NEUROENDOCRINOLOGY OF THE REPRODUCTIVE CYCLE IN THE FEMALE NATIVE THAI CHICKEN: ROLES OF DOPAMINE AND GONADOTROPIN RELEASING HORMONE

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

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การศึกษาบทบาทของโดปามีน โกนาโดโทรปินรีลิสซิงฮอร์โมน โปรแลคติน และลูติไนซิงฮอร์โมนในการควบคุมระบบสืบพันธุ์ของไก่พื้นเมืองไทยเพศเมียโดยใช้เทคนิคอิมมูโนฮิสโตเคมิสทรีพบว่าเซลล์ประสาทที่ผลิตโดปามีนกระจายอยู่ทั่วทั้งสมอง ส่วนเซลล์ประสาทที่ผลิตโกนาโดโทรปินรีลิสซิงฮอร์โมนพบในสมองตั้งแต่พรีออพติกไปจนถึงแอนทีเรียไฮโปทาลามัส จำนวนเซลล์ประสาทที่ผลิตโดปามีนที่บริเวณนิวเคลียสอินทราเมดิเอลิสและเซลล์ประสาทที่ผลิตโกนาโดโทรปินรีลิสซิงฮอร์โมนที่บริเวณนิวเคลียสคอมมิสซูรีพาลลิไอมีการเปลี่ยนแปลงตามวงจรการสืบพันธุ์ ระดับของฮอร์โมนโปรแลคตินมีการเปลี่ยนแปลงสอดคล้องกับระดับของจำนวนเซลล์ประสาทที่ผลิตโดปามีนที่บริเวณนิวเคลียสอินทราเมดิเอลิส แต่ไม่พบการเปลี่ยนแปลงของระดับของฮอร์โมนลูติไนซิงฮอร์โมน การศึกษานี้แสดงให้เห็นเป็นครั้งแรกว่าเซลล์ประสาทที่ผลิตโดปามีนมีความสัมพันธ์กับการควบคุมระบบสืบพันธุ์ในไก่พื้นเมืองไทยเพศเมีย

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NATAGARN SARTSOONGNOEN : NEUROENDOCRINOLOGY OF THE REPRODUCTIVE CYCLE IN THE FEMALE NATIVE THAI CHICKEN: ROLES OF DOPAMINE AND GONADOTROPIN RELEASING HORMONE. THESIS ADVISOR : ASST. PROF. YUPAPORN CHAISEHA, Ph.D. 397 PP.

DOPAMINE/GONADOTROPIN RELEASING HORMONE/LUTEINIZING HORMONE/NATIVE THAI CHICKEN/PROLACTIN/REPRODUCTIVE CYCLE

Roles of dopamine (DA), gonadotropin releasing hormone (GnRH), prolactin (PRL), and luteinizing hormone (LH) in the neuroendocrine regulation of reproductive system in the female native Thai chicken were investigated. Immunohistochemistry studies revealed that DA neurons were found throughout the brain. GnRH neurons were distributed in a discrete region from the preoptic area through the anterior hypothalamus. Changes in number of DA neurons in the nucleus intramedialis (nI) and GnRH neurons in the nucleus commissurae pallii were observed across the reproductive stages. Changes in PRL levels were directly correlated with variations in DA neurons within the nI. However, changes in LH levels were not observed. The results indicate, for the first time, an association exists between DA neurons and the regulation of the reproductive system in the female native Thai chicken.

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CHAPTER I

INTRODUCTION

1.1 Rational of the Study

Native Thai chickens (*Gallus domesticus*) originated from the wild jungle fowl in Southeast Asia. It was domesticated by village people approximately 3,000 years ago. Native Thai chickens have long been in Thailand for many generations. Traditionally, the native Thai chickens are raised by small farms. Raising native Thai chicken is found widespread throughout the countryside of Thailand because it is easy to raise, resistant to diseases, and acclimatized to the local environments. The main objectives of raising chickens are for consumption, competition, and recreation. Up to date, the native Thai chicken has become the new economic domestic animal of Thailand with presently growing demand and relatively high price. At present, there are about 54 millions native Thai chickens in Thailand which are raised by 2.3 millions farmer's family. It is interested that nowadays the native Thai chicken is one of the exported goods that gained income of 6.4 million baht per year. The native Thai chickens have traits of fighting cocks including strong and tough muscle, characteristics regarded as good quality when compared with the over-tenderness of broiler meat. It is resulting in high demand by consumers that preferring low fat and antibiotic-free white meat. This provides the good opportunity for production in commercial and industrial scale. Moreover, recent government policies are to encourage the development and the use of Thailand natural resources in supporting of

His Majesty the King's concept for self-sufficiency in agriculture. According to this concept, farmers tend to focus on "mixed farming" that is the strategies for helping rural farmers to increase self-sufficiency. One of the important resources that need to be developed is the native Thai chicken. However, the native Thai chickens suffer from their low productivity. One of the main causes of low reproductive performance is the incidence of maternal behaviors such as incubation behavior which is a heritable trait. The onset of incubation behavior affects the number of egg production because it terminates egg laying. Generally, the native Thai hen lays eggs 3-4 times per year, 4-17 eggs per clutch, and produces about 30-40 chicks per year which is significantly lower than that of the imported hen which produces eggs all year long. Therefore, in order to increase the production of the native Thai chicken in Thailand, it is very important to understand the basic neuroendocrinology influencing its reproductive activities.

The reproductive cycle of the native Thai chicken is divided into four reproductive stages; non-egg laying, egg-laying, incubating eggs, and rearing chicks. The primary component of integrated system that responsible for controlling the reproductive system in birds is the brain, especially the hypothalamus, the pituitary, and the gonads (testis and ovary). This system is referred to as the hypothalamo-pituitary-gonadal (H-P-G) axis. It is very well established that neurotransmitters, neurohormones, and hormones of the H-P-G axis play an important role in the reproductive cycle of avian species. The neural and neurochemical substrates regulating reproduction in birds remain vaguely defined. Two neuroendocrine systems play a pivotal role in the avian reproductive cycle. 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) and another system involves vasoactive intestinal peptide (VIP) and the subsequent secretion of prolactin (PRL). Both systems are influenced by dopamine (DA).

The period of egg laying in birds is associated with relatively high levels of LH, FSH, and gonadal steroids (e.g., estradiol and progesterone) circulating in the blood. In contrast, the onset of incubation behavior is correlated with declining levels of LH and gonadal steroids and increasing levels of PRL. FSH and LH, anterior pituitary hormones called gonadotropins, are responsible for many reproductive physiological functions. FSH induces mainly ovarian follicular growth and maintains the hierarchical size of the follicles. Subsequently, as the follicles increase in size, their production of steroid hormones, including progesterone and estradiol increases. LH stimulates progesterone production by the largest follicle. Progesterone acts on LH which then triggers ovulation. On the other hand, estradiol is intraovarian regulator of preantral follicular growth, granulosa cell proliferation, and LH receptor expression. Moreover, these steroid hormones play an important role in regulating of female secondary sex characteristics, as well as its feedback to modulate gonadotropins secretion.

It has been well established that gonadotropins secretion is regulated by the central nervous system through the hypothalamus. The hypothalamus synthesizes GnRH, a decapeptide that exerts a pivotal role in controlling of reproduction. The release of GnRH, the hypothalamic releasing factor, stimulates the secretion of gonadotropins from the pituitary. Two forms of GnRH have been isolated in chicken; cGnRH-I and cGnRH-II. Of the two forms, GnRH-I is the form that is directly involved in controlling reproduction in avian species such as chicken, mallard, King

penguin, turkey, and cockatiel. This neuropeptide increases LH and FSH secretion. Ovarian development is found to be correlated with plasma LH levels and the amount of GnRH-I content, indicating that GnRH-I expression is important to maintain pituitary-ovarian function in chicken. Changes in hypothalalamic GnRH-I content and release are correlated with several factors such as photoperiod and reproductive condition. Birds that display the reproductive activities have more GnRH immunoreactive cells and fibers when compared with the sexually inactive ones. Furthermore, the stimulatory effect of long day usually appears to be associated with an increased GnRH content, while reproductively inactive photorefractoriness is correlated with a decreased hypothalamic GnRH content.

As previously mentioned, the native Thai chickens undergo from low productivity. One of the main causes of low reproductive performance is the incidence of 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 behavior). These behaviors constrain the number of egg produced. The onset of incubation behavior is characterized by regression of the ovary and oviduct, a decline in LH secretion, and increase in PRL secretion. In birds, PRL, an anterior pituitary hormone, has been implicated as a causative factor in the onset and maintenance of incubation behavior in birds. During reproductively quiescent stages of the cycle, plasma PRL levels are very low. However, during laying and incubating stages, circulating PRL levels increase dramatically. This rising in PRL levels has been implicated as the cause for cessation of ovulation, ovarian regression, and induction of incubation behavior. PRL is widely thought to play a role in parental behaviors by mediating incubation behavior, crop milk production and secretion,

feeding of the young, and nest defense. PRL has been shown to be associated with the reproductive cycle in several avian species but no studies have been conducted on the native Thai chicken.

In mammals, it is very well established that PRL regulation is under the inhibitory control of tuberoinfundibular DAergic neurons in the hypothalamus. This is not the case in birds, where removal of hypothalamic inputs results in the complete cessation of PRL secretion. It has been established for some time that PRL secretion in birds is tonically stimulated by the hypothalamus and that the principal PRL-releasing factor (PRF) is an octacosapeptide, VIP.

DA is a neurotransmitter/neuromodulator found in both central and peripheral nervous systems and has several important physiological functions involved in a wide variety of behaviors and reproduction. The role of DA in the regulation of avian PRL secretion is unclear at present for comparing it to the mammalian DAergic strategy for PRL control. In apparent contrast with mammals, it has been established that DAergic influences are involved in both stimulating and inhibiting avian PRL secretion depending upon multiple DA receptors. DA plays an intermediary role in PRL secretion, requiring an intact VIPergic system in order to cause the release of PRL. In addition, recent evidences indicate that dynorphin, serotonin (5H-T), 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. Beside the relationship with VIP/PRL system, some other evidences provide data on an opportunity for synaptic interaction between GnRH and DA, and suggest an inhibitory role for DA on GnRH release. There are growing evidences indicating the

involvement of DA in the regulation of GnRH and the secretion of FSH and LH.

Reproduction in animals is the result of complex interaction of both external (social and physical cues) and internal (neural and endocrine signals) environmental factors. The endocrine and environmental factors associated with the reproductive cycle of avian species are complex. The transduction of environmental cues into a hormonal signal involves the hypothalamic neurotransmitter/neurohormone systems. However, their identities are not well established. In seasonal breeders, the initiation of the breeding cycle depends on the precise prediction of environmental conditions which are for health of the mating pair and the survivability of their offspring. Critical environmental stimuli include sensory information concerning photoperiod, ambient temperature, and the presence of egg and offspring. These external stimuli as well as the prevailing internal steroid milieu (estrogen and progesterone) are important in initiating and maintaining reproduction, although their relative importance varies with the stages of the reproductive cycle.

It is not only the endocrine factors that influence the reproductive cycle of birds but also the environmental factors. Most of birds are highly photoperiodic and gonadal development occurs in response to increasing day length. In seasonal breeder temperate zone birds, the effects of season and photostimulation on the H-P-G axis are well characterized. Photoperiodic cues play a pivotal role in the initiation of sexually-related neuroendocrine and behavioral changes, as they become reproductively active at the time of the greatest survival of young. Both GnRH/FSH-LH and VIP/PRL systems are dependent upon the duration of day length and involved in the transduction of photoperiodic information, resulting in either gonad recrudescence and its associated sexual activity (egg laying) or gonad regression and termination of

reproductive activity (photorefractoriness). The decline in the rate of egg production at high environmental temperature is also well recognized but the physiological basis is not well understood. Therefore, if year-round production of eggs for hatching to be achieved, it is obvious that lighting programs and environmental temperature and their interactions must be considered. The jungle fowl, the ancestor of the native Thai chicken, originated in the tropical region of Southeast Asia, where its breeding season would has been timed by both photoperiodic and non-photoperiodic factors, allowing the chick to hatch at a time of year when food is most abundant. The integrations of the endocrine system, hypothalamic neuropeptides, pituitary hormones, ovarian steroids, environmental photoperiod, ambient temperature, and the presence of egg and chick, regulate the reproductive cycle of seasonally breeding birds and this may be the case in the native Thai chicken.

As indicated above, in order to increase the production of native Thai chickens in Thailand, it is very important to understand the basic endocrinology and neuroendocrine mechanism(s) by which environmental factors influencing its reproductive activities. There are limited numbers of avian physiologists studying the reproductive endocrinology of the native Thai chicken. It has been reported that progesterone level is related to reproductive cycle of the native Thai chicken. Changes in plasma PRL levels and VIP immunoreactive neurons in the infundibular nuclear complex (INF) have been observed across the reproductive stages. In addition, the effects of photoperiod upon growth, reproductive development, laying performance, and reproductive efficiency have been reported. With paucity of information regarding the reproductive neuroendocrine regulation of this non-seasonally breeding tropical species, the focus of this dissertation research was proposed to investigate

neuroendocrine regulation of the reproductive cycle in the female native Thai chicken and the effects of photoperiod upon the neuroendocrine regulation of the reproductive system to increase reproductive efficiency by the lighting regimens. The knowledge gained will provide the information of neuroendocrine regulation including the hormonal and physiological characteristics of the reproductive cycle in the native Thai chicken. The results (lighting regimens) of the study of the role of photoperiod can be then applied commercially in poultry industry. Moreover, the results will provided the important practical data that can be implement for farmers to increase egg production of the native Thai chicken in Thailand.

1.2 Research Objectives

1.2.1 To study the role of PRL and LH in the regulation of the reproductive cycle of the female native Thai chicken.

1.2.2 To study the localization and differential expression of hypothalamic GnRH-I across the reproductive cycle of the native Thai chicken.

1.2.3 To study the localization and differential expression of hypothalamic DA across the reproductive cycle of the native Thai chicken.

1.2.4 To study the relationship between photoperiod and the neuroendocrine regulation of the female native Thai chicken’s reproductive system.

CHAPTER II

LITERATURE REVIEW

2.1 Neuroendocrine Regulation of the Avian Reproductive Cycle

The control of reproductive system in birds involves the interaction of external stimuli with endocrine mechanisms. The primary components of the integrated reproductive system in birds are the brain, especially the hypothalamus, the pituitary, and the gonad (ovary and testis). This integrated system is referred to as the hypothalamic-pituitary-gonadal (H-P-G) axis. It is very well established that neurotransmitters, neurohormones, and hormones of the H-P-G axis play an important role in the reproductive cycle of avian species. The H-P-G axis involves two neuroendocrine systems for controlling avian reproduction. These are, first the chicken gonadotropin releasing hormone-I (cGnRH-I referred to as GnRH)/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH) system and another is the vasoactive intestinal peptide/prolactin (VIP/PRL) system. The GnRH/FSH-LH system regulates the period of egg laying. GnRH stimulates the secretion of FSH and LH which in turn responsible for ovarian follicular growth and ovulation. On the other hand, the VIP/PRL system involves the maintenance of maternal behavior by which VIP stimulates PRL secretion and then the gonadal regression. Activation of GnRH/FSH-LH and VIP/PRL systems in the somatically mature photosensitive female birds initiates the transition from reproductive quiescence to reproductive activity. In addition, both systems depend on the duration of day length and 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 controlling the GnRH/FSH-LH and VIP/PRL 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).

2.1.1 Gonadotropin Releasing Hormone-I/Follicle Stimulating Hormone-Luteinizing Hormone System

The period of egg laying in birds is associated with relatively high levels of circulating FSH, LH, and gonadal steroids (e.g., estradiol and progesterone; El Halawani et al., 1988a) circulating in the blood. FSH and LH, anterior pituitary hormones called gonadotropins, are responsible for many reproductive physiological functions. At the onset of sexual maturity, the preovulatory surge of progesterone induces nesting behavior (Wood-Gush and Gilbert, 1973; El Halawani et al., 1986). FSH induces mainly ovarian follicular growth (Chaudhuri and Maiti, 1998; Rose et al., 2000) and maintains the hierarchical size of the follicles. LH stimulates progesterone production by the largest follicle (F1), leading to ovulation (Pollock and Orosz, 2002). Consequently, as the follicles increase in size, the granulosa cells and theca externa cells produce amounts of progesterone and estradiol, respectively (Porter et al., 1989). Progesterone acts on LH which then triggers ovulation, on the other hand, estradiol are intraovarian regulators of preantral follicular growth (Goldenberg et al., 1972; Richard, 1979), granulosa cell proliferation (Williams, 1940), and LH receptor expression (Knecht et al., 1985). However, it can exert both inhibitory and

stimulatory effects on gonadotropins secretion (Li et al., 1994). The combined action of estrogen, progesterone, and nesting activity further stimulates PRL secretion (El Halawani et al., 1983; 1986). Thereafter, LH levels continue to decline during incubating period (Myers et al., 1989). During laying and incubating period, circulating PRL levels increase dramatically (El Halawani et al., 1984a). These increasing PRL levels suppress the activity of the GnRH/FSH-LH system (Rozenboim et al., 1993b; You et al., 1995a) and cause of reduction of ovarian steroid secretion (Porter et al., 1991; Tabibzadeh et al., 1995), cessation of ovulation, induction of ovarian regression, and signal the transition of sexual behavior to incubation behavior (Youngren et al., 1991; Chaiseha and El Halawani, 2005). Subsequently, PRL levels decline, whereas LH levels begin to rise when incubation behavior terminates (El Halawani et al., 1988a; Knapp et al., 1988) and as soon as molting ends (Bluhm et al., 1983; Mauget et al., 1994).

It is very well documented that gonadotropins secretion is regulated by the central nervous system (CNS) through the hypothalamus. The hypothalamus synthesizes GnRH which in turn stimulates the synthesis and secretion of the pituitary gonadotropins (Ulloa-Aguirre and Timossi, 2000; Shalev and Leung, 2003). GnRH has first been isolated from porcine hypothalamus (Peczely, 1989). Two forms of GnRH have been isolated in chicken; cGnRH-I or GnRH-I and cGnRH-II (King and Millar, 1982; Miyamoto et al., 1982; 1984; Millar and King, 1984; Sherwood et al., 1988). Of the two forms, it has been suggested that only GnRH-I has a physiological role in regulating of gonadotropins secretion and directly involves in controlling reproduction in the domestic chicken (Sharp et al., 1990). GnRH perikarya and fibers are extensively distributed throughout the avian brain. In the avian hypothalamus,

GnRH neurons are found within the preoptic area (POA), anterior hypothalamus (AM), and lateral septum (LS; Mikami et al., 1988; Millam et al., 1993). However, little is known regarding the GnRH neuronal group(s) that project to the ME (Dawson and Goldsmith, 1997; Teruyama and Beck, 2000). Ovarian development is found to be correlated with plasma LH levels and the amount of GnRH-I content, indicating that the expression of the GnRH-I gene is important to maintain pituitary-ovarian function in chicken (Dunn et al., 1996). Like gonadotropins and PRL, GnRH content also changes during the reproductive cycle. During incubation, GnRH concentration is significantly elevated in the POA area (Millam et al., 1995). In addition, GnRH contents of discrete medial preoptic, infundibulum, and arcuate samples are higher in laying hens than that of non-laying hens (Advis et al., 1985). The amount of hypothalamic GnRH decreases during photorefractoriness (Dawson et al., 1985; Foster et al., 1987; Bluhm et al., 1991; Rozenboim et al., 1993a; Saldanha et al., 1994; Hahn and Ball, 1995). It has been reported that changes in GnRH mRNA expression are observed during different reproductive stages of the turkey. GnRH mRNA is predominantly expressed in the organum vasculosum laminae terminalis (OVLT) and the nucleus commissurae pallii (nCPa), and limited expression is found in the POA, nucleus preopticus medialis (POM), and nucleus septalis lateralis (SL). Hypothalamic GnRH mRNA expression is significantly increased after subjecting the non-photostimulated turkey to a 90 minute light period at Zeitgeber time (ZT) 14. GnRH mRNA abundance within the SL, OVLT, and nCPa areas is highest in laying hens, with decreasing abundance found in non-photostimulated and incubating hens, respectively. The lowest level of GnRH mRNA is observed in photorefractory hens, indicating that hypothalamic GnRH mRNA expression may be used to precisely

characterize the different reproductive stages (Kang et al., 2006). There are growing evidences that photoperiod is associated with the reproductive neuroendocrine axis in birds since the lengthening day stimulates secretion of GnRH and gonadotropins and consequent gonadal growth (Follett and Robinson, 1980; Dawson et al., 2001). All of the neural components necessary for the photoperiodic response in birds appear to lie within the diencephalon including the photoreceptor and the GnRH system. Photostimulatory inputs to GnRH neurones have the potential to increase GnRH mRNA transcription and its secretion (Dunn and Sharp, 1999), and pituitary sensitivity to GnRH in birds (Davies and Follett, 1975). In contrast, gonadal regression through photorefractoriness is related to a decrease in hypothalamic GnRH (Dawson et al., 2001; 2002).

2.1.2 Vasoactive Intestinal Peptide/Prolactin System

In birds, PRL has been implicated as a causative factor in the onset and maintenance of incubation behavior (El Halawani et al., 1997). In addition, PRL is associated with a wide range of reproductive physiology and behaviors including migration, grooming, crop milk secretion, feeding of young, nest defense, and sexual activity (Lea et al., 1981; 1986; Silver, 1984; Janik and Buntin, 1985; Buntin et al., 1991). It has been studied and well documented that gonadotropins and PRL are associated with the reproductive cycle in several avian species (Mashaly et al., 1976; Bluhm et al., 1983; El Halawani et al., 1984a; 1997; Myers et al., 1989; Wong et al., 1992b; Mauget et al., 1994). PRL gene expression and its plasma levels are highly correlated with the reproductive cycle in birds (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).

During reproductively quiescent stage of the cycle, plasma PRL levels are very low (El Halawani et al., 1984a). During egg laying period, the release of gonadotropins which induces sexual receptivity (El Halawani et al., 1986) primes the VIP/PRL system to enhance PRL secretion (El Halawani et al., 1983). Circulating PRL levels increase dramatically during incubating period (El Halawani et al., 1984a). It is this rising PRL levels which has been implicated as the cause of cessation of ovulation, ovarian regression, and induction of incubation behavior. Elevated PRL levels and incubation behavior are maintained by tactile stimuli from the nest and eggs (El Halawani et al., 1980; 1986; Opel and Proudman, 1988a). After hatching, or when eggs are replaced with poults, tactile stimuli from the young induces the emergence and maintenance of maternal response including the change from incubating eggs to brooding the young, vocalizations, nest desertion, a sharp decrease in circulating PRL (Opel and Proudman, 1989), molt, and the transition to the photorefractory state. Circulating PRL and LH levels, pituitary PRL/LH peptide contents and their mRNA levels sharply decline, eventhough long day length continues with the onset of photorefractoriness (Wong et al., 1991; 1992b; Mauro et al., 1992; El Halawani et al., 1996). A precipitous decline in PRL and FSH/LH release and their gene expression may be triggered at any time due to a lack of response to long day length or by subjecting the birds to short day lighting (Nicholls et al., 1988; El Halawani et al., 1990a).

It has been established for some time that PRL secretion in birds is tonically stimulated by the hypothalamus (Kragt and Meites, 1965; Bern and Nicoll, 1968) and that principal PRL-releasing factor (PRF) is VIP (El Halawani et al., 1997; 2000; Chaiseha and El Halawani, 2005). Avian PRL secretion and its gene expression are

regulated by VIP (Chaiseha and El Halawani, 1999; 2005). 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). In response to long day length, the VIP/PRL secretion is increased gradually, but progressive and both their release and gene expression are up-regulated (Wong et al., 1991; El Halawani et al., 1996; Tong et al., 1997; Chaiseha et al., 1998). Activation of the GnRH/FSH-LH system in photosensitive female turkey initiates the reproductive activity. When gonadotropins stimulate estrogen secretion (Wineland and Wentworth, 1975) and induce sexual receptivity (El Halawani et al., 1986), they also prime the VIP/PRL system to enhance PRL secretion (El Halawani et al., 1983). VIP stimulates PRL secretion from the anterior pituitary (Macnamee et al., 1986). This octacosapeptide is modulated by stimuli from the environment and plays a pivotal role in the control of avian reproduction (Chaiseha and El Halawani, 2005).

VIP neurons are found extensively throughout the avian hypothalamus (Yamada et al., 1982; Mikami and Yamada, 1984; Peczely and Kiss, 1988; Mauro et al., 1989; Chaiseha and El Halawani, 1999). VIP in the ME, which mediates the regulation of PRL secretion, is derived from neurons located within the infundibular nuclear complex (INF; Macnamee et al., 1986; Mauro et al., 1989; Chaiseha and El Halawani, 1999; Youngren et al., 2002a). Variations in hypothalamic VIP immunoreactivity, VIP content, VIP mRNA expression in the INF, VIP receptor mRNA, and VIP concentrations in the hypophyseal portal blood are correlated with changes in circulating PRL levels throughout the avian reproductive cycle (Mauro et al., 1989; You et al., 1995b; Youngren et al., 1996a; Chaiseha and El Halawani, 1999; Chaiseha et al., 2004).

Dopamine (DA), a neurotransmitter/neuromodulator, found extensively in both the central and peripheral nervous systems of many species, has several important physiological functions and is involved in a wide variety of behaviors and reproductive activities (Ben-Jonathan and Hnasko, 2001). In mammals, the regulation of PRL secretion is under the inhibitory control of tuberoinfundibular DAergic (TIDA) neurons in the hypothalamus (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001). Removal of this DAergic inhibition results in an increased PRL secretion and hyperprolactinemia (Nicoll and Swearingen, 1970; Nicoll, 1977). This is not the case in birds, where removal of hypothalamic inputs results in the completed cessation of PRL secretion (Chadwick et al., 1978). The role of DA in the regulation of PRL secretion is presently not as clear in birds as it is in mammals. It has been established that DAergic influences are involved in both stimulating and inhibiting avian PRL secretion depending upon multiple DA receptors. The stimulatory effect of DA on PRL secretion is regulated via D_1 DA receptors residing in the INF, where the VIP neurons are located. In contrast, DA inhibits PRL release and synthesis by blocking the action of VIP at the pituitary level through D_2 DA receptors (Youngren et al., 1996b; 1998; 2002; Chaiseha et al., 1997; 2003; Al Kahtane et al., 2003). It has been 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). Dynorphin, serotonin (5-HT), DA, and VIP all appear to stimulate avian PRL secretion along a pathway expressing κ opioid, serotonergic, DAergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator (for review, see El Halawani et al., 2000).

The following literature review will be extensively reviewed in the regulation

of GnRH/FSH-LH and VIP/PRL systems and its involvement of DAergic regulation, which is related to this dissertation research.

2.2 Gonadotropins: Structure, Function, and Regulation of Secretion

Gonadotropins (FSH and LH), anterior pituitary hormones, are the member of glycoprotein hormones. These pituitary glycoprotein hormones are derived from the family that includes LH, FSH, thyroid stimulating hormone (TSH), and placental chorionic gonadotropin (CG) that is presented only in primates and equine species. All members of the family are heterodimers, consisting of two dissimilar subunits, α- and β-subunits, which are encoded from different genes. In a given species, the α-subunit contains 92 amino acids and is identical among all glycoprotein hormones, while β-subunits are different and determine the hormonal specificity and species specificity (Pierce and Parsons, 1981). On the basic knowledge of molecular structure of mammalian pituitary hormones, it is predicted that the α- and β-subunits of the glycoprotein hormones are evolved from a common ancestral gene (Dayhoff, 1978; Fontaine and Burzawa-Gerard, 1977; Licht et al., 1977), but the mechanism(s) of evolution of the subunits of the four hormones remains to be elucidated.

Gonadotropins are synthesized and released from the anterior pituitary gland. The β-subunit confers specificity of biological action by mediating interaction with its cognated receptor. It has a pivotal role involving in a wide variety of physiological functions in vertebrates. Generally, FSH functions concurrently with LH to promote growth and differentiation of the gonads, control gametogenesis, and regulate gonadal endocrine functions (Moyle and Campbell, 1996).

2.2.1 The Structure of Follicle Stimulating Hormone

The structure, function, and regulation of FSH secretion have been studied most extensively in mammalian species (Li and Ford, 1998). FSH, a glycoprotein hormone produced by the pituitary gland, has a molecular weight (MW) of about 30 kiloDaltons (kDa). The protein dimer contains 2 polypeptide subunits, α- and β-subunits. The α-subunits of LH, FSH, TSH, and hCG are identical and contain 92 amino acids. The amino acid sequence of FSH-β-subunit is first isolated from the human pituitary by Shome and Parlow (1974). FSH-β-subunit consists of 118 amino acid residues with a predominant proportion of molecules having 108 residues due to microheterogeneity at the NH_2 and COOH termini. The amino sequence indicates an abundance of threonine, glutamic acid, and cysteine residues. The NH_2-terminal portion (up to 32 residues) shows homology with the β-subunits of other glycoprotein hormones such as LH, human CG (hCG), and TSH. However, the amino sequence differs a great deal from others in the rest of the molecule, confirming the specificity of the β-subunit of these hormones (Shome and Parlow, 1974). Complementary DNA (cDNA) cloning of the FSH-β-subunits have been cloned in many mammals species such as human, monkey, sheep, rat, mouse, pig, bovine, opossum, and others (Esch et al., 1986; Maurer, 1987; Jameson et al., 1988; Kato, 1988; Mountford et al., 1989; Kumar et al., 1995; Lawrence et al., 1997; Schmidt et al., 1999). Moreover, FSH molecules have been purified in non-mammalian species including sea turtle (Licht and Papkoff, 1985) and bullfrog (Hayashi et al., 1992). In avian species, FSH molecules have been purified in chicken (Sakai and Ishii, 1980; Krishnan et al., 1992) and ostrich (Koide et al., 1996). Furthermore, the cDNA encoding precursor molecule of FSH-β-subunit has also been cloned in Japanese quail (Kikuchi et al., 1998),

chicken (Shen and Yu, 2002), Japanese Crested ibis (Kawasaki et al., 2003), and duck (Shen et al., 2006). The deduced amino acid sequence of chicken FSH-β-subunit shows 98% homology with Japanese quail and 93% with ostrich, whereas a lower similarity (66 to 70%) is noted when compared with mammalian FSH-β-subunit. Amino acid sequences of FSH-β-subunit in various species are illustrated in Fig. 2.1.

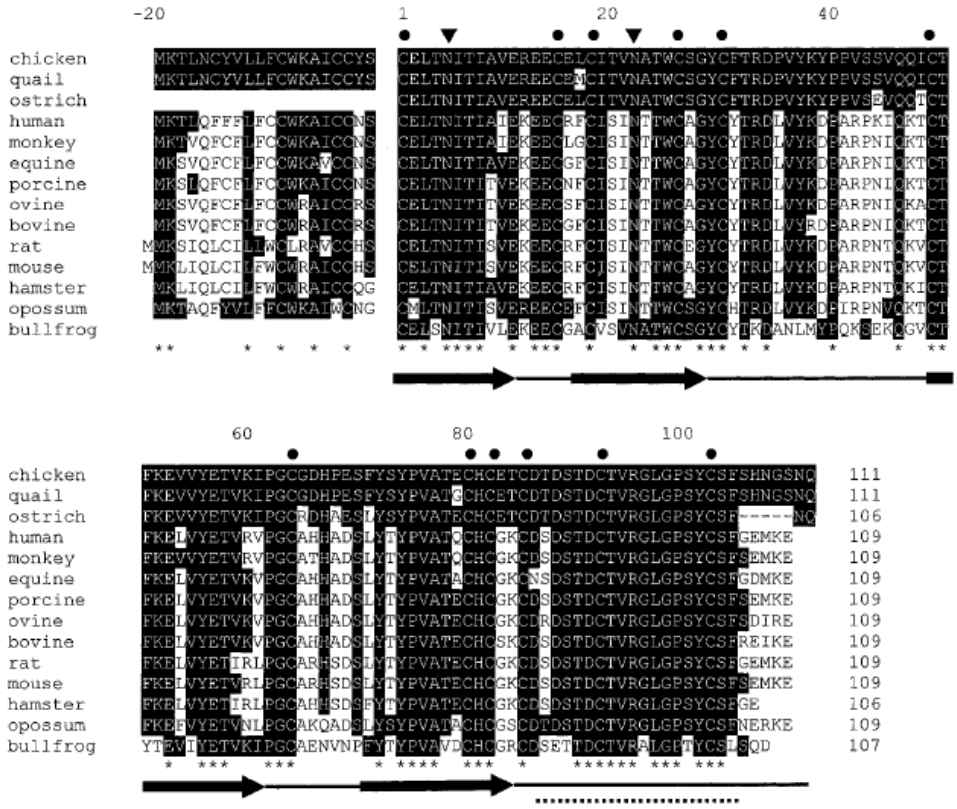

Fig. 2.1 Multiple sequence alignments of FSH-β-subunit of various species. Residues identical to chicken FSH-β are presented in white letters. Twelve conserved cysteines are denoted by ● and two putative N-linked glycosylation sites by ▼. Conserved residues are indicated by "*" under sequences. Arrows represent β-strands; single lines are loops. Line with dots corresponds to the "seat-belt" region in crystal structure of human FSH (Shen and Yu, 2002).

It is very well known that gonadotropins are secreted by gonadotrophs cells of

the anterior pituitary gland. The cellular localization of gonadotropins has been determined by immunohistochemical (IHC) techniques in a variety of species. The majority of gonadotrophs in each species contains both FSH and LH. In pigs (Dacheux, 1984) and lizards (Naik et al., 1980), all gonadotrophs reportedly contain both FSH and LH. In human, at least two thirds of gonadotrophs contain both hormones (Pelletier et al., 1976). However, the IHC evidence demonstrates that FSH and LH reside in separate populations of gonadotrophs in chicken pituitary (Proudman et al., 1999) and bovine (Bastings et al., 1991). FSH-immunopositive cells are much less numerous than that of LH positive cells in chicken. In addition, FSH is largely absented from the outer margin of the pituitary gland. In Japanese quail, pituitary cells that are bound anti-chicken FSH serum also bound anti-chicken LH serum (Mikami, 1983).

FSH acts by binding to specific receptors localized exclusively in the gonads. FSH receptor (FSHR) belongs to the family of G (guanine nucleotide-binding) protein-coupled receptors complex transmembrane proteins characterized by seven hydrophobic helices inserted into the plasma membrane and by intracellular and extracellular domains of variable dimensions depending on the type of ligands (Gudermann et al., 1995). Signals triggered by binding to the FSHR are relayed within the cells by the second messenger system, cyclic AMP (adenosine 3'5' monophosphate; cAMP). The intracellular portion of the FSHR is coupled to a G stimulatory-(Gs) protein, and upon the receptor activation by hormonal interaction with the extracellular domain, initiates the cascade of events that ultimately leads to the specific biological effects of the gonadotropins. The first sequence of a putative FSHR DNA fragment is reported by Vassart's group (Parmentier et al., 1989). Up to

date, the FSHR sequences of human, primate, equine, ovine, swine, bovine, chicken, and reptile are well characterized (for review, see Simoni et al., 1997). It is also illustrated that three human glycoprotein hormone receptor genes are huge with 70 kbp for LH receptor, 60 kbp for TSH receptor, and 54 kbp for FSHR. In birds, a cDNA corresponding to chicken FSHR (cFSHR) has been cloned (You et al., 1996). The overall sequence (693 amino acids) is determined to be considerably more homologous to the rat (71.8%) and bovine FSHR (72.2%) than that of the recently characterized chicken LH recptor (cLHR; Johnson et al., 1996). By comparison, the nucleic acids and amino acid sequence of cFSHR are 60.1% and 49.4% identical to the respective cLHR sequence.

The FSHR gene expression is highly gonad- and cell-specific, underlying its importance for oogenesis and spermatogenesis. Binding experiments have been shown that FSH binds specifically to receptors located on the membrane of Sertoli cells (Fritz, 1978; Kangasniemi et al., 1990). In female chicken, FSH binding has been localized in the granulosa cells (Richard and Midgley, 1976; Richards, 1980) and ovarian tissues (granulosa, theca, and stromal), but not in the oviduct, adrenal gland, liver, muscle, or brain (You et al., 1996). Several recent studies using molecular biology techniques confirm that granulosa cells are the only cell type expressing-FSHR. This finding is in contrast to the expression pattern observed for the LH receptor (LHR) and TSH receptor (TSHR). LHR expression can be demonstrated in a variety of organs and tissues (for review, see Simoni et al., 1997).

2.2.2 The Structure of Luteinizing Hormone

LH, like other members of the glycoprotein hormone family, is a heterodimer

consisting of a common α-subunit that contains 92 amino acids (approximate MW of 13.5 kDa) coupled to a unique β-subunit (approximate MW of 14.5 kDa); each is encoded by different genes. LH is produced by gonadotrophs in the anterior lobe of the pituitary gland. LH functions with FSH to promote growth and development of the gonads, control gametogenesis, and regulate gonadal endocrine functions (Moyle et al., 1994).

LH has a β-subunit of 121 amino acids that confers its specific biological actions and is responsible for interaction with the LHR. This subunit contains the same amino acids sequence as the β-subunit of hCG and both stimulate the same receptor. However, the hCG-β-subunit contains an additional 24 amino acids and both hormones differ in the composition of their sugar moieties. The biological half-life of LH is 20 minutes, shorter than that of FSH (3-4 hours) or hCG (24 hours). The structure, function, and regulation of LH secretion have been well investigated in mammalian species (Nakav et al., 2005). The cDNAs and deduced peptide sequences of the LH-β-subunit molecule are available for mammalian species (bovine, sheep, goat, pig, giant panda, rat, mouse, dog, hamster, rhesus monkey, human, baboon, marmoset, and zebra (for review, see Basavarajappa et al., 2008). In birds, cDNA encoding precursor molecule of the LH-β-subunit of the chicken (Noce et al., 1989), Japanese quail (Ando and Ishil, 1994), and turkey (You et al., 1995a) have been reported. Turkey and chicken LH-β-subunit sequences share approximately 92% and 93% nucleotide and amino acid sequence similarities, respectively (You et al., 1995a). Alignments of the amino acid sequences of signal peptide (a) and apoprotein (b) of the putative LH-β-subunit and homology matrices of the amino acid sequences of signal peptide and apoprotein of the LH-β-subunit of various species belonging to various

vertebrate classes are listed in Fig. 2.2 and Fig. 2.3, respectively.

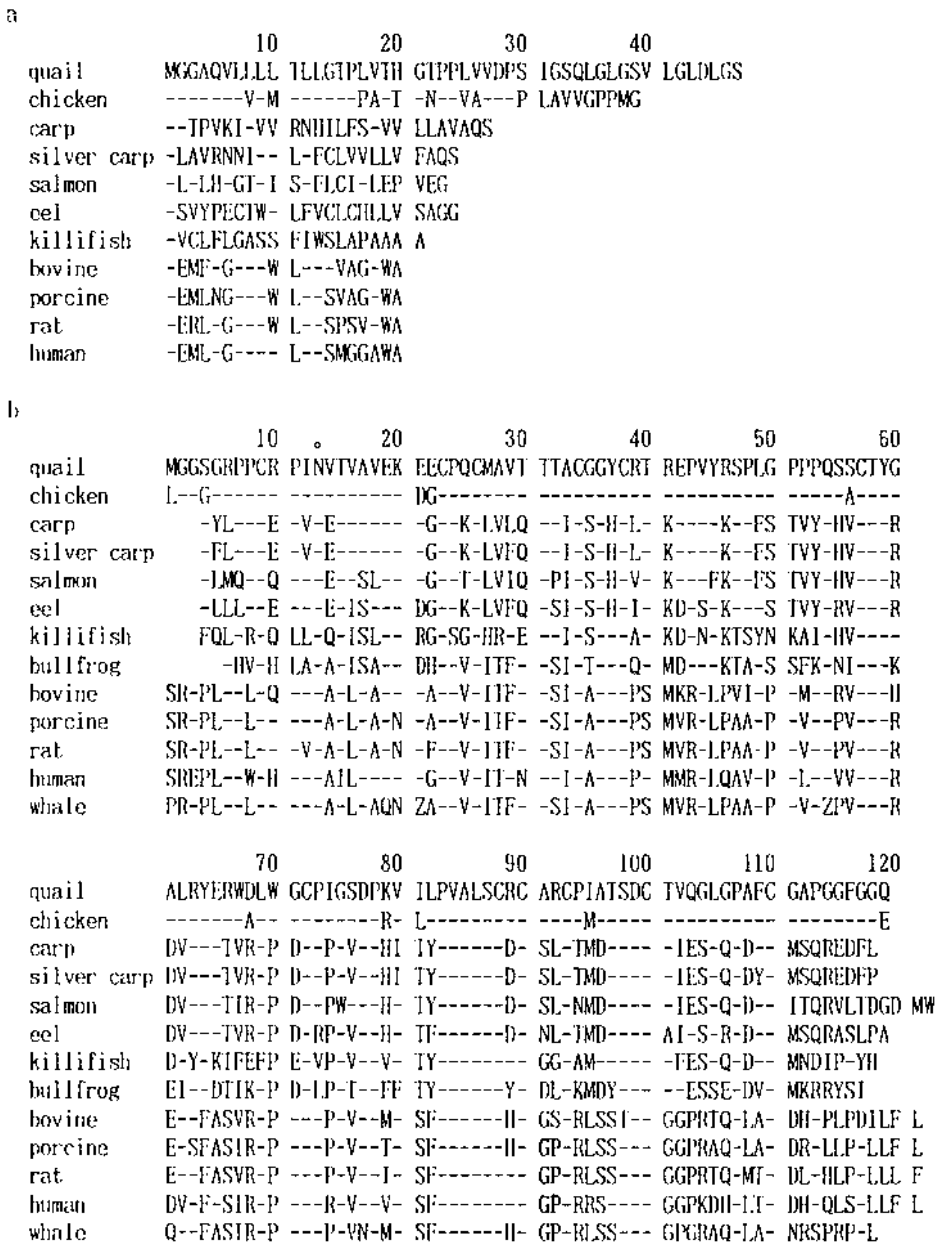

Fig. 2.2 Alignments of the amino acid sequences of signal peptide (a) and apoprotein (b) of the putative LH-β-subunit in different species. Dashes indicate amino acid residues which are indicated to those in the Japanese quail sequences (Ando and Ishii, 1994).

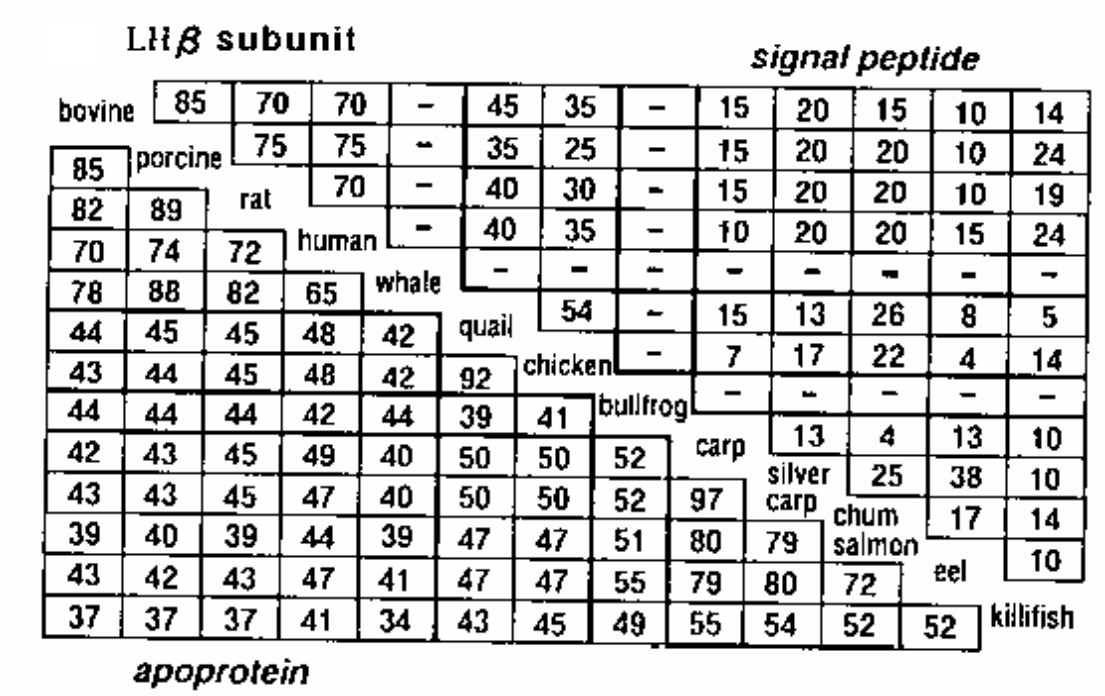

Fig. 2.3 Sequence homology (%) among LH-β-subunit of various species (Ando and Ishii, 1994).

LHR is a member of the subfamily of glycoprotein hormone receptors within the superfamily of G protein-coupled receptors. LHR interacts with both LH and hCG and is so called luteinizing hormone/choriogonadotropin receptor (LHCGR) or lutropin/choriogonadotropin receptor (LCGR). Like other G protein-coupled receptors, the LHR possess seven membrane-spanning domains or transmembrane helices (Dufau, 1998). The extracellular domain of the LHR receptor is heavily glycosylated. This transmembrane domain contains two highly conserved cysteine residues, which builds disulfide bonds to stabilize the receptor structure. With LH attached, the receptor shifts conformation and thus mechanically activates the G protein, which detaches from the receptor and activates the cAMP system (Ryu et al., 1998). The LHR consists of 674 amino acids. The size of mature LHR is 80-90 kDa,

of which about 15 kDa are contributed by carbohydrate chains (Dufau, 1990).

LHR cDNAs are cloned from human ovarian libraries and the testes of pig, rat, and mouse and the gene structure has been defined (for review, see Dufau, 1998). Recently, partial LHR cDNAs have been isolated in chicken (Johnson et al., 1996) and Japanese quail (Akazome et al., 1994). It appears to have two different LHR isoforms in chicken, one of which has 86 bp insert located in the extracellular domain of the molecule (Johnson et al., 1996). Furthermore, three different, alternatively spliced, LHR cDNA isoforms have been characterized in chicken and turkey (You, 1997; You et al., 2000). The deduced amino acid sequence of the cLHR shares 73.2% and 74.2% identity with the rat and porcine LHR sequences, respectively, with the highest homology occurring within the seven transmembrane spanning regions (86-88% identity vs. mammalian sequences). In mammalian LHRs, there are considerable evidences that after interaction of LH with the LHR, the second messenger signaling mechanisms in the ovary include the activation of adenylate cyclase/protein kinase A and phospholipase C/phosphatidylinositol systems (Mcneilly et a., 1982; El Halawani et al., 1988a).

2.2.3 The Function of Gonadotropins in Mammals

In mammals, reproduction is depended on the pulsatile release of gonadotropins, acting together to regulate gonadal functions. Biological actions of gonadotropins include stimulation of the maturation and function of the gonads (testis and ovary) and regulation of gametogenesis and steroidogenesis. FSH first primes the initial phase of follicogenesis with an increase in gene transcription that encodes growth factors, induces LHRs on granulosa cell membranes, and promotes estradiol

secretion. FSH and estradiol are required for the acquisition of LHR by granulosa cells, whereas the synthesis of androgen which is a precursor for estradiol is controlled by LH (Hsueh et al., 1984). In females, LH is responsible for follicular maturation, ovulation, and transformation of follicles into corpora lutea and the maintenance of luteal activity. After the initial phase of ovarian follicular growth, an LH surge participates in oocyte meiosis and consequent ovulation. The LH surge triggers ovulation by promoting the rupture of the preovulatory follicle and the release of the ovum. In addition, LH enhances the subsequent stages of follicular development and steroidogenesis in granulosa and luteal cells. In males, LH acts through plasma membrane receptors on the Leydig cells to maintain general metabolic processes and steroidogenic enzymes and to regulate the production and secretion of androgens (Levi-Setti et al., 2004).

FSH and LH act on the gonads to regulate folliculogenesis, ovulation, spermatogenesis, and steroidogenesis. According to the "two cells, two gonadotropins" theory (Fevold, 1941; Greep et al., 1942; Kobayashi et a., 1990; Hillier et al., 1994), both FSH and LH are necessary for ovarian follicular maturation and the syntheses of ovarian steroid hormones. LH promotes the production of androgens (dehydroepiandrosterone, androstenedione, and testosterone) from cholesterol and pregnenolone, by stimulating 17α-hydroxylase activity in the thecal cells. The androgens then diffuse to the granulosa cells, where FSH stimulates the expression of the cytochrome P450 aromatase, which converts the androgens to estrogens (Erickson et al., 1985; Richards, 1994).

