รายงานวิจัยฉบับสมบูรณ์

โครงการ การควบคุมวงจรการสืบพันธุ์ในไก่พื้นเมืองไทยเพศเมียโดยระบบประสาทและระบบต่อมไร้ท่อ:

Neuroendocrine Regulation of the Female Native Thai Chicken Reproductive Cycle

โดย ผู้ช่วยศาสตราจารย์ ดร. ยุพาพร ไชยสีหา

มีนาคม 2551

สัญญาเลขที่ RSA4780001

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ผู้วิจัย

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สังกัด

มหาวิทยาลัยเทคโนโลยีสุรนารี

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

ไก่พื้นเมืองไทยมีผลผลิตไข่ต่ำและมีพฤติกรรมความเป็นแม่สูงซึ่งส่งผลกระทบต่อผลผลิตไข่ ระบบประสาทและระบบต่อมไร้ท่อที่มีบทบาทสำคัญต่อวงจรการสืบพันธุ์ของสัตว์ปีกในเขตอบอุ่นมีสองระบบ คือ ระบบวาโซแอคทีฟอินเทสทินอลเปปไทด์/โปรแลคติน และระบบโกนาโดโทรปินรีลีสซิงฮอร์โมน/ฟอลลิเคิลสติมูเลติงฮอร์โมน-ลูติไนซิงฮอร์โมน นอกจากนี้ระบบประสาทวาโซแอคทีฟอินเทสทินอลเปปไทด์ โดปามีนและซีโรโทนิน ยังมีการทำงานร่วมกันเป็นเครือข่ายระบบประสาทในการควบคุมการหลั่งฮอร์โมนโปรแลคตินและโกนาโดโทรปินส์ แต่ยังไม่เป็นที่เข้าใจแน่ชัดในสัตว์ปีกที่อาศัยในเขตศูนย์สูตรที่มีการสืบพันธุ์ตลอดทั้งปีโดยที่ไม่ขึ้นกับช่วงแสง นอกจากปัจจัยด้านต่อมไร้ท่อแล้วปัจจัยด้านสิ่งแวดล้อม เช่น ช่วงแสงยังส่งผลกระทบต่อวงจรการสืบพันธุ์ของสัตว์ปีกอีกด้วย วัตถุประสงค์ของงานวิจัยนี้คือเพื่อศึกษาทำงานร่วมกันของระบบประสาทและระบบต่อมไร้ท่อในการควบคุมระบบสืบพันธุ์ในสัตว์ปีก การศึกษาระบบทั้งสองแบบควบคู่กันไปจะทำให้ได้ข้อมูลพื้นฐานของระบบประสาทและต่อมไร้ท่อที่เกี่ยวข้องกับระยะการสืบพันธุ์ในไก่พื้นเมืองไทย นอกจากนี้ยังได้ศึกษาบทบาทของช่วงแสงในการควบคุมระบบสืบพันธุ์อีกด้วย ผลการศึกษาพบว่าระดับโปรแลคตินมีการเปลี่ยนแปลงตามระยะการสืบพันธุ์ในขณะที่ไม่พบการเปลี่ยนแปลงระดับของลูติไนซิงฮอร์โมน เซลล์ประสาทที่ผลิตวาโซแอคทีปอินเทสทินอลเพปไทด์มีการกระจายอยู่ทั่วทั้งสมอง และพบมากในส่วนไดเอนเซฟาลอนโดยพบหนาแน่นที่สุดที่นิวเคลียสอินเฟอริออริสไฮโปทาลาไม และนิวเคลียสอินฟันดิบูไลฮโปทาลาไม การเปลี่ยนแปลงจำนวนเซลล์ประสาทที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์ภายในนิวเคลียสอินเฟอริออริสไฮโปทาลาไมและนิวเคลียสอินฟันดิบูไลไฮโปทาลาไมมีความสัมพันธ์โดยตรงกับระดับโปรแลคตินตลอดระยะวงจรการสืบพันธุ์ คาดการณ์ได้ว่าเซลล์ประสาทที่ผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์ในบริเวณดังกล่าวอาจมีบทบาทสำคัญในการควบคุมวงจรการสืบพันธุ์ของสัตว์ปีกในแถบเส้นศูนย์สูตร เซลล์ประสาทที่ผลิตโกนาโดโทรปินรีลิสซิงฮอร์โมนพบตั้งแต่พรีออพติกไปจนถึงแอนทีเรียไฮโปทาลามัสและพบหนาแน่นบริเวณนิวเคลียสคอมมิสซูรีพาลลิไอ ปริมาณของเซลล์ประสาทที่ผลิตโกนาโดโทรปินรีลิสซิงฮอร์โมนที่บริเวณนิวเคลียสคอมมิสซูรีพาลลิไอมีการเปลี่ยนแปลงตามวงรอบการสืบพันธุ์ แสดงให้เห็นว่าโกนาโดโทรปินรีลิสซิงฮอร์โมนมีความสัมพันธ์กับระบบสืบพันธุ์ในไก่พื้นเมืองไทย การกระจายตัวของโดปามีนในสมองโดยใช้เอนไซม์ไทโรซีนไฮดรอกซีเลส พบว่าเซลล์ประสาทที่มีไทโรซีนไฮดรอกซีเลสตลอดทั่วทั้งสมองและพบมากในสมองส่วนไดเอนเซฟาลอนและมีเซนเซฟาลอน มีการเปลี่ยนแปลงจำนวนของเซลล์ประสาทที่พบไทโรซีนไฮดรอกซีเลสที่บริเวณนิวเคลียสอินทราเมดิเอลิสตามวงรอบการสืบพันธุ์ และมีความสัมพันธ์โดยตรงกับการเปลี่ยนแปลงระดับของโปรแลคติน จำนวนเซลล์ประสาทที่ผลิตไทโรซีนไฮดรอกซีเลสในนิวเคลียสอินทราเมดิเอลิส จะมีการเปลี่ยนแปลงเพิ่มขึ้นในระยะการฟักไข่ ซึ่งเป็นช่วงระยะเวลาเดียวกันกับที่ฮอร์โมนโปรแลคตินมีระดับสูงสุด ผลการศึกษานี้แสดงให้เห็นเป็นครั้งแรกว่าเซลล์ประสาทที่ผลิตโดปามีนมีความสัมพันธ์กับการควบคุมระบบสืบพันธุ์ในไก่พื้นเมืองไทย ผลการศึกษานี้คาดการณ์ได้ว่าความแตกต่างของเซลล์ประสาทที่ผลิตโดปามีนในนิวเคลียสอินทราเมดิเอลิสอาจจะมีบทบาทในการควบคุมการผลิตวาโซแอคทีฟอินเทสทินอลเปปไทด์และตามมาด้วยการหลั่งฮอร์โมนโปรแลคตินในไก่พื้นเมืองไทย รูปแบบการกระจายตัวของเซลล์ประสาทวาโซแอคทีฟอินเทสทินอลเปปไทด์ โกนาโดโทรปินรีลีสซิงฮอร์โมนและไทโรซีนไฮดรอกซีเลส สอดคล้องตรงกับการศึกษาที่มีมาก่อนในสัตว์ปีกหลายชนิด การศึกษานี้สามารถยืนยันบทบาทที่สำคัญของวาโซแอคทีฟอินเทสทินอลเปปไทด์ โกนาโดโทรปินรีลีสซิงฮอร์โมนและโดปามีน ในการควบคุมระบบการสืบพันธุ์ในไก่พื้นเมืองไทย ซึ่งอาศัยอยู่ในเขตศูนย์สูตรมีระบบการควบคุมเช่นเดียวกับสัตว์ปีกที่อาศัยอยู่ในเขตอบอุ่น

Abstract

Native Thai hens have the poorest egg production and also express highly maternal behaviors; such behaviors affect egg production because the onset of incubation behavior terminates egg laying, resulting in the cessation of egg production. There are 2 major neuroendocrine systems play a pivotal role in the avian reproductive cycle of temperate zone birds; the vasoactive intestinal peptide/prolactin (VIP/PRL) and gonadotropin releasing hormone/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH) systems. In addition, there is a complex neuronal network of integrative interaction between VIPergic, dopaminergic, and serotoninergic systems regulates PRL and gonadotropins (FSH and LH) secretion but it is not well understood in birds, especially in equatorial continually breeding, non-photoperiodic avian species. It is not only endocrine factors that influence the reproductive cycle of birds but also environmental factors such as photoperiod. Birds are highly photoperiodic and gonadal development occurs in response to increasing day lengths. Therefore, the objective of this research is to investigate both systems concurrently since the two systems are functionally related, parallel investigation will provide better insight into their combined roles in the regulation of avian reproduction and to establish a baseline information on the neuroendocrine changes associated with the different reproductive stages of the native Thai chickens, an equatorial bird. The roles of photoperiod upon the neuroendocrine regulation of reproductive system were also elucidated. The results revealed that plasma PRL levels were changed across the reproductive stages, where as the changes of plasma LH levels were not observed. The results of the immunohistochemistry (IHC) studies illustrated that VIP-immunoreactive cells and fibers were found throughout the brain and predominantly located within the diencephalons with the greatest staining was found within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN). Changes in VIP-ir neurons within the IH-IN area were directly correlated with PRL throughout the reproductive cycle. This suggests that VIP neurons in the IH-IN may play a pivotal role in regulating the reproductive cycle of equatorial, continually breeding, non-photoperiodic birds. GnRH-I-ir neurons were distributed in a discrete region from the preoptic area through the anterior hypothalamus. The most abundance of GnRH-I-ir neurons was found within the nucleus commissurae pallii (nCPa). Changes in number of GnRH-I-ir neurons in the nCPa were observed across the reproductive cycle, indicating that GnRH-I are correlated with the reproductive cycle in the native Thai chicken. The distribution of dopamine (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 were elucidated. TH-ir neurons and fibers were found throughout the brain and were predominantly located within the diencephalon and mesencephalon. 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 nI increased significantly during the egg incubation period, which was also the period when circulating PRL levels were the greatest. This result indicates, for the first time, that an association exists between DA neurons and the regulation of the reproductive system in the Thai chicken. The results of this study 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. The distributions pattern of VIP-ir, GnRH-I-ir, and TH-ir observed in the present study are consistent with that reported previously in several avian species. This present study confirms the pivotal roles of VIP, GnRH, and DA in the control of avian reproduction of this equatorial, non-seasonally breeding tropical species as in the case of temperate zone birds.

Executive Summary

1. Title of the Research Project

(Thai) **การควบคุมวงจรการสืบพันธุ์ในไก่พื้นเมืองไทยเพศเมียโดยระบบประสาทและระบบต่อมไร้ท่อ**

(English) **Neuroendocrine Regulation of the Female Native Thai Chicken Reproductive Cycle**

2. Principle Investigator

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3. Research Areas

Avian Neuroendocrinology and Reproduction

4. Background, Rationale and Significance

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 millions baths 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 and the environmental factor(s) influencing its reproductive activity. Thus, the present study is proposed to investigate neuroendocrine, and photoperiod regulation of the reproductive cycle in the female native Thai chicken.

The reproductive cycle of the native Thai chicken is divided into four reproductive stages; non-egg laying, egg-laying, incubating eggs, and rearing chicks. 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).

There are several lines of evidence showing that hormones play an important role in the reproductive cycle of avian species, including maternal behavior. PRL, an anterior pituitary hormone, has been shown to be associated with the reproductive cycle in several avian species (turkey, quail, bantam, ring dove, pigeon, and mallard duck). No studies have been conducted on native Thai chickens. PRL has been implicated as a causative factor in the onset and maintenance of broodiness in birds. The onset of incubation activity in turkey hens is correlated with declining levels of gonadotropins (LH andFSH) and a dramatic rise in circulating PRL level. It is this rising PRL level which has been implicated as the cause of cessation of ovulation, ovarian regression, and induction of incubation behavior. It is very well documented that PRL is under stimulatory control by hypothalamic VIP, the avian PRL-releasing factor (PRF).

It is not only endocrine factors that influence the reproductive cycle of birds but also environmental factors. The jungle fowl, the ancestor of native Thai chickens, originated in the tropical regions of Southeastern Asia, where their breeding seasons would have been timed by both photoperiodic and non-photoperiodic factors such as temperature, allowing the chicks to hatch at a time of year when food was most abundant. Birds are highly photoperiodic and gonadal development occurs in response to increasing day lengths. The endocrine and environmental factors associated with the reproductive cycle of avian species are complex. The integration of the endocrine system, hypothalamic neuropeptides, pituitary hormones, ovarian steroids, environmental photoperiod and ambient temperature, and the presence of eggs and chicks, regulating the reproductive cycle of seasonally breeding birds and this may be the case in the native Thai chicken. It is known that light exerts a major influence on the reproductive function of birds and 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-around production of eggs for hatching to be achieved, it is obvious that lighting programs and environmental temperature and their interaction must be considered.

The goals of this research project are to characterize the neuroendocrine regulation of reproductive cycle in female native Thai chickens and the roles of photoperiod upon the

neuroendocrine regulation of reproductive system. The findings from this research project will provide an insight into the visibility of enhancing the reproductive performance of native Thai chickens. Another important outcome of this research is the increase in our knowledge into the hormonal and physiological characteristics of the reproductive system in our native Thai chicken which is not established in Thailand. The final outcome is to increase the supply high protein, healthy product which is already in demand by consumers.

5. Objectives of the Research Project

The overall objective is to characterize the neuroendocrine mechanism(s) mediating the regulation of the female native Thai chicken reproductive system. Roles of pituitary hormones (PRL and LH) and hypothalamic releasing factors (VIP, DA and GnRH) are investigated.

Specific Objectives

1. To characterize the neuroendocrine regulation and the mechanism(s) mediating the reproductive system of the female native Thai chicken.
2. To characterize the role of photoperiod in the neuroendocrine mechanism(s) regulating the reproductive system of the female native Thai chicken.

6. General Methods

7.1 Experimental Animals:

Female and male native Thai chickens, Pradoohangdum breed, 16-18 weeks old, purchased from commercial farm were used. They were reared and housed in floor pens. Food and water are constantly available. They were reared under the lighting program (12 hours of light and 12 hours of darkness; 12L:12D). At 22 weeks of age, the hens were subjected to the experiments except noted in each specific experiments. The hens were observed and classified according to their reproductive stages. Each hen had wing band number for identification. The experiments were carried out following Suranaree University of Technology Animal Care and Committee guideline.

7.2 Hypothalami and Pituitaries Preparation:

The hypothalami and pituitaries were collected Briefly, hens were killed by euthanasia injection (pentobarbital sodium). The brains were immediately dissected intact from the skull, and the pituitary glands were detached. The optic chiasma were dissected away from the ventral surface of the brain to expose the underlying hypothalamus. A block of tissue, limited rostrally by the septomesencephalic tract and caudally by posterior border of the mammillary bodies were removed. This area includes the median eminence, hypothalamus, and preoptic hypothalamus. Collected hypothalami and pituitaries were immediately frozen in liquid nitrogen, and then stored at -80 °C until used. The hypothalami will be homogenized for the determination of VIP and GnRH gene expression and the pituitaries will be homogenized for the determination of PRL, LH and FSH gene expression.

7.3 Radioimmunoassay:

Blood samples were collected from each bird and fractionated by centrifugation. The plasma were decanted and kept frozen at -20 °C until analyzed for plasma PRL, LH and FSH levels utilizing the radioimmunoassay described by Berghman et al. (1992) and Burke et al. (1979), respectively.

7.4 Enzyme-Linked ImmunoSorbent Assay:

Blood samples were collected from each bird and fractionated by centrifugation. The plasma were decanted and kept frozen at -20 °C until analyzed for plasma PRL, LH and FSH levels utilizing the Enzyme-Linked ImmunoSorbent Assay described by Proudman et al. (2001).

7.5 Northern Blot Analysis:

Total RNA will be extracted from frozen pituitaries using protocol previously described (Chaiseha et al., 1998a) and kept at –80 °C until used. Northern blot analysis will be performed as described by Sambrook et al. (1989).

7.6 In situ Hybridization:

To localize gene expression of hypothalamic factors within the brain, in situ hybridization will be performed as previously described (Chaiseha et al., 2003).

7.7 Immunohistochemistry:

Processing of Tissues for Immunohistochemistry: Prior to perfusion, each bird was intravenously injected with 3 ml of heparin (1000 unit/ml) and then euthanized with pentobarbital sodium (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. 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.

Immunohistochemistry: To localize VIP, GnRH and tyrosine hydroxylase (TH; DA marker) distribution throughout the brain of the laying hen and characterize the changes 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 and four adjacent sections in the hypothalamic areas of each bird 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.) 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.) 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.).

7.8 Image Analysis

An atlas of the chick brain (Kuenzel and Masson, 1988) was used to identify the areas of the brain that expressed VIP, GnRH, and TH immunoreactivity neurons and fibers. Microscopic images of brain sections were visualized with a fluorescence microscope at 4x, 10x, 20x, and 40x magnification. Images were captured with a digital camera and stored by (Olympus, Tokyo, Japan). To characterize the differential expression of the VIPergic, GnRH, and DAergic systems across the reproductive cycle four adjacent brain sections (eight microscopic fields) in the hypothalamic areas from each bird according to each reproductive stage were chosen and counted manually to compare the number of 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 the immunoreactivity.

7.9 Statistical Analysis

Significant differences in plasma levels of pituitary hormones and the number of VIP, GnRH, and TH 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).

Please note: To date, Suranaree University of Technology is not able to set the laboratory that can use the radioactive. Therefore, Northern blot analysis, in situ hybridization and radioimmunoassay cannot perform. However, hormonal assay are deem essential to be done for this research project. I proposed to use the facility at Prof. Dr. M.E. El Halawani at University of Minnesota which is already set up for the assays. According to the avian flu in Thailand in 2004-present, the samples cannot send to the USA for conducting the assay. Fortunately, the samples are permitted to send to Israel (Dr. Israel Rozenboim's laboratory) for conducting the hormonal assay. Eventhough, non-radioactive in situ hybridization can be conducted at the laboratory but the cost of this technique is way too expensive and the approved budget from this research project cannot afford to study this technique. Immunohistochemistry (IHC) technique was utilized to replace Northern blot analysis and in situ hybridization studies because the results of IHC will show the localization and abundance of the proteins which are the final products of the gene expression. In fact, the disadvantage of the Northern blot analysis is the results of the gene expression of total RNA from the tissue/brain but it cannot demonstrate the localization of that mRNA. Therefore, IHC technique is seemly appropriated to replace the Northern blot analysis and in situ hybridization.

7. Research Project

8.1 Objectives:

This research grant has 2 specific objectives:

<u>Objective 1:</u> Characterization of the neuroendocrine regulation and the mechanism(s) mediating the reproductive system of the female native Thai chicken.

There are 2 major neuroendocrine systems play a pivotal role in the avian reproductive cycle of temperate zone birds; the VIP/PRL and GnRH/FSH-LH systems. In addition, there is a complex neuronal network of integrative interaction between VIPergic, DAergic, and 5-HTergic systems regulates PRL and gonadotropins (FSH and LH) secretion but it is not well understood in birds, especially in equatorial continually breeding, non-photoperiodic avian species. Therefore, the objective of this study is to investigate both systems concurrently since the two systems are functionally related, parallel investigation will provide better insight into their combined roles in the regulation of avian reproduction and to establish a baseline information on the neuroendocrine changes associated with the different reproductive stages of the native Thai chickens, an equatorial bird.

<u>Objective 2:</u> Characterization of the role of photoperiod in the neuroendocrine mechanism(s) regulating the reproductive system of the female native Thai chicken.

Numerous studies in the 1950's and 1960's were conducted to investigate the effect of day length manipulation during the starting, growing, and holding periods on the reproductive performance of breeding hens. From all these studies, it was agreed that the day length had to be restricted to 6-9 hours of light per day prior to application of a gonadal-stimulatory light (14-16 hours per day). However, all aforementioned studies were conducted in temperate zone birds. Photoperiod studies on equatorial birds such as the native Thai chicken (the photoperiod; 12L:12D) are very limited. Furthermore, the role of photoperiod on the neuroendocrine regulatory mechanism(s) underlying gonadal recrudescence/regression are never been investigated in the native Thai chickens. The overall objective is to characterize the neuroendocrine regulation that governs the association of photoperiod and the gonadal recrudescence/development in the native Thai chicken. The findings from the studies will help to understand the effects of photoperiod upon the native Thai chicken reproduction. The information can be then applied commercially in poultry industry to increase reproductive efficiency and egg production of native Thai chicken in Thailand.

The results from this research grant will provide an insight the basic neuroendocrinology of native Thai chicken, which has not been established in Thailand in order to look into the visibility of enhancing the reproductive performance of native Thai chickens. In term of socio-economic and environmental benefits, if we were able to improve egg production of native Thai chicken from 40 eggs/year and produce about 30 chicks to more than double. It is possible to reach 120 eggs/hen as in imported breeds. This will increase the interest of the poultry industry to raise native Thai chickens in a large scale. Thailand has been importing chicken breeders and chicks from foreign countries, even though we have our local national breed, "native Thai chicken" which is known to have good quality meat with less fat, in addition to being resistant to diseases (use low/no antibiotics in the

diet). These characteristics make native Thai chickens very competitive with other foreign breeds, if we can raise them commercially in large numbers.

8.2 Results and Discussion

8.2.1 Objective 1:

<u>Experiment 1</u>: Circulating prolactin and gonadotropic hormones and their mRNA gene expression during the native Thai hen reproductive cycle.

The objective of this experiment is to establish a baseline information on the neuroendocrine changes associated with the different reproductive stages of the native Thai hens. The studies were divided into 2 studies.

First study, 30 female native Thai chickens at 16 weeks of age were used. The chickens were divided into 6 pens (6 female:1 male/pen) and subjected to 12 hours of light and 12 hours of darkness (12L:12D). Each pen was provided with trap nests to identify the reproductive state of each bird. Chickens were observed everyday in order to classify into 4 reproductive stages (non-laying; NL, laying hens; L, incubating hens; B, and rearing hens; R). During the experiment, blood samples from the brachial vein were collected once a week from the beginning until the end of the experiment for determining plasma PRL, LH and FSH levels by radioimmunoassay. Daily records of egg production, nesting activity, and other behaviors during the reproductive cycle were kept. The reproductive characteristics of the reproductive stages were obtained and plasma hormones were analyzed form collected blood samples from one reproductive cycle. However, in order to understand and gain more information, the experiment was extended to follow one more reproductive cycle, including collecting blood samples every week as the first reproductive cycle. The results showed that the duration of lay, number of eggs, and % hatchability per hen of the hens in the second reproductive cycle is higher than that of the hens in the first reproductive cycle. However, the differences of plasma hormones among reproductive stages, especially between the first and second reproductive cycle of the hens were not observed. Blood samples were analyzed for plasma PRL, LH, and FSH levels by enzyme-linked immunosorbent sssay (ELISA). The results revealed that pattern of circulating PRL levels were low in NL, gradually elevated in L, reached the highest levels in B, and then declined sharply in R. In contrast, there were no changes in plasma LH levels (ng/ml) across the reproductive cycles. Plasma FSH levels cannot be detected due to the sensitivity of the assay.

Second study, 30 female native Thai chickens at 16 weeks of age were used. The chickens were reared in the pens in the same condition as the first study. At each of the aforementioned reproductive stage, hens were sacrificed and brains were removed. Blood sample were collected from each chicken prior to sacrifice for determining plasma PRL, LH, and FSH levels. Hypothalami and pituitaries were isolated and immediately frozen in liquid nitrogen, then stored at -80 °C until used. The oviducts were collected and weighed. The number of F1-F5 follicles, small white follicles, and small yellow follicles were recorded after sacrificing the hens. Daily records were kept of egg production, nesting activity, and other behaviors during the reproductive cycle. The results revealed that plasma PRL levels were low in NL, gradually increased in L, reached the highest levels in B, and then markedly

declined in R. There were no changes in plasma LH levels across the reproductive cycles. Plasma FSH levels cannot be detected due to the sensitivity of the assay. Gene expression study cannot be conducted because we do not have radioactive laboratory. However, the IHC studies were conducted in **Experiment 2** to gain the same results.

The results from the study indicated that plasma PRL levels were changed across the reproductive cycle, where as changes in plasma LH levels were not observed. In addition, the ovary and oviduct weights are differences among reproductive stages. The findings that ovarian regression observed in incubating and rearing hens in the absence of a decline in LH levels is interpreted as an adaptive mechanism(s) allowing for reinitiating egg laying in the case of nest destruction at any times and irrespective of the season. The findings further suggest that the antigonadotropic effect of PRL is limited to its effect on the ovary. In conclusion, the results of this present study provide, for the first time, the baseline information of the neuroendocrine changes during the reproductive cycles in this species and support the previous studies that PRL is associated with the reproductive cycle in avian species and play a pivotal role in the incubation behavior. **(Appendix: Manuscript #1)**

<u>Experiment 2</u>: Localization and determination of VIP and GnRH mRNAs in the hypothalamus and VIP, DA and serotonin receptors mRNA gene expression in the hypothalamus and pituitary of female native Thai chickens during the different stages of their reproductive cycle.

