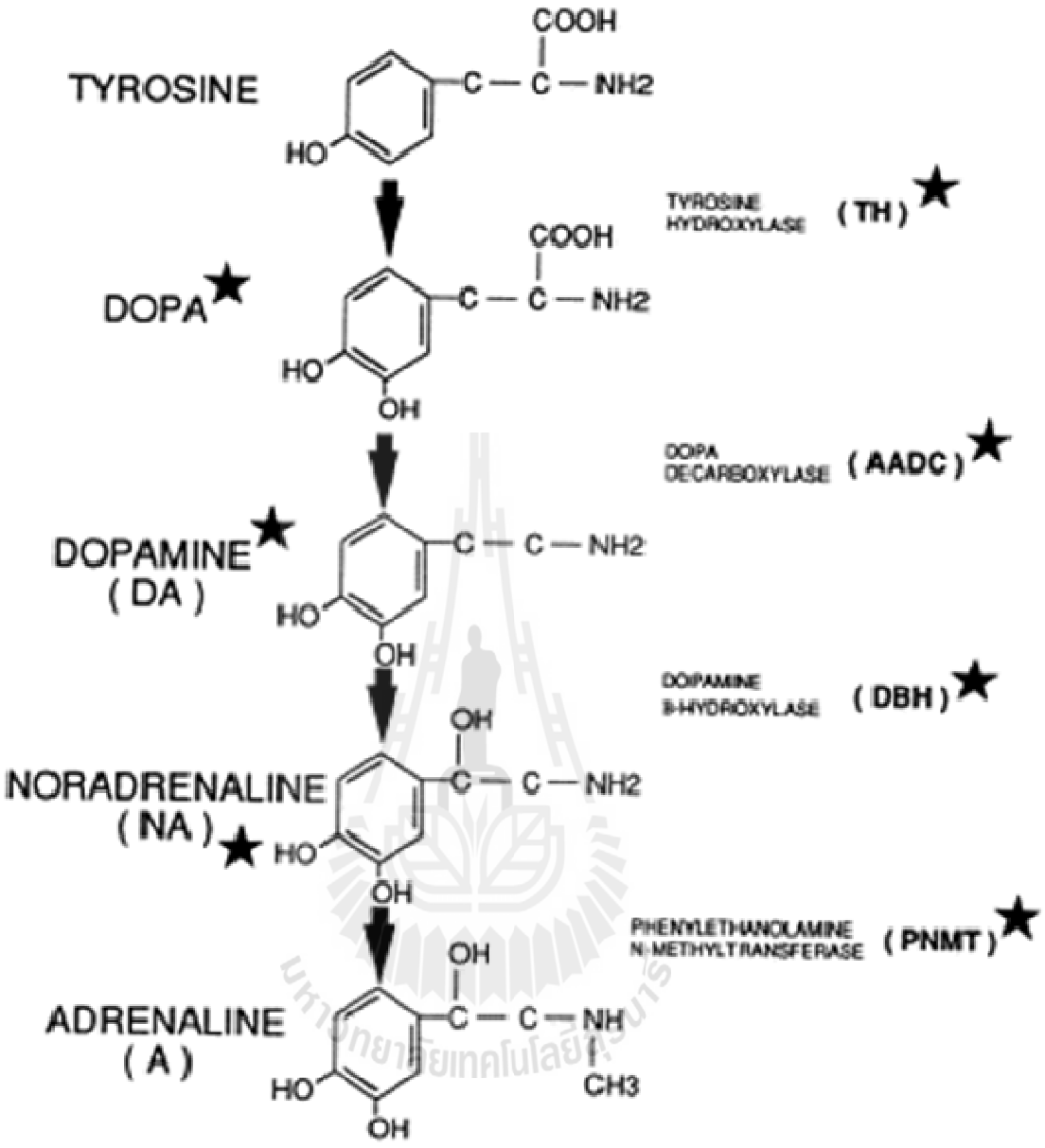

Figure 2.3 Catecholamines biosynthetic pathway and available antibodies as indicated by asterisks (Smeets and Gonzalez, 2000).

D_4 DA receptors. cDNAs characterization of these receptor subtypes reveals 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 conserved in the three receptor subtypes (Missale et al., 1998). 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 (Bates et al., 1990; Civelli et al., 1991; Sibley and Monsma, 1992; Jarvie and Caron, 1993; Jaber et al., 1996; Strange, 1996). Activation of the D_1-like DA receptors increases adenylate cyclase activity via the $G_{s\alpha}$ subunit. Activation of the D_2-like DA receptors inhibits adenylate cyclase activity via the $G_{i\alpha}$ subunit. However, the G_o and G_q proteins associated with ion channels and phosphoinositide cascade are involved as well (Stoof and Kebabian, 1984; Sidhu and Niznik, 2000).

In mammals, the distributions of DA receptor subtypes have been well investigated. 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 the brain, the D_1 and D_2 DA receptors are the most widespread and expressed at the highest levels (Dearry et al., 1990; Fremeau et al., 1991; Missale et al., 1998; Vallone et al., 2000). The D_1 DA receptor is mainly expressed in the caudate putamen, nucleus accumbens (Ac), olfactory tubercle, cerebral cortex, and amygdala (Mansour et al., 1990; Jackson and Westlind-Danielsson, 1994). The D_2 DA receptors mRNA is highly expressed in the substantia nigra (SN), ventral tegmental area (VTA), hippocampus, and in anterior and intermediate lobes of the pituitary gland. However, the amygdale contains low levels of D_2 DA receptor (Mansour et al., 1990; Bouthenet et al., 1991; Weiner et al., 1991). The D_3 DA receptor has been found in the SN and VTA, but it is expressed in a minority when compared with the D_2 DA receptor (Diaz et al., 1994; 1995). The D_4 DA receptor is highly expressed in the frontal cortex, amygdale, hippocampus, hypothalamus, and mesencephalon (Van Tol et al., 1991; O'Malley et al., 1992). The D_5 DA receptor is poorly expressed and restricted to the hippocampus, lateral mamillary nucleus, and

parafascicular nucleus of the thalamus, whereas the D_1 DA receptor is not significantly expressed (Meador-Woodruff et al., 1989; Tiberi et al., 1991). The low expression of D_1 and D_4 DA receptors in the kidney and D_5 DA receptor in the heart have been reported (Chio et al., 1994).

In birds, there are three D_1 DA receptor subtypes (D_{1A}, D_{1B}, D_{1D}) have been characterized and cloned in the chickens (Demchyshyn et al., 1995). Cloning of cDNAs encoding the D_1 and D_2 DA receptors has been reported in the 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), quails (Ball et al., 1995), chickens (Schnabel et al., 1997; Sun and Reiner, 2000), 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), quails (Levens et al., 2000), and turkeys (Schnell et al., 1999a; 1999b; Chaiseha et al., 2003a). The distributions of D_2 DA receptor mRNA has been found widespread throughout the brain, pineal gland, cortex, cerebellum, and also in the pituitary gland of the turkeys. The presences of hypothalamic D_1 DA and pituitary D_2 DA receptor mRNAs are found to increase in correlating with the reproductive stages (Chaiseha et al., 2003a).

2.4.2 The Dopaminergic System in the Mammalian Brain

DA is primarily synthesized in the CNS, but limited synthesis occurs in the adrenal medulla and non-neuronal tissues such as pancreas and anterior pituitary gland (Ben-Jonathan and Hnasko, 2001). The mammalian brain consists of several

anatomically distinct DA neuronal systems that differ in their neurochemical characteristics and physiological functions. The distribution of CA-containing cells is first described in the brain of rats. Well documentedly, the CA neurons are organized into 12 groups, namely A1 to A12 from caudal to rostral of the brain (Dahlstrom and Fuxe, 1964). These neurons are located mainly in the arcuate and the anterior periventricular nuclei of the hypothalamus. IHC studies using antibodies against the biosynthetic enzymes including TH, DBH, and PNMT to identify the CA neuronal groups have been reported (Hokfelt et al., 1984a; 1984b). In the rats, regarding to the name of CA cell groups in the CNS (Dahlstrom and Fuxe, 1964), there are 17 DAergic/NEergic (A1-A17) and 3 adrenergic (C1-C3) cell groups. Two distinct CA cell groups are found in the caudal rhombencephalon; a ventrolateral tegmental (A1, C1) and a dorsomedial group (A2, C2) in the nucleus tractus solitarri/area postrema complex. The A3 cell group is found within the dorsal accessory inferior olive. The C3 adrenergic group is found lying along the midline within and dorsal to the medial longitudinal fascicle. NEergic cells are classified into 4 groups in the pons (A4, A5, A6, A7). The A6 (locus coeruleus) is the most prominent one among these cell groups. The CA cells in the midbrain are classified into 3 groups, A8 (retrorubral), A9 (SN), and A10 (VTA) based on their localizations. At least 5 distinct CA cell groups (A11-A15) are recognized in the diencephalon, and the numbers of DA-containing neurons are comparable to those in the SN and VTA, which are generally considered to be the major loci of DA neurons in the brain (Lookingland and Moore, 2005). The A11 (caudal diencephalic group) is found in the periventricular gray matter of the thalamus, hypothalamus, and rostral midbrain. These A11 neurons project their axons toward the spinal cord (Skagerberg and Lindvall, 1985),

suggesting a role of these neurons in sensory and nociceptive processing and sensorimotor integration (van Dijken et al., 1996; Levant and McCarson, 2001). The A12 (TIDA) neurons are recognized throughout the ARC and in the adjacent part of the periventricular nucleus of the MBH. Sexual difference in the numbers of TH-ir neurons in the dorsomental and ventrolateral subdivition of the ARC has been reported (Cheung et al., 1997), implicating the role of these neurons in the regulation of pituitary hormone synthesis and secretion (Moore, 1987). It is very well established that the regulation of PRL synthesis and secretion is under the inhibitory control of TIDA neurons (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001). These TIDA neurons release DA and then DA acts directly upon the D_2 DA receptors located on pituitary lactotrophs (Civelli et al., 1991). The A13 incertohypothalamic DA neurons are found clustery in the rostral portion of the medial zona incerta, whereas the A14 DA neurons are located in the periventricular nucleus. The A15 is divided into 2 groups; A15d, a compact dorsal group locates in the ventral portion of the BNST, and more caudally, ventral to the anterior commissure, and A15v, the ventrolateral neurons find above the optic chiasm and within the supraoptic nucleus (SON). These neurons are abundance in the ventrolateral hypothalamus of seasonal breeding species and believed to mediate steroid hormones suppression of gonadotropins secretion during anestrus in ewes (Tillet and Thibault, 1989; Gayrard et al., 1994; Lehman et al., 1996). The most rostral DA cell bodies in the brain are found in the A16 (olfactory bulb) and A17 (retina).

2.4.3 The Dopaminergic System in the Avian Brain

The anatomical distribution of the avian DAergic system obviously resembles to those of mammals (Moons et al., 1994; Reiner et al., 1994). DA has been determined and visualized in several species including the 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). DA neurons are found throughout the hypothalamus (Kiss and Peczely, 1987; Reiner et al., 1994; Al-Zailaie and El Halawani, 2000) and have been shown to be immunoreactived for VIP and its mRNA (Mauro et al., 1989; 1992; Hof et al., 1991; Kuenzel et al., 1997; Chaiseha and El Halawani, 1999). The localizations of DA-ir neurons in the chicken hypothalamus and hindbrain have also been reported (Smeets and Gonzalez, 1990; Kuenzel et al., 1992). Several DA neuronal groups have been found in the preoptic hypothalamic areas of the turkeys (Al-Zailaie and El Halawani, 2000; Al-Zailaie, 2003) including the nucleus preopticus medialis (POM), nucleus anterior medialis hypothalami (AM), suprachiasmatic nucleus (SCN), nucleus ventrolateralis thalami (VLT), nucleus paraventricularis magnocellularis (PVN), regio lateralis hypothalami (LHy), nucleus ventromedialis hypothalami (VMN), nucleus dorsomedialis hypothalami (DMN), nucleus mamillaris medialis (MM), and PMM. 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). Furthermore, TH-ir neurons are predominantly located within the diencephalon and mesencephalon. Changes in the number of TH-ir neurons are observed in the nI across the reproductive stages of the native Thai chickens (Sartsoongnoen et al., 2008). The presence of DAergic fibers in the ME has been reported in the Japanese quails (Bailhache and Balthazart, 1993), chickens (Moons et al., 1994), and turkeys (Al-Zailaie, 2003). Given their widespread distributions as mentioned above, the findings that DA perikariya and terminals 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), and it is reasonable to consider whether any regional specificity exists in those DA neurons that are neuroendocrine in nature, i.e., controlling the release and expression of VIP/PRL and GnRH/FSH-LH systems. Recent findings demonstrate the existence of DA-MEL neurons in the PMM, where DA and MEL are synthesized and co-localized. These findings suggest that the pattern of 5-HT/CA neuronal distributions and their variable interaction with PMM DA-MEL neurons during different reproductive stages may offer a significant neuroanatomical basis for understanding the control of avian reproductive seasonality. It also may constitute a critical cellular process involved in the generation and expression of seasonal reproductive rhythms and suggests a previously undescribed mechanism(s) by which light signals gain access to neural targets 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).

2.4.4 The Physiological Functions of Dopamine in Birds

DA participates in several physiological functions in mammals; for example food and water intake, body homeostasis, behaviors and cognition, motor activity, regulation of milk secretion, sleep, mood, attention, learning, and reproduction (Bertolucci-D'Angio et al., 1990; Cooper and Al-Naser, 1993; Wilson et al., 1995; Velasco and Luchsinger, 1998; Ben-Jonathan and Hnasko, 2001; Hull et al., 2004; Wellman, 2005). Unlike in mammals, the role of DA in the regulation of avian PRL secretion is still large obscure for comparing it to the mammalian DAergic strategy for PRL regulation. It has been well established that DA influences are involved in both stimulating and inhibiting PRL secretion in birds. DA inhibits pituitary PRL release *in vitro* (Harvey et al., 1982; Hall and Chadwick, 1984; Hall et al., 1986; Xu et al., 1996). DA or its agonist, apomorphine, reduces PRL secretion in the pigeons and chickens, and this effect is reversed by the DA antagonist, pimozide (Hall and Chadwick, 1983). DA inhibits the release of PRL-stimulated by TRH, hypothalamic extract, or by previous exposure of the pituitary gland to estrogen in the chickens (Hall and Chadwick, 1984). 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 depended 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). These findings suggest that the stimulatory effect of DA on PRL synthesis and release is regulated via the D_1 DA receptors residing in the INF, where the VIP neurons are located. DA inhibits PRL

synthesis and release 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 been reported that DA also activates hypothalamic VIP gene expression in the INF (Bhatt et al., 2003). Additionally, it has been suggested that the signalling mechanism(s) underlying the interaction between VIP and DA in the regulation of PRL secretion involved 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).

There are some evidences suggesting an inhibitory role of DA on GnRH synthesis and release in both mammals and birds (Ramirez et al., 1984; Sharp et al., 1984). Several DA neuronal groups have been observed in the preoptic hypothalamic areas (Kiss and Peczely, 1987; Reiner et al., 1994). Exogenous DA activates hypothalamic VIP gene expression, and this increased expression is exclusively limited to 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). Furthermore, there is evidence suggesting the involvement of DA in correlating with GnRH is derived from a dense concentration of TH and GnRH-containing processes, which located in the lateral and mediobasal portion of the external layer of the ME (Contijoch et al., 1992). This finding provides an opportunity for synaptic interaction between GnRH and DA. DA inhibits GnRH release via presynaptic inputs at the ME in the chickens (Contijoch et al., 1992; Fraley and Kuenzel, 1993). Activation of the DA neurons in the ML is associated with the activation of GnRH-I and VIP neurons and the subsequent release of LH and PRL (Al-Zailaie et al., 2006). The relationship of DAergic system in the PMM and

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 that proposed to be the DA A11 group is suggested its function in controlling the reproductive seasonality in the temperate zone birds. Recently, DA-MEL co-localized neurons have been found in the PMM and shown to cycle rhythmically with photoperiodic changes (Kang et al., 2007; 2010). DA-MEL neurons may constitute a critical cellular process involved in the generation and expression of seasonal reproductive rhythms (El Halawani et al., 2009) via tOPN4x, an important component of the photoreceptive system (Kang et al., 2010). In addition, it has been reported that clock gene in the PMM can be induced by long photoperiod and light during the daily photosensitive phase to promote reproductive activity (Leclerc et al., 2010).

It is well established that DA plays an intermediary role in PRL secretion in birds, requiring an intact VIPergic system in order to release PRL (Youngren et al., 1996b). Intracranial infusions of DA are ineffective in releasing PRL 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 have been reported (Chaiseha et al., 2003b). In addition, it has been found that D_2 DA receptor agonist, puinpirole, inhibits VIP-stimulated PRL secretion and 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 release of PRL release by activating D_2 DA receptors. To date, it is concluded that dynorphin, 5-HT, 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).

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). Administration of the D_1 DA agonist increases the sexual behavior in 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 in 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 also involves in motor functions and the regulation of food and water intake in birds (Rieke, 1980; 1981; Deviche, 1984; Ravazio and Paschoalini, 1992).

2.4.5 The Regulation of Dopamine Secretion

DA neurons are originally implicated in the regulation of pituitary hormone secretion based upon the results of early receptor binding and pharmacological studies showing 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., 1977; Gibbs and Neill, 1978; Ben-Jonathan et al., 1980). The studies have demonstrated the involvement of the pituitary-specific transcription factor (Pit-1, GHF-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). It is 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 mammals, DA is regulated by estrogen in the hypothalamus, striatum, Ac, and frontal cortex in mammals (Ben-Jonathan, 1985). Estrogen enhances PRL transporter and inhibits hypothalamic DA neurons (DeMaria et al., 2000). In female

rats, estrogen modulation of striatal DA transmission influences performance on procedural memorys (Daniel et al., 2006; Quinlan et al., 2008). Results from a variety of *in vitro* and *in vivo* experiments are consistent with the hypothesis that estrogen can protect DA neurons from death that induced by a variety of toxins (Disshon and Dluzen, 1997; Miller et al., 1998; Honda et al., 2000). Numerous studies in animal models also provide evidences that estrogen influences behaviors regulated by the striatonigral and mesolimbic DA systems (Becker, 1999; 2000; Diaz-Veliz et al., 1999). Analyses of TH suggest that estrogen may regulate CA synthesis, but this is not the primary mechanism of estrogen regulation of DA neurotransmission. In the striatum, estrogen is reported to sharply increase DA synthesis, possibly via the regulation TH phosphorylation (Pasqualini et al., 1995). Increases in TH activity in the Ac following estrogen treatment have also been reported (Hernandez et al., 1991). In deed, in many cases, estrogen facilitates DA release within minutes (Ohtsuka et al., 1989; Becker and Rudick, 1999).

It has been reported that estrogen modulates postsynaptic responses to DA. Chiodo and Caggiula (1983) suggest that estrogen modulates the response of DA neurons in the SN that mediated by somatodendritic DA autoreceptors. Administration of estrogen to ovariectomized rats reduces the number of SCN neurons excited by *in vitro* DA application (Hsieh and Pan, 1990). Estrogen pretreatment increases the firing rate of rat striatal neurons in response to iontophoretcially applied DA (Arnauld et al., 1981). The studies utilized iontophoretic application of DA and of receptor subtype-selective agonists and antagonists onto striatal neurons to determine whether D_1 or D_2 receptor activity is modulated by estrogen (Demotes-Mainard et al., 1990). Estrogen has a net facilitatory

effect on DA neurotransmission by attenuating D_2 receptor inhibition of neurotransmitter release and reuptake by the DA transporter. Recently, it is suggested that estrogen might also govern postsynaptic responses to released DA, enhancing excitatory (D_1 receptor-mediated), and reducing inhibitory (D_2 receptor-mediated) components of DA actions. Furthermore, estrogen facilitation of PRL release may also involve in the regulation of both hypothalamic DA neuronal activity and pituitary responsiveness to released DA (Etgen et al., 2011).

In contrast to mammals, the studies of the regulation of DA synthesis and secretion in avian species are limited. In the Japanese quails, it is suggested that 12 hours temporal interaction of L-5-hydroxytryptophan and L-DOPA administration maintains reproductive system in stimulated condition and prevents reproductive regression in photorefractory birds, but does not prevent the onset of scotosensitivity. It is further concluded that the 12 hours temporal relationship of circadian 5-HTergic and DAergic oscillations not only eliminates photorefractoriness, but may also reestablish photosensitivity in relative photorefractory quails. These results suggest the regulatory role of neuronal oscillations and their temporal interaction in the regulation of neuroendocrine-gonadal axis with special reference to photosensitivity or photorefractoriness (Chaturvedi et al., 2006). Moreover, transcription factors, Phox2 and dHAND, are directly interacted with transactivate the promoter of the gene encoding the NEergic biosynthetic enzyme, DBH, and involved in the biosynthesis, transport, and secretion of NE (Rychlik et al., 2005). To date, it is concluded that dynorphin, 5-HT, 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 Oxytocin/Mesotocin: Structure, Functions, and Regulation of Secretion

2.5.1 The Structure of Oxytocin/Mesotocin (Oxytocin-Like Peptide)

In all vertebrate species, neurohypophysial hormones are synthesized in specific neuronal groups within the hypothalamus and released from nerve terminals of the posterior pituitary gland or into the hypophysial portal blood via the ME. The best known neurohypophysial hormones are arginine vasopressin (AVP) and oxytocin (OT) that are found in most mammalian species. These neurohypophysial hormones and other related peptides are referred to as the vasopressin (VP)/OT family. However, there are at least 14 additional neurohypophysial hormones that found in non-mammalian vertebrates (Hoyle, 1998). Cyclostomes, the most primitive vertebrates, possess a single neurohypophysial hormone, arginine vasotocin (AVT), while other vertebrates possess two neurohypophysial hormones; a VP-like hormone and an OT-like hormone. In birds, the two neurohypophysial hormones are AVT and mesotocin (MT). In addition, several peptides related to the vertebrate neurohypophysial hormones have been found in invertebrates, suggesting that the AVT gene in cyclostomes are evolved from an invertebrate neurohypophysial hormone-like gene (Murphy et al., 1998).

OT is the first peptide hormone to have its structure determined and the first to be chemically synthesized in biologically active form (Du Vigneaud et al., 1953). It is named after the “quick birth”, which it causes due to its uterotonic activity (Dale, 1906). The structure of OT gene is elucidated (Ivell and Richter, 1984), and the sequence of OT receptor is reported (Kimura et al., 1992; Gimpl and Fahrenholz, 2001). OT is found to be the most abundant of 43 identified transcripts (Gautvik et al.,

1996). To date, OT exerts a wide spectrum of central and peripheral physiological effects. The physiological actions of OT range from the modulation of neuroendocrine reflexes to the establishment of complex social and bonding behaviors related to the reproduction and care of the offspring. Some OT fibers also project from the PVN to the limbic system including the amygdala, BNST, LS, brain stem, and spinal cord, indicating that OT participates in many physiological and behavioral effects (Weindl and Sofroniew, 1981; Raggenbass, 2001). OT plays an important role in several mammalian reproductive behaviors such as sexual behaviors, induction of uterine contraction, milk ejection, and maternal behaviors (Young et al, 1997; Insel et al., 2001; Young et al., 2001). Overall, the cyclic nanopeptide OT and its structurally related peptides facilitate the reproduction in all vertebrates at several aspects (Gimpl and Fahrenholz, 2001).

The distributions of MT neurons are found in the areas of SON, PVN, and tuberomammillary area in the chickens, domestic mallards, and Japanese quails. MT fibers are found at both internal and external layer of the ME (Goossens et al., 1977; Bons, 1980). MT is also observed in areas outside the hypothalamus such as in the cerebellum, LS, optic lobe, pons, and medulla oblongata (Robinzon et al., 1988). The sequence homologies, sites of expression, and signal transduction pathways suggest close functional relationships with known mammalian and sub-mammalian AVT and OT receptors (Baeyens and Cornett, 2006).

All neurohypophysial hormones are nanopeptides with a disulfide bridge between cysteine residues 1 and 6. This results in a peptide constituted of a six-amino acid cyclic part and a carboxy-terminal a-amidated three-residue tail. Based on the amino acid at position 8, these peptides are classified into VP/OT families; the VP

family contains a basic amino acid (lysine, arginine), and the OT family contains a neutral amino acid at this position (Figure 2.4). Isoleucine in position 3 is essential for stimulating OT receptors and lysine or arginine in position 8 for acting on VP receptors. The difference in the polarity of these amino acids is believed to enable the VP and OT peptides to interact with their specific receptors (Barberis et al., 1998). MT is the OT-like hormone that found in most terrestrial vertebrates from lung fish to marsupials, which includes all non-mammalian tetrapods (amphibians, reptiles, and birds). Only two South American marsupials express OT exclusively, whereas all other marsupials have MT. In the Northern brown bandicoots (*Isoodon macrourus*; Rouille et al., 1988) and the North American opossums (*Didelphis virginiana*; Chauvet et al., 1985), OT is found together with MT. Taken together, MT has the largest distribution in vertebrates after vasotocin (VT) is found 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. The earthworm, *Eisenia foetida*, is the most primitive species from which an OT-related peptide, annetocin, has been isolated (Oumi et al., 1994).

The OT receptor is a member of the rhodopsin-type (Class I), GPCR family. The seven transmembrane a-helices are the most highly conserved among the GPCR family members. Conserved amino acid residues among the GPCRs (outlined in black in Figure 2.5) may be involved in a common mechanism for activation and signal transduction to the G protein. It is assumed that the switching from the inactive to the active conformation is associated with a change in the relative orientation of transmembrane domains 3 and 6, which then unmasks G protein binding sites.

	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).

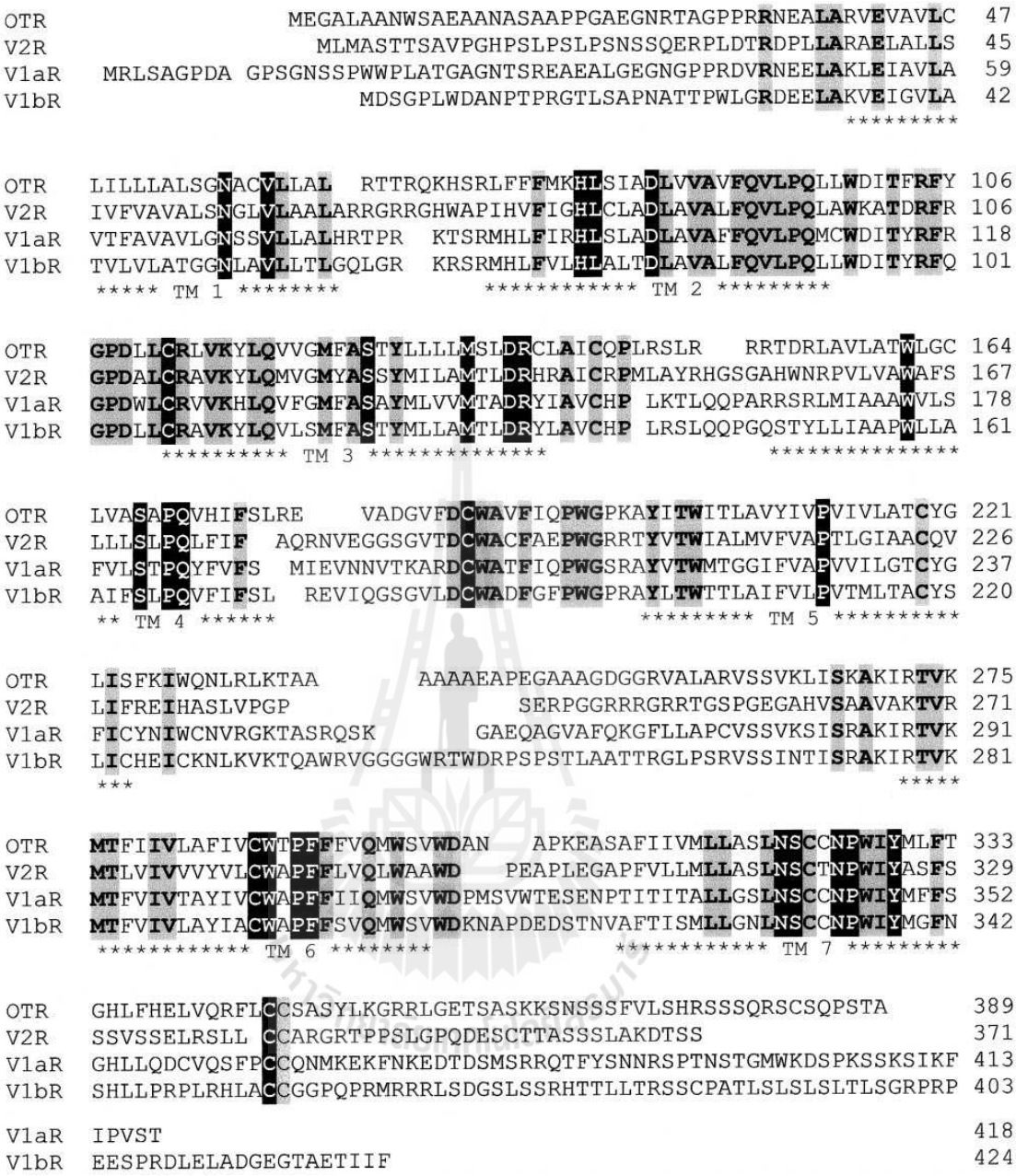

Figure 2.5 Primary sequence alignments of human OT receptor (OTR), human VP2 receptor (V2R), human VP1a receptor (V1aR), and human VP1b receptor (V1bR). The putative transmembrane helices 1-7 are underlined (asterisks). The residues conservative within the subfamily (~25% of the whole sequence) are outlined in gray, while those conservative for the whole GPCR superfamily are outlined in black (Gimpl and Fahrenholz, 2001).

OT receptors are functionally coupled to $G_{q/11}\alpha$ Class guanosine-5'-triphosphate binding proteins that stimulate together with $G\beta\gamma$ the activity of phospholipase C-β (PLC-β) isoforms. This leads to the generation of inositol trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 then triggers Ca^{2+} release from intracellular stores, whereas DAG stimulates protein kinase C, which phosphorylates unidentified target proteins. Finally, in response to an increase of intracellular Ca^{2+} levels, a variety of signal transducing events are initiated. For example, the forming Ca^{2+}-calmodulin complexes trigger activation of neuronal and endothelial isoforms of nitric oxide (NO) synthase. NO in turn stimulates the soluble guanylate cyclase to produce cyclic guanosine monophosphate. In smooth muscle cells, the Ca^{2+}-calmodulin system triggers the activation of myosin light-chain kinase activity, which initiates smooth muscle contraction, e.g., in myometrial or mammary myoepithelial cells (Sanborn et al., 1998). In neurosecretory cells, rising intracellular Ca^{2+} levels control cellular excitability, modulate their firing patterns, and lead to neurotransmitter release. Furthermore, Ca^{2+} promotes processes include gene transcription and protein synthesis.

The alignments of the three cloned avian neurohypophysial hormone receptors and the common features sharing with other receptors in the neurohypophysial hormone receptor subfamily and GPCR superfamily are shown in Figure 2.6. The first cloned avian neurohypophysial hormone receptor, the VT1 receptor in *Gullas gullas*, encodes a putative 370 amino acid protein (Tan et al., 2000). Radioligand binding experiments reveal a binding specificity with the following order of potency AVT ≅ AVP > OT ≅ MT > isotocin. The cloned receptor, expressing in COS7 cells, mediates AVT-induced phosphatidylinositol turnover and Ca^{2+}mobilization. The VT1 receptor

is expressed in the shell gland and brain. However, the expression of the VT1 receptor in the shell gland is confined to the endometrium, suggesting that any role(s) it may play in the induction of uterine contraction would be limited to a paracrine mechanism, possibly working through the release of prostaglandins (Wheatley et al., 1997). The expression of the VT1 receptor in the brain suggests a possible role in influencing sexual, maternal or social behaviors. The genes encoding the three cloned avian neurohypophysial hormone receptors consist of two exons interrupted by an intron of variable length. In the case of the VT1 receptor, the intron consists of 1073 base pairs (bp; Baeyens and Cornett, 2006).

The second cloned VT receptor subtype, the VT2 receptor also from chicken, has an open reading frame consisting of 425 amino acids (Cornett et al., 2003). The two exons are interrupted by a 708 bp intron. This receptor, when expressed in COS7 cells, results in AVT stimulated phosphatidylinositol turnover and intracellular Ca^{2+} mobilization. Northern blot analysis shows that the VT2 receptor is also expressed in the pituitary gland. The expression of VT2 receptor in the peripheral tissues could not be confirmed neither by Northern blot analysis or reverse transcription polymerase chain reaction (RT-PCR). IHC study of the anterior pituitary gland reveals the greatest expression of VT2 receptor on corticotrophs with more limited expression on lactotrophs (Jurkevich et al., 2005). Phylogenetic comparison of the VT2 amino acid sequence with those of other AVP/OT receptor family members shows that the VT2 receptor is most closely related to the mammalian V1b-VP receptor subtype (Figure 2.7). As discussed above, the V1b-VP receptor is primarily expressed on corticotrophs of the anterior pituitary gland and mediates the effect of AVP on adrenocorticotropic hormone (ACTH) release, thus playing a key role in the mammalian stress response.

Primary cultures of chicken pituitary cells when challenged with AVT also stimulate ACTH secretion. In addition, injection of AVT into the chickens results in corticosterone secretion from the adrenal cortex. Thus, the VT2 receptor and the V1b-VP receptor appear to play similar role in activating the hypothalamic-pituitary-adrenal axis in response to stress (Baeyens and Cornett, 2006).

The most recently cloned avian neurohypophysial hormone receptor is a putative 391 amino acid polypeptide (Gubrij et al., 2005). The exons of the gene are separated by a 1873 bp intron. The receptor appears to be an OT-like because of its high homology to cloned mammalian OT receptors (Figure 2.7), and because it is expressed in the myometrium of the chicken shell gland. It is believed that the receptor may be important in mediating the myometrial contractions that are characteristics of oviposition (Gubrij et al., 2005). The evidences supporting this claim come from both RT-PCR and *in situ* hybridization (ISH) analyses, which clearly show that the receptor is expressed in both myometrium and endometrium of the shell gland. Other studies have shown that the myometrium responds vigorously to AVT even after the endometrium has been stripped away, suggesting a direct effect of AVT on the myometrium (Olson and Hertelendy, 1983; Saito and Koike, 1992).

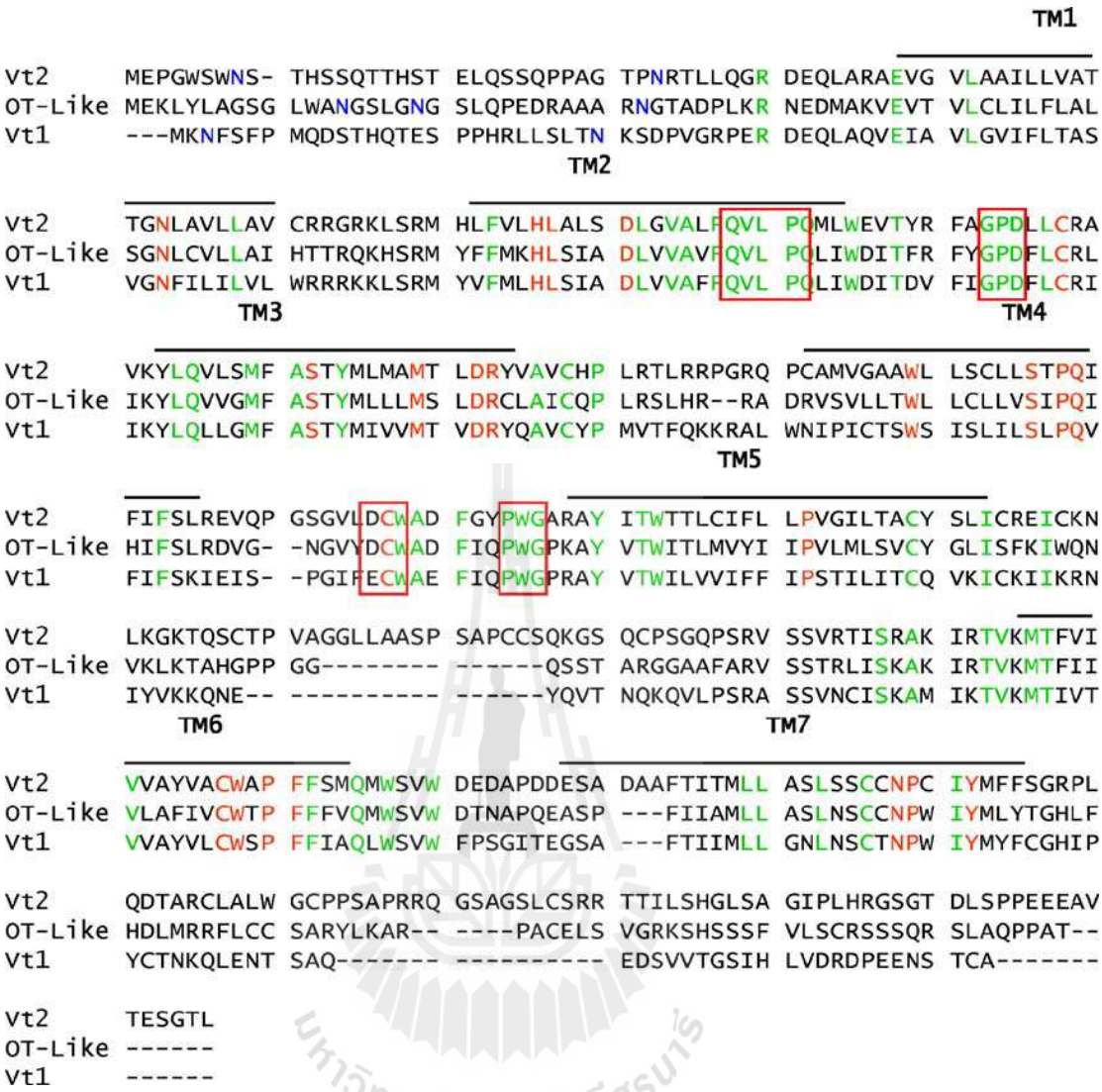

Figure 2.6 Sequence alignments of cloned avian neurohypophysial hormone receptors. The putative transmembrane helices 1-7 are underlined. Potential glycosylation sites in the amino terminus are in blue. The amino acid residues that are conserved within the three receptors are in green. The sequences conserved in the GPCR superfamily are in orange. Common sequences in the extracellular loops two and three and part of the extracellular surface of transmembrane 2, which are highly conserved among other neurohypophysial hormone receptors and may be involved in ligand binding are boxed (Baeyens and Cornett, 2006).