2.2.4 The Function of Gonadotropins in Birds

Gonadotropins are the primary regulators of follicular growth and ovulation in birds that ovulation occurs spontaneously and is hormonally controlled. Chicken ovaries are an excellent model to study follicular selection because the stage of follicular development can easily be determined by the size of the follicles. Chicken ovaries contain thousands of cortical follicles (less than 1 mm in diameter), hundreds of white follicles (1-5 mm in diameter), five or six small yellow follicles (SYF; 5-8 mm in diameter), and five or six preovulatory follicles (10-35 mm in diameter) arrange in a hierarchy (Johnson, 1993). A single follicle is selected each day from the pool of SYF to join the exclusive group of preovulatory follicles destined for ovulation. Among the most important endocrine, paracrine, and autocrine factors that mediate follicular growth and differentiation are the gonadotropins and growth factors (for review, see Johnson, 1993)

FSH is responsible for follicular recruitment and growth of the smaller follicles. Several studies indicate that FSH induces mainly ovarian follicular growth in birds and maintains the hierarchical size of the follicles (Chaudhuri and Maiti, 1998; Rose et al., 2000). It has been reported that daily injection of FSH increases the number of white follicles, small yellow follicles, and preovulatory follicles without disrupting the hierarchy in mature hens (Palmer and Bahr, 1992). On the basis of measurements of FSHR, FSH mRNA, and FSH-stimulated adenylate cyclase activity, it is found that the granulosa layer of SYF and the sixth (F6) to the third (F3) largest follicles are the primary targets of FSH (Calvo et al., 1981; Calvo and Bahr, 1983; Bahr and Calvo, 1984; Ritzhaupt and Bahr, 1987; You et al., 1996; Zhang et al., 1997). However, the FSHR and LHR decrease in the granulosa layers of the F1 and

second largest (F2) preovulatory follicles (Yamamura et al., 2001). Consistency with others studies, it has been indicated that as the follicles mature, LHR mRNA level is increased, while FSH mRNA level is decreased in granulosa layers (Johnson et al., 1996; You et al., 1996; Zhang et al., 1997; Yamamura et al., 2001). Moreover, relatively low levels of FSH binding have been detected within ovarian stroma, theca layers, and granulosa tissue, and such binding generally decreases during follicular development (Etches and Cheng, 1981; Ritzhaupt and Bahr, 1987).

As the follicles increase in size, the granulosa cells and theca externa cells produce amounts of steroid hormones which are progesterone and estradiol-17β (E2 or estrogen), respectively (Porter et al., 1989). In contrast with mammals, a three-cell model of steroidogenesis in avian follicles that takes the differential steroidogenic activities of the theca externa, theca interna, and granulosa cell layers into account has been proposed (Porter et al., 1989; Nitta et al., 1991; Velazquez et al., 1991; Kato et al., 1995). This three-cell theory is based on the facts that two kinds of steroidogenic cells are presented in the theca layers, which are testosterone and estrogen (Kato et al., 1995). However, progesterone, testosterone, and estrogen in the avian follicles are produced in the granulosa, theca interna, and theca externa layers, respectively. Granulosa tissue of F1-F4 preovulatory follicles contains significantly more progesterone than does theca tissues (Etches and Duke, 1984; Kato et al., 1995). Beside the function of FSH to regulate the growth of follicles, it has been well established that FSH can induce production of steroids from the follicles. FSH stimulates progesterone production in granulosa cells from F6-F3 follicles (Hammond et al., 1981). There is also evidence that FSH can induce modest, but significant, progesterone, androgen, and estrogen production from the theca layers of the

prehierarchical follicles *in vivo* (Kowalski et al., 1991) and prevents granulosa cells from undergoing apoptosis *in vitro* (Johnson et al., 1996). Supported by the findings from *in vitro* studies, it has been indicated that recombinant human FSH acts on granulosa cells of the prehierarchical follicles to increase the levels of cytochrome P450 cholesterol side-chain cleavage and promotes progesterone and androgen syntheses. It is proposed to occur during the final stage of differentiation of the granulosa cells following the selection of a follicle into the preovulatory hierarchy (Li and Johnson, 1993a; 1993b).

In birds, LH displays a pivotal role in the regulation of ovulation that ovulations occurs spontaneously and is hormonally controlled. The primary target for LH is the granulosa layer of the larger preovulatory follicles (Calvo et al., 1981; Calvo and Bahr, 1983; Bahr and Calvo, 1984). The cLHR mRNA transcript in granulosa cells is found to be expressed only within the preovulatory follicles (Johnson et al., 1996). LH promotes progesterone secretion by the granulosa cells of primarily F3 to F1 follicles (Hammond et al., 1981). During ovulatory cycles, ovulation is induced by a preovulatory release of LH by the anterior pituitary (Etches and Cunningham, 1976; Mashaly et al., 1976; Cunningham, 1987; Etches, 1990). The preovulatory surges of LH are associated with surges of progesterone produced from the F1 follicles (Kappauf and van Tienhoven, 1972; Mashaly et al., 1976; Bahr et al., 1983; Etches and Duke, 1984). Direct evidence for LH surge secretion controlling the ovulation in birds is given by *in vivo* studies in which injection of exogenous LH (Fraps et al., 1942; Neher and Fraps, 1950; Opel and Nalbandov, 1961) or progesterone (Fraps and Dury, 1943; Tanaka et al., 1987; Nakada et al., 1994) can induce single or multiple ovulations of the hierarchical follicles. The frequency of preovulatory LH surges is an

important determinant of ovulation and oviposition rates in birds. During preovulatory surges of LH in turkey hens, LH concentrations increase from a baseline level to a peak over 2-3 hours then decline from the peak to the baseline level over 4-6 hours (Yang et al., 1997; Liu et al., 2001a; Bacon et al., 2002). Ovulation of the F1 follicle occurs 6-8 hours after the preovulatory surge of LH (Mashaly et al., 1976; Proudman et al., 1984). Subsequently, oviposition of completely formed eggs normally occurs about 25 hours after ovulation (Wolford et al., 1964). Preovulatory surge of progesterone begins slightly earlier than or at the same time as LH surge and has a slightly longer duration (Yang et al., 1997). In turkey hens, LH concentrations increase over 1-2 hours from a baseline level to a plateau that last about 6 hours and then decline to the baseline level over 1-2 hours (Yang et al., 1997; Liu et al., 2001b). In contrast with the preovulatory surge of LH and progesterone, the concentrations of FSH and estrogen are relatively constant during the ovulatory cycle (Krishnan et al., 1993; Yang et al., 1997; Liu et al., 2001a; Bacon et al., 2002), but a significant decline in FSH occurrs prior to the preovulatory LH surge and a significant increase in FSH concentrations occurs during the 12 hours prior to oviposition (Krishnan et al., 1993).

2.2.5 Neuroendocrine Regulation of Mammals Gonadotropins Secretion

The neroendocrine control of FSH and LH syntheses and their secretions is complex and involved the interplay between the gonads, hypothalamus, and pituitary. The synthesis and secretion of FSH and LH are regulated mainly by the pulsatile release of the hypothalamic decapeptide hormone, GnRH. The release of GnRH occurs episodically from the mammalian hypothalamus. The frequency and amplitude of GnRH release from hypothalamic neuronal cells is a critical and rate-limiting step

for the control and maintenance of gonadotropins secretion from pituitary gonadotrops. It has been studied that the pulse of GnRH initiates pulsatile secretion of FSH and LH from the anterior pituitary (Clarke and Cummings, 1982; Levine et al., 1985; Levine and Duffy, 1988). In addition, changes in GnRH pulse frequency throughout the ovulatory cycle determine changes in the relative amounts of LH to FSH release (Wildt et al., 1981). GnRH is released in discrete pulses at intervals ranging from about 30 minutes to a few hours. Specially, high-frequency GnRH pulses (one pulse every 30 minutes) favor LH release, whereas low-frequency pulses (one pulse every 120 minutes) favor FSH release (Paschke et al., 1994; Kaiser et al., 1997b). Furthermore, both LH and FSH are released in a pulsatle manner into the systemic circulation, and in turn control the processes of gametogenesis and steroidogenesis. It is very well known that GnRH is the primary hypothalamic regulator of LH release in both spontaneous and induced ovulators (Conn and Crowley, 1994; Stojikovic and Catt, 1995).

In general, FSH and LH act on the ovaries and the testes to regulate folliculogenesis, ovulation, spermatogenesis, and steroidogenesis. Gonadal steroids and peptides, in turn, act upon the hypothalamus and/or pituitary to regulate either positively or negatively of the synthesis and secretion of gonadotropins. Gonadal steroids may regulate gene expression of gonadotropin subunits by affecting the level of the hypothalamus and directly at the pituitary (for review, see Burger et al., 2004).

Several lines of evidence support the importance of inhibins (INHs) in the regulation of the H-P-G axis. Negative correlation between FSH and the levels of INH-B has been reported (Anawalt et al., 1996; Jensen et al., 1997). Infusion of INH-A suppresses FSH levels without demonstrating any effects on LH secretion (Tilbrook

et al., 1993). Active immunization against INH neutralizes endogenous INH and increases the ovulation rate in several mammalian species (Brown et al., 1990; Wrathall et al., 1990; Scanlon et al., 1993). Furthermore, a variety of growth factors also modulate FSH action by autocrine and paracrine mechanisms. It is suggested that insulin-like growth factors (IGFs) and bone morphogenetic proteins (BMPs) are expressed throughout the development of follicles and oocytes and interact with gonadotropins to control maturation of follicles (for review, see Webb et al., 2007).

2.2.6 Neuroendocrine Regulation of Avian Gonadotropins Secretion

It has been very well documented that gonadotropins secretion are associated with the reproductive cycle in several avian species (mallard, Bluhm et al., 1983; King penguin, Mauget et al., 1994; turkey, Mashaly et al., 1976; El Halawani et al., 1984b; 1997; cockatiel, Myers et al., 1989; Wong et al., 1992b). During egg laying stage, plasma LH levels are elevated as compared with prelaying levels (Myers et al., 1989). The concentration of plasma LH is equally low in incubating and out-of-lay hens, while plasma PRL levels are high and low, respectively (Sharp et al., 1988). It has been reported that the onset of incubation activity is correlated with declining levels of gonadotropins and a dramatic rise in circulating PRL level in bantam hens (Sharp et al., 1979), turkeys (Burke and Dennison, 1980; El Halawani et al., 1984b), ducks (Goldsmith and Williams, 1980), canaries (Goldsmith et al., 1984), wild starlings (Dawson and Goldsmith, 1982), ring doves (Goldsmith et al., 1981), white-crowned sparrows (Hiatt et al., 1987), and spotted sandpipers (Oring et al., 1986). These increasing PRL levels during the incubation period suppress the activity of the GnRH/FSH-LH system (Rozenboim et al., 1993b; You et al., 1995a), reducing

ovarian steroids secretion (Porter et al., 1991; Tabibzadeh et al., 1995), terminating egg laying, inducing ovarian regression (Youngren et al., 1991), and signaling the transition from sexual behavior to incubation behavior (Chaiseha and El Halawani, 2005). Circulating PRL levels decline, whereas LH levels begin to rise when incubation behavior is terminated (El Halawani et al., 1988a; Knapp et al., 1988) and as soon as molting is ended (Bluhm et al., 1983; Mauget et al., 1994). Experimental manipulations of PRL levels affect the changes in circulating LH. In turkey, systemic administration of PRL decreases hypothalamic GnRH-I and GnRH-II contents and plasma LH levels (Rozenboim et al., 1993b), while incubation of anterior pituitary cells with PRL inhibits LH-β- subunit gene expression (You et al., 1995b). There is also evidence in the turkey that PRL inhibits the steroidogenic activity of LH (Camper and Burke, 1977). Injection of PRL antiserum is associated with an increase in LH levels in incubating chickens (Lea et al., 1981). Furthermore, exogenous PRL administration suppresses the increased LH secretion that occurs after ovariectomy (El Halawani et al., 1991), or when incubating chickens are nest-deprived (Sharp et al., 1988).

It is very well established that avian gonadotropins secretion is influenced by GnRH. In sexually matur active birds, GnRH is synthesized by neurosecretory cells in the hypothalamus, released from the ME into the hypophyseal portal vessels, and then transported to the pituitary gland, where it stimulates the secretion of FSH and LH. Ovarian development is found to be correlated with plasma LH level and the amount of GnRH-I content, indicating the expression of the GnRH-I gene is important to maintain pituitary-ovarian function in chicken (Dunn et al., 1996). GnRH increases LH and FSH secretion of the anterior pituitary both *in vitro* and *in vivo* (Peczely,

1989). In *in vivo* study, injection of cGnRH-I or cGnRH-II stimulates an increase in plasma LH concentrations (Guemene and Williams, 1999). Changes in pituitary responsiveness to GnRH are negatively correlated to changes in the circulating LH level (Balthazart et al., 1980). GnRH agonists imitate the native hormone and induce an endogenous LH surge (Shalev and Leung, 2003). Conversely, GnRH inhibits FSH-stimulated steroidogenesis in chickens as well as in mammals but enhances LH-stimulated progesterone production (Hertelendy et al., 1982). cGnRH-I does not affect circulating FSH levels but stimulates LH secretion when administrated to 3 weeks old cockerels (Krishnan et al., 1993). The reduced gonadotropins levels in incubating hens reflect a decrease in hypothalamic GnRH release into the hypophyseal portal system. An increase in hypothalamic GnRH content is associated with peak LH level of the preovulatory LH surge of the chicken (Johnson and Advis, 1985).

The effects of GnRH on gonadotropins secretion are well documented. Knowledge of the neurotansmitters controlling gonadotropins release in birds has been reviewed (Sharp, 1983). It has been established for sometime that adrenergic stimulation at the hypothalamic level can release GnRH and thereby increase gonadotropins secretion (Yu et al., 1991). Both stimulatory and inhibitory roles have been reported for 5-HT. 5-HT shows the inhibitory effect on GnRH (Sharp et al., 1984; 1989b). Norepinephrine (NE) has been reported to have the stimulatory effect on GnRH secretion (Knight et al., 1984). Microinjections of 5-HT into the caudal ventromedial nucleus (VMN) of the turkey hypothalamus notably impede the PRL release effected by electrical stimulation in the POA. These data suggest that 5-HT, at least at the VMN level, may be involved in the decline in circulating PRL observed during the photorefractory state (Youngren and El Halawani, 2002). Electrical

stimulation in the POA, which is known to stimulate secretion of LH and PRL, activates GnRH and VIP immunoreactive neurons (as indicated by c-fos mRNA expression) in the POA and INF areas, respectively (El Halawani et al., 2004). VIP immunoneutralization of turkey hens reveal that VIP acts as an antagonist toward both FSH and LH secretion (Ahn et al., 2001).

There are evidences suggest an inhibitory role for DA upon GnRH release in mammals as well as in birds (Ramirez et al., 1984; Sharp et al., 1984). Further evidence for the involvement of DA in correlating with GnRH is derived from dense concentration of tyrosine hydroxylase (TH; the rate limiting enzyme for DA synthesis) and GnRH-containing processes are located in the lateral and mediobasal portion of the external layer of the ME (Contijoch et al., 1992). Activation of DAergic neurons in the nucleus mamillaris lateralis (ML) is associated with the activation of GnRH-I and VIP neurons and the release of LH and PRL (Al-Zailaie et al., 2006). The results provide an opportunity for synaptic interaction between GnRH and DA. This is the first identification of a specific DA neuronal group that is associated with the stimulation of GnRH/FSH-LH and VIP/PRL systems.

It has been well demonstrated that sterioid hormones also correlate with gonadotropins secretion. Circulating LH is directly related to gonadal activity and the regulation of steroidogenesis (Robinson et al., 1988). Estrogen is intraovarian regulators of preantral follicular growth (Goldenberg et al., 1972; Richard, 1979), granulosa cell proliferation (Williams, 1940), and LHR expression (Knecht et al., 1985). However, it can exert both inhibitory and stimulatory effects on gonadotropins secretion (Li et al., 1994). In seasonally temperate zone birds, FSH and LH secretion and their gene expression are stimulated by long day length (Nicholls et al., 1988;

Dawson et al., 2001) and required the functional integrity of the GnRH neuronal system (Katz et al., 1990; Sharp et al., 1990). Both plasma FSH (Lewis et al., 1998; 1999) and LH (Dunn et al., 1990; Lewis et al., 1994; 1998; 1999; 2001; Sreekumar and Sharp, 1998; Dunn and Sharp, 1999) concentrations increase in juvenile female chickens after photostimulation. Photostimulation may advance sexual maturation by increasing gonadatropins secretion, particularly FSH, which stimulates ovarian follicular development (Palmer and Bahr, 1992). In addition, it is proposed that neuroendocrine mechamisms controlling photo-induced FSH release may involve estrogen-responsive interations between pituitary paracrine factors including activins and follistatin (Dunn et al., 2003). There are growing evidences support the importance of INHs in the regulation of H-P-G axis. Like some species of mammals (ewe, rat, heifer), active immunization of female turkeys with INH neutralizes endogenous INH and increases both circulating FSH and the number of preovulatory follicles. However, no significant increase in egg production is observed in INH-immunized hens (Ahn et al., 2001).

2.3 Prolactin: Structure, Function, and Regulation of Secretion

2.3.1 The Structure of Prolactin

PRL is a polypeptide hormone that is synthesized in and secreted from specialized cells of the anterior pituitary gland, the lactotrophs (Bern and Nicoll, 1968; Velkeniers et al., 1988; Freeman et al., 2000). PRL is also synthesized by a number of extra-pituitary tissues in mammals (Ben-Jonathan et al., 1996; Freeman et al., 2000; Soares, 2004) and in birds (Berghman et al., 1992; Ramesh et al., 2000; Chaiseha et al., 2003), but the physiological function is poorly understood. PRL is synthesized and

secreted by a broad range of other cells in the body, most prominently various immune cells, mammary epithelium, placenta, the deciduas of the pregnant uterus, and brain (Ben-Jonathan et al., 1996). Evidence for PRL production in the lacrimal gland, adrenal gland, corpus luteum, prostate gland, testis, and pancreas is also existed (Ben-Jonathan et al., 1996; Freeman et al., 2000). This prominent single-chain polypeptide hormone is discovered by Riddle and co-workers (1931; 1932). The hormone is given its name based on the fact that an extract of bovine pituitary gland would cause growth of the crop sac and stimulate the elaboration of crop milk in pigeons or promote lactation in rabbits (Riddle et al., 1933; Bern and Nicoll, 1968). PRL is one of a family of related hormones including growth hormone (GH) and placental lactogen (PL) that are hypothesized to have arisen from a common ancestral gene about 500 millions years ago. Its amino acid sequence is similar to that of GH and PL sharing genomic, structural and biological features (Boulay and Paul, 1992; Horseman and Yu-Lee, 1994). Genes encoding PRL, GH, and PL evolved from a common ancestral gene by gene duplication (Niall et al., 1971). To date, over 300 different physiological functions of PRL have been documented (Houdebine, 1983; Bole-Feysot et al., 1998; Harris et al., 2004).

PRL gene is 10 kb in size and is composed of 5 exons and 4 introns (Cooke et al., 1981; Truong et al., 1984). PRL is synthesized as a prehormone consisting of 227 amino acid residues (Miller and Eberhardt, 1983) in most mammalian species. The mature hormone is derived from this precursor molecule and contains 194-199 residues (23 kDa), depending upon species. Hormone structure is stabilized by three intramolecular disulfide bonds. The primary structure of PRL is first elucidated in the ovine (Li et al., 1970). The complete amino acid sequence of PRLs of more than 25

species have been then determined (for review, see Sinha, 1995). Comparisons of the amino acid sequence of PRL from different species are listed in Fig. 2.4. Some 32 residues seem to have been conserved among different species (Watahiki et al., 1989). The primary structures of the PRLs of various vertebrate species are presented in Fig. 2.5.

PRL receptor (PRLR), a single membrane-bound protein transmembrane receptor, is a member of Class I cytokine receptor superfamily that includes the GH receptor, leptin receptor, erythropoietin receptors, and receptors for several interleukins (Bazan, 1989; 1990; Kelly et al., 1991). Numerous PRLR isoforms have been reported in different tissues in both mammals and birds (Davis and Linzer, 1989; Ali et al., 1991; Lesueur et al., 1991; Pitts et al., 2000). Alternative spicling of the PRLR gene leads to multiple isoforms which differ in the length and composition of their cytoplasmic tail and are referred to as the short (291aa; Boutin et al., 1988) and long (591aa; Shirota et al., 1990) PRLR isoforms (for review, see Harris et al., 2004). Multiple isoforms of membrane-bound PRLR resulting from alternative splicing of the primary transcript have been identified in several species (Bole-Feysot et al., 1998). cDNAs encoding the PRLR gene have been cloned in chicken (Tanaka et al., 1992), dove, pigeon (Chen and Horseman, 1944), and turkey (Zhou et al., 1996; Pitts et al., 2000).

	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																												

Fig. 2.4 Sequence homology (%) of PRLs among different species (Sinha, 1995).

PRL is believed to activate sequentially its receptors by dimerizing two identical receptor subunits, leading to activation of Jak2-kinase asscociated with the cytoplasmic domain (for reviews, see Bole-Feysot et al., 1998; Freeman et al., 2000). Jak2 phosphorylates tyrosine residues on different target proteins, the best identified of which is the receptor itself and a family of transcription factors termed signal transducers and activators of transcription (Stats). Although the Jak2–Stat cascade is the major signaling pathway used by the PRLR, other transducing pathways are also involved in signal transduction by this receptor. Activation of mitogen-activated protein kinases (MAPK) pathway has been reported in different cellular systems under PRL stimulation (for review, see Bole-Feysot et al., 1998). In addition, activation of the nucleotide exchange protein Vav has been reported as well (Clevenger et al., 1995).

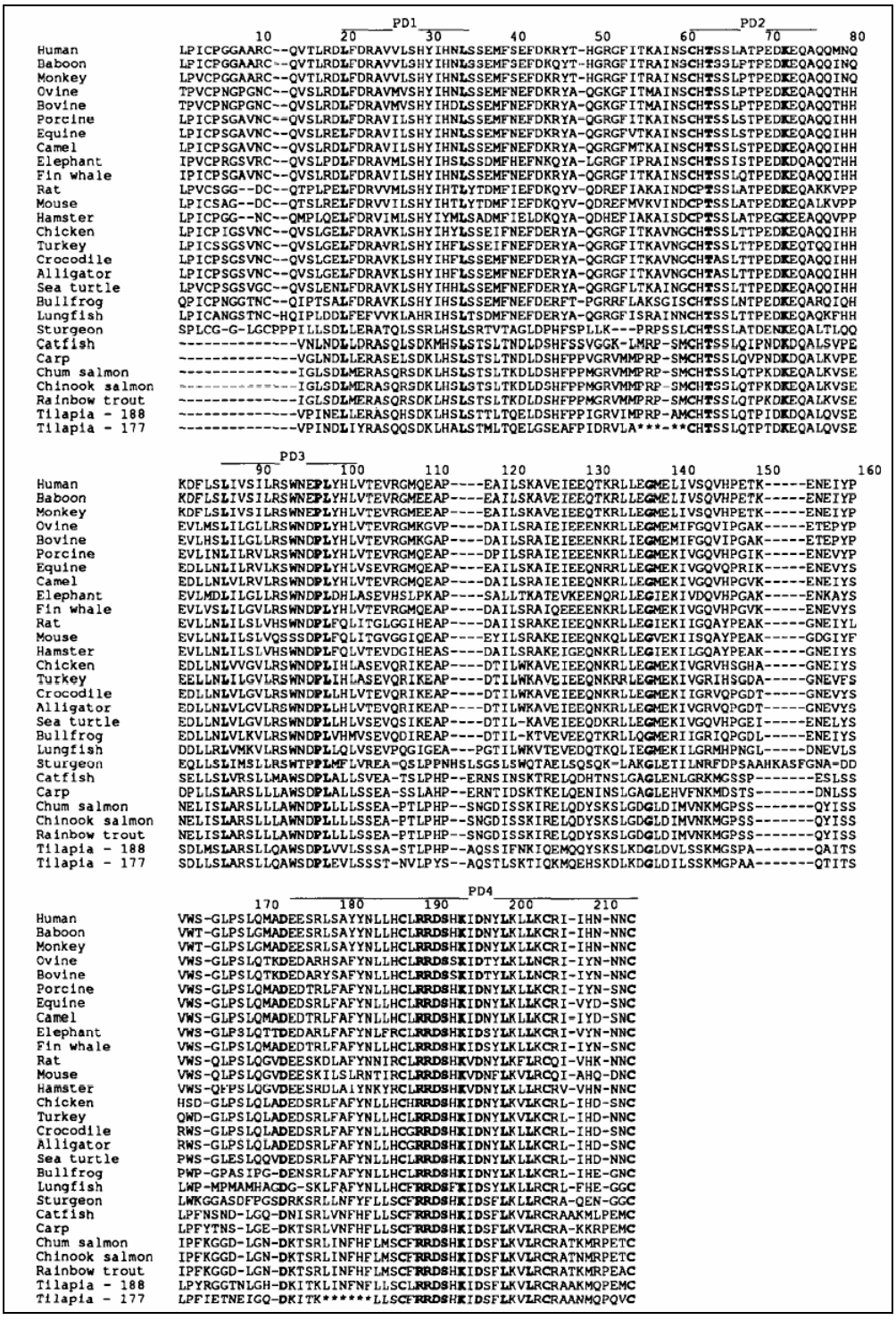

Fig. 2.5 Primary structures of PRLs of different species. (-) indicates positions left blank to optimize alignment of amino acid sequences. (*) indicates absence of residues from a genetic variant of tilapia PRL. PD is PRL domain. PDI-PD4 indicates the four highly conserved domains of the PRLs (Sinha, 1995).

PRLR and its mRNA are found in the mammary gland and ovary, two of the best characterized sites of PRL actions in mammals (Nagano and Kelly, 1994). Tissue distribution of PRLR mRNA has been characterized in rats (Nagano and Kelly, 1994; Bakowska and Morrell, 1997), turkeys (Zhou et al., 1996; Pitts et al., 2000), and chickens (Ohkubo et al., 1998). PRLR mRNA is found in the CNS of the rat and abundant message is found in the choroid plexus, bed nucleus of the stria terminalis, amygdala, central gray of the midbrain, thalamus, hypothalamus, cerebral cortex, and olfactory bulb (Freemark et al., 1995; 1996). PRL binding sites have been described in the area postrema, which is one of the main chemosensitive areas of the brain lacking the blood-brain barrier (Mangurian et al., 1999).

In mammals, PRLR are also presented in the peripheral organs such as pituitary gland, ovary, testis, liver, uterus, heart, lung, thymus, spleen, pancreas, kidney, adrenal gland, skeletal muscle, prostate gland, epithelial cells, bone, and skin (Nagano and Kelly, 1994; Nevalainen et al., 1997; Bole-Feysot et al., 1998; Clement-Lacroix et al., 1999). PRLR is extensively expressed by immune cells and some types of lymphocytes synthesize and secrete PRL, suggesting that PRL may act as an autocrine or paracrine modulator of immune activity. In birds, PRLR are detected in crop sac, brood patch, thyroid gland, liver, kidney, leg, skin, intestine (duodenum), adipose tissue, adrenal gland, thymus, spleen, heart, brain, pineal gland, ovary, testis, and in the infundibulum, magnum, and isthmus of the oviduct (Tanaka et al., 1992; Chen and Horseman, 1994; Zhou et al., 1996; Ohkubo et al., 1998; Pitts et al., 2000). In turkey, PRLR mRNA levels are the greatest in the pineal gland of laying hens and in the oviduct of incubating hens (Pitts et al., 2000).

2.3.2 The Function of Prolactin in Mammals

PRL is one of the most versatile hormones of the pituitary gland in terms of physiological functions. PRL is best known for the multiple effects exerting on the mammary gland. However, it also exerts effects on other targets important to the reproduction of mammalian species. More than 300 different and distinct physiological effects of the hormone have been documented, highlighting the importance of this adenohypophyseal hormone. PRL interacts with its receptors in a broad variety of target tissues to affect physiological processes that have been broadly grouped into those that affect reproduction, water and electrolyte balance, brain and behavior, growth and development, endocrinology and metabolism, osmoregulation, metabolism, behaviors such as migration, the nurturing of the young in different vertebrate species, and also immunoregulation (Saeki and Tanabe, 1955; Houdebine, 1983; Bole-Feysot et al., 1998; Harris et al., 2004).

Although PRL seems to be an omnipotent hormone, it is best known for the multiple effects that exerts on the mammary gland. The varied effects of PRL on the mammary gland include growth and development of the mammary gland (mammogenesis), synthesis of milk (lactogenesis), and maintenance of milk secretion (galactopoiesis). It has been well accepted that PRL is involved in the development of the mammary gland (Bern and Nicoll, 1968). Lactogenesis clearly requires pituitary PRL, since hypophysectomy during pregnancy prevents subsequent lactation. It is reported that the common absolute requirement for mike secretion in mammals is PRL. Aqueous extracts of anterior pituitary gland containing PRL initiate lactation in pseudopregnant rabbits. Replacement of PRL to hypophysectomized rabbits fully restores lactation, while hypophysectomy of rats and mice stops lactation (for review,

see Freeman et al., 2000). The initiation and maintenance of lactation following parturition is dependent on the mitogenic effects of PRL upon mammary cell development and its regulation of transcription and translation of milk proteins (Ben-Jonathan et al., 1989). It has been indicated that PRL, cortisol, and insulin act concurrently to stimulate transcription of the genes that encode milk proteins. It also seems to modulate ovulation since elevated physiological or pathological PRL levels results in cessation of cyclicity (Nicoll, 1974).

In some mammals, particularly rodents, PRL plays a critical role in copus luteum maintenance and progesterone secretion (Risk and Gibori, 2001). In most rodents, PRL acts as a luteotrophic hormone by maintaining the structural and functional integrity of corpus luteum for 6 days after mating (Morishige and Rothchild, 1974). In addition, PRL induces transcription of estrogen receptor (Frasor and Gibori, 2003) and 3β-hydorxysteroid dehydrogenase involved in progesterone synthesis (Feltus et al., 1999). Progesterone is essential for implantation of fertilized ovum (along with estrogen), maintenance of pregnancy, and inhibition of ovulation (Freeman, 1994). Aside from its luteotrophic role, there is evidence in the rat that PRL may be luteolytic as well (Malven and Sawyer, 1966; Wuttke and Meites, 1971) by inducing programmed cell death in the corpora lutea (Kanuka et al., 1997).

In addition of its essential role for lactation in mammals, PRL also plays a significant role in reproduction, maternal care, and parental behavior in birds and mammals. The best characterized PRL driven behaviors are the parental behaviors (Buntin, 1993; Schradin and Anzenberger, 1999). Enhancement of endogenous PRL secretion in response to DA antagonism has been reported to have no effect on mating behavior in females (Sodersten et al., 1983). On the contrary, when the rat is sexually

receptive in the afternoon of proestrus, suppression of the spontaneous release of PRL with a DA agonist dramatically attenuates sexual receptivity (Mena and Grosvenor, 1972). In males, PRL and its receptors are expressed in human and rat prostate epithelial cells, where their levels are increased by androgen treatment (Nevalainen et al., 1997). PRL has a subtle role in the prostate gland, whereas hyperprolactinemia has a direct on hyperplasia of the prostate gland. This is in accordance with the role of PRL on cellular proliferation. Beside its actions on reproductive processes, PRL plays a role in maintaining constancy of the internal environment by regulation of the immune system, osmotic balance, and angiogenesis as well (for review, see Freeman et al., 2000).

2.3.3 The Function of Prolactin in Birds

PRL has long been implicated in the onset and maintenance of incubation behavior in birds. Administration of exogenous PRL leads to increase parental behaviors in birds (Buntin et al., 1981; Lea and Vowles, 1986; Macnamee et al., 1986; Pedersen, 1989; Youngren et al., 1991). In addition, active immunization against recombinant-derived PRL reduces the incidence, delays the development (March et al., 1994), or prevents the occurrence (Guemene et al., 1995) of incubation behavior in birds. These results supports that PRL regulates the onset and maintenance of incubation behavior in galliform birds. PRL has been associated with incubation behavior in pigeon (Riddle et al., 1935), pheasant (Breitenbach and Meyer, 1959), cowbird (Hohn, 1959), turkey (Burke and Dennison, 1980; El Halawani et al., 1988a; Youngren et al., 1991), mallard duck (Goldsmith and Williams, 1980), and chicken (Sharp et al., 1988). Changes in pituitary PRL gene expression and its levels and

plasma PRL levels are highly correlated during different stages of the reproductive cycle in chicken and turkey (Knapp et al., 1988; El Halawani et al., 1990a; Talbot et al., 1991; Wong et al., 1991; You et al., 1995b; Karatzas et al., 1997; Tong et al., 1997). During the reproductively quiescent stage of the avian reproductive cycle, plasma PRL levels are very low (5-10 ng/ml). However, during the laying and incubating stages, circulating PRL levels increase dramatically to the level of 500-1,500 ng/ml (El Halawani et al., 1984a). This rising PRL level has been implicated as the cause of cessation of ovulation, ovarian regression, and induction of incubation behavior (Sharp et al., 1984; Buntin, 1986; Hall et al., 1986; El Halawani et al., 1988a). The levels of PRL increase at the onset of incubation behavior and are maintained at high levels through the incubation phase in both pituitary gland (Saeki and Tanabe, 1955) and circulating blood (Sharp et al., 1979; Burke and Dennison, 1980; Proudman and Opel, 1988). It has been reported that the onset of incubation behavior or continuous nesting activity is associated with 6- to 10-fold increase in circulating PRL levels, while FSH and LH drop to basal concentrations (El Halawani et al., 1988b). PRL declines when incubation behavior is terminated (El Halawani et al., 1980; Wentworth et al., 1983).

It has been suggested that an increase in the concentrations of plasma PRL during incubating period may depress LH secretion (Zadworny and Etches, 1987; Nocholas et al., 1988; El Halawani et al., 1993; Sharp et al., 1998), suggesting that PRL acts centrally to reduce LH levels by reducing hypothalamic GnRH levels (Rozenboim et al., 1993b). Furthermore, some evidence suggests that PRL also plays a role in terminating egg laying and thus regulates clutch size in species that lays more than two eggs per clutch. Cessation of egg laying is associated with an increase

concentrations of plasma PRL (Etches et al., 1979; Burke and Dennison, 1980; Lea et al., 1981; Bluhm et al., 1983; Hall and Goldsmith, 1983; Silverin and Goldsmith, 1983). In turkeys (El Halawani et al., 1991) and domestic fowl (Sharp et al., 1988), administration of exogenous PRL suppresses plasma gonadotropins. However, there is additional evidence that the suppression of gonadotropins secretion in incubating birds also involves a mechanism independent of increased PRL secretion (Sharp et al., 1988; Lea and Sharp, 1989; Sharp et al., 1989a; Lea et al., 1996). PRL may also directly inhibit ovarian steroidogenesis (Rozenboim et al., 1993b), leading to involution of the ovary with reduced ovarian steroidogenesis and regression of the oviduct. In turkeys, the abundance of LH-β subunit and PRL mRNAs shows an inverse relationship in photostimulated/laying and incubating hens (Wong et al., 1992b). Administration of exogenous PRL suppresses the photo- and ovariectomy-induced increases in LH secretion, delays the onset of egg laying, and induces incubation behavior in laying hens (El Halawani et al., 1991). Taken together, PRL has been implicated as a causative factor for the reduced circulating gonadotropins and ovarian regression, when birds shift from egg laying to incubation behavior (El Halawani et al., 1997). In addition, PRL is also insert its functions in associated with a wide range of reproductive physiology and behaviors including incubation, migration, grooming, crop milk secretion, feeding of young, nest defense, and sexual activity (Lea et al., 1981; 1986; Silver, 1984; Janik and Buntin, 1985; Buntin et al., 1991).

2.3.4 Neuroendocrine Regulation of Prolactin in Mammals

PRL secretion from the pituitary gland is governed by the hypothalamo-adenohypophysis axis. This axis includes the hypothalamus, ME, plexus of

hypophyseal portal vessels, and pituitary gland. The mammalian pituitary gland is composed of the adenohypophysis; the pars distalis and pars intermedia and neurohypophysis. In birds, the pars intermedia does not exist as a separate entity as it does in mammals. The pars distalis, the ME, and its associated capillary plexus, have distinct anterior and posterior divisions (Oksche and Farner, 1974). No anatomical separation exists between the cephalic and caudal lobes, but histological studies have defined a distinct distribution of hormone-secreting cells, indicating that the adenocorticotropin, TSH, and PRL-secreting cells are confined to the cephalic lobe, whereas GH cells are found within the caudal lobe (Kobayashi and Wada, 1973; Kansaku et al., 1995; Lopez et al., 1995; Ramesh et al., 1996).

In mammals, PRL secretion is regulated by both stimulatory and inhibitory hypothalamic factors. It is mainly under tonic inhibitory control (Neill, 1988; Ben-Jonathan et al., 1989; Lamberts and MacLeod, 1990) and the predominant mammalian PRL-inhibiting factor (PIF) is DA. DA is released from a dense network of neurons within the mediobasal hypothalamus known as the TIDA and serves as the physiological inhibitor of PRL secretion (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001). 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), which release DA that acts directly upon D_2 DA receptors located on pituitary lactotrophs (Caron et al., 1978; Civelli et al., 1991). Removal of this DAergic inhibition results in increased PRL secretion and hyperprolactinemia (Nicoll and Swearingen, 1970; Nicoll, 1977). DA or its agonists inhibits the release and gene expression of PRL, as well as proliferation of lactotrophs (Birge et al., 1970; Shaar and Clemens, 1974; Pawlikowski et al., 1978; Maurer,

1981). DA also participates in the regulation of the acute increased of PRL release. *In vivo* studies demonstrated that 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 PRL release (Grosvenor et al., 1980). Confirming by *in vitro* studies, pituitary PRL release is stimulated after short term exposure of DA (Fagin and Neil1, 1981; Denef et al., 1984). The interruption of DA and its agonists leads to an augmented PRL secretion. These studies clearly attest to the physiological relevance of DA as the PIF. However, there are evidences demonstrate that DA at concentrations much lower than those required for inhibition of PRL secretion, actually stimulates PRL secretion *in vitro* (Shin, 1978; Denef et al., 1980; Burris et al., 1991; 1992; Porter et al., 1994) and *in vivo* (Arey et al., 1993). Explanations for these phenomena suggest that all pituitary lactotrophs have the potential to respond to the inhibitory and stimulatory effects of DA (Kineman et al., 1994), and the two opposite effects of DA on PRL secretion may be mediated by distinct G-proteins (Burris et al., 1992; Niimi et al., 1993; Lew et al., 1994).

DA seems to be the predominant ongoing PIF (MacLeod and Login, 1976; Ben-Jonathan, 1985; Lamberts and MacLeod, 1990). In contrast, thyrotropin-releasing hormone (TRH) functions as a hypothalamic releasing factor of PRL in mammals. A wealth of functional data supports a role of TRH as a regulator of PRL secretion. PRL release is stimulated by TRH both *in vivo* (Grosvenor and Mena, 1980; de Greef and Visser, 1981; Lafuente et al., 1994) and *in vitro* (Maas et al., 1991). TRH also stimulates PRL gene transcription (Potter et al., 1981; Laverriere et al., 1988). PRL release by TRH occurs during a transient depression in DAergic activity (Plotsky and Neill, 1982; Martinez de la Escalera et al., 1988). However, the contradictive results

from some studies have led researchers to question its role as the PRF.

Several studies show the involvement of VIP in the regulation of PRL secretion from the pituitary (Kato et al., 1978; Rotsztejn et al., 1980; Reichlin, 1988). The presence of VIP in the hypothalamic nerve endings, the adenohypophysis (Besson et al., 1979), and the hypophyseal portal blood (Said and Porter, 1979) suggests an involvement of VIP in the regulation of pituitary secretion by a neuroendocrine pathway. VIP stimulates PRL release *in vivo* (Kato et al., 1978; Frawley and Neill, 1981) and *in vitro* (Shaar et al., 1979; Enjalbert et al., 1980; Samson et al., 1980; Matsushita et al., 1983). The administration of VIP anti-serum inhibits PRL release induced by stress, serotonin, or suckling (Shimatsu et al., 1984; Abe et al., 1985; Kaji et al., 1985a; 1985b; Ohta et al., 1985). Furthermore, VIP also appears to regulate the amount of pituitary PRL mRNA and PRL synthesis (Ben-Jonathan et al., 1989; Maas et al., 1991). Hypothalamic VIP mRNA is increased during lactation in rats (Gozes and Shani, 1986). The concentration of VIP in the hypophyseal portal blood is increased relatively to the peripheral blood (Said and Porter, 1979; Shimatsu et al., 1981). In rats and humans, VIP also promotes the entry of extracellular calcium ions into the PRL-secreting pituitary cells (Bjoro et al., 1987; Prysor-Jones et al., 1987). All of these data point VIP as the PRF in mammals. Furthermore, during the past three decades, the various PRFs and PIFs have been suggested, which are known to exert their effects at the level of pituitary may also interact at the hypothalamic level to control PRL secretion (Moog and Samson, 1990) such as TRH (Grosvenor and Mena, 1980; El Halawani et al., 1988a; Laverriere et al., 1988; Lafuente et al., 1994), 5-HT (Chaiseha and El Halawani, 2005), angiotensin II (Malarkey et al., 1987; Opel and Proudman, 1988a; Myers and Steele, 1989; Steele, 1990), oxytocin/vasopressin (Hyde

and Ben-Jonathan, 1988; 1989; Johnston and Negro-Vilar, 1988), peptide histidine isoleucine (PHI; Samson et al., 1983; Werner et al., 1983; Proudman and Opel, 1988; Kulick et al., 2005), and pituitary adenylate cyclase activating polypeptide (PACAP; Miyata et al., 1989; Yamauchi et al., 1995; Yoo et al., 2000).

2.3.5 Neuroendocrine Regulation of Prolactin in Birds

It has been established for some time that PRL secretion in birds involves a stimulatory mechanism rather than the inhibitory DAergic system found in mammals (Kragt and Meites, 1965; Bern and Nicoll, 1968; El Halawani et al., 1984a; Hall et al., 1986). PRL secretion in birds is tonically stimulated by the hypothalamus (Kragt and Meities, 1965; Bern and Nicoll, 1968) and that principal PRF is VIP (for review, see El Halawani et al., 1997; 2000). VIP meets the classic criteria for defining substances as the hypophysiotropic PRF. These criteria are (1) the localization in the hypothalamic neurons which terminate within the ME; (2) the secretion into hypophyseal portal circulation at concentrations greater than that of peripheral blood; (3) the proven biological activity *in vivo* and *in vitro*; (4) the presence of specific VIP receptors on anterior pituitary cells; and (5) the alteration of pituitary functions, due to an antagonism of the candidate substances. In the past five decades, several studies support the pivotal role of VIP as the only avian PRF. Immunoneutralization of VIP averts the rise in circulating PRL that follows photostimulation, prevents the induction of incubation behavior, up regulates LH-β- and FSH-β-subunit mRNAs, and extends the duration of reproductive activity (egg laying period), but does not prevent spontaneous gonad regression and molting (Dawson and Sharp, 1998; Sharp et al., 1989a; El Halawani et al., 1995; 1996; Ahn et al., 2001).

The role of DA in the regulation of PRL secretion in birds is unclear at present for comparing it to the mammalian DAergic strategy for PRL control. The intracerebroventricular (ICV) infusion of DA in laying turkey hens demonstrates that DA can either stimulate or inhibit PRL secretion, depending upon the concentrations used (Youngren et al., 1995). DAergic influences are involved in both stimulating and inhibiting avian PRL secretion depending upon multiple DA receptors. The stimulatory effect of DA on PRL secretion is regulated via D_1 DA receptors residing in the INF, where the VIP neurons are located. In contrast, DA inhibits PRL release and synthesis by blocking the action of VIP at the pituitary level through D_2 DA receptors (Youngren et al., 1996b; 1998; 2002a; Chaiseha et al., 1997; 2003; Al Kahtane et al., 2003). It has been 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). Dynorphin, 5-HT, DA, and VIP all appear to stimulate avian PRL secretion along a pathway expressing κ opioid, serotonergic, DAergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator (for review, see El Halawani et al., 2000).

2.4 Gonadotropin Releasing Hormone: Structure, Function, and Regulation of Secretion

2.4.1 The Structure of Gonadotropin Releasing Hormone

GnRH, a hypothalamic neuronal secretory decapeptide, is important for the control of reproduction in vertebrates. Hypothalamic GnRH, referred to as type one mammalian GnRH (mGnRH), is first isolated from porcine hypothalamus and sequenced during the early 70s by Schally, Guillemin, Yalow and co-workers, the

1977 Nobel Laureates in Medicine (Rivier, 2001). Later, a second molecular form of GnRH has been identified in the brain of several mammals and other non-mammalian vertebrate species (for review, see Bakker and Baum, 2000). This second form has been identified as chicken GnRH-II (cGnRH-II). More recently, the discovery of lamprey GnRH-III is reported (Millar et al., 2001; Bentley et al., 2004) and preferentially existed in the terminal part of the olfactory neuronal cells in the brain. To date, it has been reported that GnRH consists of a family of at least 24 isoforms, 14 of which are found in various vertebrate species (Gorbman and Sower, 2003). It is theorized that at least two or more forms of GnRH exist in every species (Troskie et al., 1998). All these forms are of classical 10 amino acid peptides, (PGlu-His-Trp-Ser-Tyr-Gly-Len-Ser-Pro-Gly-NN_2) with conserved amino acids in position 1,2,4,9, and 10 (Powell et al., 1994) and share at least 50% sequence identity (for review, see Limonta et al., 2003). The most recognized and common structural variation among the different forms of GnRH resides in amino acids between 5 and 8 in the sequence. Two forms of identified GnRH in birds are cGnRH-I ([Gln8]-GnRH) and cGnRH-II ([His5, Trp7, Ty8]-GnRH; King and Millar, 1982; Miyamoto et al., 1982; 1984; Millar and King, 1984; Sherwood et al., 1988). Of the two forms, GnRH-I is the form that is directly involved in controlling reproduction in the domestic chicken (Sharp et al., 1990). The gene encoding cGnRH-I has been cloned and characterized (Dunn et al., 1993). Evidence has been reported of ostriches having a possible third form of GnRH found in hypothalamic and extra-hypothalamic brain tissues. However, the sequence of this avian GnRH-III is not determined (Powell et al., 1987). Primary structures of GnRH forms are listed in Table 2.1.

Table 2.1 Primary structures of GnRH forms.

GnRH form	1	2	3	4	5	6	7	8	9	10
GnRH-I (mGnRH)	p-Glu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
GnRH-II (cGnRH-II)	p-Glu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH_2
cGnRH-I	p-Glu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH_2
rGnRH	p-Glu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	Gly-NH_2
lGnRH-I	p-Glu	His	Trp	Ser	Leu	Glu	Trp	Lys	Pro	Gly-NH_2
lGnRH-III	p-Glu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly-NH_2
sGnRH	p-Glu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly-NH_2
cfGnRH	p-Glu	His	Trp	Ser	His	Gly	Leu	Pro	Pro	Gly-NH_2
dfGnRH	p-Glu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly-NH_2

m, mammalian; c, chicken; r, amphibian; l, lamprey; s, salmon; cf, catfish; and df, dogfish (Limonta et al., 2003).

GnRH receptors of several mammalian species have been cloned (for review, see Ramakrishnappa et al., 2005). It is a member of the large superfamily of seven trasmembrane domain receptors that binds to the G proteins. Upon binding to its receptors, GnRH activates the G_q/G_{11} subfamily of G proteins, causing an increase in phospholipase C activity and results in phosphoinositide breakdown and generation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 releases Ca^{2+} from intracellular stores, whereas DAG activates protein kinase C. These events lead to the synthesis and release of gonadotropins (Stojilkovic and Catt, 1995). Progress has been made in defining the structure of mGnRH receptors and in identifying amino acid residues that are important for ligand binding and coupling to G proteins. The cloned of a novel GnRH receptor from chicken pituitary differs from the mGnRH receptor in its primary structure, ligand selectivity, and in the agonistic behavior of certain mGnRH receptor antagonists (Harris et al., 2004). Biological assays indicate that chicken GnRH receptor exhibits well defined differences in ligand selectivity compared with mGnRH receptors (Tanaka et al., 1980). In particular, some

antagonists of mGnRH receptors act as agonists of the chicken GnRH receptor, stimulating LH release from chicken pituitary cells (Kuo et al., 2002).

2.4.2 The Localization of Gonadotropin Releasing Hormone in the Brain

It is clear that each form of GnRH has its unique locations within the brain, which suggests a difference in developmental origin and/or adult functions (Sherwood et al., 1993; White at al., 1995; Lescheid et al., 1997; Dubois et al., 2002). In mammals, neurons producing GnRH-I are distributed in a loose rostral-caudal continuum in the ventral forebrain. The principle projection of mGnRH neurons is to the ME, where terminals of these neurons have been observed in the pericapillary space of the hypophyseal portal plexus. In contrast with GnRH-I, neurons expressing GnRH-II reside in the midbrain. The major terminal field for these GnRH-II neurons is the medial habenula, and in contrast to forebrain GnRH-I fibers, few GnRH-II fibers are visible in the ME (for review, see Bakker and Baum, 2000).