The objective of this experiment is to quantify and localize the mRNA of reproductive hormones and their releasing factors associated with the different reproductive stages.

The experimental design is similar to that described above. The IHC technique was used to localize and quantify the proteins of the hypothalamic factors. To localize VIP, GnRH and tyrosine hydroxylase (TH; DA marker) distribution throughout the brain of the laying hen and characterize the changes within individual brain areas in different reproductive stages, IHC was performed as previously described. The localization of DA and serotonin receptors cannot be detected due to the limited of the specific antibodies.

The results of the study illustrated that VIP-immunoreactive cells and fibers were found throughout the brain and predominantly located within the diencephalons with the greatest staining was found within the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN). Distribution of VIP neurons in the native Thai chicken and a comparison of VIPergic activity in the IH and IN were investigated. The pattern of VIP distribution in the native Thai chicken supports the findings reported in temperate zone species. Unlike the turkey, where there is a dissociation between VIPergic activity and PRL levels during photorefractoriness, in the native Thai chicken, which do not express photorefractoriness, changes in VIP-ir neurons within the IH-IN were directly correlated with PRL throughout the reproductive cycle. VIPergic activity reached its lowest level after hatching of the chicks in the native Thai chicken. This suggests that VIP neurons in the IH-IN may play a pivotal role in regulating the reproductive cycle and its differential expression following hatching of the young may, in part, account for the difference in

reproductive mode between equatorial, continually breeding, non-photoperiodic birds and seasonally breeding, photoperiodic birds. **(Appendix: Manuscript #2)**

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 were elucidated and 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 level. The population of TH-ir neurons in 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. **(Appendix: Manuscript #3)**

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. **(Appendix: Manuscript #4)**

The abundance of VIP, cGnRH-I, and DA neuronal network in hypothalamus of the native Thai chicken suggests of its importance in the regulation of reproductive behavior in equatorial birds.

8.2.2 Objective 2:

Environmental information initiates reproductive development prior to the onset of optimal conditions for raising offspring while other environmental factors regulate the specific timing of reproductive behaviors and the eventual termination of reproduction. The environmental cue responsible for the initiation of seasonal events is photoperiod. Most of birds are highly photoperiodic and gonadal development occurs in response to increasing day length. The endocrine and environmental factors associated with the reproductive cycle of avian species are complex. The integration of the endocrine system, hypothalamic neuropeptides/neurotransmitters, 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.The effects of photoperiod upon reproduction of the native Thai chicken, an equatorial continuous breeder, were investigated in this present study. The study was divided into three experiments.

Experiment 1: The effects of photoperiod upon circulating prolactin and gonadotropic hormones and their mRNA gene expression during the native Thai hen reproductive cycle.

The objective of this study is to characterize the effects of photoperiod upon reproductive system of native Thai hens.

Experiment 2: Alterations in PRL, LH, FSH, VIP, and GnRH mRNA expression of laying native Thai hens in response to different photoperiods.

The objective of this experiment is to study the effects of photoperiod in the regulation of PRL, LH, FSH, VIP and GnRH mRNA expression of laying native Thai hens.

Experiment 3: Characterization of the influence of photoperiod upon VIPergic, DAergic, and 5-HTergic neuronal systems.

The propose of this experiment is to identify which VIP neuronal groups is involved in the photoperiod response and further investigate the neuroendocrine regulation of dopaminergic and serotonergic systems upon the VIP neuronal systems.

Experiment 1:

Female native Thai chickens at 22 and 16 weeks of age were used. They were divided into 4 treatment groups with different photoperiodic treatments as control (CT; 12L:12D), short day (SD; 8L:16D), normal day (ND; 12L:12D), and long day (LD; 16L:8D). Blood samples were collected once a week throughout the experiment. Daily records of egg production, nesting activity, and other behaviors were kept. The chickens were observed their behaviors in order to classify them into 4 reproductive stages as non-laying (NL), laying (L), incubating (B), and rearing chicks (R). Chickens were sacrificed after conducting the experiments for 6 and 13 weeks. The ovaries and oviducts were removed and weighed, and the presence of F1-F5 follicles, small yellow follicle (SYF), and small white follicle (SWF) were recorded (Fig. 1). Means and SEM of ovary and oviduct weights in each treatment group (n=15) were shown in Fig. 2. The results showed that the ovary and oviduct weights and the presence of F1-F5 follicles, SYF, and SWF in LD group are higher than that of the other groups. In addition, the first laying hens were observed and the numbers of laying hen at the end of the experiments were highest in LD treatment group (Table 1), suggesting that long day length can enhance reproductive efficiency and increase sexual maturity of the native Thai chickens. The findings of this present study

suggested that photoperiod might play a role in reproduction of native Thai chicken. However, the results gained from the photoperiod studies are too contradictive and hardly to interpret. Originally, we expected that the photoperiod will play a largely role in reproduction of native Thai chicken. Unexpectedly, it seems like the photoperiod has no effects or play a minor role upon the reproductive system of the native Thai chicken. Therefore, blood samples were not further analyzed for the hormonal levels. It is also not worth study to further conduct the Experiment 2 and 3.

Fig. 1 The ovary of the laying native Thai hen shows the presence of F1-F5 follicles, small yellow follicles (SYF), small white follicles (SWF), and post-ovulatory follicles (POF).

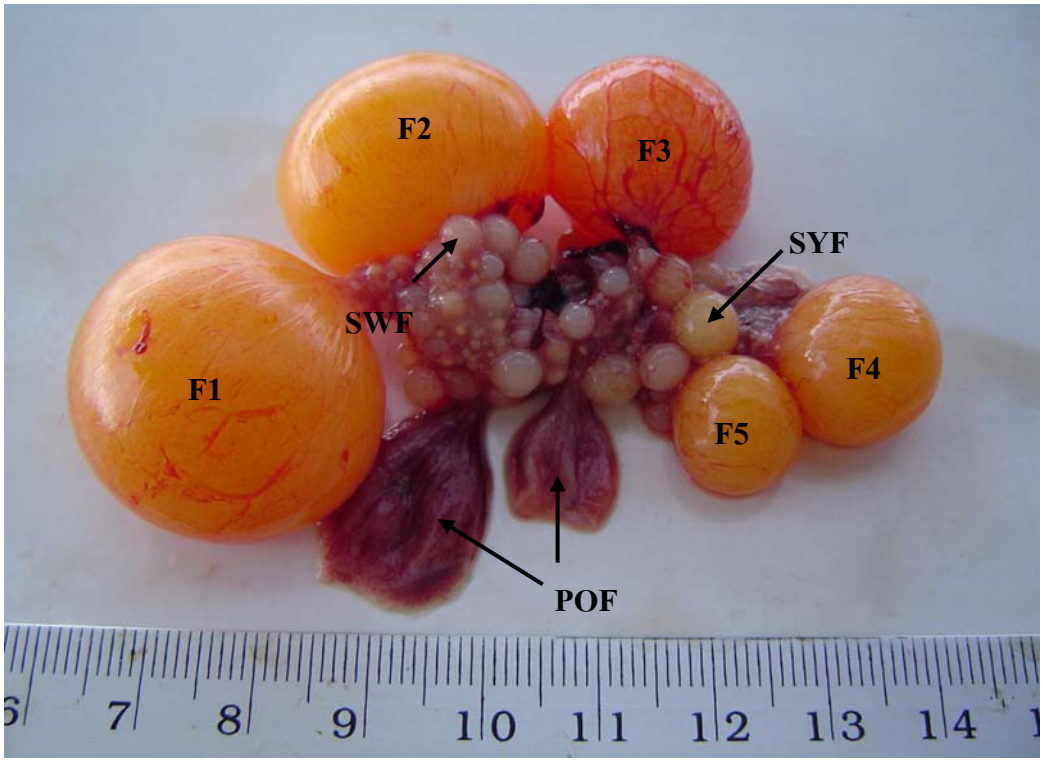

Fig. 2 Ovary and oviduct weights of the native Thai chickens in each treatment group of Experiment I (n=15). Values (Mean ± SEM) with different letters are significantly different (P<0.05).

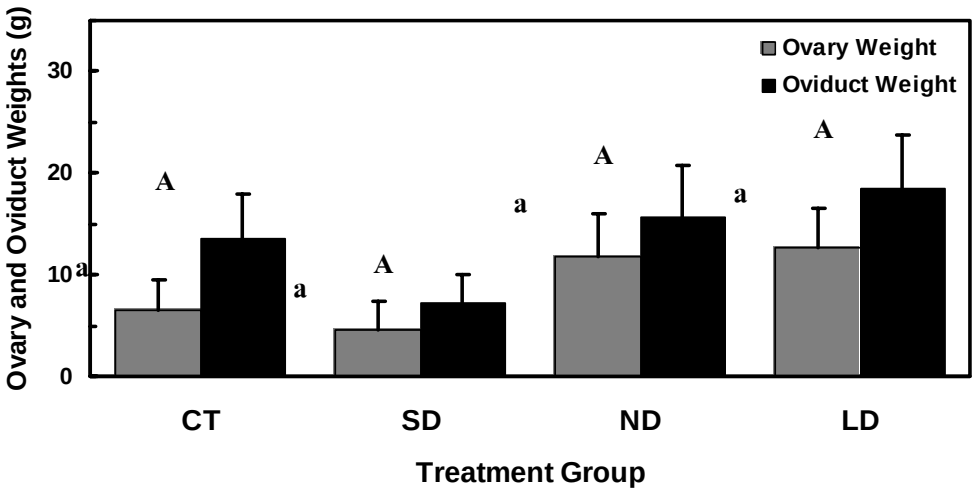

Table 1 Percentages of the number of laying hen, the presence of F1-F5 follicles, and the presence of SYF and SWF of the native Thai chickens in each treatment group of Experiment I.

Treatment Group	No. of Laying Hen (%)	The Presence of F1-F5 Follicles (%)	The Presence of SYF and SWF (%)
CT	6.67	40.00	40.00
SD	7.14	7.14	28.57
ND	13.33	40.00	40.00
LD	38.46	46.15	53.85

9. Output

9.1 Publications

1. Chaiseha Y, El Halawani ME (2005). Neuroendocrinology of the female turkey reproductive cycle. **The Journal of Poultry Science** 42: 87-100. (**Invited review**)

4 Abstracts were presented in Poultry Science Association Annual Meeting:

1. Kosonsiriluk S, Sartsoongnoen N, Prakobsaeng N, Bakken T, Songserm T, El Halawani ME, **Chaiseha Y** (2006). Distribution of vasoactive intestinal peptide-expressing Neurons in the brain of the native Thai chicken (*Gallus domesticus*). **Poultry Science 85** (Suppl.1): 45.

2. Sartsoongnoen N, Kosonsiriluk S, Prakobsaeng N, Thayananuphat, A, Songserm T, El Halawani ME, **Chaiseha Y** (2006). Immunohistochemical localization of dopamine neurons in the brain of the native Thai chicken (*Gallus domesticus*). **Poultry Science Poultry Science 85** (Suppl.1): 45.

3. Sartsoongnoen N, Kosonsiriluk S, Kang SW, Millam JR, El Halawani ME, **Chaiseha Y** (2006). Distribution of cGnRH-I immunoreactive neurons and fibers in the brain of native Thai chicken (*Gallus domesticus*). **Poultry Science 85** (Suppl.1): 45.

4. Kosonsiriluk S, Sartsoongnoen N, Prakobsaeng N, Rozenboim I, El Halawani ME, **Chaiseha Y** (2007). Prolactin and luteinizinh hormone profiles during the reproductive cycle in the native Thai chicken. **Poultry Science 86** (Suppl.1): 650.

Please Note: All manuscripts are submitted (see Appendix).

9.2 Other international publications from research collaborations (2004-2007):

1. **Chaiseha Y**, Youngren OM, El Halawani ME (2004). Expression of vasoactive intestinal peptide receptor mRNA in the hypothalamus and pituitary throughout the turkey reproductive cycle. **Biology of Reproduction** 70: 593-599.

2. Rozenboim I, Biran I, **Chaiseha Y**, Yahav S, Rosenstrauch A, Sklan D, Halevy O (2004). The effect of a green and blue monochromatic light combination on broiler growth and development. **Poultry Science** 83: 842-845.

3. Rozenboim I, Mobarky N, Heiblum R, **Chaiseha Y**, Kang SW, Biran I, Rosenstrauch A, Sklan D, El Halawani ME (2004). The role of prolactin in reproductive failure associated with heat stress in the domestic turkey. **Biology of Reproduction** 71: 1208-1213.

4. Kulick R, **Chaiseha Y**, Kang SW, Rozenboim I, El Halawani ME (2005). The relative importance of vasoactive intestinal peptide and peptide histidine isoleucine as physiological regulators of prolactin in the domestic turkey. **General and Comparative Endocrinology** 142: 267-273.

5. El Halawani ME, Proudman JA**.** Youngren OM, **Chaiseha Y** (2005). Serotonin receptor subtypes influence prolactin secretion in the turkey. **Poultry Science** 84 (Suppl.1): 45.
6. **Chaiseha Y**, El Halawani ME, Kosonsiriluk S, Sartsoongnoen N, Prakopsaeng N (2005). Neuroendocrinology of prolactin regulation in birds. **2nd Asian Reproductive Biotechnology Conference**, November 3rd-7th, Bangkok, Thailand (**Invited Talk**).

7. Al-Zailaie K, Kang SW, Youngren OM, Thayananuphat A, Bakken T, **Chaiseha Y**, Millam JR, Poundman JA, El Halawani ME (2006). Identification of dopamine, gonadotrophin-releasing hormone-I, and vasoactive intestinal peptide neurons activated by electrical stimulation to the medial preoptic area of the turkey hypothalamus: A potential reproductive neuroendocrine circuit. **Journal of Neuroendocrinology** 18: 514-525.

8. Leclerc B, Kang S, Thayananuphat A, Howell C, Kosonsiriluk S, **Chaiseha Y**, El Halawani ME (2007). Clock gene expression in the premammillary nucleus (PMM) and the pineal gland of turkey hens. **Poultry Science 86** (Suppl.1): 224.

9.3 Other academic activities

1. I was invited by **The Journal of Poultry Science** to co-write the review article with Professor M.E. El Halawani entitled "**Neuroendocrinology of the female turkey reproductive cycle**".

2. I was elected as an International Committee of International Symposium on Avian Endocrinology (ISAE) at **8th International Symposium on Avian Endocrinology**, Arizona, U.S.A, June 2004. This international committee member will be ended in 12 years. Hopefully, Thailand and/or Japan will co-hosted this symposium in the year of 2012.

3. I was awarded the **Hy-Line International Research Award** for 2005 from Poultry Science Association, USA. The award gave a plaque and monetary award ($2,500), my photograph and biographical sketch was published in ***Poultry Science*** 2005, Vol 84 (12), p 1988. This awarded details were published in Thai's newspaper 5 times and internet 9 times.

4. I was invited to give an invited talk in **2nd Asian Reproductive Biotechnology Conference**, November 3rd-7th, Bangkok, Thailand.

Appendix

1. Copy of the Reprint of Article #1:

Chaiseha Y, El Halawani ME (2005). Neuroendocrinology of the female turkey reproductive cycle. **The Journal of Poultry Science** 42: 87-100. (**Invited Review**).

The Journal of Poultry Science, 42 : 87–100, 2005

≪**Review**≫

Neuroendocrinology of the Female Turkey Reproductive Cycle

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A wealth of functional data confirms the involvement of hypothalamic vasoactive intestinal peptide (VIP) and gonadotropin releasing hormone (GnRH) in the regulation of the avian reproductive cycle. However, very little is known about the neurotransmitters or the anatomical locations of the hypophysiotropic neurons mediating the transition from one reproductive state to the next. Dopamine (DA) stimulates prolactin (PRL) and luteinizing hormone (LH) secretion by acting on VIP and GnRH neurons, respectively. DA may inhibit PRL secretion by antagonizing the action of VIP at the level of the pituitary, and limits LH secretion through presynaptic inhibition of GnRH release at the median eminence (ME) level. The stimulatory and inhibitory effects of DA are mediated via D_1 and D_2 DA receptors, respectively. However, the dopaminergic neuronal groups/subgroups which regulate the VIP/PRL and GnRH/LH systems remain to be clarified. Studies utilizing electro-pharmacological techniques in combination with radioimmunoassay, immunocytochemistry, and *in situ* hybridization histochemistry yield results suggesting the presence of a stimulatory dopaminergic pathway from the preoptic area (POA) to the infundibular nuclear complex (INF) area, where VIP neurons preside over the regulation of PRL secretion, as well as DA projections within the preoptic area-anterior hypothalamus (POA-AM) areas where the GnRH neurons reside that control LH secretion. DA neurons projecting to the ME which mediate the inhibition of the VIP/PRL and GnRH/LH systems remain to be identified.

Key words : birds, dopamine, gonadotropin releasing hormone, prolacin, vasoactive intestinal peptide

Introduction

Two neuroendocrine systems play a pivotal role in the reproductive cycle of temperate zone birds, including the domestic turkey, which is used as a model in our laboratory. One neuroendocrine system involves chicken gonadotrophin releasing hormone-I (cGnRH-I, referred to as GnRH) and the subsequent secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH ; GnRH/LH-FSH) system and the other involves the prolactin (PRL) releasing factor (PRF), vasoactive intestinal peptide (VIP) and the subsequent secretion of PRL (VIP/PRL) system. The two systems are dependent upon the duration of daylight and are involved in the transduc-

Received : August 10, 2004, Accepted : September 4, 2004
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tion of photoperiodic information resulting in either gonad recrudescence and its associated sexual activity (egg laying), or gonad regression and termination of reproductive activity (photorefractoriness).

LH and FSH secretion and gene expression are stimulated by long day length (Nicholls *et al.,* 1988 ; Dawson *et al.,* 2001) and require the functional integrity of the GnRH neuronal system (Katz *et al.,* 1990 ; Sharp *et al.,* 1990). The increase in VIP/PRL secretion in response to long day length is gradual, but progressive, and both release and gene expression are up-regulated (Wong *et al.,* 1991 ; El Halawani *et al.,* 1996 ; Tong *et al.,* 1997). Activation of the GnRH/LH-FSH and VIP/PRL systems in the somatically mature photosensitive female turkey initiates the transition from reproductive quiescence to reproductive activity. Gonadotropins stimulate estrogen secretion (Wineland and Wentworth, 1975), inducing sexual receptivity (El Halawani *et al.,* 1986), and prime the VIP/PRL system to enhance PRL secretion (El Halawani *et al.,* 1983).

At the onset of sexual maturity (first ovulation), the preovulatory surge of progesterone induces the nesting behavior associated with oviposition (Wood-Gush and Gilbert, 1973 ; El Halawani *et al.,* 1986), and the combined action of estrogen, progesterone, and nesting activity further stimulates PRL secretion (El Halawani *et al.,* 1983, 1986). These increasing PRL levels suppress the activity of the GnRH/LH-FSH system (Rozenboim *et al.,* 1993 b ; You *et al.,* 1995), reducing ovarian steroids secretion (Porter *et al.,* 1991 ; Tabibzadeh *et al.,* 1995), terminating egg laying, inducing ovarian regression (Youngren *et al.,* 1991), and signal the transition from sexual behavior to 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, 1989).

After hatching, or when eggs are replaced with poults, tactile stimuli from the young induces the emergence and maintenance of maternal responses, 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. With the onset of photorefractoriness, circulating PRL and LH levels and pituitary PRL/LH peptide and mRNA levels sharply decline, even though long day length continues (Wong *et al.,* 1991 ; Mauro *et al.,* 1992 ; Wong *et al.,* 1992 ; El Halawani *et al.,* 1996 ; Fig. 1). A rapid decrease in PRL and LH/FSH release and expression may be triggered at any time due to a lack of response to long day length (i.e. photorefractoriness) or by subjecting birds to short day lighting (Nicholls *et al.,* 1988 ; El Halawani *et al.,* 1990 a).

Immunoneutralization of VIP averts the rise in circulating PRL that follows photostimulation, prevents the induction of incubation behavior, up-regulates LHβ- and FSHβ subunit mRNAs, and extends the duration of reproductive activity (egg laying period), but does not prevent spontaneous gonad regression and molting (Sharp *et al.,* 1989 ; El Halawani *et al.,* 1995 a, 1996 ; Dawson and Sharp, 1998 ; Ahn *et al.,* 2001). Despite the well established antigonadotropic effect of PRL, it appears that the high circulating PRL level of laying, non-incubating birds is not the primary cause of

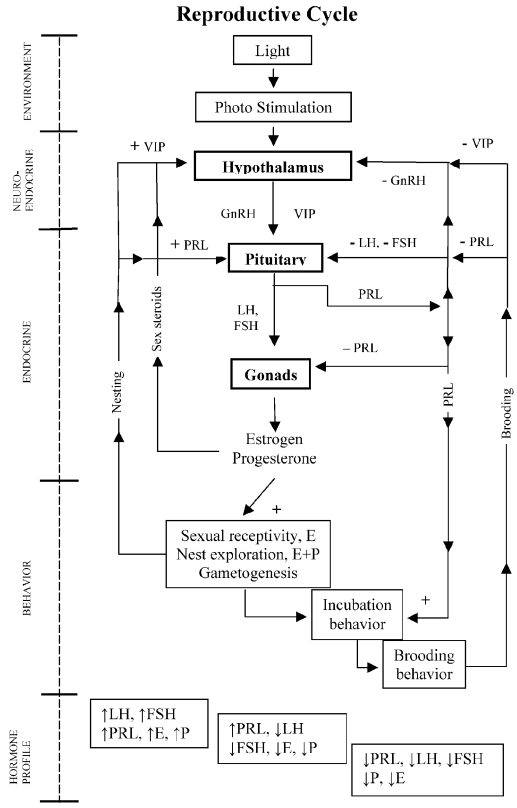

Fig. 1. Schematic diagram illustrating the interrelationships between external and internal stimuli and the neuroendocrine control of the reproductive cycle in the female turkey (bird).
+ = stimulation/positive feedback ; − = inhibition/negative feedback ; ↑ = increase ; ↓ = decrease ; GnRH = chicken gonadotropin releasing hormone-I ; VIP = vasoactive intestinal peptide ; LH = luteinizing hormone ; FSH = follicle stimulating hormone ; PRL = prolactin ; P = progesterone ; E = estrogen.

GnRH/gonadotropin suppression and the termination of reproduction (Juss,1993 ; Dawson and Sharp, 1998).

Neurohypophysiotropic Mechanisms

The final common pathway controlling the secretion of PRL, LH, and FSH is formed by a system of peptidergic neurons whose axons terminate around portal capillaries in the external layer of the median eminence (ME). VIP and GnRH are among the best characterized hypophysiotropic peptides.

GnRH neurons are found within the preoptic area (POA), anterior hypothalamus (AM) and lateral septum (LS ; Mikami *et al.,* 1988 ; Millam *et al.,* 1993). Little is known regarding the GnRH cell group(s) that project to the ME (Dawson and Goldsmith, 1997 ; Teruyama and Beck, 2000). Measurements of hypothalamic GnRH peptide in the hypothalamus during the reproductive cycle of the turkey (Millam *et al.,* 1989 ; El Halawani *et al.,* 1993 b ; Rozenboim *et al.,* 1993 a) indicate that levels do not

change in incubating birds. The amount of GnRH in the hypothalamus decreases during photorefractoriness in the turkey (Rozenboim *et al.,* 1993 a) and other avian species (Dawson *et al.,* 1985 ; Foster *et al.,* 1987 ; Bluhm *et al.,* 1991 ; Saldanha *et al.,* 1994 ; Hahn and Ball, 1995). In a recent study from our laboratory (Kang *et al.,* 2004), GnRH mRNA expression was determined utilizing *in situ* hybridization histochemistry (ISH) during the four different reproductive stages of the female turkey. GnRH mRNA was highly expressed in the organum vasculosum laminae terminalis (OVLT) and the bed nucleus of the pallial commissure (nCPA), and limited expression was observed in the POA, medial preoptic nucleus (POM), and LS. Hypothalamic GnRH mRNA expression was significantly increased after subjecting the non-photostimulated female turkey to a 90 minute light period at Zeitgeber time (ZT) 14. GnRH mRNA abundance within LS, OVLT, and nCPA areas was highest in laying hens, with decreasing abundance found in non-photostimulated and incubating hens, respectively. The lowest levels of GnRH mRNA were observed in photorefractory hens. These results indicate that hypothalamic GnRH mRNA expression may be used to precisely characterize the different reproductive states.