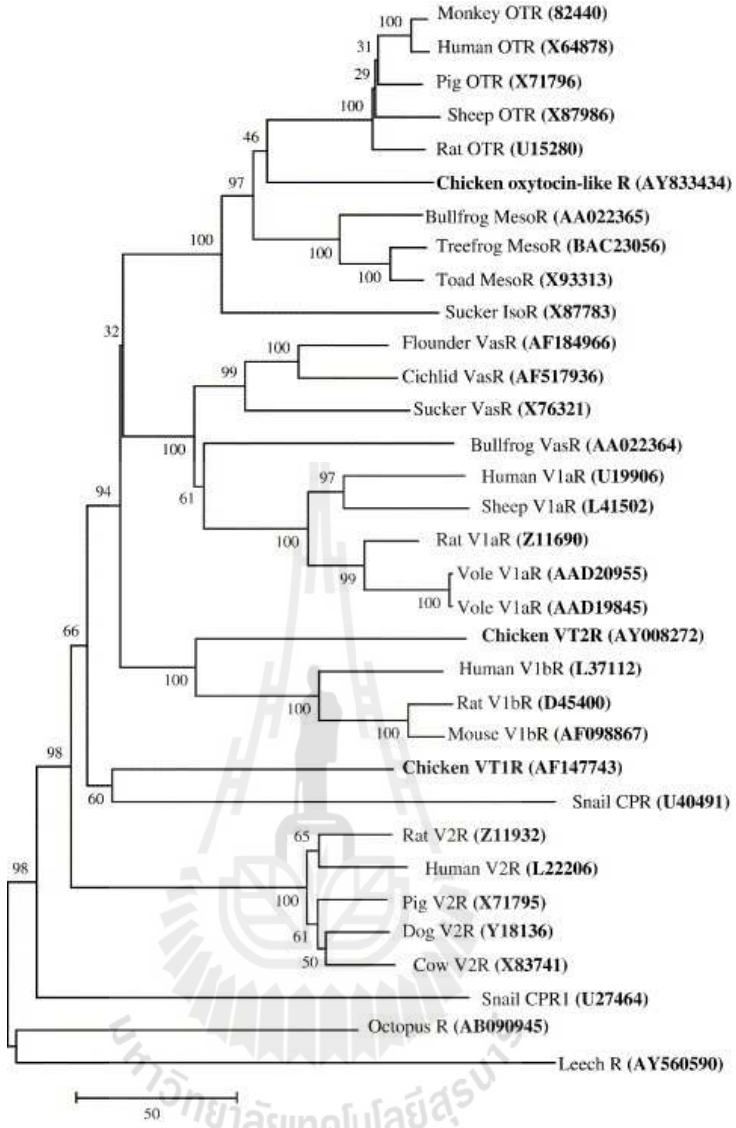

Figure 2.7 Phylogenetic relationships of AVP/OT receptor family members based on amino acid sequences. Numbers at branch points are derived from bootstrap analysis (1000 repetitions) and represent confidence limits for the positions of the branches. The proportion of differences (*p*-distance) is represented by the horizontal bar. Accession numbers are in parenthesis. The abbreviations used are OTR, oxytocin receptor; MesoR, mesotocin receptor, $V_{1a}R$, $V_{1b}R$, V2R, vasopressin receptors; VT1R, VT2R, vasotocin receptors; CPR, conopressin receptor; IsoR, isotocin receptor. The avian receptors described are in bold (Baeyens and Cornett, 2006).

2.5.2 The Physiological Functions of Oxytocin in Mammals

It is well established that OT and AVP have played a role mainly in parturition and lactation in mammals (de Wied et al., 1993; Neumann, 2007) that regulate a variety of important physiological functions. OT fulfills a large number of different physiological functions as both a circulating hormone and a neurotransmitter or neuromodulator. OT is initially discovered based upon its peripheral effects. The accidental discovery of the uterus-contracting effects of pituitary extract dates back to the beginning of the twentieth century (Dale, 1906). After that, Ott and Scott (1909) discover the milk-ejecting effects of pituitary extracts. Du Vigneaud (1953) establishes the structure of the active compounds in pituitary extracts by isolating, sequencing, and synthesizing the nanopeptide, OT and the structurally related peptide, VP. In fact, OT and VP are the first peptides to be sequenced and subsequently synthesized. Both AVP and OT have central effects on sexual, maternal and social behaviors as well as on memory and learning (Keverne and Curley, 2004; Lee et al., 2009).

OT's involvement in social memory has been evidenced by the finding that mice with a null mutation of the OT gene exhibit profound deficits in social processing and social recognition (Ferguson et al., 2000). In female prairie voles, OT appears to be especially important in the process of pair-bonding with males. Prairie voles are a monogamous species that show high levels of social behaviors including the formation of strong pair bonds with mates. OT infusion into the cerebral ventricles accelerates pair-bonding in the female prairie voles, while infusion of an OT receptor antagonist prevents pair-bond formation (Williams et al., 1994; Young and Wang, 2004). Plenty of evidences has been collected indicating the roles of OT that plays a

role in the mediation of a wide spectrum of CNS functions including the onset of maternal behaviors (Kendrick, 2000), specific mating behaviors and sexual responsiveness (Argiolas and Gessa, 1991), modulation of olfactory sensory input, modulation of memory functions, aggression, anxiety (McCarthy et al., 1996), adult pair-bond formation (Young and Wang, 2004), attachment behavior, and social recognition (Insel and Young, 2001; Ferguson et al., 2002). Recent studies in humans have also suggested that central OT modulates social cognition including increasing interpersonal trust, eye gaze, face recognition, and the ability to infer the emotions of others based on facial cues (Kosfeld et al., 2005, Domes et al., 2007, Donaldson and Young, 2008, Guastella et al., 2008, Savaskan et al., 2008). Moreover, OT also modulates social behaviors such as social memory, pair-bonding, sexual behavior, and parental behaviors when released as a neuropeptide in the CNS (McCarthy et al., 1992; Pedersen et al., 1992; Insel et al., 1997; Argiolas, 1999; Bales et al., 2004; Winslow and Insel, 2004; Lim and Young, 2006). Physiological and autonomic functions, food intake, stress response, and heart rate are also influenced by neuronal OT (de Wied et al., 1993; Verbalis et al., 1995; Neumann, 2002; Petersson, 2002). However, unlike VPergic innervation of the forebrain, OT pathways generally do not exhibit consistent sex differences (Buijs, 1978; Wang et al., 1996).

2.5.3 The Physiological Functions of Mesotocin in Birds

AVT and MT, the avian neurohypophysial hormones, are among 16 naturally occurring neuropeptides found in vertebrates (Munsick et al., 1960; Acher et al., 1970; 1997) and reported to be synthesized by neurons of the anterior hypothalamus (Acher et al., 1970), specifically magnocellular neurons of the SON and PVN. AVT

and MT are important physiological regulators of the anterior pituitary gland. It has been demonstrated that AVP and OT are involved in direct control of hormonal secretion from pituitary cells both *in vivo* and *in vitro* (Childs, 1992; Franci et al., 1993; Kjaer, 1993). 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). In birds, water deprivation is a potent osmotic stress and has significant effects on the hypothalamic machinery regulating body homeostasis. In the chickens, plasma AVT level increases during water deprivation, which provides a good correlation between plasma level of AVT and osmolality (Koike et al., 1977; 1979; Arad and Skadhauge, 1984; Arad et al., 1985; Stallone and Braun, 1986). Results from ISH studies indicate that neurons of the PVN are more sensitive than those of the SON to osmotic stimulation caused either by water deprivation (Chaturvedi et al., 1994) or injection of hypertonic saline (Jaccoby et al., 1997), and saline drink (Chaturvedi et al., 1997). Furthermore, there is a gender-related difference in osmotic control of AVT release and hypothalamic AVT gene expression in adult Japanese quails (Chaturvedi et al., 2000).

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 models, 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). Numerous studies have demonstrated that plasma levels of AVT transiently increase during oviposition in birds (Arad and Skadhauge, 1984; Nouwen et al., 1984; Tanaka et al., 1984; Rice et al., 1985; Shimada et al., 1986). Furthermore, the increase in plasma AVT levels observed at the time of oviposition has been correlated with increases in uterine contractility (Shimada et al., 1986). Indeed, AVT, but not MT, has been shown to stimulate contraction of shell gland strips *in vitro* (Koike et al., 1988).

In all non-mammalian vertebrate species that have been studied to date, AVT-containing magnocellular and parvocellular neurons are found in the POA/anterior hypothalamus in cell groups known as the SON and PVN (Moore and Lowry, 1998). In birds, using IHC and ISH methodologies, AVT-containing neurons have been observed in a number of extra-hypothalamic brain regions (Panzica, 1985; Panzica et al., 1988; Aste et al., 1996) including the lateral diencephalic region and BNST. The distribution of AVT in avian species is generally comparable to the distribution of AVP in mammalian species (Sanchez et al., 1991). This extra-hypothalamic distribution of AVT in birds suggests that in addition to its peripheral effects, AVT may influence behaviors as well. Indeed, AVT administration has been shown to modulate a number of behaviors including vocalization (Maney et al., 1997b; Goodson et al., 1999), sexual behaviors (Kihlstrom and Danninge, 1972; Castagna et al., 1998), and aggression (Goodson, 1998; Goodson and Adkins-Regan, 1999). 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 a behavioral study, MT fails to facilitate aggression or partner preference following ICV administration in zebra finches (Goodson et al., 2004).

Duplication of the VT gene in early jawed vertebrates give rise to two nanopeptides, which include the mammalian peptides, AVP and OT. Most non-mammalian vertebrates express VT and an OT-like peptide such as isotocin that found in ray-finned fish, or MT, which is ubiquitously expressed in non-mammalian tetrapods (Acher, 1972; 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). The parvocellular neurons of the PVN give rise to widespread projections in the rat brain (De Vries and Buijs, 1983), and this is almost certainly the same in other vertebrates. Lesions of the PVN in rats virtually eliminate VP projections to the caudal brainstem, but not other areas, and eliminate OT projections throughout the brain (De Vries and Buijs, 1983). 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 first evidence reported the role of

MT in avian brooding behavior has only been investigated in the turkeys. The numbers of MT-ir neurons in the PVN and the nucleus supraopticus, pars ventralis (SOv) increase in incubating hens when compared with laying hens. In addition, the induction of c-fos mRNA in the MT-ir neurons within these areas in the incubating hens stimulated with poults, and preventing poult brooding from taking place by blocking MT receptors suggest that MT is essential to the onset of maternal activities in the turkeys (Thayananuphat et al., 2011).

2.5.4 The Regulation of Oxytocin/Mesotocin Secretion

The PVN also contains parvocellular OT neurons that project to the hindbrain, brainstem, and spinal cord, where OT regulates autonomic functions (Sofroniew, 1980). Additionally, parvocellular OT neurons are found in the POA and the LHy, whereas accessory magnocellular OT neurons are found scattered across the hypothalamus. In *in vivo* studies, hypothalamic OT gene expression is stimulated during pregnancy and lactation (Van Tol et al., 1988) and in response to dehydration (Burbach et al., 1986). OT-ir fibers can be found throughout the brain including the Ac, LS, amygdala, and several structures in the hindbrain, brainstem, and spinal cord (Sofroniew, 1980; Castel and Morris, 1988). With the exception of the hindbrain and spinal cord projections, which arise from parvocellular neurons in the PVN, the source of other central OT-containing fibers has not been documented. However, lesion the PVN results in a marked reduction in OT-ir fibers throughout the brain (De Vries and Buijs, 1983). Little is known about the regulation of the release of OT from these forebrain projections, but they presumably contribute significantly to the regulation of behaviors. Gonadal steroids play an important role in mediating the

regulation of OTR expression. Most peripheral OT-binding sites are up-regulated by estrogens including the pituitary, renal, and uterine OTRs (Fuchs et al., 1983; Soloff et al., 1983; Maggi et al., 1992). The up-regulation is accompanied by an increase in OTR mRNA accumulation, suggesting that the up-regulation is consequence of a genomic estrogen effect on OTR gene transcription (Breton et al., 1995; Larcher et al., 1995).

It is very well established that gonadal steroids including estrogen influence the hypothalamo-neurohypophysial OT system at a variety of levels. Moreover, OT acts centrally to regulate a variety of steroid-regulated behaviors; male and female sexual behaviors, parental behaviors, and affiliation (McCarthy et al., 1996; Etgen, 2003; Handa et al., 2012). The promoter of the OT gene contains a consensus sequence for an estrogen response element (Richard and Zingg, 1990), suggesting that estrogen could stimulate OT-gene transcription. A more consistent view emerges for estrogen regulation of postsynaptic responses to OT in the hypothalamus. Estrogen increases OT receptor binding measured by a variety of methods in the female rat hypothalamus, especially the ventromedial nucleus. OT-binding sites also increase in response to estrogen treatment in the olfactory tubercle, Ac, OVLT, islands of Calleja, and central amygdala (Schumacher et al., 1993; Flanagan and McEwen, 1995). Maximal levels of OT-receptor binding are observed in gonadally intact female rats during the period of behavioral estrus (Insel, 1992). There are evidences that individual hypothalamic neurons express both estrogen and OT receptors (Devidze et al., 2005). Thus, estrogen may increase OT receptor density by regulating OT gene transcription. Whether estrogens are physiological regulators of OT biosynthesis in the CNS also remains to be determined, eventhough it seems likely at present.

However, the nature and functional consequences of progesterone modulation of estrogen-induced OT receptors remain to be further elucidated, especially in view of progesterone's failure to modulate hypothalamic neuronal responses to OT in brain slices from estrogen-treated female rats (Kow et al., 1991).

Recently, several observations have suggested that the GnRH agonist, triptorelin, stimulates both OT and AVP release from isolated hypothalamo-neurohypophysial system at concentrations of 10^{-9}-10^{-5} M. The strongest effect is displayed by triptorelin at a concentration of 10^{-7} M. Under the conditions of depolarization, K^+ stimulation, triptorelin affects neither OT nor AVP secretion *in vitro*. ICV infusion of triptorelin at a concentration of 10^{-7} M significantly stimulates both OT and AVP secretion into the circulation (Juszczak and Roszczyk, 2012). Moreover, CD38, a transmembrane protein with adenosine diphosphate-ribosyl cyclase activity, plays a critical role in mouse social behavior by regulating the release of OT, which is essential for mutual recognition. There is a rationale for investigating single nucleotide polymorphisms (SNPs) in the human CD38 gene in autism spectrum disorders (ASD) subjects. These results suggest that SNPs in CD38 may be possible risk factors for ASD by abrogating OT function and that some ASD subjects can be treated with OT in preliminary clinical trials (Higashida et al., 2012).

To date, there are limited data available to elucidate the the regulation of MT secretion in avian species. The first evidence reported the role of MT in avian brooding behavior shows that it is possible that PRL and the state of hyperprolactinemia may also be of importance in the increased number of MT-ir neurons in the PVN and SOv observed in late incubation in the turkey hens (Thayananuphat et al., 2011).

2.6 Maternal Behaviors in Mammals and Its Neuroendocrine Regulation

For successful reproduction, not only sexual activity is important, but also successful care of the young. Maternal behavior is crucial to the survival of fertilized eggs or offspring. The offspring need one or both parents to provide food, heat, or protection from any harm. This behavior must be performed immediately after birth or hatching of offspring (Nelson, 2000). Maternal behavior in mammals is composed of nest building, pup retrieval, crouching, exploration and sniffing of pups, licking and grooming, and placentophagia (Leckman and Herman, 2002). The onset of maternal responsivity is a prerequisite for all mother-young interaction. It brings the mother in contact with her young exposing her to a unique constellation of tactile, visual, auditory, and olfactory stimuli as well as a suckling-induced change in hormonal state (Numan and Woodside, 2010). Taken together, these promote other changes in behaviors.

Numerous neurohormones are well known to be involved in maternal behaviors in mammals. The important ovarian hormones for priming maternal behaviors are estrogen and progesterone (Pi and Grattan, 1998; Lonstein et al., 2000). Due to there is no restriction to the passage of steroid hormones from the vascular to the cerebral compartment, high-affinity binding neurons for these hormones will be activated in all parts of the brain simultaneously (Kendrick and Keverne, 1991). The pattern of secretion of steroid hormones during pregnancy is remarkably similar among all non-primate mammals and is characterized by high levels of progesterone in the postimplantation period, which decreases prior to parturition with a concomitant increase in estrogen. This prolonged priming of the brain by exposure to high

progesterone and low estrogen is important for the suppression of sexual behaviors in mammals, and for genomic activation promoting the synthesis of hypothalamic peptides such as OT, corticotropin releasing hormone, PRL, and β-endorphin (Kendrick and Keverne, 1992; Broad et al., 1995). Moreover, levels of estrogen receptor-α and estrogen receptor-β mRNA are higher in the rats exhibiting persistent licking and grooming of pups over the first week of postpartum (Champagne et al., 2003). The steroid hormones of pregnancy are integral to the induction of maternal responding, but they do not exclusively code for these events. They also influence sexual behaviors, feeding behaviors, and exploratory behaviors, and thus have the recruiting capacity for a wide range of neural systems (Kendrick and Keverne, 1992).

It is very well established that OT is the crucial neurohormone regulating maternal behaviors in mammals. The number and size of OT neurons are found to be greater in the SON, PVN, and LHy on day 1 postpartum than those of on day 29 of pregnancy or during estrus in the rabbits (Caba et al., 1996). OT receptor density is greater in the BNST, LS, and POA in the rats that display rapid maternal behaviors than those of in the slower responders (Francis et al., 2000). ICV injection of OT induces the onset of maternal behaviors (Pedersen, 1997). ICV injection of an OT antagonist or lesioning in the PVN area delays the onset of maternal behaviors, but they do not affect any aspects of this behavior after several days of postpartum (van Leengoed et al., 1987; Insel and Harbaugh, 1989). These results indicate that OT plays an important role in the onset of maternal behaviors.

In additional to regulating the peripheral physiology necessary for parturition and lactation, OT plays an important role in initiating maternal nurturing behaviors. ICV infusions of OT stimulate a rapid onset of full maternal behaviors in estrogen-

primed female rats (Pedersen and Prange, 1979; Pedersen et al., 1982). OT infusions only facilitate a rapid onset of maternal behaviors in virgin females, when tested in a novel cage (Fahrbach et al., 1986) or are rendered anosmotic (Wamboldt and Insel, 1987). However, there is a convergence of evidence from many studies that OT neurotransmission is indeed involved in regulating the onset of maternal behaviors. The rapid onset of maternal behaviors is delayed by reducing OT neurotransmission either by injecting an OTR antagonist (van Leengoed et al., 1987), infusing an anti-OT antibody (Pedersen et al., 1985), or lesioning the PVN of the hypothalamus (Insel and Harbaugh, 1989). OT also has a role in the transition from pup avoidance to maternal behaviors, but OT is less necessary for the maintenance or performance of maternal behaviors.

OT appears to be modulating maternal behaviors through actions in at least three brain regions; the MPOA, VTA, and olfactory bulb. Infusions of OTR antagonist into the MPOA and VTA prevent parturient dams from retrieving pups or from assuming a nursing posture over pups (Pedersen et al., 1994). OT receptors in both areas are increased at the time of parturition, and the levels of OT receptor mRNA are elevated in the MPOA during pregnancy (Pedersen et al., 1994; Young et al., 1997; Meddle et al., 2007).

Several studies in rodents mentioned above suggest that OT regulates maternal motivation. However, fascinating studies in sheep suggest that OT also plays a role in the establishment of the mother-infant bond, a form of social attachment. Unlike rodents, ewes develop highly selective maternal behaviors only toward their own lamps. Central infusions of OT induce short latency maternal responses to foreign lamps in estrogen-primed ewes (Kendrick et al., 1987). Maternal

behaviors in sheep develop at the onset of parturition or can be stimulated artificially with vaginocervical stimulation. In either case, the onset of maternal behaviors is associated with an increase in OT release in several brain structures including the olfactory bulb, MPOA, and PVN (Kendrick et al., 1988; 1992; da Costa et al., 1996; Arrati et al., 2006). Administration of OT directly into the PVN by retrodialysis appears to be as potent for inducing maternal bonding as vaginocervical stimulation (da Costa et al., 1996). These results suggest that OT, release within the PVN during parturition, may, via positive feedback, coordinate the release of OT in several other central sites, facilitating the onset of maternal behaviors and induction of the selective bond with the lamp.

It is well documented that PRL participates in promotion of maternal behaviors (Donner et al., 2007), and steroid hormones are required for PRL-stimulated maternal behaviors (Bridges et al., 1990). PRL plays an important role in the initiation and maintenance of lactation, regulates the production and composition of milk, and participates in maternal behavior regulation (Bridges and Ronsheim, 1990; Numan and Woodside, 2010). Neurotransmitters are also implicated in development of the nervous system (Mattson, 1988; Lipton and Kater, 1989) and of brain sex differences and maternal behaviors (Perez-Laso et al., 1994). Previous reports demonstrate that gamma-aminobutyric acid (GABA) participates via GABAA receptors in the development of sex differences in the rat brain (Segovia et al., 1996). Neonatal administration of the GABAA agonist, diazepam (DZ), to the male rats and the GABAA antagonist, picrotoxin (PTX), to the female rats alter sex differences in adult parental behaviors and in the number of accessary olfactory bulb mitral cells in adulthood (Segovia et al., 1996). 3a,5a-reduced neurosteroids regulate GABAA

receptor in a similar way to DZ, therefore it is reasonable to speculate that these neurosteroids regulate maternal behaviors in a similar way to benzodiazepines (Mellon et al., 2001). 3a,5a-reduced neurosteroids are synthesized from both progesterone and deoxycorticosterone by the steroidogenic enzymes 5a-reductase (5a-R) and 3a-hydroxysteroid dehydrogenase with the former being the rate-limiting enzyme of the reaction. 5a-R enzyme is expressed as two isozymes, 5a-R1 and 5a-R2, which are both present in different brain regions including prefrontal cortex (Sanchez et al., 2005; Torres and Ortega, 2006), and may be implicated in maternal behaviors of the rat.

The MPOA contains estrogen receptor (Shughrue et al., 1997), progesterone receptor (PR; Numan et al., 1999), PRLR (Bakowska and Morrell, 1997), and OT receptor (Champagne et al., 2004). Estrogen, PRL, and PLs have all been shown to stimulate the onset of maternal behaviors when microinjected into the MPOA (Bridges et al., 1990; 2001). OT has also been shown to act on the MPOA to stimulate maternal behaviors (Pedersen et al., 1994). Moreover, Stolzenberg and Numan (2011) propose that steroids might interact with DA within the MPOA to promote reproductive behaviors. Hull and colleagues have presented the best evidence for this idea in the context of male sexual behaviors (Bitran and Hull, 1987; Hull et al., 1999; Hull and Dominguez, 2006). In support of the idea that estrogen-DA interactions within the MPOA might also promote maternal behaviors, there are data indicating that in the absence of estradiol benzoate (EB) treatment, administration of the D_1 DA receptor agonist directly into the MPOA can promote an immediate onset of full maternal behaviors in the hysterectomy and ovariectomy rats on day 15 (Stolzenberg et al., 2007). Because of the facilitatory effect of EB action on the MPOA is replicated

by D_1 DA receptor stimulation of the MPOA, a simple interpretation is that D_1 DA receptor stimulation of the MPOA substitutes for estrogen action at the estrogen-receptor to facilitate the onset of maternal behaviors (Stolzenberg and Numan, 2011).

2.7 Maternal Behaviors in Birds

Maternal behaviors are displayed by most mothers after parturition and serve the immediate provision of care and defense for their young (Brunton and Russell, 2008). In fact, the life maintenance of each species is depended upon the presence of precise maternal care in the period that the child is dependent on the mother (Swain et al., 2007). The patterns of maternal care in mammals consist of internal incubation of eggs during gestation, delivery of the young at parturition, and maternal care until weaning (Rosenblatt, 2003). The mechanisms underlying the regulation of these behaviors may be derived from the processes involving in gestation, parturition, or the regulation of lactation including changes in circulating levels of progesterone, estrogen, OT, and PRL (Numan, 1994; Ziegler, 2000; Rosenblatt, 2002), and these hormonal activities increase in the POA, the area that involved in maternal behaviors (Featherstone et al., 2000). In addition, the experience that new mothers gain during interacting with their newborn young has long term consequences, and stimuli from the young also promote maternal responsiveness and facilitate the maternal behaviors (Fleming and Sarker, 1990).

Maternal care in birds is included 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., 1988a; Nelson, 2000).

Incubation behavior is qualified by sitting continually on their eggs until hatching, while brooding or rearing behavior is directed to the care of newly hatched chicks (Richard-Yris et al., 1983; El Halawani et al., 1988a; Ruscio and Adkins-Regan, 2004; Sharp, 2009). In general, the hens develop maternal behaviors gradually in four stages; brooding, titbitting, clucking, and normal broody behavior (Ramsay, 1953). These incidences concur with a pause in laying and a significant long term fall in the plasma levels of ovarian steroids (Richard-Yris et al., 1983; 1988). Brooding 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 that express brooding behavior allow the chicks to access and remain underneath their wings, whereas birds that do not exhibit brooding behavior avoided the chicks (Ruscio and Adkins-Regan, 2004).

2.7.1 Incubation Behavior and Its Neuroendocrine Regulation

The physiology and behavior associated with incubation behavior are the complex ones. These physiological changes include elevated circulating PRL levels, reduced circulating gonadotropins levels, reduced circulating gonaldal steroids levels, ovarian regression, cessation of laying, and altered neurotransmitter activities in the brain. The behavioral patterns that associated with incubation behavior include nesting activity, nest protection activity, and anorexia (El Halawani et al., 1988a). The initiation of incubation behavior is related to nesting frequency and egg laying. Nesting frequency increases in conjunction with the development of an increase in PRL levels until the first day of incubation. When the hens stop laying, nesting activity progressively extends to occupy the nest most of the day and has transformed

to complete incubation behavior (Lea et al., 1981). During incubation behavior, the hens sit on their clutches, persistently turn their eggs, rearrange the eggs to guarantee that they are well covered, and associate 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 accumulated 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).

In most avian species 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, so called brood patch. This brood patch develops just before the onset of the incubation behavior and functions to facilitate heat transfer from the hen to the eggs and the transmission of tactile stimuli to the hen (El Halawani et al., 1988a). Tactile stimuli at the brood patch appear to be mediated the suppression of PRL levels than by auditory or visual stimuli (Opel and Proudman, 1985). It has reported that anesthesia applied to the brood patch suppresses the PRL concentrations in the incubating ducks (Hall and Goldsmith, 1983). Evidence suggests that the brood patch formation begins about 5 days before the onset of incubation behavior (Lea et al., 1981). In the canaries and white crowned sparrows, administration of estrogen accompanied with PRL results in the development of the brood patch (Bailey, 1952; Steel and Hinde, 1963). During this behavior, birds eat and drink very little and lose their weights, and this weight loss has been reported in the

turkeys (Zadworny et al., 1985), bantam chickens (Savory, 1979), geese (Akesson and Raveling, 1981), ducks (Gatti, 1983), and native Thai chickens (Kosonsiriluk, 2007). In general, 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 that incubate their infertile eggs persist for about 50 % longer than that of normally require hatching them (Skutch, 1962). During 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 increase (Sharp, 1997).

The onset of incubation behavior is associated with declining levels of gonadotropins and gonadal steroids and increasing of PRL (Lea et al., 1981; El Halawani et al., 1988a; El Halawani and Rozenboim, 1993). This rising PRL level has been implicated as the cause of cessation of ovulation, ovarian regression, and induction and maintenance of 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), 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 presence of the chicks (Richard-Yris et al., 1987a; 1987b; 1995; Sharp et al., 1988; Leboucher et al., 1990; 1993).

Well documentedly, the increased 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 the turkeys, (El Halawani et al., 1986). In addition, stimulus of nesting maintains high circulating PRL levels in

the incubating hens. Removal of the incubation turkeys and native Thai hens from their nests results in a dramatic decrease in plasma PRL levels (El Halawani et al., 1980; Proudman and Opel, 1981; Prakobsaeng et al., 2009; 2011; Prakobsaeng, 2010). The degree of incubation behavior and the plasma levels of PRL and LH are depended 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).

2.7.2 Rearing Behavior

Maternal experiences, neurotransmitters, neurohormones, neromodulators, hormones, and stimuli from the young interact in complex events to promote maternal responsiveness in both mammals and birds. The expression of rearing behavior in birds results from the presence of chicks, 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 regularly behaviors exhibited in hens rearing of the chicks (Richard-Yris et al., 1995; 1998b; Ruscio and Adkins-Regan, 2004). In galliform birds, precocial newly hatched chicks can walk, feed, see, and hear after hatching, but they cannot effectively thermoregulate during the first 2 weeks after hatching. Brooding by the maternal hens therefore can help them to survive (Mills et al., 1997).

Brooding 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). Brooding behavior can be induced in the chickens, turkeys, and Japanese quails by introducing the newly hatched chicks to them, which the hens present immediate maternal care responses (Richard-Yris et al., 1983; Richard-Yris and Leboucher, 1986; 1987; Opel and Proudman, 1988; Leboucher et al., 1990; 1991; 1993; Ruscio and Adkins-Regan, 2004). It has been further suggested that the physical contact between hen and chicks, alone or in combination with visual and/or auditory stimuli originating from the chick, induces the brooding behavior (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 strengthen by repeated exposure of the chicks to the hen, accompanied by food, guidance, and protection (Wauters and Richard-Yris, 2001). Repeated exposure to maternal calls may be impotant for the development of post-hatch species-specific maternal call recognition during embryonic development, (Gottleib, 1976; Jain et al., 2004). The chicks are self-sufficient after hatching in precocial species, 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 occurred during brooding behavior (Zadworny et al., 1985). Precocial chicks can be filial imprinting on their parents in the first few days after hatching (Rodgers, 1995; Mills et al., 1997). The relationships

between the mothers and the precocial young has 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).

PRL's role has been well established as an incubation promoting hormone, it has been implicated in the regulation of behavior patterns associated with care of the young after hatching as well (Buntin, 1986; Vleck, 1998). PRL secretion is stimulated by exposure of the hens' tactile and visual stimuli from the chicks, and in the mean time PRL facilitates and stimulates the expression of maternal behaviors such as incubating, brooding, or feeding (Angelier and Chastel, 2009). An elevated PRL secretion is involved in the transition from sexual to parental activity (Sharp et al., 1998). Therefore, the levels of PRL are most elevated during the rearing period (Buntin, 1996). This is reflected in changes in circulating PRL levels with different maternal care behaviors in birds. Depending upon the species, either sharp declines or slow decreases of PRL concentrations after hatching have been documented (Goldsmith and Williams, 1980; Dittami, 1981; Oring et al., 1986; Hall, 1987; Oring et al., 1988, Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). In galliform birds, PRL facilitates the induction of rearing behavior. PRL injection induces the display of the maternal covering stance normally adopted during brooding behavior (Opel and Proudman, 1980). PRL concentrations generally decline shaply after the chicks are hatched in precocial species, and the presence of the chicks can modify this rate of declined PRL (Dittami, 1981; Opel and Proudman, 1989). In incubating hens, the replacement of chicks for eggs or the appearance of chicks at hatching is associated with an elevate in plasma LH levels and a marked decrease in plasma PRL levels

from the high levels presented during incubation period (Zadworny et al., 1988; Leboucher et al., 1991; Richard-Yris et al., 1998a). In fact, exposure to chicks can induce maternal behaviors in the incubating, non-incubating, and ovariectomized hens, which show markedly 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). The young of altricial species are reared jointly by both parents. The columbiforms such as pigeons and ring doves feed their newly hatched chicks by regurgitating crop milk, which is produced by epithelial mucosa cells that proliferated in response to PRL and ultimately slough from the crop sac wall (Buntin, 1996; Wang and Buntin, 1999). In addition to stimulating the production of crop milk, 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). PRL circulating levels begin to decrease after the chicks achieve their thermal independence, and they do not require constant brooding from the mothers (Goldsmith, 1991).

2.7.3 Neuroendocrine Regulation of Rearing Behavior

PRL is involved in many aspects of reproductive physiology and behaviors in both birds and mammals. It is very well established that PRL play a significant role in maternal behaviors in birds. It plays a pivotal role in parental behaviors by mediating increases in incubation behavior, crop milk production/secretion, feeding of the young, and nest defense (Silver, 1984; Janik and Buntin, 1985; Lea et al., 1986; Buntin et al., 1991). The roles of PRL in the induction and maintenance of maternal behaviors have been extensively elucidated. In birds, the circulating levels of PRL are

low before and during egg laying, increase before incubation, are maintained at high levels during incubation period, and then decrease sharply to basal levels immediately after the chicks are hatched (bantams: Sharp et al., 1979; mallard ducks: Goldsmith and Williams, 1980; Japanese bantams: Zadworny et al., 1985; 1988; Gifujidori hens: Kuwayama et al., 1992; native Thai chickens: Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008).

There are growing evidences exist to link the maternal behaviors with an increase in PRL secretion in birds. High circulating PRL levels are well known to be associated with rearing behavior in the chickens (Sharp et al., 1979; 1988; Bedrak et al., 1981; Lea et al., 1981; Hoshino and Wakita, 1989), turkeys (Burke and Dennison, 1980; Proudman and Opel, 1981), mallard ducks (Goldsmith and Williams, 1980), Auatralian black swans (Goldsmith, 1982), and native Thai chickens (Chaiyachet et al., 2012). PRL is very well accepted as a crucial factor to the onset and maintenance of broodiness in birds. In addition, it has been suggested that the patterns of PRL secretion are mediated by the exhibition of parental behaviors (Dawson and Goldsmith, 1982). PRL also acts, in part, at the gonadal level to reduce estrogen production and causes the regression of large ovarian follicles in the chickens and turkeys (Opel and Proudman, 1980; Zadworny et al., 1989). The studies in which broody chickens (Sharp et al., 1979), ducks (Goldsmith and Williams, 1980), and Auatralian black swans (Goldsmith, 1982) have been allowed to hatch and rear the chicks have shown that PRL levels decrease when the incubation period is ended. Further result from the duck suggests that this drop may occur on or right before the day of hatching (Goldsmith and Williams, 1980). It has been reported in the turkeys that the drop in PRL levels at the end of incubation period might be related to the

pipping and hatching eggs, and the consequent transition to maternal behaviors (Wentworth et al., 1983; Opel and Proudman, 1989).

Brooding 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 is involved in the onset or maintenance of brooding behavior and the possibility of an antigonaldal role in birds (Bedrak et al., 1981; Sharp et al., 1988; Zadworny et al., 1988; 1989). Previous studies have reported the relationship between circulating PRL levels and brooding behavior in the parents of precocial young, revealing that the gradual decrease of plasma PRL levels is related to the decline in brooding behavior with the age of chicks (Dittami, 1981; Opel and Proudman, 1989). In shorebirds and red-necked phalaropes, 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 the turkey hens, endocrinological parameters and production performances are changed during the expression of brooding behavior (Guemene and Williams, 1992). Plasma LH levels decrease progressively, while plasma PRL levels

increase during the hens exhibit broodiness. High levels of PRL are then maintained for a long period throughout the brooding behavior and cause the decrease in ovulation rate and egg production. Additionally, *in vitro* study illustrates that PRL synthesis and secretion are high in the pituitary gland of broody hens, and these changes are related to brooding 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 broody 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 mean time, 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 plasma estradiol levels slightly decrease. 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). Brooding behavior progressively declines, when the chicks grow older and become fledged, leading to a shap 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 broods, and then reach the 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 the 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). Taken together, these features suggest that maternal care and particularly physical contact with the young chicks 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 during the hens are caring for their young. The bantam hens stop exhibiting broody behavior between 4-10 weeks after the chicks are hatched and correspond 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 closed 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 in 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 their entire chicks exhibit an abruptly 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 PR immunoreactivity in the tuberal hypothalamic area (TR) decreases in the 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 the 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 DAergic activity and the numbers of MT-ir neurons occurred in specific neuronal regions including the PVN, nucleus dorsomedialis anterior thalami, and SOv are significantly increased in the incubating hens when compared with those of the laying hens (Lea et al., 2001; Thayananuphat et al., 2011). 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 the ring doves (Slawski and Buntin, 1995). The expression of an immediate early gene protein products, fos-like immunoreactivity (fos-ir), in the brains of brooding ring doves and Japanese quails during given tactile to their young reveals high density of fos-ir in 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, over 80 % of those hens fail to brood their poults, and they have 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 behaviors in birds (Thayananuphat et al., 2011). ICV injection of OT also causes a dose-dependent decrease in feed 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).

Up to date, there are limited data available that describe the interrelationship and the functional aspect of the changes in the DAergic and the MTergic systems with those in PRL levels during maternal behaviors in the native Thai chickens. Therefore,

this dissertation was designed to investigate an association between the DAergic and MTergic systems as well as the important role of PRL in the regulation of rearing behavior in the native Thai chickens. The findings gained from this dissertation will provide an insight into the neuroendocrine mechanism(s) underlying the regulation of maternal behaviors in the native Thai chickens. The knowledge gained from this study can be then applied commercially in poultry industry to increase egg production of native Thai chickens in Thailand.

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CHAPTER III

THE DOPAMINERGIC-PROLACTIN SYSTEM INVOLVEMENT IN BROODING BEHAVIOR OF THE NATIVE THAI CHICKEN

3.1 Abstract

Native Thai chicken (*Gallus domesticus)* is a continuously breeding species found in the equatorial zone that produces eggs all year, which is independent of photoperiodic cues. It always expresses high maternal behaviors. Maternal behaviors are hormonal dependent and initiated with the onset of incubation behavior and continue through the period when the young are taking care by parent (broody/rearing behavior). It is well established that dopaminergic (DAergic) neurotransmission is involved in the neuroendocrine regulation of the avian reproductive cycle. Previous studies have demonstrated that the DAergic input from the nucleus intramedialis (nI) and nucleus mammillaris lateralis (ML) to vasoactive intestinal peptide (VIP) neurons in the nucleus inferioris hypothalami and nucleus infundibuli hypothalami and subsequent prolactin (PRL) release are a key regulator of incubation behavior in the native Thai chickens. To date, there are limited data available that describe the interrelationship and the functional aspects of the changes in neurohormones/neurotransmitters/hormones involved in maternal behaviors during the hens take care of their chicks. It is well known that the initiation and maintenance

of maternal behaviors is correlated with plasma PRL levels. However, a role of the DA/PRL system in rearing behavior has never been elucidated in birds. Therefore, the objective of this study was to identify the DA neuronal groups that associated with the neuroendocrine regulation of rearing behavior via PRL secretion in the native Thai chickens. Incubating native Thai hens were used and divided into two groups. Hens in the first group were allowed to care for their chicks for 28 days (rearing hens; R) after hatching. In the second group, chicks were removed from the hens immediately after hatching for 28 days (non-rearing hens; NR). Blood samples were collected in the R and NR hens (n=6) for determining plasma PRL levels utilizing enzyme-linked immunosorbent assay. Immunohistochemical technique of tyrosine hydroxylase (TH, the rate-limiting enzyme for DA biosynthesis as a marker for DAergic activity) were used to compare the differential expression of DA neurons within the individual hypothalamic areas of R hens with those of NR ones. The results revealed that the TH-ir neurons and fibers were extensively distributed throughout the brain of R and NR native Thai hens and were predominantly expressed in the nI and ML areas. The expression of hypothalamic TH-ir neurons in the nucleus septalis lateralis, nucleus anterior medialis hypothalamic, organum paraventriculare, regio lateralis hypothalami, and nucleus perventricularis hypothalami areas were also observed. A dense accumulation of TH-ir neurons was found in the nI of R hens. High density of TH-ir fibers was found in the nucleus mamillaris medialis and median eminence of both treatment groups. Changes in the number of TH-ir neurons within the nI and ML areas were compared between the R and NR hens. Significant decreases in the number of TH-ir neurons of the NR hens when compared with those of the R hens were observed in the nI after the day of hatch until 14 days of the observation periods. In

both treatment groups, the difference of TH-ir neurons was not found after 14 days through 24 days of the time periods of observation. The number of TH-ir neurons in the ML was high during the rearing period, but there are no significant differences between the two groups. However, in the R hens, the number of TH-ir neurons in the ML tended to decrease after 17 days of rearing chicks. Plasma PRL levels remained at high levels on the day the chicks were hatched, and then rapidly decreased after the day of hatch, and remained at low levels throughout the 28 days of observed rearing period. Comparisons of plasma PRL levels between the R and NR hens were elucidated. In the R hens, plasma PRL levels were high when compared to those of the NR hens. The levels of plasma PRL were decreased in the hens that had their chicks removed and reached the lowest levels by the third week of deprivation from the chicks. The present results clearly indicate an association between DA neurons in the nI and ML with the degree of hyperprolactinemia. The differential expression of DA neurons in the nI and ML might play a regulatory role in rearing behavior of this species. Disruption of rearing behavior decreases the number of DA neurons in the nI and ML and accompanied with the declined plasma PRL levels, implicating that the activities of the DAergic and VIP/PRL systems are enhanced to initiate and maintain the rearing behavior. Disruption of rearing behavior suppresses the hypothalamic DAergic and VIPergic activities and reduces circulating PRL levels. In conclusion, the present findings indicate, for the first time, that the DAergic system plays a pivotal role in neuroendocrine reorganization to establish and maintain maternal behaviors in the native Thai chickens. The decline in DAergic activity and PRL levels during disrupting the rearing behavior might be related to the contribution of rearing behavior in this equatorial precocial species.