A number of previous studies have examined the distribution of cGnRH-I throughout the avian brain including chicken (Jozsa and Mess, 1982; Sterling and Sharp 1982; Mikami et al., 1988; Kuenzel and Blahser, 1991), duck (McNeill et al., 1976; Bons et al., 1978), white-crowned sparrow (Blahser et al., 1986; 1989), Japanese quail (Foster et al., 1988; Mikami et al., 1988; Perera and Follett, 1992; van Gils et al., 1993; Teruyama and Beck, 2000), European starling (Dawson et al., 1985; Foster et al., 1987; Goldsmith et al., 1989), garden warbler (Bluhm et al., 1991), great tit and ring dove (Silver et al., 1992), turkey (Millam et al., 1993), dark-eyed junco (Saldanha et al., 1994), house sparrow (Hahn and Ball, 1995), and canary (Bentley et al., 2004). IHC localization of cGnRH-I reveals three groups of immunoreactive (ir)-

cGnRH-I cells: (1) a telencephalic group medial to the lateral ventricles; (2) a basotelencephalic group located ventral to the tractus septomesencephalicua (TSM) and extending laterally and dorsocaudally; and (3) a distinctive group of cells located along the midline extending from the POA to septal regions (Foster et al., 1987; Millam et al., 1993; 1998; Teruyama and Beck, 2000). Furthermore, cGnRH-I-ir fibers are found to project into the ME from two difference sources. First, cGnRH-I-ir fiber bundles appear to originate in the preoptic and supraoptic regions, projecting along the wall of the ventricle and eventually entering the ME. The second fiber bundles originate dorsal to the preoptic region, and project along and terminate on the walls of the third ventricle. They form a neuronal network that extends throughout the infundibular regions before entering the ME (Foster et al., 1987). Specific GnRH-I-ir neurons are found in several hypothlalamic regions including the POA, POM AM, paraventricular nucleus (PVN), and nCPa. Additional scattered neurons are also found in the SL and around the OVLT. Kuenzel and Blahser (1991) reports six major groups of cGnRH-I perikarya including the olfactory bulb, olfactory tubercle, lobus parolfactorius, nucleus accumbens, septal preoptic hypothalamic region (three sub-nuclei), and lateral anterior thalamic nucleus. Several studies have reported the distributions of the cGnRH-I mRNA and its protein in the avian brains (Millam et al., 1989; Dunn and Sharp, 1999; Sun et al., 2001; Dawson et al., 2002; Kang et al., 2006). Utilizing the *in situ* hybridization indicates that cGnRH-I mRNA expressions are the greatest in the nCPa and around the OVLT. In addition, this study reports that cGnRH-I mRNAs abundance within the nCPa, OVLT, and SL are greater in laying than that of in non-photostimulated and incubating turkey hens (Kang et al., 2006).

The neuronal distribution of cGnRH-II differs from that of cGnRH-I. Studies

with turkey and Japanese quail reveal a large group of cGnRH-II ir-cells in the midbrain in the oculomotor region and basal lateral hypothalamus (Millam et al., 1993; 1998; Teruyama and Beck, 2000). cGnRH-II-ir fibers are prominent in the POA, LS, both medial and lateral to the TSM at the level of the POA and in limbic structures, olfactory areas, and forebrain (Millam et al., 1993). Although several evidences in Japanese quail, chicken (Mikami et al., 1988; Sharp et al., 1990), ostrich (Powell et al., 1987), and turkey (Millam et al., 1989) reveal that cGnRH-I and cGnRH-II are distributed differently in both brain and other tissues, the absent of GnRH-II-immunoreactivity have been observed in the ME of Japanese quail, chicken, and turkey (van Gils et al., 1993; Millam et al., 1998; D'Hondt et al., 2001; Clerens et al., 2003), suggesting that cGnRH-II does not directly promote pituitary gonadotropins secretion (Millam et al., 1993).

2.4.3 The Function of Gonadotropin Releasing Hormone in Mammals

It is well known that GnRH is the key neuropeptide controlling reproductive function in all vertebrate species. GnRH, produced in specific hypothalamic and preoptic cells, is secreted into the portal circulation to reach the anterior pituitary, where it acts by binding to specific receptors presented on the gonadotrops (Stojilkovic and Catt, 1995). GnRH release occurs episodically from the mammalian hypothalamus. The frequency and amplitude of GnRH release from hypothalamic neuronal cells is a critical and rate-limiting step for the control and maintenance of gonadotropins secretion from pituitary gonadotrops. It has been demonstrated that pulse of GnRH initiates pulsatile secretion of the FSH and LH from the anterior pituitary gland (Clarke and Cummings, 1982; Levine et al., 1985; Levine and Duffy,

1988; Moenter et al., 1991). In addition, changes in GnRH pulse frequency throughout the ovulatory cycle determine changes in the relative amounts of FSH and LH release (Wildt et al., 1981). GnRH is released in discrete pulses at intervals ranging from about 30 minutes to a few hours. High-frequency GnRH pulses (one pulse every 30 minutes) favores LH release, whereas low-frequency pulses (one pulse every 120 minutes min) favores FSH release (Wildt et al., 1981; Kaiser et al., 1997a). Both FSH and LH are released in a pulsatle manner into the systemic circulation, and in turn control the processes of gametogenesis and steroidogenesis (Conn and Crowley, 1994; Stojikovic and Catt, 1995).

The release of GnRH is strictly episodic in males and throughout the female reproductive cycle (for review, see Moenter et al., 2003). It is very well known that GnRH is the primary hypothalamic regulator of LH release in both spontaneous and induced ovulators. In spontaneously ovulating species such as rat, mouse, guinea pig, sheep, monkey, and woman, ovarian steroids secreted by mature ovarian follicles induce a pulsatile pattern of GnRH release in the ME that, in turn, stimulates a preovulatory LH surge. In the other hand, in induced ovulating species such as cat, and camel, the preovulatory release of GnRH and the resultal preovulatory LH surge, is induced by the receipt of genital somatosensory stimuli during mating (for review, see Bakker and Baum, 2000).

Although the hypothalamus and pituitary are the principal sources and targets'site for GnRH, several reports have suggested extra-hypothalamic origins of the GnRH as well as extra-pituitary presence of GnRH receptors across different type of tissues in the body such as ovay, placenta, endometrium, oviduct, testis, prostate gland, and mammary gland (for review, see Ramakrishnappa et al., 2005). This

expresses the direct effects of GnRH in an autocrine or paracrine manner eliciting a variety of responses depending on the type of target tissues and physiological conditions. In the ovary, GnRH has been shown to elicit a mixed effect of both inhibitory and stimulatory responses affecting ovarian function (for review, see Sharp, 1982; Janssens et al., 2000). In the male gonads, GnRH has been shown to cause a direct stimulatory effect on basal steriodogenesis and an inhibitory effect on gonadotropin-stimulate androgen biosynthesis (Hsueh and Jones, 1982). In adult male rats, GnRH agonist administration at a lower does for short-term duration is shown to stimulate testosterone secretion (Sharp et al., 1982), whereas the effect is opposite when the agonist is administered at higher dose or for long-term duration (Arimura et al., 1979; Hsueh and Erickson, 1979). It is also suggested that GnRH is involved in the process of fertilization. GnRH agonist has been shown to increase the cleavage rate of bovine oocyte *in vitro* (Funston and Seidel, 1995). In addition, it is suggested that GnRH expression may be regulated by PRL since GnRH mRNA has been found in the mammary gland of the pregnant and lactating rats (Palmon et al., 1994; Ikeda et al., 1995). Evidence suggests a role for GnRH in inducting follicular atresia as well as inducing apoptosis and program cell death in the ovary. However, it has been hypothesized that GnRH mRNA plays an autocrine or paracrine regulatory role in the growth of reproductive tissue tumors (ovarian carcinomas and endometrial carcinomars (for review see Ramakrishnappa et al., 2005).

2.4.4 The Function of Gonadotropin Releasing Hormone in Birds

GnRH plays a pivotal role in the control of avian reproduction. All the available evidences suggest that only GnRH-I has a physiological role in regulating of

gonadotropins secretion (Sharp et al., 1990). In sexually active birds, GnRH is synthesized by neurosecretory cells in the hypothalamus, released from the ME into the hypophysial portal vessels, and transported to the pituitary gland, where it stimulates the secretion of FSH and LH. A pulsatile pattern of GnRH release is observed from the medial basal hypothalamus and preoptic area *in vitro* (Li et al., 1994). Fully processed cGnRH-I mRNA and a variant transcript with a retained intron 1 are observed in the POA, basal hypothalamus, anterior pituitary gland, and testis of cockerel (Sun et al., 2001).

It is very well documented that avian gonadotropins secretion is influenced by GnRH. This decaneuropeptide increases FSH and LH secretion of the adenohypophysis both *in vitro* and *in vivo* (Peczely, 1989). Injection of GnRH increases plasma LH levels in white-crowned sparrow and European starling (Wingfield et al., 1979; McNaughton et al., 1995). Incubation of turkey anterior pituitary cells with GnRH results in an increase in LH-β-subunit gene expression and stimulates LH secretion (You et al., 1995a). Ovarian development is found to be correlated with increasing in circulating LH and the amount of GnRH, indicating that the expression of GnRH gene is important to maintain pituitary-ovarian function in chicken (Dunn et al., 1996). In addition, GnRH agonists may imitate the native hormone and induce an endogenous LH surge (Shalev and Leung, 2003). In contrast, GnRH inhibits FSH-stimulated steroidogenesis in chicken but enhances LH-stimulated progesterone production (Hertelendy et al., 1982). GnRH did not affect circulating FSH concentrations but stimulated LH secretion when administrated to 3 weeks old cockerels (Krishnan et al., 1993). These evidences suggest that only cGnRH-I has a physiological role in regulating of gonadotropins secretion (Sharp et

al., 1990), and cGnRH-II may not be involved in releasing avian pituitary gonadotropins. This hypothesis is confirmed by passive immunization with anti-cGnRH I, but not anti-cGnRH-II, disrupts ovulation in chicken (Sharp et al., 1990). In addition, the absent of cGnRH-II-immunoreactivity have been observed in the ME of Japanese quail, chicken, and turkey (van Gils et al., 1993; Millam et al., 1998; D'Hondt et al., 2001; Clerens et al., 2003), supporting that cGnRH-II does not directly promote pituitary gonadotropins secretion (Millam et al., 1993).

The physiological function of cGnRH-II is not clearly understood in birds. Studies showed that cGnRH-I and also cGnRH-II can stimulate the release of FSH and LH differentially from *in vitro* chicken pituitary (Millar et al., 1986). Injection of cGnRH-I or cGnRH-II stimulates an increase in plasma LH levels (Guemene and Williams, 1999). There is evidence suggests that cGnRH-II stimulates sexual behavior in avian females (Maney et al., 1997). It is hypothesized that cGnRH-II might have a role in related with courtship behavior in birds since ICV infusion of cGnRH-II enhances courtship behavior in female white-crowned sparrow, but that of cGnRH-I does not show the same effect (Maney et al., 1997). Of interest is that an antibody to lamprey GnRH-III has been shown to immunostain neurons in the PVN of the rat brain. This particular form of GnRH is also effective in releasing FSH in rat (Yu et al., 1991). It has been reported that a possible third form of GnRH has been found in the hypothalamic and extra-hypothalamic brain tissues of ostrich (Powell et al., 1987). However, the function of this form of GnRH in birds is unknown.

The role of GnRH in regulating gonadal maturation has been reported since the close association of neuropeptide Y (NPY; an important neuromodulator affecting gonadal function in mammals and birds) and GnRH perikaya in the nCPa and the

infundibular nucleus have been investigated (for review, see Kuenzel, 2000). This is supported by the results that NPY neurons migrate into the avian brain from the olfactory placode, similar to GnRH neurons (Hilal et al., 1996). In turkey hen, activation of DAergic neurons in the ML is associated with the activation of GnRH-I and VIP neurons and the release of LH and PRL (Al-Zailaie et al., 2006). Further evidence for involvement of DA in correlating with GnRH is derived from dense concentration of TH and GnRH-containing neurons are located in the lateral and mediobasal portion of the external layer of the ME (Contijoch et al., 1992). This result provides an opportunity for synaptic interaction between GnRH and DA.

2.4.5 The Regulation of Gonadotropin Releasing Hormone Secretion

The changes in GnRH content and its secretion can be correlated with internal (such as reproductive condition) and external factores (such as photoperiod). The effects of reproductive condition on GnRH secretion in avian species are well documented. Several evidences reveal that birds at the peak level of reproductive activity have more GnRH-ir cells and fibers when compared with sexually inactive or photorefractory birds (Hahn and Ball, 1995; Parry et al., 1997; Cho et al., 1998; Sharp et al., 1990). GnRH-I levels decrease when birds enter the incubating stage and this decrease in GnRH-I release is thought to be implemented by the inhibitory effect of PRL (Sharp et al., 1988). GnRH contents also change during the reproductive cycle. GnRH-I concentrations is significantly elevated in the POA of the hypothalamus during incubation (Millam et al., 1995). Measurements of GnRH peptide in the hypothalamus during the reproductive cycle of the turkey (Millam et al., 1989; El Halawani et al., 1993; Rozenboim et al., 1993a) and chicken (Dunn et al., 1996)

indicate that the peptide evels do not change. However, the amount of hypothalamic GnRH mRNA in incubating hens is lower than that of in laying hens (Dunn et al., 1996). Removal of incubating hens from their nests, which results an increase in LH secretion, is associated with an increase in the amount of GnRH mRNA in the hypothalamus (Dunn et al., 1996). In addition, GnRH contents of discrete medial preoptic, infundibulum, and arcuate samples are higher in laying hens than that of non-laying hens (Advis et al., 1985). Recently, GnRH mRNA expression is determined, utilizing *in situ* hybridization histochemistry, during the four different reproductive stages of the female turkey. GnRH mRNA abundance within the SL, OVLT, and nCPa areas is high in laying hens, with decreasing abundance found in non-photostimulated and incubating hens, respectively. The lowest levels of GnRH mRNA are observed in photorefractory hens. These results indicate that hypothalamic GnRH mRNA expression may be used to precisely characterize the different reproductive stages in birds (Kang et al., 2006).

The amount of hypothalamic GnRH decreases during photorefractoriness in starings (Dawson et al., 1985; Foster et al., 1987; Dunn et al., 1996), garden warblers (Bluhm et al., 1991), house sparrows (Hahn and Ball, 1995), dark-eyed juncos (Saldanha et al., 1994), and turkeys (Rozenbiom et al., 1993a; Kang et al., 2006). This decrease in hypothalamic GnRH occurs at a time when the amount of hypothalamic VIP is high (Mauro et al., 1992; Rozenboim et al., 1993a; Saldanha et al., 1994). Gonadal regression which is associated with decreased FSH and LH secretion through photorefractoriness is related to a decrease in hypothalamic GnRH-I (Dawson et al., 2001; 2002, Hua, 2001). An IHC study in starling (Parry et al., 1997) shows that the reduction in hypothalamic GnRH in photorefractory birds is associated with a

reduction in GnRH precursor and proGnRH-GTPase activating protein (GAP). This observation suggests that the development of photorefractoriness is promoted by the inhibition of GnRH synthesis rather than requiring inhibition of GnRH release from the ME.

Photoperiodic cue appears to be important for the onset of reproduction for most birds in temperate zones. Once environmental stimuli are transduced by the appropriate receptors, they influence the secretion of GnRH located in neurons of the preoptic and hypothalamic regions in birds and mammals. There are growing evidences that photoperiod is associated with the GnRH system. The stimulatory effect of long days appears usually to be associated with an increased GnRH contents in hypothalamus or increased immunoreactivity for GnRH in the hypothalamus and ME in avian species (Dawson et al., 1985; Foster et al., 1987; Foster et al., 1988; Goldsmith et al., 1989; Perera and Follet, 1992; Saldanha et al., 1994; Hahn and Ball, 1995). Photostimulatory inputs to GnRH neurones have the potential to increase GnRH mRNA transcription and GnRH release (Dunn and Sharp, 1999), and pituitary sensitivity to GnRH in birds (Davies and Follett, 1975). Time course analysis of changes in basal hypothalamic GnRH contents during photostimulation in the male starling provides the explanation for the photo-induced gonadal cycle (Dawson et al., 2002). In addition, it has been found that a 30 minutes light pulse provided 14 hours after the onset of light is shown to induce GnRH mRNA expression in the nCPa of reproductive quiescent turkeys maintained under a short day lighting regimen (Kang et al., 2006). Consistency with this finding, the nCPa respond to the photoperiod and a diet supplemented with sulfamethazine (SMZ), a compound that augments the effect of long days photostimulation, with a significant increase in number of GnRH cells

compared with birds fed control diets and exposed to a short days photoperiod (Kuenzel and Golden, 2006). Taken together, these findings support the role of photoperiod in correlated with GnRH to regulate the reproductive system.

It is well established that adrenergic stimulation at the hypothalamic level can release GnRH and thereby increase gonadotropins secretion (Yu et al., 1991). The roles for DA in controlling of GnRH release and LH secretion have been reported. Some other evidences suggest an inhibitory role for DA on GnRH release in mammals as well as in birds (Ramirez et al., 1984, Sharp et al., 1984). Further evidence provides an opportunity for synaptic interaction between GnRH and DA. Axons of GnRH neuronal perikaya are terminated in the external layer of the ME, which is closed proximity to terminals of TIDA neurons (Ajika, 1979; Merchenthaler et al., 1980; Ugrumov et al., 1989). However, little is known regarding the GnRH cell group(s) that project to the ME (Dawson and Goldsmith, 1997; Teruyama and Beck, 2000). These DA neurons have been implicated in mediating hypeprolactinemia-induced suppression of LH secretion (Selmanoff, 1981). DA axons 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), it is reasonable to consider whether any regional specificity exists in those DA neurons that is neuroendocrine in nature, for example, controlling the release and expression of VIP/PRL and GnRH/LH-FSH systems.

2.5 Gonadotropin Inhibiting Hormone: Structure and Function

2.5.1 The Discovery and Structure of Gonadotropin Inhibiting Hormone

It is very well established for some times that the neuropeptide control of

gonadotropins secretion at the pituitary level is primarily through the stimulatory control of the hypothalamic decapeptide, GnRH. To date, however, it has been found that the hypothalamus contains a novel hypothalamic dodecapeptide, gonadotropin inhibiting hormone (GnIH), and inhibits gonadotropins release. This is the first demonstration of a hypothalamic neuropeptide inhibiting gonadotropins release in any vertebrates. GnIH is discovered and first isolated from the brain of the Japanese quail by Tsutsui and his co-workers (2000). This isolated peptide is a dodecapeptide containing a C-terminal -Arg-Phe-NH_2 sequence (RFamide) and shown to have the sequence Ser-Ile-Lsy-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH_2 (Tsutsui et al., 2000). Subsequently, this peptide is shown to be located in the Japanese quail hypothalamo-hypophyseal system and to decrease gonadotropins release, but not PRL release from cultured anterior pituitary cells in a dose-dependent manner, therefore, it is termed as GnIH (Tsutsui et al., 2000).

The cDNA sequence encoding GnIH has been cloned in Japanese quail (Satake et al., 2001), domestic chicken (NCBI accession number AB120325), and white-crowned sparrow (Osugi et al., 2004). Similar peptides are also presented in amphibians, fish, and mammals (including humans, based on a gene database; Bentley et al., 2006). The predicted amino acid sequence of chicken GnIH differs from Japanese quail GnIH at position 3, where arginine conservatively substitutes lysine. The sequence of white-crowned sparrow GnIH differs from Japanese quail GnIH at position 5, 6, and 7 (Osugi et al., 2004) and the homology is approximately 66%. Furthermore, the GnIH precursor polypeptide is cleaved into three separate mature peptides in birds (GnIH-related peptide 1 or -PR-1, and GnIH-RP-2) and possibly two peptides in mammals (RFamide-related peptides 1 and 3; Bentley et al., 2006).

A novel GnIH receptor has been identified for the first time in Japanese quail (Yin et al., 2005). GnIH receptor is a member of G protein-coupled receptor and specifically binds to GnIH in a concentration-dependent manner. Southern blotting analysis of reverse-transcriptase-mediated PCR products reveals the expression of GnIH receptor mRNA in the pituitary gland and several brain regions including the hypothalamus in quail (Yin et al., 2005). Because of GnIH receptor is expressed in the hypothalamus, it is possible that GnIH could be acting at the level of hypothalamus via GnIH receptors. Furthermore, other brain regions, i.e., cerebrum, mesencephalon, and spinal cord, also contain GnIH receptor mRNA, suggesting multiple regulatory functions of GnIH in the avian brain (Yin et al., 2005; Bentley et al., 2006).

2.5.2 The Localization of Gonadotropin Inhibiting Hormone in the Brain

Localization of GnIH in the brain is essential to understand its physiological functions. GnIH localization in the brain of the Japanese quail has been reported utilizing immunohistochemistry (Tsutsui et al., 2000; Ubuka et al., 2003; Ukena et al., 2003). Furthermore, the distributions of GnIH have been investigated in several avian species such as song sparrows, house sparrows, Gambel's white-crowned sparrows (Bentley et al., 2003; Osugi et al., 2004), cardueline finches, and tropical sparrows (Bentley et al., 2006). Clusters of dense GnIH-ir cell bodies are found in the PVN regardless of sex and species. GnIH-ir cells in the PVN are found to be parvocellular neurons with bipolar or tripolar in the ventral potion of PVN, which show no imunoreaction with the antisera against vasotocin and mesotocin (Ukena et al., 2003). *In situ* hybridization study confirms the cellular localization of GnIH mRNA in the PVN of Japanese quail and sparrow (Ukena et al., 2003; Osugi et al., 2004). In

addition to PVN, some scattered small GnIH immunoreactive cells are located in the septal area. In contrast to the highly localized cluster of cell bodies, GnIH-ir fibers are widely distributed in the diencephalic and mesencepahlic regions. Especially, dense networks of GnIH immunoreactive fibers are found in the ventral paleostriatum, septal area, POA, hypothalamus, and optic tectum. The most prominent fibers are seen in the ME and the dorsal motor nucleus of the vagus in the medulla oblongata.

The presence of GnIH-ir fibers in the ME supports a role for GnIH in pituitary gonadotropins regulation. The distribution of GnIH-ir fibers found outside the hypothalamic area suggests the role of GnIH in participating not only in neuroendocrine functions, but also in behavioral and autonomic mechanisms (Ukena et al., 2003). It has been reported that the presence of GnIH in the PVN appears to be a conserved property among several avian species (for review, see Tsutsui et al., 2005; 2007). It has been found that GnIH fibers are observed in extremely close proximity to GnRH neurons in the POA in birds (Bentley et al., 2003). Taken together with the previous findings that GnIH fibers extend to terminals in the ME suggests that GnIH may influence the GnRH system at the neuron and fiber terminal levels. It is therefore possible that GnIH acts at the level of hypothalamus to regulate gonadotropins release, as well as at the pituitary level (Kriegsfeld et al., 2006).

Up to date, the presence of GnIH has been investigated in mammals, particularly in rodent species such as Syrian hamsters, rats, and mice (Ukena and Tsutsui, 2001; Kriegsfeld et al., 2006). GnIH cell bodies are found occupiedly a location slightly caudal to that found in birds, being confined to the rostral-caudal extent of the dorsomedial hypothalamus. GnIH fibers form an extensive network extending along a midventral and dorsal continuum from the tenia tectum to the

hindbrain. Unlike results seen in avian species, GnIH fibers found in mammals are not detected in the external layer of the ME, but are presented in the internal layer, suggesting that GnIH might not regulate gonadotropins via the traditional pathway of pituitary as it likely does in birds (Bentley et al., 2006).

2.5.3 The Function of Gonadotropin Inhibiting Hormone

As mentioned previously, GnIH is discovered and named because it inhibits gonadotropins release. Studies indicate that GnIH is a regulator of gonadotropins release *in vitro* and *in vivo*. Data collected *in vitro* indicate a direct effect of GnIH on pituitary release of LH in Japanese quail. Incubation of this peptide with anterior pituitary cells decreases plasma LH and FSH mRNA levels in does-dependent manner, but does not change plasma PRL levels (Tsutsui et al., 2000). GnIH is also effective in inhibiting circulating LH *in vivo*. Intra-peritoneal administration of GnIH into Japanese quail via osmotic pump results in significantly reducing in plasma LH levels (Ubuka et al., 2006). GnIH injected simultaneously with GnRH inhibits the surge of plasma LH above the baseline in song sparrows (Osugi et al., 2004). GnIH injections also decrease plasma levels of LH in breeding free living Gamble's white-crowned sparrows (Osugi et al., 2004). Furthermore, administration of GnIH via ICV infusion to the third ventricle causes a rapid decrease of plasma LH levels in photostimulated female white-crowned sparrows (Benley et al., 2007). In addition, it has been found that GnIH also inhibits gonadotropins common α- and β-subunits production, as well as release (Ciccone et al., 2004; Tsutsui et al., 2006a; 2006b; Ubuka et al., 2006). The effects of GnIH upon plasma LH levels are found to be similar in Syrian hamsters (Kriegsfeld et al., 2006), providing evidence for properties

of the physiological action of GnIH.

The distribution of GnIH in the several highly photoperiodic songbird species such as sparrows suggests that GnIH might play a pivotal role in the termination of breeding season in these species (Bentley et al., 2006). GnIH mRNA expression is found to be greater in reproductively inactive and incubating hens than that of in laying hens. However, administration of GnIH into nest-deprived incubating hens fails to depress plasma LH levels (Ciccone et al., 2004). It has been found that the expression of GnIH is photoperiodically controlled and increased under short day photoperiods (Ubuka et al., 2005), when the nocturnal duration of melatonin secretion increases (Cockrem and Follet, 1985). Mel_{1c}, Melatonin receptor subtype, is expressed in GnIH-ir neurons in the PVN (Ubuka et al., 2005). These findings raise the suggestion that melatonin may act directly upon GnIH neurons via its receptor to induce GnIH expression. In mature birds, chronic treatment with GnIH via osmotic pumps decreases plasma testosterone concentrations, as well as gonadotropins synthesis and release in a does-dependent manner (Ubuka et al., 2006). However, in immature birds, choronic treatment with GnIH suppresses normal testicular growth and plasma testosterone concentrations (Ubuka et al., 2006). These results show that GnIH inhibits gonadal development and maintenance by inhibiting gonadotropins synthesis and release. Based on these studies, GnIH is likely an important neuropeptide for the regulation of avian reproduction.

The presence of GnIH recptor mRNA in extra-hypothalamic regions (i.e., cerebrum, mesencephalon, spinal cord) suggests multiple regulatory functions of GnIH in the avian brain (Yin et al., 2005; Bentley et al., 2006). Recent studies have shown that GnIH stimulates feeding behavior in chicks (Tachibana et al., 2005) and

inhibits female sexual behavior in white-crowned sparrows (Bentley et al., 2006). The physiological evidence to support a role of GnIH in the regulation of reproduction in birds and the role of neurotransmitters regulating GnIH secretion is inconclusive and requires further investigatd.

2.6 Vasoactive Intestinal Peptide: Structure, Function, and Regulation of PRL Secretion

2.6.1 The Structure of Vasoactive Intestinal Peptide

VIP is a highly conserved peptide that consists of 28 amino acids. VIP is first isolated from the porcine duodenum (Said and Mutt, 1970). It is know that VIP is extensively distributed not only in the gastrointestinal tract, where it is first identified, but also in the central and peripheral nervous systems (Mutt and Said, 1974; Larsson et al., 1976; Said and Rosenberg, 1976; Giachetti et al., 1977; Rosselin et al., 1982), especially in the hypothalamus (Emson et al., 1979; Samson et al., 1979; Ceccatelli et al., 1991). It is considered to be a neurotransmitter and/or neuromodulator (Larsson et al., 1976; Marley and Emson, 1982). VIP is a neuroeptide of the VIP/glucagon/secretin family of peptides which includes secretin, glucagon, gastric inhibitory peptide (GIP), GH releasing factor, PHI, and PACAP. VIP exerts it biological effects by binding to high affinity receptors that are coupled to G proteins, whose actions are mediated to adenylate cyclase and the production of cAMP (Hokfelt et al., 1980; Couvineau et al., 1990; Lutz et al., 1995). The peptides of the VIP family are probably the results of exon duplication coupled to gene duplication. VIP gene contains 7 exons, each encoding a distinct functional domain in the final mRNA and protein. VIP and the related peptide histidine methionine (PHM) or PHI are encoded

by two adjacent exons in the genome, which exon 4 codes for peptide PHM, and exon 5 codes for VIP (Bodner et al., 1985; Yamagami et al., 1988; Giladi et al., 1990). Mammalians VIP have been cloned (Itoh et al., 1983; Nishizawa et al., 1985). The open reading frame is comprised of 165 amino acids. Both chicken and turkey VIP cDNAs have been cloned (McFarlin et al., 1995; Talbot et al., 1995; You et al., 1995b) and show a structure similar to that of the mammalian VIP gene. Chicken and turkey VIP share complete amino acid homology and are 98% homologus at the nucleotide level (You et al., 1995b). Chicken VIP is different from mammalian VIP in its amino acid sequence at position 11, 13, 26, and 28, although the number of amino acid residue is the same (Nilsson, 1975). Amino acid sequence of VIP and the member in VIP/glucagon/secretin family are presented in Fig. 2.6

	1 5 10 15 20 25
p/b VIP	HSDAVFTDNYTRLRKQMAVKKYLNSILN[a]
c VIP	HSDAVFTDNYSRFRKQMAVKKYLNSVLT[a]
pPHI	HADGVFTDDFSRLLGQLSAKKYLESLI[a]
pSECRETIN	HSDGTFTSELSRLRDSARLQRLLQGLV[a]
m GLUCAGON	HSQGTFTSDYSKYLDSRRQDFVQWLMNT
pGIP	YAEGTFISDYSIAMDKIRQQDFVNWLLA...

Fig.2.6 The amino acid sequences of VIP, PHI, secretin, glucagon, and GIP.
a: the C-terminal amino acid is in the amide form. p: porcine, b: bovine, c: chicken, m: mammalian (Rosselin et al., 1982).

Mammalian VIP receptors have been cloned and functionally characterized (Sreedharan et al., 1991; 1993; Ishihara et al., 1992; Lutz et al., 1993; Couvineau et al., 1994; Gagnon et al., 1994; Usdin et al., 1994). There are two VIP receptor subtypes (VIP1 and VIP2) and expressed in a tissue specific manner (Couvineau et al.,

1994; Usdin et al., 1994; Sherward et al., 1995). These receptors bind both VIP and PACAP. Both receptors are members of the G protein-coupled family, which biological actions are mediated via interaction with specific receptors that are coupled to adenylate cyclase and the production of cAMP (Gourdji et al., 1979; Bjoro et al., 1987; Couvineau et al., 1990; Lutz et al., 1995). VIP receptor is existed in lung, liver, small intestine, and several regions of the brain (e.g. cerebral cortex and hippocampus; Besson et al., 1986; Martin et al., 1987; Csillag et al., 1993; Usdin et al., 1994; Sherward et al., 1995). It has been suggested that a single VIP receptor is expressed and functions in non-mammalian species (Kansaku et al., 2001). VIP receptors are presented on the surface membranes of the anterior pituitary (Rozenboim et al., 1993b; Gonzales et al., 1994a; 1994b), hypothalamus (Gonzales et al., 1995), small intestine, and granulosa cells (Kawashima et al., 1995) in birds. Avian VIP receptors have been cloned and characterized in chicken (Kansaku et al., 2001) and turkey (You et al., 2001). It has been indicated that the circulating PRL variations observed across the turkey reproductive cycle are, in part, regulated by changes in VIP receptors at the pituitary level (Chaiseha et al., 2004).

2.6.2 The Function of Vasoactive Intestinal Peptide

The widespread distributions of VIP throughout the CNS and pheripheral organs, suggest the involvements of VIP in a wide variety of biological functions. VIP has been found to be distributed in the central and peripheral nervous systems (Larsson et al., 1976; Said and Rosenberg, 1976; Giachetti et al., 1977; Hokfelt et al., 1982; Rosselin et al., 1982). Furthermore, significant concentrations of VIP are detected in the gastrointestinal tract, heart, lung, thyroid gland, kidney, immune

system, urinary bladder, and genital organs. In mammals, VIP, initially considered to be a gastrointestinal hormone (Grossman, 1974), plays a roles in smooth muscle relaxation (lower esophageal sphincter, stomach, and gallbladder), stimulation the secretion of water into pancreatic juice and bile, inhibition of gastric acid secretion and absorption from the intestinal lumen, cell proliferation, and increased of gastric motility (for reviews, see Gozes et al., 1999; Gozes and Furman, 2003). VIP is believed to be presented in endocrine cells of mammalian and avian species (Polak et al., 1974). Several diverse physiological functions of VIP have been reported including inducing vasodilation (Bakken et al., 1995), increasing blood flow and exocrine gland secretion (Shimizu and Taira, 1979; Bloom and Edwards, 1980; Heistad et al., 1980; Andersson et al., 1982), energy metabolism, especially the enzymatic breakdown of glycogen to glucose (Magistretti et al., 1981), stimulation of thyroid hormone secretion (Ahren et al., 1980), and bone resorption (Hohmann et al., 1983). In addition, VIP exerts a broad spectrum of immunological functions controlling the homeostasis of the immune system such as immuno-suppression and antiinflammatory (Gomariz et al., 2001).

High concentrations of VIP have been detected in the hypothalamus (Emson et al., 1979; Samson et al., 1979; Ceccatelli et al., 1991). VIP is also found in the cerebral cortex, hippocampus, corpus striatum, and vagal centers of the medulla oblongata (Gozes et al., 1999). In the brain, VIP acts as a neurotransmitter and/or neuromodulator (Said and Rosenberg, 1976) to promote neuronal survival, induce neuronal differentiation, modulate neurotransmitter synthesis, and influence neuronal excitability (Klimaschewski, 1997). The discovery of a large population of hypothalamic VIP-immunoreactive neurons, whose axons project to the ME (Samson

et al., 1978; 1979; Polak and Bloom, 1982; Dalcik and Phelps, 1993) and a high concentration of VIP in hypophyseal portal blood (Said and Porter, 1979; Shimatsu et al., 1981; Brar et al., 1985; Mutt, 1988) suggest that VIP participates in the regulation of anterior pituitary functions. Several studies indicate that VIP regulates the release of anterior pituitary hormones such as PRL (Kato et al., 1978; Rotsztejn et al., 1980; Frawley and Neill, 1981), GH (Chihara et al., 1982), and adrenocorticotropic hormone (ACTH; Oliva et al., 1982; White et al., 1982). VIP also regulates neuroendocrine inhibition of LH (Stobie and Weick, 1989), stimulates oxytocin and vasopressin release from the neurohypophysis (Ottesen et al., 1984), stimulates male sexual behaviors (Gozes et al., 1989), and maintains the neuronal survival (Brenneman and Eiden, 1986). Moreover, it is reported that VIP is a modulator or metabolic component of circadian rhythms (Moore, 1983; Yuwiler, 1983; Card and Moore, 1984). In addition, VIP has also been shown to act comparably to acetylcholine for increasing secretion from salt glands (Gerstberger, 1988).

In mammals, several studies demonstrate the involvements of VIP in the regulation of PRL secretion from the pituitary (Kato et al., 1978; Rotsztejn et al., 1980; Reichlin, 1988). VIP stimulates PRL release *in vivo* (Kato et al., 1978; Frawley and Neill, 1981) and *in vitro* (Samson et al., 1980; Matsushita et al., 1983). Administration of VIP anti-serum inhibits PRL release induced by stress, serotonin, or suckling (Shimatsu et al., 1984; Abe et al., 1985; Ohta et al., 1985). VIP also regulates the amount of pituitary PRL mRNA and its protein (Ben-Jonathan et al., 1989; Maas et al., 1991). Hypothalamic VIP mRNA is increased during lactation in rats (Gozes and Shani, 1986). VIP receptors have been identified in the pituitary (Gourdji et al., 1979; Bjoro et al., 1987). VIP also promotes the entry of extracellular calcium ions

into the PRL-secreting pituitary cells of rat and human (Bjoro et al., 1987; Prysor-Jones et al., 1987). All of these data point VIP as the PRF in mammals.

2.6.3 Vasoactive Intestinal Peptide as the Avian PRF

Several lines of evidence support VIP as the most important PRF in birds (Macnamee et al., 1986; Opel and Proudman, 1988b; Mauro et al., 1989; El Halawani et al., 1990b; 1990c; 1997; Talbot et al., 1991). VIP meets many of the qualifications of the PRF: 1) it stimulates the *in vitro* release of PRL (the response closely correlates to the reproductive stage of the animal; 2) it stimulates the *in vivo* release of PRL from the ME of deafferentated hens; 3) high concentrations of VIP have been found in the ME, particularly in the external layer; 4) high affinity VIP receptors have been identified on anterior pituitary cells; and 5) the alteration of pituitary function, due to an antagonism of the candidate substances. Further evidence that VIP is the PRF has been provided by the findings that immunoneutralization of endogenous VIP reduces levels of both circulating PRL and pituitary PRL mRNA, and totally blocks the PRL release affected by electrical stimulation of the medial preoptic nucleus (El Halawani et al., 1990c; Youngren et al., 1994) and blocks the hormonal and behavioral characteristics of incubating hens (El Halawani et al., 1995).

Several reports have been published regarding the location of VIP perikarya and fibers within specialized brain regions of birds (Yamada et al., 1982; Mikami and Yamada, 1984; Korf and Fahrenkrug, 1984; Mikami, 1986; Macnamee et al., 1986; Peczely and Kiss, 1988; Silver et al., 1988; Mauro et al., 1989; Norgren and Silver, 1990; Hof et al., 1991; Kuenzel and Blahser, 1994; Saldanha et al., 1994; Bottjer and Alexander, 1995; Chaiseha and El Halawani, 1999). A subset of VIP-ir neurons within

the medial basal hypothalamus and septal region of the dove brain has been proposed to be encephalic photoreceptors (Silver et al., 1988).

It is very well established that avian pituitary PRL secretion is tonically stimulated by VIP, which is secreted from neurons located in the INF of the caudo-medial hypothalamus (El Halawani et al., 1997). When VIP cell bodies in the INF are lesioned, an increase in PRL normally associated with the photo-induced reproductive cycle is eliminated, and elevates levels of PRL associated with incubation behavior are suppressed (Youngren et al., 1989). These results are in accordance with the findings that variations in VIP immunoreactivity and VIP contents in the INF and ME, VIP mRNA steady-state levels in the INF (Mauro et al., 1989; Chaiseha and El Halawani, 1999), and VIP concentrations in turkey hypophyseal portal blood (Youngren et al., 1996a) are related with the levels of PRL secretion during the avian reproductive cycle. Changes in pituitary VIP receptor mRNA is observed across the reproductive stages in turkeys. Increased VIP receptor mRNA in the pituitary is observed in laying and incubating (hyperprolactinemia) hens, while much less VIP receptor mRNA is observed in the pituitary of hypoprolactinemic non-photostimulated and photorefractory turkey hens. This suggests that VIP receptors located in the INF may be involved in avian PRL secretion, and indicates that PRL secretion is principally regulated by VIP receptors at the pituitary level (Chaiseha et al., 2004).

VIP plays a prominent role in the regulation of PRL secretion. It is suggested that PRL secretion is mediated through the changes in VIP secretion and/or gene expression. VIP regulates PRL gene expression by both enhancing the transcription rate of PRL and up-regulating PRL mRNA stability (Tong et al., 1998). Variations in hypothalamic VIP immunoreactivity, VIP contents, VIP mRNA steady-state levels,

VIP mRNA expression in the INF, VIP receptor mRNA in the pituitary, and VIP concentrations in hypophyseal portal blood are correlated with changes in circulating PRL levels in turkey (Mauro et al., 1989; Youngren et al., 1996a; Chaiseha and El Halawani, 1999; Chaiseha et al., 1998; 2004), chicken (Sharp et al., 1989a), and dove (Cloues et al., 1990). Recently, changes in VIP-ir neurons in the INF area have been reported and directly correlated with plasma PRL levels across the reproductive cycle of native Thai chicken (Kosonsiriluk et al., 2006; Kosonsiriluk, 2007).

It has been suggested that VIP mediates the effects of photoperiod on PRL secretion in the turkey (El Halawani et al., 1996). Hypothalamic VIP immunoreactivity within the INF increases after photostimulation, reaching the highest levels in incubating hens. The only exception to this correlation between hypothalamic VIP and circulating PRL is found in photorefractory hens, where plasma PRL levels are extremely low in the presence of high hypothalamic VIP levels (Mauro et al., 1989). VIP contents in the ME, hypothalamic cytoplasmic VIP mRNA steady-state levels, and hypothalamic nascent VIP mRNA levels are all increased and correlated with increased PRL secretion following photostimulation (Chaiseha et al., 1998). In addition, VIP peptide and its mRNA levels in the INF increase following exposure to long days and remain elevated as long as such exposure continues, declining only when the bird is subjected to short days (Mauro et al., 1989; El Halawani et al., 1997; Chaiseha and El Halawani, 1999). These results lends support to a hypothetical scheme for photoperiodic regulation of PRL in which VIP serves as the PRF that is intimately linked to photoperiodic mechanisms. However, it remains to be established how photoperiodic information is transduced to VIP-ir neurons located in the INF of the hypothalamus (Mauro et al., 1989).

2.7 Dopamine: Structure, Localization, Function, and Regulation of PRL Secretion

2.7.1 The Structure of Dopamine

DA, found in both central and peripheral nervous systems of both vertebrates and invertebrates, is discovered by Carlsson and Hillarp (1952) at the Laboratory for Chemical Pharmacology of the National Heart Institute of Sweden. It is named DA because it is a monoamine, and its synthetic precursor is 3,4-dihydroxyphenylalanine (L-DOPA). Carlsson is awarded the 2000 Nobel Prize in Physiology or Medicine for showing that DA is not only just a precursor of NE and adrenaline or epinephrine (E) but a neurotransmitter as well (for review, see Benes, 2001). The chemical formula of DA is $C_6H_3(OH)_2$-CH_2-CH_2-NH_2. Its chemical name is 4-(2-aminoethyl) benzene-1,2-diol. DA belongs to a group of catecholamines (CA) and functios as classical neurotransmitters in the brain, thus they communicate between neurons and act within the anatomically confined space of the synapse. It has several important physiological functions involved in a wide variety of behaviors and reproduction. As a member of the CA family, DA is a precursor to NE and then E in the biosynthetic pathways for these neurotransmitters. CA and indolamines (such as serotonin) are referred to as monoamine, which are water soluble molecules that are the decarboxylated derivatives of amino acids. The distinctive structural features of CA 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, 1973).

The precursor for DA synthesis is the aromatic amino acid tyrosine. The majority of circulating tyrosine originates from dietary sources, but small amounts are derived from hydroxylation of phenylalanine by phenylalanine hydroxylase from the

liver (for review, see Missale et al., 1998). Tyrosine enters neurons by an energy-dependent uptake process and is converted to DA by two enzymes that act in sequence, TH and l-aromatic amino acid decarboxylase (AADC), also called dihydroxyphenylalanine decarboxylase (DDC). TH is considered to be the rate-limiting enzyme in this biosynthetic pathway, which converts tyrosine into L-DOPA. Consequently, ADDC catalyzes L-DOPA and produces DA. In some neurons, DA is further processed into NE by DA beta-hydroxylase (DBH), and those that also contain phenylethanolamine *N*-methyl transferase (PNMT) converts NE to E. The DA biosynthetic pathway is presented in Fig. 2.7.

TH activity is the most critical factor and considered to be the rate-limiting enzyme that controls DA synthesis. TH gene is localized at chromosome 11p in human and encodes a single form of TH that can be alternatively spliced (Powell et al., 1984). Targeted disruption of the TH gene results in perinatal lethality, which can be rescued by L-DOPA administration (Kobayashi et al., 1995). The mature enzyme is a soluble cytosolic protein composed of four subunits of approximately 60 kDa each (for review, see Kumer and Vrana, 1996). Each monomer is comprised of an inhibitory regulatory domain at the N terminus and a catalytic domain at the C terminus. The regulatory domain contains four phosphorylation sites located within the first 40 amino acids: Ser8, Ser19, Ser31, and Ser40. The catalytic domain contains protein binding region and a putative leucine zipper at the C terminus that participates in intersubunit binding.

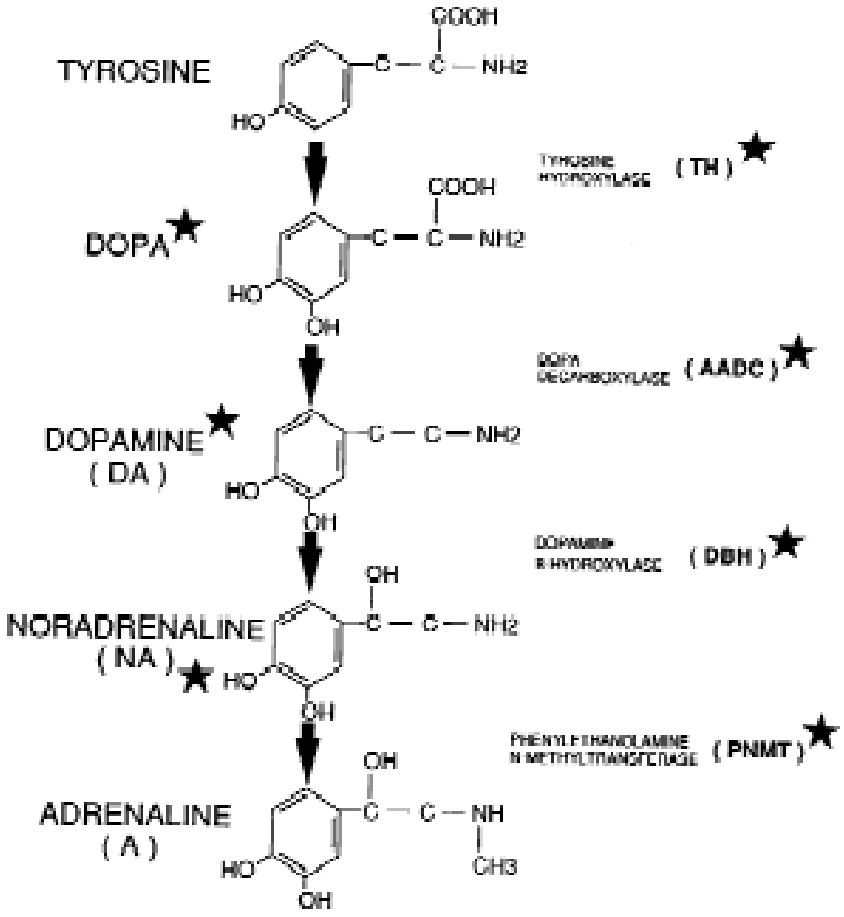

Fig. 2.7 Biosynthetic pathway of catecholamines and available antisera as indicated by asterisks (Smeets and Gonzalez, 2000).

DA exerts its biological actions by binding to the specific receptors, which belongs to the family of G protein-coupled receptors. Five distinct types of DA receptors have been isolated, characterized, and subdivided into two families based on the basis of their stimulatory or inhibitory activities on adenylate cyclase (Kebabian and Calne, 1979). The D_1-like DA subfamily comprises D_1 and D_5 DA receptors termed the D_{1A} and D_{1B} DA receptors by some investigators (Monsma et al., 1990; Sibley, 1991). The D_2-like DA receptor includes D_2, D_3, and D_4 DA receptors. Activation of D_1-like DA receptors family promotes adenylate cyclase activity, while

activation of D_2-like DA receptors family inhibits adenylate cyclase activity (Stoof and Kebabian, 1984). The D_1-like and D_2 like DA receptors are primarily associated with the $G_{s\alpha}$ and $G_{i\alpha}$ subunits, respectively. However, the G_o and G_q proteins, which are associated with ion channels and phosphoinositide metabolism, are also involved (Sidhu and Niznik, 2000). Characterization of cDNAs for all five receptor subtypes shows that D_1 and D_5 DA receptors share high homology in their transmembrane sequences; similarly, the transmembrane sequences of D_2, D_3, and D_4 DA receptors are conserved in the three receptor species (Missale et al., 1998).

The distributions of DA receptor subtypes have been well investigated in mammals utilizing isotope-labeled ligands autoradiography, *in situ* hybridization, and immonohistochemistry techniques. The five DA receptors have distinct localization within the brain and are expressed in a tissue-specific manner in the periphery. In general, D_1 and D_2 DA receptors are the most widespread and expressed at highest levels (Dearry et al., 1990; Fremeau, 1991; Missale et al., 1998; Vallone et al., 2000). D_1 DA receptor is mainly expressed in the caudate putamen, nucleus accumbens, olfactory tubercle, cerebral cortex, and amyygdala (Mansour et al., 1990, Jackson and Westlind-Danielsson, 1994). D_2 DA receptors mRNA is highly expressed in the substantia nigra (SN), ventral tegmental area (VTA), hippocampus, and in both anterior and intermediate lobes of the pituitary, whereas the amygdale contains low levels of D_2 DA mRNA (Meador-Woodruff et al., 1989; 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, where the DAergic neurons have been found, but it is expressed in a minority of DAergic neurons when compared with the D_2 DA receptor (Diaz et al., 1994; 1995). D_4 DA receptor appears to be highly expressed in the frontal cortex,

amygdale, hippocampus, hypothalamus, and mesencephalon (Van Tol et al., 1991; O'Malley et al., 1992). The distributions of poorly expressed D_5 DA receptor are restricted to the hippocampus, lateral mamillary nucleus, and parafascicular nucleus of the thalamus, where the D_1 DA receptor is not significantly expressed (Tiberi et al., 1991; Meador-Woodruff et al., 1992). In the peripheral tissues, 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).

Very few anatomical data concerning the localization of DA receptors are established in birds. Three D_1 DA receptor subtypes (D_{1A}, D_{1B}, D_{1D}) have been cloned in chicken (Demchyshyn et al., 1995). Cloning of cDNAs from turkey brain encoding D_1 and D_2 DA receptors has been reported (Schnell et al., 1999a; 1999b). The nucleotide sequence of the avian D_2 DA receptor demonstrates 75% homology to the known mammalian D_2 DA receptor. D_2 DA receptors are detected in the turkey pituitary, hypothalamus, pineal gland, cortex, and cerebellum (Schnell et al., 1999b). D_1-like DA receptors exist in the brain of the pigeon (Richfield et al., 1987; Dietl and Palacios, 1988), European starling (Casto and Ball, 1994), quail (Ball et al., 1995), chick (Schnabel et al., 1997; Sun and Reiner, 2000), and turkey (Schnell et al., 1999a; Chaiseha et al., 2003). D_2-like DA receptors have been mapped in the brain of pigeon (Richfield et al., 1987), quail (Levens et al., 2000), and turkey (Schnell et al., 1999a; 1999b; Chaiseha et al., 2003). The regional distribution of D_2 DA receptor mRNA has been found widespread throughout the brain, pineal gland, cortex, cerebellum, and also in the pituitary of the turkey. The presence of hypothalamic D_1 DA and pituitary D_2 DA receptor mRNA is found to increase in correlating with the reproductive stages, which D_1 DA receptor mRNA increases in hyperprolactinemic incubating and

D_2 DA receptor mRNA increases in hypoprolactinemic photorefractory turkey hens (Chaiseha et al., 2003). Moreover, significant increase in D_1 DA receptor mRNA during the reproductive cycle is shown in the pineal gland, suggesting that the activation of pinealocyte D_{1D} DA receptors may stimulate the release of melatonin in turkey (Schnell et al., 1999a). This receptor subtype has been classically described as being stimulatory (Bates et al., 1990; Civelli et al., 1991; Sibley and Monsma, 1992; Jarvie and Caron, 1993; Jaber et al., 1996; Strange, 1996), thus it is suggested that activation of D_1 DA receptors on pituitary lactotrophs could stimulate PRL secretion (Schnell et al., 1999a).