VIP neurons are widely distributed throughout the hypothalamus (Yamada *et al.,* 1982 ; Mikami and Yamada, 1984 ; Peczely and Kiss, 1988 ; Mauro *et al.,* 1989 ; Chaiseha and El Halawani, 1999). Studies using a combination of electrophysiology, radioimmunoassay, immunocytochemistry (ICC), and ISH suggest that VIP in the ME 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.,* 2002 a). VIP is very well accepted as the avian PRL releasing factor (PRF ; El Halawani *et al.,* 1997). VIP peptide and 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). ICC and ISH studies have shown that fluctuations in hypothalamic VIP immunoreactivity and expression within the INF parallel fluctuations in circulating PRL (Mauro *et al.,* 1989 ; Chaiseha and El Halawani, 1999). Other studies have also shown increases in the number and size of VIP immunoreactive neurons within this region in the domesticated pigeon following the initiation of crop milk secretion and feeding of off-spring, which are periods of elevated circulating PRL (Peczely and Kiss, 1988). Moreover, concentrations of VIP in portal blood plasma are significantly higher than VIP concentrations in peripheral blood plasma in all reproductive stages. VIP concentrations in portal blood plasma are lowest in non-photostimulated, reproductively quiescent turkey hens, and highest in incubating hens, with laying and photorefractory hens having intermediate levels (Youngren *et al.,* 1996 a). These differences in VIP portal blood concentrations mirror those of PRL in the general circulation, supporting the hypothesis that VIP is the avian PRF.

A decoupling of total hypothalamic VIP peptide and mRNA from circulating and pituitary PRL is seen in the VIP/PRL system of reproductively inactive photo-refractory birds (Mauro *et al.,* 1992 ; Saldanha *et al.,* 1994 ; Chaiseha *et al.,* 1998 ;

Chaiseha and El Halawani, 1999). PRL reaches its lowest level and VIP its highest level during the photorefractory stage of the reproductive cycle. This raises several questions related to the role of VIP in the initiation and termination of the avian reproductive cycle. Does the elevated hypothalamic VIP expression indicate an enhanced VIPergic system ? If so, which VIP neuron groups are involved ? VIPergic neuron ensembles are found in the INF, POA, lateral septal organ (LSO), and anterior hypothalamus-suprachiasmatic nucleus area (AM-SCN ; Mauro *et al.,* 1989 ; Chaiseha and El Halawani, 1999). We have established that VIP neurons residing in the INF area are the source of VIP regulating PRL secretion (Mauro *et al.,* 1989 ; Chaiseha and El Halawani, 1999 ; Youngren *et al.,* 2002 a). VIP axon terminals have been found in close apposition to GnRH neurons in the LSO and POA (Teruyama and Beck, 2001), and an inverse relationship between VIP in the INF and GnRH in the POA has been reported (Deviche *et al.,* 2000). Elevated hypothalamic VIP peptide and mRNA contents are associated with gonad regression and suppression of gonadotropin in photorefractory turkeys (Mauro *et al.,* 1989 ; Chaiseha *et al.,* 1998 ; Chaiseha and El Halawani, 1999). VIP immunoneutralization up-regulates LHβ- and FSHβ subunit mRNAs (Ahn *et al.,* 2001), and delays the onset of photorefractoriness and molt in starlings (Dawson *et al.,* 1998). While the functional significance of these findings remains to be clarified, they imply that VIP exerts an inhibitory influence on the gonadotropin system. There are indications that VIP has a central inhibitory influence on GnRH/LH release (Pitts *et al.,* 1994).

Monoaminergic Regulation

A wealth of functional data has confirmed the involvement of hypothalamic VIP and GnRH in the regulation of the avian reproductive cycle (El Halawani *et al.,* 1990 a, 1990 b ; Sharp *et al.,* 1998). However, very little is known about the neuro-transmitter system(s) and the anatomical location of the hypophysiotropic group/sub-group of neurons regulating GnRH/LH-FSH and VIP/PRL systems and mediating the transition from one reproductive state to the next. This review on the mono-aminergic regulation of GnRH/LH, FSH and VIP/PRL systems is limited to the recent advances in dopaminergic and serotonergic mechanisms. Earlier work on the subject has been reviewed elsewhere (El Halawani *et al.,* 1984, 1988 ; El Halawani and Rozenboim, 1993 a)

Dopamine Neurotransmission

Several DA cell groups have been identified in the preoptic-hypothalamic areas (Kiss and Peczely, 1987 ; Reiner *et al.,* 1994). DA has a stimulating effect on PRL and LH secretion by acting on their respective neuropeptidergic neurons (Bhatt *et al.,* 2002). The DAergic system has also been shown to inhibit the stimulatory effect of VIP on PRL secretion at the pituitary level (Youngren *et al.,* 1998 a), apparently by releasing DA into the capillaries of the hypophysial portal system (Youngren *et al.,* 1996 a). DAergic neurons inhibit GnRH release through presynaptic inputs at the ME level, as has been demonstrated in the chicken (Contijoch *et al.,* 1992 ; Fraley and Kuenzel, 1993).

Dopaminergic neurons are not located in a single discrete hypothalamic nucleus or

region, but instead are dispersed among a variety of hypothalamic regions (POA-AM), suprachiasmatic nucleus (SCN), lateral hypothalamic area (LHy), paraventricular nucleus (PVN), lateral mamillaris nucleus (ML), and dorsomedial nucleus (DM ; 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, GnRH neurons in the POA, and with both VIP and GnRH terminals in the external layer of the ME (preliminary data), 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/LH-FSH.

An advance in elucidating the neurochemical mechanisms came from our laboratory where using the turkey as a model, we were able to demonstrate the dual role of DA in PRL secretion and expression, stimulating via D_1 DA receptors and inhibiting via D_2 DA receptors (Youngren *et al.,* 1995 ; Xu *et al.,* 1996 ; Chaiseha *et al.,* 1997 ; Youngren *et al.,* 1998 a). Both D_1 and D_2 DA receptor mRNAs are abundant in the brain and pituitary (Schnell *et al.,* 1999 a), suggesting DA exhibits biphasic actions within the turkey hypothalamus and pituitary. In fact, tonic stimulation of PRL secretion and gene expression are regulated centrally via D_1 DA receptors on VIP neurons, where the expression of D_1 DA receptors is greater (6-fold) than that of D_2 DA receptors (Youngren *et al.,* 1995 ; Chaiseha *et al.,* 2003). DA inhibits PRL secretion and gene expression by blocking the action of VIP at the level of the anterior pituitary via D_2 DA receptors (Youngren *et al.,* 1998 a).

The increase in circulating PRL levels in response to long days is a gradual and incremental process associated with gonad recrudescence and egg laying and culminating in the dramatic augmentation observed at the onset of incubation (Goldsmith, 1985 ; El Halawani *et al.,* 1990 a). This slow progressive photostimulated increase in PRL level stands in stark contrast to the sharp and immediate decline in circulating PRL that occurs in an incubating hen following hatching of its eggs or an occurrence of nest deprivation (El Halawani *et al.,* 1980 ; Opel and Proudman, 1989). This precipitous drop in circulating PRL is most likely related to the activation of an inhibitory neural system that overrides the tonic stimulation of PRL by VIP. The cause of this inhibition is surmised to be DA, acting at the pituitary level to block the tonic stimulation of VIP upon lactotrophs (Youngren *et al.,* 1998 a), since D_2 DA receptor mRNA expression by anterior pituitary lactotrophs is up-regulated at this stage of the reproductive cycle (Chaiseha *et al.,* 2003). The identification of DAergic neurons which project to the ME and deliver DA to the anterior pituitary and those which project to the INF and stimulate VIP is the subject of ongoing studies. It remains undetermined whether the physiological processes involved in the termination of reproductive activity and the following insensitivity to long day lengths in non-incubating birds are connected to the same neural mechanisms that are responsible for the sharp decline in PRL during the transition from incubation to photorefractoriness. In the turkey, as in single brooded species in temperate zones, gonadotropins secretion may not increase after the young hatch because of the development of photorefractoriness (Follett, 1984 ; Wingfield and Farner, 1993).

The marked decrease in PRL release and gene expression and the insensitivity to long day lighting that is characteristic of photorefractory birds is apparently not attributable to the VIP/PRL system. Microinjection of a D_1 DA receptor agonist into the INF area of the hypothalamus increases plasma PRL levels equally in both laying and photorefractory hens (Youngren *et al.,* 2002 a), suggesting that the cells that secrete VIP and PRL are fully responsive at the time when photorefractoriness becomes apparent. Their inactivity presumably reflects either the inability of hypothalamic DAergic neurons (Kiss and Peczely, 1987 ; Reiner *et al.,* 1994) projecting to the INF (Youngren *et al.,* 2002 a) to stimulate the VIP/PRL system and/or the switching on of inhibitory DAergic neurons that initiate the shutting down of PRL secretion by activating D_2 DA receptors at the pituitary level (Schnell *et al.,* 1999 a ; Schnell *et al.,* 1999 b). This is substantiated by the findings that : 1) activation of D_2 DA receptors in the anterior pituitary inhibit the stimulatory effect on PRL secretion of VIP infusion into the anterior pituitary or of electrical stimulation (ES) in the POA (Youngren *et al.,* 1996 b ; Youngren *et al.,* 1998 a ; El Halawani *et al.,* 2000) ; 2) the sharp and immediate decline in PRL secretion after the young hatch or following nest deprivation occurs despite the presence of high pituitary PRL levels, which can be released at an enhanced rate by VIP *in vitro* or by *in vivo* electrical stimulation in the POA (El Halawani *et al.,* 1990 b ; Youngren *et al.,* 1993) ; and 3) the up-regulation of D_2 DA receptor mRNA in the anterior pituitary of hypoprolactinemic photorefractory hens (Chaiseha *et al.,* 2003). It can be argued that the inhibition of VIP release, and in turn PRL secretion, may be the result of down- or up-regulation of D_1 and D_2 DA receptors, respectively, on VIP neurons located in the INF area (Mauro *et al.,* 1989 ; Chaiseha *et al.,* 1997 ; Chaiseha *et al.,* 2003).

The role of DA in gonadotropic regulation remains controversial since both stimulatory and inhibitory effects have been reported (El Halawani *et al.,* 1988 ; Sharp *et al.,* 1998). Using the measurement of circulating levels of LH as the end point may in part have contributed to these inconstant results. Circulating LH levels are low and the LH response to physiological manipulation is small and variable (Youngren *et al.,* 1993), as compared to PRL, for example, making interpretation difficult. We are now able to measure both LHβ- and FSHβ subunit mRNA contents, which display a robust and stable response to physiological manipulation (Ahn *et al.,* 2001 ; Bhatt *et al.,* 2003). Recent data from our laboratory indicate that DA stimulates LHβ subunit mRNA (Bhatt *et al.,* 2003), whereas earlier data indicated that DA inhibited GnRH release at the ME level (Contijoch *et al.,* 1992 ; Fraley and Kuenzel, 1993). The possibility remains that DA may have both stimulatory and inhibitory influences on the GnRH/gonadotropic system, depending upon the site of action and/or the DAergic receptor subtypes involved, as is the case with VIP/PRL.

Serotonin Neurotransmission

Considerable evidence indicates that the serotonergic (5-HTergic) system is a potent stimulator of PRL secretion in birds (Hall *et al.,* 1986 ; El Halawani *et al.,* 1988). 5-HT seems to act centrally since ; 1) it has no effect on PRL secretion when added to pituitary cells *in vitro* (El Halawani *et al.,* 1988) ; 2) 5-HT receptors are not

present in the anterior pituitary (Macnamee and Sharp, 1989) ; and 3) intraventricular infusion of 5-HT causes plasma PRL to increase in turkeys (EI Halawani *et al.,* 1995 b ; Pitts *et al.,* 1996). We (Youngren *et al.,* 1989) have suggested that 5-HTergic fibers, traversing the hypothalamic VMN, stimulate PRL secretion through interneuronal DAergic connections to the INF where the majority of VIP immunoreactive neurons are found (Mauro *et al.,* 1989 ; Chaiseha and El Halawani, 1999). 5-HT neurons are part of a common pathway, presumable residing within the avian hypothalamus, which stimulates the secretion of PRL from the anterior pituitary. When D_1 DA receptors are blocked, the PRL-releasing efficacy of not only DA (Youngren *et al.,* 1998 a), but also 5-HT (Youngren *et al.,* 1998 b), is suppressed. 5-HT receptors appear to lie above the synapse containing D_1 DA receptors. As indicated above, the primary PRF (perhaps the only one) released from the hypothalamus into the hypothalamo-pituitary portal vessels is VIP (El Halawani *et al.,* 1997). The ability of 5-HT and DA to stimulate PRL secretion is contingent upon an intact VIPergic system. When birds are immunized against their own VIP, the central infusion of 5-HT (EI Halawani *et al.,* 1995 b), or DA (Youngren *et al.,* 1996 b) can not stimulate PRL secretion. The infusion of VIP into the ME of immunized birds results in a severely curtailed PRL response. Thus, 5-HT, DA, and VIP act to stimulate PRL secretion via a common pathway expressing 5-HTergic, DAergic, and VIPergic receptors at synapses arranged successively in that functional order.

In a preliminary study (Youngren and El Halawani, 2002 b), bilateral microinjections of 5-HT in the caudal VMN of the hypothalamus, but not the rostral part, notably impeded the PRL release effected by electrical stimulation in the POA. These data lead us to the hypothesis that 5-HT, at least at the VMN level, may be involved in the decline in circulating PRL observed during reproductive inactivity i.e. the photorefractory state (Youngren and El Halawani, 2002 b). Also, recent data from our laboratory (El Halawani *et al.,* 2004), show that electrical stimulation in the POA, which is known to stimulate LH and PRL secretion, activates GnRH and VIP immunoreactive neurons (as indicated by c-fos mRNA expression) in the POA and INF areas, respectively. This was associated with an activation of a DAergic neuronal group residing in the ML area of the hypothalamus. This is the first identification of a specific DA group that is associated with the stimulation of GnRH/LH,FSH and VIP/PRL systems.

How the hypothalamic 5-HT-DA-VIP pathway may alter PRL secretion is unknown. A hypothetical mechanism is proposed below, (Fig. 2).

1) PRL secretion is tonically stimulated by VIP neurons in the INF (El Halawani *et al.,* 1997).
2) 5-HTergic fibers, traversing the hypothalamic VMN/ML, stimulate PRL secretion through interneuronal DAergic projections, probably from the VMN/ML to the INF (Mauro *et al.,* 1989 ; Chaiseha and El Halawani, 1999 ; Youngren *et al.,* 2002 a).
3) The tonic stimulation of PRL release and expression is inhibited by DA at the pituitary level via D_2 DA receptors (Youngren *et al.,* 1995 ; El Halawani *et al.,* 1997). The source of DA is unknown. DA neurons in the VMN/ ML

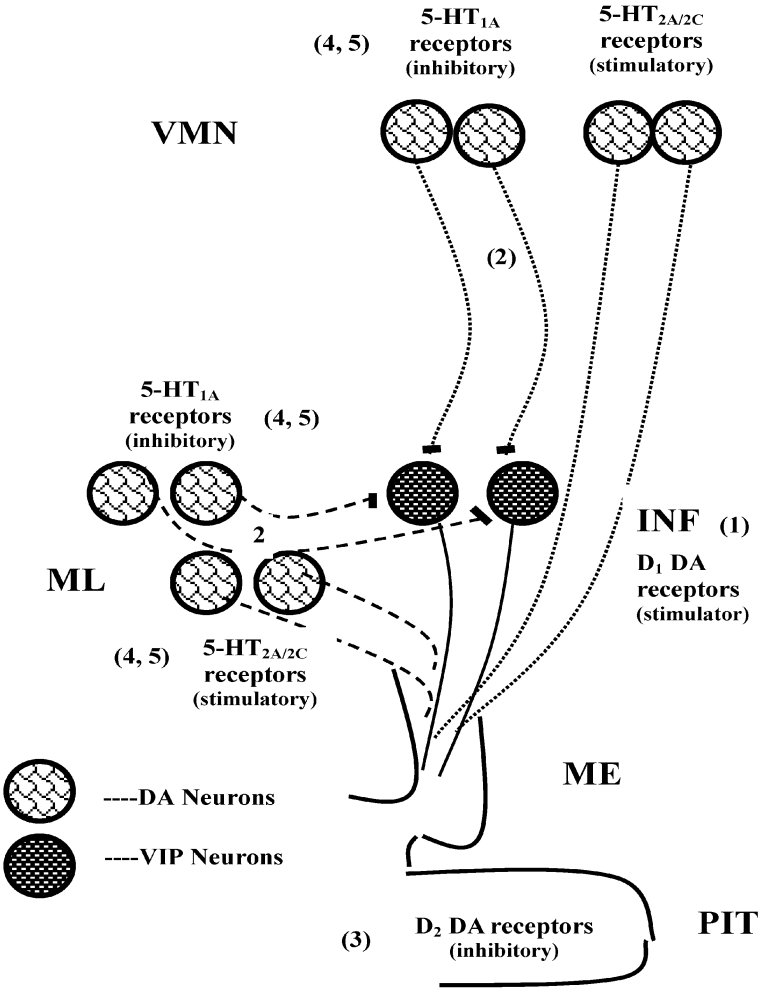

Fig. 2. Schematic diagram demonstrating the interrelationships between 5-HT, DA and the control of VIP/PRL secretion.

(Kiss and Peczely, 1987 ; Reiner *et al.,* 1994) may or may not be the source.

4) 5-HT modulation of the VIP/PRL system requires a functional DAergic system (Youngren *et al*., 1998 b).
5) 5-HT_{1A} and $5\text{-HT}_{2A/2C}$ receptors mediate the inhibitory and stimulatory influences of 5-HT on PRL secretion, respectively (unpublished preliminary results).
6) The microinjection of 5-HT in the VMN inhibits PRL release induced by electrical stimulation in the POA (Youngren and El Halawani, 2002 b).

Accordingly, the gradual increase in PRL secretion associated with photostimulation and reproductive activity is related to tonic DA stimulation of VIP neurons in the INF via D_1 DA receptors. The source of DA appears to be DAergic neurons located in the ML.

The abrupt decline in circulating PRL levels associated with the start of ovarian

regression and the ending of egg laying, as in photorefractory birds, reflect : 1) the inability of the DAergic neurons in the ML and/or VMN to stimulate the infundibular VIP system ; and/or 2) the activation of DAergic neurons projecting to the ME which turn off PRL secretion by activating D_2 DA receptors at the pituitary level. The modification in DAergic neurotransmission in the ML and/or VMN is mediated by inhibitory 5-HT_{1A} and stimulatory 5-$HT_{2A/2C}$ receptors on these DA cells.

Acknowledgement

Supported by The Thailand Research Fund (TRF) #RSA4780001 to (YC) and BARD #US-3399-03C and USDA #2004-35203-14771 to (MEH).

References

Ahn J, You SK, Kim H, Chaiseha Y and El Halawani ME. Effects of active immunization with inhibin alpha subunit on reproductive characteristics of turkey hens. Biology Reproduction, 65 : 1594–1600. 2001.

Bhatt R, Youngren OM, Kang SW and El Halawani ME. Dopamine infusion in the third ventricle increases gene expression of hypothalamic vasoactive intestinal peptide and pituitary prolactin and luteinizing hormone beta subunit in the turkey. General and Comparative Endocrinology, 130 : 41–47. 2003.

Bluhm CK, Schwabl H, Schwabl I, Perera A, Follett BK, Goldsmith AR and Gwinner E. Variations in hypothalamic gonadotrophin releasing hormone content, plasma and pituitary LH and in vitro testosterone release in a long distance migratory bird, the garden warbler (*Sylvia borin*), under constant photoperiods. Journal of Endocrinology, 128 : 339–345. 1991.

Chaiseha Y and El Halawani ME. Expression of vasoactive intestinal peptide/peptide histidine isoleucine in several hypothalami areas during the turkey reproductive cycle : Relationship to prolactin secretion. Neuroendocrinology, 70 : 402–412. 1999.

Chaiseha Y, Tong Z, Youngren OM and El Halawani ME. Transcriptional changes in hypothalamic vasoactive intestinal peptide during a photo-induced reproductive cycle in the turkey. Journal of Molecular Endocrinology, 21 : 267–275. 1998.

Chaiseha Y, Youngren OM, Al-Zailaie K and El Halawani ME. Expression of D_1 and D_2 dopamine receptors in the hypothalamus and pituitary during the turkey reproductive cycle : Colocalization with vasoactive intestinal peptide. Neuroendocrinology, 77 : 105–118. 2003.

Chaiseha Y, Youngren OM and El Halawani ME. Dopamine receptors influence vasoactive intestinal peptide release from turkey hypothalamic explants. Neuroendocrinology, 65 : 423–429. 1997.

Contijoch AM, Gonzalez C, Singh HN, Malamed S, Troncoso S and Advis JP. Dopaminergic regulation of luteinizing hormone-releasing hormone release at the median eminence level : Immunocytochemical and physiological evidence in hens. Neuroendocrinology, 55 : 290–300. 1992.

Dawson A, Follett BK, Goldsmith AR and Nicholls TJ. Hypothalamic gonadotrophin releasing hormone and pituitary and plasma FSH and prolactin during photostimulation and photorefractoriness in intact and thyroidectomized starlings (*Sturnus vulgaris*). Journal of Endocrinology, 105 : 71–77. 1985.

Dawson A and Goldsmith AR. Changes in gonadotrophin-releasing hormone (GnRH-I) in the pre-optic area and median eminence of starlings (*Sturnus vulgaris*) during the recovery of photosensitivity and during photostimulation. Journal Reproduction and Fertility, 111 : 1–6. 1997.

Dawson A, King VM, Bentley GE and Ball GF. Photoperiodic control of seasonality in birds. Journal of Biological Rhythms, 16 : 365–380. 2001.

Dawson A and Sharp PJ. The role of prolactin in the development of reproductive photorefractoriness and postnuptial molt in the European starling (*Sturnus vulgaris*). Endocrinolo-

gy, 139 : 485–490. 1998.
Deviche PJ, Saldanha CJ and Silver R. Changes in brain gonadotropin-releasing hormone- and vasoactive intestinal polypeptide-like immunoreactivity accompanying reestablishment of photosensitivity in male dark-eyed junco (*Junco hyemalis*). General and Comparative Endocrinology, 117 : 8–19. 2000.
El Halawani ME, Burke WH and Dennison PT. Effect of nest-deprivation on serum prolactin level in nesting female turkeys. Biology of Reproduction, 23 : 118–123. 1980.
El Halawani ME, Burke WH, Millam JR, Fehrer SC and Hargis BM. Regulation of prolactin and its role in gallinaceous bird reproduction. Journal of Experimental Zoology, 232 : 521–529. 1984.
El Halawani ME, Fehrer SC, Hargis BM and Porter TE. Incubation behavior in the domestic turkey : Physiological correlates. C.R.C. Critical Review in Poultry Biology, 1 : 285–314. 1988.
El Halawani ME, Kang SW, Chaiseha Y, Rozenboim I, Millam JR and Youngren OM. Dopaminergic regulation of reproduction in the female turkey. 8th International Symposium on Avian Endocrinology, 56. 2004.
El Halawani ME, Mauro LJ, Phillips RE and Youngren OM. Neuroendocrine control of prolactin and incubation in gallinacous birds. In : Progress in Comparative Endocrinology (Apple A ed.). pp 678–684. Wiley-Liss. New York. 1990 a.
El Halawani ME, Pitts GR, Sun S, Silsby JL and Sivanandan V. Active immunization against vasoactive intestinal peptide prevents photo-induced prolactin secretion in turkeys. General and Comparative Endocrinology, 104 : 76–83. 1996.
El Halawani ME and Rozenboim I. Incubation behavior in the turkey : Molecular and endocrinological implication. In : Avian Endocrinology (Sharp PJ ed.). pp. 99–109. Journal of Endocrinology Ltd. Bristol. UK. 1993 a.
El Halawani ME, Silsby JL, Behnke EJ and Fehrer SC. Hormonal induction of incubation behavior in ovariectomized female turkeys (*Meleagris gallopavo*). Biology of Reproduction, 35 : 59–67. 1986.
El Halawani ME, Silsby JL, Fehrer SC and Behnke EJ. Effects of estrogen and progesterone on serum prolactin and luteinizing hormone levels in ovariectomized turkeys (*Meleagris gallopavo*). General and Comparative Endocrinology, 52 : 67–78. 1983.
El Halawani ME, Silsby JL, Foster LK, Rozenboim I and Foster DN. Ovarian involvement in the suppression of luteinizing hormone in the incubating turkey (*Meleagris gallopavo*). Neuroendocrinology, 58 : 35–41. 1993 b.
El Halawani ME, Silsby JL and Mauro LJ. Enhanced vasoactive intestinal peptide-induced prolactin secretion from anterior pituitary cells of incubating turkeys (*Meleagris gallopavo*). General and Comparative Endocrinology, 80 : 138–145. 1990 b.
El Halawani ME, Silsby JL, Rozenboim I and Pitts GR. Increased egg production by active immunization against vasoactive intestinal peptide in the turkey (*Meleagris gallopavo*). Biology of Reproduction, 52 : 179–183. 1995 a.
El Halawani ME, Youngren OM and Chaiseha Y. Neuroendocrinology of prolactin regulation in the domestic turkey. In : Avian Endocrinology (Dawson A and Chaturvedi CM eds.). pp. 233–244. Narosa Publishing House. New Delhi. India. 2000.
El Halawani ME, Youngren OM and Pitts GR. Vasoactive intestinal peptide as the avian prolactin releasing factor. In : Perspectives in Avian Endocrinology (Harvey S and Etches RJ eds.). pp. 403–416. Journal of Endocrinology Ltd. Bristol. UK. 1997.
El Halawani ME, Youngren OM, Rozenboim I, Pitts GR, Silsby JL and Phillips RE. Serotonergic stimulation of prolactin secretion is inhibited by vasoactive intestinal peptide immunoneutra lization in the turkey. General and Comparative Endocrinology, 99 : 69–74. 1995 b.
Follett BK. Reproductive cycles of vertebrates. In : Marshall's Physiology of Reproduction. (Lamming GE ed.). pp. 283–350. Churchill Livingstone. New York. 1984.
Foster RG, Plowman G, Goldsmith AR and Follett BK. Immunocytochemical demonstration of marked changes in the luteinizing hormone releasing hormone system of photosensitive and photorefractory European starlings. Journal of Endocrinology, 115 : 211–220. 1987.