3.2 Introduction

Two neuroendocrine systems play pivotal roles in the avian reproduction. One system involves chicken gonadotropin releasing hormone-I (cGnRH-I or GnRH) and the subsequent secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH; GnRH/FSH-LH system). A further system involves vasoactive intestinal peptide (VIP) and the subsequent secretion of prolactin (PRL; VIP/PRL system). Both systems are governed by dopamine (DA: Bhatt et al., 2003; Chaiseha et al., 2003).

DA is a neurotransmitter/neuromodulator found in both central and peripheral nervous systems of many vertebrate species (Ben-Jonathan and Hnasko, 2001). In mammals, DA has the main physiological function to inhibit the release of PRL from the anterior pituitary gland as the principle PRL-inhibiting factor (PIF). The concentrations of DA in hypophysial portal blood are maintained at the physiologically active levels (Ben-Jonathan et al., 1977; Gibbs and Neill, 1978; Ben-Jonathan et al., 1980), and the pituitary lactotrophs contain DA receptors (Caron et al., 1978; Cronin et al., 1978; Goldsmith et al., 1979). It has been suggested that DA which is released from the hypothalamic tuberoinfundibular DA (TIDA) neurons serves as the hypophysiotropic PIF in mammals (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001) and is mediated through the D_2 DA receptors located on the pituitary lactotrophs (Civelli et al., 1991). DA and its agonists attenuate PRL secretion, PRL gene expression, and lactotrophs proliferation (Shaar and Clemens, 1974; Lamberts and MacLeod, 1990). Removal of this DAergic inhibition results in an increase in PRL secretion and hyperprolactinemia in mammals (Nicoll and

Swearingen, 1970; Nicoll, 1977). However, this is not the case in birds, where removal of hypothalamic inputs results in the completed cessation of PRL secretion.

In birds, it has been reported and well established that DAergic influences are involved in both stimulating and inhibiting of avian PRL secretion. DA inhibits pituitary PRL release *in vitro* (Harvey et al., 1982; Hall and Chadwick, 1983; Hall et al., 1986; Xu et al., 1996). In the turkeys, intracerebroventricular infusion of DA can either stimulate or inhibit PRL secretion depending upon the concentrations used (Youngren et al., 1995). Both stimulatory and inhibitory effects on avian PRL secretion are depended on multiple subtypes of DA receptors (Youngren et al., 1996). It is suggested that DA stimulates PRL secretion at the hypothalamic level via the D_1 DA receptors residing in the infundibular nuclear complex (INF), where the VIP neurons are located. DA inhibits PRL at the pituitary level via the D_2 DA receptors by blocking the action of VIP, the avian PRL-releasing factor (Youngren et al., 1995; 1996; Chaiseha et al., 1997; 2003; Al Kahtane et al., 2003). It is very well established that DA plays an intermediary role in PRL secretion, requiring an intact VIPergic system in order to cause the release of PRL (Youngren et al., 1996). Dynorphin, serotonin (5-HT), DA, and VIP all appear to stimulate avian PRL secretion along a pathway expressing κ opioid, 5-HTergic, 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).

Maternal care in birds is included incubation and brooding/rearing behaviors. The term incubation refers to the maternal care of unhatched eggs and brooding is the maternal care of chicks after hatching (El Halawani et al., 1988). Incubation behavior in birds is qualified by sitting continually on their eggs until they hatch, while

brooding or rearing behavior is directed to the care of newly hatched chicks (Richard-Yris et al., 1983; El Halawani et al., 1988; Ruscio and Adkins-Regan, 2004; Prakobsaeng et al., 2011; Chaiyachet et al., 2012). Generally, the hens develop maternal behaviors gradually in four stages; brooding, titbitting, clucking, and normal broody behavior (Ramsay, 1953). The incidence of maternal behaviors concurs with a pause in laying and a decrease in plasma gonadal steroid levels (Richard-Yris et al., 1983). It has been reported that, birds that exhibit brooding behavior allow chicks to access and remain underneath their wings, whereas birds that do not show brooding behavior actively avoided the chicks (Ruscio and Adkin-Regan, 2004).

It is well known that PRL regulates maternal behaviors in various species. The role of PRL in the induction and maintenance of maternal care has been extensively studied (Harris et al., 2004). In mammals, PRL begins to increase toward the end of gestation, when it is crucial for inducing milk production. In combination with progesterone and estrogen, PRL reduces the latency of onset of maternal behaviors (Bridges and Ronsheim, 1990). In several avian species such as bantams (Sharp et al., 1979; 1988), mallard ducks (Goldsmith and Williams, 1980), domestic ducks (Hall and Goldsmith, 1983), Japanese bantams (Zadworny et al., 1988), and native Thai chickens (Kosonsiriluk et al., 2008), it has been shown that PRL concentrations are low before egg laying, increase slightly during egg laying, increase sharply before incubation and are maintained at high levels during incubation, and then decrease rapidly to basal levels immediately after hatching the young. Abundant evidences have linked maternal behaviors in several avian species with increased PRL secretion. High PRL levels are known to be associated with brooding behavior in the chickens (Sharp et al., 1979; 1988; Bedrak et al., 1981; Lea et al., 1981; Hoshino and Wakita,

1989), turkeys (Burke and Dennison, 1980; Proudman and Opel, 1981), mallard ducks (Goldsmith and Williams, 1980; Boos et al., 2007), swans (Goldsmith, 1982), and native Thai chickens (Chaiyachet et al., 2012). The studies in which broody chickens (Sharp et al., 1979), ducks (Goldsmith and Williams, 1980), and swans (Goldsmith, 1982) have been allowed to hatch and rear the young have shown that PRL concentrations decline at the end of the incubation period. Tactile stimuli from poults decrease circulating PRL in incubating hens without eggs and nests (Opel and Proudman, 1988). Physical contact, as well as visual and/or auditory stimuli from the young chicks, is clearly involved in the appearance and maintenance of maternal behaviors (Richard-Yris and Leboucher, 1987; Opel and Proudman, 1989). Furthermore, PRL has been implicated as a causative factor for reduced circulating gonadotropins and ovarian regression when birds shift from egg laying to incubation behavior in the chickens, turkeys, pigeons, pheasants, mallard ducks, cow birds, and native Thai chickens (Breitenbach and Meyer, 1959; Goldsmith and Williams, 1980; Bluhm et al., 1983; Lea and Sharp, 1989; Zadworny et al., 1989; El Halawani et al., 1997; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). It is apparent that PRL is involved in many aspects of reproductive physiology and behaviors. It plays a pivotal role in parental behavior by mediating increases in incubation, crop milk production and secretion, feeding of young, and nest defense (Silver, 1984; Janik and Buntin, 1985; Lea et al., 1986; Buntin et al., 1991; Prakobsaeng et al., 2011).

In contrast of the temperate zone seasonal breeding species, the native Thai chicken (*Gallus domesticus)* is a continuously breeding species found in the equatorial zone that produces eggs all year, which is independent of photoperiodic cues (Kosonsiriluk, 2007; Sartsoongnoen, 2007; Kosonsiriluk et al., 2008). The native Thai

chicken is the domesticated chicken without genetic selection. The reproductive cycle of the native Thai chicken is divided into four reproductive stages; non-egg laying, egg laying, incubating eggs, and rearing chicks (Kosonsiriluk, 2007). It always expresses high maternal behaviors, which is a heritable trait from the ancestor, the wild jungle fowl (Austic and Nesheim, 1990; Hillel et al., 2003; Sawai et al., 2010). Maternal behaviors are hormonal dependent and initiated with the onset of incubation behavior and continue through the period when the young are taking care by parent (broody/rearing behavior; Prakobsaeng, 2010). However, there are limited data regarding the neuroendocrine regulation of rearing behavior in this non-temperate zone gallinaceous bird. Recently, it has been well established that incubation behavior in this species is regulated by the VIP/PRL, GnRH/FSH-LH, and DAergic systems (Sartsoongnoen et al., 2006; 2008; 2012; Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011). Plasma PRL and LH levels across the reproductive cycle of the native Thai chickens have been reported. Changes in numbers of VIP-ir neurons within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) areas are directly correlated with changing plasma PRL levels throughout the reproductive cycle, suggesting that VIP expression in the IH-IN of the native Thai chickens plays a regulatory role in year-round reproductive activity (Kosonsiriluk, 2007; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). Moreover, it has been demonstrated that changes in the number of VIP-immunoreactive (ir) neurons in the IH-IN are associated with DAergic neurons within the nucleus intramedialis (nI) and nucleus mamillaris (ML) areas, resulting in PRL release to induce and maintain incubation behavior in the native Thai chickens. It is further suggested that nesting activity stimulates PRL secretion through activation of the DAergic system, which in turn stimulates VIPergic

system. The elevated PRL levels increase nesting activity and maintain incubation behavior (Prakobsaeng et al., 2011; Sartsoongnoen et al., 2012). Recently, disruption of rearing behavior in the native Thai hens by removing the chicks from the hens markedly decreases plasma PRL levels, a parallel decline in the number of VIP-ir neurons in the IH-IN, and an accompanying increase in the number of GnRH-I-ir neurons in the nucleus commissurae pallii (nCPa), suggesting that the VIPergic system in the IH-IN and the GnRH system in the nCPa may be involved in the regulation of the reproductive neuroendocrine system and the initiation and maintenance of rearing behavior in this non-seasonal breeding, equatorial precocial species (Chaiyachet et al., 2010; 2012).

To date, there are limited data available that describe the interrelationship and the functional aspects of the changes in neurohormones/neurotransmitters/hormones involved in maternal behaviors during the hens take care of their chicks. Therefore, the objective of this study was to identify the DA neuronal groups that associated with the neuroendocrine regulation of rearing behavior via PRL secretion in the native Thai chickens. Immunohistochemistry (IHC) was conducted to reveal the distributions of tyrosine hydroxylase (TH)-ir (as a marker for DA) neurons and fibers in the brain of native Thai chickens. Differences in the number of TH-ir neurons within individual hypothalamic areas at the different time periods of rearing and non-rearing hens after the incubating eggs were hatched were compared. Blood samples were collected from the rearing and non-rearing hens for determining plasma PRL levels by an enzyme-linked immunosorbent assay (ELISA). The findings of differential expression of DA neurons within the individual hypothalamic areas and subsequence of PRL secretion may provide an insight into the mechanism(s) underlying the neuroendocrine

regulation of brooding behavior in the native Thai chickens, which could help to improve the productivity of the native Thai chickens in Thailand.

3.3 Materials and Methods

3.3.1 Experimental Animals

Female and male native Thai chickens (*G. domesticus*), Pradoohangdam breed, were used. They were reared and housed (7-8 females: 1 mature rooster) in floor pens equipped with basket nests under natural light (approximately 12 hrs of light and 12 hrs of darkness; 12L: 12D). Each hen was identified by wing band number. Feed and water were given *ad libitum*. One hundred and two female native Thai chickens, 22-24 weeks old, were used. After hatching their chicks, the hens were divided into 2 groups; rearing (R) and non-rearing (NR) hens. The R hens were allowed to rear the chicks naturally. The NR hens were disrupted from rearing behavior and not allowed to rear their chicks by removing them from their chicks to another pen. Both groups were reared with the mature roosters. All the hens were checked four times per day and classified as broody/rearing hens throughout the experiment. During rearing behavior, the hens remained with their chicks, displayed aggressive behavior, and emitted a characteristic high-pitched vocalization, when they were approached by humans and/or the male. The hen fluffed her feathers and crouched over the chicks, whether she encouraged the chicks to go under her wings, and the types of vocalizations to protect and/or feed the chicks (Opel and Proudman, 1989; Edgar et al., 2011; Thayananuphat et al., 2011). The animal protocols described in this study were approved by Suranaree University of Technology Animal Care and Use Committee.

3.3.2 Experimental Design

3.3.2.1 Experiment I

To determine the effects of removing chicks from the hens on plasma PRL levels at different time periods of the R hens compared with those of the NR hens, one hundred and two female and twelve male native Thai chickens, 24 weeks old, were used. The chickens were randomly divided into 12 floor pens (8-9 hens: 1 rooster) and observed their behaviors daily. After hatching their chicks, all hens were divided into two groups; R and NR groups as described above. Blood samples were collected from the brachial vein of each hen prior to euthanize with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France) at different time periods (day 4, 7, 10, 14, 17, 21, 24, and 28) of the R and NR groups (n=6). Blood samples were fractionated by centrifugation, and the plasma samples were stored at -20 °C until used to determine the plasma PRL levels by an ELISA. A postmortem examination of each hen was performed to confirm its reproductive status.

3.3.2.2 Experiment II

To determine the distributions of TH-ir neurons and fibers in the brain of the native Thai hens at the different time periods of the R and NR hens, twenty four female and four male native Thai chickens, 22-24 weeks old, were used. After hatching, all the hens were divided into two groups; R and NR groups (n=6). In the R group, the hens were allowed to take care of their chicks for 4 and 10 days, whereas in the NR groups, the hens were deprived from their chicks at the same time periods of the R group. Both groups were raised in floor pens with the roosters and observed their daily behaviors. The brain of each hen was pressure-perfused, sectioned with a

cryostat, and processed by IHC to visualize and analyze the changes in the number of TH-ir neurons throughout the brain. A postmortem examination of each hen was performed to confirm its reproductive status.

3.3.2.3 Experiment III

To study the association of DA neurons with the neuroendocrine regulation of rearing behavior, one hundred and two female and twenty five male native Thai chickens, 22-24 weeks old, were used. After hatching, the hens were divided into two groups; R and NR groups. The R hens were allowed to rear the chicks naturally. The NR hens were disrupted from rearing behavior and not allowed to rear their chicks by removing them from the chicks to another pen. Both groups were raised in floor pens with the roosters and observed their daily behaviors. All the hens were checked four times per day and classified as broody/rearing hens throughout the experiment. To compare the time courses in changes in the number of TH-ir neurons in the nI and ML areas, both R and NR hens were then sacrificed at different time periods (day of hatch, day 4, 7, 10, 14, 17, 21, 24, and 28; n=6) after they started to rear their chicks or after the chicks were removed. The brains were fixed by pressure perfusion prior to sectioning in a cryostat and further processed for IHC to visualize and analyze the changes in the number of TH-ir neurons in the nI and ML areas. A postmortem examination of each hen was performed to confirm its reproductive status.

3.3.3 PRL Hormone Assay

Plasma PRL levels were determined using an ELISA according to a previously described method (Kosonsiriluk et al., 2008). Briefly, plates were coated

with 100 µl of AffiniPure goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), which was diluted in 0.05 M potassium phosphate buffer (pH 7.4) at the dilution of 1:2,000 and incubated overnight at 4 °C. Surfaces were blocked by addition of blocking solution (100 µl per well of 0.4 % casein in 0.15 M phosphate buffered saline (PBS; pH 7.2) containing 1.0 mM ethylenediaminetetraacetic acid (EDTA) and 0.02 % thimerosal). After incubation, the plates were washed three times in 0.03 M PBS (pH 7.2) 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 microliters of samples (10 µl plasma diluted in 40 µl of assay buffer) or standards containing chicken PRL (kindly provide by Dr. A.F. Parlow, National Hormone and Peptide Program, USA) were added and 25 µl each of biotinylated-PRL (1:50,000 dilution), and rabbit anti-chicken PRL (kindly provide by Dr. John Proudman, USDA, USA) at 1:20,000 dilution were then added into the reaction and incubated overnight at 4 °C. After incubation, the plates were washed and 0.1 ml of streptavidin horseradish peroxidase (1:5,000 dilution) were added. After incubation at room temperature for 2 hrs, the 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 4.0) were added. After 1 hr of incubation at room temperature, the color reaction was then measured at 405 nm in an ELISA reader (Tecan Group Ltd., Mannedorf, Switzerland). The assay of plasma PRL levels in native Thai chickens was validated as follows. Pooled plasma samples of the native Thai chickens produced a dose-response curve that paralleled with the chicken PRL standard curve. The plasma samples were determined in duplicate within a single assay. The intra-assay coefficient of variation was 9.165 % and the

sensitivity was 3.9 ng/ml.

3.3.4 Processing of Tissues for Immunohistochemistry

Prior to perfusion, each hen was intravenously injected with 3,000 units of heparin (Baxter Healthcare Corporation, Deerfield, IL, USA), and then euthanized with pentobarbital sodium (Nembutal, Ceva Sante Animale). The head was removed and immediately fixed by pressure perfusion via the carotid arteries with 100 ml of PBS (pH 7.4) for 3-5 min, followed by 650 ml of a freshly prepared 4 % paraformaldehyde in 0.1 M PBS (pH 7.4) for 30 min according to a method previously described by Sartsoongnoen et al. (2008). The brain was then dissected intact from the skull and soaked in 20 % sucrose in the PBS (pH 7.4) at 4 °C for 48 hrs or until it is saturated for cryoprotection. The brain was then frozen in powdered dry ice for 1 hr, and stored at -35 °C until sectioned. Frozen brains were sectioned in the coronal plane at a thickness of 16 µm using a cryostat (Microtome cryostat HM525, Microm International GMbH, Walldorf, Germany). Sections were mounted onto chrome alum-gelatin-coated glass slides with two sections per slide and stored desiccated at -20 °C until used for IHC. Six adjacent sections of each individual brain area were processed by IHC to visualize and analyze the changes in the number of TH-ir neurons.

3.3.5 Immunohistochemistry

Changes in the number of TH-ir neurons in the brain of R and NR hens by IHC were conducted according to a previously described method (Sartsoongnoen et al., 2008; Prakobsaeng et al., 2011). The primary and secondary antibodies used for

detecting the TH-ir neurons were primary mouse monoclonal antibody raised directly against TH (ImmunoStar, Inc., Hudson, WI, USA) and Cy^{TM}3-conjugated AffiniPure donkey anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc.), respectively. Six adjacent sections of the individual hypothalamic areas from the R and NR hens at different time periods were thawed to room temperature prior to use. The sections were rehydrated in the PBS (pH 7.4) for 30 min at room temperature. After removing from the PBS, the sections were then incubated with 60 µl of primary antibody at 1:1,000 dilution in the PBS (pH 7.4) containing 1 % bovine serum albumin and 0.3 % Triton-X 100 at 4 °C overnight in a moist chamber. Subsequently, the sections were then washed three times with the PBS (pH 7.4) for 5 min each. After washing, 60 µl of secondary antibody at 1:500 dilution in the PBS (pH 7.4) was applied under dark conditions onto the sections. The slides were further incubated in a moist dark chamber at room temperature for 1 hr, washed with the PBS (pH 7.4) three times for 5 min each, and then mounted with DPX mountant (Sigma-Aldrich, Inc., Steinheim, Germany). Microscopic images of the brain sections were visualized and further analyzed.

3.3.6 Image Analysis

Microscopic images of the brain sections of the hens were visualized under a fluorescence microscope (Nikon ECLIPSE80i, Tokyo, Japan) using a cooled digital color camera (Olympus DP72, Tokyo, Japan). The images were captured and stored by DP72-BSW Software (Olympus, Tokyo, Japan). The differential expression of TH-ir neurons and fibers in each individual area of the brain was visualized and analyzed. The number of TH-ir neurons of six adjacent sections was counted manually to

determine changes in the numbers of TH-ir neurons in the individual hypothalamic areas. The TH-ir neurons counted from the six adjacent sections of each hen (6 hens per area) for each treatment group were averaged to determine the numbers of TH-ir neurons counted per section in each brain area. The mean values were compared between the R and NR hens at different time periods. To avoid double-counting neurons with cell bodies that appeared on two adjacent sections, sections were viewed under 400x 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 TH immunoreactivity. The specificity of the anti-TH antibody was tested by omission of the primary antibody during that step of IHC. No immunostaining of TH was observed in the control sections.

3.3.7 Statistical Analysis

Significant differences in the plasma PRL levels (means ± SEM) according to each treatment group and the number of TH-ir neurons (means ± SEM) in the individual hypothalamic areas according to each treatment group were compared utilizing one-way analysis of variance (ANOVA). Significant differences between the treatment groups were computed utilizing the Tukey's HSD Test. Differences were considered significant if the P-value was less than 0.05. All statistical tests were analyzed using the SPSS for Windows Software (version 13.0, SPSS Inc., Chicago, IL, USA).

3.4 Results

3.4.1 Experiment I

The plasma PRL levels of R and NR hens at different time periods are shown in Table 3.1 and Figure 3.1. On the day the chicks were hatched, the plasma PRL levels remained at high levels ($P<0.05$; 138.74 ± 24.58 ng/ml), then rapidly decreased after 4 days of hatching (50.43 ± 11.40 ng/ml), and remained at low levels throughout the observed rearing period (day 7; 27.83 ± 3.99, day 10; 27.29 ± 4.64, day 14; 27.51 ± 2.98, day 17; 31.44 ± 11.36, day 21; 31.72 ± 2.83, day 24; 30.52 ± 3.84, day 28; 32.61 ± 5.50 ng/ml). In the NR group, when the chicks were removed from the hens after hatching, the plasma PRL levels (ng/ml) were low at day 4 (26.73 ± 2.34), day 7 (28.86 ± 4.55), day 10 (25.62 ± 3.81), day 14 (22.34 ± 5.60), day 17 (23.31 ± 2.29), day 21 (16.03 ± 2.20), day 24 (18.03 ± 2.39), and day 28 (18.29 ± 5.70). During the observed rearing period, plasma PRL levels of the R hens were compared with those of the NR hens. The levels of plasma PRL significantly decreased at 21 days after the chicks were removed from the hens ($P<0.05$; R21 vs NR21; 31.72 ± 2.83 vs 16.03 ± 2.20 ng/ml) and 24 days ($P<0.05$; R24 vs NR24; 30.52 ± 3.84 vs 18.03 ± 2.39 ng/ml). However, the plasma PRL levels were not different between the R and NR hens at day 4 to day 17 and day 28. Taken together, in the R hens, plasma PRL levels were high when compared to those of the NR hens. Disruption of rearing behavior by removing the chicks was accompanied by a precipitous decline in plasma PRL levels.

3.4.2 Experiment II

As revealed by IHC, the expressions of hypothalamic TH-ir neurons were observed at day 4 and day 10 of the R and NR native Thai hens across the

hypothalamic areas; nucleus septalis lateralis (SL), nucleus anterior medialis hypothalami (AM), nucleus suprachaiasmaticus, pars medialis (SCNm), organum paraventriculare (PVO), regio lateralis hypothalami (LHy), nucleus perventricularis hypothalami (PHN), nI, and ML (Table 3.2, Figures 3.2-3.5). The greatest density of TH-ir neurons was observed in the nI and ML areas (Figure 3.5). A few number of TH-ir neurons were also found in the SL and PHN of both R and NR hens. A dense accumulation of TH-ir fibers were found in the median eminence (ME) and nucleus mamillaris medialis (MM) as shown in Figure 3.6. The distributions of TH-ir neurons were also found in the other areas of day 4 and day 10 in both groups. Some of the TH-ir neurons were found in the AM, LHy, and PVO in both groups (Table 3.3, Figures 3.7 and 3.8). The results revealed that the highest accumulation of TH-ir neurons was found within the nI of R hens at day 4 (Table 3.3; Figures 3.7 and 3.8; R4; 39.17 ± 3.45 cells), and day 10 (Table 3.3; Figures 3.7 and 3.8; R10; 39.83 ± 1.82 cells), whereas the number of TH-ir neurons in nI was decreased in the NR hens ($P<0.05$; NR4; 24.71 ± 1.12 cells and NR10; 27.79 ± 2.42 cells). TH-ir neurons abundance was also observed in the ML (Table 3.3; Figures 3.7 and 3.8; R4 vs NR4; 39.71 ± 2.05 vs 32.38 ± 3.43 cells, R10 vs NR10; 42.04 ± 5.94 vs 39.13 ± 4.24 cells), but the difference between treatment groups was not statistically significant ($P>0.05$).

3.4.3 Experiment III

The differential expression of TH-ir neurons in the nI of R and NR hens are illustrated in Figures 3.9-3.11. The number of TH-ir neurons in the nI of R and NR hens are shown in Figure 3.9 and Table 3.4. When compared between the R and NR groups, TH-ir neurons counted (cells) significantly decreased in the NR hens at days

4, 7, 10, and 14 ($P<0.05$; R4 vs NR4; 39.17 ± 3.45 vs 24.71 ± 1.12, R7 vs NR7; 42.88 ± 1.56 vs 22.83 ± 1.51, R10 vs NR10; 39.83 ± 1.82 vs 27.79 ± 2.42, R14 vs NR14; 35.50 ± 1.92 vs 24.46 ± 3.22). The number of TH-ir neurons showed no difference after 14 days at the time periods of observation through 24 days in both groups. However, at day 28, the number of TH-ir neurons was significantly different between the R and NR hens ($P<0.05$; R28 vs NR28; 21.46 ± 2.26 vs 13.63 ± 0.89 cells)

The differential expression of TH-ir neurons in the ML of R and NR hens are also shown in Figures 3.12-3.14. The numbers of TH-ir neurons in the ML of R and NR hens are shown in Figure 3.12 and Table 3.4. The number of TH-ir neurons (cells) in the ML markedly declined after the day of hatch and through 14 days of the rearing period, but there are no significantly different between both groups. ($P>0.05$; R4 vs NR4; 39.67 ± 2.02 vs 32.38 ± 3.43, R7 vs NR7; 41.13 ± 6.25 vs 32.79 ± 1.81, R10 vs NR10; 42.04 ± 5.94 vs 39.13 ± 4.24, R14 vs NR14; 51.50 ± 5.92 vs 44.08 ± 5.74). In the R group, the number of TH-ir neurons in the ML tended to decrease after 17 days of rearing chicks. In the NR group, TH-ir neurons in the ML stayed at the same levels from day 4 throughout day 14 and then sharply decreased at day 17 during the observation time periods. The distribution patterns of TH-ir neurons in the nI and ML areas were consistent in every R hens. When the hens were removed from their chicks, the number of TH-ir neurons decreased in the same discrete patterns.

Table 3.1 Mean ± SEM of plasma PRL concentrations (ng/ml) of rearing and non-rearing native Thai hens at different days of rearing periods (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group at a given time point.

Group	**Days Following of Chicks' Deprivation from Hens**				
	Day of Hatch	**4**	**7**	**10**	**14**
Rearing	138.74 ± 24.58^{a}	50.43 ± 11.40^{b}	27.83 ± 3.99^{b}	27.29 ± 4.64^{b}	27.51 ± 2.98^{b}
Non-rearing	N/A	26.73 ± 2.34^{A}	28.86 ± 4.55^{A}	25.62 ± 3.81^{A}	22.34 ± 5.60^{A}

Table 3.1 Mean ± SEM of plasma PRL concentrations (ng/ml) of rearing and non-rearing native Thai hens at different days of rearing periods (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group at a given time point (Continued).

Group	**Days Following of Chicks' Deprivation from Hens**			
	17	**21**	**24**	**28**
Rearing	31.44 ± 11.36^{b}	$31.72 \pm 2.83^{b*}$	$30.52 \pm 3.84^{b*}$	32.61 ± 5.50^{b}
Non-rearing	23.31 ± 2.29^{A}	16.03 ± 2.20^{A}	18.03 ± 2.39^{A}	18.29 ± 5.70^{A}

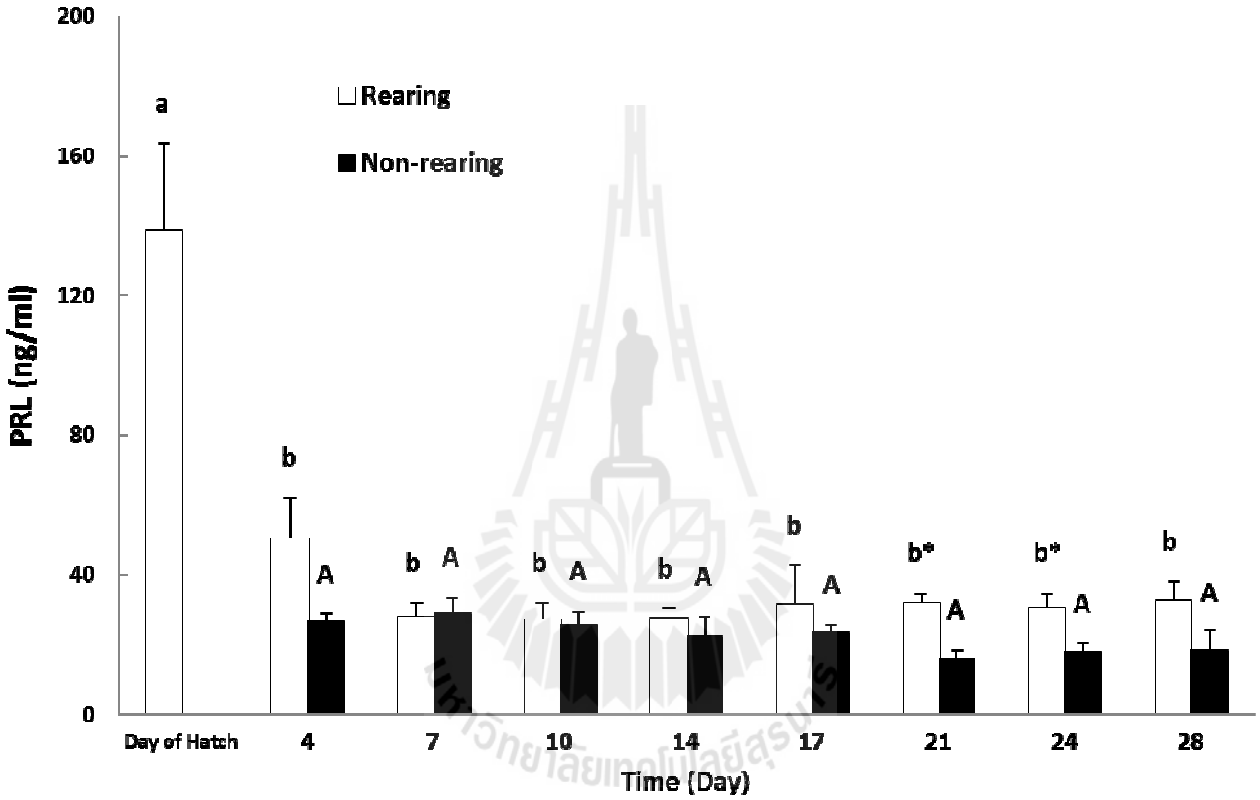

Figure 3.1 Changes in plasma PRL concentrations of rearing and non-rearing native Thai hens after the day of hatch through 28 days of observation period (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group at a given time point.

Table 3.2 Abbreviations of brain areas. Nomenclature and abbreviations are from a stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988).

SL	Nucleus septalis lateralis
AM	Nucleus anterior medialis hypothalami
SCNm	Nucleus suprachaiasmaticus, pars medialis
PVO	Organum paraventriculare
LHy	Regio lateralis hypothalami
PHN	Nucleus perventricularis hypothalami
ME	Eminentia mediana (Median eminence)
nI	Nucleus intramedialis
MM	Nucleus mamillaris medialis
ML	Nucleus mamillaris lateralis
V III	Ventriculus tertius (Third ventricle)

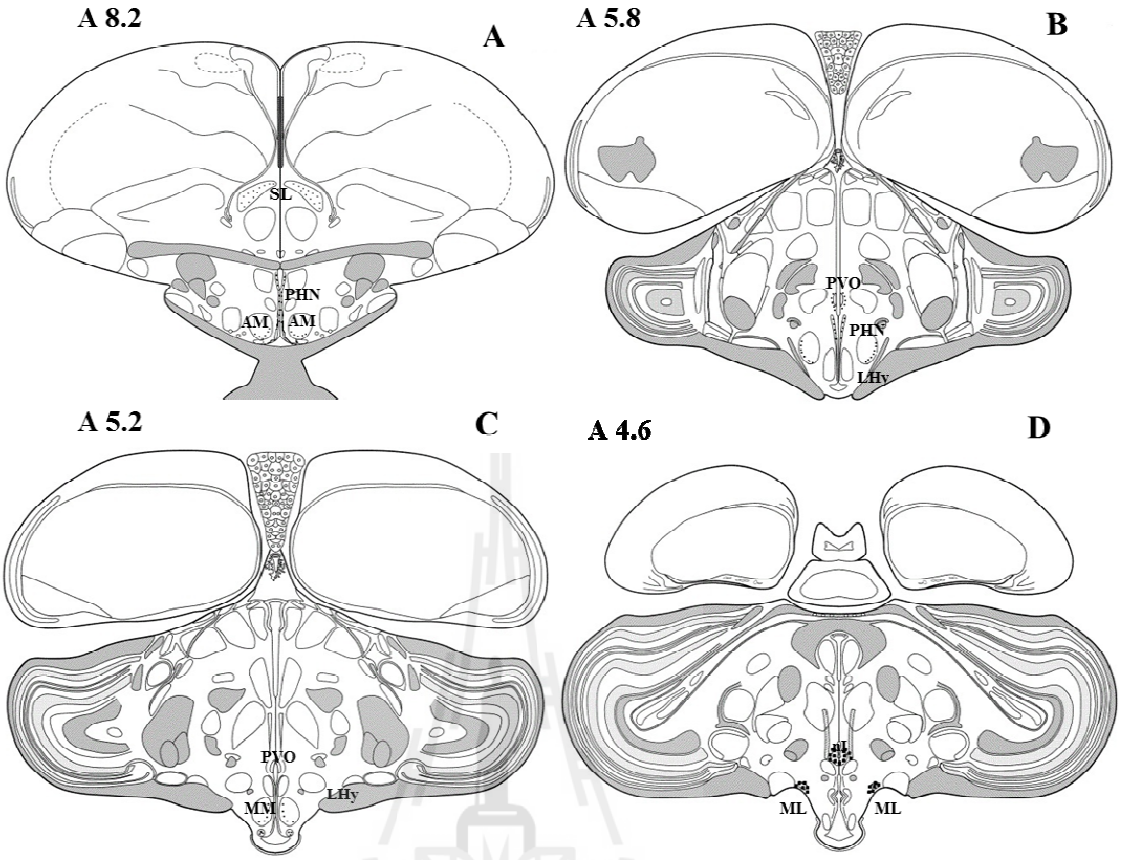

Figure 3.2 Schematic coronal brain sections illustrating the areas where the expression of TH-ir (black dot) was observed (**A-D**). The sampling regions for counting the number of TH-ir neurons in the nI and ML (**D**) are represented by rectangles. Coronal illustrations were redrawn from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988).

Table 3.3 The number of TH-ir neurons in the individual hypothalamic areas of rearing (R) and non-rearing (NR) native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different areas are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group in each area.