2.7.2 The Localization of Dopaminergic System in Mammals

DA is synthesized primarily in the CNS, but limited production also occurs in the adrenal medulla. DA is also detectable in a few non-neuronal tissues such as pancreas and anterior pituitary gland (for review, see Ben-Jonathan and Hnasko, 2001). The mammalian brain contains 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 by Dahlstrom and Fuxe (1964). The CA neuron in the brain is organized into 12 groups, labeled A1 to A12 from caudal to rostral utilizing the Falck-Hillarp histofluorescence technique (Dahlstrom and Fuxe, 1964). In the hypothalamus, these cells are located mainly in the arcuate and the anterior periventricular nuclei. Later, these CA cell groups are reanalyzed and additional CA cell groups are identified by using IHC methods with antibodies against the various biosynthetic enzymes such as TH, DBH, and PNMT by Hokfelt and colleagues (Hokfelt et al., 1984a; 1984b). Following that,

the CA systems in the brain of Murinae (rat, Fuxe et al., 1969; Lindvall and Bjorklund, 1974; Bjorklund and Lindvall, 1984; Chan-Palay et al., 1984; Van Den Pol et al., 1984; mouse, D'Este et al., 2007; grass rat, Mahoney et al., 2007; guinea pig, Mulders and Roberston, 2005) and other mammalian species have been reported. (cat, Cowchock et al., 1974; Cheung and Sladek, 1975; Luppi et al.,1986; Kitahama et al., 1987; squirrel monkey, Felten et al., 1974; opossum, Crutcher and Humbertson, 1978; rabbit, Blessing et al, 1981; highveld gerbil, Moon et al, 2007; sheep, Tillet and Thibault, 1989; cattle, Leshin et al., 1995; pig, Ruggiero et al., 1992; Leshin et al., 1996; human, Spencer et al., 1985; Tillet and Kitahama, 1998; giraffe, Badlangana, 2007; monkey, Thind and Goldsmith, 1986; Kohama et al., 1992)

According to the nomenclature of CA cell groups in the CNS of rats described by Hokfelt and colleagues, there are 17 DAergic/NEergic (A1-A17) and three adrenergic (C1-C3) cell groups in the brain of rats. Two distinct CA cell groups are recognized 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 (Dahlstrom and Fuxe, 1964). The C3 adrenergic group lies along the midline within and dorsal to the medial longitudinal fascicle. In the pons, NEergic cells are classified into four groups (A4, A5, A6, and A7). Of these cell groups, the A6 (locus coeruleus) is the most prominent one. The CA cells in the midbrain are classified into three groups, A8 (retrorubral), A9 (SN), and A10 (VTA), on the basis of their localizations. At least five different CA cell groups are recognized in the diencephalon of rats, labeled A11-A15. These cell groups constitute two rostrocaudally oriented columns on each side of the brain, i.e., dorsolateral and ventrolateral group. The numbers of DA-containing

neurons in the rat diencephalon 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) are located in the periventricular gray matter of thalamus, hypothalamus, and rostral midbrain. Axons from these neurons project to the spinal cord (Skagerberg and Lindvall, 1985), suggesting a role of these neurons in sensory and nociceptive processing, as well as sensorimotor integration (van Dijken et al., 1996; Levant and McCarson, 2001). The A12 (TIDA) neurons are distributed throughout the arcuate nucleus (ARC) and in the adjacent part of the periventricular nucleus of the mediobasl hypothalamus. There is sexual difference in the number of TH-ir neurons in the dorsomental and ventrolateral subdivision of the ARC (Cheung et al., 1997). This DA neuronal group is originally implicated in the regulation of pituitary hormone secretion based on the results of early receptor binding and pharmacological studies (Moore, 1987). The regulation of PRL secretion is under the inhibitory control of TIDA neurons in the hypothalamus (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001), which release DA that acts directly upon D_2 DA receptors located on pituitary lactotrophs (Civelli et al., 1991). The DA perikaya of incertohypothalamic DA neurons (A13) are clustered in the rostral portion of the medial zona incerta. The A14 DA neurons are located in the periventricular nucleus. The A15 can be divided into two distinct groups. The first one is a compact dorsal group (A15d) located in the ventral portion of the bed nucleus of the stria terminalis, and more caudally, ventral to the anterior commissure. Another group is A15 ventrolateral neurons (A15v) which is found above the optic chiasm and within the supraoptic nucleus. These neurons are prominent in the ventrolateral hypothalamus of seasonal breeding species such as sheep (Tillet and Thibault, 1989),

and are believed to mediate steroid hormones suppression of gonadotropins secretion during anestrus in ewes (Gayrard et al., 1994; Lehman et al., 1996). The most rostral DA cell bodies in the brain are found in the olfactory bulb (A16) and retina (A17).

2.7.3 The Localization of Dopaminergic System in Birds

The anatomical distribution of the avian DAergic system apparently resembles to that of mammals (Moons et al., 1994; Reiner et al., 1994). The use of specific antibodies against mammalian catecholaminergic enzymes; TH, AADC, DBH, PNMT, and utilizing specific antibodies to the neurotransmitter themselves in immunocytochemical studies has also contributed to the present knowledge of the neuroanatomical aspects of catecholaminergic systems in the avian CNS. DA has been measured and visualized in many avian species including domestic fowl (Knigge and Piekut, 1985), quail (Ottinger et al., 1986; Balthazart et al., 1992; 1998; Bailhache and Balthazart, 1993; Absil et al., 2001), pigeon (Kiss and Peczely, 1987; Berk 1991; Divac et al., 1994; Durstewitz et al., 1998), zebra finch (Barclay and Harding, 1990; Bottjer, 1993; Mello et al., 1998), chicken (Contijoch et al., 1992; Moons et al., 1994; 1995), budgerigar (Roberts et al., 2001), collared dove (den Boer-Visser and Dubbeldam, 2002), turkey (Al-Zailaie and El Halawani, 2000), and canary (Appeltants et al., 2001). DA neurons are found throughout the avian hypothalamus (Kiss and Peczely, 1987; Reiner et al., 1994; Al-Zailaie and El Halawani, 2000) and have been shown to be immunoreactive for VIP (Mauro et al., 1989; Hof et al., 1991; Mauro et al., 1992) and VIP mRNA (Kuenzel et al., 1997; Chaiseha and El Halawani, 1999). The localizations of DA-ir cells in the chicken hypothalamus (Smeets and Gonzalez, 1990) and hindbrain (Kuenzel et al., 1992) have been described previously.

DA neurons are not located in a single discrete hypothalamic nucleus or origin, but instead are dispersed among a variety of hypothalamic regions. Several DA neuronal groups have been identified in the preoptic hypothalamic areas of the turkey (Al-Zailaie and El Halawani, 2000; Al-Zailaie, 2003). These areas include the POM, AM, suprachiasmatic nucleus (SCN), nucleus ventrolateralis thalami (VLT), PVN, hypothalamus pars lateralis (LHy), VMN, nucleus dorsomedialis hypothalami (DMN), ML, nucleus mamillaris medialis (MM), and nucleus premamillaris (PMM). The distribution of TH-ir positive DBH negative cells is found in the turkey hypothalamus and other avian species (Kiss and Peczely, 1987; Bailhache and Balthazart, 1993; Moons et al., 1994; Reiner et al., 1994; den Boer-Visser and Bubbeldam, 2002). Given their widespread distributions, the findings that DA axons 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), it is reasonable to consider whether any regional specificity exists in those DA neurons that is neuroendocrine in nature, i.e., controlling the release and expression of VIP/PRL and GnRH/FSH-LH systems. The existence of DAeric fibers in the ME has been reported in quail (Bailhache and Balthazart, 1993), chicken (Moons et al., 1994), and turkey (Al-Zailaie, 2003). This suggests the importance of this structure as a possible source of DA, which plays a role in the regulation of PRL in birds. Recently, DA neurons found in the turkey hypothalamus including the POA, ML, PMM have been suggested being a potential reproductive neuroendocrine circuit controlling reproductive seasonality in temperate zone birds, which is highly photoperiodic and gonadal development occurs in response to increasing day length (Al-Zailaie et al., 2006; Kang et al., 2007; Thayananuphat et al.,

2007a; 2007b).

2.7.4 The Function of Dopamine in Mammals

DA participates in several physiological functions in mammals including food and water intake, body homeostasis, behaviors and cognition, motor activity, regulation of milk secretion, sleep, mood, attention, learning, and reproductive regulation (Bertolucci-D'Angio et al., 1990; Cooper and Al-Naser, 1993; Wilson et al., 1995; Velasco and Luchsiner, 1998; Ben-Jonathan and Hnasko, 2001; Hull et al., 2004; Wellman, 2005). Some findings link DA to putative drive systems for hunger and thirst. It has been suggestedthat DA controls different aspects of basic motivation (Stellar, 1954). In domesticated farm animals such as cattle, there is a small but growing literature which implicates CA as important neuroendocrine regulators. These include studies of thermoregulation, hormonal secretion, feeding behavior, physiological/psychological indicators of stress, animal well-being, and the etiology of some disorders caused by grazing endophyte-infected fescue (Leshin et al., 1995).

The importance of DA in the control of movements is well demonstrated in pathological conditions such as Parkinson's disease. Indeed, this disease is characterized by a strong reduction of circulating DA due to the degeneration of DAergic neurons. The effects of DA on motor activity have been extensively investigated (for review, see Clark and White, 1987; Jackson and Westlind-Danielsson, 1994). DA agonists and antagonists have been used to determine DA functions associated with locomotion in rats, which DA agonists increase DA functions, thus increasing motor activity, while DA antagonists have the opposite effect. Taken together with the generation of genetically modified mice for different

components of the DAergic pathway constitutes a powerful toll for studying the *in vivo* roles of these proteins. Utilizing these techniques, D_1, D_2, and D_3 DA receptors have been implicated to have a role in controlling the degree of locomotion. Activation of D_2 DA receptors, which results in decreased DA release, has been shown to decrease locomotor activity. This finding is supported by the injection of D_1 and D_2 DA antagonists show the decrease in motor activity (for review, see Jackson and Westlind-Danielsson, 1994). D_2 DA receptor knock out mice clearly reveals a motor impairment in mutant mice (Baik et al., 1995). Another disease can also involve DA dysfunction such as Attention-Deficit Hyperactivity Disorder (ADHD). ADHD is a condition of childhood, symptoms of which include inattention, hyperactivity, and distraction. This reveals the role of DA in regulating cognitive and attention functions (Nieoullon, 2002). There is some evidence that polymorphisms in the D_4 DA receptor and DA reuptake transporter (DAT) genes are associated with ADHD (Cook et al., 1995; Gill et al., 1997; La Hoste et al., 1996; Swanson et al., 1998). The impairment of emotional processes in neurologic and psychiatric pathologies involving the DAergic system (Parkinson disease, schizophrenia, autism, ADHD, Huntington disease, frontal lobe lesions), as well as the influence that administration of DAergic agonist/antagonists exert on the processing of emotion, suggests a role for DA in emotional process (for review, see Salgado-Pineda et al., 2005). The effects of DA on blood pressure, cardiac output, and regional blood flow have been reported since the DA receptor subtypes have been found in the peripheral organs. DA receptors found in blood vessels, adrenal gland, kidney, and heart have several physiological functions related to vasodilatation, regulation of CA release, sodium reabsorption, renin and aldosterone secretion, as well as vasopressin action. It is well recognized that DA

plays a major role in the control of various aspects of reproduction including the secretion of gonadotropic hormones and activation of male and female sexual behaviors. DA neurons are found prominently in the ventrolateral hypothalamus of seasonal breeding species such as sheep (Tillet and Thibault, 1989), and are believed to mediate steroid hormones suppression of gonadotropins secretion during anestrus in ewes (Gayrard et al., 1994; Lehman et al., 1996).

2.7.5 The Function of Dopamine in Birds

DA in avian brain has been shown to be involved in several physiological functions that are quite similar to those of mammals. DA plays a role in many aspects of avian sexual activity and reproduction. DA in the medial POM facilitates male sexual behavior (Hull et al., 1995; Dominguez and Hull, 2005; Bharati and Goodson, 2006). D_1 DA agonist administration is found to increase the aspects of sexual behavior in quail (Balthazart et al., 1997). DA perikaya in the ML represent a discrete subset of neurons that control reproduction in birds including GnRH-I and VIP perikaya. Activation of DAergic cells in the ML is linked to activation of GnRH-I and VIP neurons and the release of LH and PRL (Al-Zailaie et al., 2006). DA in the PMM, proposed to be the DA A11 groups, is suggested its function in controlling reproductive seasonality in the temperate zone birds. The expressions of c-fos mRNA within the PMM are differentially activated by light and corresponded with a rhythm of photosensitivity (Thayananuphat et al., 2007a; 2007b). The relationship of DAergic system in the PMM and GnRH-I system in the nCPa during the photo-induction reproductive activity has been reported. Recently, DA-melatonin colocalized neurons have been found in the PMM and shown to cycle rhythmically with photoperiodic

changes (Kang et al., 2007). Another aspect considering the function of A11 DA groups is a hypothesis that DA within the posterior hypothalamus, particularly from the nucleus intramedialis (nI) may be play a role in the onset of puberty (Fraley and Kuenzel, 1993). In contrast with this study, Kuenzel (2000) suggests DAergic neurons that might be possible to influence gonadal maturation are DA neurons located in the PVN and ML. In addition, it has been suggested the rostral A11 neurons of the caudal hypothalamus involve in courtship singing in songbirds that sing and court females for breeding, such as zebra finch (Bharati and Goodson, 2006).

The involvement of DA in motor functions in birds that exists similarly as in mammals has been reported. Unilateral kainic acid lesions of the paleostriatum or the SCN (its source of DAergic input) in pigeons induces persistent turning in one direction, postural problems, arrhythmic movements, and head or whole body tremors (Rieke, 1980; 1981). Apomorphine, a relatively non-selective D_2 DA receptor agonist, has also been shown to facilitate stereotypic pecking bouts in a dose-dependent manner and the avian basal ganglia is a likely site for these DAergic effects (Zarrindast and Amin, 1992). Like mammals, DA has been known to regulate aspect of food and water intake in birds. This is supported by the results that apomorphine and DA administration in pigeons can reduce food consumption (Deviche, 1984; Ravazio and Paschoalini, 1992).

2.7.6 Dopamine as the PIF in Mammals

DA neurons are originally implicated in the regulation of pituitary hormone secretion on the basis of the results of early receptor binding and pharmacological studies showing that: (1) DA receptors are located in hypophysiotropic regions of the

hypothalamus and pituitary gland and (2) activation or blockade of these DA receptors alters pituitary hormone secretion both *in vivo* and *in vitro* (Moore, 1987). DA concentrations in hypophyseal portal blood are maintained at physiologically active levels (Ben-Jonathan et al., 1977; Gibbs and Neill, 1978; Ben-Jonathan et al., 1980). The direct biological actions of DA on pituitary hormone secretion are largely inhibitory, maintaining basal secretion of PRL from anterior pituitary. It is well established that PRL secretion is under tonic inhibitory control exerted by DA. DA or its agonists inhibit the release and gene expression of PRL, as well as lactotrophs proliferation (Birge et a., 1970; Shaar and Clemens, 1974). DA receptors have been found in the pituitary lactotrops (Caron et al., 1978; Cronin et al., 1978; Goldsmith et al., 1979). DA also participates in the regulation of the acute increase of PRL release. *In vivo* studies demonstrate that 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 release of PRL. Confirming by *in vitro* data that PRL release in anterior pituitary is stimulated after short term exposure of DA (Fagin and Neil1, 1981; Denef et al., 1984). DA that is released from hypothalamic TIDA neurons serves as the physiological inhibitor of PRL secretion (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001) and is mediated through D_2 DA receptors residing on plasma membrane of pituitary lactotrophs (Civelli et al., 1991). Removal of this DAergic inhibition results in increased PRL secretion and hyperprolactinemia (Nicoll and Swearingen, 1970; Nicoll, 1977). 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 possible that PRL surges which occur 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). D_2 DA receptors on pituitary lactotrophs inhibit PRL release in both mammals (Ben-Jonathan and Hnasko, 2001) and birds (El Halawani et al., 2000). Although TIDA is very well known in regulating of PRL secretion (DeMaria et al., 1998), suggested three populations of hypothalamic DAergic neurons collectively referred to as neuroendocrine DAergic neurons contribute to the tonic inhibition of PRL secretion. These are (1) TIDA with axons terminating in the external zone of the ME, (2) tuberohypophyseal DAergic neurons (THDA) with soma in the rostral region of the ARC and axons terminating in the neural and intermediate lobes of the pituitary gland, and (3) periventricular hypophyseal DAergic neurons (PHDA) with soma in the periventricular nucleus (A14) and axons terminating in the intermediate lobe of the pituitary gland. In contrast with the function of D_2 DA receptors to inhibit PRL release, it has been reported that D_1 and/or D_5 DA receptors may actually stimulate PRL release from pituitary in rats (Porter et al., 1994).

In mammals, several studies show the involvement of VIP in the regulation of PRL secretion from the pituitary (Kato et al., 1978; Rotsztejn et al., 1980; Reichlin, 1988). VIP fibers are found intermingle with DAergic neurons in the ARC and periventricular nucleus. This study also shows 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 (Gerhold et al., 2001). DA and 5-HT appear to have a complementary interaction regarding PRL secretion. DA and 5-HT are colocalized

within neurons in the hypothlalamus of baboon (Thind et al., 1987), and synaptic junctions between serotonergic 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 serotonergic activity (King et al., 1985) and intraventricular injections of 5-HT reduce DA levels in portal blood of rats (Pilotte and Porter, 1981). Indeed, the primary function of TIDA neurons is to suppress the secretion of PRL from anterior pituitary gland. However, it has been found that the synthesis and release of α-MSH and multiple acetylated form of β-endorphin from the melanotrophs is tonically suppressed by DA acting on inhibitory D_2 DA receptors located directly on these cells (Cote et al., 1982; Tilders et al., 1985). DA also has controversial roles in the regulation of gonadotropins secretion since both stimulatory and inhibitory roles for DA in control of GnRH release and LH secretion have been reported. Axons of GnRH neuronal perikaya are terminated in the external layer of the ME, which is closed proximity to terminals of TIDA neurons (Ajika, 1979; Ugrumov et al., 1989). These DA neurons have been implicated in mediating hypeprolactinemia-induced suppression of LH secretion (Selmanoff, 1981). Finally, previous data suggest that DA inhibits GH and thyrotropin release via direct actions in the anterior pituitary, but under normal baseline conditions, these DA systems are not tonically active (Lookingland and Moore, 2005). These results clearly support function of DA as the inhibitory factor for regulating pituitary hormone secretion.

Aside from providing the role of DA as the PIF, several studies providing additional support for the role of DA as the PRF. Attenuation of DAergic tone results in an augmentation of PRL release which is small compared to PRL response *in vivo* following physiological stimuli. DA at low concentrations stimulates PRL secretion *in*

vitro (Shin, 1978; Denef et al., 1980; Burris et al., 1991; 1992; Porter et al., 1994) and *in vivo* (Arey et al., 1993). These phenomena suggest that pituitary lactotrophs have the potential to respond to the inhibitory and stimulatory effects of DA (Kineman et al., 1994). A subpopulation of lactotrophs sensitive to the stimulatory effect of DA exists (Burris et al., 1992; Burris and Freemen, 1993) and the two opposite effects of DA upon PRL secretion may be mediated by distinct G proteins (Burris et al., 1992; Niimi et al., 1993; Lew et al., 1994). In mammals, the hypothalamic control of PRL secretion is dependent on a balance between VIP and TRH stimulation on one hand, and DA inhibition on the other hand. Those three factors, as well as the peripheral hormones, estradiol, and glucocorticoids act directly on PRL-secreting cells by different receptors (Gourdji et al., 1973; Rotsztejn et al., 1980).

2.7.7 Dopamine Regulation of Avian PRL Secretion

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 control. It has been well established that DAergic influences are involved in both stimulating and inhibiting avian PRL secretion. *In vitro* studies on the effects of DA on the pituitary cells demonstrate that DA inhibits PRL release (Harvey et al., 1982; Hall and Chadwick, 1984; Hall et al., 1986; Xu et al., 1996). DA or its agonist, apomorphine, reduces PRL secretion caused by the co-incubation of chicken and pigeon pituitary glands with hypothalamic extract. This effect is reversed by the DA receptor antagonist, pimozide (Hall and Chadwick, 1983). In chicken, DA inhibits the release of PRL stimulated by TRH, hypothalamic extract, or by previous exposure of the pituitary gland to estrogen (Hall and Chadwick, 1984). However, other data clearly show that DA is not an

inhibitor but a stimulator of PRL secretion in non-laying turkey (Harvey et al., 1982). *In vivo* experiments produce supporting results. ICV infusion of DA in laying turkey hens demonstrates that DA can either stimulate or inhibit PRL secretion depending upon the concentrations used (Youngren et al., 1995). Subsequent ICV infusion study suggests that DAergic influences are involved in both stimulating and inhibiting avian PRL secretion depending upon multiple DA receptors (Youngren et al., 1996b). The presence of both D_1 and D_2 DA receptor mRNAs in the turkey brain and pituitary confirm that DA exhibits biphasic actions within the turkey hypothalamus and pituitary gland (Schnell et al., 1999a; 1999b, Chaiseha et al., 2003). The stimulatory effect of DA on PRL secretion is regulated via D_1 DA receptors residing in the INF, where the VIP neurons are located. In contrast, DA inhibits PRL release and synthesis by blocking the action of VIP at the pituitary level through D_2 DA receptors (Youngren et al., 1996b; 1998; 2002a; Chaiseha et al., 1997; 2003; Al Kahtane et al., 2003). DA activates hypothalamic VIP gene expression in the INF (Bhatt et al., 2003).

It is well studies 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, an increase that 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., 2003). 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. It is suggested that the inhibitory effects of DA on VIP-induced PRL gene transcription may result from DA suppression of the transcriptioning fraction 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 turkey (Wong et al., 1992a; Kurima et al., 1998) and chicken (Tanaka et al., 1991). It is known that the secretion of avian PRL also requires an intact serotonergic system (El Halawani et al., 1988c). To date, it is concluded that dynorphin, serotonin, DA, and VIP all appear to stimulate avian PRL secretion along a pathway expressing κ opioid, serotonergic, DAergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator (for review, see El Halawani et al., 2000).

It has been well studied that 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). Furthermore, 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., 2003). The DAergic expression during the turkey reproductive cycle parallels the changes in plasma PRL levels and

VIP immunoreactivity, VIP content, and VIP mRNA expression within the INF area (El Halawani et al., 1980; 1984a; Mauro et al., 1989; Wong et al., 1991; Chaiseha et al., 2003; 2004).

2.8 Photoperiodic Control of Reproductive Cycle in Birds

2.8.1 The Role of Photoperiod in Seasonal Reproduction

In birds, environmental information initiates reproductive development prior to the onset of optimal condition for raising offspring, while other environmental informations regulate the specific termination of reproductive activity (Wingfield et al., 2000). Most of birds show annual cycles of physiological and behavioral events including specific times for molt, body weight changes, migration behavior, gonadal development, and breeding behavior. Breeding seasons can occur at the same time of the year (seasonal or predictable breeders) and there are birds that rely on unpredictable food availability (opportunistic breeders). In seasonal breeder birds, the initiation of breeding cycle depends upon the precise prediction of environmental conditions, which are optimal for health of the mating pair and the survivability of its offspring. The environmental cue which is the predominant proximal factor for the initiation of seasonal events is photoperiod. Most birds breeding outside the tropical zone use seasonal changes in day length to provide predictive information for the optimal time to initiate breeding (for review, see Sharp, 1996). In most species at mid and high latitudes, gonadal maturation occurs during spring, as photoperiod increases and so called long day breeders. The exact time and rate of maturation varies between species depending on their breeding seasons, and it can be varied within species depending upon latitude and local non-photoperiodic factors. Rowan (1925) is the first

to demonstrate that an increase in the photoperiod could advance gonadal maturation and the longer the (constant) photoperiod to which birds are exposed, the greater the rate of maturation (Burger, 1947). In addition, increased photoperiod may maintain reproductive system of some species in a continuous state of activity (Nicholls et al., 1988; Chaturvedi et al., 1993). In most of long day breeding photoperiodic species, period of reproduction is terminated abruptly by the rapid collapse of gonad during late summer when days are still long. During this period, birds stop responding to light after prolonged exposure to long days (usually several weeks; Hamner, 1968; Follett and Pearce-Kelly, 1990; Saldanha et al., 1994; Bentley et al., 1998). At this time, gonads regress, reproductive hormones decline, and breeding behavior ceases (Dawson et al., 2001). Birds exhibiting this complete loss of sensitivity to long days as a reproductive stimulus are said to become absolutely photorefractory (Hamner, 1968; Dawson and Sharp, 2007). The degree of photorefractoriness, absolute or relative, is related to the length and symmetry of breeding season. Absolute photorefractoriness is characterized by an apparently spontaneous gonadal regression during continued exposure to long photoperiods and that, following regression; a further increase in photoperiod does not induce renewed gonadal maturation (Hamner, 1968). Unlike absolute photorefractory birds, some birds remain continuously in breeding condition under constant long days but gonads regress if they are shifted to relatively short days (>15 hours). This type of refractoriness is so called relative refractoriness and it is seen in various species of Indian weaver bird (Thapliyal and Tewary, 1964; Chakravorty et al., 1985) and quail (Anthony, 1970; Follett, 1984). Relative photorefractoriness requires a decrease in the photoperiod to induce regression, but regression occurs under a photoperiod that is still longer than that which induced

maturation earlier in the year (Robinson and Follett, 1982). In general, birds with short predictable breeding seasons, particularly those with breeding seasons relatively early in the year, become absolutely photorefractory. Those with later, longer or less predictable breeding seasons are thought more likely to show relative photorefractoriness (Hahn et al., 2004). Although most birds living at non-equatorial latitudes are long day breeders but some tropical and sub-tropical birds which do not experience variations in annual day length are also reported to use photoperiod as an environmental factor to time their seasonal reproduction and exhibit the phenomena of absolute photorefractoriness such as common myna, red headed bunting, red vented bulbul, and Indian rose finch (Chaturvedi and Thapliyal, 1983; Thapliyal and Gupta, 1989; Rani and Kumar, 2000).

The site of photorefractoriness is believed to be at the hypothalamic or higher level (Farner and Lewis, 1971; Storey and Nicholls, 1976) because some photorefractory birds are able to respond readily and with essentially normal gonad development to exogenous stimulatory agents such as gonadotropins (Stetson et al., 1973). It has been demonstrated that post reproductive gonadal regression is due to markedly decrease in LH and FSH (Nicholls, 1974; Mattocks et al., 1976; Dawson and Goldsmith, 1982). Photorefractory birds usually do not regain responsiveness to increased day length (i.e., become photosensitive) until they are exposed to short days (usually less than 12 hours of light per day) for several weeks (Boulakoud and Goldsmith, 1995; Dawson, 1991; Wilson, 1992).

Although photoperiod is the primary proximate factor used to initiate reproductive development in many seasonally breeding temperate zone birds, non-photoperiodic environmental signals also influence the timing of reproduction in birds.

These signals include temperature (Wada, 1993; Wingfield et al., 2003; Perfito et al., 2005), food availability (Hahn, 1998; Hau et al., 2000; Deviche and Sharp, 2001), increased green vegetation (Priedkalns et al., 1984), humidity (Cynx, 2001; Vleck and Priedkalns, 1985), water availability (Vleck and Priedkalns, 1985), rain (Zann et al., 1995), and presence of mates (Wingfield and Monk, 1994; Eda-Fujiwara et al., 2003). Non-photoperiodic cues are thought to accelerate or inhibit the effects of photoperiod on reproductive development and behaviors, thus ensuring that the timing of breeding is synchronized with optimal local environmental conditions (Wingfield et al., 2000). In some environments such as the tropics, deserts, and near the Arctic, birds may breed at different times in different years (Hahn et al., 1997; Deviche and Sharp, 2001) and in these environments the timing of reproductive behaviors appears to be predominantly controlled by non-photoperiodic stimuli (Hahn et al., 1997; Zann et al., 1995; Hau, 2001) such as increased rainfall (Zann et al., 1995; Lloyd, 1999; Grant et al., 2000).

2.8.2 Light Detection and Circadian Rhythms

In most animals living in temperate regions, reproduction is under photoperiodic control. This ensures the birth of young in spring or summer, the optimal seasons for survival. The chicken eye, similar to the human eye, is capable of seeing in a narrow part of the light spectrum (380-760 nm). However birds, unlike mammals, do not need the eyes. Photoperiodic information is detected by an extra-retinal encephalic photoreceptor, while the supplementary information is processed by the ears and eyes (Yokoyama and Farner, 1978; Yokoyama et al., 1978; Glass and Lauber, 1981; Foster et al., 1994; Saldanha et al., 1994; Wada et al., 2000; Li et al.,

2004). Blinded, or pinealectomized, or both blinded and pinealectomized, tree sparrows show no difference in photosensitivity (LH secretion, testicular growth and regression, and photorefractoriness) compared with intact individuals (Wilson, 1991). The role of light in biological activities associated with egg production is well known. Light quality can be defined by three criteria: 1) pattern of light-dark exposure (photoperiod), 2) light intensity (brightness), and 3) spectra composition (Andrews and Zimmerman, 1990). In birds from subtropical and temperate latitudes, the gradual or abrupt increase in day length initiates gonad recrudescence and egg laying. Conversely, reduction in day length delays the onset of sexual maturity or terminates egg laying activity in birds (Benoit, 1964; Woodard et al., 1969).

The photoperiodic regulation of reproduction appears to depend entirely on putative encephalic photoreceptors. It has long been known that extra-retinal photoreception has been shown to be necessary for the photoperiodic induction of gonadal development in some avian species (Menaker, 1971). Homma and Sakkakibara (1971) have demonstrated the presence of deep brain photoreceptors in Coturnix quail. The immunocytochemical study, using an antibody raised against a membrane fraction of rat retina, known to bind to the *N*-terminal of rhodopsin (Hargrave et al. 1986), demonstrates that opsin-ir cells are located in the lateral septum organ (LSO) and the INF in dove (Silver et al. 1988; Saldanha et al. 1994). This hypothesis is in agreement with the recent finding indicated that a population of cerebrospinal fluid-contacting neurons (CSFcn) in the LSO may serve as an encephalic photoreceptors functioning to signal the onset of seasonal reproductive development in birds (Li et al., 2004). On the other hand, numerous reports have suggested that the mediobasal hypothalamus (MBH), including the infundibular

nucleus (IN), ME, and nucleus hypothalamicus posterior medialis (NHPM) is an important center for the regulation of photoperiodism in birds. This suggestion is derived from the results that lesions of the IN, the ME, or NHPM within the MBH can block the rise of LH and testicular growth under long photoperiods (Sharp and Follett, 1969; Davies and Follett, 1975; Ohta and Homma, 1987). In addition, electrical stimulation of the IN increases LH secretion (Konishi et al., 1987) and induces testicular growth (Ohta et al., 1984). Furthermore, the expression of fos-like immunoreactivity in the IN and ME has been shown to result from the photostimulation of one long-day photoperiod (Meddle and Follett, 1995; 1997). Taken together, these results suggest that deep brain photoreceptors are thought to be localized in the the IN (Silver et al., 1988).

It is an established fact that the circadian clock is involved in the photoperiodism of various organisms (Pittendrigh, 1972). Circadian (~24 hours) rhythms are a fundamental property of living systems and impose a 24 hours temporal organization regulating the physiology and biochemistry of most organisms. Circadian systems are composed of multiple inputs, oscillators, and outputs. Timing cues are transmitted via input pathways to oscillators, which then drive rhythms and entrain suboscillators through output pathways. In vertebrates, these components comprise at least the retina, the SCN of the hypothalamus, and the pineal gland (Menaker and Tosini, 1995). In the mammal circadian system, the SCN is the primary circadian pacemaker (Moore, 1979). Light signals reach the SCN via a dedicated retinal pathway, the retinohypothalamic tract (RHT; Moore and Lenn, 1972; Moore, 1973; Goel et al., 1999). The information of these retinal cells receive on day length is interpreted by the SCN and passed onto the pineal gland. In the pineal gland,

photoperiodic information is translated into a daily cycle of melatonin and the duration of the night is reflected in the length of the nocturnal melatonin secretion. In long day breeders, short day-induced melatonin suppresses gonadal activity, while it induces gonadal activity in short day breeders such as the sheep and goat (Arendt, 1995). Although melatonin has a determining role in regulating photoperiodic response of gonads in mammals, no effect of melatonin manipulation is observed in the seasonal regulation of gonads in birds (Gwinner et al., 1997). Avian species do exhibit robust melatonin rhythms like mammals, but they do not seem to use them for photoperiodic time measurement. Decades of experiments manipulating either the pineal or melatonin in a variety of avian species found no effect on the photoperiodic regulation of reproductive physiology (for review, see Gwinner and Hau, 2000). The reasons for these differences between avian and mammalian photoperiodism has been a mystery for several decades.

Extra-retinal photoreceptors involve in the transduction of photoperiodic information for the control of reproduction in birds. These receptors are believed to provide input to the biological clock that generates a circadian rhythm of photoinducibility of reproduction (Simpson and Follett, 1982; Follett and Pearce-Kelly, 1990). Clock genes are expressed in the hypothalamus (Brandstatter et al., 2001; Yoshimura et al., 2001) and photoreceptors are closely associated with GnRH neurons and its terminals (Saldanha et al., 2001). Indeed, the SCN is the dominant biological clock regulating circadian rhythms in mammals (Klein, et al., 1991). In birds, however, two nuclei, the medial SCN (mSCN) and the visual SCN (vSCN), have been reported to be the avian SCN. Cassone's group has proposed that the vSCN is the avian homologus of the mammalian SCN (Cassone, 1991). On the other hand,

several studies have suggested that the mSCN is anatomically homologous to the mammalian SCN. Circadian clock genes have been cloned in the Japanese quail (qClock, qPer2, and qPer3; Yoshimura et al., 2000) and house sparrow (qPer2; Brandstatter et al., 2001). qClock, qPer2, and qPer3 clock genes are expressed in the mSCN but not in the vSCN (Yoshimura et al., 2001), indicating that the mSCN is a circadian oscillator in birds.

2.8.3 Photoperiodic Regulation of Avian Reproduction

The control of reproductive system in birds involves the interaction of external stimuli with endocrine mechanisms. Photoperiodic cues appear to be important for the onset of reproduction for most birds in temperate zones, as they become reproductively active in spring when day length increases. Photoperiod has been associated with the reproductive neuroendocrine axis in birds since the lengthening day stimulates secretion of GnRH and gonadotropins and consequent gonadal growth (Follett and Robinson, 1980, Dawson et al., 2001). The stimulatory effect of long days appears usually to be associated with an increase in GnRH content in hypothalamus or increased immunoreactivity for GnRH in the hypothalamus and ME in avian species: European starling (Dawson et al. 1985; Foster et al. 1987; Goldsmith et al. 1989), garden warbler (Bluhm et al. 1991), dark-eyed junco (Saldanha et al. 1994), house sparrow (Hahn and Ball, 1995), and Japanese quail (Foster et al. 1988; Perera and Follett 1992). It has been established that photosensitive males have more and larger POA GnRH-ir cells and fibers in this region than that of photorefractoriness males (Deviche et al., 2000). Follet and co workers (1977) demonstrate that all necessary photoperiodic information must be received by 14.7 hours from the light on (dawn).

GnRH release after long days stimulation occurs 22 hours from dawn (Perera and Follett, 1992).

cGnRH-I is thought to be directly control gonadal maturation by stimulating the release of gonadotrophins (Sharp et al., 1990). Exposing photosensitive birds to day length longer than approximately 12 hours (defined as long days), as is naturally the spring, stimulates the release of GnRH, LH, and FSH (Lewis and Farner, 1973; Wingfield et al., 1980; Dawson and Goldsmith, 1983; Fehrer et al., 1985; Wilson, 1985; Foster et al., 1987; McNaughton et al., 1995; Meddle and Follett, 1997). In turkey hen, increase in LH generally occurs within 2 to 3 days after the photoperiod is changed from short days to long days. Photosensitive turkey hens secrete LH in low frequency, high amplitude pulses superimposed on a low baseline (Chapman et al., 1994). A period of lower and relatively stable LH concentrations then follows during the time of prelaying gonadal growth (Godden and Scanes, 1997; Burke and Dennison, 1980; El Halawani et al., 1983; Siopes and El Halawani, 1989). This sustained increase in plasma LH levels associated with a change from short day to long day has been reported in Japanese quail (Follett et al., 1977; Perera and Follett, 1992), white-crowned sparrow (Yokoyama and Farner, 1976, Wingfield et al., 1980), and domestic fowl (Bonney et al., 1974; Sharp, 1993). Preovulatory LH surge that cause ovulation in birds is normally occured during the dark period (scotophase) in laying turkey (Chapman et al., 1994; Yang et a., 2000) and chicken (Etches and Schoch, 1984) exposed to diurnal lighting. Plasma LH levels remain elevated during the subsequent laying phase of reproduction, but drop to prelaying concentrations during incubating or if photorefractoriness is occured (Kuwayama et al., 1992). Dunn and Sharp (1990) reported that the shortest photoperiod required to stimulate LH

release (critical daylength) in 8 weeks old dwarf broiler breeders is between 8 and 10.5 hours, while the shortest photoperiod required to maximize LH release (saturation daylength) is between 10.5 and 12.5 hours. This pattern of secretion is stimulatory to the ovary and continues until egg laying begins when it is modified by ovulatory surges of LH.

It has been found that hypothalamic neurotransmitter such as CA plays a role in the light-stimulated the regulation of avian gonadotropic activity (Menaker, 1971). This finding is supported with finding that hypothalamic NE turnover is increased in light-stimulated quail (Campbell and Wolfson, 1974). Moreover, photoperiodically-induced gonadal growth in quail is accompanied by elevated serum LH (Nicholls et al., 1973) and it can be blocked by lesioning the hypothalamus (Sharp and Follett, 1969) or intraventricular infusion of 6-hydroxydopamine (Davies and Follett, 1974). Recent studies using turkey as a model show that DA neurons in the PMM are differentially sensitive to photostimulation depending upon circadian time. A relationship between the DAergic system in the PMM and the GnRH system in the nCPa during the photoinduction of avian reproductive activity is also reported (Thayananuphat et al., 2007a).

It is well established that photostimulation activates the GnRH/FSH-LH and VIP/PRL systems. PRL secretion is increased after photostimulation and particularly high in birds incubating eggs or caring for their young (Goldsmith, 1985). The vernal photo-induced increase in LH secretion is accompanied by an increase in PRL secretion (for review, see Sharp et al., 1998). It has been suggested that the increase in concentrations of plasma PRL during incubation (El Halawani et al., 1993; Sharp et al., 1988) and the development of photorefractoriness may play a role in depressing

LH secretion and inducing gonadal regression (for review, see Nicolls et al., 1988). During the development of photorefractoriness, decreased plasma LH and increased plasma PRL are correlated with decreased hypothalamic GnRH-I and increased hypothalamic VIP. In birds exposed to seasonal changes in day length, the seasonally maximal concentrations of plasma PRL levels associated with the development of photorefractoriness can be explained, in part, by the saturation day length for photo-induced PRL release being reached in late spring/mid summer. During the development of photorefractoriness, high concentrations of plasma PRL play a role in ensuring the rapid regression of the gonads, but are not essential for the maintenance of the condition (for review, see Sharp et al., 1998).

Most birds in temperate zones undergo gonadal regression to become reproductively inactive in summer, even though day length is still long (Farner and Follett, 1979; Farner and Gwinner, 1980). Photorefractoriness is associated with decreased GnRH production and circulating LH levels, rapid gonadal involution, and molt (Wingfield et al., 1980; Foster et al., 1987; Kubokawa et al., 1994; Hahn and Ball, 1995; Parry et al., 1997; Cho et al., 1998). This photorefractoriness has been shown to be correlated with great reductions in immunoreactivity for GnRH in the hypothalamus and ME in European starling (Foster et al., 1987; Goldsmith et al., 1989; Dawson et al., 1985), garden warbler (Bluhm et al., 1991), dark-eyed junco (Saldanha et al., 1994), and house sparrow (Hahn and Ball, 1995). The onset of photorefractoriness is accompanied with decreased hypothalamic expression of the GnRH precursor peptide, proGnRH-GAP (Parry et al., 1997). This decrease, which precedes a change in hypothalamic GnRH immunostaining, indicates that photorefractoriness results from a reduction in GnRH synthesis rather than release of

this peptide at the ME level. Induction of photorefractoriness is associated with increased VIP (Mauro et al., 1992, Saldanha et al., 1994; Deviche et al., 2000) and decreased GnRH expression, whereas dissipation of photorefractoriness concurs with decreased VIP and increased GnRH brain expression (Deviche et al., 2000). It has been found that, unlike the absolute photorefractoriness, the relative photorefractoriness is not associated with a reduction in GnRH (Foster et al., 1988; Follett and Pearce-Kelly, 1990). However, the study also suggests that the relative refractoriness may induce gonadal regression through a cessation of GnRH secretion, whereas absolute refractoriness involves down-regulation of peptide production as well (Hahn and Ball, 1995). Prolong exposure to a stimulatory photoperiod at least in part result of photorefractoriness in house sparrow results in a reduction in the concentrations of plasma LH (Sharp et al., 1992; Ciccone et al., 2005) and FSH (Ciccone et al., 2005).

In most species, photosensitivity can be restored only by exposure to short days as would naturally occur during the fall and winter (Nicholls et al., 1988; Wilson, 1992). Restoration of photosensitivity in adults (Dawson et al., 1986; Dawson and Goldsmith, 1997), as well as acquisition of photosensitivity in juvenile birds (Goldsmith et al., 1989), is associated with an increased production of hypothalamic GnRH, an increased number and sized of GnRH-containg neurons, as well as with increased GnRH-containing nerve fiber numbers in the POA (Deviche et al., 2000). Dawson and Goldsmith (1997) report that GnRH concentration in the POA increases after transfer of long day-exposed photorefractory European starlings to short day. Moreover, it has been found that the state of photorefractoriness can also be dissipated by the removal of circulating thyroid hormones, i.e., thyroidectomy. This technique

has shown that both the initiation and maintenance of photorefractoriness are dependent upon the presence of thyroxine (Wieselthier and van Tienhoven, 1972; Goldsmith and Nicholls, 1984). Termination of the photorefractory condition by thyroidectomy has also been observed in tree sparrows (Wilson and Reinert, 1993; 1995).

It has been established for sometimes that not only photoperiod that affects reproductive activities in birds, but also the effect of different wavelength of light. Photostimulation, as affected by different wavelength, has been discussed previously regarding chickens (Harrison et al., 1970), turkeys (Scott and Payne, 1937), sparrows (Ringoen, 1942), ducks (Benoit, 1964), and quails (Phogat et al., 1985). In general, red light stimulates egg production efficiently, whereas green or blue light has little or no effect. In commercial layers, during the first and second seasons, total egg production is significantly influenced by light color, with the greatest number of eggs produced in the group treated with red light. Furthermore, eggs laid under blue or green light is consistently larger then those laid under red light (Pyrzak et al., 1987). In addition, source, spectra, intensity, and regimen of light supplementations become major factors in modern poultry management.

2.9 The Studies of the Native Thai Chicken Reproduction

Native Thai chickens (*Gallus domesticus*), originated from the wild jungle fowl in Southeast Asia, is domesticated by village people approximately 3,000 years ago. Native Thai chickens have long been in Thailand for many generations. Traditionally, the native Thai chickens are raised by small farms. Raising native Thai chicken is found widespread throughout the countryside of Thailand because it is easy

to raise, resistant to diseases, and acclimatized to the local environments. The main objectives of raising chickens are for consumption, competition, and recreation. Up to date, the native Thai chicken has become the new economic domestic animal of Thailand with presently growing demand and relatively high price. At present, there are about 54 millions native Thai chickens in Thailand which are raised by 2.3 millions farmer's family (Department of Livestock Development, 2006). It is interested that nowadays the native Thai chicken is one of the exported goods that gained income of 6.4 millions baths per year (Department of Livestock Development, 2006). The native Thai chickens have traits of fighting cocks including strong and tough muscle, characteristics regarded as good quality when compared with the over-tenderness of broiler meat. It is resulting in high demand by consumers that preferring low fat and antibiotic-free white meat. This provides the good opportunity for production in commercial and industrial scale. Moreover, recent government policies are to encourage the development and the use of Thailand natural resources in supporting of His Majesty the King's concept for self-sufficiency in agriculture. According to this concept, farmers tend to focus on "mixed farming" that is the strategies for helping rural farmers to increase self-sufficiency. One of the important resources that need to be developed is the native Thai chicken. However, the native Thai chickens suffer from their low productivity. Some characteristics inherited from the wild jungle fowl and still expressed in native Thai chicken are maternal and incubation behaviors (Beissinger et al., 1998). The onset of incubation behavior affects the number of egg production because it terminates egg laying. Generally, the native Thai hen lays eggs 3-4 times per year, 4-17 eggs per clutch, and produces about 30-40 chicks per year which is significantly lower than that of the imported hen which

produce eggs all year long. Therefore, in order to increase the production of the native Thai chicken in Thailand, it is very important to understand the basic neuroendocrinology influencing its reproductive activities.

In seasonal breeder temperate zone birds, the effects of season and photostimulation on the H-P-G axis are well characterized. Contrary to the temperate zone seasonal breeding species, the native Thai chicken is an equatorial zone continuously breeding species that produces eggs all year long. The jungle fowl is the ancestor of the native Thai chicken, originated in the tropical region of Southeast Asia, where its breeding season would has been timed by both photoperiodic and non-photoperiodic factors, allowing the chick to hatch at a time of year when food is most abundant. The integration of the endocrine system, hypothalamic neuropeptides/neurotransmitters, pituitary hormones, ovarian steroids, environmental photoperiod, ambient temperature, and the presence of egg and chick regulates the reproductive cycle of seasonally breeding birds and this may be the case in the native Thai chicken.

There are a limited number of studies providing data about neuroendocrine regulation in this non-temperate zone gallinaceous bird. Studies of growth and reproductive performance in the native Thai chicken when comparing with those of some other pure-breed imported chickens have been investigated (Kongruttananun, 1992; Chotesangasa et al., 1993). The studies demonstrate that plasma progesterone level is related to reproductive cycle of the native Thai chicken (Katawatin et al., 1997; Aangkaew, 1999; Sangkaew et al., 2000; Chotesangasa et al., 1993). The effects of photoperiod upon growth, reproductive development, laying performance, and reproductive efficiency in native Thai chickens have been reported (Chotesangasa et

al., 1992; Chotesangasa and Gongruttananun, 1994; 1995; 1997; Choprakarn et al., 1998). However, the results of these studies are contradictive and far from understood. Recently, plasma PRL and LH levels across the reproductive cycle of the native Thai chicken have first been reported (Kosonsiriluk, 2007; Kosonsiriluk et al., 2007). In addition, the distributions and changes in number of VIP-ir neurons in the brain of the native Thai chicken have been investigated and found to mirror the changes in plasma PRL levels across the reproductive stages (Kosonsiriluk, 2008). As indicated above, in order to increase the production of native Thai chickens in Thailand, it is very important to understand the basic endocrinology and neuroendocrine mechanism(s) by which environmental factors influencing its reproductive activities. Thus, the focus of this dissertation research is proposed to investigate neuroendocrine regulation of the reproductive cycle in the female native Thai chicken and the effects of photoperiod upon the neuroendocrine regulation of the reproductive system. The knowledge gained will provide the information of neuroendocrine regulation including the hormonal and physiological characteristics of the reproductive cycle in the native Thai chicken. The results (lighting regimens) of the study of the role of photoperiod can be applied commercially in poultry industry. Moreover, the results will provided the important practical data that can be then implement for farmers to increase egg production of the native Thai chicken in Thailand.