Fraley GS and Kuenzel WJ. Immunocytochemical and histochemical analyses of gonadotrophin releasing hormone, tyrosine hydroxylase, and cytochrome oxidase reactivity within the hypothalamus of chicks showing early sexual maturation. Histochemistry, 99 : 221–229. 1993.

Goldsmith AR. Prolactin in avian reproduction : Incubation and the control of seasonal breeding. In : Prolactin : Basic and Clinical Correlates (MacLeod RM, Scapagini U and Thorner M eds.). pp. 410–425. Springer. Padua. 1985.

Hahn TP and Ball GF. Changes in brain GnRH associated with photorefractoriness in house sparrows (*Passer domesticus*). General and Comparative Endocrinology, 99 : 349–363. 1995.

Hall TR, Harvey S and Chadwick A. Control of prolactin secretion in birds : A review. General and Comparative Endocrinology, 62 : 171–184. 1986.

Juss TS. Neuroendocrine and neural changes associated with the photoperiodic control of reproduction. In : Avian Endocrinology (Sharp PJ ed.) pp. 47–60. Journal of Endocrinology Ltd. Bristol. UK. 1993.

Kang SW, Thayananuphat A, Rozenboim I, Millam JR, Proudman JA and El Halawani ME. Differential expression of gonadotropin releasing hormone-I mRNA in the hypothalamus during the turkey reproductive cycle. 8th International Symposium on Avian Endocrinology, 85. 2004.

Katz LA, Millar RP and King JA. Differential regional distribution and release of two forms of gonadotrophin releasing hormone in the chicken brain. Peptides, 11 : 443–450. 1990.

Kiss JZ and Peczely P. Distribution of tyrosine-hydroxylase (TH)-immunoreactive neurons in the diencephalons of the pigeon (*Columba livia domestica*). Journal of Comparative Neurology, 257 : 333–346. 1987.

Macnamee MC and Sharp PJ. The functional activity of hypothalamic 5-hydroxytryptamine neurons in broody bantam hens. Journal of Endocrinology, 120 : 125–134. 1989.

Macnamee MC, Sharp PJ, Lea RW, Sterling RJ and Harvey S. Evidence that vasoactive intestinal polypeptide is a physiological prolactin-releasing factor in the bantam hen. General and Comparative Endocrinology, 62 : 470–478. 1986.

Mauro LJ, Elde RP, Youngren OM, Phillips RE and El Halawani ME. Alterations in hypothalamic vasoactive intestinal peptide-like immunoreactivity are associated with reproduction and prolactin release in the female turkey. Endocrinology, 125 : 1795–1804. 1989.

Mauro LJ, Youngren OM, Proudman JA, Phillips RE and El Halawani ME. Effects of reproductive status, ovariectomy, and photoperiod on vasoactive intestinal peptide in the female turkey hypothalamus. General and Comparative Endocrinology, 87 : 481–493. 1992.

Mikami S and Yamada S. Immunohistochemistry of the hypothalamic neuropeptides and anterior pituitary cells in the Japanese quail. Journal of Experimental Zoology, 232 : 405–417. 1984.

Mikami S, Yamada S, Hasegawa Y and Miyamoto K. Localization of avian LHRH- immunoreactive neurones in the hypothalamus of the domestic fowl, (*Gallus domesticus*) and the Japanese quail, (*Coturnix japonica*). Cell and Tissue Research, 251 : 51–58. 1988.

Millam JR, Craig-Veit CB, Adams TE and Adams BM. Avian gonadotrophin-releasing hormones I and II in the brain and other tissues in turkey hens. Comparative Biochemistry and Physiology, 94A : 771–776. 1989.

Millam JR, Faris PL, Youngren OM, El Halawani ME and Hartman BK. Immunohistochemical localization of chicken gonadotrophin-releasing hormones I and II (cGnRH-I and II) in turkey hen brain. Journal of Comparative Neurology, 333 : 68–82. 1993.

Nicholls TJ, Goldsmith AR and Dawson A. Photorefractoriness in birds in comparison with mammals. Physiological Review, 68 : 133–176. 1988.

Opel H and Proudman JA. Plasma prolactin levels in incubating turkey hens during pipping of the eggs and after introduction of poults into the nest. Biology of Reproduction, 40 : 981–987. 1989.

Peczely P and Kiss JZ. Immunoreactivity to vasoactive intestinal polypeptide (VIP) and thyrotropin-releasing hormone (TRH) in hypothalamic neurons of the domesticated pigeon (*Columba livia*). Alterations following lactation and exposure to cold. Cell and Tissue Research, 251 : 485–494. 1988.

Pitts GR, Youngren OM, Silsby JL, Foster LK, Foster DN, Rozenboim I, Phillips RE and El Halawani ME. Role of vasoactive intestinal peptide in the control of prolactin-induced turkey incubation behavior. I. Acute infusion of vasoactive intestinal peptide. Biology of Reproduction, 50 : 1344–1349. 1994.

Pitts GR, Youngren OM, Phillips RE and El Halawani ME. Photoperiod mediates the ability of serotonin to release prolactin in the turkey. General and Comparative Endocrinology, 104 : 265–272. 1996.

Porter TE, Silsby JL, Behnke EJ, Knapp TR and El Halawani ME. Ovarian steroid production in vitro during gonadal regression in the turkey. I. Changes associated with incubation behavior. Biology of Reproduction, 45 : 581–586. 1991.

Reiner A, Karle DJ, Adderson KD and Medina L. Catecholaminergic perikarya and fibers in the avian nervous system. In : Phylogeny and Development of Catecholamine Systems in the CNS of Vertebrates (Smeets WJ and Reiner AJ eds.). pp 135–181. Cambridge University Press. Cambridge. England. 1994.

Rozenboim I, Silsby JL, Tabibzadeh C, Pitts GR, Youngren OM and El Halawani ME. Hypothalamic and posterior pituitary content of vasoactive intestinal peptide and gonadotrophin releasing hormones I and II in the turkey hen. Biology of Reproduction, 49 : 622–626. 1993 a.

Rozenboim I, Tabibzadeh C, Silsby JL and EL Halawani ME. Effect of ovine prolactin administration on hypothalamic vasoactive intestinal peptide (VIP), gonadotropin releasing hormone I and II content, and anterior pituitary VIP receptors in laying turkey hens. Biology of Reproduction, 48 : 1246–1250. 1993 b.

Saldanha CJ, Deviche PJ and Silver R. Increased VIP and decreased GnRH expression in photorefractory dark-eyed juncos (*Junco hyemalis*). General and Comparative Endocrinology, 93 : 128–136. 1994.

Schnell SA, You SK and El Halawani ME. D_1 and D_2 dopamine receptor messenger ribonucleic acid in brain and pituitary during the reproductive cycle of the turkey hen. Biology of Reproduction, 60 : 1378–1383. 1999 a.

Schnell SA, You SK, Foster DN and El Halawani ME. Molecular cloning and tissue distribution of an avian D_2 dopamine receptor mRNA from the domestic turkey (*Meleagris gallopavo*). Journal and Comparative Neurology, 407 : 543–554. 1999 b.

Sharp PJ, Dawson A and Lea RW. Control of luteinizing hormone and prolactin secretion in birds. Comparative Biochemistry and Physiology C : Pharmacology Toxicology and Endocrinology, 119 : 275–282. 1998.

Sharp PJ, Sterling RJ, Talbot RT and Huskinsson NS. The role of hypothalamic vasoactive intestinal polypeptide in the maintenance of prolactin secretion in incubating bantam hens : Observations using passive immunization, radioimmunoassay and immunohistochemistry. Journal of Endocrinology, 122 : 5–16. 1989.

Sharp PJ, Talbot RT, Main GM, Dunn IC, Fraser HM and Huskinsson NS. Physiological roles of chicken LHRH-I and-II in the control of gonadotrophin release in the domestic chicken. Journal of Endocrinology, 124 : 291–299. 1990.

Tabibzadeh C, Rozenboim I, Silsby JL, Pitts GR, Foster DN and El Halawani ME. Modulation of ovarian cytochrome P450 17 alpha-hydroxylase and cytochrome aromatase messenger ribonucleic acid by prolactin in the domestic turkey. Biology of Reproduction, 52 : 600–608. 1995.

Teruyama R and Beck MM. Changes in immunoreactivity to anti-cGnRH-I and -II are associated with photostimulated sexual status in male quail. Cell and Tissue Research, 300 : 413–426. 2000.

Teruyama R and Beck MM. Double immunocytochemistry of vasoactive intestinal peptide and cGnRH-I in male quail : Photoperiodic effects. Cell and Tissue Research, 303 : 403–414. 2001.

Tong Z, Pitts GR, Foster DN and El Halawani ME. Transcriptional and post-transcriptional regulation of prolactin during the turkey reproductive cycle. Journal of Molecular Endocrinology, 18 : 223–231. 1997.

Wineland MJ and Wentworth BC. Peripheral serum levels of 17 beta estradiol in growing turkey hens. Poultry Science, 54 : 381–387. 1975.

Wingfield JC and Farner DS. Endocrinology of reproduction in wild species. In : Avian Biology IX (Farner DS, King JR and Parkes KC eds.). pp. 163–327. Academic Press. London. UK. 1993.

Wong EA, Ferrin NH, Silsby JL and El Halawani ME. Cloning of a turkey prolactin cDNA : Expression of prolactin mRNA throughout the reproductive cycle of the domestic turkey (*Meleagris gallopavo*). General and Comparative Endocrinology, 83 : 18–26. 1991.

Wong EA, J.L. Silsby JL, Ishii S, and. El Halawani ME. Pituitary luteinizing hormone and prolactin messenger ribonucleic acid levels are inversely related in laying and incubating turkey hens. Biology of Reproduction, 47 : 598–602. 1992.

Wood-Gush DG and Gilbert AB. Some hormones involved in the nesting behavior of hens. Animal Behavior, 21 : 98–103. 1973.

Xu M, Proudman JA, Pitts GR, Wong EA, Foster DN and El Halawani ME. Vasoactive intestinal peptide stimulates prolactin mRNA expression in turkey pituitary cells : Effects of dopaminergic drugs. Proceeding Society of Experimental Biology and Medicine, 212 : 52–62. 1996.

Yamada S, Mikami S and Yanaihara N. Immunohistochemical localization of vasoactive intestinal polypeptide (VIP)-containing neurons in the hypothalamus of the Japanese quail, *Coturnix coturnix*. Cell and Tissue Research, 226 : 13–26. 1982.

You SK, Foster LK, Silsby JL, EL Halawani ME and Foster DN. Sequence analysis of the turkey LH beta subunit and its regulation by gonadotrophin-releasing hormone and prolactin in cultured pituitary cells. Journal of Molecular Endocrinology, 14 : 117–129. 1995.

Youngren OM, Chaiseha Y, Al-Zailaie K, Whiting S, Kang SW and El Halawani ME. Regulation of prolactin secretion by dopamine at the level of the hypothalamus in the turkey. Neuroendocrinolgy, 75 : 185–192. 2002 a.

Youngren OM, Chaiseha Y and El Halawani ME. Regulation of prolactin secretion by dopamine and vasoactive intestinal peptide at the level of the pituitary in the turkey. Neuroendocrinology, 68 : 319–325. 1998 a.

Youngren OM, Chaiseha Y and EI Halawani ME. Serotonergic stimulation of avian prolactin secretion requires an intact dopaminergic system. General and Comparative Endocrinology, 112 : 63–68. 1998 b.

Youngren OM, Chaiseha Y, Phillips RE and El Halawani ME. Vasoactive intestinal peptide concentrations in turkey hypophysial portal blood differ across the reproductive cycle. General and Comparative Endocrinology, 103 : 323–330. 1996 a.

Youngren OM and El Halawani ME. Inhibitory and stimulatory effects of serotonin (5-HT) on prolactin (PRL) secretion in the domestic turkey. Poultry Science, 80 (Supplement 1) : 44. 2002 b.

Youngren OM, EI Halawani ME, Phillips RE and Silsby JL. Effects of preoptic and hypothalamic lesions in female turkeys during a photo-induced reproductive cycle. Biology of Reproduction, 41 : 610–617. 1989.

Youngren OM, El Halawani ME, Silsby JL and Phillips RE. Effect of reproductive condition on luteinizing hormone and prolactin release induced by electrical stimulation of the turkey hypothalamus. General and Comparative Endocrinology, 89 : 220–228. 1993.

Youngren OM, El Halawani ME, Silsby JL and Phillips RE. Intracranial prolactin perfusion induces incubation behavior in turkey hens. Biology of Reproduction, 44 : 425–431. 1991.

Youngren OM, Pitts GR, Phillips RE and EI Halawani ME. Dopaminergic control of prolactin secretion in the turkey. General and Comparative Endocrinology, 104 : 225–230. 1996 b.

Youngren OM, Pitts GR, Phillips RE and El Halawani ME. The stimulatory and inhibitory effects of dopamine on prolactin secretion in the turkey. General and Comparative Endocrinology, 98 : 111–117. 1995.

2. Copy of the Manuscript #1:

Kosonsiriluk S, Sartsoongnoen N, Prakobsaeng N, Rozenboim I, El Halawani ME, **Chaiseha Y** (2007). Prolactin and luteinizing hormone profiles during the reproductive Cycle in the native Thai chicken. **Poultry Science** (Submitted; **Impact factor** = 1.656).

Prolactin and Luteinizing Hormone profiles during the Reproductive Cycle in the Native Thai Chicken

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Key words: Birds, Luteinizing hormone, Native Thai chicken Prolactin, Reproductive cycle

Running Title: PRL and LH in the Native Thai Chicken

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ABSTRACT

Unlike Gallinacous-temperate zone birds, native Thai chicken, an equatorial non-photoperiodic continuous breeder consists of four reproductive stages including non-laying (NL), laying (L), incubating (broodiness: B), and rearing of young (R). In temperate zone birds, prolactin (PRL) and luteinizing hormone (LH) levels vary during reproductive stages with the high PRL levels observed during the incubation phase are responsible for the suppression of gonadotropic hormones and ovarian steroids, follicular atresia, termination of egg laying activity, and induction of incubation behavior. The objective of this study was to establish baseline information of the neuroendocrine regulation emphasizing the plasma levels of PRL and LH profiles associated with reproductive cycles of the native Thai chickens. Chickens were divided into two experimental studies and classified into four reproductive stages: NL, L, B, and R. Daily records of egg production, nesting activity, and other behaviors were recorded during the reproductive cycle. During the experiments, blood samples were collected for determining plasma PRL and LH levels for two reproductive cycles and in each reproductive stage by Enzyme-Linked ImmunoSorbent Assay. The results revealed that pattern of circulating PRL levels were low in NL, gradually elevated in L, reached the highest levels in B, and then declined sharply in R. The mean of plasma PRL levels (ng/ml) were significantly higher in B (351.97±37.08, $P<0.05$) than that of in L (40.40±12.60), NL (25.92±1.39), and R (23.80±2.17). In contrast, there were no changes in plasma LH levels (ng/ml) across the reproductive cycles (NL: 3.86±0.36, L: 3.44±0.09, B: 3.74±0.13, and R: 3.22±0.04). The ovary weights (g) were significantly higher in L (35.92±2.12, $P<0.05$) than that of in NL, B, and R (1.47±0.18, 3.07±0.23, and 1.92±0.19). The findings that ovarian regression observed in incubating and rearing hens in the absence

of a decline in LH levels is interpreted as an adaptive mechanism(s) allowing for reinitiating egg laying in the case of nest destruction at any times and irrespective of the season. The findings further suggest that the antigonadotropic effect of PRL is limited to its effect on the ovary. In conclusion, the results of this present study provide, for the first time, the baseline information of the neuroendocrine changes during the reproductive cycles in this species and support the previous studies that PRL is associated with the reproductive cycle in avian species and play a pivotal role in the incubation behavior.

INTRODUCTION

In birds, changes in concentrations of luteinizing hormone (LH) and prolactin (PRL) during the reproductive cycles are well documented (Follett, 1984; El Halawani et al., 1988; Nicholls et al., 1988). 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 (Follett, 1984). There are two neuroendocrine systems that play a pivotal role in the avian reproductive cycle. First, gonadotropin releasing hormone-I/follicle stimulating hormone-luteinizing hormone (GnRH/FSH-LH) system which involves GnRH-I and the subsequent secretion of FSH and LH. Second, vasoactive intestinal peptide/prolactin (VIP/PRL) system which involves VIP and leading to the subsequent secretion of PRL. In temperate zone birds, both systems depend upon 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 secretion of PRL, LH, and FSH is formed by a system of peptidergic neurons whose axons terminate around portal capillaries in the external layer of the ME. VIP and GnRH are among the best characterized hypophysiotropic neuropeptides (Chaiseha and El Halawani, 2005).

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. High concentrations of LH have been shown to be associated with initiation of egg production (Bacon and Long, 1995; Liu et al., 2002). FSH induces mainly ovarian follicular growth in birds (Chaudhuri and Maiti, 1998; Rose et al., 2000) and maintains the hierarchical size of the bird follicles. Circulating LH is directly related to gonadal activity and the control of steroidogenesis (Robinson et al., 1988). LH stimulates progesterone production by the largest follicle (F1), leading to ovulation (Pollock and Orosz, 2002). Ovarian development is found to correlate with plasma LH and the amount of GnRH-I indicating that the expression of the GnRH-I gene is important to maintain pituitary-ovarian function in chicken (Dunn et al., 1996). GnRH-I 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).

In birds, PRL is 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; Silver, 1984; Janik and Buntin, 1985; Buntin et al., 1991). Changes in PRL gene expression and its plasma levels are highly correlated with the reproductive cycle in birds (Talbot et al., 1991; Tong et al., 1997). Plasma PRL levels are very low (5-10 ng/ml) during the reproductively quiescent stages of the turkey reproductive cycle. However, during the laying and incubating stages, circulating PRL levels increase dramatically (500-1500 ng/ml; El Halawani et al., 1984a). It is very well established that PRL is 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). PRL is widely thought to play a role in parental behavior. PRL has

been shown to be associated with the reproductive cycle in several avian species (for review, see: El Halawani et al., 1997) but no studies have been conducted on the native Thai chicken.

The onset of incubation behavior is correlated with declining plasma levels of LH and gonadal steroids and increasing plasma levels of PRL in birds (Lea et al., 1981; El Halawani and Rozenboim, 1993). LH levels begin to increase continuously and reach a peak before ovulation (Mashaly et al., 1976) and plasma concentration of FSH is low throughout the ovulatory cycle but a significant decline in FSH occurred prior to the pre-ovulatory LH surge (Krishnan et al., 1993). Thereafter, LH levels continue to decline during incubating period (Myers et al., 1989). On the contrary, during laying and incubating period, circulating PRL levels increase dramatically (El Halawani et al., 1984a). It is this rising PRL level which 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., 1988). These increasing PRL levels suppress the activity of the GnRH/FSH-LH system (Rozenboim et al., 1993; You et al., 1995), reduce ovarian steroids secretion (Porter et al., 1991; Tabibzadeh et al., 1995), terminate egg laying, induce ovarian regression (Youngren et al., 1991), and signal the transition from sexual behavior to incubation behavior. Elevated PRL levels and incubation behavior are maintained by tactile stimuli from the nest and eggs (El Halawani et al., 1980; Opel and Proudman, 1988). After hatching, or when eggs are replaced with poults, tactile stimuli from the young induces the emergence and maintenance of maternal responses, a sharp decrease in circulating PRL (Opel and Proudman, 1989), and the transition to the photorefractory state.

It is very well documented that PRL is under stimulatory control by the VIP, the avian PRL-releasing factor (PRF; for review, see: El Halawani et al., 1997; 2000; Chaiseha and El Halawani, 2005). Variations in hypothalamic VIP immunoreactivity, VIP content, and VIP mRNA steady-state levels in the infundibular nuclear complex (INF), VIP-immunoreactive (VIP-ir) fibers in the ME, and VIP concentrations in hypophyseal portal blood are correlated with changes in the amount of circulating PRL throughout the turkey reproductive cycle (Mauro et al., 1989; Youngren et al., 1996a; Chaiseha and El Halawani, 1999). Changes in pituitary VIP receptor mRNA were also observed across the reproductive stages in turkeys (Chaiseha et al., 2004). VIP and PRL release and gene expression are up-regulated in response to long day length (Wong et al., 1991; Tong et al., 1997; Chaiseha et al., 1998). The role of dopamine (DA) in the regulation of avian PRL secretion is unclear at present for comparing it to the mammalian DAergic strategy for PRL control. Recent evidences suggested that DA plays an intermediary role in PRL secretion, requiring an intact VIPergic system in order to cause the release of PRL (Youngren et al., 1996b). In addition, recent evidences indicate that dynorphin, serotonin (5-HT), DA, and VIP all appear to stimulate avian PRL secretion along a common 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 (El Halawani et al., 2000).

PRL and gonadotropin secretions are controlled by closely related mechanisms. From a number of physiological or experimental reproductive conditions, an inverse relationship between PRL and gonadotropins secretion seems to emerge. The onset of incubation activity is associated with greatly enhanced circulating PRL levels and diminished LH levels in birds (Sharp et al., 1979; Burke and Dennison, 1980; Goldsmith and Williams, 1980; Goldsmith et al., 1981; 1984; Dawson and Goldsmith, 1982; Bluhm et al., 1983; El Halawani et al., 1984b; Oring et al., 1986; Hiatt et al., 1987). There are only a limit number of scientists studying the neuroendocrine regulation of reproduction in the native Thai chicken. Compared with other domestic animals, relatively little is known about the changes in reproductive hormones during the reproductive cycles of the native Thai chicken. Thus, this study was proposed to establish baseline information of the neuroendocrine changes across the reproductive cycles

and the association with the different reproductive stages of the native Thai chicken. The findings from this study will provide the baseline information of the hormonal profiles and physiological characteristics of the reproductive system in the native Thai chicken, which has never been studied. The knowledge gained will help to understand the basic neuroendocrine regulation of their reproductive cycle.

MATERIALS AND METHODS

Experimental Animals

Female native Thai chickens, Pradoohangdam breed, at 16 weeks of age were used. They were reared and housed in floor pens under natural daylight (approximately 12 hours of light and 12 hours of dark; 12L:12D). Each pen was provided with nests. Food and water were constantly available. Each hen was identified by wing band number. Chickens were randomly divided in floor pens (6-7 females:1 male/pen). The chickens were observed their behaviors in order to classify them into 4 reproductive stages as non-laying (NL), laying (L), incubating (broodiness: B), and rearing chicks (R). The criteria for the reproductive stages classification were: 1) NL: the chickens that had not reached the sexual maturity and did not lay or express incubation and maternal behaviors, 2) L: the hens that laid regularly, 3) B: the hens that exhibited persistent nesting activity, no egg production, and aggressive nest protection behavior, and 4) R: the hens that stopped nesting and laying eggs, and reared the chicks after hatching. Daily records of egg production, nesting activity, and other behaviors were recorded throughout the experiments. The animals were treated in accordance with Suranaree University of Technology Animal Care and Use Committee Guidelines.

Experimental Design

Experiment I:

30 female native Thai chickens were used. The chickens were randomly divided into 5 pens (6 females:1 male/pen). Chickens were observed their behaviors everyday in order to classify them into 4 reproductive stages: NL, L, B, and R. During the experiment, blood samples of 30 female native Thai chickens were collected from the brachial vein in heparinized tubes once a week from the beginning until the end of the experiment (two reproductive cycles). Plasma samples were separated by centrifugation and stored at -35°C until used for determining plasma PRL and LH levels by enzyme-linked immunosorbent assay (ELISA). Daily records of egg production, nesting activity, and other behaviors were recorded during the reproductive cycle.