Group	**Hypothalamic Area**						
	AM	**LHy**	**ML**	**nI**	**PHN**	**PVO**	**SL**
R4	10.67 ± 0.26^{b}	10.29 ± 2.89^{b}	39.71 ± 2.05^{a}	$39.17 \pm 3.45^{a*}$	5.25 ± 0.62^{b}	11.58 ± 1.33^{b}	6.79 ± 0.49^{b}
NR4	10.17 ± 0.89^{C}	5.17 ± 0.60^{C}	32.38 ± 3.43^{A}	24.71 ± 1.12^{B}	4.00 ± 0.41^{C}	8.79 ± 1.05^{C}	5.58 ± 0.69^{C}
R10	10.71 ± 0.99^{b}	6.25 ± 0.54^{b}	42.04 ± 5.94^{a}	$39.83 \pm 1.82^{a*}$	3.38 ± 0.20^{b}	9.92 ± 0.68^{b}	7.88 ± 1.04^{b}
NR10	10.96 ± 1.21^{C}	4.79 ± 0.62^{C}	39.13 ± 4.24^{A}	27.79 ± 2.42^{B}	4.04 ± 0.30^{C}	11.21 ± 0.84^{C}	6.00 ± 0.62^{C}

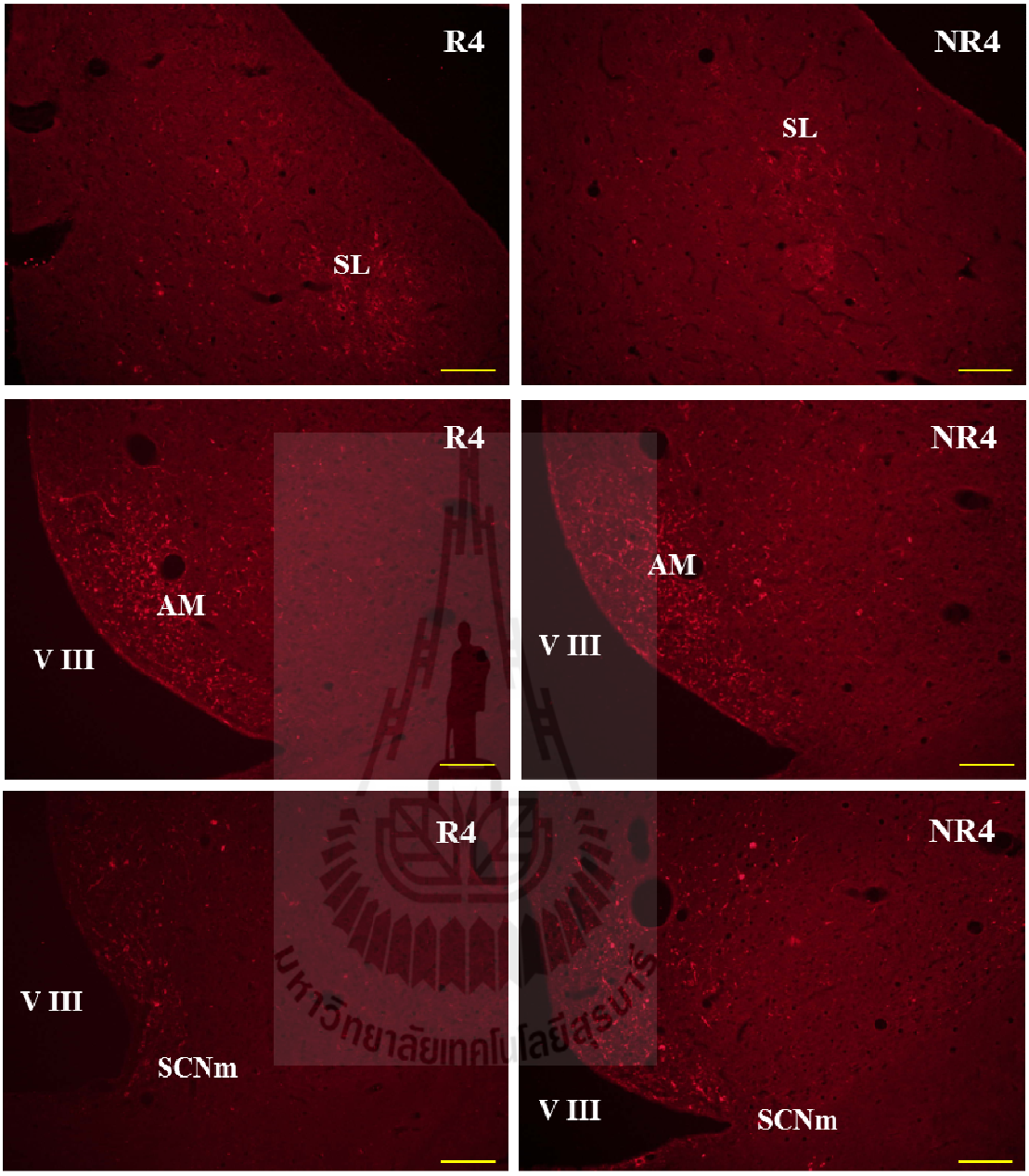

Figure 3.3 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the hypothalamus of rearing (R) and non-rearing (NR) native Thai hens at day 4. For abbreviations, see Table 3.2. Scale bar = 100 µm.

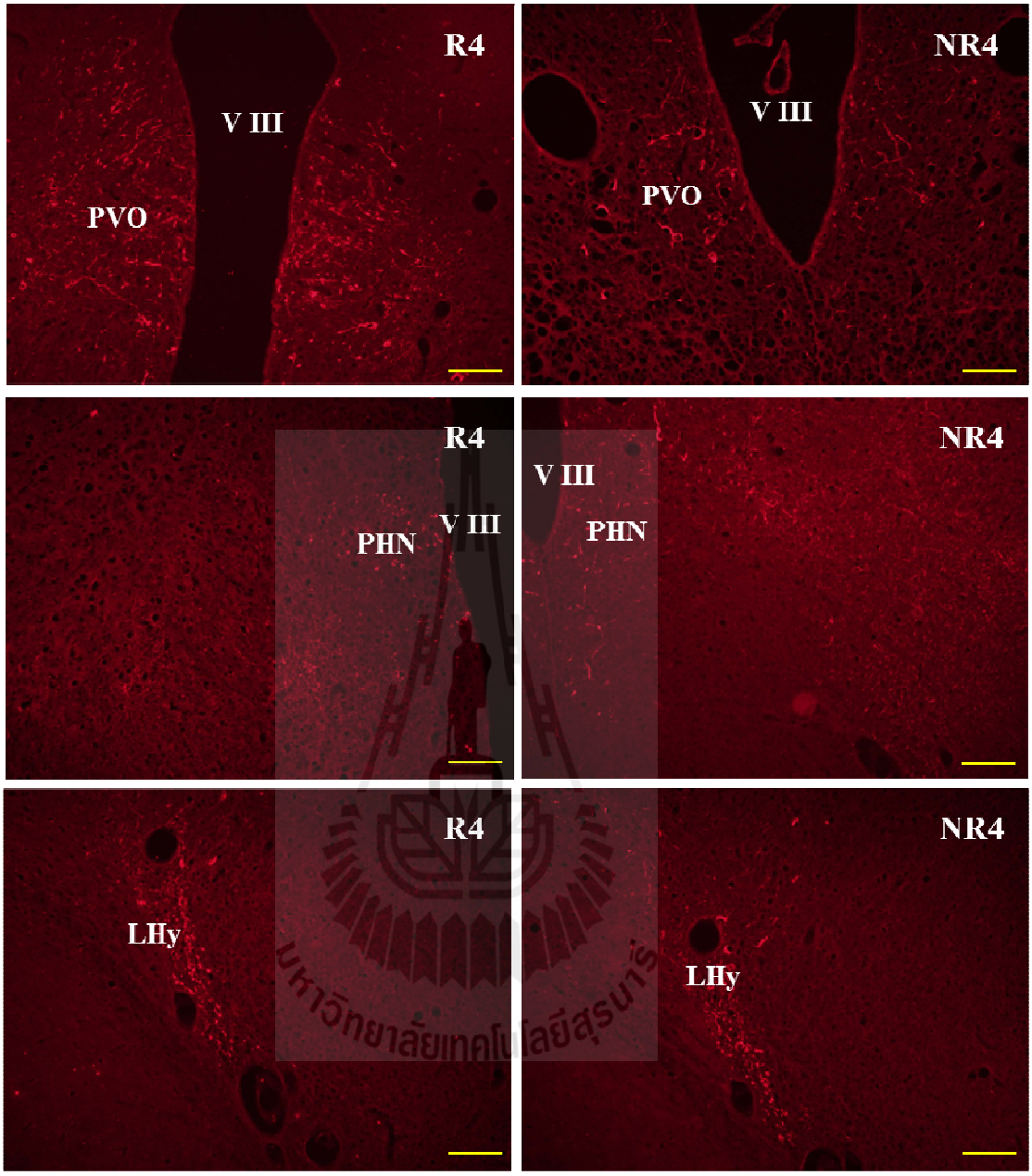

Figure 3.3 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the hypothalamus of rearing (R) and non-rearing (NR) native Thai hens at day 4. For abbreviations, see Table 3.2. Scale bar = 100 µm. (Continued).

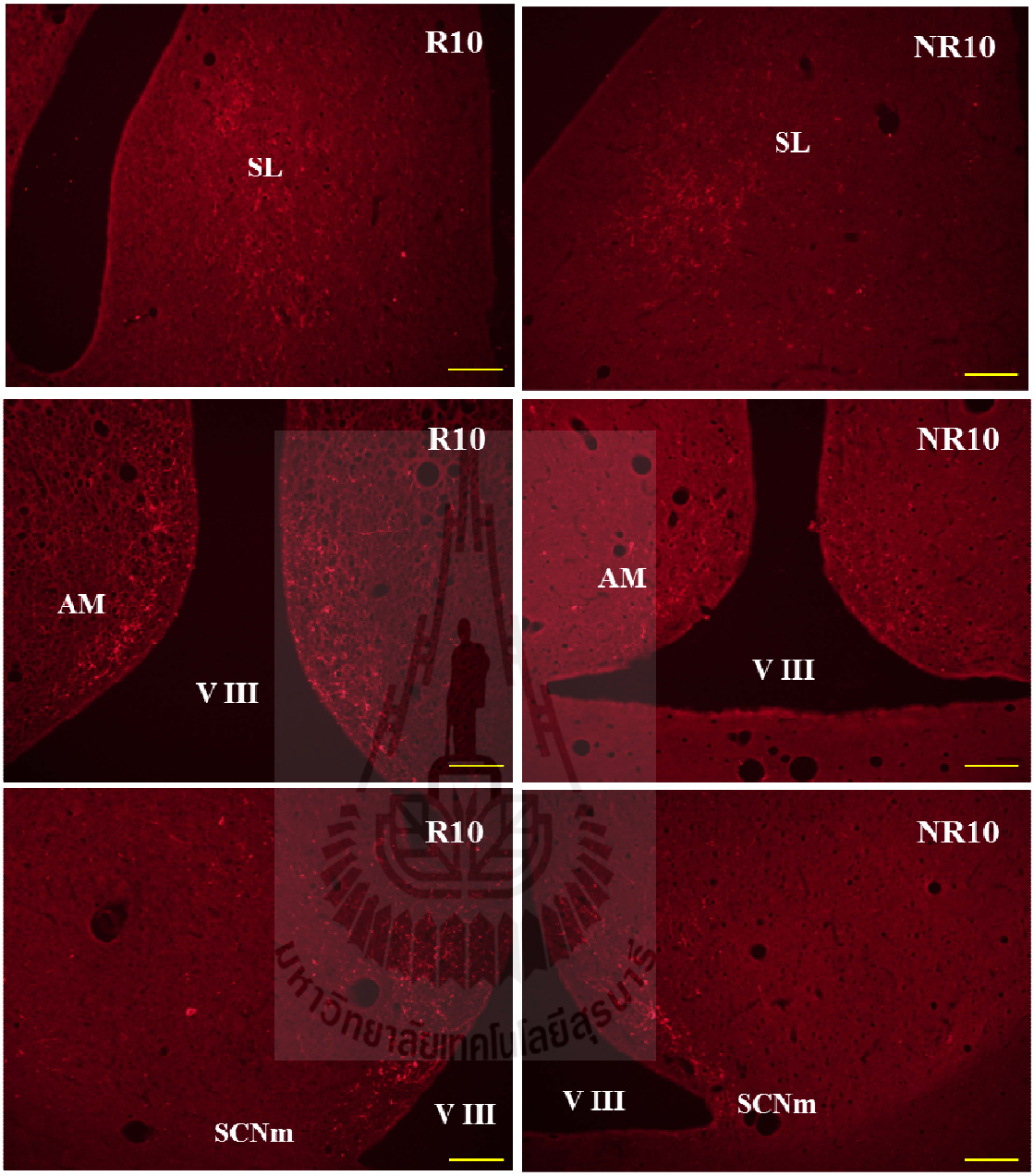

Figure 3.4 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the hypothalamus of rearing (R) and non-rearing (NR) native Thai hens at day 10. For abbreviations, see Table 3.2. Scale bar = 100 µm.

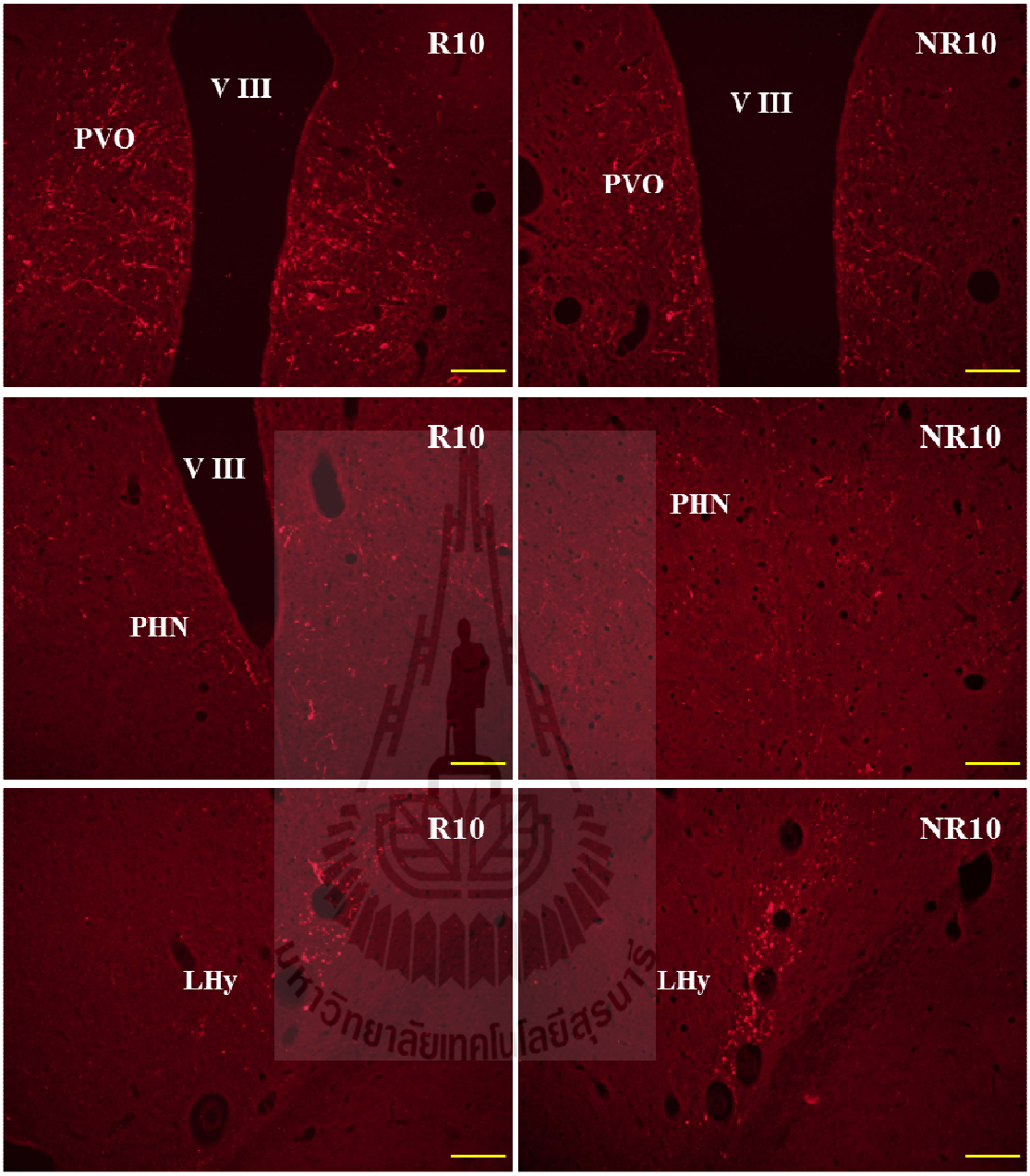

Figure 3.4 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the hypothalamus of rearing (R) and non-rearing (NR) native Thai hens at day 10. For abbreviations, see Table 3.2. Scale bar = 100 µm. (Continued).

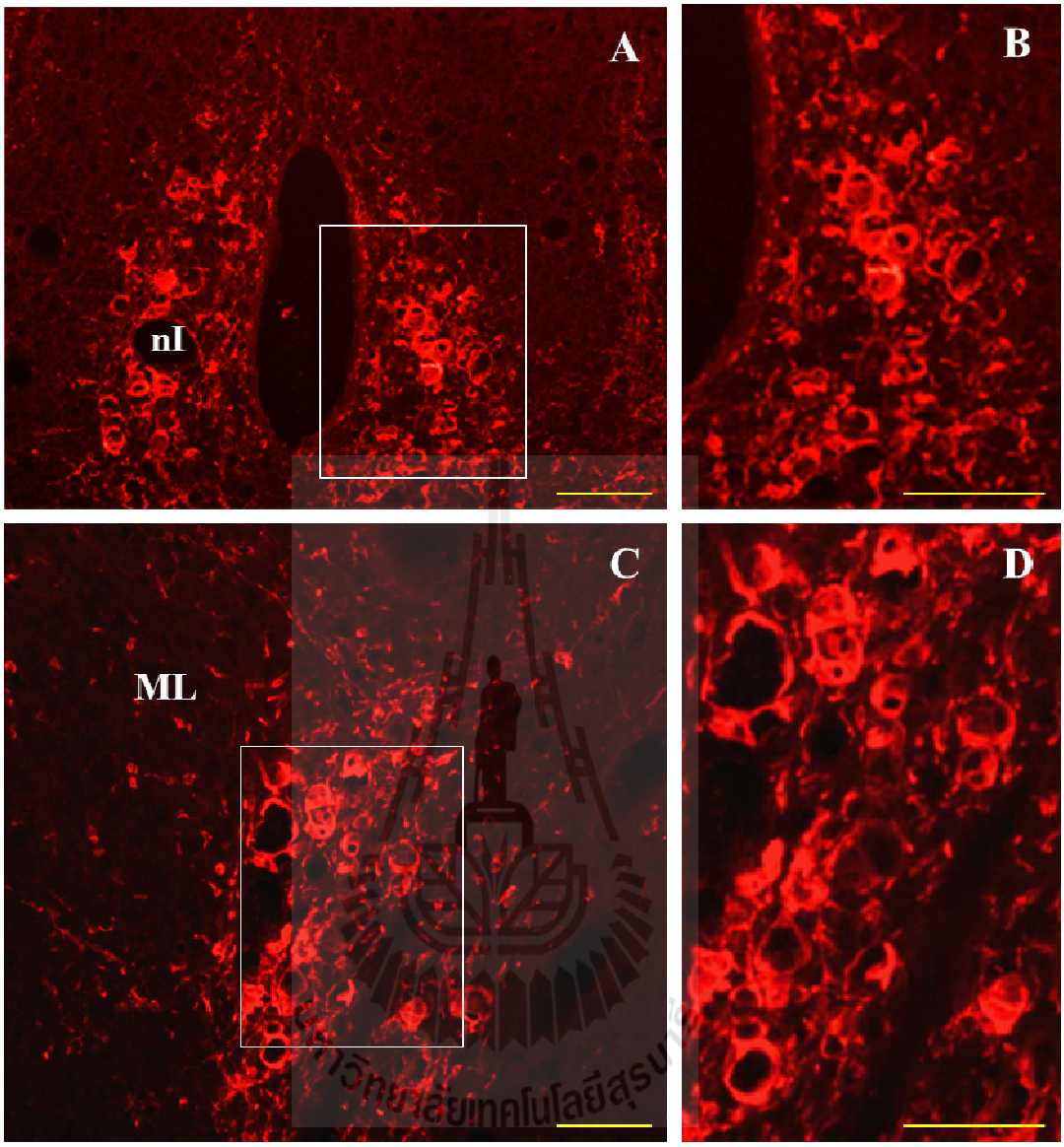

Figure 3.5 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the nucleus intramedialis (nI; **A**) and nucleus mamillaris lateralis (ML; **C**) of the native Thai chicken. Rectangles indicate areas from which following photomicrographs are taken. Higher magnification of the TH-ir neurons in the nI (**B**) and ML (**D**). For abbreviations, see Table 3.2. Scale bar = 50 µm.

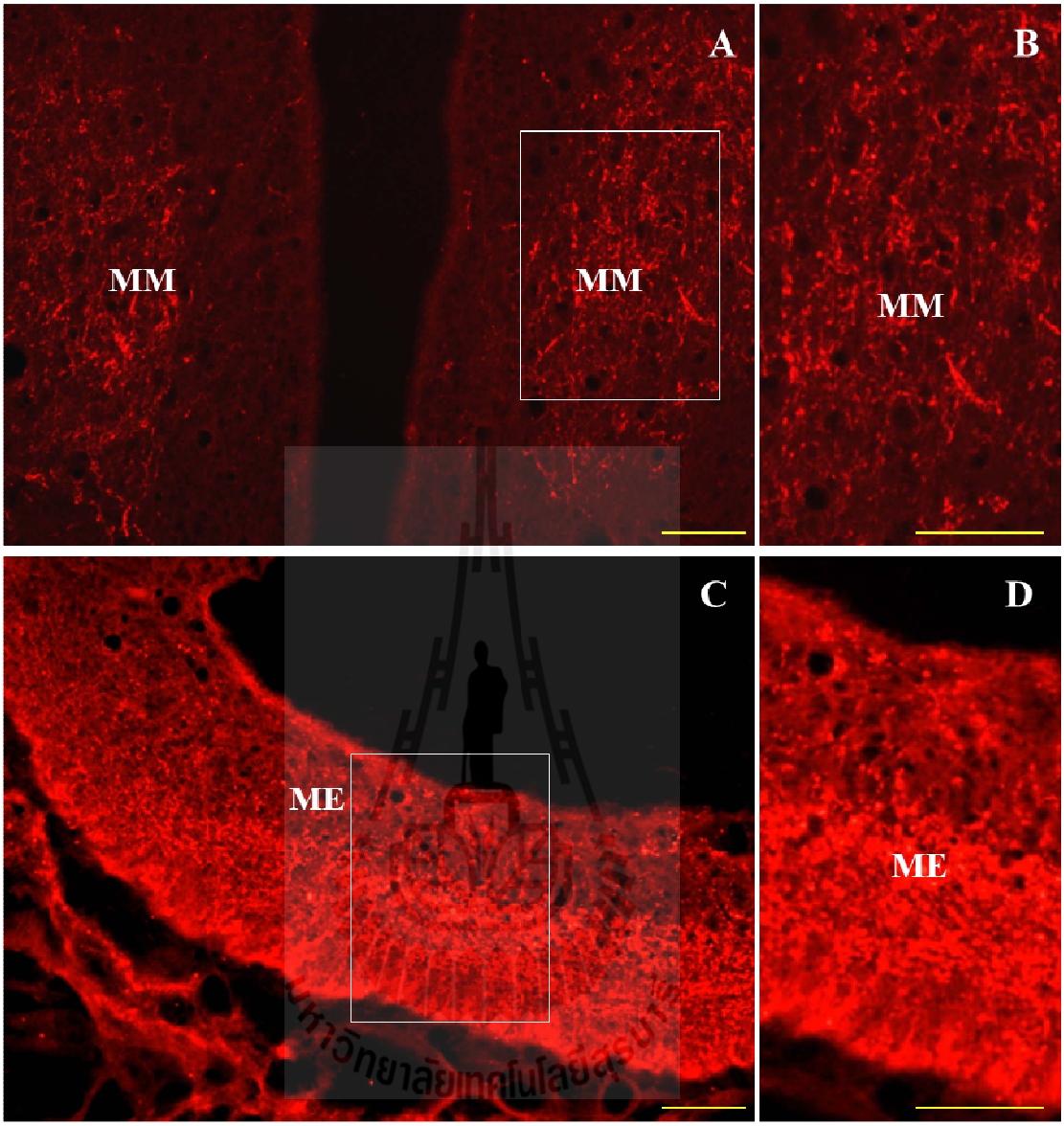

Figure 3.6 Photomicrographs showing the accumulations of TH-ir fibers in the nucleus mamillaris medialis (MM; **A**) and median eminence (ME; **C**) of the rearing native Thai hens. Rectangles indicate areas from which following photomicrographs are taken. Higher magnification of the TH-ir neurons in the MM (**B**) and ME (**D**). For abbreviations, see Table 3.2. Scale bar = 50 µm.

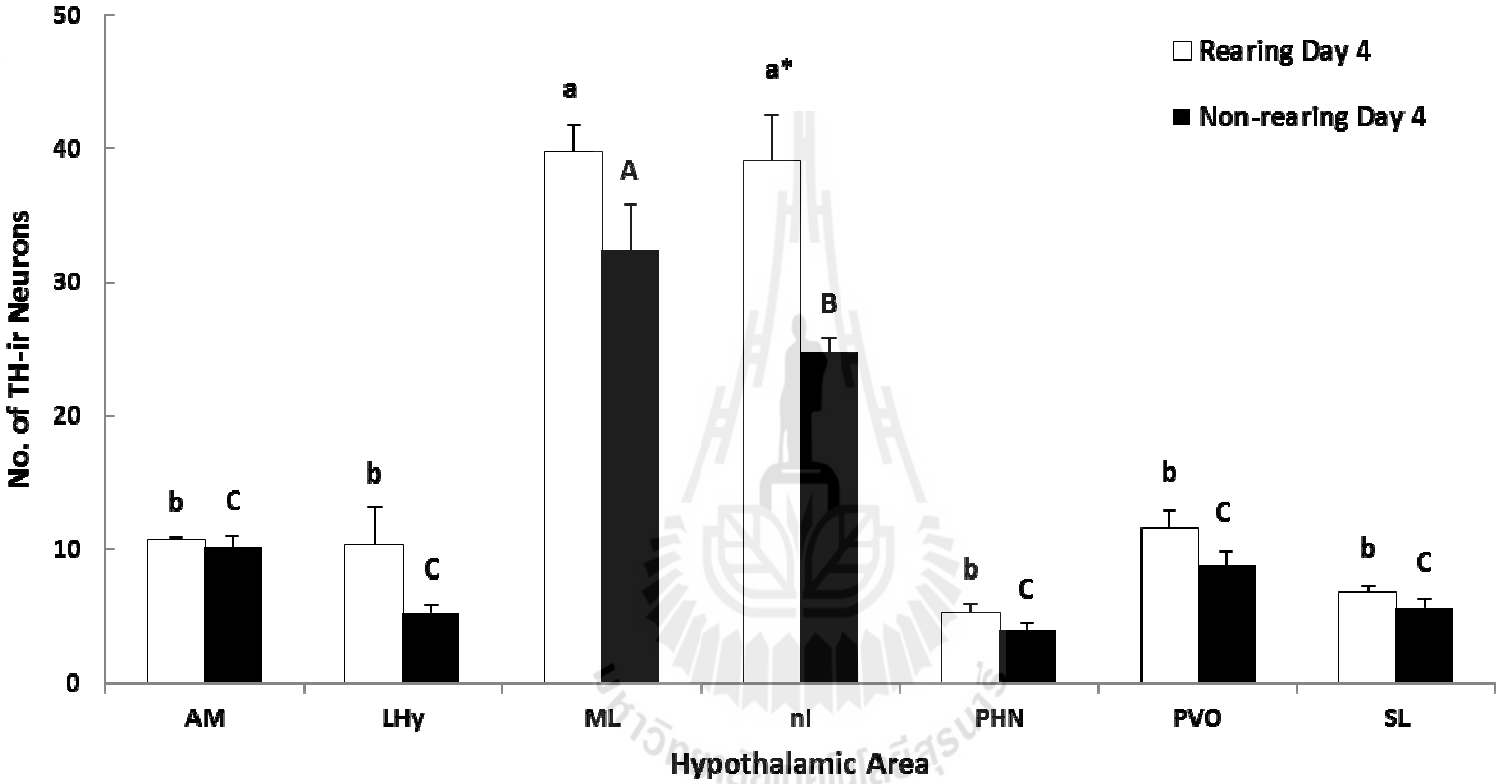

Figure 3.7 Changes in the number of TH-ir neurons in the individual hypothalamic areas (AM, LHy, ML, nI, PHN, PVO, and SL) of rearing and non-rearing native Thai hens at day 4 (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different areas are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group in each area.

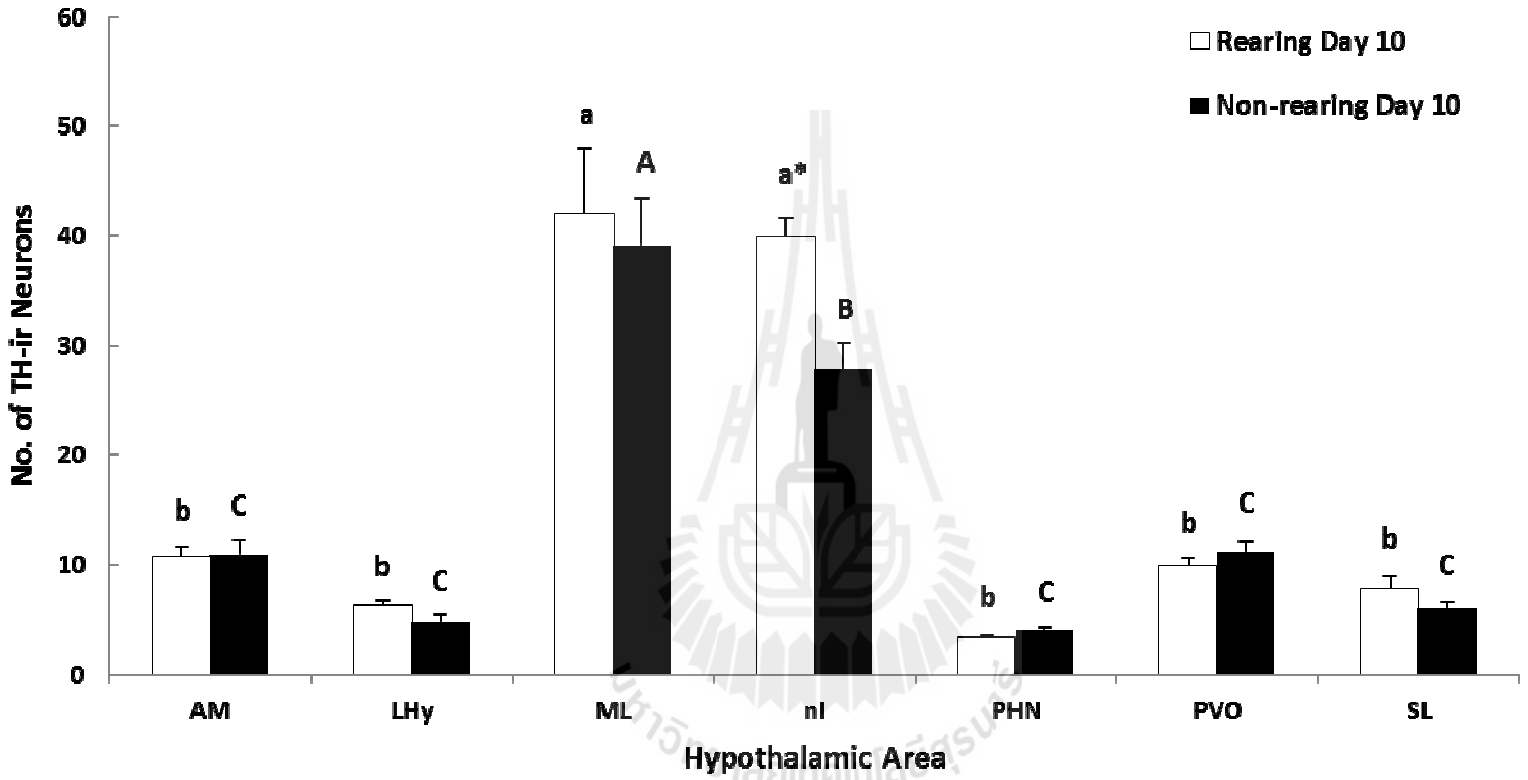

Figure 3.8 Changes in the number of TH-ir neurons in the individual hypothalamic areas (AM, LHy, ML, nI, PHN, PVO, and SL) of rearing and non-rearing at day 10 of native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different areas are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group in each area.

Table 3.4 The number of TH-ir neurons (Mean ± SEM) in the nI and ML of rearing and non-rearing native Thai hens at different days of the observation periods (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group at a given time point.

Area	Group	Days Following of Chicks' Deprivation from Hens				
		Day of Hatch	**4**	**7**	**10**	**14**
nI	**Rearing**	39.96 ± 1.44^{a}	$39.17 \pm 3.45^{a*}$	$42.88 \pm 1.56^{a*}$	$39.83 \pm 1.82^{a*}$	$35.50 \pm 1.92^{a*}$
	Non-rearing	N/A	24.71 ± 1.12^{AB}	22.83 ± 1.51^{ABC}	27.79 ± 2.42^{A}	24.46 ± 3.22^{AB}
ML	**Rearing**	38.13 ± 4.47^{ab}	39.67 ± 2.02^{ab}	41.13 ± 6.25^{a}	42.04 ± 5.94^{a}	51.50 ± 5.92^{a}
	Non-rearing	N/A	32.38 ± 3.43^{AB}	32.79 ± 1.81^{AB}	39.13 ± 4.24^{A}	44.08 ± 5.74^{A}

Table 3.4 The number of TH-ir neurons (Mean ± SEM) in the nI and ML of rearing and non-rearing native Thai hens at different days of the observation periods (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group at a given time point (Continued).

Area	Group	Days Following of Chicks' Deprivation from Hens			
		17	**21**	**24**	**28**
nI	**Rearing**	18.92 ± 2.02^{b}	16.96 ± 2.19^{b}	16.79 ± 2.11^{b}	$21.46 \pm 2.26^{b*}$
	Non-rearing	13.88 ± 1.82^{C}	17.67 ± 2.42^{BC}	16.17 ± 2.12^{BC}	13.63 ± 0.89^{C}
ML	**Rearing**	21.00 ± 1.76^{bc}	16.79 ± 3.16^{c}	15.50 ± 1.65^{c}	20.96 ± 3.49^{bc}
	Non-rearing	21.71 ± 4.39^{BC}	18.38 ± 2.23^{BC}	19.83 ± 2.56^{BC}	13.13 ± 0.92^{C}

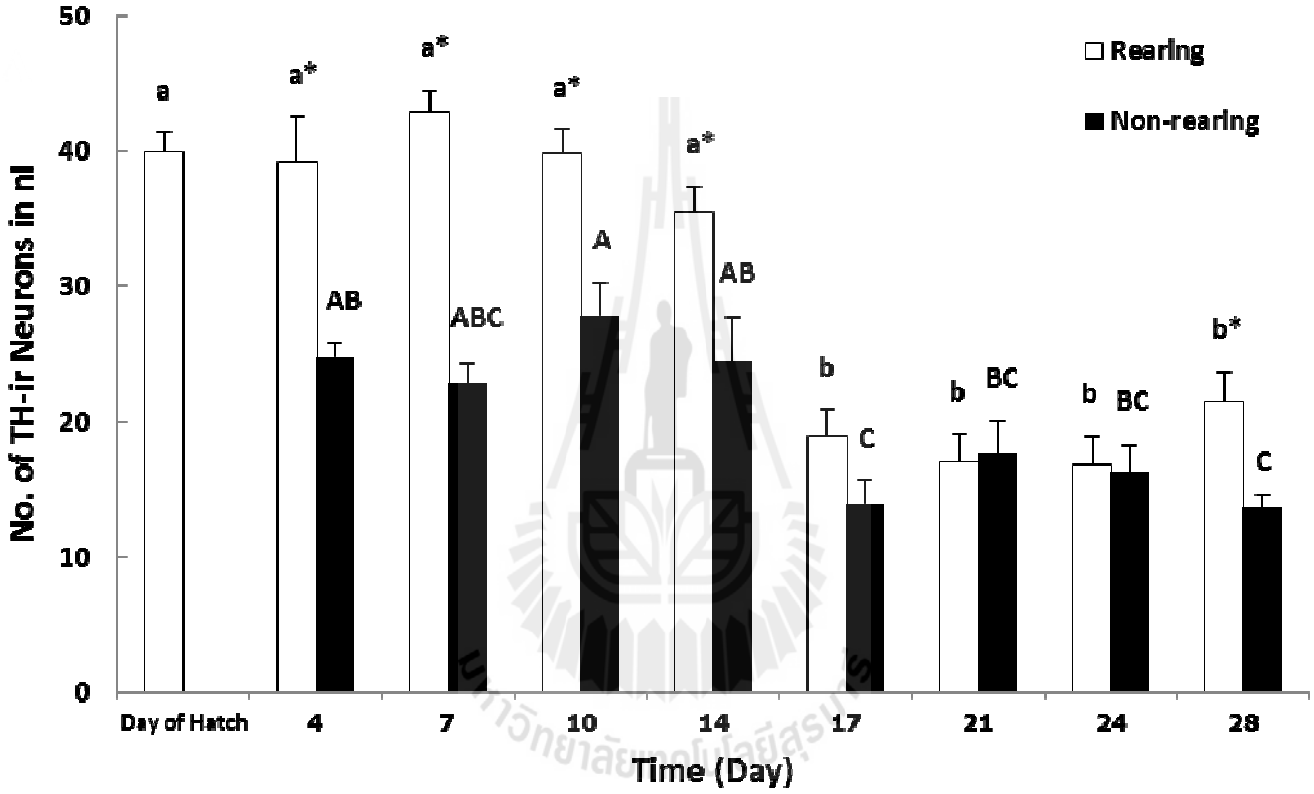

Figure 3.9 Changes in the number of TH-ir neurons in the nucleus intramedialis (nI) of rearing and non-rearing native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group at a given time point.

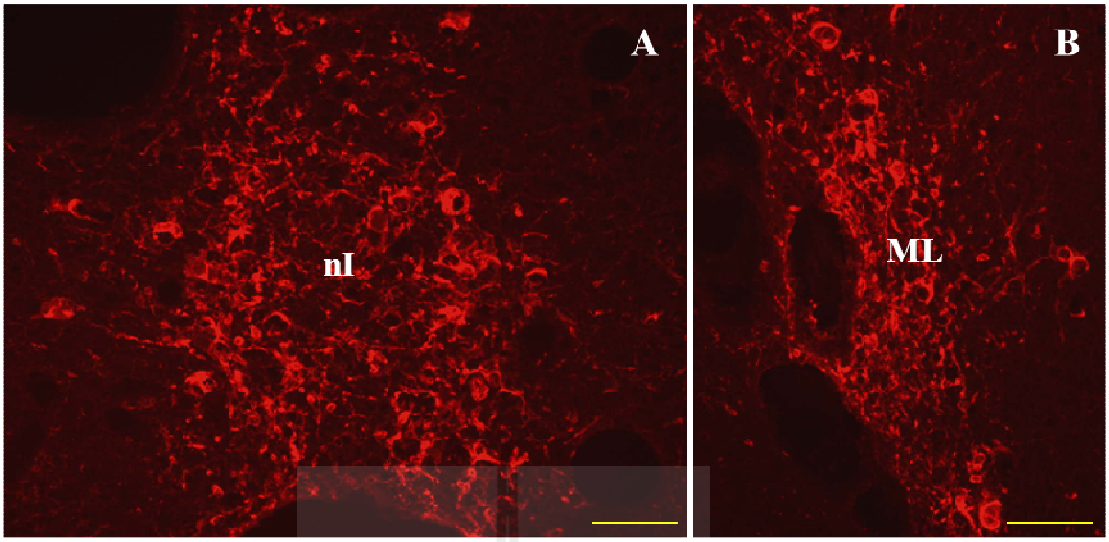

Figure 3.10 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the nucleus intramedialis (nI; **A**) and nucleus mamillaris lateralis (ML; **B**) at the day of hatch in the native Thai hens. For abbreviations, see Table 3.2. Scale bar = 50 µm.