2.10 References

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CHAPTER III

REGULATION OF REPRODUCTIVE CYCLE IN THE NATIVE THAI CHICKEN: ROLE OF PROLACTIN AND LUTEINIZING HORMONE

3.1 Abstract

The regulation of reproductive system in birds involves the interaction of external stimuli with endocrine mechanisms. Luteinizing hormone (LH) and prolactin (PRL) are anterior pituitary hormones that play an important role in the regulation of avian reproduction in temperate zone birds. Increased in day length stimulates the secretion of LH to regulate the period of egg laying. PRL also releases as the consequence of light stimulation to regulate and maintenance of incubation behavior. Contrary to temperate zone seasonal breeding species, the native Thai chicken is an equatorial zone continuously breeding species that produces eggs all year long independent of photoperiodic cues. The reproductive cycle of the native Thai hen may be divided into four reproductive stages; non-egg laying (NL), egg laying (L), incubating eggs (B), and rearing chicks (R). This study was designed to investigate the role of PRL and LH in the regulation of the reproductive cycle of the native Thai chicken. Blood samples were taken from each bird to determine plasma PRL and LH levels according to the reproductive stages utilizing enzyme-linked immunosorbent assay. In addition, the reproductive characteristics were recorded throughout three

reproductive cycles. The results revealed that plasma PRL levels changed according to the reproductive stages. Plasma PRL levels (ng/ml) were low in NL (26.46 $\pm$ 0.65), slightly increased in L (cycle 1 = 49.97 $\pm$ 6.90; cycle 2 = 39.60 $\pm$ 4.81), sharply increased to the maximum levels in B (cycle 1 = 312.10 $\pm$ 27.18; cycle 2 = 274.57 $\pm$ 16.66), and then declined in R (cycle 1 = 41.75 $\pm$ 8.07; cycle 2 = 32.19 $\pm$ 1.37). Plasma LH levels (ng/ml) were essentially the same throughout the reproductive cycle (cycle 1; NL: 3.99 $\pm$ 0.14, L: 3.77 $\pm$ 0.27, B: 3.59 $\pm$ 0.10, R: 3.96 $\pm$ 0.16; cycle 2; L: 3.86 $\pm$ 0.24, B: 4.24 $\pm$ 0.37, R: 4.23 $\pm$ 0.19). The durations of incubating and rearing were significantly lower in the second reproductive cycle, corresponding with the slightly decreased in plasma PRL levels and increased in plasma LH levels. However, there was no significant different between the plasma PRL and LH levels and the number of egg production among the reproductive cycles. Unlike the temperate zone avian species, it might be possible that instead of using the photoperiod as the environmental cues, the regulation of the reproductive system in the native Thai chicken might be correlated with the period of incubating and rearing of the young. In conclusion, the results of this study support the pivotal role of PRL in associated with reproductive cycle of the equatorial gallinaceous avian species.

3.2 Introduction

The control of reproductive system in birds involves two neuroendocrine systems. These are, first the chicken gonadotropin releasing hormone-I (cGnRH-I referred to as GnRH)/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH) system and another is the vasoactive intestinal peptide/prolactin (VIP/PRL) system. The GnRH/FSH-LH system regulates the period of egg laying. On the other

hand, the VIP/PRL system involves the maintenance of maternal behaviors by which VIP stimulates PRL secretion and then gonadal regression. In temperate zone birds, activation of GnRH/FSH-LH and VIP/PRL systems in the somatically mature photosensitive female birds initiates the transition from reproductive quiescence to reproductive activity (Chaiseha and El Halawani, 2005). FSH and LH, anterior pituitary hormones called gonadotropins, are responsible for many reproductive physiological functions. The hypothalamic control of LH and PRL secretion in birds is mediated by a hypophyseal portal vascular system which transports regulatory neuropeptides and neurotransmitters released from the median eminence (ME) to the anterior pituitary gland (Follet, 1984).

The period of egg laying in birds is associated with relatively high levels of FSH, LH, and gonadal steroids (estradiol and progesterone) circulating in the blood. It is generally considered that LH is an important hormone in controlling reproduction of the hen. LH displays a pivotal role in the regulation of ovulation that in birds, ovulation occurs spontaneously and is hormonally controlled. Ovulation is induced by a preovulatory release of LH (Etches and Cunningham, 1976; Mashaly et al., 1976; Cunningham, 1987; Etches, 1990). The frequency of preovulatory LH surge is an important determinant of ovulation and oviposition rates in birds. In turkey hen, ovulation of the largest (F1) follicle occurs 6-8 hours after the preovulatory surge of LH (Mashaly et al., 1976; Proudman et al., 1984). This LH surge is associated with surge of progesterone produced from the F1 follicle (Kappauf and van Tienhoven, 1972; Mashaly et al., 1976; Bahr et al., 1983; Etches and Duke, 1984). FSH induces mainly ovarian follicular growth (Chaudhuri and Maiti, 1998; Rose et al., 2000) and maintains the hierarchical size of the follicles. In contrast with the preovulatory surge

of LH and progesterone, concentrations of FSH and estrogen are relatively constant during the ovulatory cycle (Krishnan et al., 1993; Yang et al., 1997; Liu et al., 2001; Bacon et al., 2002), but a significant decline in FSH occurs prior to the preovulatory LH surge and a significant increase in FSH concentrations occurs during the 12 hours prior to oviposition (Krishnan et al., 1993).

PRL is a polypeptide hormone that is synthesized in and secreted from specialized cells of the anterior pituitary gland, the lactotrophs (Bern and Nicoll, 1968; Velkeniers et al., 1988; Freeman et al., 2000). It has been implicated as a causative factor in the onset and maintenance of incubation behavior in birds (El Halawani et al., 1997). PRL gene expression and its plasma levels are highly correlated with the reproductive cycle in birds (Knapp et al., 1988; El Halawani et al., 1990; Talbot et al., 1991; Wong et al., 1991; You et al., 1995b; Tong et al., 1997). Changes in pituitary PRL gene expression and its plasma PRL levels are highly correlated during different stages of the reproductive cycle in chicken and turkey (Knapp et al., 1988; El Halawani et al., 1990; Talbot et al., 1991; Wong et al., 1991; You et al., 1995b; Karatzas et al., 1997; Tong et al., 1997). During reproductively quiescent stage of the cycle, plasma PRL levels are very low (El Halawani et al., 1984). During egg laying period, the release of gonadotropins which induces sexual receptivity (El Halawani et al., 1986) primes the VIP/PRL system to enhance PRL secretion (El Halawani et al., 1983a). Circulating PRL levels increase dramatically during incubating period (El Halawani et al., 1984). It is this rising PRL levels which has been implicated as the cause of cessation of ovulation, ovarian regression, and induction of incubation behavior. It has been suggested that an increase in circulating PRL during incubation period may depress LH secretion (Nicholas et al., 1988; El Halawani et al., 1993;

Sharp et al., 1998). After hatching, or when eggs are replaced with poults, tactile stimuli from the young induces the emergence and maintenance of maternal response including the change from incubating eggs to brooding the young, vocalizations, nest desertion, a sharp decrease in circulating PRL (Opel and Proudman, 1989), molt, and the transition to the photorefractory state. Circulating PRL and LH levels, pituitary PRL/LH peptide contents and their mRNA levels sharply decline, even though long day length continues with the onset of photorefractoriness (Wong et al., 1991; 1992; Mauro et al., 1992; El Halawani et al., 1996). A precipitous decline in PRL and FSH/LH release and their gene expression may be triggered at any time due to a lack of response to long day length or by subjecting the birds to short day lighting (Nicholls et al., 1988; El Halawani et al., 1990).

It is well studied that in birds, the secretion of LH and PRL is controlled by the releasing hormone, GnRH and VIP, respectively. Ovarian development is found to be correlated with increasing in circulating LH and the amount of GnRH, indicating that the expression of GnRH gene is important to maintain pituitary-ovarian function in chicken (Dunn et al., 1996). Several evidences reveal that birds at the peak level of reproductive activity have more GnRH immunoreactive (-ir) cells and fibers when compared with sexually inactive or photorefractory birds (Sharp et al., 1990; Hahn and Ball, 1995; Parry et al., 1997; Cho et al., 1998). GnRH levels decrease when birds enter the incubating stage and this decrease is thought to be implemented by the inhibitory effect of PRL, which reaches its highest level during this stage (Sharp et al., 1988). It has been established for some time that PRL secretion in birds is tonically stimulated by the hypothalamus (Kragt and Meites, 1965; Bern and Nicoll, 1968) and that principal PRL-releasing factor (PRF) is VIP (El Halawani et al., 1997; 2000;

Chaiseha and El Halawani, 2005). Avian PRL secretion and its gene expression are regulated by VIP (Chaiseha and El Halawani, 1999; 2005). Variations in hypothalamic VIP immunoreactivity, VIP content, VIP mRNA expression in the infundibular nuclear complex (INF), VIP receptor mRNA, and VIP concentrations in hypophyseal portal blood are correlated with changes in circulating PRL levels throughout the turkey reproductive cycle (Mauro et al., 1989; Youngren et al., 1996a; Chaiseha et al., 1998; 2004; Chaiseha and El Halawani, 1999). Additional evidences support the role of dopamine (DA) in the regulation of PRL in birds. DA has a pivotal role in avian PRL secretion, acting centrally through D_1 DA receptors to stimulate PRL secretion. DA effects PRL secretion by operating through the VIP system, causing VIP to be dispersed to the pituitary gland. DA also inhibits PRL secretion by activating D_2 DA receptors at the level of the pituitary gland, antagonizing the effect of VIP (Youngren et al., 1996b; 1998; 2002; Chaiseha et al., 1997; 2003; Al Kahtane et al., 2003). Up to date, it has been known that dynorphin, serotonin, DA, and VIP all appear to stimulate avian PRL secretion along a pathway expressing κ opioid, serotonergic, DAergic, and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator (for review, see El Halawani et al., 2000).

It has been studied and well documented that gonadotropins and PRL are associated with the reproductive cycle in several avian species (Mashaly et al., 1976; Bluhm et al., 1983; El Halawani et al., 1984; 1997; Myers et al., 1989; Wong et al., 1992; Mauget et al., 1994). The onset of incubation activity is associated with greatly enhanced circulating PRL levels and diminished LH levels in bantam hen (Sharp et al., 1979), turkey (Burke and Dennison, 1980; El Halawani et al., 1984), duck (Goldsmith and Williams, 1980; Bluhm et al., 1983), ring dove (Goldsmith et al., 1981), wild

starling (Dawson and Goldsmith, 1982), spotted sandpiper (Oring et al., 1986), canary (Goldsmith et al., 1984), and white-crowned sparrow (Hiatt et al., 1987). It has been attributed to the fact that increased concentration of PRL plays a role in the cessation of egg laying and broodiness during the active period of laying (Sharp et al., 1998). An elevated level of serum PRL has a negative effect on the reproductive performance, resulting in decreased egg production and broodiness in domestic hen (Lea et al., 1981; Sharp et al., 1988) and turkey (Burke and Dennison, 1980; El Halawani et al., 1980; 1988; Burke et al., 1981; Zadworny et al., 1985; Sharp et al., 1989). The elevated PRL levels and depressed LH levels of incubating hens are maintained by tactile stimuli from the nest and eggs (El Halawani et al., 1980; 1986; Opel and Proudman, 1988) and can be reversed by nest deprivation (El Halawani et al., 1980; Proudman and Opel, 1981; Zadwomy and Etches, 1987; Sharp et al., 1988) or the introduction of chicks (LeBoucher et al., 1990).

In temperate zone birds, the activation of GnRH/FSH-LH and VIP/PRL systems depend upon the duration of day length (photoperiod) and the transduction of photoperiodic information, resulting in either gonad recrudescence and its associated sexual activity or gonad regression and the termination of reproductive activity. Contrary to the temperate zone seasonal breeding species, the native Thai chicken is an equatorial zone continuously breeding species that produces eggs all year long independent of photoperiodic cues. There are a limited number of studies providing data about neuroendocrine regulation in this non-temperate zone gallinaceous bird. The studies demonstrate that plasma progesterone level is related to reproductive cycle of the native Thai chicken (Chotesangasa et al., 1993; Katawatin et al., 1997; Sangkaew et al., 2000). Recently, plasma PRL and LH levels across the reproductive

cycle of the native Thai chicken have first been reported (Kosonsiriluk, 2007; Kosonsiriluk et al., 2007). In addition, the distribution and changes in number of VIP-ir neurons in the brain of the native Thai chicken have been investigated and found to mirror the changes in plasma PRL levels across the reproductive stages (Kosonsiriluk et al., 2006; Kosonsiriluk, 2007). As indicated above, in order to increase the production of native Thai chickens in Thailand, it is very important to understand the basic endocrinology that regulates its reproductive system. Thus, the focus of this study was proposed to investigate the role of PRL and LH in the regulation of the reproductive cycle in the female native Thai chicken. The knowledge gained will provide the information of neuroendocrine regulation including the hormonal and physiological characteristics of the reproductive cycle in the native Thai chicken that will help to improve the productivity of the native Thai chicken.

3.3 Materials and Methods

3.3.1 Experimental Animals

Female native Thai chickens (*Gallus domesticus*), 16-18 weeks of age, Pradoohangdam breed were used. Each hen was identified by wing band number. They were reared and housed together with a mature male (1 male: 8 females) in floor pens under natural light (approximately 12 hours of light and 12 hours of dark; 12L:12D). Feed and water were provided *ad libitum.* The baskets for egg laying were provided. Birds were divided into 4 reproductive stages: non-egg laying (NL), egg laying (L), incubating eggs (B), and rearing chicks (R). The four reproductive stages were identified by behavioral observation. Briefly, NL were birds that had not reached the sexual maturity and had never been laid eggs, L hens, had been laying regularly, B

hens stopped laying and sat on the nest to incubate their eggs, and R hens, after the chicks hatched, stopped incubating eggs, and taking care of the chick. The chickens were observed during their reproductive cycles. Daily records of egg production, nesting activity, and other behaviors during the reproductive cycle were kept. The animal protocols described in this study were approved by Suranaree University of Technology Animal Care and Use Committee Guidelines.

3.3.2 Experimental Design

15 Female native Thai chickens, 16-18 weeks of age, were used. Chickens were observed during their reproductive cycle and were classify into 4 reproductive stages: NL, L, B, and R. Daily records of egg production, nesting activities and other behaviors during reproductive cycle from the beginning until the third reproductive cycle were recorded. Body weights of the birds were recorded weekly. During the experiment, blood samples were collected from the brachial vein in each reproductive stage for two reproductive cycles for determining plasma PRL and LH levels (n=10). Blood samples were fractionated by centrifugation. The plasma samples were stored at -20°C until assayed. Plasma PRL and LH levels were determined utilizing an enzyme-linked immunosorbent assay (ELISA) according to a previously described method (Proudman et al., 2001).

3.3.3 Measurement of Plasma PRL Concentrations

Plasma PRL levels were measured by ELISA technique (Proudman et al., 2001). 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 at the dilution 1:2,000. The plates were incubated at 4°C overnight and then blocked with blocking solution (100 µl of 0.4% casein, 0.01% thimerosal, 1 mM EDTA). 20 µl of samples, 30 µl of the assay buffer (0.1% casein, 0.01% thimerosal, 1 mM EDTA), 25 µl of anti-PRL (1:20,000, provided by Dr. John Proudman, USDA, USA), and 25 µl of β-PRL tracer (1:50,000) were added into the reaction, then incubated at 4°C overnight. The reactions were measured the absorbent at 405 nm. The plasma samples were measured in duplicate within a single assay.

3.3.4 Measurement of Plasma LH Concentrations

Plasma LH levels were measured by ELISA technique (Proudman et al., 2001). 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 at the dilution 1:2,000. The plates were incubated at 4°C overnight and then blocked with blocking solution (100 µl of 0.4% casein, 0.01% thimerosal, 1 mM EDTA). 20 µl of samples, 30 µl of the assay buffer (0.1% casein, 0.01% thimerosal, 1 mM EDTA), and 50 µl of anti-LH (1:10,000, provided by Dr. John Proudman, USDA, USA) diluted in the assay buffer were added into the reactions. The plates were incubated plate overnight at 4°C. The plates were washed and then added 100 µl of LH tracer diluted in the assay buffer at the dilution 1:500 and then incubated at 4°C overnight. The reactions were measured the absorbent at 405 nm. The samples were determined in duplicate within a single assay.

3.3.5 Statistical Analysis

Reproductive characteristics including egg production, hatchability, and the duration of laying, incubating, and rearing were compared among three reproductive cycles. Differences in plasma PRL and LH levels among 4 reproductive stages in each reproductive cycle were compared. Results were expressed as mean ± SEM. Significant differences between reproductive stages were analyzed employing one way analysis of variance (ANOVA) with multiple comparisons were determined using Tukey's HSD test. P value (P) < 0.05 was considered statistically significant. All statistical tests were analyzed employing the SPSS for Windows software (version 13.0, SPSS Inc., Chicago, IL, USA).

3.4 Results

3.4.1 Reproductive characteristic during the reproductive cycle of the native Thai chicken

The reproductive characteristics of the native Thai chicken were observed during three reproductive cycles (Table 3.1). The hens started to lay, in the first laying cycle, at 30.93 $\pm$ 0.88 weeks old. The duration of laying period were compared among three reproductive cycles. In the first laying cycle, the duration of laying period were low (14.67 $\pm$ 1.16 days) when compared with the second and the third laying cycle (27.40 $\pm$ 7.85 days and 18.00 $\pm$ 5.15 days, respectively). However, there was no significantly different between the duration of laying in each reproductive cycle. The number of egg production in each hen was lowest in the first laying cycle (11.00 $\pm$ 0.53 eggs). An increase in the number of egg production was found in the second laying cycle (18.27 $\pm$ 3.35 eggs), and the third laying cycle (15.75 $\pm$ 1.15 eggs). However, no significant difference of egg production was observed among the three

reproductive cycles. The duration of incubating period was essentially the same between the first and second reproductive cycle (19.73 $\pm$ 0.91 and 19.33 $\pm$ 0.33 days), but the duration of incubation period significantly decreased in the third laying cycle (16.88 $\pm$ 0.64 days, $P<0.05$). Although the percentage of hatchability was not significantly different between the first and the second cycle, the durations of rearing period between these two reproductive cycles were significantly different. Apparently, the hens took time for rearing their chicks in the first reproductive cycle (52.87 $\pm$ 5.30 days) longer than that of in the second reproductive cycle (40.73 $\pm$ 2.48 days, $P<0.05$).

3.4.2 Plasma PRL and LH levels during the reproductive cycle of the native Thai chicken

Plasma PRL and LH levels were determined across the reproductive cycle of the native Thai chicken (n=10, Table 3.2). In the first reproductive cycle, plasma PRL levels were low in NL (26.46 $\pm$ 0.65 ng/ml), gradually augmented in L (49.97 $\pm$ 6.90 ng/ml), significantly higher in B (312.10 $\pm$ 27.18 ng/ml, $P<0.05$), and declined dramatically to the same level of L in R (41.75 $\pm$ 8.07 ng/ml). Consequently, as the hens started to lay in the second laying cycle, plasma PRL levels were slightly decreased (39.60 $\pm$ 4.81 ng/ml). Plasma PRL levels sharply increased again in B (274.57 $\pm$ 16.66 ng/ml, $P<0.05$) and markedly declined in R (32.19 $\pm$ 1.37 ng/ml). Overall, plasma PRL levels in the second reproductive cycle were lower than that of in the first reproductive cycle, but there is no significant difference between the two reproductive cycles (Fig. 3.1).

Plasma LH levels was essentially the same throughout the reproductive cycles of the native Thai chicken (n=10, Table 3.2, Fig 3.2). Changes in plasma LH levels

were not observed across the reproductive stages of the first reproductive cycle (NL: 3.99 $\pm$ 0.14, L: 3.77 $\pm$ 0.27, B: 3.59 $\pm$ 0.10, R: 3.96 $\pm$ 0.16 ng/ml). Plasma LH levels slightly increased in the second reproductive cycle (L: 3.86 $\pm$ 0.24, B: 4.24 $\pm$ 0.37, R: 4.23 $\pm$ 0.19 ng/ml). However, there was no significantly different between the first and the second reproductive cycles.

Table 3.1 Reproductive characteristics of the native Thai chickens during the reproductive cycles.

Reproductive Characteristics	Cycle 1 (n=15)	Cycle 2 (n=15)	Cycle 3
Age at First Lay (wk)	30.93 ± 0.88^{a}	43.47 ± 1.38^{b}	55.93 ± 1.78^{c} (n=15)
Duration of Laying (day)	14.67 ± 1.16^{a}	27.40 ± 7.85^{a}	18.00 ± 5.15^{a} (n=10)
Egg Production (egg/hen)	11.00 ± 0.53^{a}	18.27 ± 3.35^{a}	15.75 ± 1.15^{a} (n=10)
Duration of Incubating (day)	19.73 ± 0.91^{a}	19.33 ± 0.33^{ab}	16.88 ± 0.64^{b} (n=5)
Hatchability (%)	63.42 ± 6.78^{a}	59.86 ± 6.08^{a}	-
Duration of Rearing (day)	52.87 ± 5.30^{a}	40.73 ± 2.48^{b}	-

Values are expressed as the mean ± SEM. Values with different superscripts within the same row are significantly different ($P<0.05$).

Table 3.2 Plasma PRL and LH concentrations in each reproductive stage (n=10) during the reproductive cycles of the native Thai chickens.

Reproductive Stage	PRL Concentration (ng/ml)		LH Concentration (ng/ml)	
	Cycle 1	Cycle 2	Cycle 1	Cycle 2
NL	26.46 ± 0.65^{a}	-	3.99 ± 0.14^{a}	-
L	49.97 ± 6.90^{a}	39.60 ± 4.81^{A}	3.77 ± 0.27^{a}	3.86 ± 0.24^{A}
B	312.10 ± 27.18^{b}	274.57 ± 16.66^{B}	3.59 ± 0.10^{a}	4.24 ± 0.37^{A}
R	41.75 ± 8.07^{a}	32.19 ± 1.37^{A}	3.96 ± 0.16^{a}	4.23 ± 0.19^{A}

Values are expressed as the mean ± SEM. Values with different superscripts within the same column are significantly different ($P<0.05$).

Fig. 3.1 Plasma PRL levels in the native Thai chickens in each reproductive stage during the two reproductive cycles (n=10). Values are expressed as the mean ± SEM. Values with different letters are significantly different (P<0.05).

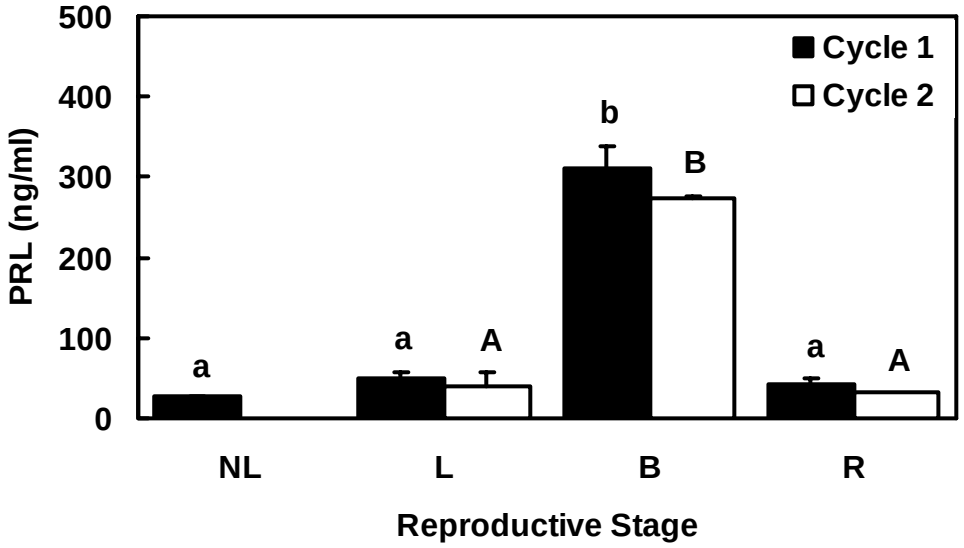
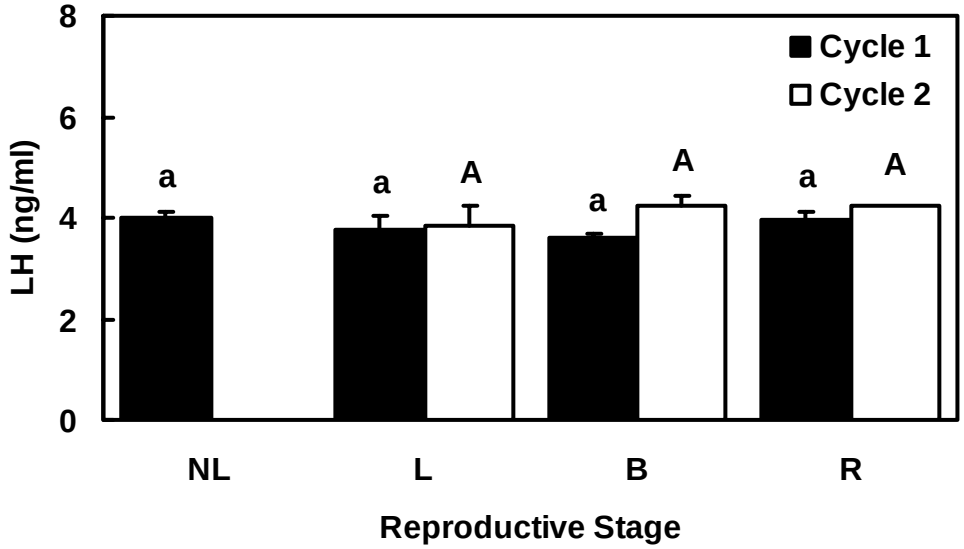


Fig. 3.2 Plasma LH levels in the native Thai chickens in each reproductive stage during the two reproductive cycles (n=10). Values are expressed as the mean ± SEM. Values with different letters are significantly different (P<0.05).



3.5 Discussion

The results of the present study showed that plasma PRL levels changed during the reproductive stages of the native Thai chicken. However, plasma LH levels were essential the same during the reproductive cycle. In addition, the female reproductive characteristics were recorded and compared throughout the three reproductive cycles. Plasma PRL levels were low when birds had not reached sexual maturity. When the chicken started to lay, at the first laying cycle, plasma PRL levels slightly increased. After the hen stopped laying and sat on the nest during incubating stage, circulating PRL was at the highest levels. Consequently, as the chick hatched, plasma PRL levels dropped to the same levels of egg-laying period. When the birds began to lay at the second laying cycle, the pattern of plasma PRL occurred similarly with the first reproductive cycle. However, changes in plasma LH levels were not observed during the reproductive stages. The durations of rearing and duration of incubating were significantly lower in the second and the third reproductive cycles, respectively when compared with the first reproductive cycles.

Changes in concentration of PRL levels were found varied according to the reproductive stages of the native Thai chicken. Corresponding with this present result, it has been studied and well documented that gonadotropins (LH and FSH) and PRL are associated with the reproductive cycle in several avian species (Mashaly et al., 1976; Bluhm et al., 1983; El Halawani et al., 1984; 1997; Myers et al., 1989; Wong et al., 1992; Mauget et al., 1994; Kosonsiriluk et al., 2007). The changes observed in plasma PRL levels in this equatorial species are in good agreement with observations made previously in temperate zone birds. The hormonal profile of PRL during the reproductive cycle of the native Thai chicken was similar to the turkey (El Halawani et

al., 1984). In the turkey hen, circulating PRL levels were very low during the reproductive quiescent stage of the cycle. During egg laying period, the release of gonadotropins which induced sexual receptivity (El Halawani et al., 1986) primed the VIP/PRL system to enhance PRL secretion (El Halawani et al., 1983a). Circulating PRL levels increased dramatically during incubating period (El Halawani et al., 1984) and associated with decreased in the concentrations of plasma LH and ovarian steroids and with the regression of the ovary and oviduct (Sharp, 1980). Furthermore, it has been suggested that this rising PRL level has been implicated as the cause of cessation of ovulation, ovarian regression, and induction of incubation behavior. In addition, this finding is supported by the studies found that changes in pituitary PRL gene expression and its plasma PRL levels were highly correlated during different stages of the reproductive cycle in chicken and turkey (Knapp et al., 1988; El Halawani et al., 1990; Talbot et al., 1991; Wong et al., 1991; You et al., 1995b; Tong et al., 1997; Karatzas et al., 1997). By contrast with this result, in ring dove (*Streptopelia risoria*) in which PRL stimulated crop sac development, PRL levels were not attained in adults until after the young have hatched (Goldsmith, 1983). This study demonstrated that plasma PRL were at the highest levels during incubating stage. Correspondence with this result, PRL has been associated with incubation behavior in pigeon (Riddle et al., 1935), pheasant (Breitenbach and Meyer, 1959), cowbird (Hohn, 1959), turkey (Burke and Dennison, 1980; El Halawani et al., 1988; Youngren et al., 1991), mallard duck (Goldsmith and Williams, 1980), and chicken (Sharp et al., 1988). Administration of exogenous PRL leaded to increase parental behaviors in birds (Buntin et al., 1981; Lea and Vowles, 1986; Macnamee et al., 1986; Pedersen, 1989; Youngren et al., 1991). In addition, active immunization against recombinant-derived PRL reduced the incidence,

delayed the development (March et al., 1994), or prevented the occurrence of incubation behavior in birds. These results, including the present study, support the role of PRL in the regulation and maintenance of incubation behavior in galliform birds.

After hatching, plasma PRL levels sharply decreased from the peak levels in B to the basal levels in R. Correspondent with the present study, a sharp decrease in circulating PRL were found in the temperate zone birds that their eggs were replaced with poults during the transition from incubating eggs to brooding of the young (Opel and Proudman, 1989). Consequently, the hens started to molt and transition to the photorefractory state. At the onset of the photorefractoriness, circulating PRL and LH levels, pituitary PRL/LH peptide contents and their mRNA levels sharply declined, even though long day length continued (Wong et al., 1991; 1992; Mauro et al., 1992; El Halawani et al., 1996). A precipitous decline in PRL and FSH/LH release and their gene expression may be triggered at any time due to a lack of response to long day length or by subjecting the birds to short day lighting (Nicholls et al., 1988; El Halawani et al., 1990). This is not the case in the native Thai chicken in which the duration of day length does not change during the year. It has been reported that long day length may, in parts, play a role on the reproduction of the native Thai chicken by enhance the sexual maturity and the reproductive system of the native Thai chickens. However, it is not clearly to state that the photoperiod regulates the reproduction of native Thai chicken since the development of the gonad was still observed in the short day treatment group (Kosonsiriluk, 2007). In this study, plasma PRL levels also declined after the chick hatched and continued to decrease during R period. However, after rearing the chicks, the native Thai chicken came back to lay in the second laying

cycle without molting and did not need to subject with short day photoperiod like as happened in temperate zone birds. It might be possible that the reproductive cycle of the native Thai chicken is regulated in part by the present of the chick during the rearing period. Thus, the present and constant of maternal behavior, but not the photoperiodic cues, is the factor that might regulates the reproductive system in this non-photoperiodic avian species.

In birds, LH displays a pivotal role in the regulation of ovulation that occurs spontaneously and is hormonally controlled. In most birds for which data existed, circulating LH levels tend to be high during gonadal growth and egg laying and decline during incubation. During ovulatory cycle, plasma levels of LH and other reproductive hormones increased from a basal level to a peak level before ovulation in chicken (Kappauf and van Tienhovan, 1972; Furr et al., 1973; Lague et al., 1975; Etches and Cunningham, 1976; Proudman et al., 1984), turkey (Mashaly et al., 1976; Proudman et al., 1984), Japanese quail (Doi et al., 1980), and duck (Tanabe et al., 1980). During the preovulatory surges of LH in turkey hens, LH concentrations increased from a baseline level to a peak over 2-3 hours then declined from the peak to the baseline level over 4-6 hours (Yang et al., 1997; Liu et al., 2001; Bacon et al., 2002). Increased in plasma LH levels that coincided with the initiation of egg laying were reported in chicken (Furr et al., 1973; Shodono et al., 1975; Wilson and Sharp, 1975), herring gull (Scanes et al., 1974), turkey (Mashaly et al., 1976), white-crowned sparrow (Mattocks et al., 1976; Wingfield and Farner, 1978b), snow geese (Campbell et al., 1978), mallard (Donham et al., 1976; Donham, 1979; Tanabe et al., 1980), and Japanese quail (Doi et al., 1980). In cockatiels, nest inspection and laying were characterized by high LH levels (Myers et al., 1989). A fall in LH secretion at the

onset of incubation has also been reported in ring dove (Cheng and Follett, 1976; Silver et al., 1980), snow geese (Campbell et al., 1978), white-crowned sparrow (Wingfield and Farner, 1978a; 1978b), bantam hen (Sharp et al., 1979), turkey (Cogger et al., 1979), pied flycatchers (Silverin and Goldsmith, 1983), zebra finches (Vleck and Priedkalns, 1985), spotted sandpipers (Oring et al., 1986), and cockatiels (Myers et al., 1989). Although in most birds circulating LH levels tend to be high during gonadal growth and egg laying, there are some exception like as presented in this study. In male white-crowned sparrow, the pattern of increase and decrease of both LH and FSH are more gradual (Hiatt et al., 1987). In ring dove and wild starling, plasma LH levels increased during the nesting period and declined during egg-laying, incubating and after hatching (Cheng and Follett, 1976; Dawson and Goldsmith, 1982). In contrast to the hormonal profile of PRL, changes in concentration of plasma LH were not observed throughout the reproductive stages of the native Thai chicken. It is possible that the sampling times failed to detect occasions when LH levels were higher in this species. However, this result is corresponding with the previous studies indicated that serum LH showed no significant changes for the different reproductive phases (El Halawani et al., 1984; Wong et al., 1992). An increase in serum LH has been shown to precede the decline in circulating PRL in hyperprolactinemic, incubating birds (El Halawani et al., 1983b). In addition, it was shown that changes in plasma LH and PRL are not always synchronous in nest-deprived and re-nesting bantam hens (Sharp et al., 1988). These finding supports the present results that circulating LH levels are not always inversely related to PRL levels in birds.

In birds, PRL and gonadotropins secretions are controlled by closely related mechanisms. From a number of physiological or experimental reproductive

conditions, an inverse relationship between PRL and gonadotropins secretions seems to emerge. It has been suggested that an increase in the concentrations of plasma PRL during incubating period may depress LH secretion (Zadworny and Etches, 1987; Nicholas et al., 1988; El Halawani et al., 1993; Sharp et al., 1998), suggesting that PRL acts centrally to reduce LH levels by reducing hypothalamic GnRH levels (Rozenboim et al., 1993). It is well studied that an elevated level of serum PRL has a negative effect on the reproductive performance, resulting in decreased egg production and broodiness in domestic hen (Lea et al., 1981; Sharp et al., 1988) and turkey (Burke and Dennison, 1980; El Halawani et al., 1980; 1988; Burke et al., 1981; Zadworny et al., 1985; Sharp et al., 1989). In this present study, although plasma PRL levels were not significantly different between the two reproductive cycles, the concentrations of plasma PRL during the second reproductive cycle were slightly lower than that of in the first reproductive cycle. In contrast with plasma PRL levels, plasma LH concentrations were slightly higher during the second reproductive cycle. These results is supported by the finding that the duration of laying and the number of egg production in the second reproductive cycle is higher than that of in the first laying cycle. Taken together, the reproductive performance of the chickens in the second reproductive cycle were better than that of the first cycle. Thus, it might be possible that the duration of rearing in the second reproductive cycle decreased because of an effects of the decreased PRL levels and increased LH levels during the second reproductive cycle. These findings is supported the pivotal role of PRL in the regulation and maintenance of maternal behavior in this species.

It is well studied that in birds, the secretion of LH and PRL is controlled by the releasing hormone, GnRH and VIP, respectively. Several evidences revealed that birds

at the peak level of reproductive activity have more GnRH-ir cells and fibers when compared with sexually inactive or photorefractory birds (Sharp et al., 1990; Hahn and Ball, 1995; Parry et al., 1997; Cho et al., 1998). GnRH levels decreased when birds enter the incubating stage and this decreased is thought to be implemented by the inhibitory effect of PRL, which reached its highest level during this stage (Sharp et al., 1988). The results in the native Thai chicken from this study is in good accordance with the study demonstrating that the number of GnRH-ir neurons were highest during L period of the reproductive cycle. In addition, it has been reported that changes in plasma PRL levels across the reproductive cycle were found to be paralleled with the changes in the number of VIP-ir neurons in the INF area of the native Thai chicken (Kosonsiriluk et al., 2006, Kosonsiriluk, 2007). These findings, taken together, support the roles of GnRH and VIP in correlating with plasma hormonal levels in the regulation of the reproductive cycle of the non-photoperiodic continuous breeding avian species, the native Thai chicken.

In summary, changes in plasma PRL levels were found throughout the reproductive stages of the native Thai chicken. However, plasma LH levels were essentially the same during the reproductive cycle. Significantly different in the durations of incubating and rearing period were observed between each reproductive cycle. However, plasma PRL and LH levels, the duration of egg-laying, the number of egg production, and percentage of hatchability were not significantly different between each reproductive cycle. These findings support the pivotal roles of PRL and LH in the regulation of reproductive system in this equatorial avian species.

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CHAPTER IV

GONADOTROPIN RELEASING HORMONE: LOCALIZATION AND DIFFERENTIAL EXPRESSION ACROSS THE REPRODUCTIVE CYCLE OF THE NATIVE THAI CHICKEN

4.1 Abstract

Avian reproduction is primarily regulated by gonadotropin releasing hormone-I (GnRH-I) which is synthesized by neurosecretory cells in the hypothalamus. This decaneuropeptide stimulates the synthesis and release of pituitary gonadotropins. However, the data of the neuroendocrine regulation of native Thai chicken, a non-seasonally breeding tropical species are limited and need to be further investigated. The expression of gonadotropin releasing hormone/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH) system in temperate zone birds is regulated by a gonad stimulating photoperiod and vary during reproductive stages. The distribution of GnRH-I neurons has been reported in many temperate zone species. The GnRH-I neuronal system needs to be clarified in the native Thai chicken. Differential GnRH-I expression may give us insight into the mechanism(s) underlying the regulation of the reproductive cycle in this species. The distribution of GnRH-I neurons of native Thai chicken brain was elucidated utilizing immunohistochemical technique. In addition, the differential expression of GnRH-I immunoreactive (ir) neurons were compared

across the reproductive cycle. The results revealed that GnRH-I-ir neurons were distributed in a discrete region lying close to the third ventricle from the level of preoptic area through the anterior hypothalamus. The most abundance of GnRH-I-ir neurons was found within the nucleus commissurae pallii (nCPa). Additional GnRH-I-ir neurons were observed in the nucleus preopticus medialis, nucleus anterior medialis hypothalami, nucleus paraventricularis magnocellularis, regio lateralis hypothalami, nucleus septalis lateralis, nucleus ventrolateralis thalami, and nucleus dorsolateralis anterior thalami, pars magnocellularis. GnRH-I-ir fibers were mainly bilaterally located along the third ventricle with more abundance around the organum vasculosum lamina terminalis and very dense fibers were observed in the external layer of the median eminence, which has been reported for other avian species. Changes in number of GnRH-I-ir neurons in the nCPa were observed across the reproductive cycle. The number of GnRH-I-ir neurons in the nCPa was the highest in laying hens when compared with other reproductive stages. These results indicated that GnRH-I are correlated with the reproductive cycle in the native Thai chicken. This present study confirms a pivotal role of GnRH-I in the control of avian reproduction of this non-seasonally breeding tropical species.

4.2 Introduction

Gonadotropin releasing hormone (GnRH) is a hypothalamic neuronal secretory decapeptide that is important for controlling of reproduction in birds. GnRH is synthesized by the hypothalamus, released from the median eminence (eminentia mediana; ME) into the hypophyseal portal vessels, and then transported to the pituitary gland, where it stimulates the secretion of gonadotropins (luteinizing

hormone; LH, follicle stimulating hormone; FSH, Ulloa-Aguirre and Timossi, 2000; Shalev and Leung, 2003). It has been stated that this decapeptide plays a pivotal role in the control of avian reproduction. Two forms of GnRH are identified in birds which are chicken GnRH-I or cGnRH-I as referred to GnRH-I ([Gln8]-GnRH) and cGnRH-II ([His5, Trp7, Ty8]-GnRH; King and Millar, 1982; Miyamoto et al., 1982; Millar and King, 1984; Sherwood et al., 1988). All the available evidences suggest that only GnRH-I has a physiological role in regulating of gonadotropins secretion (Sharp et al., 1990). Ovarian development is found to be correlated with plasma LH levels and the amount of GnRH-I content, indicating the expression of the GnRH-I is important to maintain pituitary-ovarian function in chicken (Dunn et al., 1996). It has been reported for sometime that GnRH-I increases FSH and LH secretion of the adenohypophysis both *in vitro* and *in vivo* (Peczely, 1989). Incubation of turkey anterior pituitary cells with GnRH-I results in an increase in LH-β-subunit gene expression and stimulates LH secretion (You et al., 1995). GnRH-I and GnRH-II release FSH and LH differentially from *in vitro* chicken pituitary (Millar et al., 1986). Injection of GnRH-I increases plasma LH levels in the white-crowned sparrow, European starling, and chicken (Wingfield et al., 1979; McNaughton et al., 1995; Guemene and Williams, 1999). GnRH-I stimulates LH secretion, but not affect FSH concentration, when administrated to 3 weeks old cockerels (Krishnan et al., 1993). In addition, GnRH agonists may imitate the native hormone and induces an endogenous LH surge (Shalev and Leung, 2003).

A number of previous studies have examined the distribution of GnRH-I neurons/fibers throughout the avian brain including chicken (Jozsa and Mess, 1982; Sterling and Sharp, 1982; Mikami et al., 1988; Kuenzel and Blahser, 1991), duck

(McNeill et al., 1976; Bons et al., 1978), white-crowned sparrow (Blahser et al., 1986; 1989), Japanese quail (Foster et al., 1988; Mikami et al., 1988; Perera and Follett, 1992; van Gils et al., 1993; Teruyama and Beck, 2000), European starling (Dawson et al., 1985; Foster et al., 1987; Goldsmith et al., 1989), garden warbler (Bluhm et al., 1991), great tit and ring dove (Silver et al., 1992), turkey (Millam et al., 1993), dark-eyed junco (Saldanha et al., 1994), and house sparrow (Hahn and Ball, 1995). Immunohistochemical localization studies of GnRH-I reveal three groups of GnRH-I immunoreactive (ir) cells: (1) a telencephalic group medial to the lateral ventricles; (2) a basotelencephalic group located ventral to the tractus septomesencephalicua (TSM) and extending laterally and dorsocaudally; and (3) a distinctive group of cells located along the midline extending from the preoptic area to septal regions (Foster et al., 1987; Millam et al., 1993; 1998; Teruyama and Beck, 2000). Furthermore, GnRH-I-ir fibers are found to project into the ME from two difference sources. First, GnRH-I-ir fiber bundles appear to originate in the preoptic and supraoptic regions, projecting along the wall of the ventricle and eventually entering the ME. The second fiber bundles originate dorsal to the preoptic area and project along and terminate on the walls of the third ventricle (V III). They form a neuronal network that extends throughout the infundibular regions before entering the ME (Foster et al., 1987). Specific GnRH-I-ir neurons are found in several hypothlalamic regions including the preoptic-anterior hypothalamus, nucleus preopticus medialis (POM), nucleus anterior medialis hypothalami (AM), nucleus paraventricularis magnocellularis (PVN), and nucleus commissurae pallii (nCPa). Additional scattered neurons are also found in the nucleus septalis lateralis (SL) and around the organum vasculosum laminae terminalis (OVLT). Several studies have reported the distribution of the GnRH-I mRNA and

protein in the avian brains (Millam et al., 1989; Dunn and Sharp, 1999; Sun et al., 2001; Dawson et al., 2002; Kang et al., 2006). In cockerel, fully processed GnRH-I mRNA and a variant transcript with a retained intron 1 are observed in the preoptic area, the basal hypothalamus, the anterior pituitary gland, and testis (Sun et al., 2001). Utilizing the *in situ* hybridization indicates that GnRH-I mRNA expression is greatest in the nCPa and around the OVLT of the turkey brain (Kang et al., 2006).

Changes in hypothalamic GnRH content and release are correlated with several factors, such as photoperiod and reproductive condition. The effects of reproductive condition on GnRH secretion in avian species are well documented. Several evidences reveal that birds at the peak level of reproductive activity have more GnRH-ir cells and fibers when compared with sexually inactive or photorefractory birds (Sharp et al., 1990; Hahn and Ball, 1995; Parry et al., 1997; Cho et al., 1998). Measurements of hypothalamic GnRH peptide in the hypothalamus during the reproductive cycle of the turkey (Millam et al., 1989; El Halawani et al., 1993; Rozenboim et al., 1993) and chicken (Dunn et al., 1996) indicate that there is no change or a decrease in incubating birds. Moreover, GnRH contents of discrete medial preoptic, infundibulum, and arcuate samples are in laying hens than that of non-laying hens (Advis et al., 1985). During incubation, GnRH concentration is significantly elevated in the POA area (Millam et al., 1995). The amount of GnRH in the hypothalamus decreases during photorefractoriness (Dawson et al., 1985; Foster et al., 1987; Bluhm et al., 1991; Rozenboim et al., 1993; Saldanha et al., 1994; Hahn and Ball, 1995). Regarding GnRH-I mRNA expression, it has been reported that the hypothalamic GnRH-I mRNA expression is greater in laying hen than that of in incubating (Dunn et al., 1996; Kang et al., 2006) and lowest in photorefractoriness

hens (Kang et al., 2006).

Photoperiodic cue appears to be important for the onset of reproduction for most birds in temperate zones. The stimulatory effect of long days appears usually to be associated with an increased GnRH content or increased immunoreactivity for GnRH in the hypothalamus and ME in avian species (Dawson et al., 1985; Foster et al., 1987; 1988; Goldsmith et al., 1989; Perera and Follet, 1992; Saldanha et al., 1994; Hahn and Ball, 1995). In the other hand, the photorefractoriness has been shown to correlate with great reduction in GnRH-ir structures in birds (Foster et al., 1987; Dawson et al., 1985; Goldsmith et al., 1989; Bluhm et al., 1991; Saldanha et al., 1994; Hahn and Ball, 1995; Cho et al., 1998; Marsh et al., 2002). These findings support the role of photoperiod in correlated with GnRH to regulate the reproductive system in temperate zone birds. Contrary to the temperate zone seasonal breeding species, the native Thai chicken is an equatorial zone continuously breeding species that produces eggs all year long independent of photoperiodic cues. There are a limited number of studies providing data regarding neuroendocrine regulation in this non-temperate zone gallinaceous bird. Importantly, there is no study delineating the anatomical distribution and functional aspect of the GnRH system in the native Thai chicken. To further understand the neuroendocrine regulation of reproduction in the native Thai chicken, this present immunohistochemistry study was designed to investigate the distribution of GnRH-I-ir neurons throughout the brain. In addition, changes in number of GnRH-I-ir neurons were measured at different reproductive stages. The findings from this proposed study will help to understand the basic neuroendocrine regulation of the native Thai chicken reproductive cycle.

4.3 Materials and Methods

4.3.1 Experimental Animals

30 Female native Thai chickens (*Gallus domesticus*), 16-18 weeks of age, Pradoohangdam breed were used. They were reared and housed together with a mature male (1 male: 7-8 females) in floor pens under natural light (approximately 12 hours of light and 12 hours of dark; 12L:12D). Feed and water were provided *ad libitum.* Birds were divided into 4 reproductive stages: non-egg laying (NL), egg laying (L), incubating eggs (B), and rearing chicks (R). The four reproductive stages were identified by behavioral observation and postmortem examination. Birds were sacrificed according to their reproductive stages. Briefly, NL were birds that had never been laid eggs, L hens, in their first laying cycle, had been laying for 7 days, B hens stopped laying and exhibited incubating behavior for 10 days, and R hens had been rearing chicks for 2 weeks. Blood samples were withdrawn from a brachial vein to analyze plasma PRL levels by enzyme-linked immunosorbent assay (ELISA) as an aid to confirming reproductive condition. The animal protocols described in this study were approved by Suranaree University of Technology Animal Care and Use Committee Guidelines.

4.3.2 Experimental Design

4.3.2.1 Experiment I

To determine the distribution of GnRH-I system in the brain of the native Thai chicken, laying hens (n=6) were used to study the localization of GnRH-I-ir neurons and fibers throughout the brain. The brains were fixed by pressure-perfused prior to section and used for further processed by immunohistochemistry. Plasma PRL levels

and a postmortem examination of each hen were performed to confirm its reproductive status.

4.3.2.2 Experiment II

To determine the changes in number of GnRH-I-ir neurons within the nCPa across the reproductive stage, native Thai chickens in each reproductive stage (NL, L, B, R) were used (n=6). The brains were fixed by pressure-perfused prior to section and used for further processed by immunohistochemistry. Plasma PRL levels and a postmortem examination of each hen were performed to confirm its reproductive status.

4.3.3 Processing of Tissues for Immunohistochemistry

After collecting a blood sample and prior to perfusion, each bird was intravenously injected with 3 ml of heparin (Baxter Healthcare Corporation, Deerfield, IL, USA; 1000 unit/ml) and then euthanized with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France; 2 ml/kg). The head was removed and immediately pressure-perfused via the carotid arteries with 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 3-5 minutes followed by a freshly prepared 4% paraformaldehyde (pH 7.4) for 30 minutes according to a previously described method (Al-Zailaie et al., 2006). The brain was then removed from skull with the pituitary attached and placed in 20% sucrose in PBS at 4^{o}C for 48 hours or until saturated for cryoprotection. The brain was then frozen in powdered dry ice for 1 hour, and stored at -35^{o}C until sectioned. Frozen brains were sectioned in coronal plane at a thickness of 16 μm using a cryostat (Leica CM1850, Leica Instruments

GMbH, Nussioch, Germany). Sections were mounted onto a gelatin-subbed slide with 2 sections per slide and stored desiccated at -20^{o}C until further processed for immunohistochemistry.