Experiment II:

100 female and 15 male native Thai chickens at 16 weeks of age were used. The chickens were randomly divided into 15 floor pens (6-7 females:1 male/pen). Blood samples of native Thai chickens were collected in each reproductive stage (n=25): NL, L: 7 days after the first egg was laid and hens laid egg regularly, B: 10 days after hens stopped laying and were sitting on nests continuously, and R: 14 days after the first chick was hatched and hens were rearing their chicks. The blood samples were collected from the brachial vein in heparinized tubes prior to euthanize with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France). After chickens were sacrificed, ovaries and oviducts were removed and weighed. Plasma samples were separated by centrifugation and stored at -35°C until used for determining plasma PRL and LH concentrations by ELISA. Daily records of egg production, nesting activity, and other behaviors were kept during the reproductive cycle.

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.

Measurement of Plasma LH Concentrations

Plasma LH levels of Experiment I (n=10) and II (n=10) 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.

Statistical Analyses

Differences of plasma PRL and LH levels, ovary and oviduct weights among 4 reproductive stages (treatment groups) were analyzed. Egg production, hatchability, and the duration of laying, incubating, and rearing were compared between two reproductive cycles. Results were expressed as mean ± SEM. Significant differences of mean were statistically analyzed using one way analysis of variance (ANOVA). Significance differences among treatment groups were computed utilizing Tukey's HSD test. Differences were considered as statistically significant if a P value was less than 0.05. All statistical analyses were performed using SPSS Windows Software (SPSS Windows Software, version 13.0, SPSS Inc., Chicago, IL, USA).

RESULTS

The reproductive characteristics during the reproductive cycles of native Thai chickens were recorded. The age at the first lay, egg production, hatchability, and the duration of laying, incubating, and rearing during two reproductive cycles were shown in Table 1. The duration of lay, number of egg produced, and % hatchability per hen of the hens in the second reproductive cycle are higher than that of the hens in the first reproductive cycle. The significant different ($P<0.05$) of egg production and the duration of rearing between the first and the second reproductive cycle were observed (12.45±0.99 vs. 18.37±2.15 eggs and 54.83±3.53 vs. 41.93±1.88 days, respectively).

Blood samples of the native Thai chickens were collected weekly throughout two reproductive cycles and the concentrations of plasma PRL and LH levels were determined by ELISA. The results showed that hormonal profiles of plasma PRL and LH levels during the reproductive cycles were similar in all birds (n=10). In the first reproductive cycle, plasma PRL levels were low in NL, gradually increased in L, continued to rise and reached the highest in B, and immediately declined to the basal levels in R, whereas plasma LH levels were fluctuated throughout the two reproductive cycles. Similar patterns of plasma PRL levels were observed in the second reproductive cycle. The profiles of plasma PRL and LH levels in individual bird are illustrated in Figure 1.

Blood samples of the native Thai chickens in each reproductive stage were collected prior to sacrifice the chickens for determining the plasma PRL and LH levels by ELISA. The findings from this study clearly support that plasma PRL levels (Figure 2A, n=10, ng/ml) were low in NL (25.92±1.39), gradually elevated in L (40.40±12.60), significantly increased in B (351.97±37.08, $P<0.05$), and declined sharply to the basal level in R (23.80±2.17). The changes in plasma LH levels were not observed across the reproductive stages. (Figure 2B, n=10, ng/ml, NL: 3.86±0.36, L: 3.44±0.09, B: 3.74±0.13, and R: 3.22±0.04). However, the differences of plasma hormones between the first and second reproductive cycle of the hens were not observed.

The ovary and oviduct were removed and weighed after sacrifice all the chickens (n=25). The highest ovary weights (g) was observed in laying hens (35.92±2.12) compared to other reproductive stages (NL: 1.47±0.18, B: 3.07±0.23, and R: 1.92±0.19). The oviduct weights (g) were low in NL (2.32±0.80) and R (3.79±0.63), increased in B (6.70±0.35, $P<0.05$), and significantly higher in L (39.27±2.03, $P<0.05$; Figure 3).

DISCUSSION

This study reports, for the first time, the hormonal profiles of PRL and LH during the reproductive cycles, as well as the reproductive characteristics of the female native Thai chickens. During the two reproductive cycles, plasma PRL levels were low in non-laying birds, gradually increased when birds started to lay, continued to rise and reached the highest levels when birds entered incubation phase, and immediately declined to the same levels of non-laying birds in birds that rearing chicks. Plasma LH levels were fluctuated throughout reproductive cycles. The plasma PRL levels were determined in each reproductive stage. The low levels of plasma PRL were observed in NL and tended to be higher in L. However, significant different of the mean of plasma PRL levels were observed in B and plasma PRL levels were then declined in R. The changes in plasma LH levels were not observed across the reproductive stages. The ovary weights were significantly higher in L than that of in NL, B, and R. The finding that ovarian regression observed in incubating and rearing hens in the absence of a decline in LH levels is interpreted as an adaptive mechanism(s) allowing for reinitiating egg laying in the case of nest destruction at any times and irrespective of the season. The results of this present study provide the baseline information of the neuroendocrine changes during the reproductive cycle in this equatorial species and support the previous studies that PRL is associated with the reproductive cycle in avian species and play an important role in the incubation behavior. These findings further suggest that the antigonadotropic effect of PRL is limited to its effect on the ovary.

Plasma PRL levels were low in NL when the chickens had not reached their sexual maturity and the reproductive systems were not fully developed. The transition of the reproductive stage from NL to L was associated with an increase in basal plasma PRL levels and accompanied with the start of the development of ovary and oviduct without a concomitant rise in basal plasma LH levels. The sharply increased in plasma PRL levels were observed during birds shift their reproductive stages from L to B and associated with the regression of ovaries and oviducts without the absence of a decline in plasma LH levels. In contrast to the pattern of plasma PRL, the fluctuated plasma LH levels were observed throughout the two reproductive stages. These results suggest that PRL is released in substantial amounts into the circulation according to the reproductive stage of the chicken. LH may probably arise entirely in the peripheral circulation. The duration of lay, number of egg produced, and % hatchability per hen of the hens in the second reproductive cycle is higher than that of the hens in the first reproductive cycle. The significant different of egg production and the duration of rearing between the first and the second reproductive cycle

were observed. However, the differences of plasma hormones between the first and the second reproductive cycle of the hens were not observed. Taken together, the reproductive performance of the chickens in the second reproductive cycle were better than that of the first cycle.

The results of this study are in good agreement with previous studies in temperate zone birds. In temperate zone birds, PRL and LH levels vary during the four reproductive stages with the high PRL levels observed during the incubation phase are responsible for the suppression of gonadotropic hormones and ovarian steroids, follicular atresia, termination of egg laying activity, and induction of incubation behavior. PRL action on the reproductive neuroendocrine system has been shown to be mediated by its feedback effects on the hypothalamus, pituitary, and ovary (Chaiseha and El Halawani, 2005). These evidences correlated very well with the findings of this study that plasma PRL levels were reached the highest levels in incubating hens. The hormonal profile of PRL during the reproductive cycle of the native Thai chicken was similar to the turkey (El Halawani et al., 1984a) which the minor changes of plasma PRL levels were observed in the native Thai chickens, equatorial non-photoperiodic continuous breeders.

In birds, it is very well documented that PRL is 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; Silver, 1984; Buntin et al., 1991). It has been established that PRL is 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). Changes in 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., 1995; Tong et al., 1997). It has been indicated that PRL concentrations in the blood rises as egg laying proceed and reach the highest in incubation and these were similar to the results of this present study. In addition, it has been suggested that PRL may also directly inhibit ovarian steroidogenesis (Rozenboim et al., 1993). Thus, these actions of PRL would lead to involution of the ovary with reduced ovarian steroidogenesis and regression of the oviduct. High levels of PRL are instrumental for regression of reproductive system in birds (Dawson and Sharp, 1998), and molting of feathers in birds or pelage in mammals (Lincoln, 1990; Dawson and Sharp, 1998), which also coincides with demise of reproductive system. It is very well accepted that during incubation, high levels of PRL directly inhibit hypothalamic secretion of GnRH, which in turn reduces pituitary secretion of LH and leads to regression of the gonads (Curlewis, 1992; El Halawani and Rozenboim, 1993).

In birds, it has been very well documented that PRL secretion is tonically stimulated by VIP (for reviews, see: El Halawani et al., 1997; 2000; Chaiseha and El Halawani, 2005). Variations in hypothalamuc VIP immunoreactivity, VIP content, and VIP mRNA steady-state levels in the INF, VIP-ir fibers in the ME, and VIP concentrations in hypophyseal portal blood are correlated with changes in the amount of circulating PRL throughout the turkey reproductive cycle (Mauro et al., 1989; Youngren et al., 1996a; Chaiseha and El Halawani, 1999). Pituitary VIP receptor mRNAs were changed across the reproductive stages with the highest expression found in the incubating turkey hens (Chaiseha et al., 2004). The result from this present study is in good accordance with the previous reports, 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 chickens (Kosonsiriluk et al., 2006).

It is generally considered that LH is one of the important hormones in controlling reproduction of hens. During ovulatory cycle, plasma levels of LH and other reproductive

hormones increase from a basal level to a peak level before ovulation in hen (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). However, the changes of plasma LH levels were not observed during the reproductive cycle of the native Thai chicken. Due to relatively long intervals (one time per week) between collected samples, it might not be possible to determine whether LH concentrations increase during ovulatory cycle of the native Thai hens. Thus, a precise conclusion concerning temporal changes in LH associated with ovulation was not possible to be established in this present study and needed to be further investigated.

The fluctuation of plasma LH levels was observed throughout the reproductive cycles of the native Thai chicken. In contrast, 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). 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), and turkey (Cogger et al., 1979). It has been established that the onset of incubation behavior is correlated with declining plasma levels of LH and gonadal steroids and increasing plasma levels of PRL in bantam (Lea et al., 1981) and turkey hens (El Halawani and Rozenboim, 1993). A suppressive effect of PRL on gonadotropin secretion is suggested by the association between high PRL levels and low LH levels in incubating birds. This is supported by the findings that the administration of PRL antiserum to incubating chicken results in an increase in circulating LH (Lea et al., 1981), and exogenous PRL suppresses LH and induces gonadal regression (Opel and Proudman, 1980; Buntin and Tesch, 1985; Sharp et al., 1988; El Halawani et al., 1991).

No significant difference was observed in plasma LH levels of the native Thai chickens across four reproductive stages examined. This result is consistent with the LH levels previously reported in turkeys which serum LH in laying and incubating hens was not significantly different (Harvey et al., 1981; El Halawani et al., 1984b; Wong et al., 1992). Previous findings indicated that the LH-releasing mechanism is not impaired under the conditions of hyperprolactimia in birds, since the high PRL levels in incubating hens do not depress LH secretion (Sharp et al., 1986; El Halawani et al., 1987). These findings, taken together with the findings of this present study that plasma LH levels were not suppressed by the high levels of plasma PRL, suggest that the interaction of PRL with LH appears to be variable and is species-specific.

In conclusion, the results of this present study provide baseline information of the neuroendocrine changes emphasizing the plasma levels of PRL and LH profiles associated with reproductive stages of the native Thai chicken and support the previous studies that PRL is associated with the reproductive cycle in avian species and play a pivotal role in the incubation behavior.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support from the Thailand Research Fund (TRF) through the Royal Golden Jubilee (RGJ) Ph.D. program (Grant No. PHD/0226/2544 to SK and YC), the TRF (Grant No. RSA4780001 to YC), and the Research and Development Grant of Suranaree University of Technology to YC. The authors are grateful to University Farm of Suranaree University of Technology for providing the chicken barns.

REFERENCES

Bacon, W. L., and D. W. Long. 1995. Changes in plasma luteinizing hormone concentration in turkey hens after switching from short-day to long-day photoperiods. Domest. Anim. Endocrinol. 12:257-267.

Bluhm, C. K., R. E. Phillips, and W. H. Burke. 1983. Serum levels of luteinizing hormone (LH), prolactin, estradiol, and progesterone in laying and nonlaying canvasback ducks (*Aythya valisineria*). Gen. Comp. Endocrinol. 52:1-16.

Breitenbach, R. P., and R. K. Meyer. 1959. Pituitary prolactin levels in laying, incubating and brooding pheasants (*Phasianus colchicus*). Proc. Soc. Exp. Biol. Med. 101:16-19.

Buntin, J. D. 1986. Role of prolactin in avian incubation behavior and care of young: Is there a casual relationship? Pages 252-267 in Reproduction: A Behavioral and Neuroendocrine Perspective. B. R. Komisaruk, H. I. Seigel, M. F. Cheng, and H. H. Feder, eds. NewYork Academy of Science, NewYork.

Buntin, J. D., G. M. Becker, and E. Ruzycky. 1991. Facilitation of parental behavior in ring doves by systemic or intracranial injections of prolactin. Horm. Behav. 25:527-534.

Buntin, J. D., and D. Tesch. 1985. Effects of intracranial prolactin administration on maintenance of incubation readiness, ingestive behavior and gonadal condition in ring doves. Horm. Behav. 19:188-203.

Burke, W. H., and P. T. Dennison. 1980. Prolactin and luteinizing hormone level in female turkeys (*Meleagris gallopavo*) during a photoinduced reproductive cycle and broodiness. Gen. Comp. Endocrinol. 41:92-100.

Campbell, R. R., S. A. Ashton, B. K. Follet, and J. F. Leatherland. 1978. Seasonal changes in plasma concentrations of LH in the Lesser Snow Goose (*Anser caerulescens caerulescens*). Biol. Reprod. 18:663-668.

Chaiseha, Y., and M. E. El Halawani. 1999. Expression of vasoactive intestinal peptide/peptide histidine isoleucine in several hypothalamic areas during the turkey reproductive cycle: Relationship to prolactin secretion. Neuroendocrinology 70:402-412.

Chaiseha, Y., and M. E. El Halawani. 2005. Neuroendocrinology of the female turkey reproductive cycle. J. Poult. Sci. 42:87-100.

Chaiseha, Y., Z. Tong, O. M. Youngren, and M. E. El Halawani. 1998. Transcriptional changes in hypothalamic vasoactive intestinal peptide during a photo-induced reproductive cycle in the turkey. J. Mol. Endocrinol. 21:267-275.

Chaiseha. Y., O. M. Youngren, and M. E. El Halawani. 2004. Expression of vasoactive intestinal peptide receptor messenger RNA in the hypothalamus and pituitary throughout the turkey reproductive cycle. Biol. Reprod. 70:593-599.

Chaudhuri, S., and B. R. Maiti. 1998. Effects of gonadotropins and prolactin on ovarian activity of a wild avian species, the tree pie *Dendrocitta vagabunda*. Indian J. Exp. Biol. 36:790-795.

Cheng, M. F., and B. K. Follett. 1976. Plasma luteinizing hormone during the breeding cycle of the female ring dove. Horm. Behav. 7:199-205.

Cogger, E. A., W. H. Burke, and L. A. Ogren. 1979. Serum luteinizing hormone, progesterone, and estradiol levels in relation to broodiness in the turkey (*Meleagris gallopavo*). Poult. Sci. 58: 1355-1360.

Curlewis, J. D. 1992. Seasonal prolactin secretion and its role in seasonal reproduction: A review. Reprod. Fert. Dev. 4:1-23.

Dawson, A., and A. R. Goldsmith. 1982. Prolactin and gonadotrophin secretion in wild starlings (*Sturnus vulgaris*) during the annual cycle and in relation to nesting, incubation, and rearing young. Gen. Comp. Endocrinol. 48:213-221.

Dawson, A., and P. J. Sharp. 1998. The role of prolactin in the development of reproductive

photorefractoriness and postnuptial molt in the European starling (*Sturnus vulgaris*). Endocrinology 139:485-490.
Doi, O., T. Takai, T. Nakanura, and Y. Tanabe. 1980. Changes in the pituitary and plasma LH, plasma and follicular progesterone and estradiol, and plasma testosterone and estrogen concentrations during the ovulatory cycle of the quail (*Cotunix cotunix japonica*). Gen. Comp. Endocrinol. 41:156-163.
Donham, R. S. 1979. Annual cycle of plasma luteinizing hormone and sex hormones in male and female mallards (*Anas platyrhynchos*). Biol. Reprod. 21:1273-1285.
Donham, R. S., C. W. Dane, and D. S. Farner. 1976. Plasma luteinizing hormone and the development of ovarian follicles after loss of clutch in female mallards (*Anas platyrhynchos*). Gen. Comp. Endocrinol. 29:152-155.
Dunn, I. C., K. K. Beattie, D. Maney, H. M. Sang, R. T. Talbot, P. W. Wilson, and P. J. Sharp. 1996. Regulation of chicken gonadotropin-releasing hormone-I mRNA in incubating, nest-deprived and laying bantam hens. Neuroendocrinology 63:504-513.
El Halawani, M. E., W. H. Burke, and P. T. Dennison. 1980. Effect of nest-deprivation on serum prolactin level in nesting female turkeys. Biol. Reprod. 23:118-123.
El Halawani, M. E., W. H. Burke, J. R. Millam, S. C. Fehrer, and B. M. Hargis. 1984a. Regulation of prolactin and its role in gallinaceous bird reproduction. J. Exp. Zool. 232:521-529.
El Halawani, M. E., S. C. Fehrer, B. M. Hargis, and T. E. Porter. 1988. Incubation behavior in the domestic turkey: Physiological correlates. CRC Crit. Rev. Poult. Biol. 1:285-314.
El Halawani, M. E., S. C. Fehrer, and J. L. Silsby. 1987. Enhanced luteinizing hormone release by luteinizing hormone-releasing hormone in incubating female turkeys (*Meleagris gallopavo*). Biol. Reprod. 36:884-889.
El Halawani, M. E., L. J. Mauro, R. E. Phillips, and O. M. Youngren. 1990. Neuroendocrine control of prolactin and incubation behavior in gallinaceous birds. Prog. Clin. Biol. Res. 342:674-684.
El Halawani, M. E., and I. Rozenboim. 1993. The ontogeny and control of incubation behavior in turkeys. Poult. Sci. 72:906-911.
El Halawani, M. E., J. L. Silsby, E. J. Behnke, and S. C. Fehrer. 1984b. Effect of ambient temperature on serum prolactin and luteinizing hormone levels during the reproductive life cycle of the female turkey (*Meleagris gallopavo*). Biol. Reprod. 30:809-815.
El Halawani, M. E., J. L. Silsby, O. M. Youngren, and R. E. Phillips. 1991. Exogenous prolactin delays photo-induced sexual maturity and suppresses ovariectomy-induced luteinizing hormone secretion in the turkey (*Meleagris gallopavo*). Biol. Reprod. 44:420-424.
El Halawani, M. E., O. M. Youngren, and Y. Chaiseha. 2000. Neuroendocrinology of PRL regulation in the domestic turkey. Pages 233-244 in Avian Endocrinology. A. Dawson, and C. M. Chaturvedi, eds. Narosa Publishing House, New Delhi.
El Halawani, M. E., O. M. Youngren, and G. R. Pitts. 1997. Vasoactive intestinal peptide as the avian prolactin-releasing factor. Pages 403-416 in Perspectives in Avian Endocrinology. S. Harvey, and R. J. Etches, eds. Journal of Endocrinology, Bristol.
Etches, R. J., and F. J. Cunningham. 1976. The interrelationship between progesterone and luteinizing hormone during the ovulatory cycle of the hen (*Gullus domesticus*). J. Endocrinol. 71:51-58.
Follett, B. K. (1984). Reproductive cycles of vertebrates. Pages 283-350 in Marshall's Physiology of Reproduction. G. E. Lamming, ed. Churchill Livingstone, NewYork.
Furr, B. J. A., R. C. Bonney, R. T. England, and F. J. Cunningham. 1973. Luteinizing

hormone and progesterone in peripheral blood during the ovulatory cycle of the hen, *Gallus domesticus*. J. Endocrinol. 57:159-169.
Goldsmith, A. R., S. Burke, and J. M. Prosser. 1984. Inverse changes in plasma prolactin and LH concentrations in female canaries after disruption and reinitiation of incubation. J. Endocrinol. 103:251-256.
Goldsmith, A. R., C. Edwards, M. Koprucu, and R. Silver. 1981. Concentrations of prolactin and luteinizing hormone in plasma of doves in relation to incubation and development of the crop gland. J. Endocrinol. 90:437-443.
Goldsmith, A. R., and D. M. Williams. 1980. Incubation in mallards (*Anas platyrhnchos*): Changes in plasma levels of prolactin and luteinizing hormone. J. Endocrinol. 86:371-379.
Hall, T. R., S. Harvey, and A. Chadwick. 1986. Control of prolactin secretion in birds: A review. Gen. Comp. Endocrinol. 62:171-184.
Harvey, S., E. Bedrak, and A. Chadwick. 1981. Serum concentrations of prolactin, luteinizing hormone, growth hormone, corticosterone, progesterone, testosterone, and oestradiol in relation to broodiness in domestic turkeys (*Meleagris gallopavo*). J. Endocrinol. 89: 187-195.
Hiatt, E. S., A. R. Goldsmith, and D. S. Farner. 1987. Plasma levels of prolactin and gonadotropins during the reproductive cycle of white-crowned sparrows (*Zonotrichia leucophrys*). Auk 104:208-217.
Hohn, E. O. 1959. Prolactin in the cowbird's pituitary in relation to avian brood parasitism. Nature 184:2030.
Janik, D. S., and J. D. Buntin. 1985. Behavioural and physiological effects of prolactin in incubating ring dove. J. Endocrinol. 105:201-209.
Kappauf, B., and A. van Tienhoven. 1972. Progesterone concentrations in peripheral plasma of laying hens in relation to the time of ovulation. Endocrinology 90:1350-1355.
Knapp, T. R., S. C. Fehrer, J. L. Silsby, T. E. Porter, E. J. Behnke, and M. E. El Halawani. 1988. Gonodal steroid modulation of basal and vasoactive intestinal peptide-stimulated prolactin release by turkey anterior pituitary cells. Gen. Comp. Endocrinol. 76:1141-1144.
Kosonsiriluk, S., N. Sartsoongnoen, N. Prakobsaeng, T. Bakken, T. Songserm, M. E. El Halawani, and Y. Chaiseha. 2006. Distribution of vasoactive intestinal peptide-expressing neurons in the brain of the native Thai chicken (*Gallus domesticus*). Poult. Sci. 85 Suppl 1:45.
Krishnan, K. A., J. A. Proudman, D. J. Bolt, and J. M. Bahr. 1993. Development of an homologous radioimmunoassay for chicken follicle-stimulation hormone and measurement of plasma FSH during the ovulatory cycle. Comp. Biochem. Physiol. Comp. Physiol. 105:729-734.
Lague, P. C., A. van Tienhoven, and F. J. Cunningham. 1975. Concentrations of estrogens, progesterone and LH during the ovulatory cycle of the laying chicken. Biol. Reprod. 12:590-598.
Lea, R. W., A. S. M. Dods, P. J. Sharp, and A. Chadwick. 1981. The possible role of prolactin in the regulation of nesting behavior and the secretion of luteinizing hormone in broody bantams. J. Endocrinol. 91:89-97.
Lincoln, G. A. 1990. Correlation with changes in horns and pelage, but not reproduction, of seasonal cycles in the secretion of prolactin in rams of wild, feral and domesticated breeds of sheep. J. Reprod. Fert. 90:285-296.
Liu, H. K., D. W. Long, and W. L. Bacon. 2002. Interval between preovulatory surges of luteinizing hormone increases late in the reproductive period in turkey hens. Biol. Reprod. 66:1068-1075.

Mashaly, M. M., G. P. Birrenkott, M. M. El Begearmi, and B. C. Wentworth. 1976. Plasma LH and progesterone concentrations in the turkey hen during the ovulatory cycle. Poult. Sci. 55:1226-1234.

Mattocks, P. W., D. S. Farner, and B. K. Follett. 1976. The annual cycle of luteinizing hormone in the plasma of intact and castrated White-crowned Sparrows, *Zonotrichia leucophrys gambelii*. Gen. Comp. Endocrinol. 30:156-161.

Mauro, L. J., R. P. Elde, O. M. Youngren, R. E. Phillips, and M. E. El Halawani. 1989. Alterations in hypothalamic vasoactive intestinal peptide-like immunoreactivity are associated with reproduction and prolactin release in the female turkey (*Meleagris gallopavo*). Endocrinology 125:1795-1804.

Myers, S. A., J. R. Millam, and M. E. El Halawani. 1989. Plasma LH and prolactin levels during the reproductive cycle of the cockatiel (*Nymphicus hollandicus*). Gen. Comp. Endocrinol. 73:85-91.

Nicholls, T. J., A. R. Goldsmith, and A. Dawson. 1988. Photorefractoriness in birds and comparison with mammals. Physiol. Rev. 68:133-176.