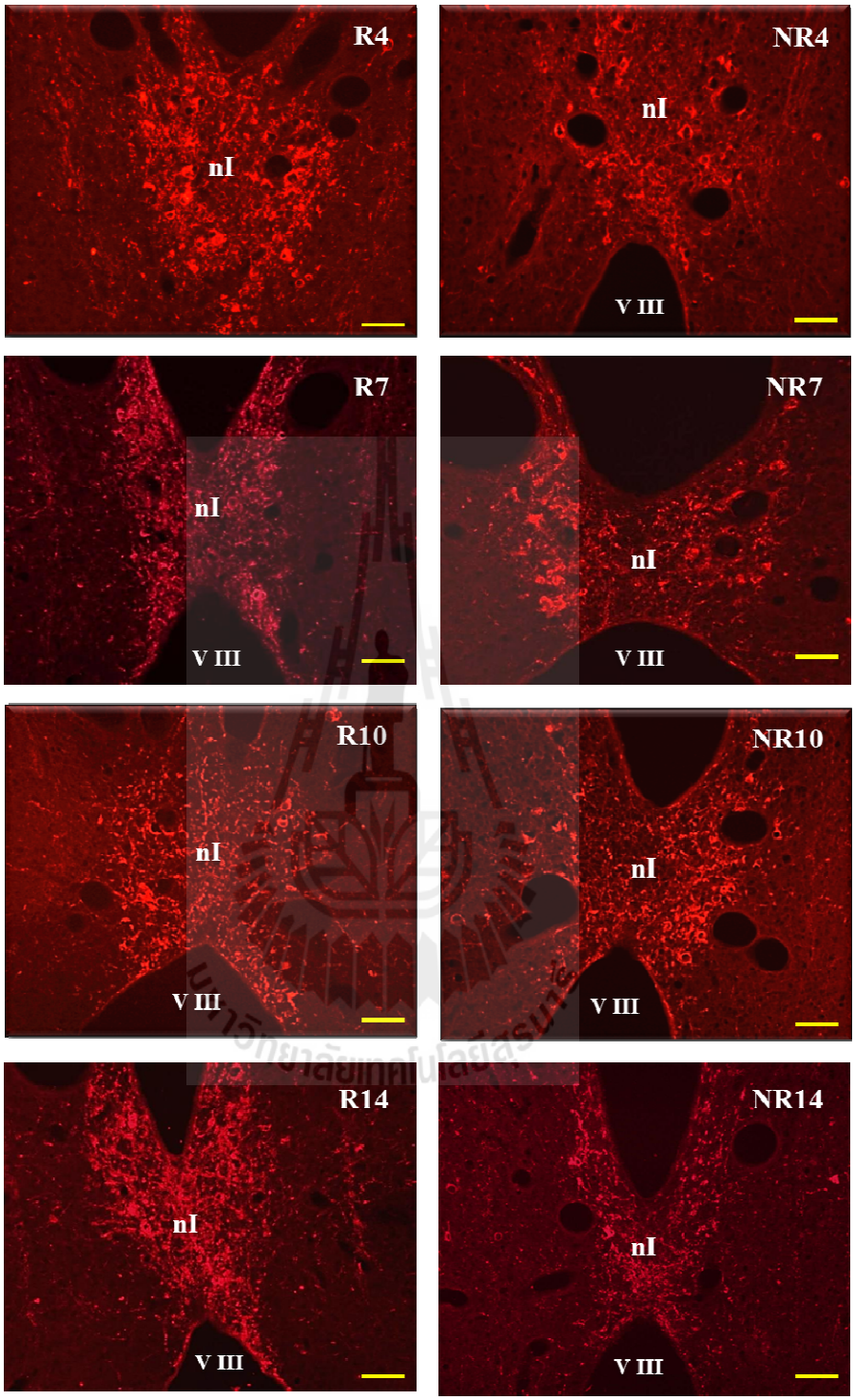

Figure 3.11 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the nucleus intramedialis (nI) of rearing (R) and non-rearing (NR) native Thai hens on different days following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 3.2. Scale bar = 100 µm.

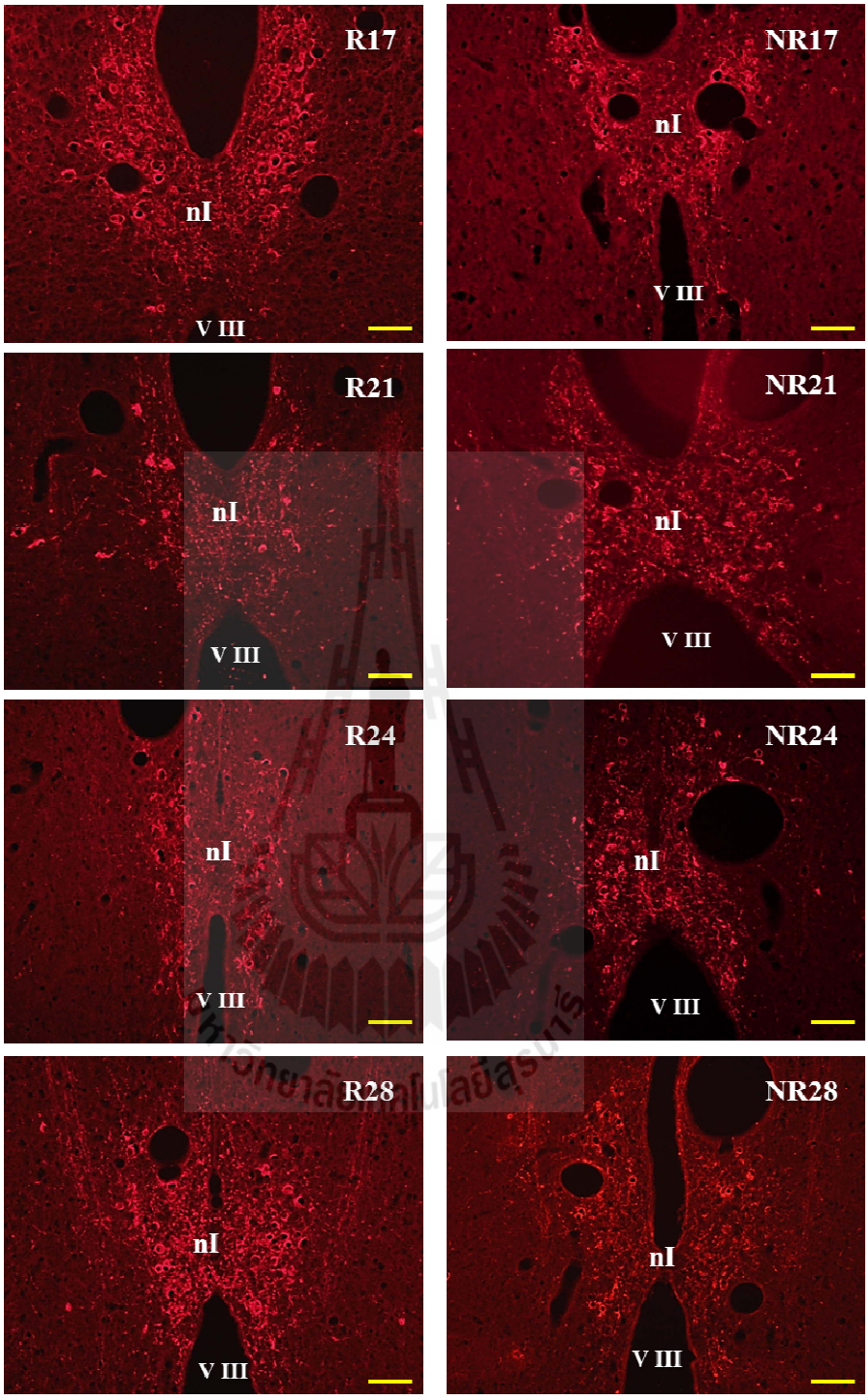

Figure 3.11 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the nucleus intramedialis (nI) of rearing (R) and non-rearing (NR) native Thai hens on different days following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 3.2. Scale bar = 100 µm (Continued).

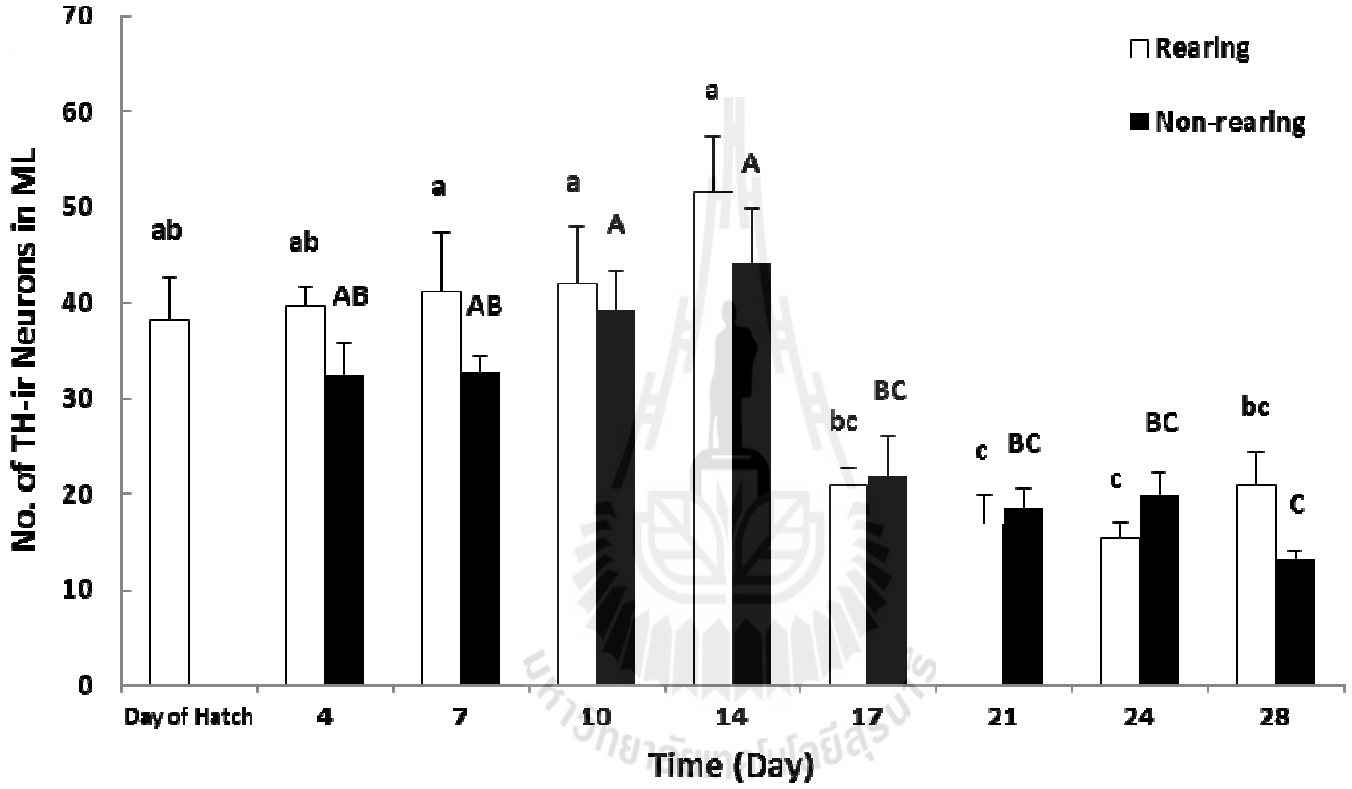

Figure 3.12 Changes in the number of TH-ir neurons in the nucleus mamillaris lateralis (ML) of rearing and non-rearing native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group at a given time point.

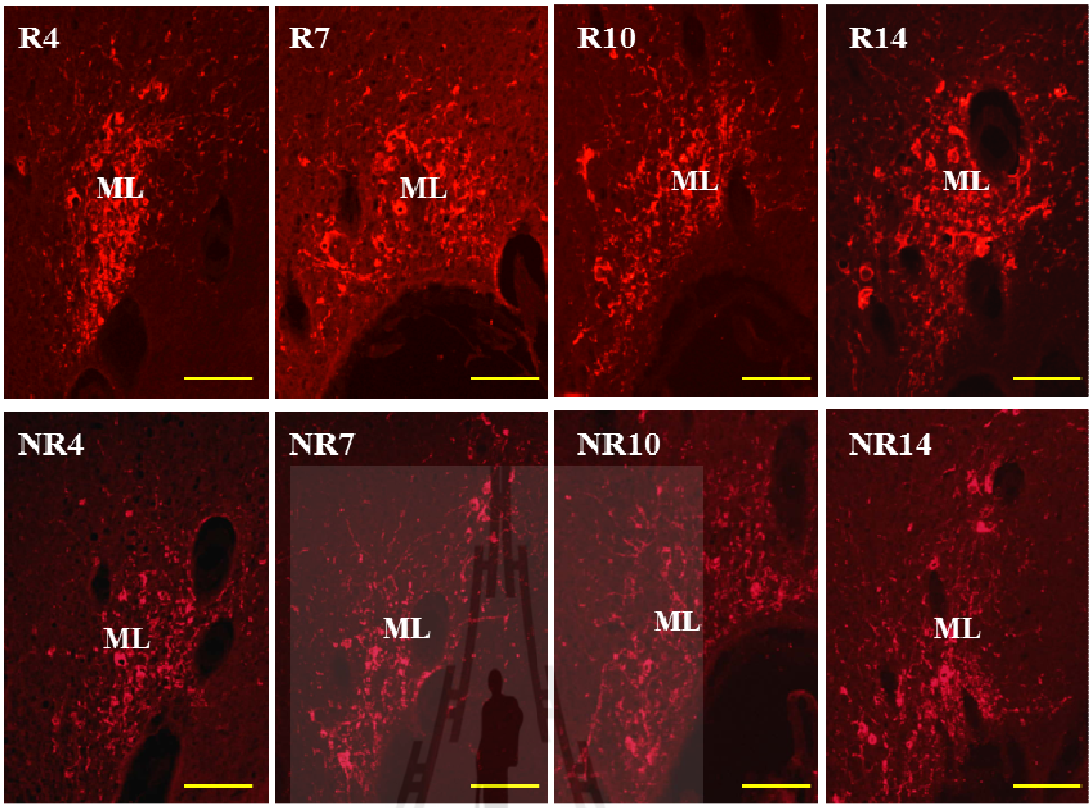

Figure 3.13 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the nucleus mamillaris lateralis (ML) of rearing (R) and non-rearing (NR) native Thai hens at day 4, day 7, day 10, and day 14 following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 3.2. Scale bar = 100 µm.

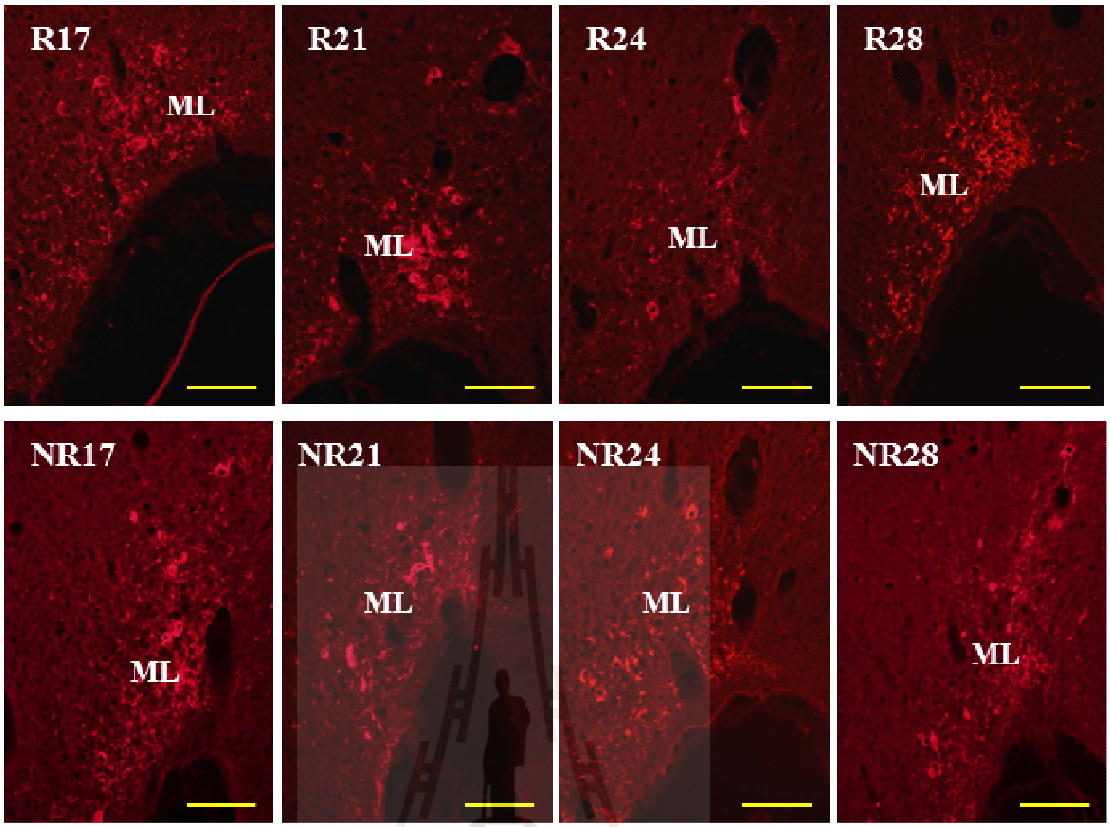

Figure 3.14 Photomicrographs illustrating the distributions of TH-ir neurons and fibers in the nucleus mamillaris lateralis (ML) of rearing (R) and non-rearing (NR) native Thai hens at day 17, day 21, day 24, and day 28 following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 3.3. Scale bar = 100 µm.

3.5 Discussion

The results of this present study clearly demonstrate an association between the hypothalamic DAergic system and circulating PRL levels during rearing behavior in the native Thai chickens. The results reveal marked differences in the number of TH-ir neurons in the nI and ML and plasma PRL levels between hens rearing chicks and non-rearing chicks, indicating a pivotal role of the DAergic and VIP/PRL systems in the initiation and maintenance of rearing behavior in this tropical and continuous breeding species.

The findings from this present study reveal that the TH-ir neurons and fibers were extensively distributed throughout the brain of R and NR native Thai hens and were predominantly expressed in the nI and ML areas. The expression of hypothalamic TH-ir neurons in the SL, AM, PVO, LHy, and PHN areas were also observed. A dense accumulation of TH-ir neurons was found in the nI of R hens. High density of TH-ir fibers was found in the MM and ME of both treatment groups. Changes in the number of TH-ir neurons within the nI and ML areas were compared between the R and NR hens. Significance decreases in the number of TH-ir neurons of the NR hens when compared with those of the R hens were observed in the nI after the day of hatch until 14 days of the observation periods. The number of TH-ir neurons showed no difference after 14 days at the time periods of observation through 24 days in both groups. The number of TH-ir neurons in the ML was high during the rearing period, but there was no significantly different between both groups. Decreasing of TH-ir neurons in the nI and ML areas of the R and NR hens after 14 days after hatching were the same pattern. The plasma PRL levels remained at high levels on the day the chicks were hatched, and then sharply decreased after the day of

hatch, and remained at low levels throughout the 28 days rearing period. Comparisons of plasma PRL levels between the R and NR hens were elucidated. In the R hens, plasma PRL levels were high when compared to those of the NR hens. The levels of plasma PRL were decreased in hens that had their chicks removed and reached the lowest levels by the third week of separation from the chicks. Taken together with the above findings of hypothalamic TH-ir distributions, this study clearly indicates an association between DA neurons in the nI and ML with the degree of hyperprolactinemia. These present findings are implicated an enhanced activity of the DAergic and VIP/PRL systems in the initiation and maintenance of rearing behavior. Disrupting the rearing behavior suppresses the hypothalamic DAergic and VIPergic activities and reduces circulating PRL levels.

In this present study, the plasma PRL levels remained at high levels on the day chicks were hatched, and then significantly decreased by day 4 after hatching and remained at low levels throughout the observed rearing period. The levels of plasma PRL were low in the NR hens when compared to those of the R hens and reached the lowest levels until 28 days of rearing period. This result corresponds with previous findings that the decreases in the number of VIP-ir neurons in the IH-IN of nest-deprived hens (Prakobsaeng et al., 2011) and chick-deprived hens (Chaiyachet et al., 2012) mirror the decreases in plasma PRL levels, and they come back into lay within 18 days after nest deprivation (Prakobsaeng et al., 2011) and 21 days of removing chicks (Chaiyachet et al., 2012), which is corresponded with increases in ovary and oviduct weights at the time when the hens return to lay (Prakobsaeng et al., 2011; Chaiyachet, 2012; Chaiyachet et al., 2012). Further evidences supporting the above findings include the demonstrated changes in the number of GnRH-I-ir neurons

observed in the nCPa of native Thai hens are correlated with the reproductive stages. The greatest number of GnRH-I-ir neurons is found in laying hens, then slightly decreases in incubating hens, and declines to the lowest levels during the rearing stage (Sartsoongnoen et al., 2012). Furthermore, studies the effects of nest-deprived and chicks removing, the native Thai hens show a significant increase in the number of GnRH-I-ir neurons in the nCPa (Prakobsaeng, 2010; Chaiyachet, 2012; Sartsoongnoen et al., 2012), while the number of VIP-ir neurons in the IH-IN decreases and ovary and oviduct weights increase as hens start the new laying cycle after nest deprivation or removal the hatched chicks (Prakobsaeng et al., 2011; Chaiyachet et al., 2012). These findings clearly demonstrate that PRL is involved in the initiation and maintenance of rearing behavior in this galliform species. The external cues such as physical contact with chicks and the presence of chicks are also involved in the maintenance of plasma PRL levels and rearing behavior. Removal of chicks from caring of the hens disrupts rearing behavior, decreases plasma PRL levels, induces ovarian recrudescence, increases ovary and oviduct weights, and finally induces the hens to come back to lay in the new cycle (Prakobsaeng et al., 2011; Chaiyachet, 2012; Chaiyachet et al., 2012).

The results in this present study are consistent with previous findings that plasma PRL levels begin to fall immediately after hatching and continue to decline for about one week after the day of the chicks are hatched (Sharp et al., 1979; Lea et al., 1981; Zadworny et al., 1985; 1988; Leboucher et al., 1990; Kuwayama et al., 1992; Kosonsiriluk et al., 2008). As demonstrated in previous studies of gallinaceous species, changes in plasma PRL levels are observed across the reproductive cycle. Plasma PRL levels are found to be low in non-laying hens, gradually increase in

laying hens, continue to rise and reach the highest levels in incubating hens, and immediately decline to the basal levels, when the hens are caring for their young (El Halawani et al., 1984; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). In several avian species, circulating PRL levels decline dramatically at the time of hatching (Goldsmith and Williams, 1980; Dittami, 1981; Goldsmith, 1982; Hall and Goldsmith, 1983; Wentworth et al., 1983). In the pigeons and doves, PRL stimulates the growth and development of specialized epithelial cells lining the crop sac, leading to formation of crop milk, which is fed to the newly hatched chicks. The rise in PRL concentrations is associated with the onset or maintenance of egg incubation and care for the young in a number of free-living passerine species (Goldsmith, 1991; Buntin, 1996). In addition, the expression of incubation behavior changes endocrinological parameters and production performances in the turkey hens (Guemene and Williams, 1992). When the hens exhibit incubation behavior, plasma LH levels decrease progressively, while plasma PRL levels increase (Porter et al., 1991). High circulating levels of PRL are maintained for a long period throughout incubation behavior and cause the decrease in ovulation rate and egg production. Moreover, *in vitro* studies demonstrate that PRL synthesis and release are high in the pituitary gland of incubating hens, and these changes are related to incubating behavior (Hoshino and Wakita, 1989).

In this present study, circulating PRL levels of the R hens were compared with those of the NR hens during 28 days of the observed rearing period. The results show differences in plasma PRL levels between the R and NR hens. In the R hens, plasma PRL levels were higher than those of the NR hens, indicating the secretion of PRL in the R hens is facilitated by the presence of chicks and stimulates maternal behaviors,

which in turn suppress the gonadal activity (Sharp et al., 1988; Richard-Yris et al., 1995). Furthermore, it has been reported that the hens that are allowed to rear their chicks return to lay later than the hens that are not allowed to rear their chicks (Chaiyachet, 2012; Chaiyachet et al., 2012). Several studies provide evidences that PRL is not released at an increased rate, while the hens are caring for their young, but it is involved in the initiation and/or maintenance of maternal behaviors and has an antigonadal role (Sharp et al., 1979; 1988; Bedrak et al., 1981; Kuwayama et al., 1992; Leboucher et al., 1993; Richard-Yris et al., 1995; Boos et al., 2007; Kosonsiriluk et al., 2008). In galliform birds, PRL is involved in maternal behaviors. 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). Replacement of the eggs by chicks induces maternal behaviors in incubating, non-incubating, and ovariectomized hens (Richard-Yris et al., 1987; 1995; 1998; Leboucher et al., 1990; 1993; Lea et al., 1996). Physical contact with newly hatched chicks during brooding bouts slows down the decrease of PRL secretion and inhibits LH and estradiol release in maternal hens (Leboucher et al., 1993). Thus, it is possible that coexistence of the newly hatched chicks may suppress LH secretion of the hen in the natural breeding cycle (Kuwayama et al., 1992). Furthermore, it is very well demonstrated that the presence of chicks inhibits the pituitary-ovarian axis (Richard-Yris et al., 1987; Sharp et al., 1988) and non-brooders (Richard-Yris et al., 1983; 1987).

It has been further suggested that the physical contact between the hen and chicks, alone or in combination with visual and/or auditory stimuli originating from the chick, induces the brooding behavior (Maier, 1963; Richard-Yris and Leboucher,

1987; Richard-Yris et al., 1998). 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 strengthen by repeated exposure of the chicks to the hen, accompanied by food, guidance, and protection (Wauters and Richard-Yris, 2001). Repeated exposure to maternal calls may be important for the development of post-hatch species-specific maternal call recognition during embryonic development (Gottleib, 1976; Jain et al., 2004). The chicks are self-sufficient after hatching in precocial species, 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 the PRL levels may be related to the large changes in intermediary and water metabolism that occurred during brooding behavior (Zadworny et al., 1985). Precocial chicks can be filial imprinting on their parents in the first few days after hatching (Rodgers, 1995; Mills et al., 1997). The relationships between the mothers and the precocial young has 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).

In the current study, utilizing TH as a marker for DA neurons, no significant differences in the number of TH-ir neurons in the SL, AM, PVO, PHN, and LHy of R and NR hens were observed during 4 and 10 days of rearing versus non-rearing chicks after the day of hatch. These results are consistent with the previous findings studied in the native Thai chickens indicating the changes in the number of TH-ir neurons in the AM, nucleus paraventricularis magnocellularis, and ML are less

dramatic during the reproductive cycle, and no significant differences are observed in these hypothalamic areas in non-laying, laying, incubating, and R hens. The TH-ir neurons are found to be abundant in the nI and ML and the numbers of TH-ir neurons in the nI are low in non-laying and significantly increase in incubating birds (Sartsoongnoen et al, 2008; Prakobsaeng et al., 2011). The highest density of TH-ir neurons found within the nI increases during incubation and decreases when the hens are deprived from their nests (Prakobsaeng et al., 2011). Previous studies suggest that the expression of VIP neurons in the IH-IN following hatching of the young may, in part, account for the difference in reproductive neuroendocrine responses of this equatorial bird (Kosonsiriluk et al., 2008). It has been demonstrated that changes in the number of VIP-ir neurons in the IH-IN are associated with alterations in DAergic neurons within the nI and ML, the release of PRL, and induction and maintenance of incubation behavior in the native Thai chickens. It has further been suggested that nesting activity stimulates PRL secretion by the activation of the DAergic system, which in turn stimulates the VIP system. The elevated PRL levels increase nesting activity and maintain incubation behavior (Prakobsaeng 2010; Prakobsaeng et al., 2011). In addition, nest deprivation results in decreased TH-ir neurons in these hypothalamic areas, paralleling decreased VIP-ir neurons in the IH-IN, and subsequence decreased PRL secretion (Prakobsaeng et al., 2011).

Recently, it has been suggested that the VIP/PRL system plays a significant role in neuroendocrine reorganization to establish maternal behaviors in the native Thai chickens. It is possible that the decline in number of VIP-ir neurons and in turn the decrease in VIPergic activity and the decrease in PRL secretion in the NR hens are related to their contribution to rearing the chicks. Both the number of VIP-ir

neurons and circulating PRL levels decline sharply after the chicks are hatched, and these declines are further accelerated if the hens are deprived of their chicks. These findings taken together with the previous findings in altricial birds that circulating PRL levels remain elevated after the young are hatched suggest that the activity of the VIP/PRL system and its contribution to maternal behaviors are related to the extent of maternal care required for the hatched young. Thus, these data well support an association of the neuronal interactions between the GnRH-Iergic, VIPergic, and DAergic systems in the neuroendocrine regulation of reproductive activity in the native Thai chickens.

The present findings reveal a dense accumulation of TH-ir fibers located within the MM and the external layer of ME. These results are consistent with the previous studies in mammals, which have been suggested that these areas are involved in the regulation of PRL secretion. PRL secretion is regulated by the inhibitory control of TIDA neurons (A12 DA group) residing in the INF (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001), which release DA that acts directly upon the D_2 DA receptors located on pituitary lactotrophs (Civelli et al., 1991). However, these results are not in a good agreement with the studies in birds, since it has been suggested that the TIDA neurons in birds are absent (Reiner et al., 1994), and the DAergic system in the avian hypothalamus may not be the primary PIF (Kiss and Peczely, 1987). It has been reported that the TIDA is lack of hypothalamic TH-ir neurons in birds (Kiss and Peczely, 1987; Bailhache and Balthazart, 1993; Moons et al., 1994; Appeltants et al., 2001). Furthermore, previous study has reported that TH immunoreactivity found in the tuberal hypothalamus is limited to a single discrete area of the MM and to the external layer of ME, where

only TH-ir fibers are found (Sartsoongnoen et al., 2008; Prakobsaeng et al., 2011). In the chickens, TH-ir neurons in the nI is corresponded to the mammalian A11 DA group (Moons et al., 1994; Lookingland and Moore, 2005), which consists of neurons that may play a role in the onset of puberty (Fraley and Kuenzel, 1993). Also, the A11 DA group is shown to be involved in the regulation of reproductive seasonality in the turkeys (Thayananuphat et al., 2007), and its activity reflects the performance of courtship singing in zebra finches (Bharati and Goodson, 2006). No double-labeled immunoreactive neurons for both TH and DA-ß-hydroxylase, the enzyme for noradrenalin biosynthesis, are found in the hypothalamus of quails (Bailhache and Balthazart, 1993), turkeys (Al-Zailaie, 2003), and other avian species (Reiner et al., 1994). Therefore, it is suggested that TH-ir neurons found in the nI of the native Thai chickens could be the DAergic neurons.

It is well known that steroid hormones are involved in the regulation of maternal behaviors in mammals within the medial preoptic area (MPOA). The MPOA area contains estrogen receptors (Shughrue et al., 1997), progesterone receptors (Numan et al., 1999), PRLR (Bakowska and Morrell, 1997), and oxytocin (OT) receptors (Champagne et al., 2004). Estrogen, PRL, and placental lactogens have all been shown to stimulate the onset of maternal behaviors when microinjected into the MPOA (Bridges et al., 1990; 2001). OT has also been shown to act on the MPOA to stimulate maternal behaviors (Pedersen et al., 1994). In this present study, DA neurons that found in the nI may be interact with OT within the MPOA to initiate and/or promote reproductive behaviors (Stolzenberga and Numan, 2011), and this is supported by the idea in the context of male sexual behaviors (Bitran and Hull, 1987; Hull et al., 1999; Hull and Dominguez, 2006). In support of the idea that estrogen-DA

interactions within the MPOA might also promote maternal behaviors, there are data indicating that in the absence of estradiol benzoate treatment, administration of the D_1 DA receptor agonist directly into the MPOA can promote an immediate onset of full maternal behaviors in the hysterectomy and ovariectomy rats on day 15 (Stolzenberg et al., 2007).

In this present study, the number of TH-ir neurons in the nI was high after the day of hatch and remained at high levels until 14 days of taking care of chicks, indicating the possibility that DA is the crucial neurotransmitter/neurohormone to initiate the maternal behaviors in the native Thai chickens. In order to maintain the maternal behaviors, it may be involved with other neurohormones/hormones and/or external stimuli such as eggs and chicks. In some avian species, stimuli from the young or from parent-young interactions may promote or sustain elevated circulating PRL levels (Buntin, 1996), suggesting that physical contact familiar with auditory, and/or visual stimuli from chicks during the rearing period slows down the decrease of PRL secretion and inhibits gonadotropins and ovarian steroid hormones (Richard-Yris and Leboucher, 1987; Richard-Yris et al., 1987; 1998; Leboucher et al., 1993). It has been further suggested that the presence of chicks had no effect on PRL secretion but tends to maintain its level (Sharp et al., 1988; Richard-Yris et al., 1995). A definite threshold in circulating PRL levels is necessary to promote and/or maintain post-hatching maternal behaviors in the precocial birds (Boos et al., 2007). Indeed, depending on the species, either a sharp decline or a slow decrease of PRL concentrations after hatching has been reported (Goldsmith and Williams, 1980; Dittami, 1981; Oring et al., 1986; 1988; Hall, 1987; Opel and Proudman, 1989; Richard-Yris et al., 1995; 1998; Setiawan et al., 2006). In those species, although

PRL concentration during rearing period is lower than that of incubation period, the level remains higher than those of non-rearing ones (Boos et al., 2007), suggesting that PRL is likely involved in parental care after hatching (Criscuolo et al., 2002). Moreover, the recent findings reveal the number of VIP-ir neurons in the IH-IN area is high in the R hens, whereas the number of VIP-ir neurons decreases in the NR hens as compared to their respective R hens. During the rearing period, changes in the VIP-ir neurons within the IH-IN are correlated with plasma PRL levels, suggesting that the VIP/PRL system plays a significant role in neuroendocrine reorganization to establish rearing behavior in the native Thai chickens (Chaiyachet et al., 2012).

In conclusion, the present findings indicate, for the first time, that the DAergic system plays a pivotal role in neuroendocrine reorganization to establish and maintain maternal behaviors in the native Thai chickens. The differential expression of DA neurons in the nI and ML might play a regulatory role in rearing behavior of this bird. Disruption of rearing behavior decreases the number of DA neurons in the nI and ML and accompanied with the declined plasma PRL levels, implicating that the activities of the DAergic and VIP/PRL systems are enhanced to initiate and maintain the rearing behavior. Disrupting the rearing behavior suppresses the hypothalamic DAergic and VIPergic activities and subsequent reduced circulating PRL levels. The decline in DAergic activity and circulating PRL levels during disrupting the rearing behavior might be related to the contribution of rearing behavior in this equatorial precocial species.

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CHAPTER IV

ROLE OF MESOTOCIN ON MATERNAL CARE OF CHICKS IN THE NATIVE THAI HEN

4.1 Abstract

Native Thai chickens (*Gallus domesticus)* is a continuously breeding species found in the equatorial zone that produces eggs all year, which is independent of photoperiodic cues. It always expresses high maternal behaviors. Maternal behaviors are hormonal dependent and initiated with the onset of incubation behavior and continue through the period when the young are taking care by parent (broody/rearing behavior). Oxytocin (OT) is known to induce and regulate maternal behaviors in mammals via supraoptic nucleus and paraventricular nucleus (PVN), whereas the physiological function(s) of mesotocin (MT; the avian homolog of OT) is poorly understood in birds. The mechanisms underlying the regulation of maternal behaviors in mammals may be derived from the processes involving in the gestation, parturition, or the regulation of lactation including changes in circulating levels of progesterone, estrogen, OT, and prolactin (PRL). To date, there are limited data available that describe the interrelationship and the functional aspects of the changes in neurohormones/neurotransmitters/hormones involved in maternal behaviors during the hens take care of their chicks. It is well shown that the initiation and maintenance of maternal behaviors is associated with the VIP/PRL system. However, a role of the

MTergic system in rearing behavior has never been elucidated in this species. Therefore, the aim of this study was to identify the MT neuronal groups that may be associated with the reproductive regulatory system(s) and also involved with the neuroendocrine regulation of rearing behavior in the native Thai chickens. Immunohistochemistry (IHC) was conducted to reveal the distributions of MT-immunoreactive (ir) neurons and fibers in the brain of native Thai chickens. Changes in the number of MT-ir neurons of the individual hypothalamic areas were compared across the reproductive cycle. Differences in the number of MT-ir neurons within the individual hypothalamic areas at the different time periods of rearing (R) and non-rearing hens (NR) after the incubating eggs were hatched were compared as well. As revealed by IHC, the MT-ir neurons and fibers were distributed in a discrete region lying close to the third ventricle through the anterior hypothalamus with the greatest abundance found within the nucleus preopticus medialis (POM), nucleus supraopticus; pars ventralis (SOv), and PVN, nucleus ventrolateralis thalami, and regio lateralis hypothalami. Small numbers of MT-ir neurons also found in the nucleus preopticus periventricularis, nucleus perventricularis hypothalami, nucleus anterior medialis hypothalami, nucleus suprachiasmaticus; pars medialis, tractus septomesencephalicus, and nucleus dorsolateralis anterior thalami; pars magnocellularis. Small groups of MT-ir fibers were also found in the organum vasculosum lamina terminalis, organum subseptale; organum interventriculare, and the external layer of the eminentia mediana. To determine the changes in the number of MT-ir neurons within the SOv, POM, and PVN areas across the reproductive stages, the native Thai chickens were divided into four reproductive stages: non-egg laying (NL), egg laying (L), incubating eggs (B), and rearing chicks (R). The results revealed that there were more MT-ir

neurons presented in the POM and PVN than that of in the SOv. The numbers of MT-ir neurons were low in the SOv, POM, and PVN of the NL group, then gradually increased in the L hens, and peaked in the B and R groups. Most notably, the numbers of MT-ir neurons in these hypothalamic areas displayed fluctuations across the reproductive cycle and appeared to be the highest when the hens had shifted from egg laying period to rearing chicks. Changes in the number of MT-ir neurons within the SOv, POM, and PVN areas were compared between the R and NR hens. Significant decreases in the number of MT-ir neurons of the NR hens when compared to those of the R hens were observed in the POM and PVN after the day of hatch throughout 28 days of the observation periods, while within the SOv, the MT-ir neurons were significantly different between the R and NR hens after seven days through 28 days of the observation periods. However, the numbers of MT-ir neurons in SOv, POM, and PVN were higher in the R hens when comparing with the NR hens. The numbers of MT-ir neurons were sharply decreased in the NR hens. These findings indicate, for the first time, that the MTergic system plays a pivotal role in neuroendocrine reorganization to establish and maintain maternal behaviors in this equatorial precocial species. The decline in MTergic activity during disrupting rearing behavior might be related to the contribution of rearing behavior in this bird. The results further suggest the role of MT in these hypothalamic nuclei during the period of rearing chicks resembles that of OT in these similar brain structures during the parturition and lactation in mammals.

4.2 Introduction

For successful reproduction, not only sexual activity is important but also successful care of the young. Maternal behavior is crucial to the survival of fertilized eggs or offspring. The offspring need one or both parents to provide food, heat, or protection from any harm. This behavior must be performed immediately after birth or hatching of the offspring (Nelson, 2000). Maternal behaviors in mammals are composed of nest building, pup retrieval, crouching, exploration and sniffing of pups, licking and grooming, and placentophagia (Leckman and Herman, 2002). The onset of maternal responsibity is a prerequisite for all mother-young interaction. It brings the mother in contact with her young exposing her to a unique constellation of tactile, visual, auditory, and olfactory stimuli as well as a suckling-induced change in hormonal state (Numan and Woodside, 2010). Taken together, these promote other changes in behaviors.