4.3.4 Immunohistochemistry

In order to localize GnRH-I distribution throughout the brain of the laying hen and characterize the changes in GnRH-I-ir neurons within the nCPa in different reproductive stages, immunohistochemistry was performed as previously described (Al-Zailaie et al., 2006). Briefly, tissue sections of different areas throughout the brains of laying hens (n=6) and four adjacent sections of the nCPa area in each bird (n=6) according to each reproductive stage were placed in PBS for 30 minutes at room temperature. After PBS removal, each section was incubated with 60 µl primary rabbit monoclonal antibody directed against GnRH-I (generously provided by Dr. J.R. Millam, University of California, Davis, USA) diluted 1:1000 with PBS (pH 7.4) containing 1% bovine serum albumin and 0.3% triton-X at 4^{o}C in a moist chamber for 24 hours. The next day, after removal of excess antibody, the sections were then washed 3 times in PBS for 5 minutes each. After washing, 60 µl of secondary antibody Cy^{TM}3-conjugated AffiniPure Donkey Anti-Rabbit IgG (diluted 1:500, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was applied on each section. The sections were further incubated in a moist dark chamber at room temperature for 1 hour. The slides were then rinsed with PBS to stop the reaction, washed again 3 times in PBS for 5 minutes each, and finally coverslipped using DPX mountant (Sigma-Aldrich, Inc., Steinheim, Germany).

4.3.5 Image Analysis

An atlas of the chick brain (Kuenzel and Masson, 1988) was used to identify the areas of the brain that expressed GnRH-I-ir neurons and/or fibers. Microscopic images of brain sections were visualized with a fluorescence microscope (Olympus IX71, Tokyo, Japan) at 4x, 10x, 20x, and 40x magnification. Images were captured with a digital camera (Olympus DP70, Tokyo, Japan), and stored by DP70-BSW software (Olympus, Tokyo, Japan). To characterize the differential expression of the GnRH-I system across the reproductive cycle, four adjacent brain sections corresponding to the nCPa from each bird according to each reproductive stage (n=6) were chosen and counted manually to compare the number of GnRH-I-ir neurons in the nCPa area. The specificity of the antibody used in this study was tested by omission of the primary antibody during that step of immunohistochemistry, resulting in the absence of GnRH-I immunoreactivity.

4.3.6 Statistical Analysis

Significant differences in the number of GnRH-I-ir neurons (mean ± SEM) in the nCPa area across the reproductive stage were compared employing one way analysis of variance (ANOVA). Significant differences between reproductive stages with multiple comparisons were determined using Tukey's HSD test. $P<0.05$ was considered statistically significant. All statistical tests were analyzed employing the SPSS for Windows software (version 13.0, SPSS Inc., Chicago, IL, USA).

4.4 Results

4.4.1 Experiment I

Immunohistochemistry localization study of GnRH-I in the laying native Thai chicken revealed that the distribution and appearance of GnRH-I-ir neurons were spanned the length of the hypothalamus from the preoptic region, anterior hypothalamus to the end of the septal region. GnRH-I fibers were mainly bilaterally located along the third ventricle with more abundance around the OVLT and very dense fibers were observed in the external layer of the ME. Schematic representations of the distribution of GnRH-I-ir neurons and fibers throughout the brain are shown in Fig. 4.1.

4.4.1.1 The prepotic region

The first group of GnRH-I-ir neurons occurred at the most rostral extent of the preoptic area in the region ventral to the TSM (Fig. 4.1A). At this level, a small amount of GnRH-I-ir neurons were found extending from the V III into the POM (Fig 4.2A). With in the POM, the oval shape with a monopolar process neurons containing GnRH-I immunoreactivity were observed (Fig. 4.2B). Another group of GnRH-I-ir neurons were observed at more laterally. It is a sparse population of GnRH-I-ir neurons, spindle shape-liked, formed a narrow and elongated group forming a line adjacent and parallel to the floor of the brain (Fig 4.2C). Very dense GnRH-I-ir fibers were observed in and around the OVLT (Fig. 4.3A). Just dorsal to the OVLT, a moderate number of GnRH-I-ir neurons and fibers occurred, in and adjacent to the nucleus preopticus periventricularis (POP; Fig 4.2D, 4.3B). Some of GnRH-I-ir fibers were observed around the ventral tips of the ventriculus lateralis (lateral ventricle,

VL), near the nucleus accumbens (Ac), but not all were in the well-defined area of this nucleus.

4.4.1.2 The hypothalamic region

At the rostral part of the hypothalamus, a few GnRH-I-ir neurons, mainly bipolar cells were located along the midline in the AM (Fig. 4.4A) and PVN (Fig. 4.4B). The OVLT, where a discrete group of GnRH-I-ir fibers were innervated, was now position more dorsally than on its first appearance at the base of the brain. Other identified GnRH-I-ir neurons appear to be part of the nucleus preopticus medianus (POMn). The POMn is a sexually dimorphic nucleus well described in the Japanese quail (Adkins-Regan and Watson, 1990), lies very close to the V III and ventral to the anterior commissure (CA). At more laterally, many sparsely scattered GnRH-I-ir neurons were found in and around the regio lateralis hypothalami (lateral hypothalamic area, LHy; Fig. 4.5A). All perikaya immunostained in the LHy were bipolar, fusiform in shape, and less immunoreactive fibers were found coexisted (Fig. 4.5B). Other groups of GnRH-I-ir neurons were seen more lateral in the nucleus ventrolateralis thalami (VLT; Fig. 4.5C), as well as to the tip of the TSM in the nucleus dorsolateralis anterior thalami and pars magnocellularis (DLAmc; Fig. 4.5D). GnRH-I-ir neurons in the DLAmc immunostained less intensely when compared with larger GnRH-I-ir neurons were found in all other groups. GnRH-I-ir fibers were line symmetrically along the V III, in the nucleus periventricularis hypothalami (PHN; Fig. 4.6A). The more extensive GnRH-I-ir fibers were observed at the base of the V III in the nucleus suprachiasmaticus, pars medialis (SCNm; Fig. 4.6B). Moving in caudal hypothalamus, very intense GnRH-I-ir fibers were innervated in the external layer of

the ME (Fig. 4.7A, 4.7B). Small numbers of GnRH-I-ir fibers were found in the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN; Fig. 4.7C). There were no GnRH-I-ir neuron observed in the IH-IN area, and also no immunostaining observed in the pituitary (Pit; Fig. 4.7A).

4.4.1.3 The septal region

The clearest group of GnRH-I-ir neuron and fibers was found within and about the nCPa. GnRH-I-ir neurons were most abundance in the region from the nCPa to the caudal most septal area. At the rostral part, GnRH-I-ir neurons and fibers found in the nCPa started to appear in a triangular group of fibers just dorsal to the CA (Fig 4.8A). More caudally, GnRH-I-ir neurons and fibers in the dorsal region of the nCPa changed their overall pattern from a triangular to an oval shape. These neurons were large and found close to the midline on the both side of the V III (Fig. 4.8B, 4.8D). At this level of the brain, a number of GnRH-I-ir fibers was found within the organum subseptale (supseptal organ, SSO; Fig. 4.8C), the proposed circumventricular organ (CVO) in birds.

Another dense group of GnRH-I-ir fibers was located near the nCPa and extended dorsally and laterally toward the caudal end of the SL. A dense plexus of GnRH-I-ir fibers in the SL were coursed very close to the ventral horn of the VL (Fig. 4.9A). Some of GnRH-I-ir fibers found in the SL was found to overlap slightly within the nucleus septalis medialis (SM). However, only a limited number of GnRH-I-ir neurons was seen in the SL. GnRH-I-ir neurons in the SL were very small and immunostained less intensely compared with larger GnRH-I-ir neurons found in all other groups (Fig. 4.9B). The last group of GnRH-I-ir fibers occurred at the end of the

septal region at the level at which it separated from the thalamus.

4.4.2 Experiment II

The present study indicated that GnRH-I-ir neurons were found distributed throughout the hypothalamus, from the rostral part of the preoptic region to the end of the septal region. The most dense GnRH-I-ir neurons were observed in the nCPa. Changes in number of GnRH-I-ir neurons within the nCPa were found across the reproductive cycle of the native Thai chicken (Fig. 4.10, 4.11). The number of GnRH-I-ir neurons in the nCPa was low in pre-pubertal NL stage (2.29 $\pm$ 1.24 cells). When the hens reached sexual maturity and started laying, the number of GnRH-I-ir neurons sharply increased (14.90 $\pm$ 1.93 cells, $p<0.05$) to the highest levels. The number of GnRH-I-ir neurons slightly decreased after the hen stop laying and become incubating the eggs, (5.63 $\pm$ 2.40 cells). Finally, the number of GnRH-I-ir neurons decline to the lowest level during rearing stage (0.38 $\pm$ 0.24 cells). These relationships were not observed within other areas of the hypothalamus.

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)

Ac	Nucleus accumbens
AM	Nucleus anterior medialis hypothalami
CA	Anterior commissure
DLAmc	Nucleus dorsolateralis anterior thalami, pars magnocellularis
HL	Nucleus habenularis lateralis
HM	Nucleus habenularis medialis
IH	Nucleus inferioris hypothalami
IN	Nucleus infundibuli hypothalami
LHy	Regio lateralis hypothalami (Lateral hypothalamic area)
LSO	Organum septi laterale (Lateral septal organ)
ME	Eminentia mediana (Median eminence)
nCPa	Nucleus commissurae pallii
OVLT	Organum vasculosum lamina terminalis
PHN	Nucleus periventricularis hypothalami
Pit	Pituitary
PVN	Nucleus paraventricularis magnocellularis
POM	Nucleus preopticus medialis
POMn	Nucleus preopticus medianus
POP	Nucleus preopticus periventricularis
SCNm	Nucleus suprachiasmaticus, pars medialis

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), continued.

SL	Nucleus septalis lateralis
SM	Nucleus septalis medialis
SSO	Organum subseptale (Supseptal organ)
TSM	Tractus septomesencephalicus
V III	Ventriculus tertius (Third ventricle)
VL	Ventriculus lateralis
VLT	Nucleus ventrolateralis thalami
VMN	Nucleus ventromedialis hypothalami

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

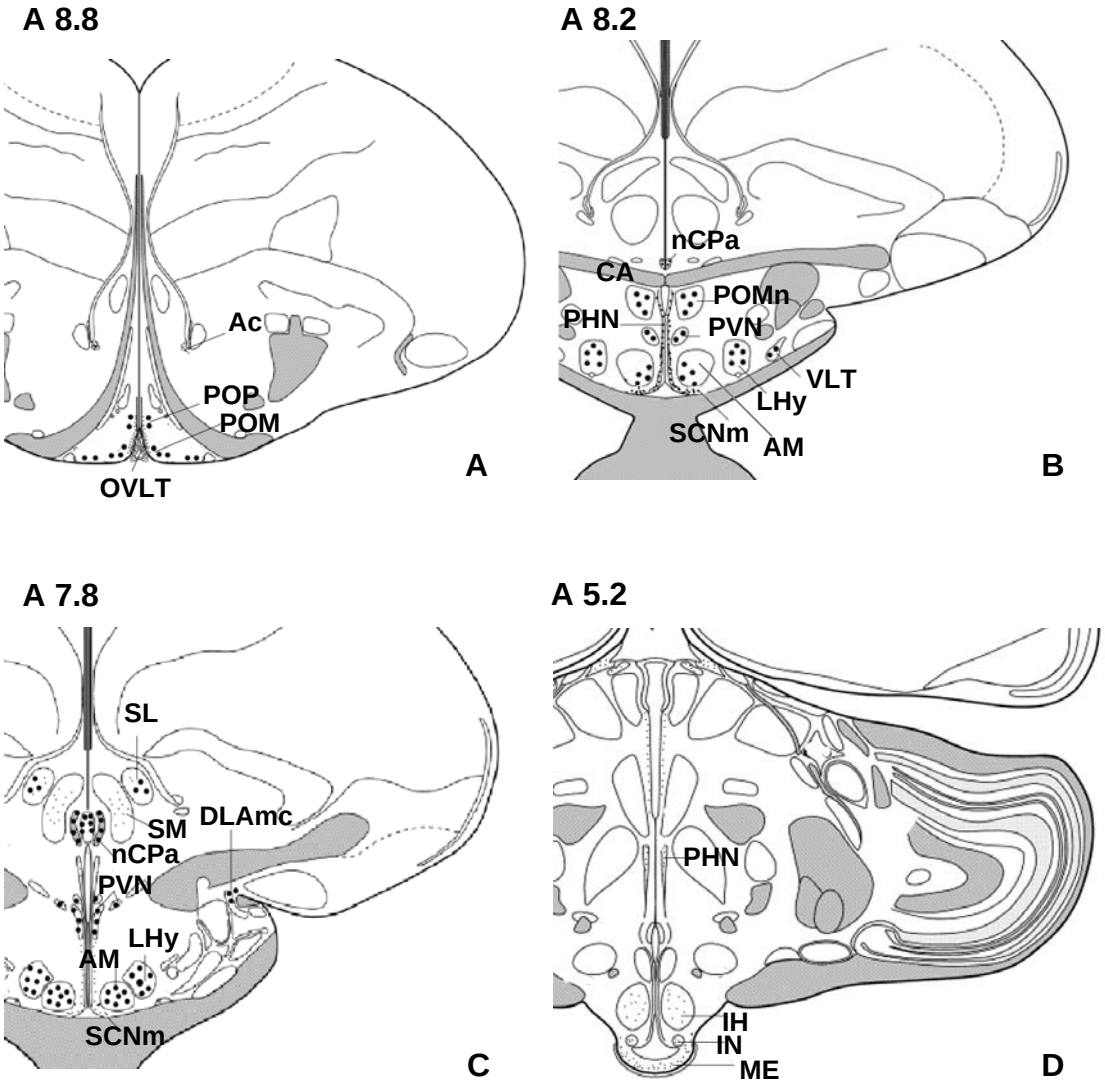


Fig. 4.2 Photomicrographs illustrating the distribution of GnRH-I-ir neurons in the preoptic area. (**A**) GnRH-I-ir neurons in the POM. Rectangles indicate areas from which following photomicrographs were taken. (**B**) Higher magnification from (**A**) showed an oval shape with monopolar process neurons in the POM. (**C**) A sparse population of GnRH-I-ir neurons, spindle shape-liked, formed a narrow and elongated group forming a line adjacent and parallel to the floor of the brain. (**D**) Higher magnification of the GnRH-I-ir neurons in the POP. Bar=50 μm. For abbreviations, see Table 4.1.

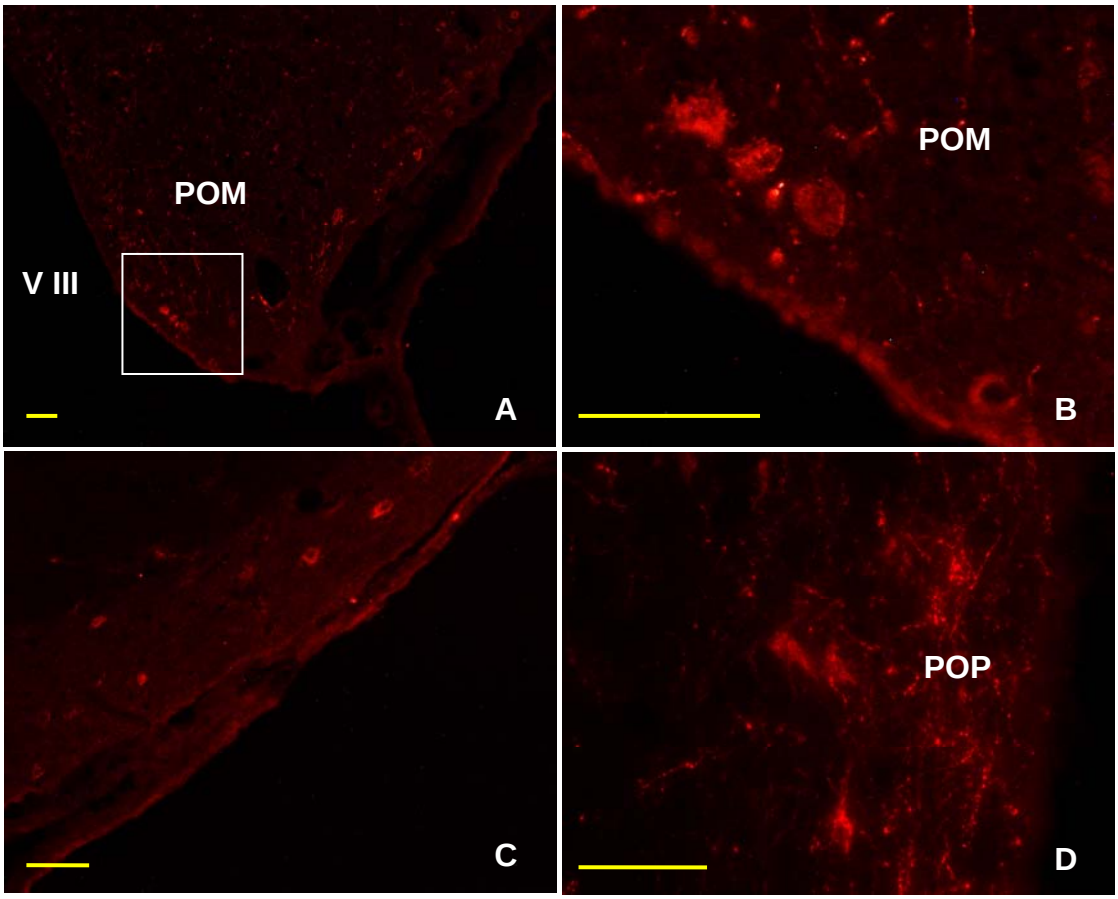


Fig. 4.3 Photomicrographs illustrating the distribution of GnRH-I-ir fibers in the OVLT (**A**) and POP (**B**). Bar=50 µm. For abbreviations, see Table 4.1.

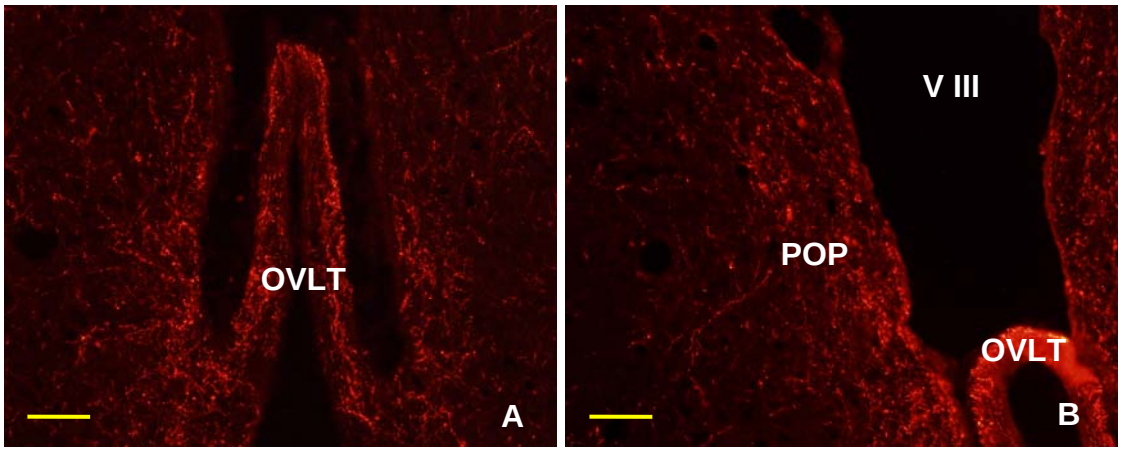


Fig. 4.4 Photomicrographs illustrating the distribution of GnRH-I-ir neurons in the AM (**A**) and PVN (**B**). Bar=50 µm. For abbreviations, see Table 4.1.

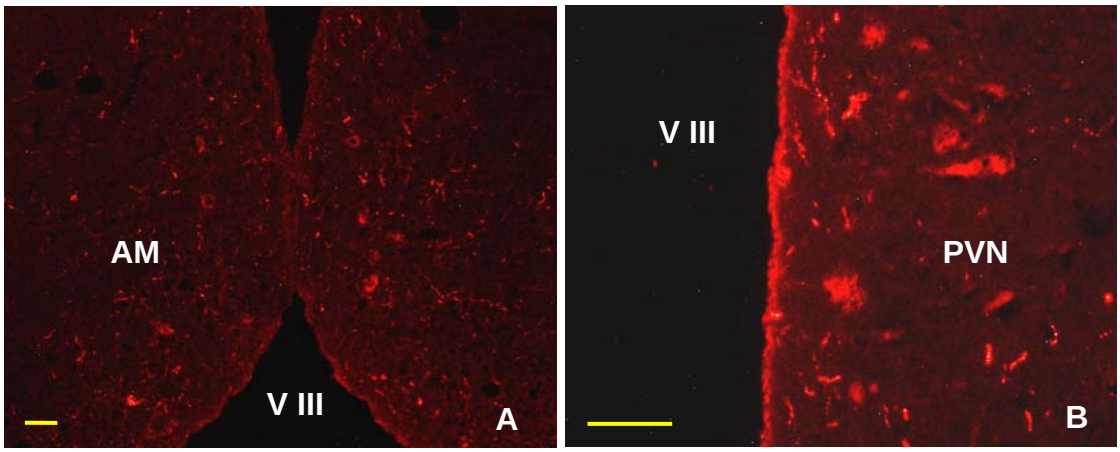


Fig. 4.5 Photomicrographs illustrating the distribution of GnRH-I-ir neurons in the hypothalamic region and at more laterally region. Scattered GnRH-I-ir neurons were distributed in the LHy (**A**). The characterization of GnRH-I-ir neurons observed in the LHy were usually spindle and bipolar in shape (**B**). At more laterally, small groups of oval shape GnRH-I-ir neurons were found in the VLT (**C**) and DLAmc (**D**). Bar=50 μm. For abbreviations, see Table 4.1.

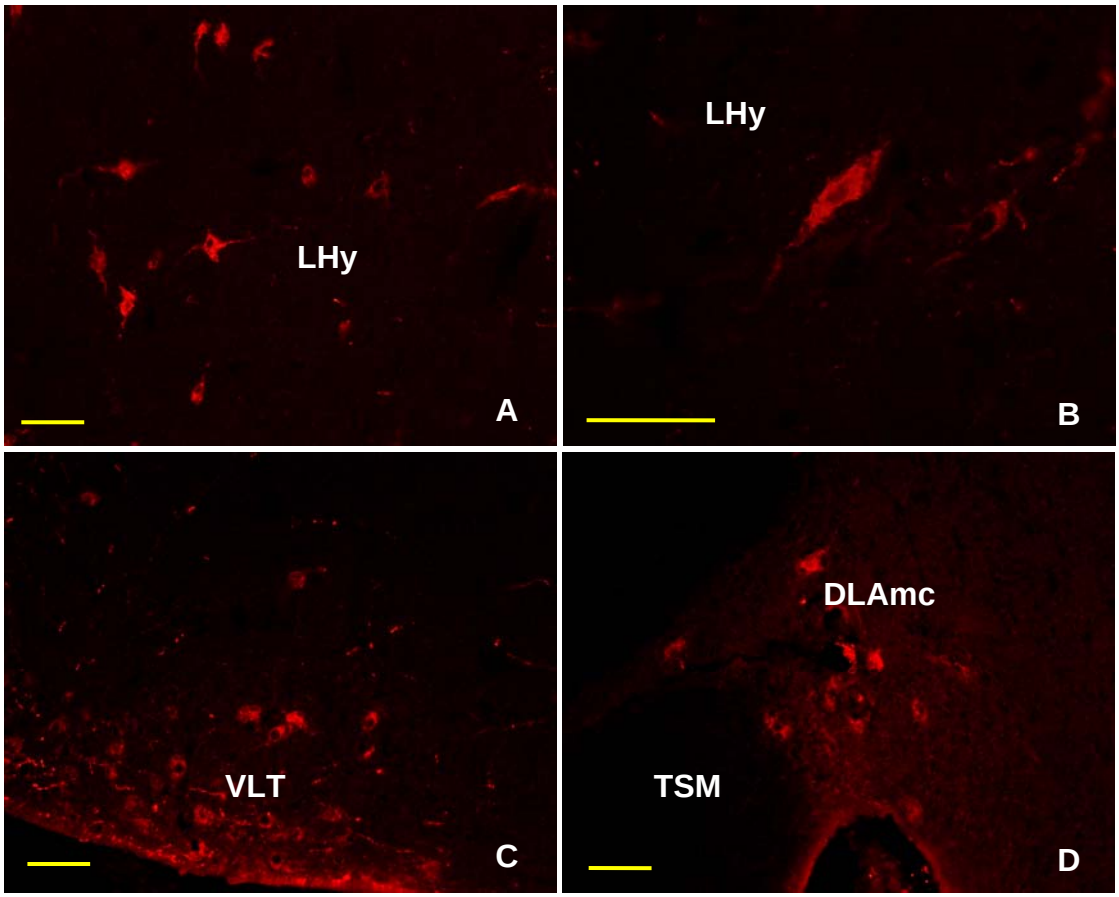


Fig. 4.6 Photomicrographs illustrating the distribution of GnRH-I-ir fibers in the lateral hypothalamic region. GnRH-I-ir fibers were lined symmetrically along the V III in the PHN (**A**). The more extensive GnRH-I-ir fibers were observed at the base of the V III in the SCNm (**B**). Bar=50 μm. For abbreviations, see Table 4.1.

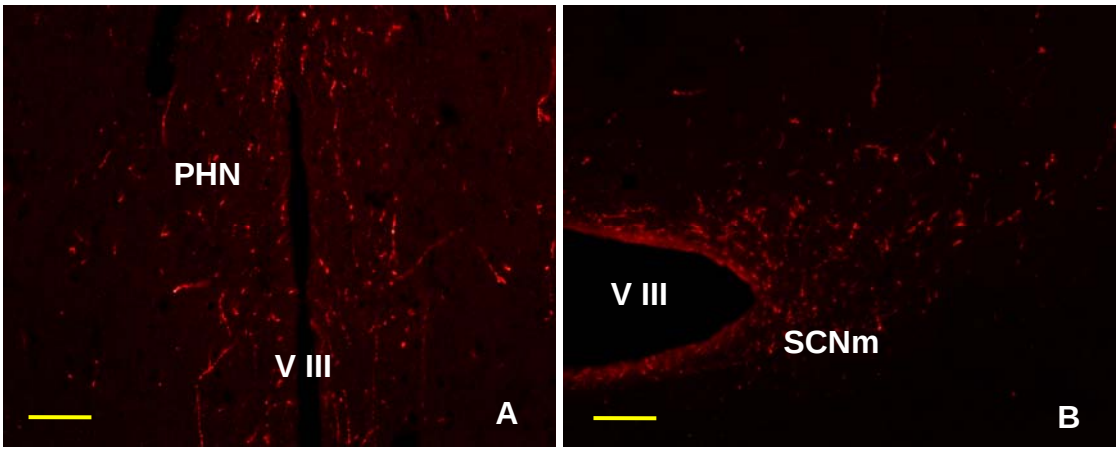


Fig. 4.7 Photomicrographs illustrating the distribution of GnRH-I-ir fibers in the IN-IH area. Very intense GnRH-I-ir fibers were innervated at the external layer of the ME, note that there was no GnRH-I immunoreacitivty observed in the pituitary (**A**). Bar=100 µm. (**B**) Higher magnification of GnRH-I-ir fibers in the ME. Small numbers of GnRH-I-ir fibers were found in the IH (**C**). Bar=50 µm. For abbreviations, see Table 4.1.

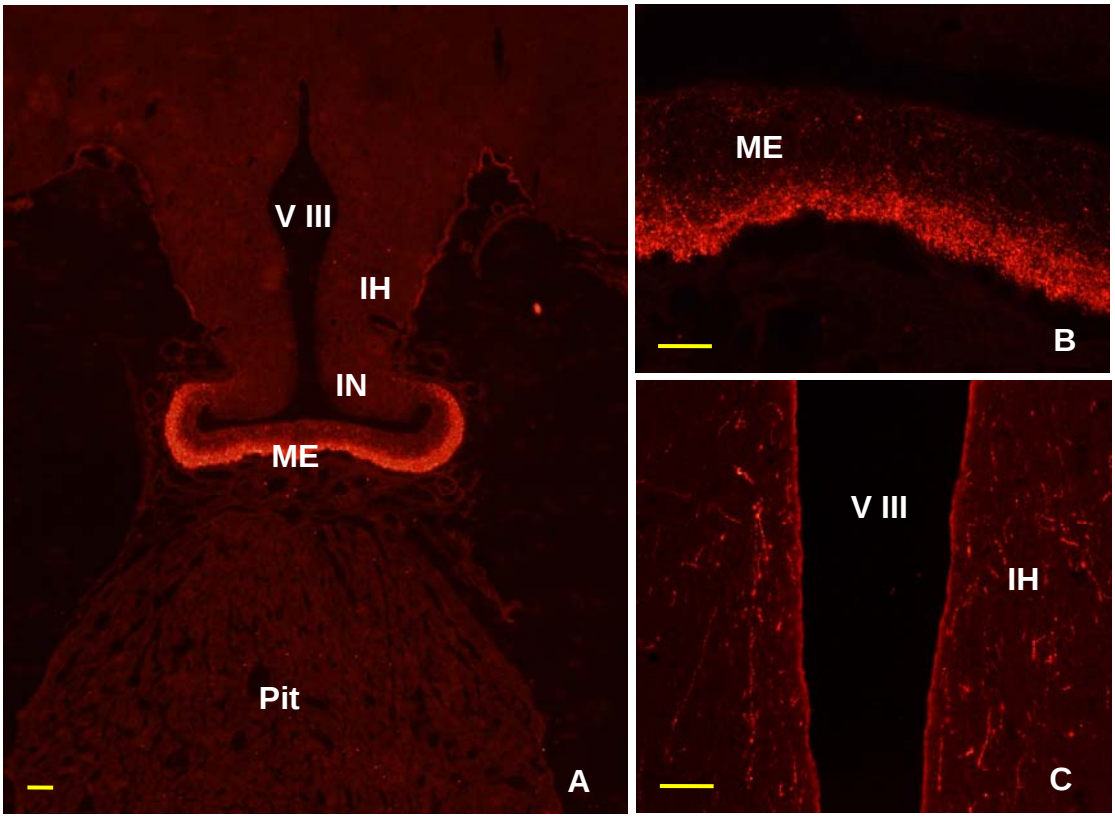


Fig. 4.8 Photomicrographs illustrating the distributions of GnRH-I-ir neurons and fibers in the nCPa. In the rostral part, GnRH-I-ir fibers in the nCPa appeared in a triangular group of fibers just dorsal to the CA (**A**). GnRH-I-ir neurons were large and found close to the midline on the both side of the V III when moving more caudally (**B** and **C**). At this plane of section, GnRH-I-ir fibers were found within the SSO (**C**). (**D**) Higher magnification of GnRH-I-ir neurons found in the nCPa. Bar=50 µm. For abbreviations, see Table 4.1.

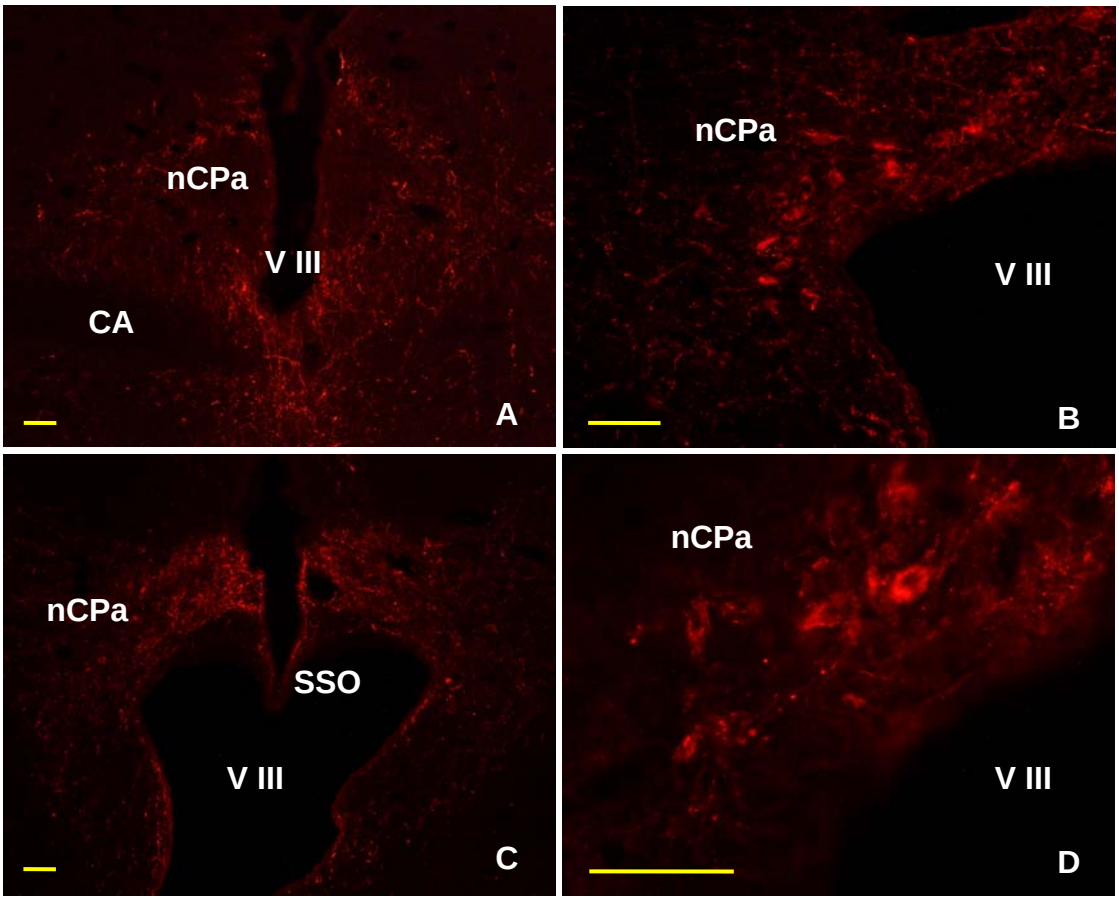


Fig. 4.9 Photomicrographs illustrating the distributions of GnRH-I-ir neurons and fibers in the SL. A dense plexus of GnRH-I-ir fibers was located near the nCPa and extended dorsally and laterally toward the lateral ventricle in the SL (**A**). Higher magnification of GnRH-I-ir neurons in the SL showed a very small and immunostained less intensely compared with larger GnRH-I-ir neurons found in all other groups (**B**). Bar=50 μm. For abbreviations, see Table 4.1.

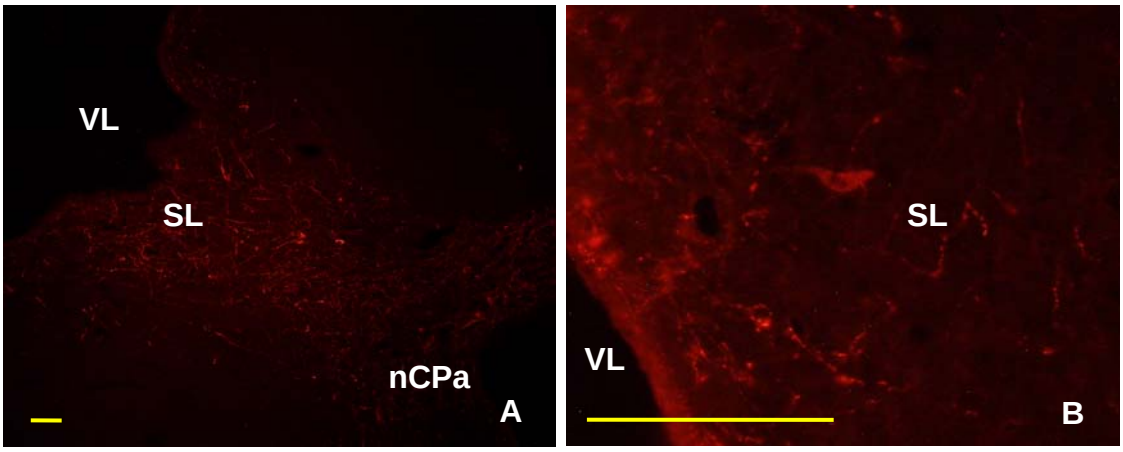


Fig. 4.10 Photomicrographs illustrating the expression of GnRH-I-ir neurons in the nCPa during different reproductive stages. Bar=100 μm. For abbreviations, see Table 4.1.

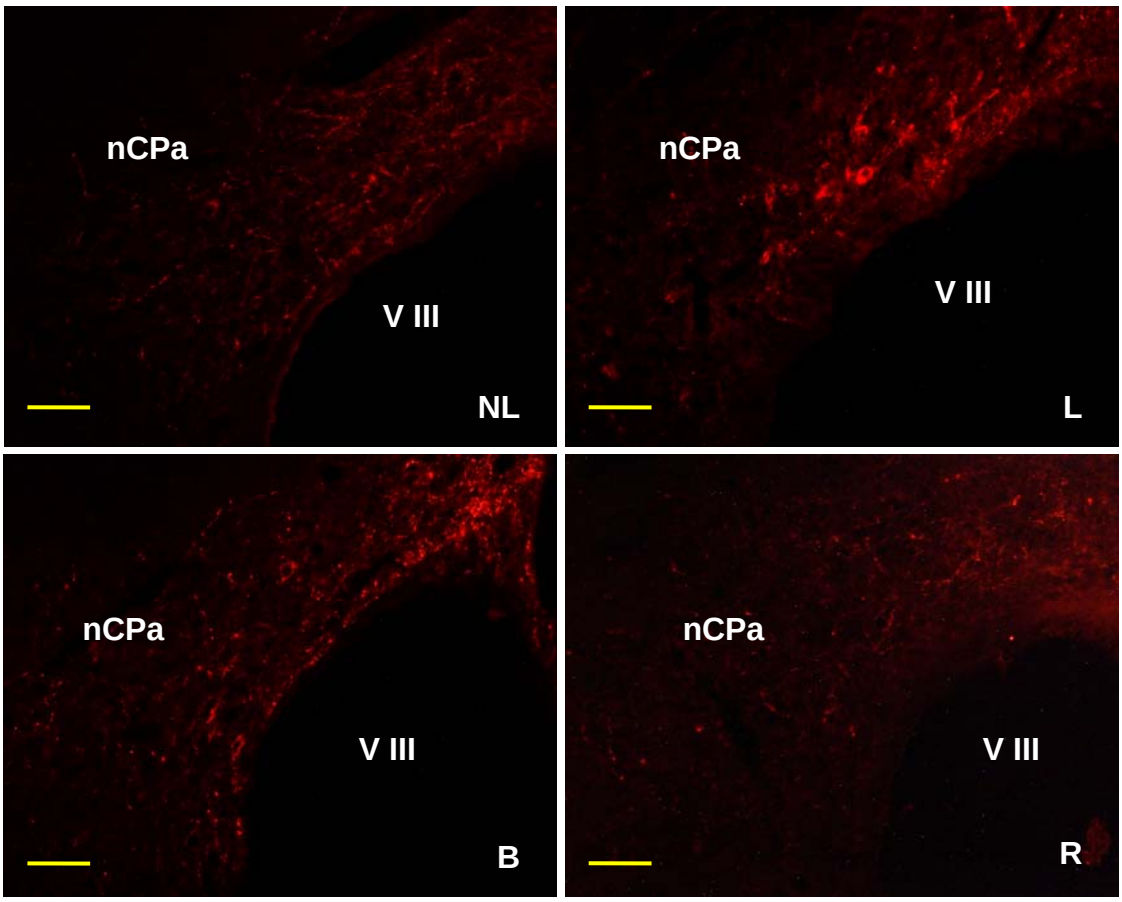
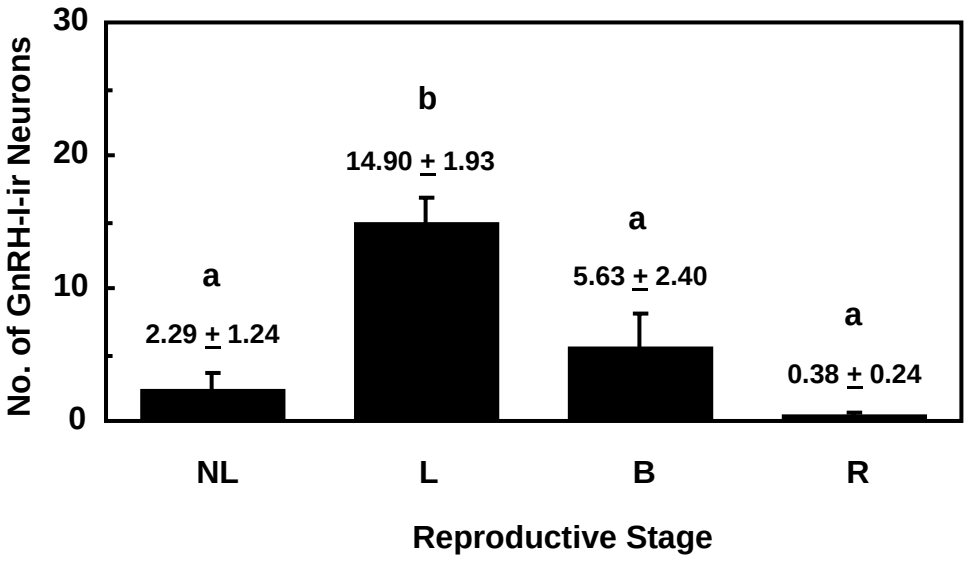


Fig. 4.11 Changes in number of GnRH-I-ir neurons in the nCPa of the native Thai chicken at different reproductive stages. Values (number of GnRH-I-ir neurons/section) are presented as the mean ± SEM (n=6). Values with different letters are significantly different (P<0.05).



4.5 Discussion

The results of the present study revealed that GnRH-I-ir neurons and fibers were distributed lying close to the V III within the length from preoptic region throughout the caudal end of the hypothalamus. The greatest density of GnRH-I-ir neurons was found within the nCPa. Changes in number of GnRH-I-ir neurons in the nCPa were observed across the reproductive cycle. Small numbers of GnRH-I-ir neurons were also observed in the POM, AM, PVN, LHy, VLT, and DLAmc. Dense clusters of GnRH-I-ir fibers were innervated within the discrete region of the OVLT, SL, and in the external layer of ME. Small numbers of GnRH-I-ir fibers were also found lying symmetry adjacent to the V III. The results of the present study indicated that GnRH-I were correlated with the reproductive cycle in the native Thai chicken, confirming a pivotal role of GnRH-I in the control of avian reproduction of this non-seasonally breeding tropical species.

The distributions of GnRH-I-ir neurons and fibers in this present study are in accordance with previous studies that indicated the distributions of GnRH-I neurons and/or fibers throughout the avian brain including chicken (Jozsa and Mess, 1982; Sterling and Sharp, 1982; Mikami et al., 1988; Kuenzel and Blahser, 1991), duck (McNeill et al., 1976; Bons et al., 1978), white-crowned sparrow (Blahser et al., 1986; 1989), Japanese quail (Foster et al., 1988; Mikami et al., 1988; Perera and Follett, 1992; van Gils et al., 1993; Teruyama and Beck, 2000), European starling (Dawson et al., 1985; Foster et al., 1987; Goldsmith et al., 1989), garden warbler (Bluhm et al., 1991), great tit and ring dove (Silver et al., 1992), turkey (Millam et al., 1993), dark-eyed junco (Saldanha et al., 1994), and house sparrow (Hahn and Ball, 1995). The results correspond with other immunohistochemical studies indicated the three groups

of GnRH-I-ir cells: (1) GnRH-I-ir neurons in the prepotic area that lined close to the V III and extended medial and ventral to the TSM; (2) a distinctive group of cells located along the midline in the hypothalamic region; (3) GnRH-I-ir neurons group in the septal region including the nCPa and SL. It has been established previously that neurons of the septal prepotic hypothalamic system are a very heterogeneous population that is the major group of perikaya that project to the ME and OVLT (Sterling and Sharp, 1982). The present study confirms that the majority of GnRH-I-ir neurons and fibers were distributed close proximately to the VL and V III. The results are supported by previous studies and suggested that GnRH-I may exert a biological effect through the ventricular system. In the other hand, this study could not detect the GnRH-I-ir structure in the olfactory bulb, olfactory tubercle/lobus paralfactorius, and in the oculomotor complex, which have been found in the brain of the 3 weeks old chick (Kuenzel and Golden, 2006). However, it has been reported that GnRH-I-ir fibers were found in the olfactory bulb of the quail, but not in chicken (Mikami et al., 1988). Taken together, it is possible that GnRH-I distribution in this area is species and age differences. The characterization of GnRH-I-ir neurons observed in this study were usually small in size, spindle, and bipolar in shape. However, an oval shape with monopolar process neurons could be detected in the POM and also in the DLAmc.

Only a few GnRH-I-ir neurons were seen in the preoptic region. The most anterior group of GnRH-I-ir neurons was found within the POM and POP. GnRH-I-ir neurons in the POP and fibers in the PHN were identified as the POMn (Kuenzel and Blashser, 1991). The POM and POMn is a sexually dimorphic nucleus well described in the Japanese quail (Adkins-Regan and Watson, 1990), as well as in other bird species (Viglietti-Panzica et al., 1986; Panzica et al., 1991; 1996). Furthermore, it has

been reported that the main traditional group of GnRH neurons that are found in and around the POM of mammals is the neurons that projects to the ME (Goldsmith et al., 1990; Silverman et al., 1994). Dense discrete clusters of GnRH-I-ir fibers were found in the OVLT. The structure of the OVLT in the hen was originally described in detail by Dellmann (1964). Importantly, the OVLT is a major terminal projection site for GnRH neurons in the chick as it has been reported for mammals (Barry, 1979; Shivers et al., 1983; Rothfeld and Gross, 1985).

In the present study, although the GnRH-I-ir neurons occurred most commonly close to the midline, they may also found as far from the midline. The ventrolateral groups of GnRH-I-ir neurons were observed at the tip of the TSM in the DLAmc. These findings are correspond well with the results of previous studies indicating the distributions of GnRH-I and II in the quail and chicken brain (van Gils et al., 1993). However, the recent studies reported the only group of GnRH-I-ir neurons that was not position near the lateral or the third ventricles is in the nucleus lateralis anterior thalami (LA; Kuenzel and Blahser, 1991). As previously reported, the LA is a primary retinal recipient area (Ehrlich and Mark, 1984), which has a different origin from that of the larger traditional GnRH neurons shown to originate from the olfactory region (Norgren and Gao, 1994). In addition, it has been found that the activation of the photostimulated long day has no effect on the changes in number of GnRH-I-ir neurons in this region (Kuenzel and Golden, 2006). In the caudal hypothalamus, very intense GnRH-I-ir fibers were detected in the external layer of the ME. Only a limit number of GnRH-I-ir fibers were found in the IH and IN. Interestingly, no GnRH-I-ir neurons have been observed in and around this region, which is equivalent to the mammalian arcuate nucleus (Kuenzel and Blahser, 1991).

In the septal region, the greatest density of GnRH-I-ir neurons was observed in the nCPa. These results correspond with the study in turkeys (Teruyama and Beck, 2001) indicating that GnRH-I-ir cell were most numerous in the region from nCPa to the caudal most septal area. Moreover, it has been reported that the major GnRH-I-ir neurons in the group found in and around the nCPa increase in number, reaching a peak near the caudal end of the septum, and rapidly decrease after the separation of the septum from the thalamus (Kuenzel and Golden, 2006). This present study confirmed that an abrupt termination of GnRH-I-ir neurons was observed at the end of the sepal region at the point at which it separated from the thalamus. At the level of nCPa, GnRH-I-ir fibers were also found in the SSO, a proposed CVO in birds (Kuenzel and Blahser, 1994). Another dense group of GnRH-I-ir fibers was located near the nCPa and extended dorsally and laterally toward the caudal end of the SL. Densely labeled fibers and a small number of GnRH-I-ir neurons were located in and around the SL. The area of SL is also known as the lateral septal organ (LSO). The presence of GnRH-I-ir neurons and fibers in the LSO has been reported in accordance with this study (Teruyama and Beck, 2000; 2001). The LSO of the chick has been found to have an ependymal specialization characterized by multiple layers of columnar ependymal cells (Kuenzel and Blahser, 1994). Therefore, the LSO has been suggested as an additional CVO in birds and reptiles (Kuenzel and van Tienhoven, 1982; Korf and Fahrenkrung, 1984; Hirunagi et al., 1993; Kuenzel and Blahser, 1994). In accordance with the present study is the result that GnRH-I-ir bipolar cells were found in the ependymal in and about the LSO (Teruyama and Beck, 2000). In addition, the study showed that GnRH-I-ir cells were found closely distributed along the lateral ventricle with VIP-ir cerebrospinal fluid containing cells (CSF; Teruyama

and Beck, 2001). The ultrastructural demonstration that VIP nerve terminals in the lateral septum contact putative secretory GnRH neurons (Hirunagi et al., 1994), the coexistence of VIP-ir CSF-contacting cells and GnRH-I-ir cells (Teruyama and Beck, 2001), and by the synaptic connections of chicken GnRH-I-ir and VIP-ir cells in this region (Kiyoshi et al., 1998), suggesting the involvement of the ventricular system in GnRH-I and VIP functions. The presence of GnRH in the CSF has also been documented in mammalian species (Joseph et al., 1975). Moreover, injection of GnRH into the CSF may reach the portal blood (Porter et al., 1975), and stimulate the release of LH (Ben-Jonathan et al., 1974). It has been suggested that the CSF-contacting neurons of the avian LSO might represent a component of the extra-retinal encephalic photoreceptor involved in photoperiodic regulation (Oliver and Bayle, 1982; Foster et al., 1985). The localization of GnRH-I-ir structures in the ependyma of the septal area suggested their interactions with putative photoreceptors and made the septal area a likely candidate for the site of the integration of photoperiodic cues and regulation of the GnRH system in quail (Teruyama and Beck, 2000).