Opel, H., and J. A. Proudman. 1980. Failure of mammalian prolactin to induce incubation behavior in chickens and turkeys. Poult. Sci. 59:2550-2558.

Opel, H., and J. A. Proudman. 1988. Effects of poults on plasma concentrations of prolactin in turkey hens incubating without eggs or a nest. Br. Poult. Sci. 29:791-800.

Opel, H., and J. A. Proudman. 1989. Plasma prolactin levels in incubating turkey hens during pipping of the eggs and after introduction of poults into the nest. Biol. Reprod. 40:981-987.

Oring, L. W., A. J. Fivizzani, M. E. El Halawani, and A. R. Goldsmith. 1986. Seasonal changes in prolactin and luteinizing hormone in the polyandrous spotted sandpiper, *Actitis macularia*. Gen. Comp. Endocrinol. 62:394-403.

Pollock, C. G., and S. E. Orosz. 2002. Avian reproductive anatomy, physiology and endocrinology. Vet. Clin. North Am. Exot. Anim. Pract. 5:441-474.

Porter, T. E., J. L. Silsby, E. J. Behnke, T. R. Knapp, and M. E. El Halawani. 1991. Ovarian steroid production *in vitro* during gonadal regression in the turkey. I. Changes associated with incubation behavior. Biol. Reprod. 45:581-586.

Proudman, J. A., C. G. Scanes, H. Opel, and M. A. Ottinger. 1984. Two avian luteinizing hormone radioimmunoassay procedures compared by measurements of changes during the ovulatory cycle of turkey and broiler hens. Poult. Sci. 63:1269-1275.

Proudman, J. A., T. D. Siopes, F. Vandesande, and L. R. Berghman. 2001. Thyroid hormone and prolactin profiles in male and female turkeys following photostimulation: Validation of an ELISA for turkey prolactin. Poult. Sci. 80 Suppl 1:469.

Riddle, O., R. W. Bates, and E. L. Lahr. 1935. Prolactin induces broodiness in fowl. Am. J. Physiol. 111:352-360.

Robinson, F. E., R. J. Etches, C. E. Anderson-Langmuir, W. H. Burke, K. W. Cheng, F. J. Cunningham, S. Ishii, P. J. Sharp, and R. T. Talbot. 1988. Steroidogenic relationships of gonadotrophin hormones in the ovary of the hen (*Gallus domesticus*). Gen. Comp. Endocrinol. 69:455-466.

Rose, M. P., R. E. Gaines Das, and A. H. Balen. 2000. Definition and measurement of follicle stimulating hormone. Endocr. Rev. 21:5-22.

Rozenboim, I., C. Tabibzadeh, J. L. Silsby, and M. E. El Halawani. 1993. Effect of ovine prolactin administration on hypothalamic vasoactive intestinal peptide (VIP), gonadotropin releasing hormone I and II content, and anterior pituitary VIP receptors in laying turkey hens. Biol. Reprod. 48:1246-1250.

Scanes, C. G., P. Cheeseman, J. G. Phillips, and B. K. Follett. 1974. Seasonal and age variation of circulating immunoreactive leutinizing hormone in capture Herring gulls,

Larus argentatus. J. Zool. 174:369-375.
Sharp, P. J., M. C. Macnamee, R. J. Sterling, R. W. Lea, and H. C. Pedersen. 1988. Relationships between prolactin, LH and broody behavior in bantam hens. J. Endocrinol. 118:279-286.
Sharp, P. J., M. C. Macnamee, R. T. Talbot, R. J. Sterling, and T. R. Hall. 1984. Aspects of the neuroendocrine control of ovulation and broodiness in the domestic hen. J. Exp. Zool. 232:475-483.
Sharp, P. J., C. G. Scanes, J. B. Williams, S. Harvey, and A. Chadwick. 1979. Variations in concentrations of prolactin, luteinizing hormone, growth hormone, and progesterone in the plasma of broody bantams (*Gallus domesticus*). J. Endocrinol. 80:51-57.
Sharp, P. J., R. J. Sterling, R. C. de L Milton, and R. P. Millar. 1986. Effect of luteinizing hormone releasing hormone and its analogues on plasma luteinizing hormone concentrations in incubating bantam hens. Br. Poult. Sci. 27:129-135.
Shodono, M., T. Nakamura, Y. Tanabe, and K. Wakabayashi. 1975. Simultaneous determinations of oestradiol-17β, progesterone, and luteinizing hormone in the plasma during the ovulatory cycle of the hen. Acta. Endocrinol. 78:565-573.
Silver, R. 1984. Prolactin and parenting in the pigeon family. J. Exp. Zool. 232:617-625.
Silver, R., A. R. Goldsmith, and B. K. Follett. 1980. Plasma luteinizing hormone in male ring doves during the breeding cycle. Gen. Comp. Endocrinol. 42:19-24.
SPSS Inc. 2004. SPSS Base 13.0 Users Guide. Prentice Hall, NJ.
Tabibzadeh, C., I. Rozenboim, J. L. Silsby, G. R. Pitts, D. N. Foster, and M. E. El Halawani. 1995. Modulation of ovarian cytochrome P450 17 alpha-hydroxylase and cytochrome aromatase messenger ribonucleic acid by prolactin in the domestic turkey. Biol. Reprod. 52:600-608.
Talbot, R. T., M. C. Hanks, R. J. Sterling, H. M. Sang, and P. J. Sharp. 1991. Pituitary prolactin messenger ribonucleic acid levels in incubating and laying hens: Effects of manipulating plasma levels of vasoactive intestinal polypeptide. Endocrinology 129:496-502.
Tanabe, Y., T. Nakamura, Y. Omiya, and T. Yano. 1980. Changes in the plasma LH, progesterone, and estradiol during the ovulatory cycle of the duck (*Anas platyrhnchos domestica*) exposed to different photoperiods. Gen. Comp. Endocrinol. 41:378-383.
Tong, Z., G. R. Pitts, D. N. Foster, and M. E. El Halawani. 1997. Transcriptional and post-transcriptional regulation of prolactin during the turkey reproductive cycle. J. Mol. Endocrinol. 18:223-231.
Wilson, S. C., and P. J. Sharp. 1975. Effects of progesterone and synthetic luteinizing hormone releasing hormone on the release of luteinizing hormone during sexual maturation in the hen (*Gallus domesticus*). J. Endocrinol. 67:359-369.
Wingfield, J. C., and D. S. Farner. 1978a. The endocrinology of a natural breeding populations of the White-crowned Sparrow (*Zonotrichia leucophrys pugetensis*). Physiol. Zool. 51:189-205.
Wingfield, J. C., and D. S. Farner. 1978b. The annual cycle of plasma irLH and steroid hormones in feral populations of the White-crowned Sparrow, *Zonotrichia leucophrys gambelii*. Biol. Reprod. 19:1046-1056.
Wong, E. A., N. H. Ferrin, J. L. Silsby, and M. E. El Halawani. 1991. Cloning of a turkey prolactin cDNA: Expression of prolactin mRNA throughout the reproductive cycle of the domestic turkey (*Meleagris gallopavo*). Gen. Comp. Endocrinol. 83:18-26.
Wong, E. A., J. L. Silsby, S. Ishii, and M. E. El Halawani. 1992. Pituitary luteinizing hormone and prolactin messenger ribonucleic acid levels are inversely related in laying and incubating turkey hens. Biol. Reprod. 47:598-602.
You, S., J. L. Silsby, J. Farris, D. N. Foster, and M. E. El Halawani. 1995. Tissue-specific

alternative splicing of turkey preprovasoactive intestinal peptide messenger ribonucleic acid, its regulation, and correlation with prolactin secretion. Endocrinology 136:2602-2610.
Youngren, O. M., M. E. El Halawani, J. L. Silsby, and R. E. Phillips. 1991. Intracranial prolactin perfusion induces incubation behavior in turkey hens. Biol. Reprod. 44:425-431.
Youngren, O. M., Y. Chaiseha, R. E. Phillips, and M. E. El Halawani. 1996a. Vasoactive intestinal peptide concentrations in turkey hypophysial portal blood differ across the reproductive cycle. Gen. Comp. Endocrinol. 103:323-330.
Youngren, O. M., G. R. Pitts, R. E. Phillips, and M. E. El Halawani. 1996b. Dopaminergic control of prolactin secretion in the turkey. Gen. Comp. Endocrinol. 104:225-230.

Figure and Table Legends

Table 1. Reproductive characteristics of the native Thai chickens during the reproductive cycles.

Figure 1A-D. Representative of Plasma PRL and LH concentrations during the reproductive cycles in the native Thai chickens

Figure 2. Plasma PRL (**A**) and LH (**B**) levels in the native Thai chickens in each reproductive stage (n=10). Values are expressed as the Mean ± SEM. Values with different letters are significantly different ($P<0.05$).

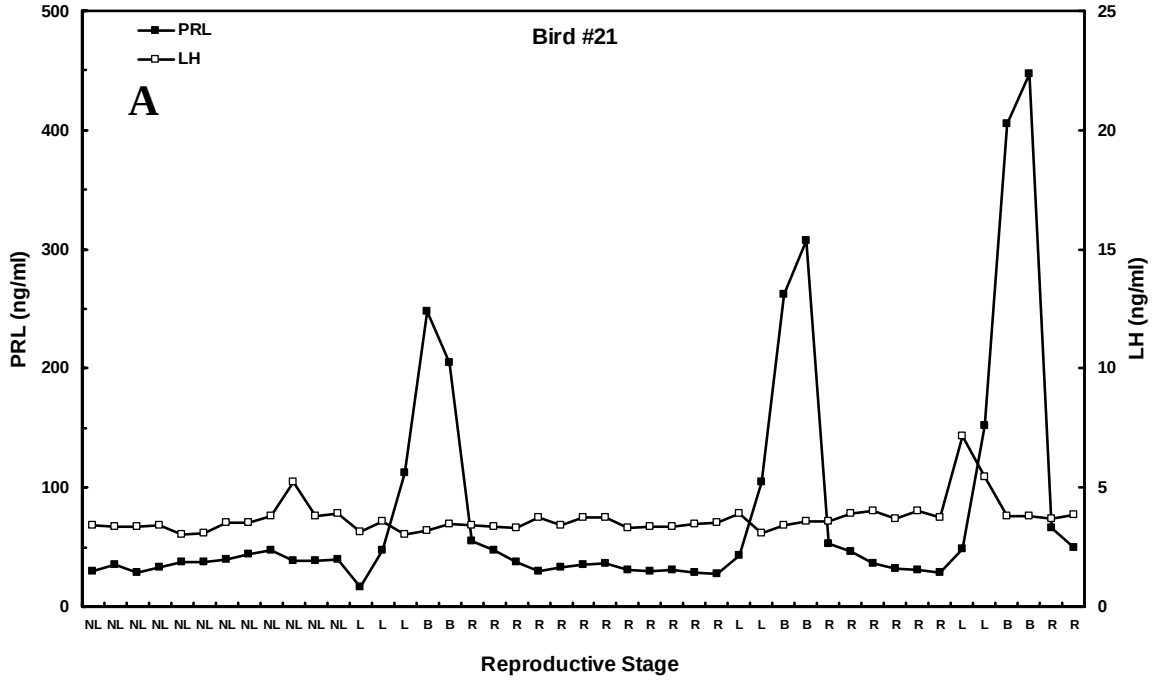
Figure 3. Ovary and oviduct weights of the native Thai chickens in each reproductive stage (n=25). Values are expressed as the Mean ± SEM. Values with different letters are significantly different ($P<0.05$).

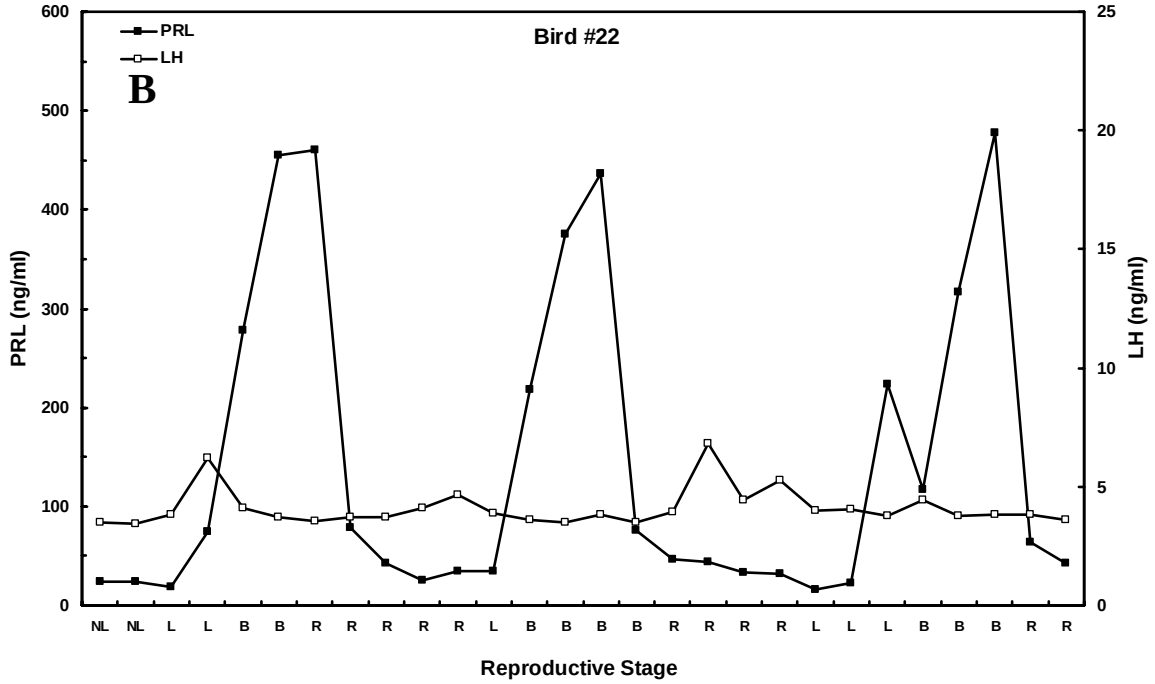
Table 1.

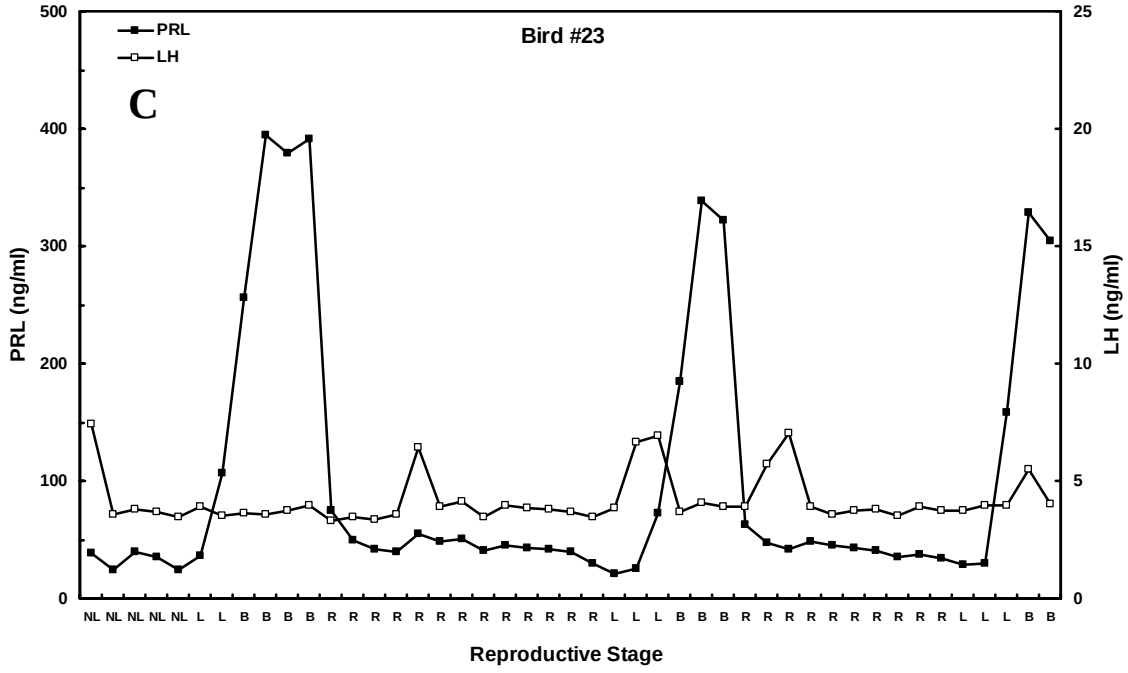
Reproductive Cycle	Age at First Lay (wk)	Duration of Laying (day)	Egg Production (egg)	Duration of Incubating (day)	Hatchability (%)	Duration of Rearing (day)
Cycle 1 (n=29)	32.31 ± 0.75	18.55 ± 2.48[a]	12.45 ± 0.99[a]	20.00 ± 0.65[a]	58.10 ± 5.12[a]	54.83 ± 3.53[a]
Cycle 2 (n=27)	45.56 ± 1.13	28.59 ± 5.08[a]	18.37 ± 2.15[b]	19.00 ± 0.28[a]	65.93 ± 4.29[a]	41.93 ± 1.88[b]

Values are expressed as the Mean ± SEM.
Values with different letters are significantly different ($P<0.05$).

Figure 1.


PRL
LH
Bird #21
A
PRL (ng/ml)
LH (ng/ml)
Reproductive Stage
NL NL NL NL NL NL NL NL NL NL NL L L L B B R R R R R R R R R R R L L B B R R R R R R L L B B R R


PRL
LH
Bird #22
B
PRL (ng/ml)
LH (ng/ml)
Reproductive Stage
NL NL L L B B R R R R R L B B B B R R R R L L L B B B R R


PRL
LH
Bird #23
C
PRL (ng/ml)
LH (ng/ml)
Reproductive Stage

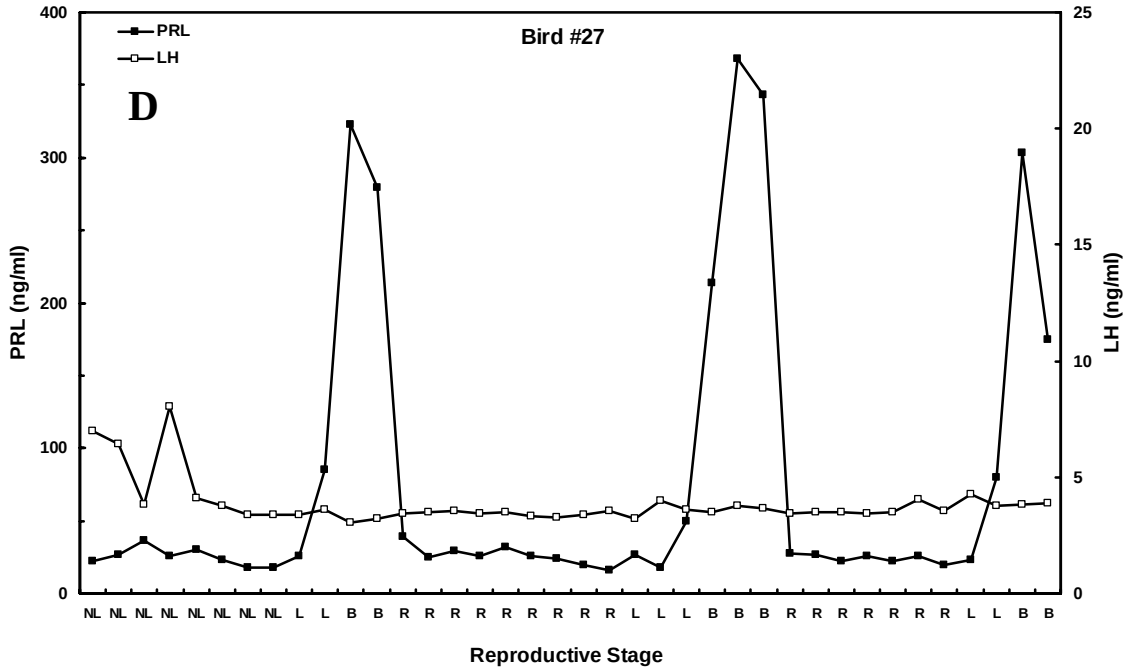

PRL
LH
Bird #27
D
PRL (ng/ml)
LH (ng/ml)
Reproductive Stage

Figure 2.

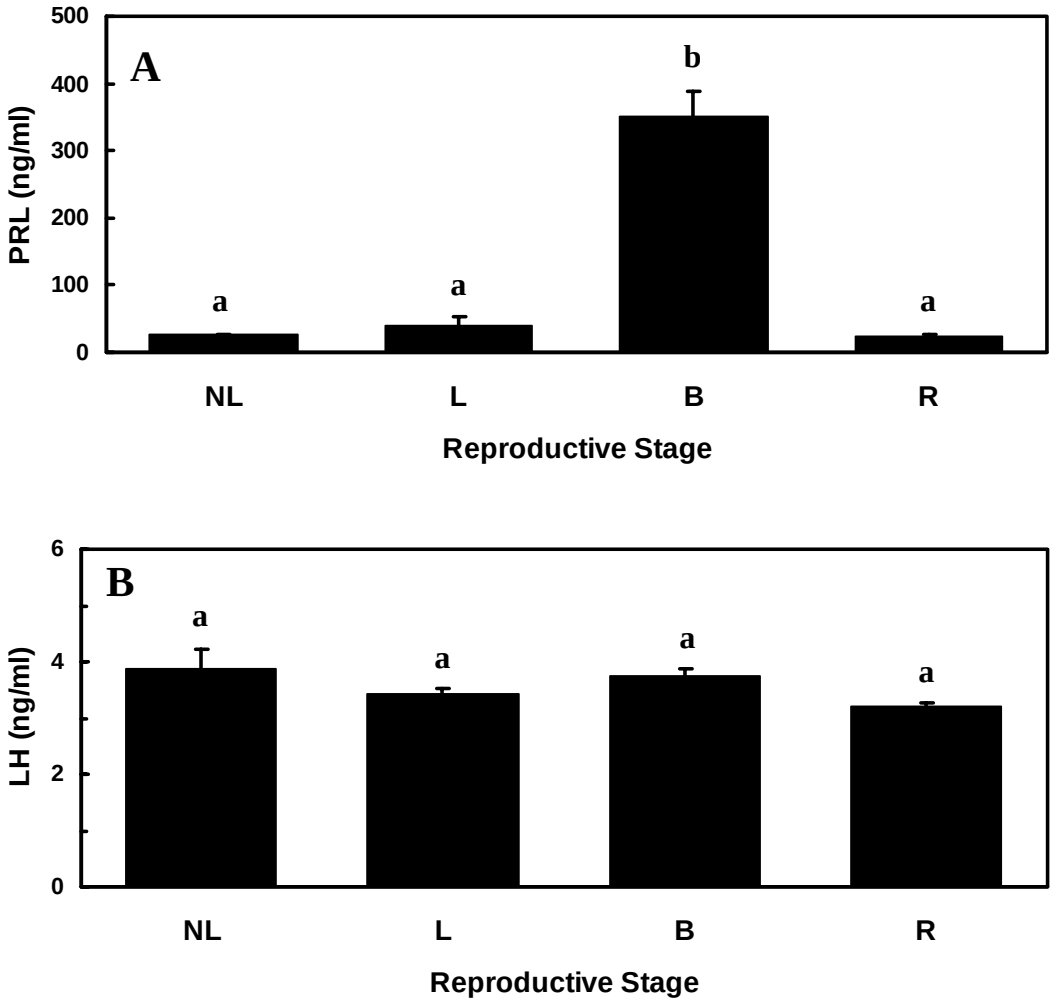
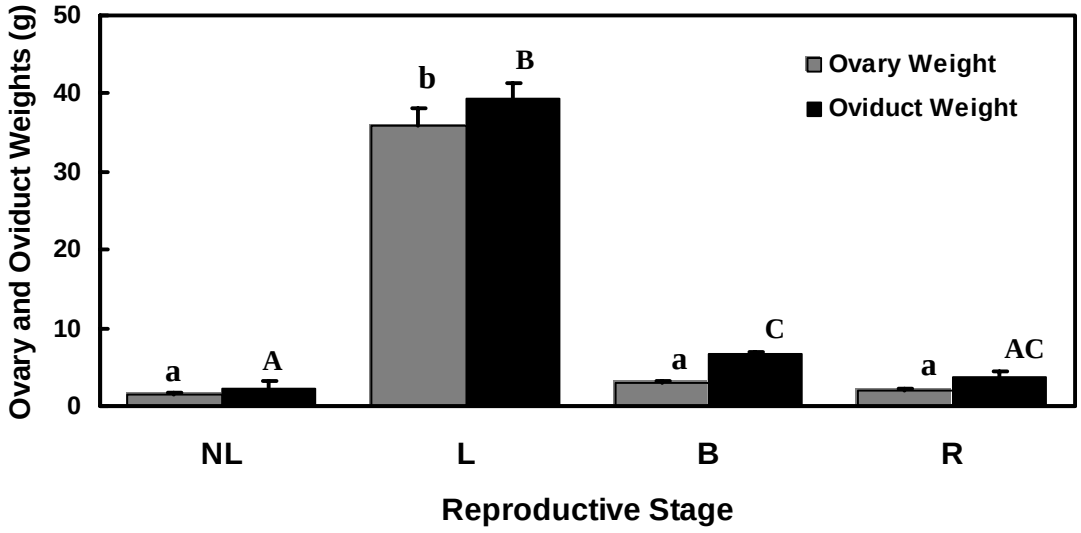

A
PRL (ng/ml)
500
400
300
200
100
0
a
a
b
a
NL
L
B
R
Reproductive Stage
B
LH (ng/ml)
6
4
2
0
a
a
a
a
NL
L
B
R
Reproductive Stage

Figure 3.