Most mothers display maternal behaviors after parturition and serve the immediate provision of care, defense for their offspring, and maintain in the period that the child is dependent on the mothers (Swain et al., 2007; Brunton and Russell, 2008). In mammals, the patterns of maternal care consist of internal incubation of embryos during gestation, delivery of the young at parturition, and maternal care until weaning (Rosenblatt, 2003). The mechanisms underlying the regulation of maternal behaviors may be derived from the processes involving in the gestation, parturition, or the regulation of lactation including changes in circulating levels of progesterone, estrogen, oxytocin (OT), and prolactin (PRL). These hormonal activities increase in the medial preoptic area (MPOA) of the hypothalamus during the expression of maternal behaviors (Ziegler, 2000). Apparently, some neuropeptides, neurohormones,

and hormones, most notably OT and PRL that play a key role in the onset of maternal behaviors, are prominent in the reorganization of the neuronal systems controlling energy balance, stress response, anxiety, and aggression in postpartum females as well (Numan and Woodside, 2010).

Maternal care in birds is included incubation and brooding/rearing behaviors. The term incubation refers to the maternal care of unhatched eggs and brooding is the maternal care of chicks after hatching (El Halawani et al., 1988). Incubation behavior in birds is qualified by sitting continually on their eggs until they hatch, while brooding or rearing behavior is directed to the care of newly hatched chicks (Richard-Yris et al., 1983; El Halawani et al., 1988; Ruscio and Adkins-Regan, 2004; Prakobsaeng et al., 2011; Chaiyachet et al., 2012). Generally, the hens develop maternal behaviors gradually in four stages; brooding, titbitting, clucking, and normal broody behavior (Ramsay, 1953). The incidence of maternal behaviors concurs with a pause in laying and a decrease in plasma gonadal steroid levels (Richard-Yris et al., 1983). It has been reported that, birds that exhibit brooding behavior allow chicks to access and remain underneath their wings, whereas birds that do not show brooding behavior actively avoided the chicks (Ruscio and Adkin-Regan, 2004).

It has been well known that OT plays a pivotal role in parturition and lactation in mammals (de Wied et al., 1993). However, it has been reported that OT is both a hormone, released from the neurohypophysis, and a neurotransmitter/neuromodulator, released at synapses in the brain (Numan, 1994), involved during the onset of maternal behaviors. Hypothalamic neurons of the paraventricular nucleus (PVN) and supraoptic nucleus (SON) are capable of releasing OT into various neural sites at the time of birth. Furthermore, there are evidences showing that OT can stimulate the onset of

maternal behavior at the levels of MPOA, ventral tegmental area (VTA), or nucleus accumbens (Ac; Pedersen et al., 1994; Olazabal and Young, 2006).

Mesotocin (MT) is the avian homolog of OT (Acher et al., 1970). MT-immunoreactive (ir) neurons were found in several brain areas such as the nucleus supraopticus; pars ventralis (SOv), and PVN (Goossens et al., 1977; Bons, 1980). However, little is known regarding the physiological function(s) of MT in birds. It does not appear to be involved in aggression, partner preference, cardiovascular function as well as plasma osmolarity (Robinzon et al., 1994; Goodson et al., 2004), but it may participate in renal blood flow (Bottje et al., 1989). The first evidence reported the role of MT in avian brooding behavior has only been investigated in the turkeys. The numbers of MT-ir neurons in the PVN and SOv increase in incubating hens when compared with laying hens. In addition, the induction of c-fos mRNA in the MT-ir neurons within these brain nuclei in incubating hens stimulated with poults, and preventing poult brooding from taking place by blocking MT receptors suggest that MT is essential to the onset of maternal activities in the turkeys (Thayananuphat et al., 2011).

Native Thai chicken (*Gallus domesticus)* is domesticated without genetic selection. The reproductive cycle of the native Thai chicken is divided into four reproductive stages; non-egg laying, egg laying, incubating eggs, and rearing chicks (Kosonsiriluk, 2007). It always expresses high maternal behaviors which is a heritable trait from the ancestor, the wild jungle fowl (Austic and Nesheim, 1990; Hillel et al., 2003; Sawai et al., 2010). Maternal behaviors are hormonal dependent and initiated with the onset of incubation behavior and continue through the period when the young are taking care by parent (broody/rearing behavior; Prakobsaeng, 2010). However,

there are limited data regarding the neuroendocrine regulation of rearing behavior in this non-temperate zone gallinaceous species. Recently, it has been well established that incubation behavior in this species is regulated by the vasoactive intestinal peptide (VIP)/PRL system, chicken gonadotropin releasing hormone-I (cGnRH-I or GnRH) and the subsequent secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH; GnRH/FSH-LH system), and dopaminergic (DAergic) system (Sartsoongnoen et al., 2006; 2008; 2012; Kosonsiriluk et al., 2008; Prakobsaeng et al., 2011). Plasma PRL and LH levels across the reproductive cycle of the native Thai chickens have been reported (Kosonsiriluk, 2007; Sartsoongnoen et al, 2008). Changes in the numbers of VIP-ir neurons within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) areas are directly correlated with changing in plasma PRL levels throughout the reproductive cycle, suggesting that VIPergic neurons in the IH-IN plays a regulatory role in year-round reproductive activity in the native Thai chickens (Kosonsiriluk, 2007; Kosonsiriluk et al., 2008; Sartsoongnoen et al., 2008). Moreover, it has been demonstrated that changes in the number of VIP-ir neurons in the IH-IN are associated with DAergic neurons within the nucleus intramedialis and nucleus mamillaris areas, resulting in PRL release to induce and maintain incubation behavior in the native Thai chickens. It is further suggested that nesting activity stimulates PRL secretion through activation of the DAergic system, which in turn stimulates the VIPergic system. The elevated PRL levels increase nesting activity and maintain incubation behavior (Prakobsaeng et al., 2011; Sartsoongnoen et al., 2012). Recently, disruption of rearing behavior in the native Thai hens by removing the chicks from the hens markedly decreases plasma PRL levels, a parallel decline in the number of VIP-ir neurons in the IH-IN, and an

accompanying increase in the number of GnRH-I-ir neurons in the nucleus commissurae pallii (nCPa), suggesting that the VIPergic system in the IH-IN and the GnRH system in the nCPa may be involved in the regulation of the reproductive neuroendocrine system and the initiation and maintenance of rearing behavior in this precocial species (Chaiyachet et al., 2010; 2012).

To date, there are limited data available that describe the interrelationship and the functional aspects of the changes in neurohormones/neurotransmitters/hormones involved in maternal behaviors during the hens take care of their chicks. Therefore, the aim of this study was to identify the MT neuronal groups that may be associated with the reproductive regulatory system(s) and also involved with the regulation of maternal behaviors in the native Thai chickens. Immunohistochemistry (IHC) was conducted to reveal the distributions of MT-ir neurons and fibers in the brain of native Thai chickens. Changes in the number of MT-ir neurons of individual hypothalamic areas were compared across the reproductive cycle. Differences in the number of MT-ir neurons within individual hypothalamic areas at the different time periods of rearing (R) and non-rearing hens (NR) after the incubating eggs were hatched were compared. The findings of differential expression of MT within individual hypothalamic areas may provide an insight into the mechanism(s) underlying the regulation of brooding behavior in the native Thai chickens, non-seasonal breeding, equatorial precocial species.

4.3 Materials and Methods

4.3.1 Experimental Animals

Female and male native Thai chickens (*G. domesticus*), Pradoohangdam breed, ranging between 22-24 weeks of age, were used. They were reared and housed (7-8 females: 1 mature rooster) in floor pens with nest baskets under natural light (approximately 12 hrs of light and 12 hrs of darkness; 12L: 12D). Feed and water were provided *ad libitum*. The animal protocols described in this study were approved by Suranaree University of Technology Animal Care and Use Committee.

4.3.2 Experimental Design

4.3.2.1 Experiment I

To determine the distributions of the MT-ir neurons and fibers in the brain of the native Thai chicken, laying hens (hens that were in their first laying cycle and had been laying for 7 days; n = 6), were used. The brains were pressure-perfused prior to sectioning in a cryostat and further processing by IHC. A postmortem examination of each hen was performed to confirm its reproductive status.

4.3.2.2 Experiment II

To determine the changes in the number of MT-ir neurons within the SOv, nucleus preopticus medialis (POM), and PVN areas across the reproductive stages, twenty-four female and three male native Thai chickens, 22-24 weeks old, were used and were divided into four reproductive stages (n = 6): non-egg laying (NL; hens that had never laid eggs), egg laying (L; hens in their first laying cycle and that had been laying for 7 days), incubating eggs (B; hens that had stopped laying and exhibited

incubating behavior for 10 days), and rearing chicks (R; hens that had been rearing chicks for 14 days). The brains were fixed by pressure perfusion prior to sectioning in a cryostat and further processed for IHC. A postmortem examination of each hen was performed to confirm its reproductive status.

4.3.2.3 Experiment III

To study the association of MT-ir neurons with the neuroendocrine regulation of rearing behavior, one hundred and two female native Thai chickens, 22-24 weeks old, were used. After hatching, the hens were divided into two groups; rearing (R) and non-rearing (NR) hens. The R hens were allowed to rear the chicks naturally. The NR hens were disrupted from rearing behavior and not allowed to rear their chicks by removing them from the chicks to another pen. All hens in both groups were reared in floor pens with the roosters and observed their daily behaviors. All the hens were checked four times per day and classified as broody/rearing hens throughout the experiment. During rearing behavior, the hens remained with their chicks, displayed aggressive behavior, and emitted a characteristic high-pitched vocalization when they were approached by humans and/or the male. The hen fluffed her feathers and crouched over the chicks, whether she encouraged the chicks to go under her wings, and the types of vocalizations to protect and/or feed the chicks (Opel and Proudman, 1989; Edgar et al., 2011; Thayananuphat et al., 2011). To compare the time courses in changes in the number of MT-ir neurons in the individual brain nuclei, both R and NR hens were then sacrificed at different time periods (day of hatch, day 4, 7, 10, 14, 17, 21, 24, and 28; n=6) after they started to rear their chicks or after the chicks were removed. The brains were fixed by pressure perfusion prior to sectioning in a cryostat

and further processed for IHC. A postmortem examination of each hen was performed to confirm its reproductive status.

4.3.3 Processing of tissues for immunohistochemistry

Prior to perfusion, each bird was intravenously injected 3 ml of heparin (Baxter Healthcare Corporation, Deerfield, IL, USA; 1000 U/ml) and then euthanized with pentobarbital sodium (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 650 ml of a freshly prepared 4 % paraformaldehyde in 0.1 M PBS (pH 7.4) for 30 min according to a method previously described by Prakobsaeng et al. (2011). The brain was then dissected intact from the skull and soaked in 20 % sucrose in the PBS (pH 7.4) at 4 °C for 48 hrs or until saturated for cryoprotection. The brain was then frozen in powdered dry ice for 1 hr, and stored at -35 °C until sectioned. Frozen brains were sectioned in the coronal plane at a thickness of 16 µm using a cryostat (Microtome cryostat HM525, Microm International GMbH, Walldorf, Germany). Sections were mounted onto chrome alum-gelatin-coated glass slides, with two sections per slide, and stored desiccated at -20 °C. Six adjacent sections were processed by IHC to visualize changes in the number of MT-ir neurons in the SOv, POM, and PVN areas. 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 chosen starting with the most rostral section that contained each nucleus and every sections 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 previously published (Thayananuphat et al., 2011).

4.3.4 Immunohistochemistry

The methods used to determine the MT distributions throughout the brain of the laying hens and within individual brain regions of hens in different reproductive states has been previously described (Thayananuphat et al., 2011). The primary and secondary antibodies used for detecting the MT-ir neurons and fibers were primary rabbit polyclonal antibody directed against OT (ImmunoStar, Inc., Hudson, WI, USA) and Cy^{TM}3-conjugated AffiniPure donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), respectively. Briefly, tissue sections of the individual hypothalamic areas from the hens were thawed to room temperature prior to use. They were rehydrated with the PBS (pH 7.4) for 30 min at room temperature. After the PBS removal, the sections were then incubated with 60 µl of primary antibody diluted 1:1000 with the PBS (pH 7.4) containing 1 % bovine serum albumin (BSA) and 0.3 % Triton-X 100 at 4 °C overnight in a moist chamber. Subsequently, the slides were then washed three times with the PBS (pH 7.4) for 5 min each. After washing, 60 µl of secondary antibody at 1:500 dilution in the PBS (pH 7.4) were applied under dark conditions onto the sections. Slides were further incubated in a moist dark chamber at room temperature for 1 hr, washed with the PBS (pH 7.4) 3 times for 5 min each, and then mounted with DPX mountant (Sigma-Aldrich, Inc., Steinheim, Germany). Microscopic images of the brain sections were visualized and further analyzed. The numbers of MT-ir

neurons were counted according to previously described methods (Prakobsaeng et al., 2011; Thayananuphat et al., 2011).

The specificity of used antibody is tested by pre-absorption control and was performed to verify the specificity of these peptides to MT. Briefly, tissue sections were incubated overnight with normal rabbit serum, OT antibody diluted 1:1000 with the PBS (pH 7.4), and 1 % BSA pre-absorbed with 10 µg/ml of MT (Bachem, Torrance, CA, USA) or vasotocin (VT; Bachem) diluted 1:1000 with the PBS (pH 7.4) and 1 % BSA. Pre-absorption of OT antibody with MT completely abolished the staining of neuronal cells compared to tissues stained with non-preabsorbed MT. Immunostaining with normal rabbit antisera also abolished the staining (Figure 4.2).

4.3.5 Image analysis

Microscopic images of the brain sections were visualized with a fluorescence microscope (Nikon ECLIPSE80i, Tokyo, Japan) using a cooled digital color camera (Olympus DP72, Tokyo, Japan). The images were captured and stored by DP72-BSW Software (Olympus, Tokyo, Japan). The differential expression of MT-ir neurons and fibers in each individual area of the brain was visualized and analyzed. The numbers of MT-ir neurons in six adjacent sections were counted manually to determine changes in the numbers of MT-ir neurons in the individual hypothalamic areas. The MT-ir neurons counted from the six adjacent sections for each hen (6 hens per area) for each treatment group were averaged to determine the numbers of MT-ir neurons counted per section in each brain area. The mean values were compared across the reproductive stages and between the R and NR hens at different time periods. To avoid double-counting neurons with cell bodies that appeared on two adjacent

sections, sections were viewed under 400x 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.

4.3.6 Statistical Analysis

Significant differences in the number of MT-ir neurons (means ± SEM) in the individual hypothalamic areas according to each treatment group were compared utilizing one-way analysis of variance (ANOVA). Significant differences between the treatment groups were computed utilizing the Tukey's HSD Test. Differences were considered significant if the P-value was less than 0.05. All statistical tests were analyzed using the SPSS for Windows Software (version 13.0, SPSS Inc., Chicago, IL, USA).

4.4 Results

4.4.1 Specificity of Antibody to Mesotocin

The MT-ir neurons are shown (Figures 4.2A and 4.2B). Pre-absorption of OT antibody with 10 µg/ml of MT completely abolished the staining of neuronal cells (Figures 4.2C and 4.2D) comparing with tissues stained with non-preabsorbed MT (Figures 4.2A and 4.2B) or tissues stained with pre-absorbed VT (Figures 4.2E and 4.2F). Immunostaining with normal rabbit antisera also abolished the MT staining. This result appeared to be consistent in all tissues including the SOv (Figures 4.2A,

4.2C, and 4.2E) and PVN areas (Figures 4.2B, 4.2D, and 4.2F). These results indicated that OT antibody used in this experiment is cross-reactive to MT, but not VT.

4.4.2 Experiment I

Localization study of MT by IHC in the laying native Thai chickens revealed that the distribution and appearance of MT-ir neurons were spanned the length of the hypothalamus from the preoptic region, the anterior hypothalamus to the end of the septal region. MT fibers were mainly bilaterally located along the third ventricle and also distributed in a discrete region lying close to the third ventricle from the level of preoptic area (POA) through the anterior hypothalamus (Table 4.1; Figures 4.1 and 4.3-4.6), and very dense fibers were observed in the external layer of the eminentia mediana (ME; Figure 4.7). Schematic representations of the distributions of MT-ir neurons and fibers throughout the brain are shown in Figure 4.1. The distributions of MT-ir neurons in Figures 4.4-4.6 are shown by schematic diagrams of coronal sections in the hypothalamic regions from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988).

As revealed by IHC, the MT-ir neurons and fibers were distributed in a discrete region lying close to the third ventricle through the anterior hypothalamus (Figures 4.3-4.6), with the greatest abundance found within the SOv (Figures 4.4A and 4.4F), POM (Figures 4.4A-4.4E), nucleus ventrolateralis thalami (VLT; Figures 4.5A and 4.5E), regio lateralis hypothalami (LHy; Figure 4.5A, 4.5F, 4.5G, 4.6A, and 4.6G), and PVN (Figures 4.5A, 4.5B, 4.5D, 4.6A, 4.6C, and 4.6D). Higher magnification of the MT-ir neurons is shown in Figure 4.3, illustrating an oval shape with monopolar process neurons in the POM and PVN. Small numbers of the MT-ir

neurons also found in the nucleus preopticus periventricularis (POP; Figure 4.4A), nucleus perventricularis hypothalami (PHN; Figures 4.5A and 4.5D), nucleus anterior medialis hypothalami (AM; Figures 4.5A, 4.5C, 4.6A, and 4.6F), nucleus suprachiasmaticus; pars medialis (SCNm; Figures 4.5A and 4.5C), tractus septomesencephalicus (TSM; Figure 4.5H), and nucleus dorsolateralis anterior thalami; pars magnocellularis (DLAmc; Figure 4.6E). A small group of MT-ir neurons was found at the end of PVN area (Figure 4.7A). Small groups of the MT-ir fibers were also found in the organum vasculosum lamina terminalis (OVLT; Figures 4.4A-4.4D), organum subseptale; organum interventriculare (SSO; Figure 4.6B), and the external layer of the eminentia mediana (ME; Figures 4.7B-4.7D).

4.4.3 Experiment II

The numbers of counted MT-ir neurons in three hypothalamic areas including the SOv, POM, and PVN were compared across the reproductive stages (Table 4.2, Figures 4.8-4.11). In all areas examined, the differential distributions of MT-ir neurons were observed across the reproductive stages. The results revealed that there were more MT-ir neurons presented in the POM (Figures 4.8B and 4.10) and PVN (Figures 4.8C and 4.11) than that of in the SOv (Figures 4.8A and 4.9). Within the SOv, the numbers of MT-ir neurons were low in the NL group (24.00 ± 4.31 cells), then markedly increased in the L group (37.87 ± 4.28 cells), and reached the highest density in both B ($P<0.05$; 54.54 ± 5.43 cells) and R groups (38.50 ± 4.96 cells). However, the numbers of MT-ir neurons were slightly decreased when the hens made the transition from incubating to rearing behaviors (Figures 4.8A and 4.9). In contrast, within the POM and PVN areas, the numbers of MT-ir neurons (cells) were low

during the reproductively quiescent stage (POM; 55.58 ± 7.65, PVN; 33.29 ± 2.85), then markedly increased when the hens began to lay (POM; 72.08 ± 4.56, PVN; 44.21 ± 3.28) and further incubate their eggs ($P<0.05$; POM; 90.08 ± 3.21, PVN; 75.00 ± 6.73), and reach the highest level at the rearing period ($P<0.05$; POM; 96.83 ± 3.81, PVN; 81.00 ± 3.89). The MT-ir neurons in the POM are shown in Figures 4.8B and 4.10, whereas the MT-ir neurons in the PVN are shown in Figures 4.8C and 4.11. Most notably, the numbers of MT-ir neurons in these hypothalamic areas displayed fluctuations across the reproductive cycle and appeared to be the highest when the hens had shifted from egg laying period to rearing chicks.

4.4.4 Experiment III

To elucidate the association of MT-ir neurons with the neuroendocrine regulation of rearing behavior, changes in the numbers of MT-ir neurons within the SOv (Table 4.3, Figures 4.12 and 4.13), POM (Table 4.3, Figures 4.14 and 4.15), and PVN (Table 4.3, Figures 4.16 and 4.17) areas of the R hens were compared with those of the NR hens at different time periods. The results revealed that the numbers of MT-ir neurons within the SOv (Table 4.3 and Figure 4.12), POM (Table 4.3 and Figure 4.14), and PVN (Table 4.3 and Figure 4.16) of the R hens were significantly ($P<0.05$) higher than that of the NR hens. Changes in the number of MT-ir neurons in the POM of R and NR hens at different time of rearing periods are shown in Figures 4.14 and 4.15. Within the POM, the MT-ir neurons were higher in the R hens than that of the NR hens after the day of hatch (91.63 ± 8.32 cells) through the observed rearing periods ($P<0.05$; R4 vs NR4; 84.88 ± 6.07 vs 48.63 ± 3.85, R7 vs NR7; 88.79 ± 13.04 vs 51.42 ± 5.23, R10 vs NR10; 94.17 ± 9.38 vs 46.96 ± 5.29, R14 vs NR14;

85.33 ± 3.32 vs 35.13 ± 6.01, R17 vs NR17; 73.83 ± 2.79 vs 42.63 ± 5.37, R21 vs NR21; 74.75 ± 2.03 vs 48.21 ± 3.18, R24 vs NR24; 84.42 ± 5.07 vs 48.29 ± 2.92, R28 vs NR28; 80.29 ± 2.58 vs 42.96 ± 2.47 cells). Within the SOv, the MT-ir neurons were not significantly different between the R and NR hens after four days of rearing periods ($P>0.05$; R4 vs NR4; 43.54 ± 8.33 vs 34.00 ± 9.92, R7 vs NR7; 41.83 ± 9.49 vs 21.17 ± 3.50 cells), but the differences of the MT-ir neurons were observed between the R and NR hens after seven days of rearing periods ($P<0.05$; R10 vs NR10; 40.17 ± 4.41 vs 20.38 ± 2.80, R14 vs NR14; 38.50 ± 4.96 vs 20.42 ± 3.65, R17 vs NR17; 33.75 ± 2.66 vs 14.63 ± 1.99, R21 vs NR21; 42.58 ± 8.32 vs 19.88 ± 1.79, R24 vs NR24; 39.83 ± 8.13 vs 16.33 ± 1.21, R28 vs NR28; 32.79 ± 3.11 vs 15.13 ± 3.15 cells). The changes of MT-ir neurons in the PVN were significantly greater than that of the NR hens after day of hatch ($P<0.05$; 117.46 ± 8.72 cells) and through 28 days of rearing time periods ($P<0.05$; R4 vs NR4; 105.79 ± 2.60 vs 53.54 ± 2.98, R7 vs NR7; 110.00 ± 5.90 vs 52.92 ± 2.87, R10 vs NR10; 88.88 ± 3.77 vs 51.71 ± 1.15, R14 vs NR14; 85.33 ± 3.32 vs 55.58 ± 1.62, R17 vs NR17; 88.00 ± 8.13 vs 39.21 ± 2.62, R21 vs NR21; 80.63 ± 2.96 vs 38.63 ± 2.51, R24 vs NR24; 75.67 ± 5.02 vs 40.79 ± 2.48, R28 vs NR28; 84.38 ± 4.16 vs 33.33 ± 3.62 cells). Interestingly, the numbers of MT-ir neurons in the PVN of the R hens remained high after day of hatch and until 7 days, and then slightly declined through 28 days of rearing periods (Figures 4.16 and 4.17). All R hens reared their chicks (after hatching), immediately display brooding/rearing behavior, including vocalization, feather fluffing, crouching posture and slightly holding their wings away from the body, pulling chicks with their beaks under their body and between the body and the wings, during the period of observation.

Table 4.1 Abbreviations of brain areas. Nomenclature and abbreviations are from a stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988).

POM	Nucleus preopticus medialis
POP	Nucleus preopticus periventricularis
OVLT	Organum vasculosum lamina terminalis
SOv	Nucleus supraopticus; pars ventralis
CA	Commissura anterior
PHN	Nucleus perventricularis hypothalami
PVN	Nucleus paraventricularis magnocellularis
TSM	Tractus septomesencephalicus
VLT	Nucleus ventrolateralis thalami
LHy	Regio lateralis hypothalami
AM	Nucleus anterior medialis hypothalami
SCNm	Nucleus suprachiasmaticus; pars medialis
SSO	Organum subseptale, Organum interventriculare
DLAmc	Nucleus dorsolateralis anterior thalami; pars magnocellularis
ME	Eminentia mediana (Median eminence)
V III	Ventriculus tertius (Third ventricle)
Pit	Pituitary

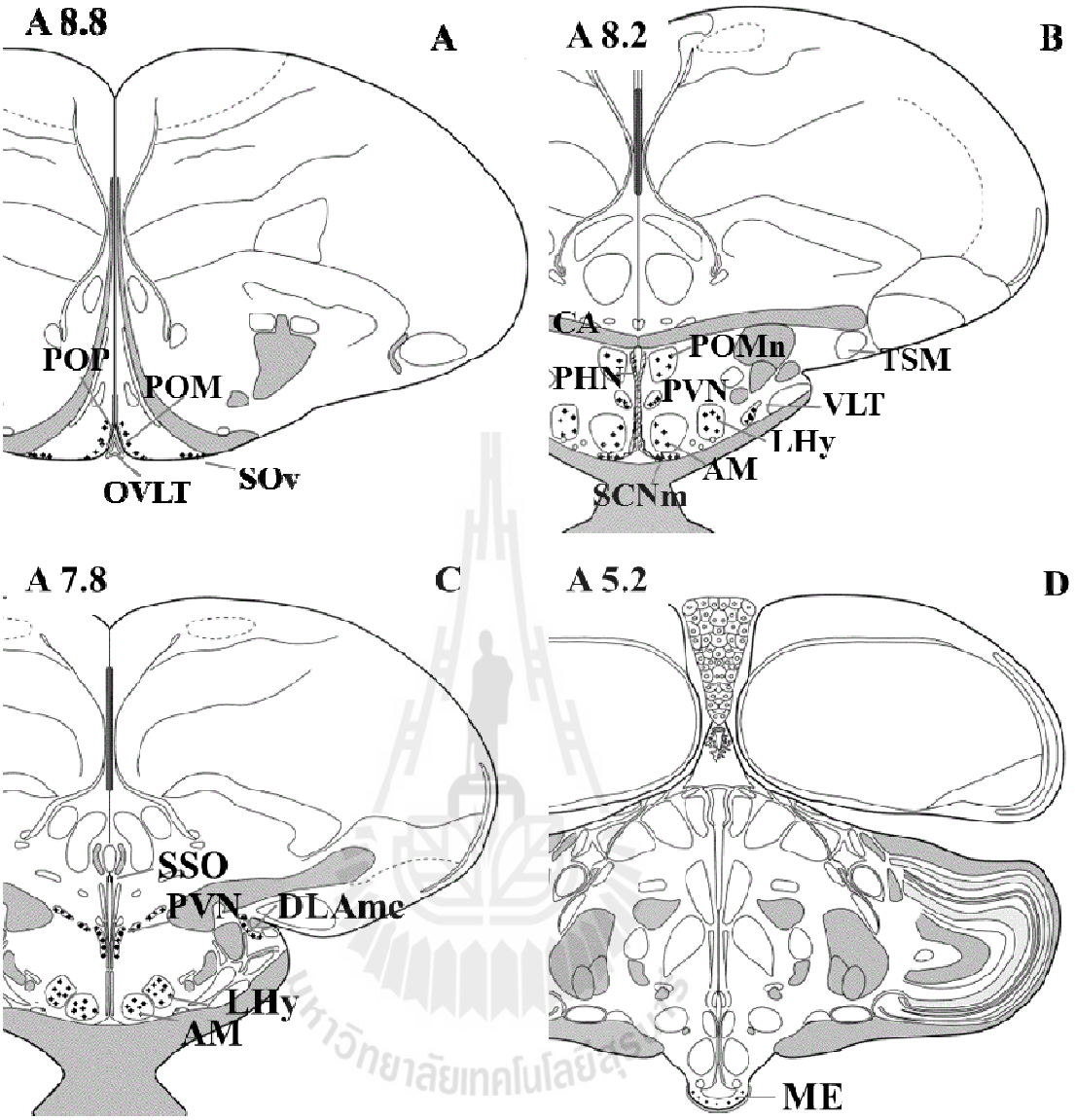

Figure 4.1 Schematic diagrams of coronal sections illustrating the distributions of MT-ir neurons (black dot) and fibers (small black dot) throughout the brain of the native Thai chicken. Sections are presented in a rostral to caudal order from **A** to **D**. Coronal illustrations are redrawn, with the given coordinates, from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988).

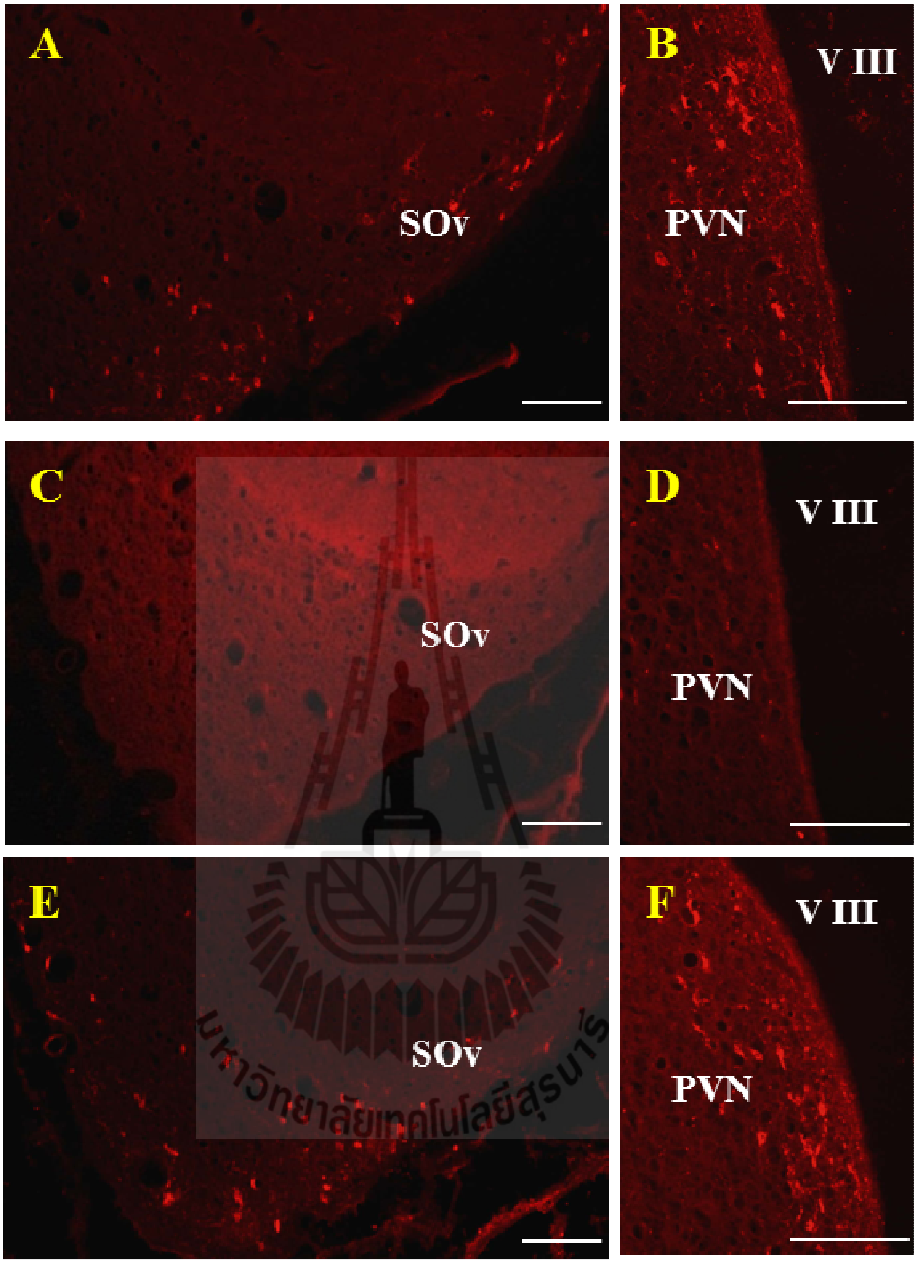

Figure 4.2 Photomicrographs demonstrating the MT-ir neurons in the SOv (**A**, **C**, **E**) and PVN (**B**, **D**, **F**) areas. Brain sections in **A** and **B** stained with OT antibody, whereas sections in **C** and **D** are stained with OT antibody pre-absorbed with 10 µg/ml of MT, whereas sections in **E** and **F** are stained with OT antibody pre-absorbed with 10 µg/ml of VT. For abbreviations, see Table 4.1. Scale bar = 100 µm (**A**, **C**, **E**), scale bar = 50 µm (**B**, **D**, **F**).

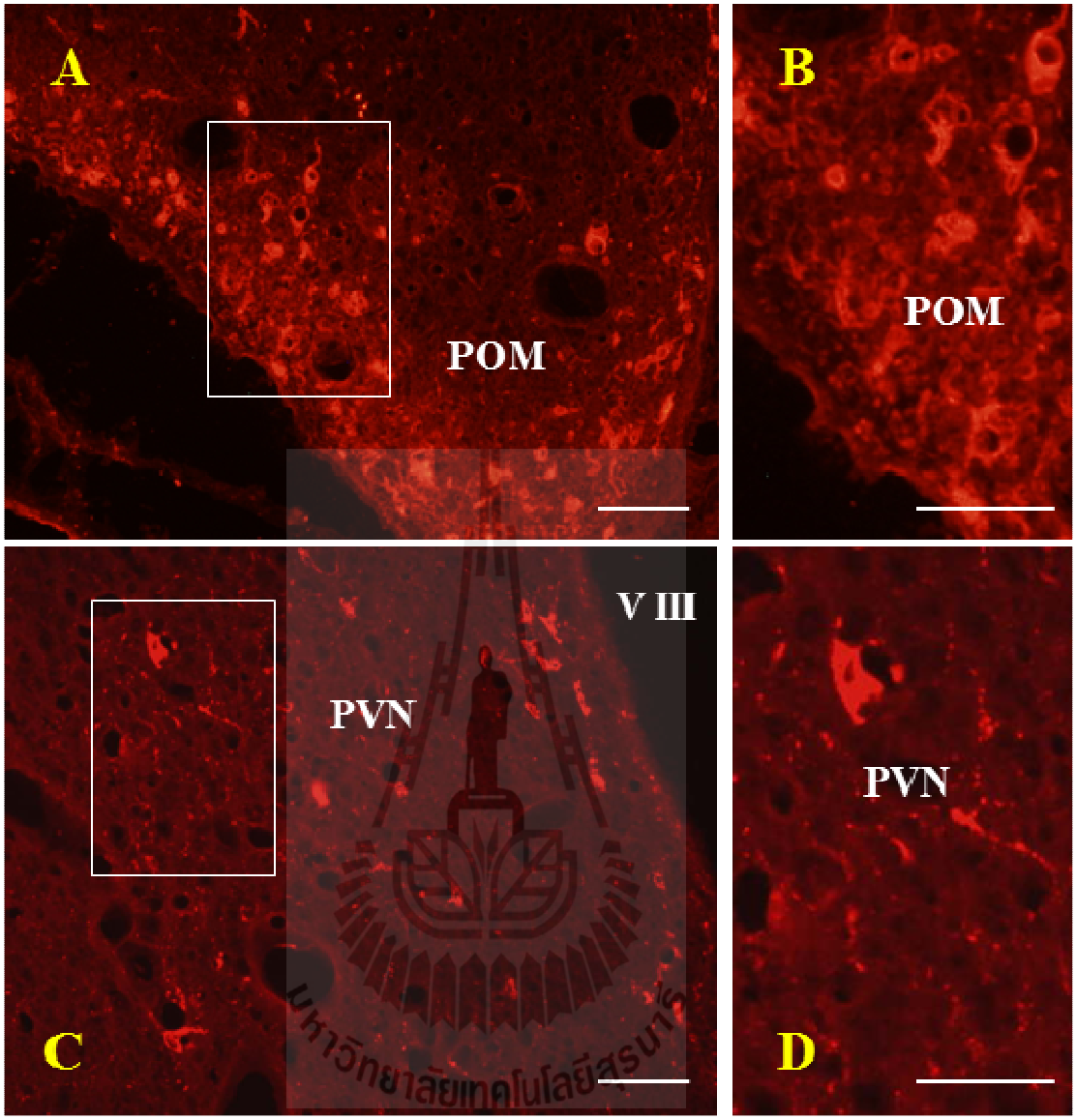

Figure 4.3 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the nucleus preopticus medialis (POM; **A**) and nucleus paraventricularis magnocellularis (PVN; **C**) of the native Thai chicken. Rectangles indicate areas from which following photomicrographs are taken. (**B**) Higher magnification of the MT-ir neurons from (**A**) showed an oval shape with monopolar process neurons in the POM. (**D**) Higher magnification of MT-ir neurons in the PVN. For abbreviations, see Table 4.1. Scale bar = 100 µm (**A**, **B**), scale bar = 50 µm (**C**, **D**).