The present study indicated that the number of GnRH-I-ir neurons in the nCPa changed across the reproductive stage of the native Thai chicken. The greatest number of GnRH-I-ir neurons was found in L stage. The numbers were decreased in B and NL stages. The lowest numbers of GnRH-I-ir neurons was found in R. Similar results showed that GnRH mRNAs abundance within the nCPa, OVLT, and SL were greater in laying than that of in non-photostimulated and incubating hens (Kang et al., 2006). Corresponding with this present results is the finding that GnRH-I-ir cells in the caudal most septal area where it begins to separated from what becomes the habenular region of the photosimulated sexual actively male quail was higher than that of

sexually inactive short day male (Teruyama and Beck, 2000). In addition, although there was no change in the number of GnRH-I-ir neurons within the nCPa of the turkey hen, the intensity of GnRH-I-ir neurons in this region was found to change across the reproductive cycle (Al-Zailaie, 2003). Taken together, these findings support that changes in GnRH-I-ir neurons in the nCPa are correlated with the reproductive cycle of birds.

Consistent with the present findings, several evidences revealed that birds at the peak level of reproductive activity have more GnRH-I-ir cells and fibers when compared with sexually inactive or photorefractory birds (Stevenson and MacDougall-Shackleton, 2005; Sharp et al., 1990; Hahn and Ball, 1995; Parry et al., 1997; Cho et al., 1998). Moreover, GnRH contents of discrete medial preoptic, infundibulum, and arcuate samples were higher in laying hens than that of non-laying hens (Advis et al., 1985). In addition, the levels of hypothalamic GnRH mRNA and its peptide were highest in laying and depressed in photorefractoriness, incubating, and non-photostimulating turkey hens (Rozenboim et al., 1993). It has been reported that hypothalamic GnRH mRNA expression was greater in laying hen than that of in incubating (Dunn et al., 1996; Kang et al., 2006), and lowest in photorefractoriness hens (Kang et al., 2006). In contrast with the result of the present study, measurements of GnRH peptide in the hypothalamus during the reproductive cycle of the turkey (Millam et al., 1989; El Halawani et al., 1993; Rozenboim et al., 1993) and chicken (Dunn et al., 1996) indicated that there is no change or a decrease in incubating birds.

Beside the fact that changes in GnRH synthesis and secretion were observed according the reproductive condition. It has been found that photoperiodic cues appear to be important to regulate the GnRH system as well. The stimulatory effect of long

days appears usually to be associated with an increased GnRH content or increased immunoreactivity for GnRH in the hypothalamus and ME in birds (Dawson et al., 1985; Foster et al., 1987; 1988; Goldsmith et al., 1989; Perera and Follet, 1992; Saldanha et al., 1994; Hahn and Ball, 1995). Photostimulatory inputs to GnRH neurones have the potential to increase GnRH mRNA transcription and GnRH release (Dunn and Sharp, 1999), and pituitary sensitivity to GnRH (Davies and Follett, 1975). Time-course analysis of changes in basal hypothalamic GnRH content during photostimulation in the male starling provided the explanation for the photo-induced gonadal cycle (Dawson et al., 2002). In addition, it has been found that a 30 minutes light pulse provided 14 hours after the onset of light was shown to induce GnRH mRNA expression in the nCPa of reproductive quiescent turkeys maintained under a short day lighting regimen (Al-Zailaie et al., 2006). Consistency with this finding, the number of GnRH-I-ir cells in the nCPa increased in long day photostimulated birds (Kuenzel and Golden, 2006). In the other hand, the photorefractoriness has been shown to be correlated with great reduction in GnRH-I-ir structures in the hypothalamus and ME in European starling (Dawson et al., 1985; Foster et al., 1987; Goldsmith et al., 1989), garden warber (Bluhm et al., 1991), dark-eyed junco (Saldanha et al., 1994), house sparrow (Hahn and Ball, 1995), house finch (Cho et al., 1998), and American goldfinch (Marsh et al., 2002), supporting the role of photoperiod in correlated with GnRH to regulate the reproductive system.

In summary, GnRH-I-ir neurons and fibers were found distributed in the discrete region of the brain of the native Thai chicken. The present study demonstrated that changes in the number of GnRH-I-ir neurons were observed in the nCPa during the reproductive cycle with the highest numbers observed in egg laying

stages. These findings are presumed to suggest that GnRH neurons in the prepotic, anterior hypothalamus and septal region, especially the nCPa are involved in the reproductive regulatory system in this non-photoperiodic species. The differential expression of GnRH neurons in the nCPa may affect the changes in gonadotropins release and secretion that consequently affects egg production.

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CHAPTER V

THE DOPAMINERGIC SYSTEM IN THE BRAIN OF THE NATIVE THAI CHICKEN: LOCALIZATION AND DIFFERENTIAL EXPRESSION ACROSS THE REPRODUCTIVE CYCLE

5.1 Abstract

Dopamine (DA) has a pivotal role in avian prolactin (PRL) secretion, acting centrally through D_1 DA receptors to stimulate PRL secretion. DA effects PRL secretion by operating through the vasoactive intestinal peptide (VIP) system, causing VIP, the avian PRL-releasing factor, to be dispersed to the pituitary gland. DA also inhibits PRL secretion by activating D_2 DA receptors at the level of the pituitary gland, antagonizing the effect of VIP. This immunohistochemical study was designed to investigate the distribution of DA in the brain of the native Thai chicken, utilizing tyrosine hydroxylase (TH, the rate limiting enzyme in the DA pathway) as a marker for dopaminergic activity. In addition, the differential expression of TH immunoreactive (TH-ir) neurons in the hypothalamus were compared across the reproductive cycle. The results revealed that TH-ir neurons and fibers were found throughout the brain of laying hens and were predominantly located within the diencephalon and mesencephalon. The distribution pattern of TH immunoreactivity observed in this study was consistent with that reported previously in several avian

species. However, changes in the number of TH-ir neurons in the nucleus intramedialis (nI) were observed across the reproductive cycle and correlated directly with variations in PRL levels. The population of TH-ir neurons in the nI increased significantly during the egg incubation period, which was also the period when circulating PRL levels were the greatest. This study indicates, for the first time, that an association exists between DA neurons and the regulation of the reproductive system in the Thai chicken. There is a paucity of information about the reproductive neuroendocrine regulation of tropical non-seasonally breeding avian species and it is suggested 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 such birds.

5.2 Introduction

Dopamine (DA), a neurotransmitter/neuromodulator, is found extensively in both the central and peripheral nervous systems of many species, has several important physiological functions and is involved in a wide variety of behaviors and reproductive activities (Ben-Jonathan and Hnasko, 2001). DA plays a prominent role in prolactin (PRL) secretion in both birds and mammals, and PRL secretion from the pituitary is closely correlated with the reproductive cycle in birds. During the reproductively quiescent stages of the avian cycle, plasma PRL levels are very low (5-10 ng/ml); however, during the laying and incubating stages, circulating PRL levels increase dramatically (500-1500 ng/ml; El Halawani et al., 1984). In mammals, although PRL secretion is regulated by both stimulatory and inhibitory factors, it is mainly under tonic inhibitory control (Neill, 1988; Ben-Jonathan et al., 1989; Lamberts and MacLeod, 1990) by tuberoinfundibular dopaminergic neurons in the

hypothalamus (Ben-Jonathan et al., 1989; Ben-Jonathan and Hnasko, 2001), which release DA that acts directly upon inhibitory D_2 DA receptors located on pituitary lactotrophs (Civelli et al., 1991). Removal of this dopaminergic inhibition results in increased PRL secretion and hyperprolactinemia (Nicoll and Swearingen, 1970; Nicoll, 1977). This is not the case in birds, where removal of hypothalamic inputs results in the complete cessation of PRL secretion (Tixier-Vidal and Bayle, 1966; Chadwick et al., 1978). It has been established for some time that PRL secretion in birds is tonically stimulated by the hypothalamus (Kragt and Meites, 1965; Bern and Nicoll, 1968) and that the principal PRL-releasing factor (PRF) is vasoactive intestinal peptide (VIP; for review, see El Halawani et al., 1997).

The role of DA in the regulation of PRL secretion is presently not as clear in birds as it is in mammals. The intracerebroventricular infusion of DA in laying turkey hens demonstrated that DA can either stimulate or inhibit PRL secretion, depending upon the concentrations used (Youngren et al., 1995). It has been established that dopaminergic influences are involved in both stimulating and inhibiting avian PRL secretion depending upon multiple DA receptors. The stimulatory effect of DA on PRL secretion is regulated via D_1 DA receptors residing in the infundibular nuclear complex (INF), where the VIP neurons are located. In contrast, DA inhibits PRL release and synthesis by blocking the action of VIP at the pituitary level through D_2 DA receptors (Youngren et al., 1996b; 1998; 2002; Chaiseha et al., 1997; 2003; Al Kahtane et al., 2003). It has been 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). Dynorphin, serotonin, DA, and VIP all appear to stimulate avian PRL secretion along a pathway expressing κ opioid, serotonergic, dopaminergic,

and VIPergic receptors at synapses arranged serially in that functional order, with the VIPergic system as the final mediator (for review, see El Halawani et al., 2000).

In birds, it has been shown that dopaminergic activity and receptor mRNA expression are changed according to different physiological behaviors and reproduction. Dopaminergic 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). Furthermore, stimulatory D_1 DA receptor mRNA expression has been found to increase in the hypothalamus of hyperprolactinemic incubating hens and in the pituitary of laying hens. However, inhibitory D_2 DA receptor mRNA expression is increased in the pituitary of hypoprolactinemic photorefractory hens (Schnell et al., 1999a; 1999b; Chaiseha et al., 2003). Changes in dopaminergic expression during the turkey reproductive cycle parallel the changes in plasma PRL levels and VIP immunoreactivity, content, and mRNA expression within the INF area (El Halawani et al., 1980; 1984; Mauro et al., 1989; Wong et al., 1991; Chaiseha et al., 2003; 2004).

DA is produced in several areas of the brain, activating the five types of DA receptors (D_1-D_5, and their variants; Contreras et al., 2002). Dopaminergic neurons are present in the ventral tegmental area of the midbrain, substantia nigra pars compacta, and arcuate nucleus of the hypothalamus in mammals. The anatomical distribution of the avian dopaminergic system apparently resembles that of mammals (Moons et al., 1994; Reiner et al., 1994), as DA neurons are found throughout the avian hypothalamus (Kiss and Peczely, 1987; Reiner et al., 1994; Al-Zailaie and El Halawani, 2000) and have been shown to be immunoreactive for VIP (Mauro et al., 1989; 1992; Hof et al., 1991) and VIP mRNA (Kuenzel et al., 1997; Chaiseha and El

Halawani, 1999). DA has been measured and visualized in various bird species, including domestic fowl (Knigge and Piekut, 1985), quail (Ottinger et al., 1986; Balthazart et al., 1992; 1998; Bailhache and Balthazart, 1993; Absil et al., 2001), pigeon (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., 1998a), chicken (Contijoch et al., 1992; Moons et al., 1994; 1995), budgerigar (Roberts et al., 2001), collared dove (den Boer-Visser and Dubbeldam, 2002), turkey (Al-Zailaie and El Halawani, 2000), and canary (Appeltants et al., 2001). In birds, dopaminergic neurons are widely dispersed throughout the forebrain, midbrain, and hindbrain. Dopaminergic neurons are not located in a single discrete hypothalamic nucleus or region, but instead are dispersed among a variety of hypothalamic regions. These areas include the preoptic areas (POA), nucleus anterior medialis hypothalami (AM), nucleus suprachiasmaticus (SCN), the regio lateralis hypothalami (LHy), nucleus paraventricularis magnocellularis (PVN), nucleus mamillaris lateralis (ML), and nucleus dorsomedialis hypothalami (DMN; Kiss and Peczely, 1987; Reiner et al., 1994). Given their widespread distributions, and the findings that DA axons and terminals are found intermingled with VIP neurons in the INF, gonadotropin releasing hormone (GnRH) neurons in the POA, and with both VIP and GnRH terminals in the external layer of the eminentia mediana (median eminence, ME; Contijoch et al., 1992; Fraley and Kuenzel, 1993a), it is reasonable to consider whether any regional specificity exists in those DA neurons that is neuroendocrine in nature, i.e., controlling the release and expression of the VIP/PRL and GnRH/luteinizing hormone-follicle stimulating hormone (GnRH/LH-FSH) systems. Recently, DA neurons found in the turkey hypothalamus, including the POA, ML, and nucleus premamillaris (PMM),

have been proposed as a potential reproductive neuroendocrine circuit that controls reproductive seasonality in temperate zone birds, which are highly photoperiodic and whose gonadal development occurs in response to increasing day length (Al-Zailaie et al., 2006; Kang et al., 2007; Thayananuphat et al., 2007a; 2007b).

The neural and neurochemical substrates regulating reproduction in birds remain vaguely defined. Two neuroendocrine systems play a pivotal role in the reproductive cycle of temperate zone birds, such as the domestic turkey. One system involves chicken gonadotropin releasing hormone-I (cGnRH-I) and the subsequent secretion of LH and FSH (Sharp et al., 1998) and the other system involves the PRF, VIP and the subsequent secretion of PRL (Chaiseha and El Halawani, 2005). Both systems are influenced by DA. Contrary to the temperate zone seasonal breeding species, the native Thai chicken is an equatorial zone continuously breeding species that produces eggs all year long independent of photoperiodic cues. There is a limited number of studies providing data about neuroendocrine regulation in this non-temperate zone gallinaceous bird. Importantly, there is no study delineating the anatomical distribution and functional aspect of the dopaminergic system in the native Thai chicken. To further understand the neuroendocrine regulation of reproduction in the native Thai chicken, this present immunohistochemical study was designed to investigate the distribution of dopaminergic neurons throughout its brain utilizing tyrosine hydroxylase (TH, the rate limiting enzyme for DA synthesis) antibody as a marker for dopaminergic activity. In addition, in order to investigate whether or not specific DA neuronal groups in the hypothalamic area may be correlated with the reproductive cycle of the native Thai chicken, the change in numbers of TH immunoreactive (TH-ir) neurons in the hypothalamus were measured at different

reproductive stages. The results of this study may identify DA neuronal groups that are associated with the reproductive regulatory system in this equatorial species.

5.3 Materials and Methods

5.3.1 Experimental Animals

Female native Thai chickens (*Gallus domesticus*), 16-18 weeks of age were used. They were reared and housed together with a mature male (1 male: 8 females) in floor pens under natural light (approximately 12 hours of light and 12 hours of dark; 12L:12D). Feed and water were provided *ad libitum.* Birds were divided into 4 reproductive stages: non-egg laying (NL), egg laying (L), incubating eggs (B), and rearing chicks (R). The four reproductive stages were identified by behavioral observation and postmortem examination. Birds were sacrificed according to their reproductive stages. Briefly, NL were birds that had never laid eggs, L hens, in their first laying cycle, had been laying for 7 days, B hens stopped laying and exhibited incubating behavior for 10 days, and R hens had been rearing chicks for 2 weeks. Blood samples were withdrawn from a brachial vein to analyze plasma PRL levels as an aid to confirming reproductive condition. Laying hens were used to study the localization of TH-ir neurons and fibers throughout the brain. Changes in the number of TH-ir neurons in individual brain areas following reproductive stages were investigated using five birds per each reproductive group. The animal protocols described in this study were approved by Suranaree University of Technology Animal Care and Use Committee Guidelines.

5.3.2 Experimental Design

5.3.2.1 Experiment I

To determine the distribution of DA system in the brain of the native Thai chicken, laying hens (n=5) were used to study the localization of TH-ir neurons and fibers throughout the brain. The brains were fixed by pressure-perfused prior to section and used for further processed by immunohistochemistry. Plasma PRL levels and a postmortem examination of each hen were performed to confirm its reproductive status.

5.3.2.2 Experiment II

To determine the changes in number of TH-ir neurons in the hypothalamic areas corresponding to the AM, PVN, nucleus intramedialis (nI), and ML across the reproductive stage, native Thai chickens in each reproductive stage (NL, L, B, R) were used (n=5). Blood samples of the native Thai chickens in each reproductive stage were collected from the brachial vein in heparinized tubes prior to sacrifice the chickens (n=5). Plasma samples were separated by centrifugation and stored at -35°C until used for determining plasma PRL levels by enzyme-linked immunosorbent assay (ELISA). The brains were fixed by pressure-perfused prior to section and used for further processed by immunohistochemistry. A postmortem examination of each hen was performed to confirm its reproductive status.

5.3.3 Measurement of Plasma PRL Concentrations

A blood sample was collected from each bird and fractionated by centrifugation. The plasma was stored at -20°C until assayed. Plasma PRL levels were

determined utilizing an enzyme-linked immunosorbent assay according to a previously described method (Proudman et al., 2001). 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 at the dilution 1:2,000. The plates were incubated at 4°C overnight and then blocked with blocking solution (100 µl of 0.4% casein, 0.01% thimerosal, 1 mM EDTA). 20 µl of samples, 30 µl of the assay buffer (0.1% casein, 0.01% thimerosal, 1 mM EDTA), 25 µl of anti-PRL (1:20,000, provided by Dr. John Proudman, USDA, USA), and 25 µl of β-PRL tracer (1:50,000) were added into the reaction, then incubated at 4°C overnight. The reactions were measured the absorbent at 405 nm. The plasma samples were measured in duplicate within a single assay.

5.3.4 Processing of Tissues for Immunohistochemistry

After collecting a blood sample and prior to perfusion, each bird was intravenously injected with 3 ml of heparin (Baxter Healthcare Corporation, Deerfield, IL, USA; 1000 unit/ml) and then euthanized with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France; 2 ml/kg). The head was removed and immediately pressure-perfused via the carotid arteries with 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 3-5 min, followed by a freshly prepared 4% paraformaldehyde (pH 7.4) for 30 min according to a previously described method (Al-Zailaie et al., 2006). The brain was then removed from skull with the pituitary attached and placed in 20% sucrose in PBS at 4^{o}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^{o}C until sectioned. Frozen brains were sectioned in coronal plane at a thickness of

16 μm using a cryostat (Leica CM1850, Leica Instruments GMbH, Nussioch, Germany). Sections were mounted onto a gelatin-subbed slide with 2 sections per slide and stored desiccated at -20^{o}C until further processed for immunohistochemistry.

5.3.5 Immunohistochemistry

In order to localize TH distribution throughout the brain of the laying hen and characterize the changes in TH-ir neurons within individual brain areas in different reproductive stages, immunohistochemistry was performed as previously described (Al-Zailaie et al., 2006). Briefly, tissue sections of different areas throughout the brains of laying hens (n=5) and four adjacent sections in the hypothalamic areas corresponding to the AM, PVN, nI, and ML of each bird (n=5) according to each reproductive stage were placed in PBS for 30 min at room temperature. After PBS removal, each section was incubated with 60 μl primary mouse monoclonal antibody directed against TH (ImmunoStar, Inc., Hudson, WI, USA) diluted 1:1000 with PBS (pH 7.4) containing 1% bovine serum albumin and 0.3% triton-X at 4^{o}C in a moisture chamber for 24 hrs. The next day, after removal of excess antibody, the sections were then washed 3 times in PBS for 5 min each. After washing, 60 μl of secondary antibody Cy^{TM}3-conjugated AffiniPure Donkey Anti-Mouse IgG (diluted 1:500, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was applied on each section. The sections were further incubated in a moist dark chamber at room temperature for 1 hr. The slides were then rinsed with PBS to stop the reaction, washed again 3 times in PBS for 5 min each, and finally coverslipped using DPX mountant (Sigma-Aldrich, Inc., Steinheim, Germany).

5.3.6 Image Analysis

An atlas of the chick brain (Kuenzel and Masson, 1988) was used to identify the areas of the brain that expressed TH-ir neurons and fibers. Microscopic images of brain sections were visualized with a fluorescence microscope (Olympus IX71, Tokyo, Japan) at 4x, 10x, 20x, and 40x magnification. Images were captured with a digital camera (Olympus DP70, Tokyo, Japan), and stored by DP70-BSW software (Olympus, Tokyo, Japan). To characterize the differential expression of the dopaminergic system across the reproductive cycle, four adjacent brain sections in the hypothalamic areas corresponding to the AM, PVN, nI, and ML from each bird according to each reproductive stage (n=5) were chosen and counted manually to compare the number of TH-ir neurons in individual hypothalamic areas. The specificity of the antibody used in this study was tested by omission of the primary antibody during that step of immunohistochemistry, resulting in the absence of TH immunoreactivity.

5.3.7 Statistical Analysis

Significant differences in plasma PRL levels and the number of TH-ir neurons (mean ± SEM) in the individual hypothalamic areas according to each reproductive stage were compared employing one way analysis of variance (ANOVA). Significant differences between reproductive stages with multiple comparisons were determined using Tukey's HSD test. $P<0.05$ was considered statistically significant. All statistical tests were analyzed employing the SPSS for Windows software (version 13.0, SPSS Inc., Chicago, IL, USA).

5.4 Results

5.4.1 Experiment I

As revealed by immunohistochemistry, TH-ir neurons and fibers were distributed throughout the brain (telencephalon, diencephalon, mesencephalon, and rhombencephalon) of laying hens. The majority of the TH-ir neurons were predominantly located within the diencephalon and mesencephalon (Fig. 5.1).

5.4.1.1 Telencephalon

The most rostral group of TH-ir fibers was observed within lobus parolfactorius (LPO) in the ventral telencephalon. In the areas of nucleus septalis medialis (SM) and nucleus septalis lateralis (SL) the majority population of neurons were TH immunonegative cells that showed pericellular arrangements of TH-ir fibers surrounding them (Fig. 5.2A). This type of neuron can also be seen scattered within the dorsal zone of the telencephalon along the hyperstriatum ventrale (HV). At the ventral end of the ventriculus lateralis (VL), a small group of TH-ir fibers was found in the nucleus accumbens (Ac, Fig. 5.2B) and the organum septi laterale (lateral septum organ; LSO).

5.4.1.2 Diencephalon

The main concentration of TH immunoreactivity was located in the diencephalon. The most rostral group of TH-ir neurons was found throughout the entire preoptic area immediately caudal to the tractus septomesencephalicus (TSM), in the AM (Fig. 5.3A) and nucleus suprachiasmaticus, pars medialis (SCNm, Fig. 5.3B). The bipolar TH-ir cells in the AM were co-expressed with a compact group of TH-ir

fibers that lay along the third ventricle (V III). A small group of TH-ir neurons and fibers was observed more laterally in the LHy. A few TH-ir neurons were also identified around the nucleus preopticus periventricularis (POP). TH immunoreactivity was not observed within the organum vasculosum lamina terminalis (OVLT). Immediately posterior to the commissura anterior (anterior commissure; CA), the distribution of TH-ir neurons extended in a more dorsal periventricular position into the PVN. Small to moderate sized unipolar and bipolar neurons with long fibers extending parallel to the ventricle were found in this area (Fig. 5.3C and 5.3E). In the organum paraventriculare (paraventricular organ; PVO), a rather dense number of TH-ir neurons and fibers were accumulated bilaterally close to the midline (Fig. 5.3D and 5.3F). In apposition to the PVO, some scattered TH-ir neurons were expressed. This group of neurons sent long fibers to the TH-ir neurons in the LHy (Fig. 5.3G). In addition, a compact group of TH-ir fibers were innervated in the DMN.

In the caudal hypothalamus, the greatest density of TH-ir neurons was located in the nI. The neurons in this area, which were mainly large, ovoid, and highly immunostained, were clustered bilaterally where the brain fuses together across the VIII (Fig. 5.4A and 5.4B). A tight band of highly labeled fibers formed a connection between the two compact groups of neurons. This group of neurons was also found when moving caudally, however the density tended to be less than those appearing more rostrally. Moreover, TH-ir neurons in the nI appeared to combine into a single group. A few TH-ir neurons and fibers were also observed within the PMM, tractus quintofrontalis (QF), and tractus infundibularis (IF). More ventrally, a small group of TH-ir neurons and fibers were observed at the ML (Fig. 5.4C and 5.4D). In the INF, an apparently compact group of TH-ir fibers could be detected in the nucleus

mamillaris medialis (MM). Some scattered labeled fibers were found in the nucleus inferioris hypothalami (IH). There was a dense accumulation of TH-ir fibers in the ME, limited only to the external layer (Fig. 5.4E). In the dorsal zone of the hypothalamus, TH-ir neurons were noted within the substantia grisea centralis (GCt, Fig. 5.4F). The TH-ir neurons in the GCt were usually multipolar appearing with branching. There were a few TH labeled neurons and fibers dispersed along the midline from the GCt to the nI in the nucleus of Darkschewitsch (D).

5.4.1.3 Mesencephalon

Dense, heavily-stained TH-ir neurons and fibers were found in the area ventralis (AVT, Fig. 5.5A), where a cluster of TH-ir fibers coexisted with TH-ir neurons. The nucleus interpeduncularis (IP) was devoid of any immunoreactivity. There was a large group of TH-ir neurons and fibers found in the nucleus tegmenti pedunculo-pontinus, pars compacta (substantia nigra; TPc, Fig. 5.5B) and tractus occipitomesencephalicus (OM). TH-ir neurons in the TPc and OM were multipolar with many dendritic processes. The compact groups of TH-ir neurons were independently expressed with strongly labeled fibers. This characteristic of these TH-ir neurons and fibers was apparently different from the TH-ir neurons in the aforementioned brain areas. A small group of TH-ir neurons and fibers were also found in the brachium conjunctivum ascendens (BCA) and brachium conjunctivum descendens (BCD).

5.4.1.4 Rhombencephalon

Diffused TH-ir neurons and fibers were found in the locus ceruleus (LoC, Fig. 5.5C) and nucleus subceruleus ventralis (SCv). The TH-ir neurons in these nuclei were similar in shape and size when compared with the neurons in the TPc, although the number of the TH-ir neurons in the LoC and SCv were markedly less and with only a few coincident fibers. A few TH-ir neurons and fibers were found in the nucleus decussationis brachiorum conjunctivorum (nDBC), and extending further caudally to the nucleus subceruleus dorsalis (SCd). In the pons and medulla, TH immunonegative cells surrounded by TH-ir fibers were identified as a discrete nucleus in the nucleus magnocellularis cochlearis (MCC). Only a limited number of TH-ir fibers were observed in the nucleus vestibularis medialis (VeM) and nucleus vestibularis descendens (VeD). There was a single spindle shape-liked cell with no dendritic process and axons found in the dorsal edge of the midbrain in the nucleus mesencephalicus nervi trigemini (nVm) and cerebellum (Cb, Fig. 5.5D) but the intensity of the TH immunoreactivity was markedly low.

5.4.2 Experiment II

5.4.2.1 Plasma PRL levels across the reproductive stages

Plasma PRL levels were determined across the reproductive stage of the native Thai chicken (n=5, Fig. 5.6A). Plasma PRL levels were low in NL (23.33 $\pm$ 2.42 ng/ml), gradually augmented in L (45.15 $\pm$ 8.39 ng/ml), significantly higher in B (240.13 $\pm$ 35.10 ng/ml, $P<0.05$), and declined dramatically to the same level of NL in R (27.63 $\pm$ 2.17 ng/ml).

5.4.2.2 Changes in number of TH-ir neurons in the hypothalamus

The number of TH-ir neurons in four hypothalamic areas, including the AM, PVN, nI, and ML, were compared across the reproductive stages (n=5, Table 5.2). The results revealed that in all areas examined, the number of TH-ir neurons was high in the AM and nI, but low in the PVN and ML. The greatest density of TH-ir neurons was observed in the nI where differential expression of TH-ir neurons was seen across the reproductive stages (Fig. 5.6B and 5.7). The number of TH-ir neurons was low in NL (31.60 $\pm$ 2.43 cells) and slightly increased in L (38.10 $\pm$ 3.57 cells). When the hens began to incubate eggs, the number of TH-ir neurons markedly increased to the highest level (48.70 $\pm$ 5.32 cells). The increase in TH-ir neurons in B was significantly greater (1.54-fold) as compared to NL ($P<0.05$). Subsequently, the number of TH-ir neurons decreased slightly during the transition from B to R (35.00 $\pm$ 2.14 cells). A high density of TH-ir neurons was also observed in the AM (Table 5.2). The number of TH-ir neurons in the AM displayed some fluctuation across the reproductive cycle and appeared to be highest in hens that had shifted from incubation to the rearing of chicks (B = 33.12 $\pm$ 5.29 vs R = 41.50 $\pm$ 7.49 cells), but the difference was not statistically significant. The least number of TH-ir neurons were observed in the PVN and ML, and these numbers remained essentially the same in all reproductive stages ($P>0.05$, Table 5.2, Fig. 5.8).

Table 5.1 Abbreviations of brain areas. Nomenclature and abbreviations are from a stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988)

Ac	Nucleus accumbens
AM	Nucleus anterior medialis hypothalami
AVT	Area ventralis
BCA	Brachium conjunctivum ascendens
BCD	Brachium conjunctivum descendens
Cb	Cerebellum
D	Nucleus of Darkschewitsch
DMN	Nucleus dorsomedialis hypothalami
GCt	Substantia grisea centralis
IF	Tractus infundibularis
LHy	Regio lateralis hypothalami
LoC	Locus ceruleus
ME	Eminentia mediana (Median eminence)
ML	Nucleus mamillaris lateralis
nDBC	Nucleus decussationis brachiorum conjunctivorum
nI	Nucleus intramedialis
nVm	Nucleus mesencephalicus nervi trigemini
NIII	Nervus oculomotorius
OM	Tractus occipitomesencephalicus

Table 5.1 Abbreviations of brain areas. Nomenclature and abbreviations are from a stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988), continued.

PMM	Nucleus premamillaris
PVN	Nucleus paraventricularis magnocellularis
PVO	Organum paraventriculare
QF	tractus quintofrontalis
SCNm	Nucleus suprachiasmaticus, pars medialis
SCv	Nucleus subceruleus ventralis
SL	Nucleus septalis lateralis
SM	Nucleus septalis medialis
TPc	Nucleus tegmenti pedunculo-pontinus, pars compacta
V III	Ventriculus tertius (third ventricle)
VL	Ventriculus lateralis

Fig. 5.1 Schematic diagrams of coronal sections illustrating the distribution of TH-ir neurons (black dot) throughout the brain of the laying native Thai chicken. Sections are presented in a rostral to caudal order from **A**-**F**. Coronal illustrations are redrawn, with the given coordinates, from the stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988).

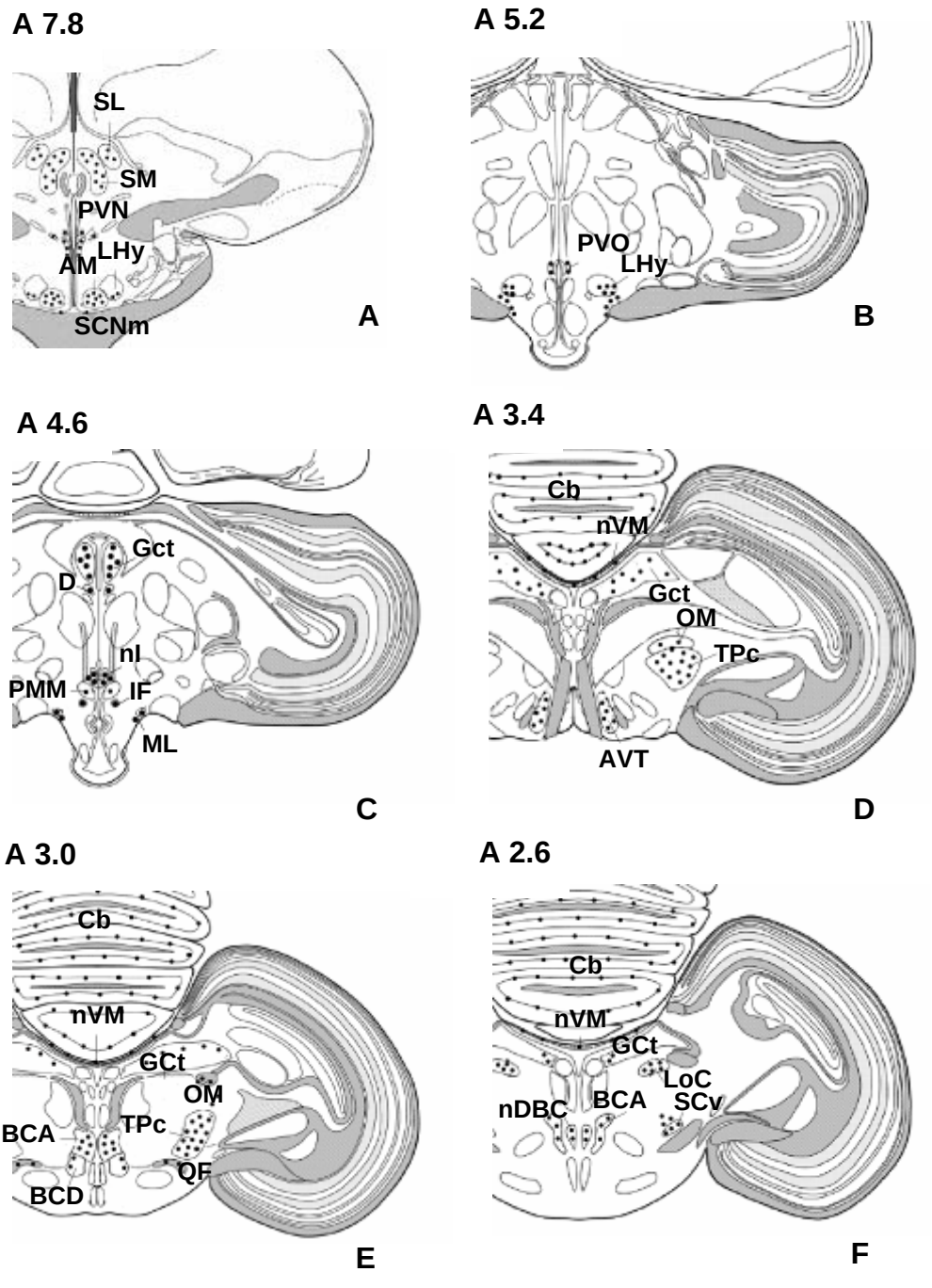


Fig. 5.2 Photomicrographs showing TH-ir structures in the telencephalon. (**A**) A TH immunonegative cell surrounded by TH-ir positive fibers in the SM. (**B**) TH-ir fibers at the ventral terminus of the VL. Bar=50 µm. For abbreviations, see Table 5.1.

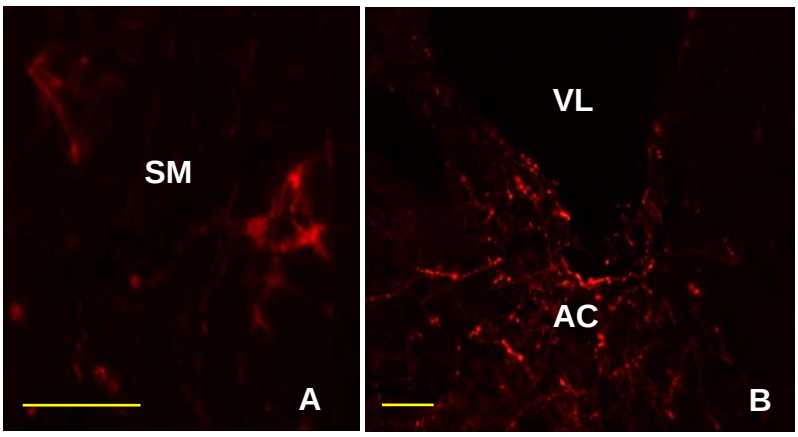


Fig. 5.3 Photomicrographs illustrating the distribution of TH-ir neurons in the diencephalon. TH-ir neurons are found in (**A**) AM, (**B**) SCNm, and (**C**) PVN (Bar=100 µm). Insert in (**A**) at higher magnification (Bar=50 µm) of a bipolar cell in the AM. (**D**) A dense number of TH-ir neurons situated bilaterally close to the third ventricle in the PVO (Bar=100 µm). (**E**) Higher magnification in the PVN, showing a neuron with an elongated fiber (Bar=50 µm). (**F**) Scattered TH-ir fibers between the PVO and LHy (Bar=100 µm). (**G**) Higher magnification of a compact group of TH-ir neurons in the LHy (Bar=50 µm). For abbreviations, see Table 5.1.

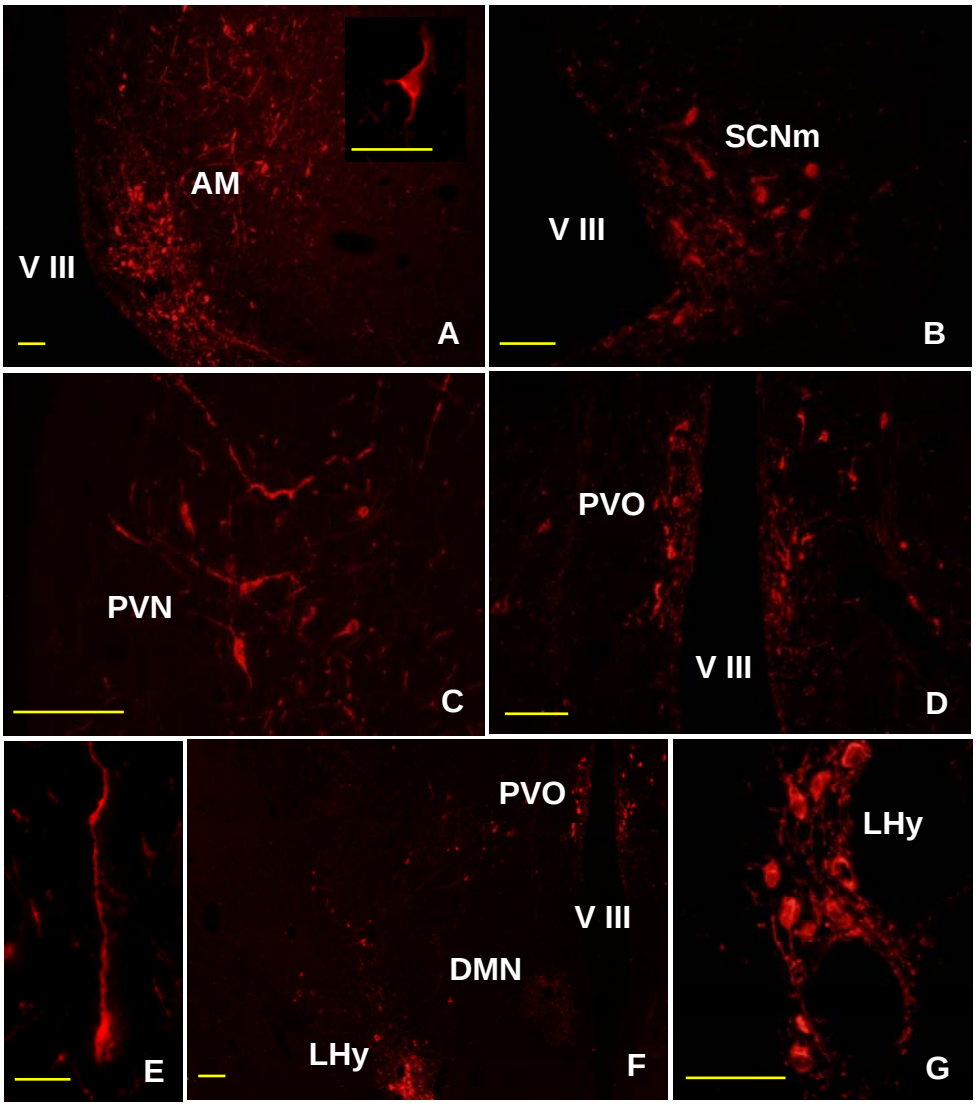


Fig. 5.4 Photomicrographs showing TH-ir neurons and fibers in the caudal hypothalamus. (**A**) A compact group of TH-ir neurons in nI, located on both sides of the fused ventricle. (**B**) Large, ovoid, and intensely labeled TH-ir neurons are found in the nI. (**C**) In the tuberal hypothalamus, TH-ir cells are observed in the ML; only TH-ir fibers are found in the MM and ME. (**D**) Higher magnification of TH-ir neurons in the ML and (**E**) TH-ir fibers in the external layer of the ME. (**F**) Small numbers of TH-ir neurons are scattered within the GCt. Bar=100 µm. For abbreviations, see Table 5.1.

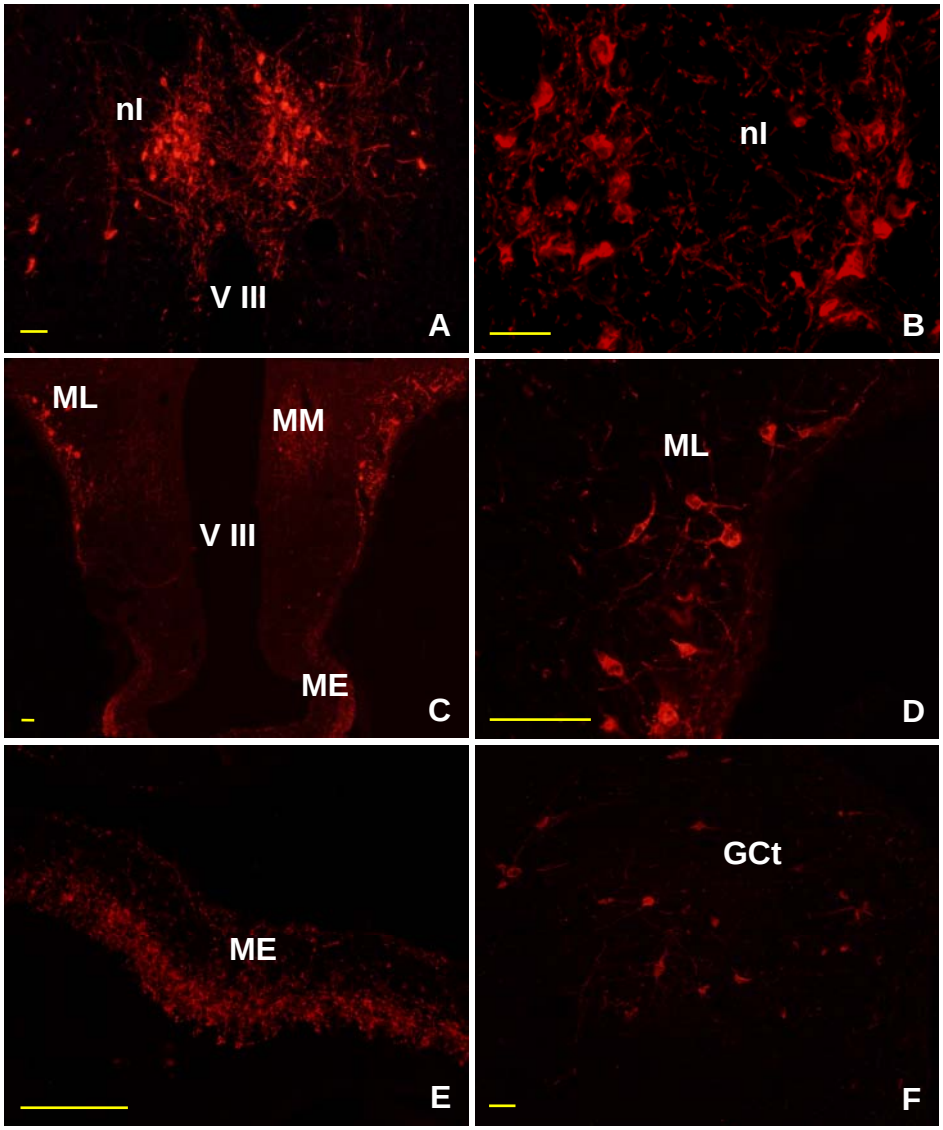


Fig. 5.5 Photomicrographs illustrating the TH-ir neurons and fibers in the mesencephalon. (**A**) A cluster of TH-ir fibers and TH-ir neurons in the AVT, adjacent to the NIII. (**B**) A large group of TH-ir neurons and intensely labeled fibers are co-localized within the TPc. (**C**) TH-ir neurons in the LoC are multipolar cells with many dendritic processes. (**D**) Some weakly labeled TH-ir neurons are found in the Cb. Bar=100 µm. For abbreviations, see Table 5.1.

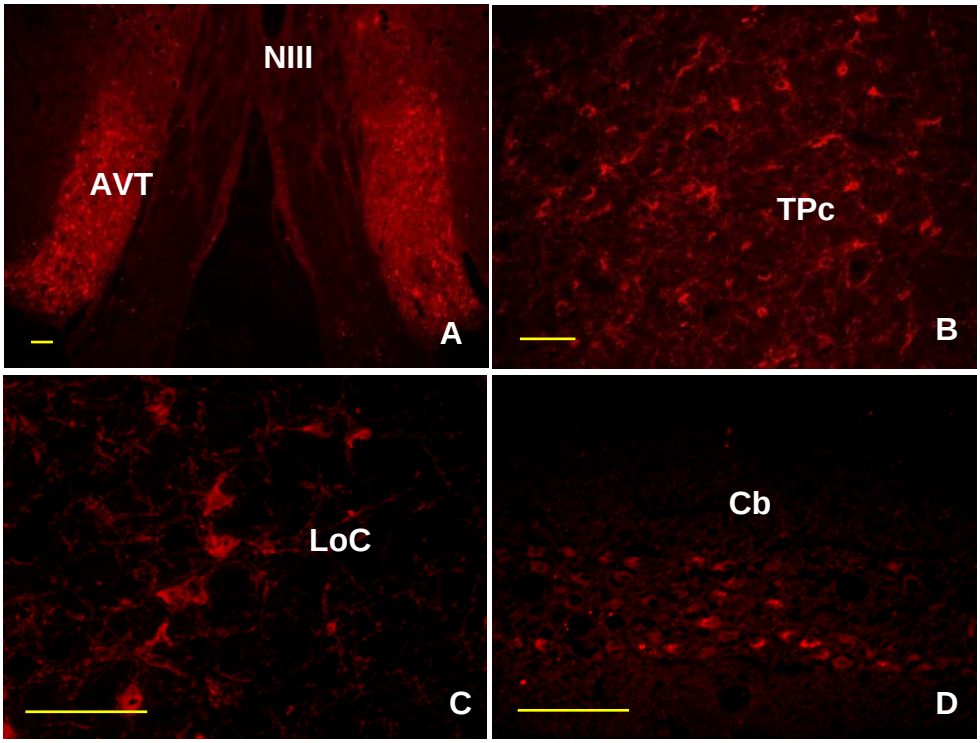


Table 5.2 The number of TH-ir neurons within individual hypothalamic areas (AM, PVN, nI, and ML) in the native Thai chicken at different reproductive stages.

Hypothalamic Area	Reproductive Stage			
	NL	L	B	R
AM	33.83 ± 6.13^{a}	27.10 ± 3.68^{a}	33.12 ± 5.29^{a}	41.50 ± 7.49^{a}
PVN	15.80 ± 1.20^{a}	15.60 ± 2.38^{a}	17.20 ± 3.34^{a}	17.25 ± 1.56^{a}
nI	31.60 ± 2.43^{a}	38.10 ± 3.57^{ab}	48.70 ± 5.32^{b}	35.00 ± 2.14^{ab}
ML	21.80 ± 1.30^{a}	22.90 ± 1.61^{a}	19.80 ± 2.98^{a}	19.10 ± 0.93^{a}

Values (number of TH-ir neurons/section) are presented as the mean ± SEM (n=5).

Values with different superscripts within the same row are significantly different ($P<0.05$).

Fig. 5.6 (**A**) Plasma PRL concentrations and (**B**) numbers of TH-ir neurons in the nI in the native Thai chicken at different reproductive stages. Values are presented as the mean ± SEM (n=5). Values with different letters are significantly different (P<0.05).

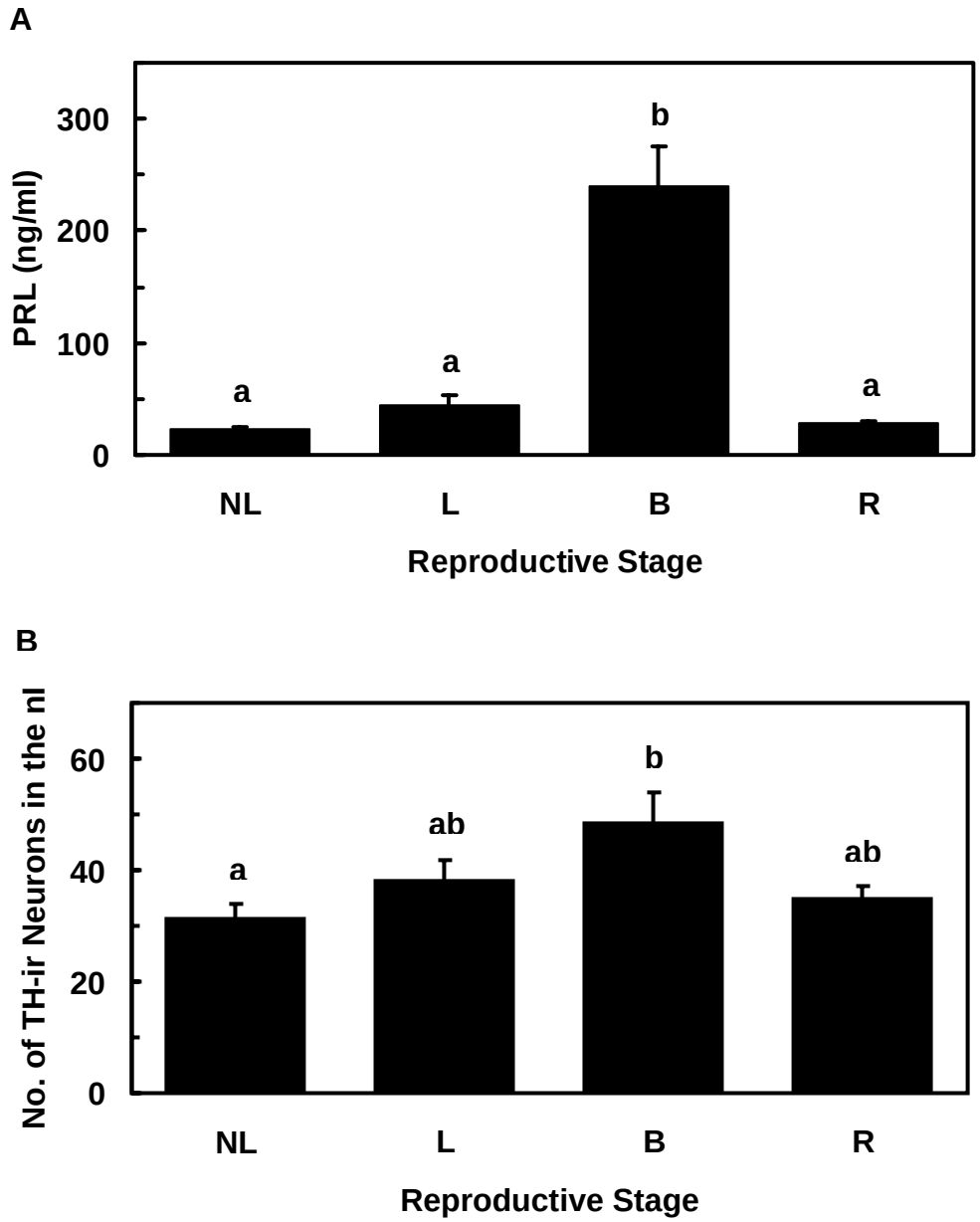


Fig. 5.7 Photomicrographs illustrating the expression of TH-ir neurons in the nI during different reproductive stages. Bar=100 µm. For abbreviations, see Table 5.1.