Ovary and Oviduct Weights (g)
50
40
30
20
10
0
Ovary Weight
Oviduct Weight
a
A
b
B
a
C
a
AC
NL
L
B
R
Reproductive Stage

3. Copy of the Manuscript #2:

Kosonsiriluk S, Sartsoongnoen N, Chaiyachet O, Prakobsaeng N, Songserm T, Rozenboim I, El Halawani ME, **Chaiseha Y** (2007). Vasoactive intestinalpPeptide and its role in continuous and seasonal reproduction in birds. **General and Comparative Endocrinology** (Submitted; **Impact factor** = 2.487).

Vasoactive Intestinal Peptide and Its Role in Continuous and Seasonal Reproduction in Birds

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Running title: Reproduction and VIP in non-seasonal and seasonal birds

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Abstract

Native Thai chicken is an equatorial species that breeds throughout the year, unaffected by photoperiod, whereas in the domestic turkey, a seasonal breeder, both photoperiod and the reproductive cycle alter the profile of vasoactive intestinal peptide (VIP), the avian prolactin releasing factor. This study investigated VIPergic activity throughout a complete reproductive cycle in both the native Thai chicken and the domestic turkey, hypothesizing that the differential expression of VIP would provide insight into the differing reproductive strategies of the two species. The investigation focused on 1) the distribution of VIP neurons in the native Thai chicken brain and 2) a comparison of VIPergic activity in the nucleus inferioris hypothalami (IH) and the nucleus infundibuli hypothalami (IN) where VIP levels and VIP neuron concentrations have been shown to vary with the reproductive cycles of the two species. VIP immunoreactivity was found throughout the brain of the native Thai chicken, but was predominantly located within the IH and IN. VIP immunoreactive (VIP-ir) neurons were also observed within the organum septi laterale, nucleus anterior medialis hypothalami, and regio lateralis hypothalami. A dense accumulation of VIP-ir fibers was found in the external layer of the eminentia mediana. The pattern of VIP distribution in the native Thai chicken supports the findings reported in temperate zone avian species. Unlike the turkey, where there is a dissociation between VIPergic activity and prolactin levels during the photorefractory stage, in native Thai chickens, which do not express photorefractoriness, changes in the number of VIP-ir neurons within the IH-IN were directly correlated with varying concentrations of circulating prolactin throughout the reproductive cycle. In the native Thai chicken; VIPergic activity reached its lowest level after hatching of the chicks, while in the turkey VIP was lowest only after exposure to a short day photoperiod and the

acquisition of photosensitivity. This suggests that the VIP neurons in the IH-IN may play a pivotal role in regulating the reproductive cycle in avian species and its differential expression following hatching of the young may, in part, account for the difference in reproductive mode between equatorial, continually breeding, non-photoperiodic birds (native Thai chickens) and seasonally breeding , photoperiodic birds (turkeys).

Key words: Equatorial birds; Immunohistochemistry; Native Thai chicken; Photoperiod; Photorefractory; Turkey; Vasoactive intestinal peptide

1. Introduction

In birds, changes in concentrations of luteinizing hormone (LH) and prolactin (PRL) during the reproductive cycles are well documented (Follett, 1984; El Halawani et al., 1988). The onset of incubation behavior is correlated with declining plasma levels of LH and increasing plasma levels of PRL (Lea et al., 1981). The hypothalamic control of PRL and LH secretion is mediated by a hypophyseal portal vascular system which transports regulatory neuropeptides and neurotransmitters released from the median eminence (eminentia mediana; ME) to the anterior pituitary gland (Follett, 1984). Avian PRL secretion is tonically stimulated (Kragt and Meites, 1965; Bern and Nicoll, 1968) by vasoactive intestinal peptide (VIP) which is secreted from neurons located in the infundibular nuclear complex (INF) of the caudo-medial hypothalamus (Sharp et al., 1989; El Halawani et al., 1997).

Pituitary PRL secretion is strongly correlated with the avian reproductive cycle. During the reproductively quiescent stages of the cycle, plasma PRL levels are extremely 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; Sharp et al., 1989). In turkeys, tactile stimuli from poults decrease circulating PRL in hens incubating without eggs and nest (Opel and Proudman, 1988). Physical contact as well as visual and/or auditory stimuli from young chicks are clearly involved in the appearance and maintenance of maternal behavior (Richard-Yris and Leboucher, 1987; Opel and Proudman, 1989).

In temperate zone birds, expression and secretion within the collective VIP/PRL system are activated by an escalating photoperiod which stimulates the gonad. VIP mRNA steady-state level in the hypothalamus and VIP content in the ME increase following photostimulation and are closely correlated with increased PRL secretion in turkeys (Chaiseha et al., 1998). Immunocytochemical studies have shown that hypothalamic VIP-immunoreactive (VIP-ir) neurons within the INF and VIP-ir fibers in the ME correspond to enhanced circulating PRL levels (Mauro et al., 1989). Other studies have also shown increases in the number and size of VIP-ir neurons within this region in the domesticated pigeon and ring dove during periods of elevated circulating PRL levels (Peczely and Kiss, 1988; Cloues et al., 1990). VIP localization studies have been conducted in the brains of several avian species, such as the Pekin duck (Korf and Fahrenkrug, 1984), Japanese quail (Peczely and Kiss, 1988), turkey (Mauro et al., 1989; Chaiseha and El Halawani, 1999), pigeon (Cloues et al., 1990), ring dove (Norgren and Silver, 1990), chicken (Kuenzel and Blahser, 1994; Kuenzel et al., 1997), dark-eyed junco (Saldanha et al., 1994), and male zebra finch (Bottjer and Alexander, 1995).

To date, there has been no report on the VIPergic system of the native Thai chicken, a continuously breeding equatorial species that is unaffected by photoperiod. One objective of this study was to delineate the VIPergic system in the native Thai chicken brain and investigate its relationship to PRL secretion and other related reproductive activities. Another objective was to compare changes in VIP neurons, located in the infundibular area of the

caudal hypothalamus, between a non-photoperiodic species, the native Thai chicken, and a photoperiodic species, the domestic turkey, across a complete reproductive period. Differential VIP expression and function in the area of the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN) may provide further insight into the neural mechanisms underlying the regulation of the reproductive cycle in a non-photoperiodic avian species.

2. Materials and methods

2.1. Experimental animals

Female native Thai chickens, 16-18 weeks of age, were used. They were housed with mature roosters (8 females:1 male) in floor pens with nests under natural light (approximately 12 hours of light and 12 hours of dark; 12L:12D). Feed and water were available *ad libitum*. The hens were classified into four reproductive stages: non-egg laying (NL), egg laying for 1 week (LAY), incubating eggs for 10 days (INC), and rearing chicks for 2 weeks (R). A postmortem examination of each hen was performed to confirm its reproductive status. Somatically mature large white female Hybrid turkeys, 33 weeks of age, were used. The birds were housed in floor pens under a light regimen of 6L:18D. Three groups (n=6) were photostimulated (15L:9D) at successive three week intervals, while a fourth group remained at 6L:18D (non-photostimulated). This provided birds of the same age in four reproductive stages: 1) non-photostimulated (NPS); 2) laying eggs for 3 to 4 weeks (LAY); 3) incubating eggs for 3 to 4 weeks (INC); and 4) photorefractory (REF), in which brooding behavior was induced in incubating hens by replacing eggs with one week old poults. Poults were removed after the hens abandoned their nests and started brooding behavior, which included vocalizations, feather fluffing, crouching posture and guiding poults beneath the body and wings. After the poults were removed the hens began to molt. These four physiological states were confirmed by observation of sexual behavior, incubation behavior and molting and by postmortem examination of the reproductive organs. The animal protocols described in this study were approved by Suranaree University of Technology Animal Care and Use Committee guideline and the University of Minnesota Institutional Animal Care and Use Committee guideline.

2.2. Experimental procedures

2.2.1. Experiment 1: Changes in plasma PRL levels across the reproductive cycle of the native Thai chicken

Blood samples (n=10) were collected from native Thai chickens at different reproductive stages (NL, LAY, INC, and R). Plasma PRL levels were determined in duplicate within a single assay utilizing an enzyme-linked immunosorbent assay according to a previously described method (Proudman et al., 2001).

2.2.2. Experiment 2: Changes in VIP-ir neurons in the IH-IN areas of the native Thai chicken and the INF of the turkey across the reproductive cycle

Native Thai chickens and turkeys from each of the four reproductive stages (n=6) were used. The brains were pressure-perfused, sectioned with a cryostat, and processed by immunohistochemistry to localize and identify VIP-ir neurons.

2.2.3. Experiment 3: Effects of removing chicks on ovary and oviduct recrudescence in the native Thai hen

For comparison of ovary and oviduct recrudescence between hens rearing chicks and hens that had their chicks removed, native Thai hens were divided into two groups. In the first group, hens were sacrificed after rearing chicks for 1, 2, 3, 4, or 5 weeks (n=6) following hatching. In the second group, chicks were removed from the hens immediately after hatching. The hens were then sacrificed at 1, 2, 3, 4, or 5 weeks (n=6) after chick-removal. Ovaries and oviducts were removed and weighed at the time of hen sacrifice.

2.2.4. Experiment 4: The distribution of VIP immunoreactivity throughout the brain of the laying native Thai hen

Laying native Thai hens (n=6) were used. The brains were pressure-perfused, sectioned with a cryostat, and processed by immunohistochemistry to localize and identify VIP-ir neurons and fibers.

2.3. Processing of tissues for immunohistochemistry

Prior to perfusion, native Thai chickens and turkeys were intravenously injected with 3,000 and 5,000 units of heparin, respectively (Baxter Healthcare Corporation, Deerfield, IL, USA), and euthanized with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France). The head was removed and immediately pressure-perfused via the carotid arteries with phosphate buffered saline (PBS, pH 7.4) for 3-5 min, followed by a freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min according to a previously described method (Al-Zailaie et al., 2006). The brain was then dissected intact from the skull, and soaked in 20% sucrose in PBS at 4°C for 48 hrs or until saturated for cryoprotection. The brain was then frozen in powdered dry ice for 1 hr, and stored at -20°C until sectioned in the coronal plane at a thickness of 16 µm utilizing a cryostat. The sections were mounted onto subbed slides and stored desiccated at -20°C until further processed for immunohistochemistry.

2.4. Immunohistochemistry

In order to localize VIP distribution throughout the brain of the laying native Thai hen and to visualize changes in VIP-ir neurons within the IH-IN/INF area of the Thai chicken and the turkey at different reproductive stages, immunohistochemistry was performed as previously described (Al-Zailaie et al., 2006). Briefly, brain tissue sections of different areas throughout laying Thai hen brains and four adjacent sections from each reproductive stage in the IH-IN/INF area of Thai chickens and turkeys were thawed to room temperature prior to use. The sections were rehydrated in PBS for 30 min at room temperature. After PBS removal, the sections were then covered with 100 µl of VIP primary antibody (polyclonal anti-chicken VIP antiserum (VIP4-DYC8); generously provided by Dr. M.E. El Halawani, University of Minnesota, USA) diluted 1:1,000 with PBS (pH 7.4) containing 1% bovine serum albumin and 0.3% triton-X. Tissue section slides were then incubated in a moist chamber at 4°C overnight, and then washed three times with PBS (pH 7.4) for 5 min each. After washing, 100 µl of secondary antibody diluted 1:500 Cy^{TM}3-conjugated AffiniPure Donkey Anti-Rabbit IgG and 1:100 FITC-donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) were applied for chicken and turkey sections under dark conditions. Slides were further incubated in a moist dark chamber at room temperature for 1 hr, washed with PBS (pH 7.4) three times for 5 min each, and then mounted with DPX mountant (Sigma-Aldrich, Inc., Steinheim, Germany). Microscopic images of brain sections throughout the brain of the hens were visualized with a fluorescence microscope (Olympus IX71, Tokyo, Japan) at 4x, 10x, 20x, and 40x magnification using a

cooled digital color camera (Olympus DP70, Tokyo, Japan). The images were then captured and stored by DP70-BSW software (Olympus, Tokyo, Japan). Sixteen microscopic fields of four adjacent sections correspond to the IH-IN and INF areas of each bird were taken at 10x magnification. The total number of VIP-ir cells was counted manually to visualize the changes of VIP-ir cells within the IH-IN and INF areas throughout reproductive stages. To aid in the documentation of neuroanatomical results illustrating VIP immunoreactivity, the nomenclature and schematic diagrams from the stereotaxic atlases of the brain of the chick (Kuenzel and Masson, 1988), the chicken hypothalamus (Kuenzel and van Teinhoven, 1982), and the turkey hypothalamus (Youngren, unpublished) were utilized. The specificity of the anti-VIP serum was tested by omitting the anti-VIP serum from the primary antiserum. No immunostaining of VIP was observed in control sections.

2.5. Statistical analysis

Significant differences (mean±SEM) in plasma PRL levels in the native Thai chicken, the number of VIP-ir neurons per section among reproductive stages of the native Thai chicken and turkey, and the ovary and oviduct weights of rearing and chick-removed native Thai hens were compared by one way analysis of variance (ANOVA) with 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).

3. Results

3.1. Changes in plasma PRL levels across the reproductive cycle of the native Thai chicken

Plasma PRL levels (Fig. 1), measured across the reproductive cycle (n=10), were found to be low in non-layers (NL, 28.3±3.9 ng/ml), somewhat augmented, but not significantly in layers (LAY, 42.8±8.9 ng/ml), and highest in hens incubating eggs (INC, 238.4±19.8 ng/ml, $P<0.05$). When hens began rearing chicks, PRL levels quickly dropped to a low point again (R, 25.7±1.3 ng/ml), equaling that of non-laying birds.

3.2. Changes in VIP-ir neurons in the IH-IN areas of the native Thai chicken and the INF of the turkey across the reproductive cycle

Changes in the VIP-ir neuron populations within the IH and IN areas were observed across the reproductive cycle of the native Thai chicken (Fig. 2A, 3A). VIP-ir neuron counts were low in non-layers (NL) when compared to laying hens (LAY, $P<0.05$). There was a marked and significant increase in VIP-ir neurons during the incubating phase (INC, $P<0.05$) when cell count reached its highest point, more than double that of non-laying birds. When the hens shifted from incubating eggs to rearing chicks (R), the number of VIP-ir neurons in the IH-IN area returned to non-laying levels.

In the turkey INF, in the area of the IH and IN, VIP-ir cells counts were very low in the non-photostimulated birds (Fig. 2B, 3B) and increased significantly ($P<0.05$) when the hens began to lay eggs (LAY). When the turkey hens were incubating eggs (INC) there was another significant increase ($P<0.05$) in VIP-ir neurons above that of the layers. During the period when the incubating hens had their eggs removed and poults given to them (REF), the VIP-ir neuron count in the IH-IN area decreased. However, it only decreased to egg laying levels ($P>0.05$) and remained well above levels observed in the non-photostimulated turkeys ($P<0.05$).

3.3. Effects of removing chicks on ovary and oviduct recrudescence in the native Thai hen

When the native Thai hens were rearing their young (Fig. 4A, B), their ovary and oviduct weights (g) showed no increase across a five week period (mean ovary weight/week: 2.6±0.2, 1.9±0.1, 1.6±0.2, 2.5±0.5, 2.3±0.7; mean oviduct weight/week: 4.7±0.5, 3.5±0.3, 3.1±0.2, 3.9±1.3, 4.8±1.9). In contrast, ovary and oviduct weight in the native Thai hens that had their chicks removed at hatching displayed a significant increase ($P<0.05$) by the third week (mean ovary weight/week: 4.4±1.5, 15.0±7.3, 47.3±6.8, 43.1±4.3, 27.0±10.8; mean oviduct weight/week: 9.4±4.2, 22.2±5.0, 45.0±4.7, 55.7±3.5, 34.5±7.1).

3.4. The distribution of VIP immunoreactivity throughout the brain of the laying native Thai hen

Immunohistochemistry revealed that VIP-ir cells and fibers were distributed throughout the brain (telencephalon, diencephalon, mesencephalon, and rhombencephalon) of the female native Thai chicken (Fig. 5), with predominant accumulation occurring within the diencephalon.

3.4.1. Telencephalon

Beginning with the anterior portion of the brain, the most rostral VIP-ir fibers were found in the lobus parolfactorius (LPO). In the septal area, a cluster of cerebrospinal fluid (CSF)-contacting VIP-ir neurons with bulb-like processes projecting into the ventriculus lateralis (VL) was observed in the medial portion of the organum septi laterale, pars medialis; LSOm (Fig. 6A). A group of VIP-ir fibers was also found in and about the nucleus accumbens (Ac). There was a dense accumulation of VIP-ir fibers present in the nucleus septalis lateralis (SL) and the nucleus septalis medialis (SM), but few VIP-ir cells were seen in the SL (Fig. 6B). Furthermore, a few uniformly dark-stained VIP-ir neurons were located in the nucleus taeniae (Tn).

3.4.2. Diencephalon

The greatest expression of VIP-ir neurons occurred in the diencephalon, with the highest accumulation found within the IH-IN (Fig. 7A, 7B). Most of the VIP-ir neurons were found in the caudal portion of the IH-IN area, whereas there were only a few VIP-ir neurons found in the rostral portion. None of the VIP-ir neurons in this area had bulb-like processes and did not appear to protrude into the third ventricle. Dense accumulations of VIP-ir fibers were found in the external layer of the ME (Fig. 7C), where beaded fibers were distributed specifically in a palisade arrangement. A large group of VIP-ir neurons was observed outside the hypothalamus in the nucleus rotundus (ROT, Fig. 8A, 8B). The cluster of VIP-ir cells in this area showed stained coarse-grained granules in the cytoplasm and neurite. Scattered VIP-ir neurons were also found within the nucleus anterior medialis hypothalami (AM, Fig. 8C), regio lateralis hypothalami (LHy, Fig. 8D), and nucleus ventromedialis hypothalami (VMN). A very dense accumulation of VIP-ir fibers was also observed in the LHy. Within the boundaries of the nucleus periventricularis hypothalami (PHN, Fig. 8E), there were beaded VIP-ir fibers, some of which extended in parallel with the third ventricle to the IH-IN. VIP-ir fibers were observed in the nucleus paraventricularis magnocellularis (PVN) as well. In the thalamus, some VIP-ir fibers were present in the ventral portion of the tractus cortico-habenularis et cortico-septalis (CHCS). Additional VIP-ir fibers were also found in the nucleus commissurae pallii (nCPa, Fig. 8F).

3.4.3. Mesencephalon and rhombencephalon

There were VIP-ir neurons scattered in the substantia grisea centralis (GCt, Fig. 9A), nucleus intercollicularis (ICo), area ventralis (AVT, Fig. 9B), nucleus tegmenti pedunculo-pontinus, pars compacta (TPc, Fig. 9C, 9D), nucleus subceruleus ventralis (SCv), locus ceruleus (LoC), and nucleus interpeduncularis (IP). Moving caudally to the beginning of the pons, a dense plexus of VIP-ir fibers was observed in the area of the nucleus parabrachialis, pars ventralis (PBv), nucleus tractus solitarii (S) and some in the nucleus linearis caudalis (LC) and nucleus reticularis paragiganto-cellularis lateralis (Rpgl). Additionally, VIP-ir cells were found lining the cortex layer of the cerebellum (Cb, Fig. 10A). Conversely, there was no immunostaining observed in the pituitary (Fig. 10B).

4. Discussion

VIP-ir neurons and fibers were extensively distributed throughout the brain of the native Thai chicken and were predominantly expressed in the diencephalon, where VIP-ir neurons were concentrated within the IH-IN area of the infundibulum. VIP-ir neurons were also found in the AM, LHy, PVN, and VMN. The findings of this study correspond with previous studies regarding the distribution of VIP-containing neurons and fibers within the brains of several avian species (Yamada et al., 1982; Macnamee et al., 1986; Peczely and Kiss, 1988; Mauro et al., 1989; Norgren and Silver, 1990; Kuenzel and Blahser, 1994) and provides additional evidence that VIP is also the PRL releasing factor (PRF) in non-photoperiodic, continuously breeding avian species. Changes in VIP-ir neurons within the IH-IN area, but not other areas, were directly correlated with changing concentrations of circulating PRL throughout the reproductive cycle of the native Thai chicken.

The results of the present study provide evidence suggesting that hypothalamic VIP expression in the IH-IN of the non-photoperiodic native Thai chicken plays a regulatory role in its year-round reproductive activity. VIP is well accepted as the avian PRF (Sharp et al., 1989; El Halawani et al., 1997). Immunocytochemistry and in situ hybridization studies have shown fluctuations in hypothalamic VIP immunoreactivity and expression within the native Thai chicken IH-IN and the turkey INF parallel fluctuations in circulating PRL (this study; Mauro et al., 1989; Chaiseha and El Halawani, 1999). Unlike photoperiodic birds (i.e. the turkey), where the VIP/PRL neuroendocrine system is photoperiodic dependent (Mauro et al., 1989; Dawson and Sharp, 1998; Chaiseha and El Halawani, 1999), in the native Thai chicken, a non-photoperiodic, continuous breeder, the VIP/PRL system may initially be activated by ovarian steroids, as the number of VIP-ir neurons in the IH-IN and plasma PRL levels start to increase during sexual activity and breeding. At the onset of sexual maturity (first ovulation), the preovulatory surge of progesterone has been shown to induce the nesting behavior associated with oviposition (Wood-Gush and Gilbert, 1973; El Halawani et al., 1986), and the combined action of estrogen and progesterone stimulates the VIP/PRL system (El Halawani et al., 1983; 1986). The increased number of VIP-ir neurons which have been shown to be correlated with the up-regulation of VIP peptide and its mRNA (Mauro et al., 1989; 1992; Chaiseha and El Halawani, 1999), and the maximum plasma PRL levels reached in incubating hens could result from the presence of eggs and persistent nesting activity (El Halawani et al., 1980).

The increased neuroendocrine activity of the VIP/PRL system has been shown to suppress the release of gonadotropin-releasing hormone (GnRH)/LH-follicle stimulating hormone (FSH; Sharp et al., 1998), reduce ovarian steroids secretion (Zadworny et al., 1988), terminate egg laying, induce ovarian regression (Zadworny et al., 1988; Youngren et al., 1991), and commence nest protective behavior and anorexia (Zadworny and Etches, 1987). These behavioral and neuroendocrine changes have been attributed to increased PRL levels and the state of hyperprolactinemia to initiate and establish incubation behavior (El Halawani

et al., 1986; Opel and Proudman, 1989; Chaiseha and El Halawani, 2005). It is possible that PRL and the state of hyperprolactinemia may also be of importance in the increased number of VIP-ir neurons in the IH-IN observed in late incubation.

The termination of incubation behavior following hatching of chicks or by the introduction of day old poults (present study; Opel and Proudman, 1989), induced a decline in plasma PRL level in both the native Thai chicken and the turkey which could have resulted from the abandonment of the nest, absence of the nesting stimulus and the presence of chicks/poults (El Halawani et al., 1980; Opel and Proudman, 1988; 1989). In the native Thai chicken and unlike the turkey, the decline in circulating PRL levels was associated with a reduction in the number of VIP-ir neurons in the IH-IN, and, in turn, reduction of VIPergic activity to that of pre-laying levels, whereas ovarian regression continued. The lack of ovarian recrudescence, the initiation of reproductive activity and the start of a new reproductive cycle despite the down regulation of the VIP/PRL system in the native Thai chicken appears to be related to an inhibitory influence of chicks on the reproductive neuroendocrine system. In the present study, removal of chicks from the care of chickens induced ovarian recrudescence within one week, appearance of yellow follicles within two weeks and egg laying by the third week of separation from chicks. This finding is consistent with the earlier suggestion that maternal behavior, including physical contact, as well as visual and/or auditory stimuli originating from the chicks, have an inhibitory effect on the reproductive neuroendocrine system (Richard-Yris and Leboucher, 1987; Richard-Yris et al., 1987; Sharp et al., 1988). 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).