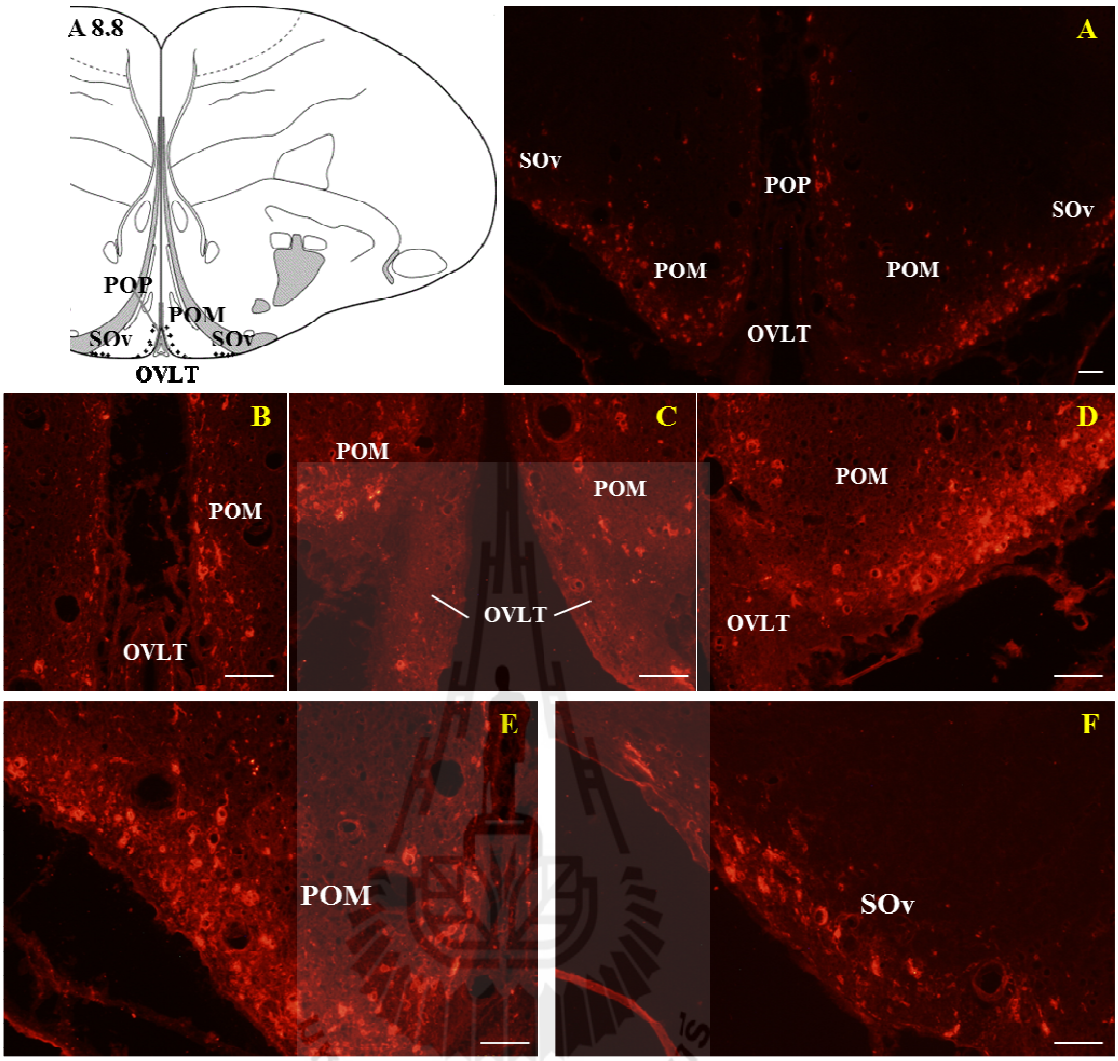

Figure 4.4 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the preoptic area at A8.8 of schematic diagrams of coronal sections of the hypothalamic regions of the native Thai chicken. For abbreviations, see Table 4.1. Scale bar = 200 µm (**A**), scale bar = 100 µm (**B**, **C**, **D**, **E**, **F**).

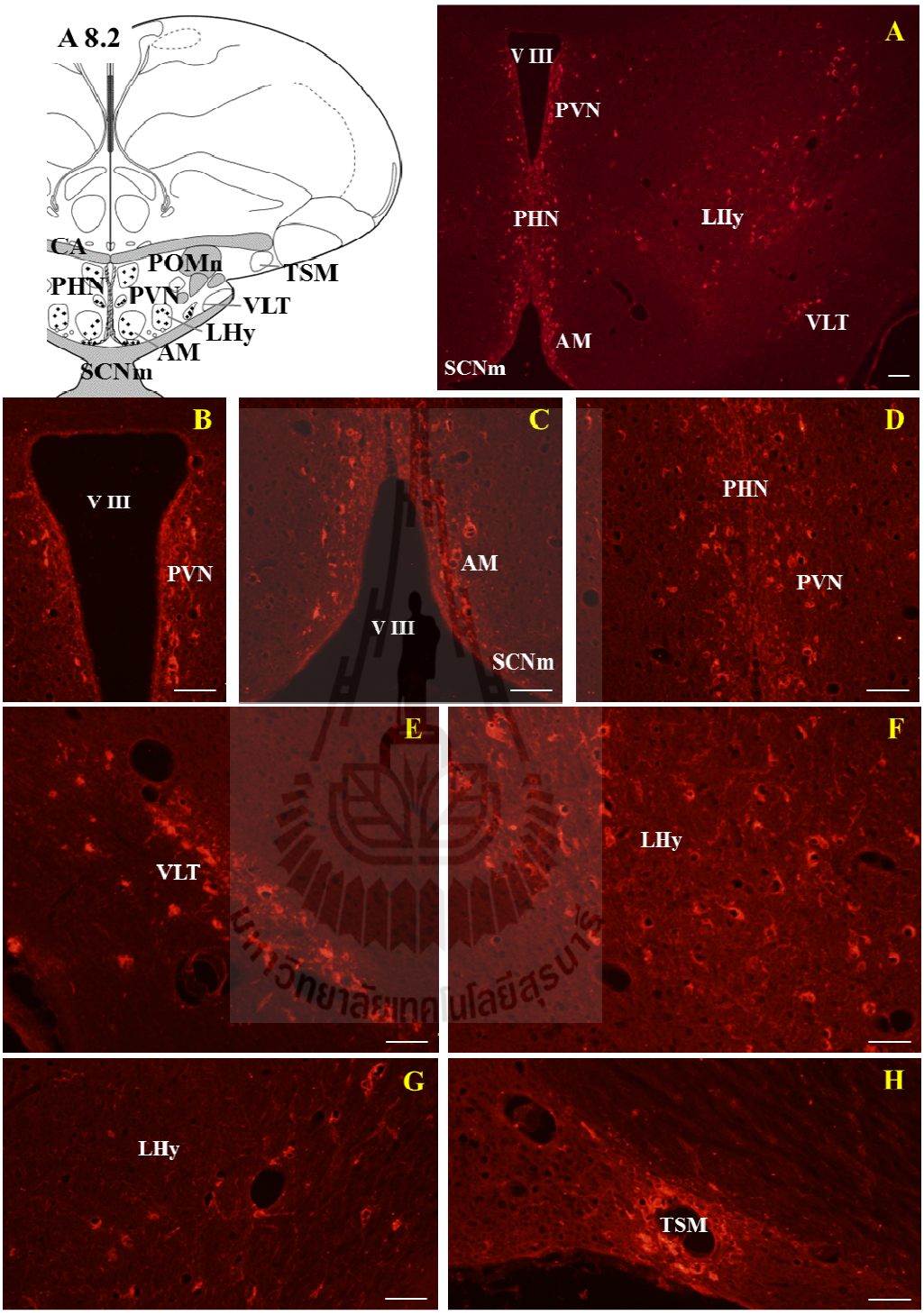

Figure 4.5 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the preoptic area at A8.2 of schematic diagrams of coronal sections of the hypothalamic regions of the laying native Thai chicken. For abbreviations, see Table 4.1. Scale bar = 200 µm (**A**), scale bar = 100 µm (**B**, **C**, **D**, **E**, **F**, **G**, **H**).

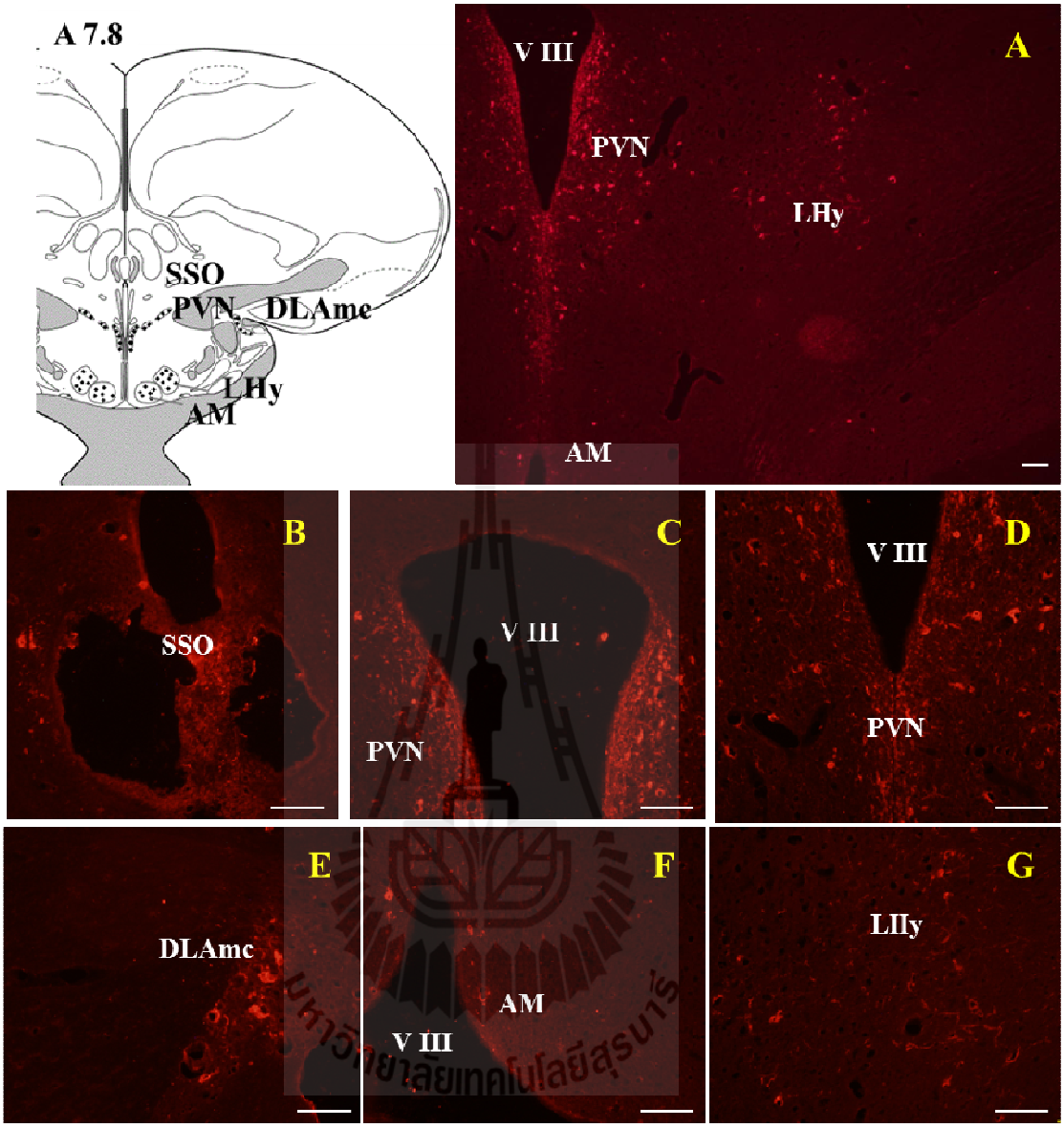

Figure 4.6 Photomicrographs illustrating the distributions of MT-ir neurons and fibers at A7.8 of schematic diagrams of coronal sections of the hypothalamic regions and more lateral regions of the native Thai chicken. For abbreviations, see Table 4.1. Scale bar = 200 µm (**A**), scale bar = 100 µm (**B**, **C**, **D**, **E**, **F**, **G**).

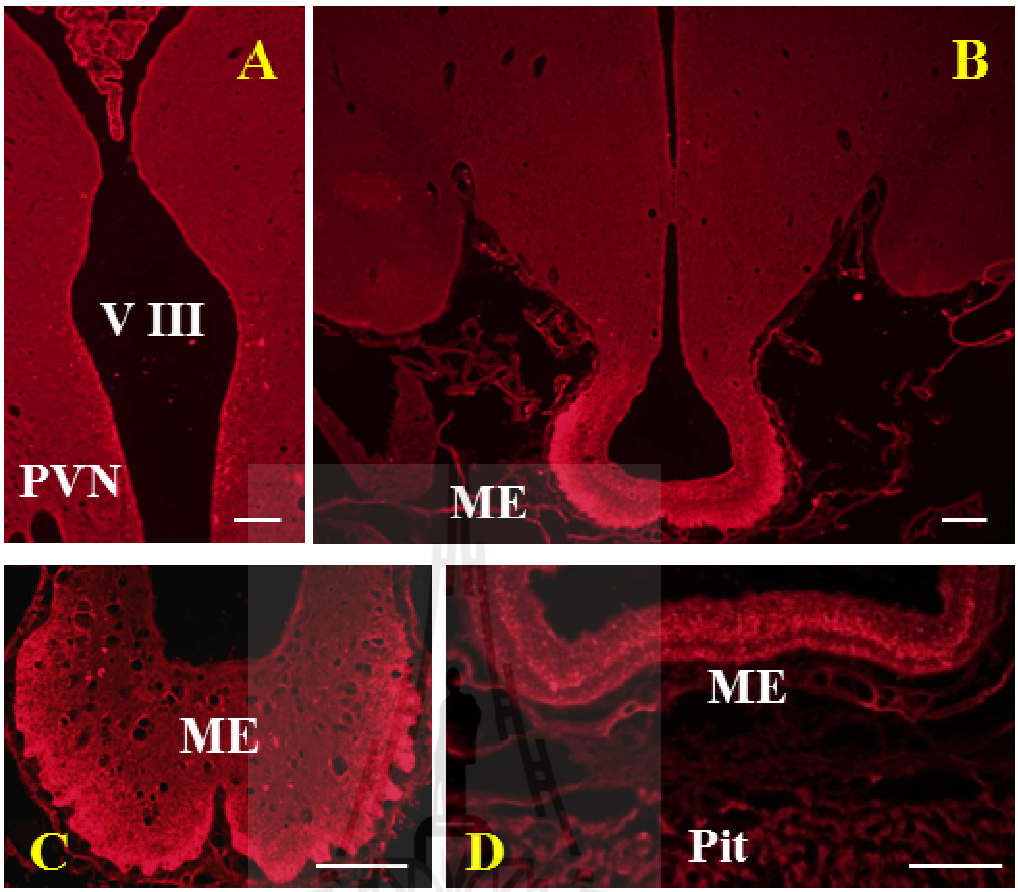

Figure 4.7 Photomicrographs illustrating the distribution of MT-ir neurons in the end of nucleus paraventricularis magnocellularis (PVN; **A**) in the native Thai chicken. MT-ir fibers were found in the external layer of the median eminence (ME; **B**, **C**, **D**). There was no MT-immunoreactivity observed in the pituitary (Pit). For abbreviations, see Table 4.1. Scale bar = 200 µm (**A**, **B**), scale bar = 100 µm (**C**, **D**).

Table 4.2 The number of MT-ir neurons within the individual hypothalamic areas (SOv, nucleus supraopticus, pars ventralis; POM, nucleus preopticus medialis; PVN, nucleus paraventricularis magnocellularis) in the native Thai chicken at different reproductive stages (NL, non-egg laying; L, egg laying; B, incubating eggs; R, rearing chicks). Values represent the means ± SEM (n=6*)*. Values with different superscripts are significantly different (P<0.05) within each group.

Hypothalamic Area	**Reproductive Stage**			
	NL	**L**	**B**	**R**
SOv	24.00 ± 4.31^{b}	37.87 ± 4.28^{ab}	54.54 ± 5.43^{a}	38.50 ± 4.96^{ab}
POM	55.58 ± 7.65^{c}	72.08 ± 4.56^{bc}	90.08 ± 3.21^{ab}	96.83 ± 3.81^{a}
PVN	33.29 ± 2.85^{b}	44.21 ± 3.28^{b}	75.00 ± 6.73^{a}	81.00 ± 3.89^{a}

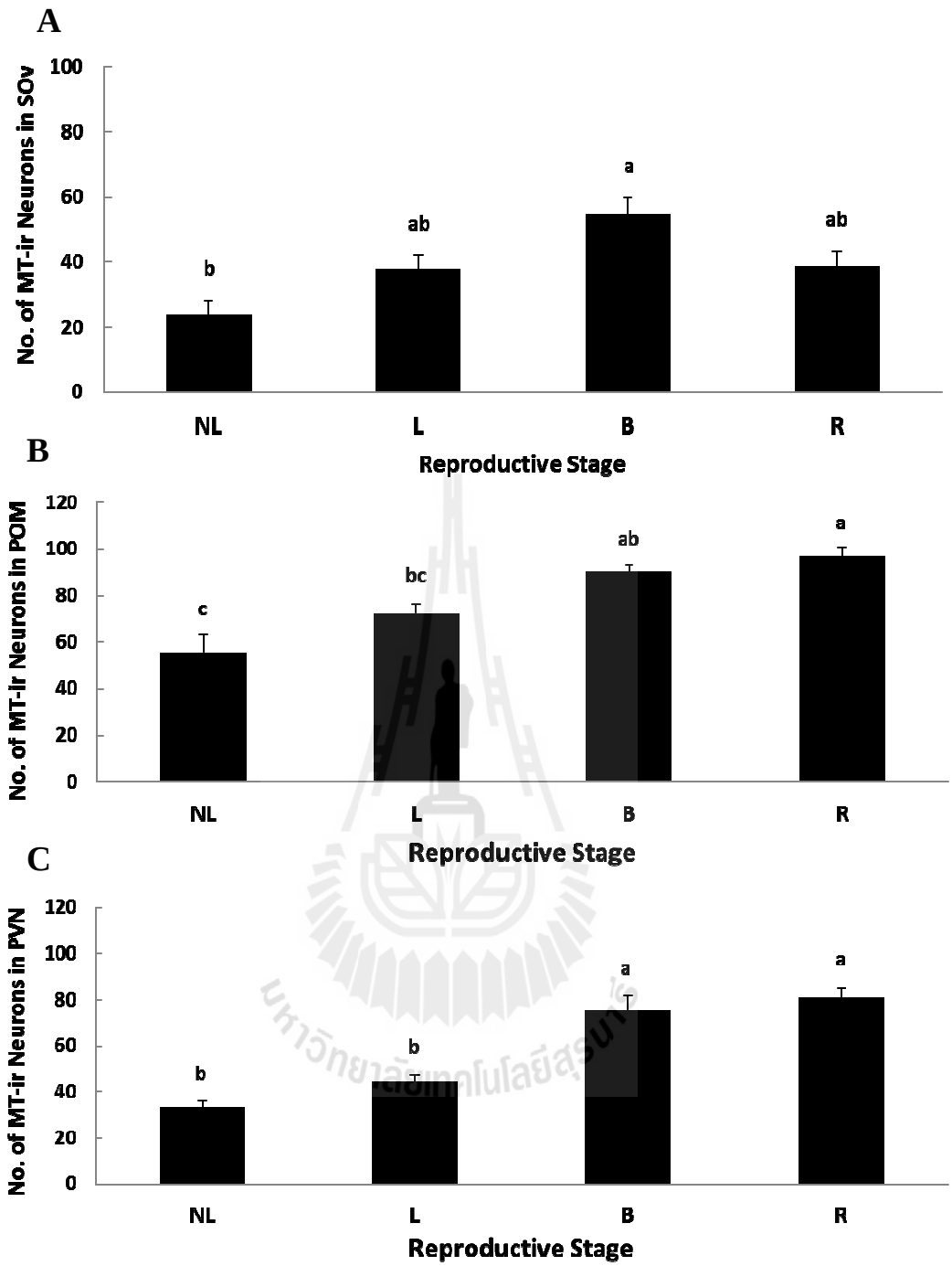

Figure 4.8 Number of MT-ir neurons in the nucleus supraopticus; pars ventralis (SOv; **A**), nucleus preopticus medialis (POM; **B**), and nucleus paraventricularis magnocellularis (PVN; **C**) in the native Thai chicken at different reproductive stages (NL, non-egg laying; L, egg laying; B, incubating eggs; R, rearing chicks). Values with different letters are significantly different ($P<0.05$; $n=6$).

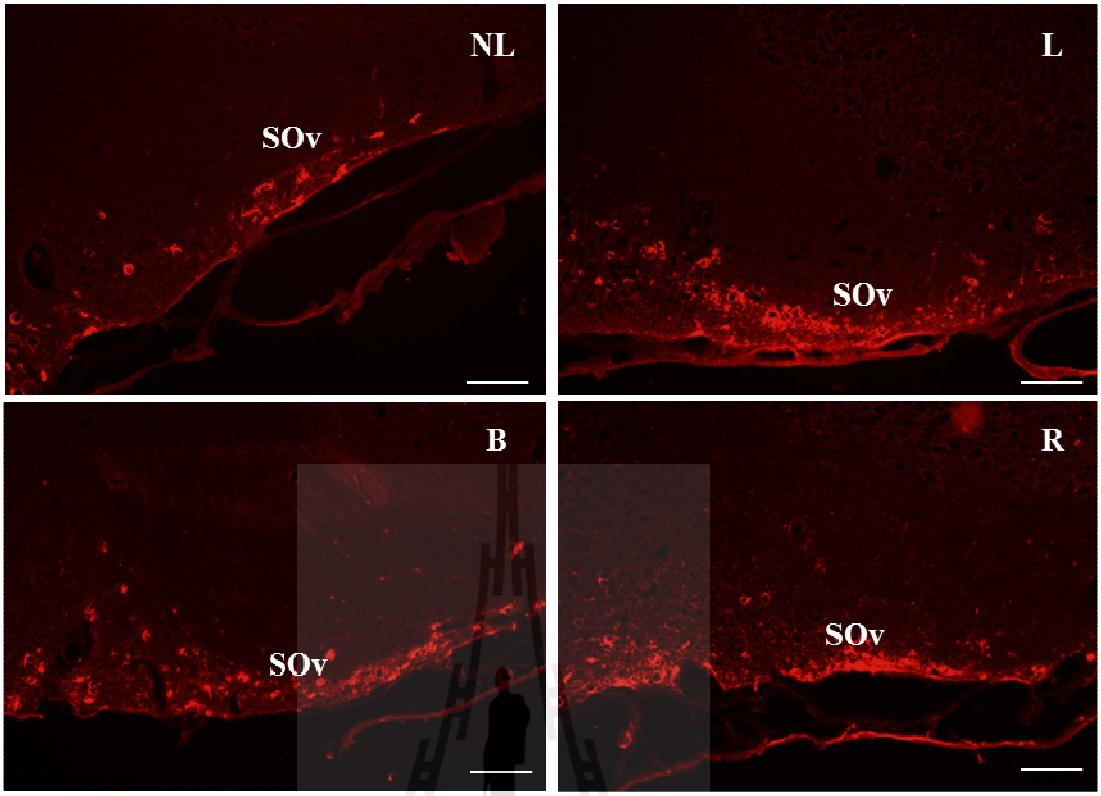

Figure 4.9 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the nucleus supraopticus; pars ventralis (SOv) during different reproductive stages (NL, non-egg laying; L, egg laying; B, incubating eggs; R, rearing chicks). For abbreviations, see Table 4.1. Scale bar = 100 µm.

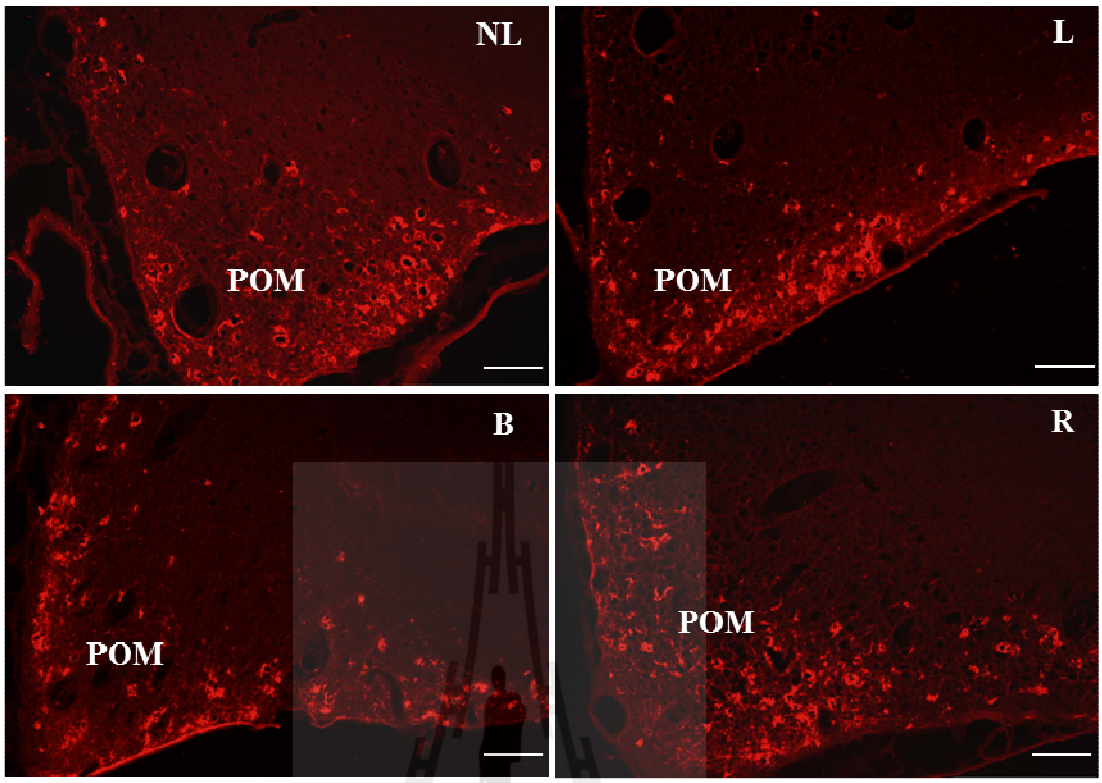

Figure 4.10 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the nucleus preopticus medialis (POM) during different reproductive stages (NL, non-egg laying; L, egg laying; B, incubating eggs; R, rearing chicks). For abbreviations, see Table 4.1. Scale bar = 100 µm.

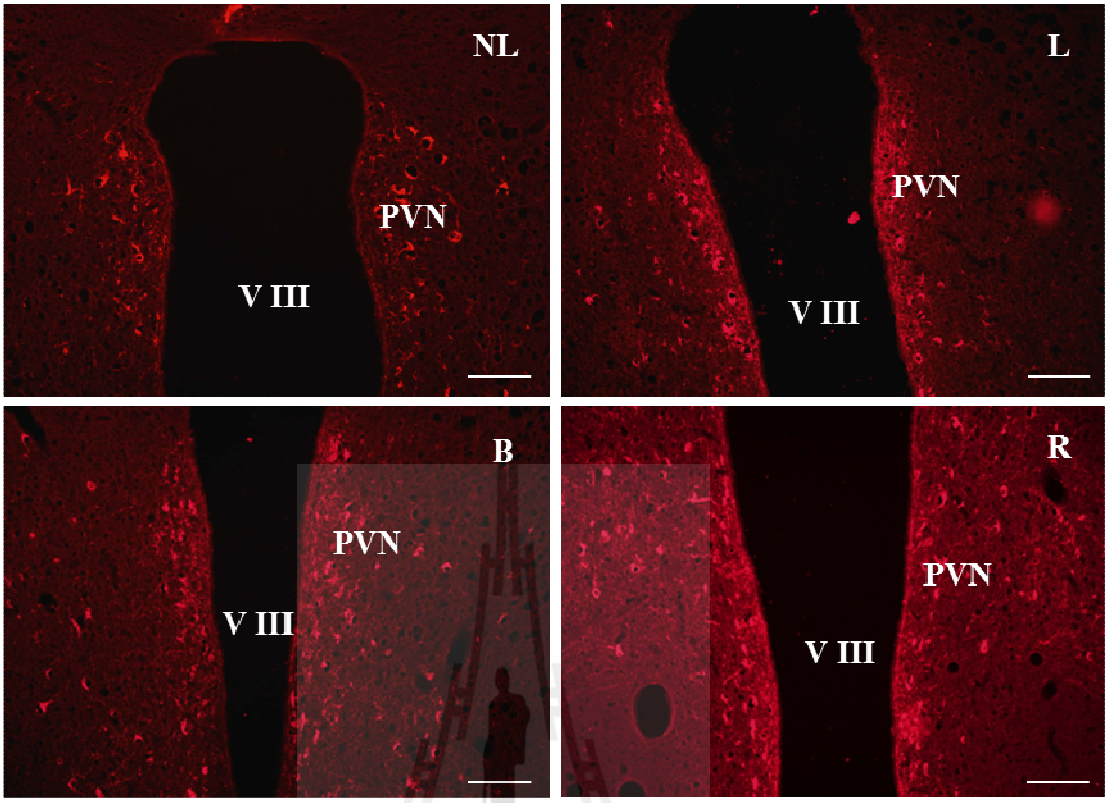

Figure 4.11 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the nucleus paraventricularis magnocellularis (PVN) during different reproductive stages (NL, non-egg laying; L, egg laying; B, incubating eggs; R, rearing chicks). For abbreviations, see Table 4.1. Scale bar = 100 µm.

Table 4.3 The number of MT-ir neurons (Mean ± SEM) in the the nucleus supraopticus; pars ventralis (SOv), nucleus preopticus medialis (POM), and nucleus paraventricularis magnocellularis (PVN) of rearing and non-rearing native Thai hens at different days of the observation periods (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group at a given time point.

Area	Group	Days Following of Chicks' Deprivation from Hens				
		Day of Hatch	**4**	**7**	**10**	**14**
SOv	**Rearing**	48.00 ± 7.01^{a}	43.54 ± 8.33^{a}	41.83 ± 9.49^{a}	$40.17 \pm 4.41^{a*}$	$38.50 \pm 4.96^{a*}$
	Non-rearing	N/A	34.00 ± 9.92^{A}	21.17 ± 3.50^{A}	20.38 ± 2.80^{A}	20.42 ± 3.65^{A}
POM	**Rearing**	91.63 ± 8.32^{a}	$84.88 \pm 6.07^{a*}$	$88.79 \pm 13.04^{a*}$	$94.17 \pm 9.38^{a*}$	$85.33 \pm 3.32^{a*}$
	Non-rearing	N/A	48.63 ± 3.85^{A}	51.42 ± 5.23^{A}	46.96 ± 5.29^{A}	35.13 ± 6.01^{A}
PVN	**Rearing**	117.46 ± 8.72^{a}	$105.79 \pm 2.60^{abc*}$	$110.00 \pm 5.90^{ab*}$	$88.88 \pm 3.77^{bcd*}$	$85.33 \pm 3.32^{bcd*}$
	Non-rearing	N/A	53.54 ± 2.98^{A}	52.92 ± 2.87^{A}	51.71 ± 1.15^{AB}	55.58 ± 1.62^{A}

Table 4.3 The number of MT-ir neurons (Mean ± SEM) in the nucleus supraopticus; pars ventralis (SOv), nucleus preopticus medialis (POM), and nucleus paraventricularis magnocellularis (PVN) of rearing and non-rearing native Thai hens at different days of the observation periods (n=6). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group at a given time point (Continued).

Area	Group	Days Following of Chicks' Deprivation from Hens			
		17	**21**	**24**	**28**
SOv	**Rearing**	$33.75 \pm 2.66^{a*}$	$42.58 \pm 8.32^{a*}$	$39.83 \pm 8.13^{a*}$	$32.79 \pm 3.11^{a*}$
	Non-rearing	14.63 ± 1.99^{A}	19.88 ± 1.79^{A}	16.33 ± 1.21^{A}	15.13 ± 3.15^{A}
POM	**Rearing**	$73.83 \pm 2.79^{a*}$	$74.75 \pm 2.03^{a*}$	$84.42 \pm 5.07^{a*}$	$80.29 \pm 2.58^{a*}$
	Non-rearing	42.63 ± 5.37^{A}	48.21 ± 3.18^{A}	48.29 ± 2.92^{A}	42.96 ± 2.47^{A}
PVN	**Rearing**	$88.00 \pm 8.13^{bcd*}$	$80.63 \pm 2.96^{d*}$	$75.67 \pm 5.02^{d*}$	$84.38 \pm 4.16^{cd*}$
	Non-rearing	39.21 ± 2.62^{C}	38.63 ± 2.51^{C}	40.79 ± 2.48^{BC}	33.33 ± 3.62^{C}

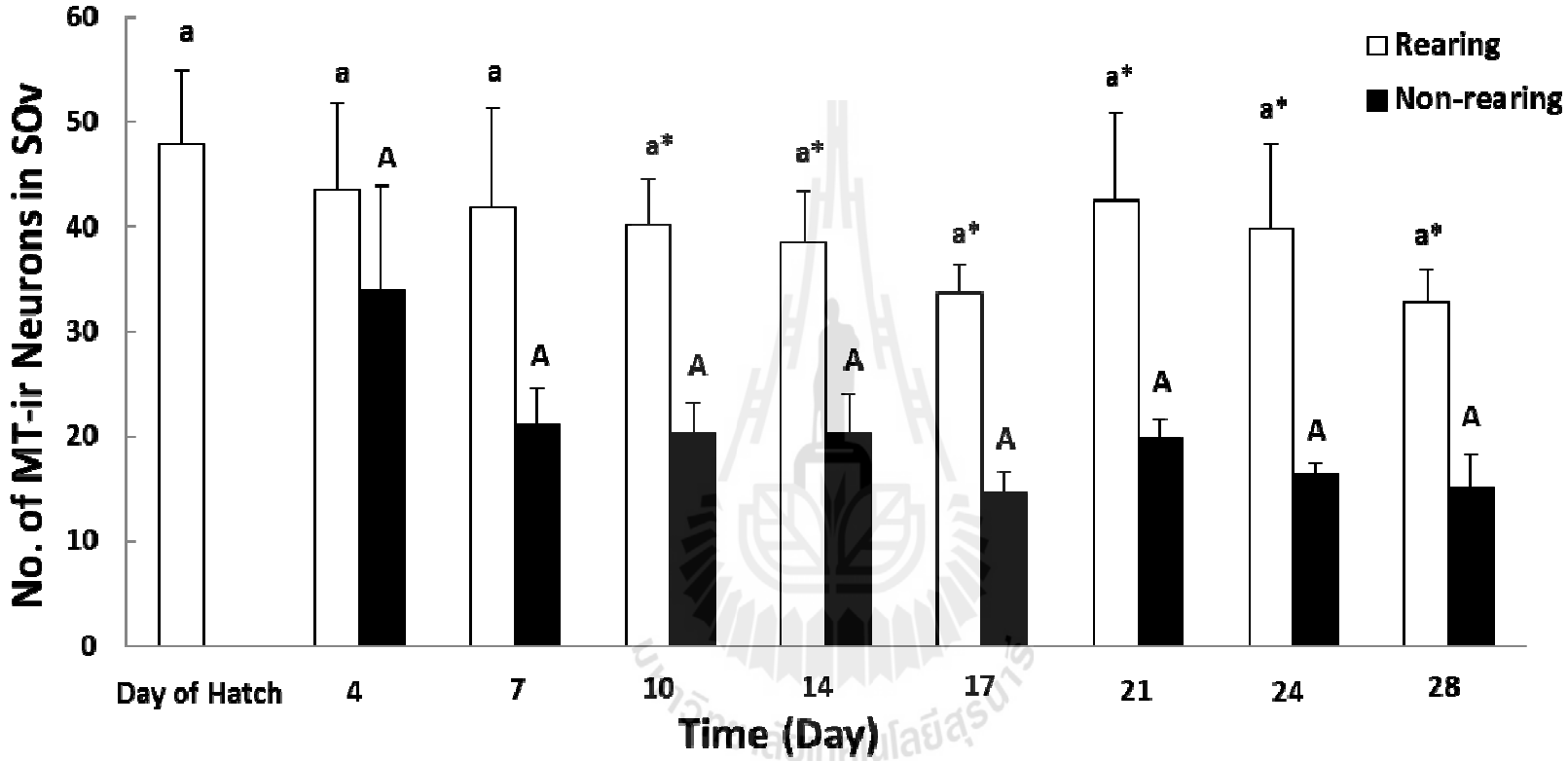

Figure 4.12 Changes in the number of MT-ir neurons in the nucleus supraopticus; pars ventralis (SOv) of rearing and non-rearing native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group at a given time point.

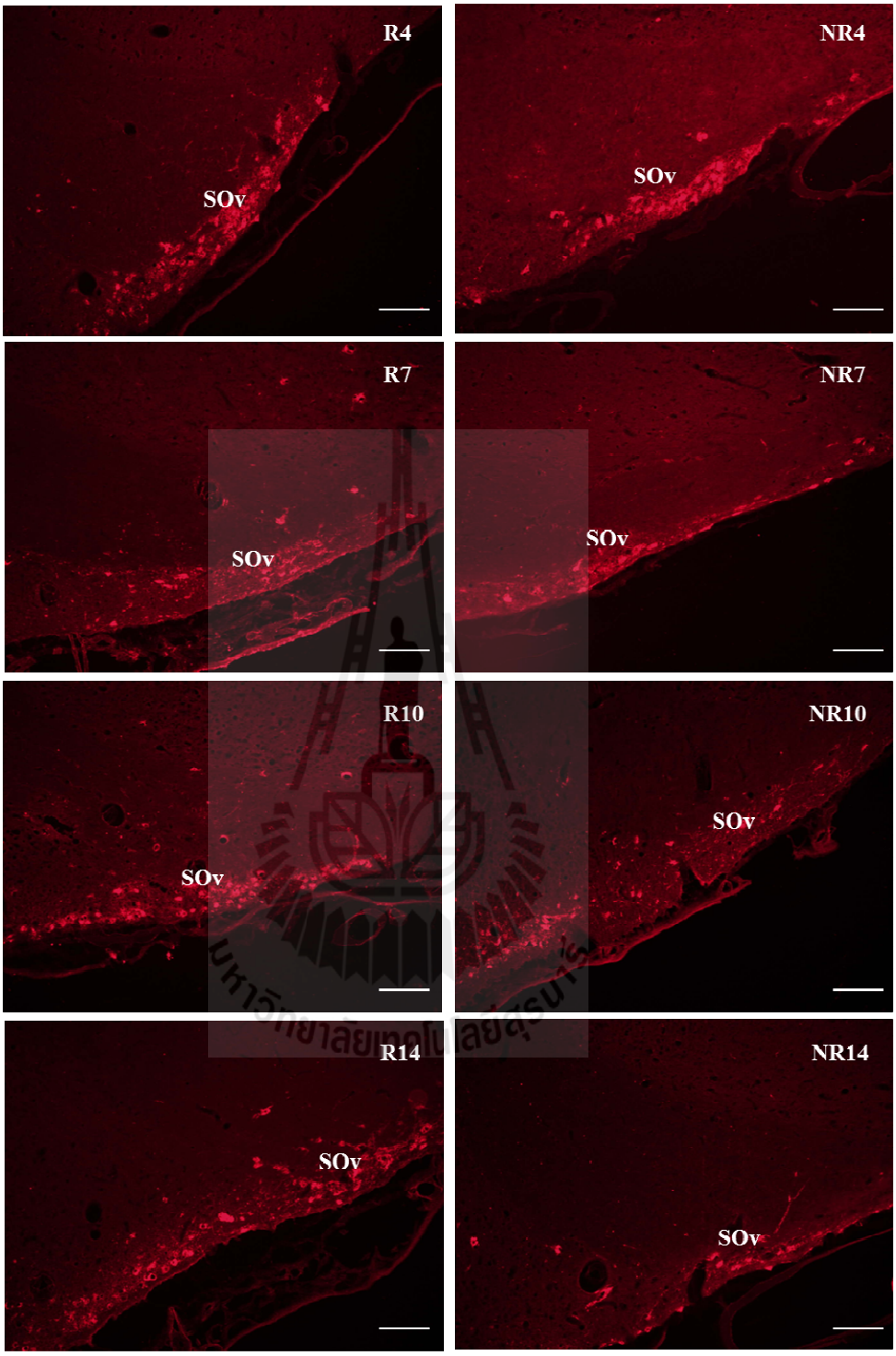

Figure 4.13 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the nucleus supraopticus; pars ventralis (SOv) of rearing (R) and non-rearing (NR) native Thai hens on different days following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 4.1. Scale bar = 100 µm.