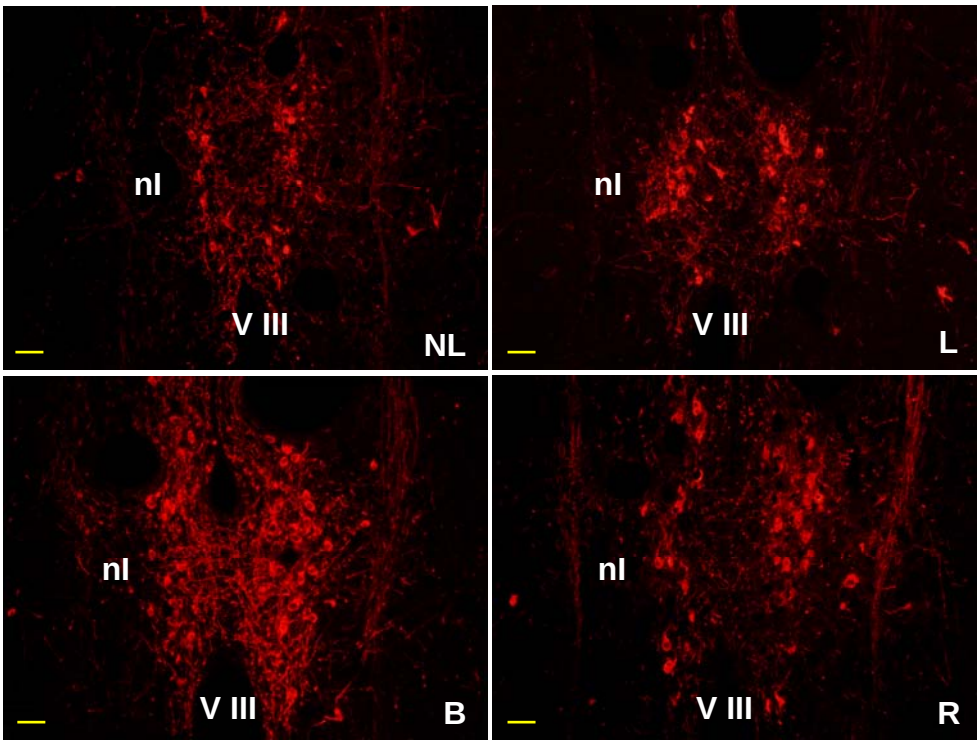
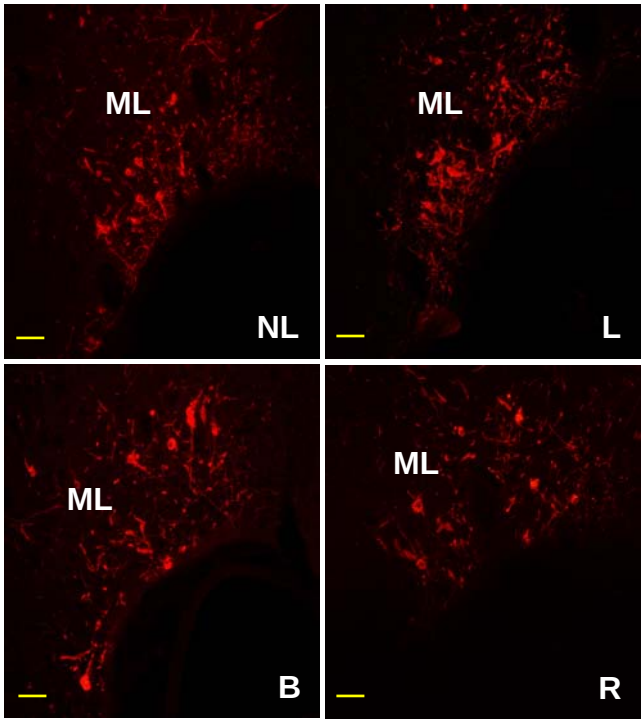


Fig. 5.8 Photomicrographs illustrating the expression of TH-ir neurons in the ML during different reproductive stages. Bar=100 μm. For abbreviations, see Table 5.1.



5.5 Discussion

The present study demonstrates for the first time in the native Thai chicken that changes taking place in dopaminergic neurons in the caudal hypothalamus may be related to reproductive activity in this nonseasonal, continuous breeding tropical species. These changes occur in nI, a cluster of neurons close to, and perhaps spanning, the V III. This nucleus lies dorsal to and in fairly close proximity to the infundibular area where VIP, the avian PRF, is secreted and released to the ME, and hence to the anterior pituitary to cause the release of PRL. This study also shows that circulating PRL varies across the reproductive cycle of the Thai chicken and that this

variation is closely correlated with dopaminergic changes in the nI. And lastly, the study charts the distribution of dopaminergic cells and fibers within the brain of the Thai chicken.

Immunohistochemistry revealed that the number of TH-ir neurons in nI were significantly increased during the period of egg incubation as compared to non-laying hens. These neurons showed a tendency to increase in quantity as egg laying occurred, reaching maximum numbers during incubation, and then decreasing slightly when chick rearing was taking place. Plasma PRL showed the same tendency of rising during egg laying to a maximum level during incubation, and then dropping off after hatching has taken place. These results in the Thai chicken are also in accordance with previous studies in which the number of TH-ir cells in the periventricular mid-hypothalamic regions was higher in brooding than that of non-brooding birds (Lea et al., 2001). Also consistent with the present findings is the existence of specific DA-binding sites in the anterior hypothalamus which markedly increased in incubating bantam hens when compared with laying or nest deprived hens (Macnamee and Sharp, 1989). Results also correspond with previous studies demonstrating that high levels of PRL are associated with incubation behavior in birds (Riddle et al., 1935; Breitenbach and Meyer, 1959; Burke and Dennison, 1980; Goldsmith and Williams, 1980; El Halawani et al., 1988; Sharp et al., 1988; Chaiseha et al., 1998; Kosonsiriluk et al., 2007). PRL has been implicated as a causative factor in the onset and maintenance of parental behavior and it has been very well established that the onset of incubation activity is correlated with a dramatic rise in circulating PRL levels and declining levels of gonadotropins (El Halawani et al., 1988; Knapp et al., 1988). These increasing PRL levels reduce ovarian steroid secretion (Porter et al., 1991), terminate egg laying, and

induce ovarian regression (Youngren et al., 1991). PRL secretion in birds is tonically stimulated by the hypothalamus (Kragt and Meites, 1965; Bern and Nicoll, 1968) and the principal PRF is VIP (El Halawani et al., 1997; 2000; Chaiseha and El Halawani, 2005). Variations in hypothalamic VIP immunoreactivity, VIP content, VIP mRNA expression in the INF, VIP receptor mRNA, and VIP concentrations in hypophyseal portal blood are correlated with changes in circulating PRL levels throughout the turkey reproductive cycle (Mauro et al., 1989; Youngren et al., 1996a; Chaiseha and El Halawani, 1999; Chaiseha et al., 1998; 2004). Recently, changes in VIP-ir neurons in the INF area have been reported and directly correlated with the plasma PRL levels across the reproductive cycle of the native Thai chicken (Kosonsiriluk, 2007). Furthermore, preliminary work from our laboratory indicates that TH-ir neurons in the nI of incubating nest-deprived hens are sharply reduced when compared with incubating hens (unpublished data). Taken together, these findings seem to suggest that the differential expression of DA neurons in the nI of this equatorial species are correlated with, and could be responsible for, alterations in VIP release and the subsequent variations in PRL secretion. DA stimulates PRL secretion at the hypothalamic level via D_1 DA receptors and inhibits at the pituitary level via D_2 DA receptors (Youngren et al., 1996b; Chaiseha et al., 1997; Al Kahtane et al., 2003). The distribution of D_1 and D_2 DA receptor mRNA expression in the hypothalamus and pituitary supports this supposition (Schnell et al., 1999a; Chaiseha et al., 2003). Further support is found with the fact that stimulatory D_1 DA receptor mRNA expression increases in hyperprolactinemic incubating hens and inhibitory D_2 DA receptor mRNA expression increases in hypoprolactinemic photorefractory hens, along with the finding that VIP mRNA is co-localized with D_1 and D_2 DA receptors in

the INF, where VIP-ir neurons and mRNA expression increase during the incubation stages.

It has been proposed that TH-ir neurons in the nI of the chicken correspond to the mammalian DA A11 group (Moons et al., 1994; Lookingland and Moore, 2005). In mammals, the hypothalamic DA A11 group has a putative role in sensory and nociceptive processing, as well as sensorimotor integration (van Dijken et al., 1996; Levant and McCarson, 2001). In birds, the results of the present study in conjunction with a previous study (Lea et al., 2001) suggests TH-ir neurons in this area correlate with the reproductive regulatory system, especially during the incubation period. In the temperate zone bird, it has been suggested that DA neurons in the PMM constitute the avian A11 group and that it functions in controlling reproductive seasonality. The expression of c-fos mRNA within the PMM is differentially activated by light and corresponds with the rhythm of photosensitivity (Thayananuphat et al., 2007a; 2007b). A relationship between the dopaminergic system in the PMM and the GnRH-I system at the nucleus commissurae pallii (bed nucleus pallial commissure; nCPa) during photo-induced reproductive activity has been reported. Recently, neurons have been found in the PMM that express both DA and melatonin and have been shown to cycle rhythmically with photoperiodic changes (Kang et al., 2007).

Another aspect regarding the function of A11 DA groups is the hypothesis that DA within the posterior hypothalamus, particularly from the nI, may play a role in the onset of puberty (Fraley and Kuenzel, 1993a). In contrast with this study, it has been suggested that dopaminergic neurons located in the PVN and ML might be possibly influencing gonadal maturation (Kuenzel, 2000). In addition, it has been put forward that the more rostral of the A11 neurons in the caudal hypothalamus may be involved

in courtship singing in song birds that sing and court females for breeding, such as zebra finches (Bharati and Goodson, 2006). In the remainder of brain areas examined (AM, PVN, and ML), changes in number were less dramatic during the reproductive cycle, with no significant differences observed between groups. No significant differences in the number of TH-ir neurons in the AM, PVN, and ML of the turkey hypothalamus during the reproductive cycle have been reported. Furthermore changes in the number of TH-ir neurons and stain intensity in the nucleus preopticus medialis (POM) have been observed between reproductive stages (Al-Zailaie et al., 2003). Curiously, in this study, only a limited number of DA neurons were found in the POM, which is consistent with previous studies of DA- and TH-ir neuron distribution in the chicken and canary (Moons et al., 1994, Appeltants et al., 2001). Activation of dopaminergic cells in the A14 and A15 groups is a critical link leading to seasonal shifts in the sensitivity of estrogen negative feedback in the ewe (Lehman et al., 1996), and it has been suggested that these groups are represented by the AM in birds (Moons et al., 1994; Appeltants et al., 2001) A previous study indicated that DA in the medial preoptic area facilitated male sexual behavior (Hull et al., 1995; Dominguez and Hull, 2005; Bharati and Goodson, 2006). The PVN has been shown to control the hormonal secretions of anterior and posterior pituitary in mammals (Swanson and Sawchenko, 1983). In addition, PVN seems to play a pivotal role in the onset of puberty in rats (Gellert and Ganong, 1960) as well as in birds (Fraley and Kuenzel, 1993a; 1993b; Kuenzel, 2000). DA perikaya in the ML represent a discrete subset of neurons that control reproduction in birds, including control of GnRH-I and VIP perikaya. Activation of dopaminergic cells in the ML are linked to activation of GnRH-I and VIP neurons and the release of LH and PRL (Al-Zailaie et al., 2006). Plasma LH

levels in the native Thai chicken did not change during the reproductive cycle (Kosonsiriluk et al., 2007). It might be possible that, in the native Thai chicken, the TH-ir neurons in the ML are not involved with the regulation of the PRL/VIP system, but may be partially correlated with the DA neuronal circuit that regulates the GnRH/LH system.

The present study also investigated the distribution of TH-ir neurons and fibers in the brain of the native Thai chicken. In general, the distribution of TH-ir neurons and fibers corresponded with previous studies, including the domestic fowl (Knigge and Piekut, 1985), pigeon (Kiss and Peczely, 1987; Berk, 1991), Japanese quail (Bailhache and Balthazart, 1993), and zebra finch (Bottjer, 1993). The anatomical distribution is also in accordance with studies using the catecholamic enzyme for determining the distribution of the dopaminergic system in the bird brain (Contijoch et al., 1992; Moons et al., 1994; 1995; Durstewitz et al., 1998; Mello et al., 1998a; Roberts et al., 2001; Absil et al., 2001; Appeltants et al., 2001; den Boer-Visser and Dubbeldam, 2002; Al-Zailaie et al., 2006). However, there are minor species differences in the abundance and distribution of DA neurons when compared with the chicken brain. In the laying native Thai chicken, TH-ir neurons and fibers were found distributed throughout the entire brain and predominantly located within the diencephalon and mesencephalon. Contradictory to these present results, the majority of L-3,4-dihydroxyphenylalanine (L-DOPA) and DA-ir cells in the chicken are found widely within the midbrain and the brainstem (Moons et al., 1994). In the diencephalon, the densest TH-ir neurons were observed in the nI. The result corresponds with the finding that the chick nI contained dopaminergic neurons (Kuenzel et al., 1992). L-DOPA and DA-ir neurons have been observed in the nI of

the chicken brain (Moons et al., 1994). On the other hand, a few noradrenaline-ir (NA-ir) fibers were observed in the chicken nI (Moons et al., 1995). A number of TH-ir neurons and fibers were also found in the AM, SCNm, PVN, LHy, PVO, PMM, and ML. A dense group of cerebrospinal fluid (CSF)-contacting cells immunoreactive for L-DOPA and DA were observed in the PVO of the chicken brain (Moons et al., 1994). As in this study, these neurons have extended bipolar processes running perpendicularly to the wall of the third ventricle. However, this previous result is not in good agreement with this present study, since although a compact group of TH-ir neurons was found in the PVO, none of them were CSF-contacting cells. In the pigeon, the PVO does not appear to contain TH-ir cells (Kiss and Peczely, 1987). The PVO has been proposed to be a circumventricular organ (CVO) in the avian brain (Kuenzel and van Tienhoven, 1982) that is suspected of endocrine activity and possibly affects hypothalamic function (Weindl, 1973). In the INF area, a small group of TH-ir fibers were located within the MM and in the external layer of ME. In mammals, the regulation of PRL secretion is under the inhibitory control of tuberoinfundibular DA (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 D_2 DA receptors located on pituitary lactotrophs (Civelli et al., 1991). The lack of hypothalamic TH-ir cells in the tuberoinfundibular area has been reported in birds (Kiss and Peczely, 1987; Bailhache and Balthazart, 1993; Moons et al., 1994; Appeltants et al., 2001).The present study confirms 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 were found. This result supports the earlier suggestion that TIDA neurons in birds are absent (Reiner et al., 1994) and that

DA in the avian hypothalamus may not be the primary prolactin inhibiting factor (Kiss and Peczely, 1987). A large group of TH-ir neurons and fiber were observed in the mesencephalic area in the AVT, TPc, and OM. The DA neurons in the AVT and TPc appeared to be homologous to the ventral tegmental area (A10) and substrantia nigra (A9; Moons et al., 1994; Bentivoglio and Morelli, 2005). In good agreement with the present study, the DA neuronal group in substantia nigra is the major locus of dopaminergic cells in the rat brain (van den Pol et al., 1984). A10 DA neurons in the AVT are known to exhibit immediate early gene responses to sexual interaction in male Japanese quail (Charlier et al., 2005). Notably, the characteristic of TH-ir neurons found in this area is their apparent difference from the neurons found in the diencephalon. Moreover, the number of TH-ir neurons was markedly decreased when compared with the previous areas.

Scattered TH-ir neurons were observed in the LoC, SCv, nDBC, SCd, and MCC and TH-ir fibers were observed in VeM and VeD. In contrast with this study, the majority of the L-DOPA and DA-ir cells are found extensively within the midbrain and the brainstem (Moons et al., 1994). Unlike the TH-ir structures found in the diencephalon, it might be possible that the TH-ir neurons and fibers found in rhombencephalon are NA neurons, not DA neurons. Employment of the TH antibody for indicating the distribution of the dopaminergic system was used in this present study because TH is the rate-limiting enzyme in DA synthesis. Indeed, the presence of TH-ir structures could not distinguish between DA and NA. However, previous data reported that the density of DA-beta hydroxylase (DBH), an enzyme responsible for NA synthesis in the avian brains, is much lower than that of the density of TH-ir fibers (Mello et al., 1998a; 1998b). In addition, it has been reported that double-labeled

neurons immunoreactive for TH and DBH were not found in the turkey hypothalamus (Al-Zailaie et al., 2000). These results are supported by the data that DBH-ir neurons and positive fibers seem to be confined to the lower brain stem, pons, and medulla (Reiner et al., 1983; von Bartheld and Bothwell, 1992; Bailhache and Balthazart, 1993). However, the distribution of DA-ir neurons has been reported in the AVT, TPc, LoC, BCA, VeM and SCv of the chicken (Moons et al., 1994). Thus, it might be possible that TH immunoreactivity observed in these areas of the native Thai chicken could be dopaminergic neurons.

In summary, TH-ir neurons and fibers are found distributed throughout the brain of the native Thai chicken. The present study demonstrated, for the first time, that the number of TH-ir neurons in the nI changes during the reproductive cycle, with the highest numbers observed in incubating hens and correlated with the levels of plasma PRL. These findings are presumed to suggest that dopaminergic neurons in the nI are involved in the reproductive regulatory system in this non-photoperiodic species. The differential expression of TH-ir neurons in the nI may affect the changes occurring in VIP release and ensuing PRL secretion.

5.6 References

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CHAPTER VI

THE INFLUENCES OF PHOTOPERIOD ON THE REPRODUCTIVE SYSTEM OF THE NATIVE THAI CHICKEN

6.1 Abstract

In seasonal breeder birds, the initiation of the breeding cycle depends upon the precise prediction of environmental conditions which are optimal for health of the mating pair and the survivability of its offspring. The environmental cue which is the predominant proximal factor for the initiation of seasonal events in birds is photoperiod. The reproductive system of most birds living over a wide range of latitudes from the arctic regions to the tropics can presumably respond to changes in day length. The integration of the endocrine system, hypothalamic neuropeptides/neurotransmitters/ neuromodulators, pituitary hormones, ovarian steroids, environmental photoperiod, ambient temperature, and the presence of egg and chick, regulating the reproductive cycle of seasonally breeding birds and this may be the case in the native Thai chicken. This study was designed to investigate the effects of photoperiod upon the neuroendocrine regulation of the reproductive system in the native Thai chicken, a non-seasonally breeding tropical species. Female chickens at 22 (Experiment I) and 16 (Experiment II) weeks old were subjected into 4 different photoperiodic treatments as control (CT), short day (SD), normal (ND), and long day (LD). The results showed that

the age at first laying was tend to lower in birds kept in long day photoperiod than that of other treatment groups. In addition, the number of laying hens and egg productions were highest in this treatment group (Experiment I, CT: 52, SD: 50, ND: 56, and LD: 76 eggs; Experiment II, CT: 55, SD: 42, ND: 21, and LD: 95 eggs). The results indicated that long day photoperiod can enhance sexual maturity and laying performance of the native Thai chickens, suggesting that photoperiod may, in parts, has an effect on the reproduction of the native Thai chicken.

6.2 Introduction

In birds, environmental information initiates reproductive development prior to the onset of optimal condition for raising offspring, while another environmental information regulates the specific termination of reproductive activity (Wingfield et al., 2000). Breeding seasons can occur at the same time of the year (seasonal or predictable breeders) and there are birds that rely on unpredictable food availability (opportunistic breeders). In seasonal breeder birds, the initiation of the breeding cycle depends upon the precise prediction of environmental conditions which are optimal for health of the mating pair and the survivability of its offspring (Curlewis, 1992). The environmental cue which is the predominant proximal factor for the initiation of seasonal events is photoperiod. Most birds breeding outside the tropical zone use seasonal changes in day length to provide predictive information for the optimal time to initiate breeding (for review, see Sharp, 1996). In most avian species at mid and high latitudes, gonadal maturation occurs during spring, as photoperiod increases and so called long day breeders. In contrast, in most of long day breeding photoperiodic species, period of reproduction is terminated abruptly by the rapid collapse of gonad

during late summer when days are still long. During this period, called photorefractoriness, birds stop responding to light after prolonged exposure to long days (usually several weeks; Hamner, 1968; Follett and Pearce-Kelly, 1990; Saldanha et al., 1994; Bentley et al., 1998). At this time, gonads regress, reproductive hormones decline, and breeding behavior ceases (Dawson et al., 2001).

Birds have extra-retinal photoreceptors which they use, in conjunction with a circadian clock, to measure photoperiod (Dawson et al., 2001). Day length plays an essential role in the control of seasonal reproduction in many avian species (Nicholls et al., 1988; Dawson et al., 2001; Kubokawa et al., 1994). All of the neural components necessary for the photoperiodic response in birds appear to lie within the diencephalons including the photoreceptor and the gonadotropins releasing hormone (GnRH) system. Photoperiod has been associated with the reproductive neuroendocrine axis in birds since the lengthening day stimulates secretion of GnRH and gonadotropins and consequent gonadal growth (Follett and Robinson, 1980; Dawson et al., 2001). There are growing evidences that photoperiod associated with the GnRH system. The stimulatory effect of long days appears to be associated with an increase in GnRH content in hypothalamus or increase immunoreactivity for GnRH in the hypothalamus and median eminence in avian species such as European starling (Dawson et al., 1985; Foster et al., 1987; Goldsmith et al., 1989), garden warbler (Bluhm et al., 1991), dark-eyed junco (Saldanha et al., 1994), house sparrow (Hahn and Ball, 1995), and Japanese quail (Foster et al., 1988; Perera and Follett, 1992). On the other hand, gonadal regression through photorefractoriness is related to a decrease in hypothalamic GnRH-I (Dawson et al., 2001; 2002).

There are evidences demonstrating the relationship of the hormones varies

depending upon the stage of the reproductive cycle and the seasonal photostimulation. Exposing photosensitive birds to day length longer than approximately 12 hours (defined as long days), as is naturally the spring, stimulates the release of GnRH, luteinizing hormone (LH), and follicle stimulating hormone (FSH; Lewis and Farner, 1973; Wingfield et al., 1980; Dawson and Goldsmith, 1983; Fehrer et al., 1985; Wilson, 1985; Foster et al., 1987; McNaughton et al., 1995; Meddle and Follett, 1997). In turkey hen, increase in LH generally occurs within 2 to 3 days after the photoperiod is changed from short days to long days. Photosensitive turkey hens secrete LH in low frequency, high amplitude pulses superimposed on a low baseline (Chapman et al., 1994). This sustained increase in plasma LH levels associated with a change from short day to long day has been reported in Japanese quail (Follett et al., 1977; Perera and Follett, 1992), white-crowned sparrow (Yokoyama and Farner, 1976; Wingfield et al., 1980), and domestic fowl (Bonney et al., 1974; Sharp, 1993). Preovulatory LH surge that cause ovulation in birds is normally occurred during the dark period (scotophase) in laying turkey (Chapman et al., 1994; Yang et a., 2000) and chicken (Etches and Schoch, 1984) exposed to diurnal lighting. Plasma LH levels remain elevated during the subsequent laying phase of reproduction, but drop to prelaying concentrations during incubating or if photorefractoriness is occurred (Kuwayama et al., 1992). Dunn and Sharp (1990) reported that the shortest photoperiod required to stimulate LH release (critical day length) in 8 weeks old dwarf broiler breeders is between 8 and 10.5 hours, while the shortest photoperiod required to maximize LH release (saturation daylength) is between 10.5 and 12.5 hours. This pattern of secretion is stimulatory to the ovary and continues until egg laying begins when it is modified by ovulatory surges of LH.

In seasonal breeder temperate zone birds, the effects of season and photostimulation on the hypothalamic-pituitary-gonadal axis are well characterized. On the other hand, seasonal reproduction in tropical birds has not been as well studied as in temperate zone species (Hau, 2001). At the equator, there is no annual change in photoperiod. However, breeding in many avian species remains seasonal (Lofts and Murton, 1968). Non-photoperiodic cues are thought to accelerate or inhibit the effects of photoperiod on reproductive development and behaviors, thus ensuring that the timing of breeding is synchronized with optimal local environmental conditions (Wingfield et al., 2000). These conditions include temperature (Wada, 1993; Wingfield et al., 2003; Perfito et al., 2005), food availability (Hahn, 1998; Hau et al., 2000; Deviche and Sharp, 2001), increased green vegetation (Priedkalns et al., 1984), humidity (Cynx, 2001; Vleck and Priedkalns, 1985), water availability (Vleck and Priedkalns, 1985), rain (Zann et al., 1995), and presence of mates (Wingfield and Monk, 1994; Eda-Fujiwara et al., 2003). In some environments such as the tropics, deserts, and near the Arctic, birds may breed at different times in different years (Hahn et al., 1997; Deviche and Sharp, 2001) and in these environments the timing of reproductive behaviors appears to be predominantly controlled by non-photoperiodic stimuli (Zann et al., 1995; Hahn et al., 1997; Hau, 2001) such as increased rainfall (Zann et al., 1995; Lloyd, 1999; Grant et al., 2000).

It is not known how tropical birds detect and translate their apparently important local cues into neuroendocrine signals that govern reproduction. Interestingly, tropical avian species experiencing relatively smaller amplitude of annual photoperiodic cycle also appear responding to sufficiently small changes in light hours, and they use this cue to time their breeding (Chandola et al., 1985;

Gwinner and Dittami, 1985; Hau et al., 1998; Hau, 2001; Styrsky et al., 2004). Both high- and low-latitude species possess sensitivity to even very small changes in photoperiods (Chandola et al., 1985; Gwinner and Dittami, 1990; Wingfield and Farner, 1993; Deviche and Small, 2001; Hau, 2001). A study on blue tits supports this by showing that a long photoperiod can override non-photoperiodic factors in timing of the reproduction (Lambrechts and Perret, 2000). In addition, it has been reported that even small changes in photoperiod can be used to time reproduction for some species such as spotted antbird *(Hylophylax naevioides)* in Panama (Hau et al., 1998; Beebe et al., 2005). It has been hypothesized that many birds living in the deserts of Mexico and the American Southwest use a combination of photoperiod and cues associated with rain to time reproduction (Delesantro, 1978; Miller, 1958; Vleck, 1993). Some tropical and sub tropical birds which do not experience variations in annual day length are also reported to use photoperiod as an environmental factor to time their seasonal reproduction and exhibit the phenomena of absolute photorefractoriness such as common myna, red headed bunting, red vented bulbul, and Indian rose finch (Chaturvedi and Thapliyal, 1983; Thapliyal and Gupta, 1989; Rani and Kumar, 2000).

In contrast to the temperate zone seasonal breeding species, native Thai chicken is an equatorial zone continuously breeding species that produces eggs all year long. The jungle fowl is the ancestor of the native Thai chicken, originated in the tropical region of Southeast Asia, where its breeding season would has been timed by both photoperiodic and non-photoperiodic factors, allowing the chick to hatch at a time of year when food is most abundant. The integration of the endocrine system, hypothalamic neuropeptides/neurotransmitters, pituitary hormones, ovarian steroids,

environmental photoperiod, ambient temperature, and the presence of egg and young regulates the reproductive cycle of seasonally breeding birds and this may be the case in the native Thai chicken. Although the effects of photoperiod upon the reproductive function are well recognized in seasonal breeder temperate zone bird, there is evidence supported that reproductive function in breeding opportunistically species is partly dependent on photoperiod as demonstrated by several findings in male red crossbills (Tordoff and Dawson, 1965; Hahn, 1995; 1998). There are a limited number of studies providing data about the effect of photoperiod upon the reproductive system in this non-temperate zone gallinaceous bird. The effects of photoperiod upon growth, reproductive development, laying performance, and reproductive efficiency in native Thai chickens have been reported (Chotesangasa et al., 1992; Chotesangasa and Gongruttananun, 1994; 1995; 1997; Choprakarn et al., 1998). However, the results of these studies are too contradictive and far from understood. Thus, this study was designed to investigate the effects of photoperiod upon the neuroendocrine regulation of the reproductive system in the native Thai chicken. The knowledge gained will provide the information of neuroendocrine regulation including the physiological characteristics of the reproductive cycle in the native Thai chicken when treated with different photoperiod. The results (lighting regimens) of the study can be applied commercially in poultry industry to increase egg production of the native Thai chicken in Thailand.

6.3 Materials and Methods

6.3.1 Experimental Animals

116 Female native Thai chickens (*Gallus domesticus*), Pradoohangdam breed,

were used. Each hen was identified by wing band number. The birds were reared and housed in 4 separate rooms for each lighting program. Feed and water were provided *ad libitum.* The baskets for egg laying were provided. Birds were divided into 4 reproductive stages: non-egg laying (NL), egg laying (L), incubating eggs (B), and rearing chicks (R). The four reproductive stages were identified by behavioral observation. Briefly, NL were birds that not reached the sexual maturity and had never been laid eggs, L hens, had been laying regularly, B hens stopped laying and sit on the nest to incubate their eggs, and R hens, after the chicks hatched, stopped incubating eggs, and taking care of the chick. The chickens were observed during their reproductive cycle. Daily records of egg production, nesting activity, and other behaviors during the reproductive cycle were recorded. The animal protocols described in this study were approved by Suranaree University of Technology Animal Care and Use Committee Guidelines.

6.3.2 Experimental Design

6.3.2.1 Experiment I

To determine the effects of photoperiod upon the laying performance of native Thai hens, 76 female native Thai chickens at 22 weeks of age were used. They were reared and housed together with a mature male in separate rooms for each lighting program. The chickens were randomly divided into 4 treatment groups (19 females:1 male/pen) as the followings:

1) Control (CT): chickens were housed in floor pen under the natural light (approximately 12 hours of light and 12 hours of dark; 12L:12D; Hydrographic Department, 2006).

2) Short day (SD): chickens were housed in floor pen under the lighting regimen of 8L:16D.

3) Normal day (ND): chickens were housed in floor pen under the lighting regimen of 12L:12D.

4) Long day (LD): chickens were housed in floor pen under the lighting regimen of 16L:8D.

The chickens were reared in separate rooms under four different photoperiodic treatments for 6 weeks. Each pen was provided with nests. Chickens were observed during their reproductive cycle in order to classify them into 4 reproductive stages: NL, L, B, and R. Daily records of egg production, nesting activity, and other behaviors during the reproductive cycle were kept. At the end of the experiment, chickens were euthanized with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France). After chickens were sacrificed, ovaries and oviducts were removed and weighed. The presence of the ovary that contains a hierarchy of yellow yolky follicles were classified according to their diameters as F1 (larger than 3.5 cm), F2 (3.0-3.4 cm), F3 (2.0-2.9 cm), F4 (1.5-1.9 cm), and F5 (1.0-1.4 cm) were recorded. The present of the F1-F5 follicles was the criteria for considering sexual maturity in NL birds.

6.3.2.2 Experiment II

To determine the effects of photoperiod upon the sexual maturation of native Thai chicken, 40 female native Thai chickens at 16 weeks of age were used. They were reared and housed together with a mature male in separate rooms for each lighting program. The chickens were randomly divided into 4 treatment groups (10

females:1 male/pen) as the followings:

1) Control (CT): chickens were housed in floor pen under the natural light (approximately 12 hours of light and 12 hours of dark; 12L:12D; Hydrographic Department, 2006).

2) Short day (SD): chickens were housed in floor pen under the lighting regimen of 8L:16D.

3) Normal day (ND): chickens were housed in floor pen under the lighting regimen of 12L:12D.

4) Long day (LD): chickens were housed in floor pen under the lighting regimen of 16L:8D.

The chickens were reared in separate rooms under four different photoperiodic treatments for 13 weeks. Each pen was provided with nests. Chickens were observed during their reproductive cycle in order to classify them into 4 reproductive stages: NL, L, B, and R. Daily records were kept of egg production, nesting activity, and other behaviors during the reproductive cycle. At the end of the experiment, chickens were euthanized with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France). After chickens were sacrificed, ovaries and oviducts were removed and weighed. The presence of the ovary that contains a hierarchy of yellow yolky follicles were classified according to their diameters as F1 (larger than 3.5 cm), F2 (3.0-3.4 cm), F3 (2.0-2.9 cm), F4 (1.5-1.9 cm), and F5 (1.0-1.4 cm) were recorded. The present of the F1-F5 follicles was the criteria for considering sexual maturity in NL chicken. NL chicken that observed for the F1-F5 follicles were considered to be sexual maturity birds. On the other hand, pre-sexual maturity bird were identified by the absent of F1-F5 hierarchical follicle in NL chickens.

6.3.3 Statistical Analysis

Differences in age at first laying and egg production among 4 photoperiodic groups (treatment groups) were compared. Results were expressed as mean ± SEM. Significant differences between reproductive stages were analyzed employing one way analysis of variance (ANOVA) with multiple comparisons were determined using Tukey's HSD test. P value (P) < 0.05 was considered statistically significant. All statistical tests were analyzed employing the SPSS for Windows software (version 13.0, SPSS Inc., Chicago, IL, USA).

6.4 Results

6.4.1 Experiment I

Means and SEM of the age at first laying in each treatment group (n=19) were shown in Fig. 6.1 and Table 6.1. The results showed that the first groups that laying were the chicken in the LD group (25.22 $\pm$ 0.44 weeks old). The last groups that laying were the chicken in SD group (30.75 $\pm$ 1.25 weeks old). However, the age at first laying was not significant different among treatment groups. The number of egg production was greater in LD group (76 eggs) than that of in CT, ND, and SD groups (52, 56 and 50 eggs, respectively; Table 6.1). The results showed that the number of laying hen were found highest in LD group (9 hens), intermediate in ND group (7 hens), low in CT group (5 hens), and the least in SD group (4 hens; Table 6.3). At the end of the experiment, the ovaries were removed and recorded for the presence of F1-F5 follicles (Table 6.2). The sexual maturity chickens were observed greater in CT group (5 chickens) and ND group (4 chickens) than that of in SD group (2 chickens)

and LD group (1 chicken). In contrast, the SD group had the greatest number of pre-sexual maturity birds (13 chickens; Table 6.3).

6.4.2 Experiment II

Means and SEM of the age at first laying in each treatment group (n=10) were shown in Fig. 6.2 and Table 6.4. The results showed that the chicken in LD group started to lay eggs earlier (23.86 $\pm$ 0.88 weeks old) than chickens in CT, SD and ND groups (27.60 $\pm$ 0.62, 26.67 $\pm$ 0.37, and 28.50 $\pm$ 0.22 weeks old), respectively. However, there was no significant different among treatment groups. The number of egg production was highest in LD group (95 eggs), high in CT group (55 eggs), and low in SD group (42 eggs). The lowest egg production was found in ND group (21 eggs; Table 6.4). The ovaries were removed and recorded for the presence of F1-F5 follicles (Table 6.5). The greatest number of laying hen was found in LD group (7 hens), intermediate in CT group (5 hens), low in SD and ND groups (3 and 2 hens, respectively). The numbers of sexual maturity birds were essentially the same in every treatment groups. However, the greatest number of pre-sexual maturity birds was observed in ND group (Table 6.6).

Fig. 6.1 Age at first laying (week) of the native Thai chicken in each treatment group of Experiment I (n=19). Values are expressed as the mean ± SEM. Values with different letters are significantly different (P<0.05).

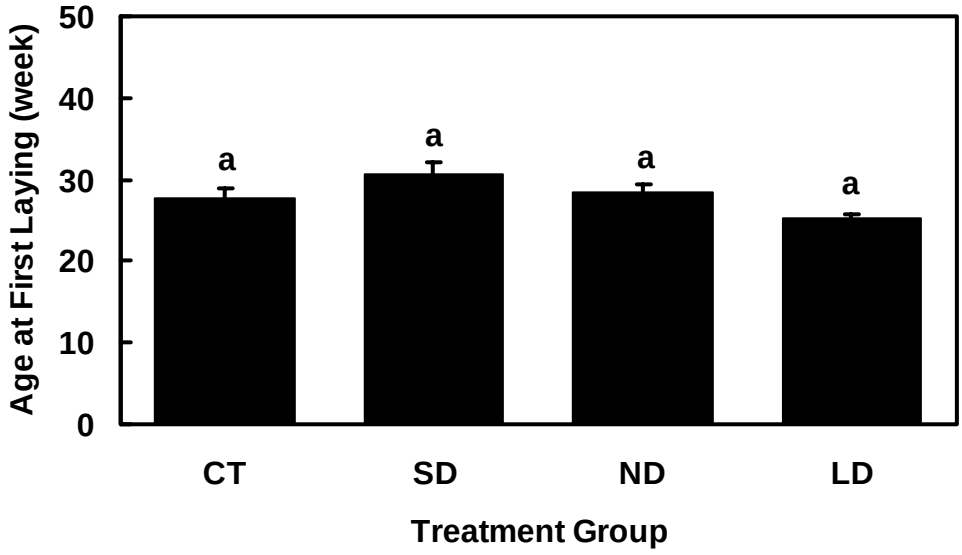

Table 6.1 Mean ± SEM of the age at first laying, total number of laying hen, and egg production of the native Thai chicken in each treatment group of Experiment I (n=19).

Treatment Group	Age at First Laying (week)	Egg Production (egg)
CT	27.60 ± 1.22	52
SD	30.75 ± 1.25	50
ND	28.33 ± 1.04	56
LD	25.22 ± 0.54	76

Table 6.2 The presence of F1-F5 follicles of the native Thai chicken in each treatment group of Experiment I (n=19).

Treatment Group	F1	F2	F3	F4	F5	Total
CT	0	2	6	4	7	19
SD	1	4	12	5	4	26
ND	3	5	21	8	9	46
LD	1	1	8	5	7	22

Table 6.3 Total number of chickens in each reproductive stage in each treatment group of Experiment I (n=19).

Treatment Group	Number of Chicken		
	Laying Hen	Sexual Maturity	Pre-Sexual Maturity
CT	5	5	9
SD	4	2	13
ND	7	4	8
LD	9	1	9

Fig. 6.2 Age at first laying (week) of the native Thai chicken in each treatment group of Experiment II (n=10). Values are expressed as the mean ± SEM. Values with different letters are significantly different (P<0.05).

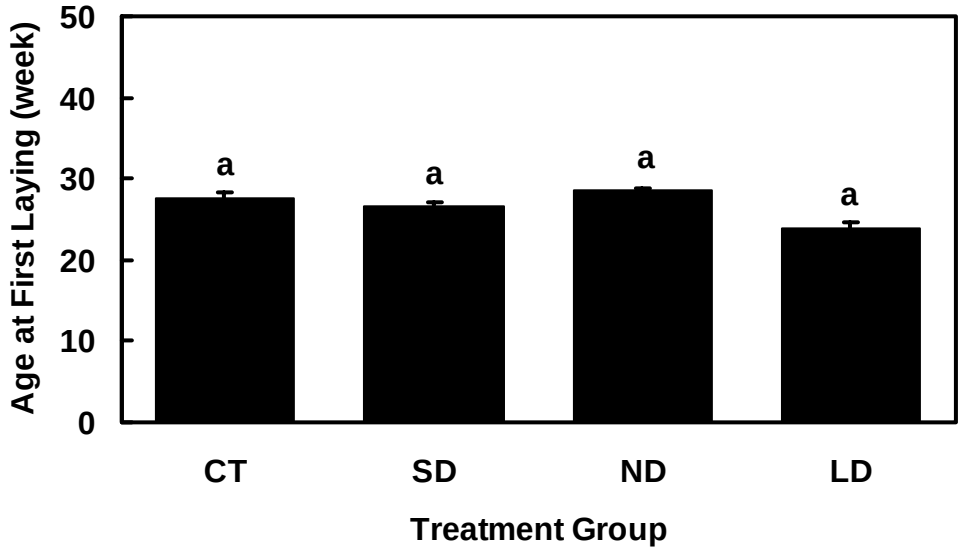

Table 6.4 Mean ± SEM of the age at first laying, total number of laying hen, and egg production of the native Thai chicken in each treatment group of Experiment II (n=10).

Treatment Group	Age at First Laying (week)	Egg Production (egg)
CT	27.60 ± 0.62	55
SD	26.67 ± 0.37	42
ND	28.50 ± 0.22	21
LD	23.86 ± 0.88	95

Table 6.5 The presence of F1-F5 follicles of the native Thai chicken in each treatment group of Experiment II (n=10).

Treatment Group	F1	F2	F3	F4	F5	Total
CT	2	15	5	4	0	34
SD	0	7	11	7	2	33
ND	0	2	8	4	4	23
LD	1	4	4	6	2	25

Table 6.6 Total number of chickens in each reproductive stage in each treatment group of Experiment II (n=10).

Treatment Group	Number of Chicken		
	Egg Laying	Sexual Maturity	Pre-Sexual Maturity
CT	5	3	2
SD	3	3	4
ND	2	3	5
LD	7	1	2

6.5 Discussion

The results of the present study showed that birds kept in LD photoperiod start to lay earlier than that of birds that were kept in CT, ND, and SD photoperiod. In addition, the number of laying hens and egg productions were highest in LD groups. These findings suggested that photoperiod has an effect upon the laying performance and the sexual maturity of the native Thai chickens.

The effects of photoperiod upon the regulation of sexual maturity in the native Thai chicken were investigated. When birds were subjected into LD photoperiod, they started to lay earlier than that of the control group. On the other hand, the age at first laying was delayed in birds subjected into SD photoperiod. This result corresponds with previous studies in temperate zone birds indicated that an increase in the photoperiod could advance gonadal maturation and the longer the (constant) photoperiod to which birds are exposed, the greater the rate of maturation (Burger, 1947). In addition, increased photoperiod may maintain reproductive system of some avian species in a continuous state of activity (Nicholls et al., 1988; Chaturvedi et al., 1993). The physiological processes underlying the timing of breeding in birds have been studied extensively in temperate zone avian species (Murton and Westwood, 1977; Wingfield et al., 1992; Ball, 1993; Cockrem, 1995; Hahn et al., 1997). Most information on the relationship between changes in photoperiod and ovarian functions comes from the domestic hen, where photo-induced changes in FSH or LH are indicators of the rate of ovarian development, as measured by mean age at first egg (Lewis et al., 1994; 1998; 1999). Photoperiod has been associated with the reproductive neuroendocrine axis in birds since the lengthening day stimulates secretion of GnRH and gonadotropins and consequent gonadal growth (Follett and

Robinson, 1980; Dawson et al., 2001). It has been found that increasing in day length stimulates the release of GnRH, FSH, and LH in birds (Lewis and Farner, 1973; Wingfield et al., 1980; Dawson and Goldsmith, 1983; Fehrer et al., 1985; Wilson, 1985; Foster et al., 1987; McNaughton et al., 1995; Meddle and Follett, 1997). Conversely, reduction in day length (short photoperiod) delays the onset of sexual maturity or terminate egg laying activity in birds (Benoit, 1964; Woodard et al., 1969).

The total number of laying hens at the end of the experiment was highest in LD groups of both experiments. In addition, the total number of egg production was the highest in LD group and lowest in SD group of Experiment II. These results support the effect of photoperiod upon the laying performance in this species. It is suggested that LD photoperiod can enhance the reproductive system of the native Thai chicken. This result is in good agreement with previous studies indicated that egg production in laying hen was at the highest levels when exposing photosensitive hens to an appropriated day length (approximately 14-16 hours/day) during the egg laying period (North and Bell, 1990). The results of this study indicated that birds kept at shot day photoperiod still laid eggs. This was not the case in temperate zone seasonal breeders such as turkey in which the elevated activity of the reproductive system that characterized the breeding period is absolutely stimulated by long day photoperiod. However, the result in the native Thai chicken corresponds with studies reported that although full ovarian maturation occurs in domestic hens in the absence of photostimulation, it is greatly accelerated by photostimulation (Morris, 1979; Sharp, 1992). Moreover, it has been suggested that the reproductive system of most, and perhaps all, birds living over a wide range of latitudes from the arctic regions to the tropics can presumably respond to changes in day length (Deviche and Small, 2001).

Clearly, the activity of the ovary as was observed by the presence of F1-F5 follicles was changed according to the photoperiodic treatments. It was seen that at the end of the experiment, birds in SD groups had less number of F1-F5 follicles than that of observed in other groups. This data indicated that the degree of ovarian development reduced when subjected birds into short day photoperiod. This data is supported by the result that at the end of the experiment, the numbers of birds that reached sexual maturity and had been laying egg were higher in LD group than that of other groups. These results, taken together, indicated that the gonads of the chickens in LD group tended to develop earlier than that of the other treatment groups.

It has been reported that other non-photoperiodic cues (i.e. climatic factors, food availability) in addition to chicks may also be important in determining the onset of egg laying and breeding (Hahn et al., 1997). It has been reported that maternal behavior including physical contact as well as visual and/or auditory stimuli originating from the chicks has inhibitory effect on the reproductive neuroendocrine system (Richard-Yris and Leboucher, 1987; Richard-Yris et al., 1987; Sharp et al., 1988). Recently, it has been found that differential expression following hatching of the young may in part explain the difference in reproductive strategies between an equatorial non-photoperiodic continually breeding native Thai chickens and photoperiodic seasonally breeding turkeys (Kosonsiriluk, 2007).

The integration of the endocrine system, hypothalamic neuropeptides and neurotransmitters, pituitary hormones, ovarian steroids, environmental photoperiod, ambient temperature, and the presence of egg and young regulates the reproductive cycle of seasonally breeding birds and this may be the case in the native Thai chicken. A positive correlation between ambient temperature and the rate of gonadal

development has been shown to exist in birds (Farner and Mewaldt, 1952; Engles and Jenner, 1956; Farner and Wilson, 1957). The results from this study are supported by the previous studies revealed that exposure to thermal stress (acute or chronic) adversely affects ovulation rate and decreases reproductive performance in the laying hen (Miller and Sunde, 1975; DeAndrade et al., 1976; 1977; Jones et al., 1976; Wolfenson et al., 1979; Cowan and Michie, 1980; Tanor et al., 1984; Scott and Balnave, 1988). It has been suggested that the decline in ovulation rate may be an indirect consequence of reduced feed intake and subsequent body weight loss experienced by the hyperthermic hen (DeAndrade et al., 1976; Austic, 1985). However, in this study, body weights of birds in each treatment group were not significantly different (data not shown). Support this result is the suggestion that the effects of high environmental temperatures upon the rate of egg laying appeared largely unrelated to food intake (Smith and Oliver, 1972; Donoghue et al., 1990; Servatius et al., 2001). Increased prolactin (PRL) levels in response to heat stress have been suggested as a mechanism involved in this reproductive malfunction (El Halawani et al., 1984; Donoghue et al., 1989).

The effects of photoperiod upon the reproductive function are well recognized in seasonal breeder temperate zone birds. However, the results from this study suggested the role of photoperiod upon the reproductive function of the native Thai chicken, a non-temperate zone continuous breeding species. The results were in good agreement with previous studies indicated that reproductive function in breeding opportunistically species is partly dependent on photoperiod (Tordoff and Dawson, 1965; Hahn, 1995; 1998). The effects of photoperiod upon growth, reproductive development, laying performance, and reproductive efficiency in native Thai chickens

have been reported (Chotesangasa et al., 1992; Chotesangasa and Gongruttananun, 1994; 1995; 1997; Choprakarn et al., 1998; Kosonsiriluk, 2007). In addition, the results were in good agreement with studies conducted in some tropical and sub tropical birds which do not experience variations in annual day length such as common myna, red headed bunting, red vented bulbul, and Indian rose finch (Chaturvedi and Thapliyal, 1983; Thapliyal and Gupta, 1989; Rani and Kumar, 2000) and support the role of photoperiod upon the reproduction of native Thai chicken.

In summary, the reproductive characteristics including sexual maturity and laying performance of the native Thai chicken were improved by rearing birds in long day photoperiod. The results support the role of photoperiod upon the reproduction of native Thai chicken. It is suggested that although the native Thai chicken was not used photoperiod as a pivotal environmental cue to timing of breeding as in temperate zone birds, photoperiod may, in parts, has an effect on the reproduction of the native Thai chicken.

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