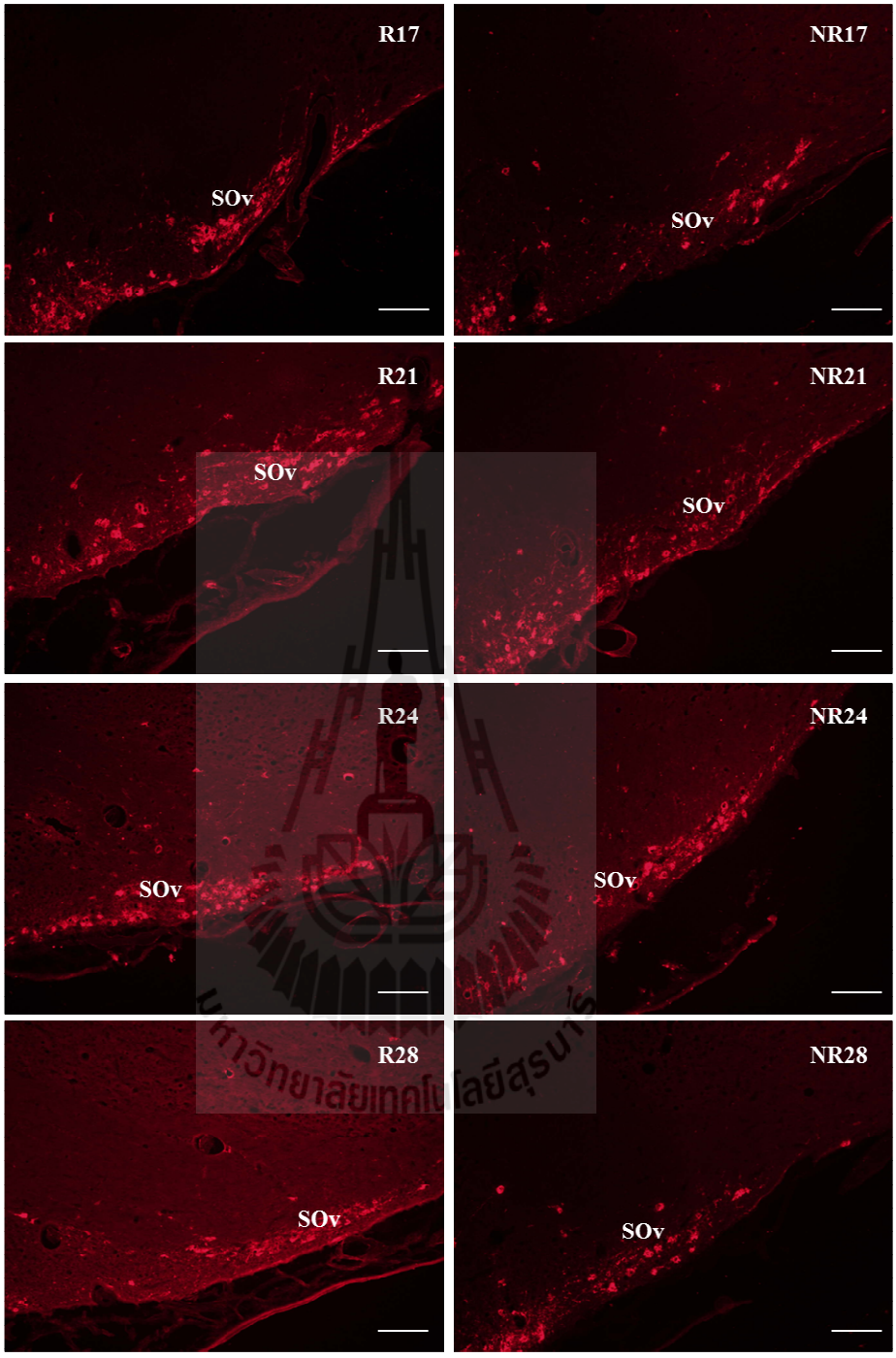

Figure 4.13 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the nucleus supraopticus; pars ventralis (SOv) of rearing (R) and non-rearing (NR) native Thai hens on different days following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 4.1. Scale bar = 100 µm. (Continued).

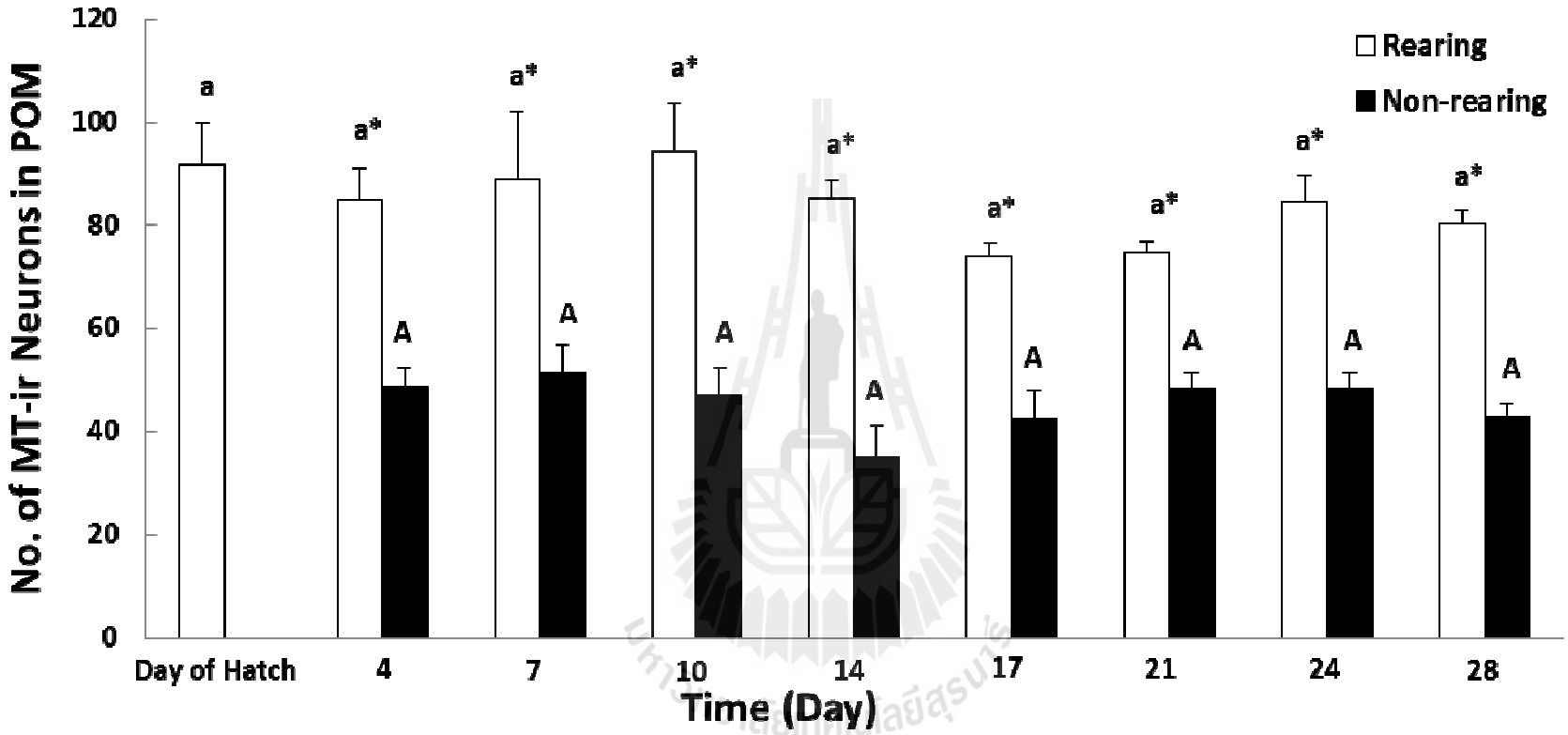

Figure 4.14 Changes in the number of MT-ir neurons in the nucleus preopticus medialis (POM) of rearing and non-rearing native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group at a given time point.

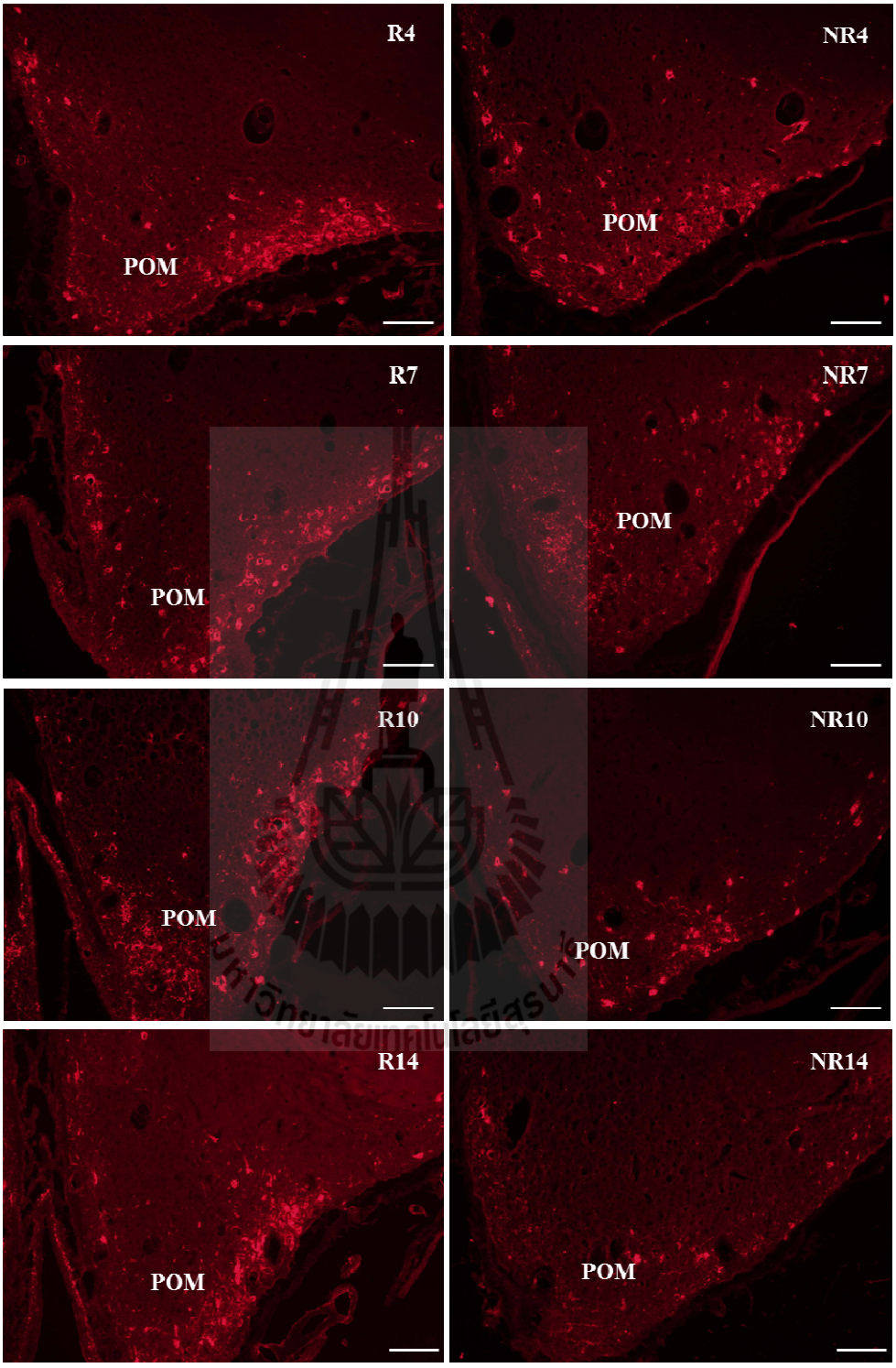

Figure 4.15 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the nucleus preopticus medialis (POM) of rearing (R) and non-rearing (NR) native Thai hens on different days following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 4.1. Scale bar = 100 µm.

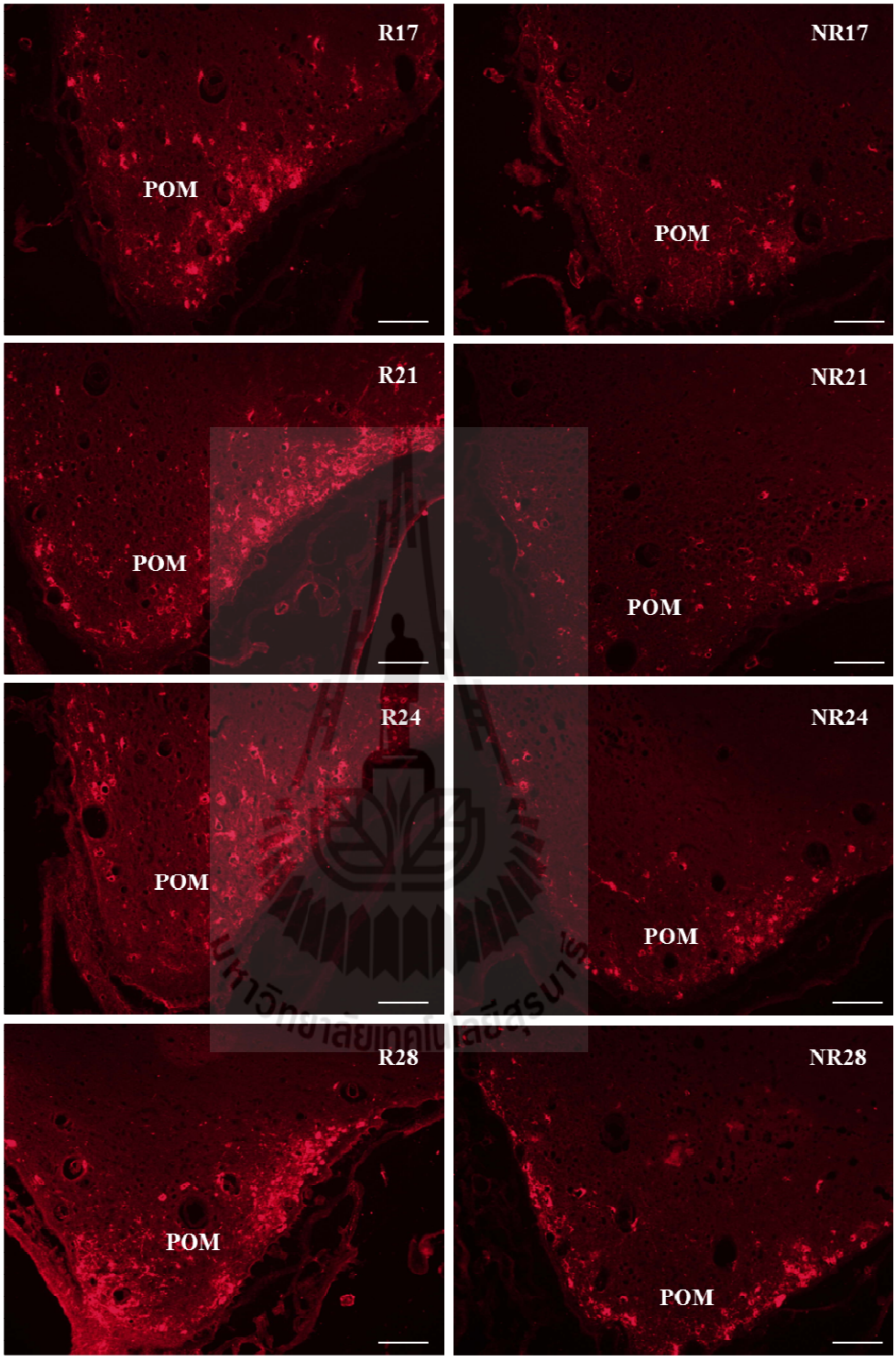

Figure 4.15 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the nucleus preopticus medialis (POM) of rearing (R) and non-rearing (NR) native Thai hens on different days following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 4.1. Scale bar = 100 µm (Continued).

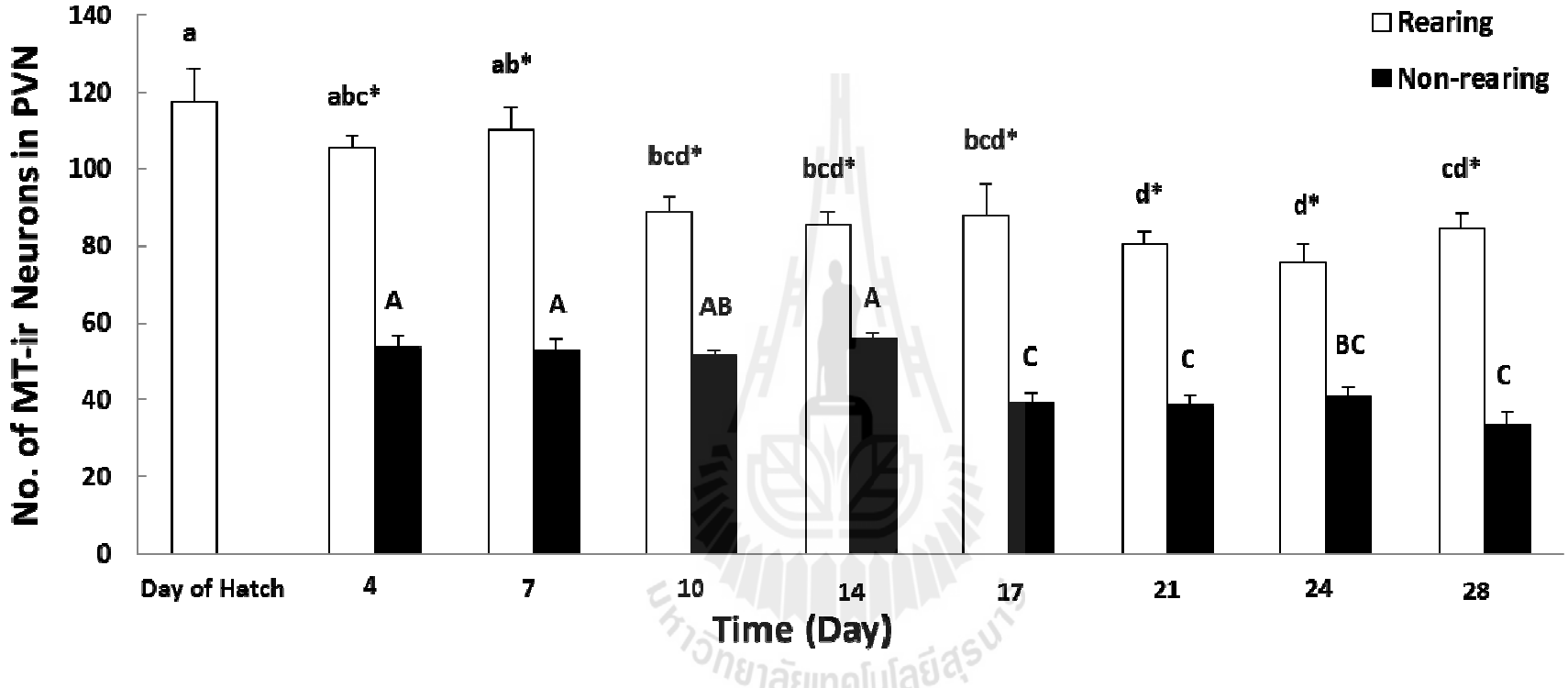

Figure 4.16 Changes in the number of MT-ir neurons in the nucleus paraventricularis magnocellularis (PVN) of rearing and non-rearing native Thai hens (n=6). Values are presented as mean ± SEM. Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and $^{*}P<0.05$ for a comparison between group at a given time point.

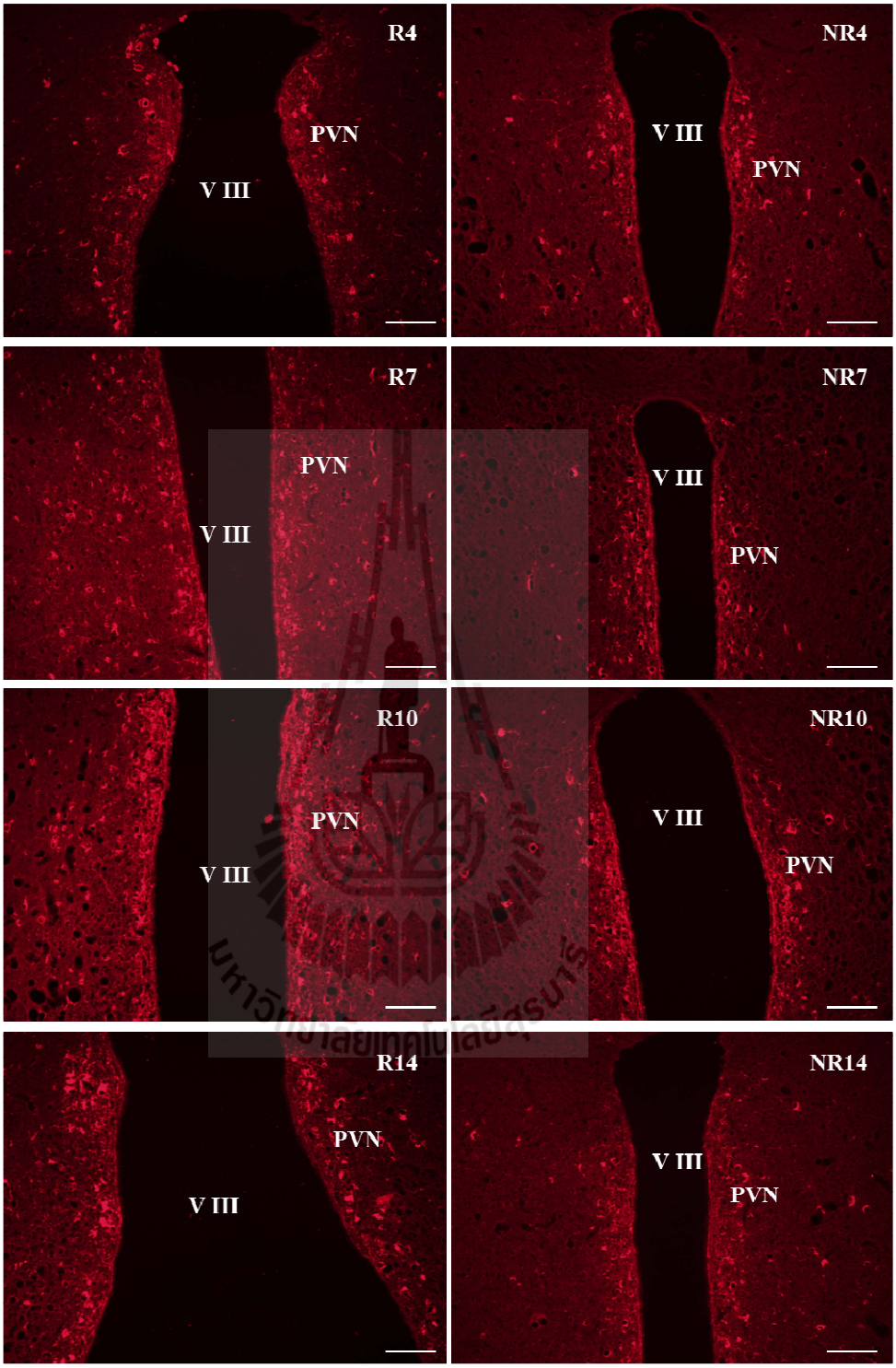

Figure 4.17 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the nucleus paraventricularis magnocellularis (PVN) of rearing (R) and non-rearing (NR) native Thai hens on different days following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 4.1. Scale bar = 100 µm.

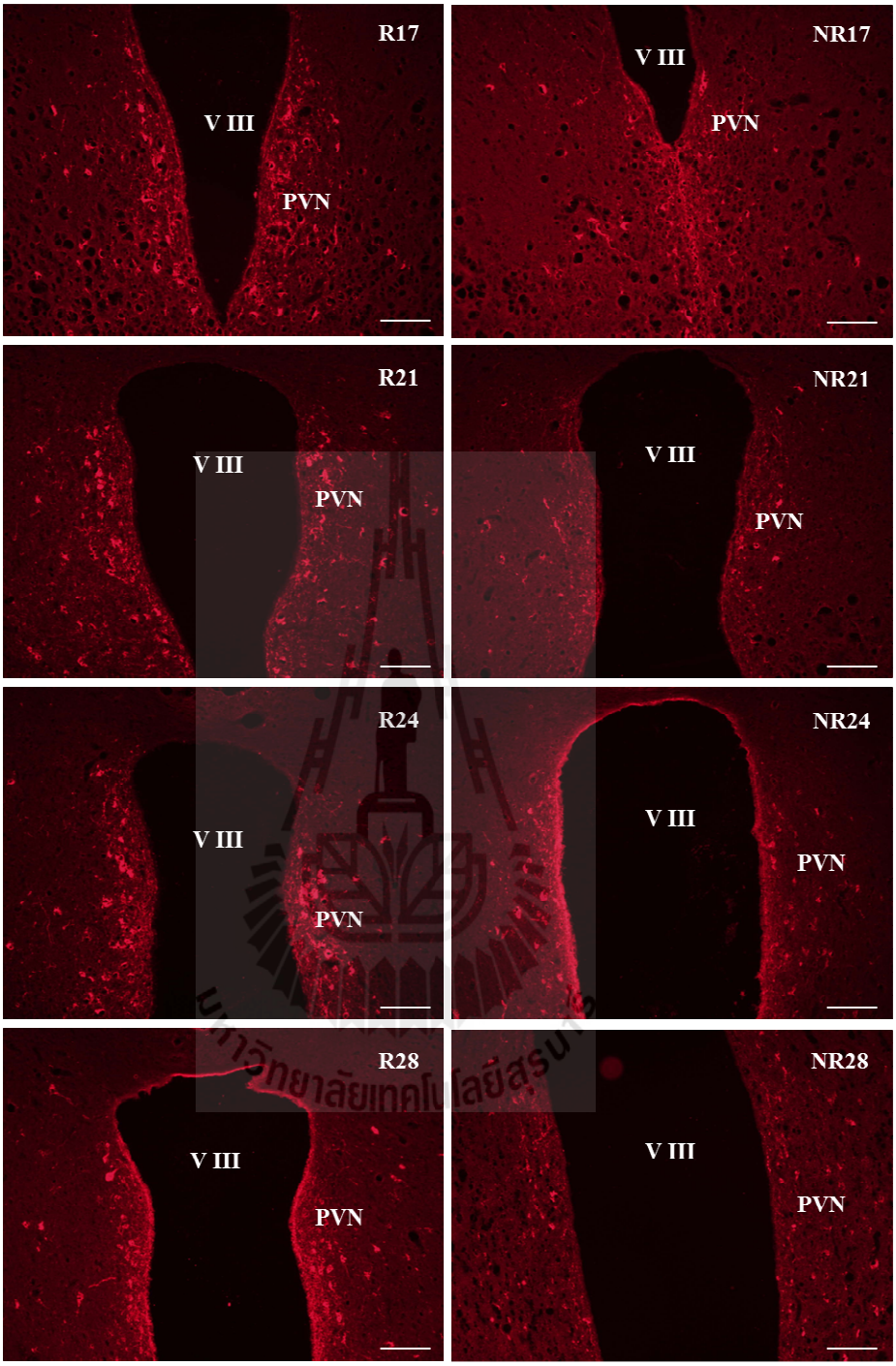

Figure 4.17 Photomicrographs illustrating the distributions of MT-ir neurons and fibers in the nucleus paraventricularis magnocellularis (PVN) of rearing (R) and non-rearing (NR) native Thai hens on different days following the initiation of rearing or non-rearing their chicks. For abbreviations, see Table 4.1. Scale bar = 100 µm. (Continued).

4.5 Discussion

This study investigates the association of the MTergic system with rearing/brooding behavior in the native Thai chickens. The results reveal that the MT-ir neurons and fibers were found predominantly in the diencephalon with the highest abundance in the SOv, POM, and PVN. The results demonstrate marked differences in the number of MT-ir neurons in the SOv, POM, and PVN across the reproductive cycle, indicating that the MTergic system within these nuclei is associated with the reproductive cycle in this tropical species. Furthermore, the MT-ir neurons in the SOv, POM, and PVN were higher in the R hens when compared to those of the NR hens. The MT-ir neurons were sharply decreased in the NR hens. These findings indicate, for the first time, that MTergic system plays a pivotal role in neuroendocrine reorganization to establish and maintain maternal behaviors. The decline in MTergic activity during disrupting rearing behavior might be related to the contribution of rearing behavior in this equatorial precocial species. The results further suggest the role of MT in these nuclei during the period of rearing chicks resembles that of OT in these similar brain structures during the parturition and lactation in mammals.

In the present study, the distributions of MT-ir neurons and fibers in the brain of the native Thai chickens were revealed. The majority of MT-ir neurons were distributed closely to the third ventricle within the POA area of the hypothalamus. The greatest density of MT-ir neurons were found prominently within the SOv, POM, VLT, LHy, and PVN areas. Small numbers of the MT-ir neurons were also observed within the POP, AM, PHN, SCNm, DLAmc, and TSM. Small numbers of the MT-ir fibers were found in the OVLT, SSO, and the external layer of the ME. The anatomical distributions of MT immunoreactivity from this present study were similar

to those of reported previously in chickens, domestic mallards, and Japanese quails (Goossens et al., 1977; Bons, 1980). Furthermore, the MT-ir neurons were also found in areas outside the hypothalamus such as the cerebellum, LS, optic lobe, pons, and medulla oblongata in the chickens (Robinzon et al., 1988). However, previous studies in mammals found that OT-ir fibers can be found throughout the brain including the Ac, lateral septum, amygdala, and several structures in the hindbrain, brainstem, and spinal cord (Sofroniew, 1980; Castel and Morris, 1988). With the exception of the hindbrain and spinal cord projections, which arise from parvocellular neurons in the PVN, the source of other central OT-containing fibers has not been documented. However, lesion of the PVN results in a marked reduction in OT-ir fibers throughout the brain (De Vries and Buijs, 1983). Little is known regarding to the regulation of the release of OT from these forebrain projections, but they presumably contribute significantly to the regulation of behaviors. Gonadal steroids play an important role in mediating the regulation of OT receptor (OTR) expression. Most peripheral OT-binding sites are up-regulated by estrogen including the pituitary, renal, and uterine OTRs (Fuchs et al., 1983; Soloff et al., 1983; Maggi et al., 1992). This up-regulation is accompanied by an increase in OTR mRNA accumulation, suggesting that the up-regulation is consequence of a genomic estrogen effect on OTR gene transcription (Breton et al., 1995; Larcher et al., 1995).

The results of this present study provide evidence that the MTergic system is associated with the reproductive cycle in the native Thai chicken, a tropical species. There were more MT-ir neurons presented in the POM and PVN than that of in the SOv. The numbers of MT-ir neurons were gradually increased in the POM and PVN, when birds make transition from eggs laying to incubating period and remained at the

highest level in the rearing chick period. These results are consistent with previous study in the turkeys, the seasonally breeding birds, reporting that the MT-ir neurons increase in the PVN and SOv of the incubating hens when compared to the laying ones (Thayananuphat et al., 2011). Similarly, it has been reported in mammals that OT mediates complex social and reproductive behaviors including mating behavior, pair-bond formation, and maternal behaviors. In addition to the role in regulating the peripheral physiology necessary for parturition and lactation, OT plays a significant role in initiating maternal nurturing behavior as well. Intracerebroventricular infusions of OT stimulate a rapid onset of full maternal behaviors in estrogen-primed female rats (Pedersen and Prange, 1979; Pedersen et al., 1982). Moreover, the studies in mammals demonstrate that the MPOA contains estrogen receptor (Shughrue et al., 1997), progesterone receptor (Numan et al., 1999), PRL receptor (Bakowska and Morrell, 1997), and OTR (Champagne et al., 2003). Estrogen, PRL, and placental lactogens have all been shown to stimulate the onset of maternal behaviors when microinjected into the MPOA (Bridges et al., 1990; 2001). OT has also been shown to act on the MPOA to stimulate maternal behaviors (Pedersen et al., 1994). Moreover, Stolzenberga and Numan (2011) propose that steroids might interact with DA within the MPOA to promote reproductive behaviors. Hull and colleagues have presented the best evidence for this idea in the context of male sexual behaviors (Bitran and Hull, 1987; Hull et al., 1999; Hull and Dominguez, 2006). In support of the idea that estrogen-DA interactions within the MPOA might also promote maternal behaviors, there are data indicating that in the absence of estradiol benzoate (EB) treatment, administration of a D_1 DA receptor agonist directly into the MPOA can promote an immediate onset of full maternal behaviors in the hysterectomy and ovariectomy rats

on day 15 (Stolzenberg et al., 2007). Because of the facilitatory effect of EB action on the MPOA is replicated by D_1 DA receptor stimulation of the MPOA, a simple interpretation is that D_1 DA receptor stimulation of the MPOA substitutes for estrogen action at the estrogen receptor to facilitate the onset of maternal behaviors (Stolzenberga and Numan, 2011).

Changes in the number of MT-ir neurons were observed in the SOv, POM, and PVN of R and NR hens, and the numbers of MT-ir neurons in these hypothalamic nuclei were compared between the R and NR hens. In all these hypothalamic nuclei, the MT-ir neurons were significantly higher in the hens presented with chicks than those of the hens deprived their chicks. In addition to find that the MT-ir neurons in the POM vary across the reproductive stages, the highest density of MT-ir neurons were observed in the R hens when compared to those of the NR hens. It has been suggested that these MT neuronal groups in the POM may be conserved for the maternal care for the chicks during the rearing behavior. This result supports those reported in mammalian species (Numan and Sheehan, 1997; Numan and Insel, 2003). OT appears to be regulating maternal behaviors though physiological actions in at least three brain regions; the MPOA, the VTA, and the olfactory bulb (OB). Infusions of OTR antagonist into the MPOA and VTA prevent parturient dams from retrieving pups or from assuming a nursing posture over pups (Pedersen et al., 1994). OTRs are found to be increased in the MPOA and VTA at the time of parturition, and the mRNA expression of OTR is elevated in the MPOA during pregnancy (Pedersen et al., 1994; Young et al., 1997; Meddle et al., 2007). It has been further suggested that OT regulates maternal motivation in rodents. However, fascinating studies in sheep suggest that OT plays a role in the establishment of the mother-infant bond, a form of

social attachment as well. Unlike rodents, ewes develop highly selective maternal behavior toward their own lambs only and reject foreign lambs. Infusions of OT induce short latency maternal responses to foreign lamps in estrogen-primed ewes (Kendrick et al., 1987). In sheep, maternal behaviors develop at the onset of parturition or can be artificially stimulated with vaginocervical stimulation. Additionally, high concentration of estrogen receptor is found at OT neurons in the MPOA in the rabbits (Caba et al., 2003). In the rats, OTRs mRNA is increased during pregnancy and/or at parturition in the SON, OB, MPOA, bed nucleus of the stria terminalis, and medial amygdala (Young et al., 1997; Meddle et al., 2007). However, some evidences suggest that the POA is important for the expression of maternal behaviors (Featherstone et al., 2000). In birds, it has emerged as a crucial site for physiological action of PRL in relation to parental care based upon the binding studies. Lesions to the POA specifically disrupt PRL-induced parental feeding, while sparing PRL-induced hyperphagia in the ring doves (Slawski and Buntin, 1995). Concordant with this result is the fact that expression of the immediate early gene, c-fos, is enhanced in the POA of doves in association with 2 weeks of incubation behavior (Sharp et al., 1996).

The numbers of MT-ir neurons were compared in the SOv and PVN of R and NR hens. As mentioned above, the numbers of MT-ir neurons within these nuclei of the R hens were significantly higher than that of the NR hens. In the R hens, the MT-ir neurons were found the greatest within both nuclei after the chicks hatched and remained at the high level through the observed rearing period. Within the PVN, the MT-ir neurons were higher in the R hens in comparison to their respective NR hens through the observation period. However, the MT-ir neurons in the SOv were significantly different between the R and NR hens after day 7 of rearing period. These

results are in good agreement with previous studies in mammals (van Leengoed et al., 1987). An increase in the number of OT-ir neurons is observed in the PVN of the rats during the parturition (Jirikowski et al., 1989). Within the PVN, OT concentrations increase in post-parturient ewes and maternal behavior is induced after OT retrodialysis in the PVN of ewes treated with estrogen and progesterone (Da Costa et al., 1996). The number of OT neurons expressing heterogeneous nuclear RNA in the SON increases at the end of parturition in lactating rats comparing with virgin or pregnant rats (Douglas et al., 1998). Fos-ir neurons in the PVN and SON were more abundance in the parturient and lactating rats than those in the virgin or pregnant rats. High numbers of these Fos-ir neurons were co-localized with OT neurons in the SON and PVN in the parturient rats (Lin et al., 1998). The results of this present study imply the role of MT in the SOv and PVN during brooding behavior in birds is similar to the role of OT in the SON and PVN during parturition and lactation in mammals. It has been suggested that OT, released within the PVN during parturition, may coordinate the release of OT in other central sites via positive feedback mechanism, facilitating the onset of maternal behaviors as well as the induction of bonding (Da Costa et al., 1996; 1999). Thus, it might be the case in birds that the presented of chicks mediate the hens to maintain their maternal care via the MTergic system situated in the PVN area as similar in mammals.

In conclusion, the present findings clearly demonstrate a relationship between the MTergic system and maternal behaviors in the native Thai chicken, a non-seasonal breeding, equatorial precocial species. The results reveal markedly different in the numbers of MT-ir neurons in the SOv, POM, and PVN across the reproductive cycle, indicating that the MTergic system is associated with the reproductive state of the

birds. The differential expressions of the MT-ir neurons were observed within the SOv, POM, and PVN between the hens rearing chicks and hens deprived of their chicks. Furthermore, the numbers of MT-ir neurons in the SOv, POM, and PVN were higher in the hens rearing their young, whereas the numbers of MT-ir neurons were sharply decreased in the NR hens. These results indicate a pivotal role of the MTergic system in the initiation and maintenance of brooding/rearing behavior through the same nuclei that mainly regulated maternal behaviors in mammals.

4.6 References

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CURRICULUM VITAE

NAME: Miss Duangsuda Chokchaloemwong

DATE OF BIRTH: January 05, 1985

PLACE OF BIRTH: Nakhon Pathom, Thailand

NATIONALITY: Thai

EDUCATION:

2007-present Ph.D. Candidate (Environmental Biology)
Suranaree University of Technology, Thailand

2003-2006 B.Sc. (1st Class Honors, Animal Production Technology)
Suranaree University of Technology, Thailand

AWARDS AND HONORS:

1. Scholarship for excellence undergraduate student
2. Scholarship from the Royal Golden Jubilee Ph.D. Program, the Thailand Research Fund, Thailand

PUBLICATIONS:

5 international journals and 6 international meeting abstracts