In the present study, the introduction of poults to incubating turkeys disrupted incubation, initiated maternal behavior, then induced molting and reduced the number of VIP-ir neurons from that observed during incubation, but VIP expression remained significantly greater than that of short day photosensitive turkeys. The finding in the present study that the acquisition of photosensitivity, by pretreatment of photorefractory turkey hens with a short day light treatment, was associated with a decline in the number of VIP-ir neurons supports an earlier suggestion which implicates elevated hypothalamic VIP peptide and mRNA content in the insensitivity of seasonal breeders to long day lighting and the transition to photorefractoriness (Mauro et al., 1989; Saldanha et al., 1994; Chaiseha et al., 1998). There are indications that VIP has a central inhibitory influence on GnRH/LH release (Pitts et al., 1994). These findings, taken together with the present study results in the native Thai chicken, suggest that the reduced expression of VIP to the pre-laying levels observed following termination of incubation in continuously breeding native Thai chickens may allow for the resumption of reproductive activity and breeding following the hatching of chicks. However, caring for the chicks appears to override the facilitator effect of this low VIPergic activity, preventing it from starting a new reproductive cycle.

It is further suggested that the VIP neural group in the IH-IN/INF may play a pivotal role in regulating the reproductive cycle in avian species and its differential expression following hatching of the young may, in part, explain the difference in reproductive strategies between equatorial, non-photoperiodic, continually breeding native Thai chickens (birds) and photoperiodic, seasonally breeding turkeys (birds). The importance of the central (IH-IN/INF) VIPergic system in the regulation of the reproductive cycle, independent of circulating PRL, may be supported by the results of VIP immunoneutralization studies in seasonally breeding birds (El Halawani et al., 1995; 1996; Dawson and Sharp, 1998; Ahn et al., 2001). In these studies, VIP immunoneutralization prevents the rise in circulating PRL that follows photostimulation and the induction of incubation behavior, up-regulates LHβ- and FSHβ-subunit mRNA, and extends the duration of reproductive activity, but does not prevent

spontaneous gonad regression, molting or the onset of photorefractoriness. These functional changes in the reproductive neuroendocrine system in response to immunization against VIP appear to be related to the immunoneutralization of peripherally released VIP acting at the pituitary level without affecting the up-regulated hypothalamic VIP expression associated with photorefractoriness and the termination of reproductive activity.

In contrast to the sharp decline in circulating PRL levels, as well as in the number of VIP-ir neurons, in incubating native Thai chickens following termination of incubation and hatching of eggs, the immediate decline in circulating PRL that occurs in incubating turkey hens following hatching of the young/introduction of poults and the transition to photorefractoriness is not accompanied by a corresponding decline in the number of VIP-ir neurons and VIPergic activity (Mauro et al., 1989; 1992; Chaiseha and El Halawani, 1999; present study), suggesting an alternate mechanism(s) is responsible for the observed decline in PRL secretion and termination of incubation behavior. In the turkey, it has been shown that tonic stimulation of PRL by VIP can be overridden by activation of the DAergic system acting directly at the pituitary level via D_2 DA receptors (Xu et al, 1996; Youngren et al., 1998). These receptors are found to be up-regulated during the photorefractory state of the reproductive cycle (Chaiseha et al., 2003). It is suggested that the DAergic system and its regulation of PRL, in addition to VIP, may be a component of a neuroendocrine mechanism(s) underlying the differential regulation of the reproductive cycles between the native Thai chicken (equatorial non-photoperiodic) and the turkey (seasonally photoperiodic). Whether the increased VIP expression at the termination of incubation in the turkey plays a regulatory role in maintaining the state of photorefractoriness and reproductive inactivity in seasonally breeding temperate zone birds remains an open question. It is of interest to note that the termination of photorefractoriness and the acquisition of photosensitivity by short day treatment in the turkey are associated with a significant decline in VIP expression.

In summary, the present findings, for the first time, characterize the VIP/PRL system and its relationship to reproduction in the native Thai chicken, which is a continuous breeding species that does not exhibit photoperiodic cycles. VIP-ir neurons and fibers are extensively distributed throughout the brain. Changes in the number of VIP-ir cells within the IH-IN areas were observed and directly correlated with concentrations of circulating PRL, suggesting that hypothalamic VIP expression in the IH-IN of the non-photoperiodic native Thai chicken plays a regulatory role in year-round reproductive activity. In addition, the abundance of VIP neuronal networks in the native Thai chicken hypothalamus suggests its importance in the regulation of reproductive activities in this equatorial bird.

Acknowledgements

The authors would like to acknowledge the financial support from the Thailand Research Fund (TRF) through the Royal Golden Jubilee (RGJ) Ph.D. program (Grant No. PHD/0226/2544 to SK and YC), the TRF (Grant No. RSA4780001 to YC), and the Research and Development Grant of Suranaree University of Technology to YC. The authors are grateful to University Farm of Suranaree University of Technology for providing the chicken barns.

References

Ahn, J., You, S.K., Kim, H., Chaiseha, Y., El Halawani, M.E., 2001. Effects of active immunization with inhibin alpha subunit on reproductive characteristics of turkey hens. Biol. Reprod. 65, 1594-1600.

Al-Zailaie, K.A., Kang, S.W., Youngren, O.M., Thayananuphat, A., Bakken, T., Chaiseha, Y., Millam, J.R., Proudman, J.A., El Halawani, M.E., 2006. Identification of dopamine, gonadotrophin-releasing hormone-I, and vasoactive intestinal peptide neurons activated by electrical stimulation to the medial preoptic area of the turkey hypothalamus: A potential reproductive neuroendocrine circuit. J. Neuroendocrinol. 18, 514-525.

Bern, H.A., Nicoll, C.S., 1968. The comparative endocrinology of prolactin. Recent Prog. Horm. Res. 24, 681-720.

Bottjer, S.W., Alexander, G., 1995. Localization of met-enkephalin and vasoactive intestinal polypeptide in the brains of male zebra finches. Brain Behav. Evol. 45, 153-177.

Chaiseha, Y., El Halawani, M.E., 1999. Expression of vasoactive intestinal peptide/peptide histidine isoleucine in several hypothalamic areas during the turkey reproductive cycle: Relationship to prolactin secretion. Neuroendocrinology 70, 402-412.

Chaiseha, Y., El Halawani, M.E., 2005. Neuroendocrinology of the female turkey reproductive cycle. J. Poult. Sci. 42, 87-100.

Chaiseha, Y., Tong, Z., Youngren, O.M., El Halawani, M.E., 1998. Transcriptional changes in hypothalamic vasoactive intestinal peptide during a photo-induced reproductive cycle in the turkey. J. Mol. Endocrinol. 21, 267-275.

Chaiseha, Y., Youngren, O.M., Al-Zailaie, K., El Halawani, M.E., 2003. Expression of D_1 and D_2 dopamine receptors in the hypothalamus and pituitary during the turkey reproductive cycle: Colocalization with vasoactive intestinal peptide. Neuroendocrinology 77, 105-118.

Cloues, R., Ramos, C., Silver, R., 1990. Vasoactive intestinal polypeptide-like immunoreactivity during reproduction in doves: Influence of experience and number of offspring. Horm. Behav. 24, 215-231.

Dawson, A., Sharp, P.J., 1998. The role of prolactin in the development of reproductive photorefractoriness and postnuptial molt in the European starling (*Sturnus vulgaris*). Endocrinol. 139, 485-490.

El Halawani, M.E., Burke, W.H., Dennison, P.T., 1980. Effect of nest-deprivation on serum prolactin level in nesting female turkeys. Biol. Reprod. 23, 118-123.

El Halawani, M.E., Burke, W.H., Millam, J.R., Fehrer, S.C., Hargis, B.M., 1984. Regulation of prolactin and its role in gallinaceous bird reproduction. J. Exp. Zool. 232, 521-529.

El Halawani, M.E., Fehrer, S.C., Hargis, B.M., Porter, T.E., 1988. Incubation behavior in the domestic turkey: Physiological correlates. CRC. Crit. Rev. Poult. Biol. 1, 285-314.

El Halawani, M.E., Pitts, G.R., Sun, S., Silsby, J.L., Sivanandan, V., 1996. Active immunization against vasoactive intestinal peptide prevents photo-induced PRL secretion in turkeys. Gen. Comp. Endocrinol. 104, 76-83.

El Halawani, M.E., Silsby, J.L., Behnke, E.J., Fehrer, S.C., 1986. Hormonal induction of incubation behavior in ovariectomized female turkeys (*Meleagris gallopavo*). Biol. Reprod. 35, 59-67.

El Halawani, M.E., Silsby, J.L., Fehrer, S.C., Behnke, E.J., 1983. Effects of estrogen and progesterone on serum prolactin and luteinizing hormone levels in ovariectomized turkeys (*Meleagris gallopavo*). Gen. Comp. Endocrinol. 52, 67-78.

El Halawani, M.E., Silsby, J.L., Rozenboim, I., Pitts, G.R., 1995. Increased egg production by active immunization against vasoactive intestinal peptide in the turkey (*Meleagris gallopavo*). Biol. Reprod. 52, 179-183.

El Halawani, M.E., Youngren, O.M., Pitts, G.R., 1997. Vasoactive intestinal peptide as the avian prolactin releasing factor. In: Harvey, S., Etches, R.J. (Eds.), Perspectives in Avian Endocrinology, Journal of Endocrinology Ltd, Bristol, pp. 403-416.

Follett, B.K., 1984. Reproductive cycles of vertebrates. In: Lamming, G.E. (Ed.), Marshall's Physiology of Reproduction. Churchill Livingstone, New York, pp. 283-350.

Hahn, T.P., Boswell, T., Wingfield, J.C., Ball, G.F., 1997. Temporal flexibility in avian reproduction: Patterns and mechanisms. In: Nolan, V., Ketterson, E.D., Thompson, C.F. (Eds.), Current Ornithology. Plenum Press, New York, pp. 39-80.

Korf, H.W., Fahrenkrug, J., 1984. Ependymal and neuronal specializations in the lateral ventricle of the Pekin duck, *Anas platyrhynchos*. Cell Tissue Res. 236, 217-227.

Kragt, C.L., Meites, J., 1965. Stimulation of pigeon pituitary prolactin release by pigeon hypothalamic extract *in vitro*. Endocrinology 76, 1169-1176.

Kuenzel, W.J., 2002. Chicken Brain Atlas. [On line]. Available: *http://avianbrain.org/nomen/Chicken_Atlas.html.*

Kuenzel, W.J., Blahser, S., 1994. Vasoactive intestinal peptide (VIP)-containing neurons: Distribution throughout the brain of the chick (*Gallus domesticus*) with focus upon the lateral septal organ. Cell Tissue Res. 275, 91-107.

Kuenzel, W.J., Masson, M., 1988. A stereotaxic atlas of the brain of chick (*Gallus domesticus*). Johns Hopkins University Press, Baltimore.

Kuenzel, W.J., Mccune, S.K., Talbot, R.T., Sharp, P.J., Hill, J.M., 1997. Sites of gene expression for vasoactive intestinal polypeptide throughout the brain of the chick (*Gallus domesticus*). J. Comp. Neurol. 381, 101-118.

Kuenzel, W.J., van Tienhoven, A., 1982. Nomenclature and location of avian hypothalamic nuclei and associated circumventricular organs. J. Comp. Neurol. 206, 293-313.

Lea, R.W., Dods, A.S.M., Sharp, P.J., Chadwick, A., 1981. The possible role of prolactin in the regulation of nesting behavior and the secretion of luteinizing hormone in broody bantams. J. Endocrinol. 91, 89-97.

Macnamee, M.C., Sharp, P.J., Lea, R.W., Sterling, R.J., Harvey, S., 1986. Evidence that vasoactive intestinal polypeptide is a physiological prolactin-releasing factor in the bantam hen. Gen. Comp. Endocrinol. 62, 470-478.

Mauro, L.J., Elde, R.P., Youngren, O.M., Phillips, R.E., El Halawani, M.E., 1989. Alterations in hypothalamic vasoactive intestinal peptide-like immunoreactivity are associated with reproduction and prolactin release in the female turkey. Endocrinology 125, 1795-1804.

Mauro, L.J., Youngren, O.M., Proudman, J.A., Phillips, R.E., El Halawani, M.E., 1992. Effects of reproductive status, ovariectomy, and photoperiod on vasoactive intestinal peptide in the female turkey hypothalamus. Gen. Comp. Endocrinol. 87, 481-493.

Norgren, R.B., Silver, R., 1990. Distribution of vasoactive intestinal peptide-like and neurophysin-like immunoreactive neurons and acetylcholinesterase staining in the ring dove hypothalamus with emphasis on the question of an avian suprachiasmatic nucleus. Cell Tissue Res. 259, 331-339.

Opel, H., Proudman, J.A., 1988. Effects of poults on plasma concentrations of prolactin in turkey hens incubating without eggs or a nest. Br. Poult. Sci. 29, 791-800.

Opel, H., Proudman, J.A., 1989. Plasma prolactin levels in incubating turkey hens during pipping of the eggs and after introduction of poults into the nest. Biol. Reprod. 40, 981-987.

Peczely, P., Kiss, J.Z., 1988. Immunoreactivity to vasoactive intestinal polypeptide (VIP) and thyreotropin-releasing hormone (TRH) in hypothalamic neurons of the domesticated pigeon (*Columba livia*). Alterations following lactation and exposure to cold. Cell Tissue Res. 251, 485-494.

Pitts, G.R., Youngren, O.M., Silsby, J.L., Rozenboim, I., Chaiseha, Y., Phillips, R.E., Foster, D.N., El Halawani, M.E., 1994. Role of vasoactive intestinal peptide in the control of prolactin-induced turkey incubation behavior. II. Chronic infusion of vasoactive intestinal peptide. Biol. Reprod. 50, 1350-1356.

Proudman, J.A., Siopes, T.D., Vandesande, F., Berghman, L.R., 2001. Thyroid hormone and prolactin profiles in male and female turkeys following photostimulation: Validation of an ELISA for turkey prolactin. Poult. Sci. 80, 469.
Richard-Yris, M.A., Leboucher, G., 1987. Effects of exposure to chicks on maternal behavior in domestic chickens. Bird Behav. 7, 31-36.
Richard-Yris, M.A., Leboucher, G., Chadwick, A., Garnier, D.H., 1987. Induction of maternal behavior in incubating and non-incubating hens: Influence of hormones. Physiol. Behav. 40, 193-199.
Saldanha, C.J., Deviche, P.J., Silver, R., 1994. Increased VIP and decreased GnRH expression in photorefractory dark-eyed juncos (*Junco hyemalis*). Gen. Comp. Endocrinol. 93, 128-136.
Sharp, P.J., Dawson, A., Lea, R.W., 1998. Control of luteinizing hormone and prolactin secretion in birds. Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol. 119, 275-282.
Sharp, P.J., Macnamee, M.C., Sterling, R.J., Lea, R.W., Pedersen, H.C., 1988. Relationships between prolactin, LH and broody behaviour in bantam hens. J. Endocrinol. 118, 279-286.
Sharp, P.J., Sterling, R.J., Talbot, R.T., Huskisson, N.S., 1989. The role of hypothalamic vasoactive intestinal polypeptide in the maintenance of prolactin secretion in incubating bantam hens: Observations using passive immunization, radioimmunoassay and immunohistochemistry. J. Endocrinol. 122, 5-13.
Wood-Gush, D.G., Gilbert, A.B., 1973. Some hormones involved in the nesting behavior of hens. Anim. Behav. 21, 98-103.
Xu, M., Proudman, J.A., Pitts, G.R., Wong, E.A., Foster, D.N., El Halawani, M.E., 1996. Vasoactive intestinal peptide stimulates prolactin mRNA expression in turkey pituitary cells: Effects of dopaminergic drugs. Proc. Soc. Exp. Biol. Med. 212, 52-62.
Yamada, S., Mikami, S., Yanaihara, N., 1982. Immunohistochemical localization of vasoactive intestinal polypeptide (VIP)-containing neurons in the hypothalamus of the Japanese quail, *Coturnix coturnix*. Cell Tissue Res. 226, 13-26.
Youngren, O.M., Chaiseha, Y., El Halawani, M.E., 1998. Regulation of prolactin secretion by dopamine and vasoactive intestinal peptide at the level of the pituitary in the turkey. Neuroendocrinology 68, 319-325.
Youngren, O.M., El Halawani, M.E., Silsby, J.L., Phillips, R.E., 1991. Intracranial prolactin perfusion induces incubation behavior in turkey hens. Biol. Reprod. 44, 425-431.
Zadworny, D., Etches, R.J., 1987. Effects of ovariectomy or force feeding on the plasma concentrations of prolactin and luteinizing hormone in incubating turkey hens. Biol. Reprod. 36, 81-88.
Zadworny, D., Shimada, K., Ishida, H., Sumi, C., Sato, K., 1988. Changes in plasma levels of prolactin and estradiol, nutrient intake, and time spent nesting during the incubation phase of broodiness in the Chabo hen (*Japanese bantam*). Gen. Comp. Endocrinol. 71, 406-412.

Figure legends

Fig. 1. Changes in plasma PRL concentrations across the reproductive cycle of the native Thai chicken. Values are presented as the mean±SEM (n=10). Significant differences between means are denoted by different letters ($P<0.05$).

Fig. 2. Changes in the number of VIP-ir neurons in the IH-IN of the native Thai chicken (A) at different reproductive stages (NL, LAY, INC, and R). Changes in the number of VIP-ir neurons in the INF of the turkey hypothalamus (B) at different reproductive stages (NPS,

LAY, INC, and REF). Values are presented as the mean±SEM (n=6). Significant differences between means are denoted by different letters ($P<0.05$).

Fig. 3. Photomicrographs of coronal sections of the native Thai chicken hypothalamus showing the distribution of VIP-ir cells and fibers within the IH-IN of the native Thai chicken (A) at different reproductive stages (NL, LAY, INC, and R). Photomicrographs of coronal sections of the turkey hypothalamus showing the distribution of VIP-ir cells and fibers within the INF of the turkey (B) at different reproductive stages (NPS, LAY, INC, and REF). Bar 100 μm. The following abbreviations are used in the figure legends and in the text:

Ac	Nucleus accumbens
AM	Nucleus anterior medialis hypothalami
AVT	Area ventralis
Cb	Cerebellum
CHCS	Tractus cortico-habenularis et cortico-septalis
GCt	Substantia grisea centralis
ICo	Nucleus intercollicularis
IH	Nucleus inferioris hypothalami
IN	Nucleus infundibuli hypothalami
INF	Infundibular nuclear complex
IP	Nucleus interpeduncularis
LC	Nucleus linearis caudalis
LHy	Regio lateralis hypothalami (Lateral hypothalamic area)
LoC	Locus ceruleus
LPO	Lobus parolfactorius
LSO	Organum septi laterale (Lateral septal organ)
LSOm	Organum septi laterale, pars medialis
ME	Eminentia mediana (Median eminence)
nCPa	Nucleus commissurae pallii
PBv	Nucleus parabrachialis, pars ventralis
PHN	Nucleus periventricularis hypothalami
Pit	Pituitary
PVN	Nucleus paraventricularis magnocellularis
ROT	Nucleus rotundus
Rpgl	Nucleus reticularis paragiganto-cellularis lateralis
S	Nucleus tractus solitarii
SCv	Nucleus subceruleus ventralis
SL	Nucleus septalis lateralis
SM	Nucleus septalis medialis
Tn	Nucleus taeniae
TSM	Tractus septomesencephalicus
TPc	Nucleus tegmenti pedunculo-pontinus, pars compacta (Substantia nigra)
V III	Ventriculus tertius (Third ventricle)
VL	Ventriculus lateralis
VMN	Nucleus ventromedialis hypothalami

Fig. 4. Changes in the ovary (A) and oviduct (B) weight of rearing and chick-removed native Thai hens. Values are presented as the mean±SEM (n=5). Significant differences between means in each group at different time points are denoted by different letters ($P<0.05$) and *$P<0.05$ for a comparison between groups at a given time point.

Fig. 5. Schematic diagrams of coronal sections from rostral to caudal (A-F) showing the distribution of VIP-ir cells (black dots) throughout the brain of the laying native Thai chicken. Coronal illustrations were redrawn from the chicken brain atlas (Kuenzel, 2002) with nomenclature taken from Kuenzel and Masson (1988). The number in the upper right hand corner shows the anterior distance in mm from the zero coordinates given in the stereotaxic atlas of the chick brain. For abbreviations, see Fig. 3.

Fig. 6. Photomicrographs of coronal sections in the septal area of the laying native Thai chicken brain demonstrating the distribution of CSF-contacting neurons located in the LSOm (A), while the SL contains a dense plexus of VIP-ir fibers and a few VIP-ir cells (B). *Bar* 50 μm. For abbreviations, see Fig. 3.

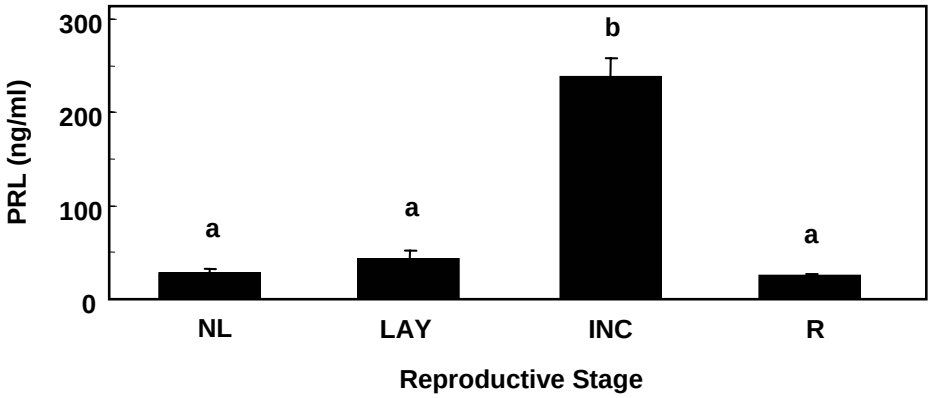
Fig. 7. Photomicrographs of coronal sections within the IH-IN illustrating numerous VIP-ir cells in the IH-IN area and a dense accumulation of VIP-ir fibers in the ME of the laying native Thai chicken brain (A; *bar* 100 μm). Higher magnification of the VIP-ir neurons was demonstrated in the IH-IN area (B). Enlargement of a dense arrangement of VIP nerve terminals in the external layer of the ME (C). *Bar* 50 μm. For abbreviations, see Fig. 3.

Fig. 8. Photomicrographs of coronal sections in the hypothalamus and surrounding areas of the laying native Thai chicken brain demonstrating the cluster of VIP-ir cells with coarse-grained granules in the cytoplasm and neurites in the ROT (A; *bar* 50 μm). Higher magnification of VIP-ir cells in the ROT (B; *bar* 20 μm). Scattered VIP-ir cells located in the AM and LHy (C, D). VIP-ir fibers in the PHN and some fibers oriented parallel to the third ventricle (E). VIP-ir fibers found in nCPa (F). *Bar* 50 μm. For abbreviations, see Fig. 3.

Fig. 9. Photomicrographs of coronal sections demonstrating the distribution of VIP-ir cells in the mesencephalon of the laying native Thai chicken brain. The specific binding of VIP antibody was observed within the GCt (A), AVT (B), and TPc (C). Higher magnification of VIP-ir cells from Fig. 9C (D). *Bar* 50 μm. For abbreviations, see Fig. 3.

Fig. 10. Photomicrographs of coronal sections of the laying native Thai chicken brain demonstrating VIP-ir cells lining the cortex layer of the Cb (A), whereas no immunostaining was observed in the pituitary (B). *Bar* 200 μm. Insert: higher magnification of VIP-ir cells in the Cb. *Bar* 20 μm. For abbreviations, see Fig. 3.

Fig. 1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b
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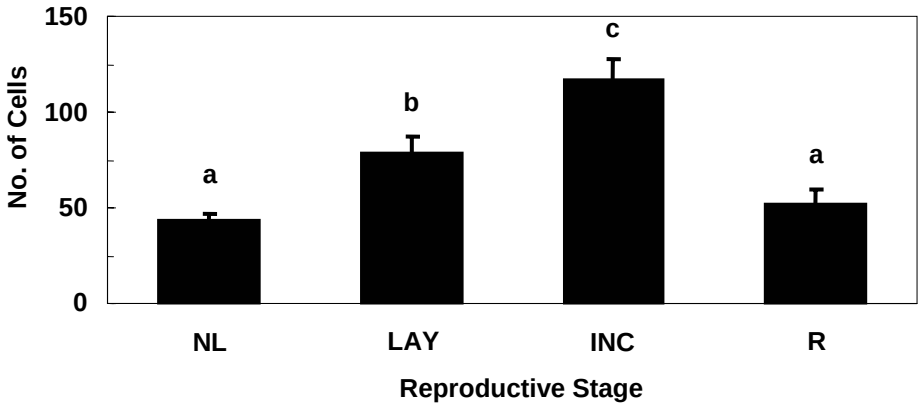
NL
LAY
INC
R
Reproductive Stage

Fig. 2

A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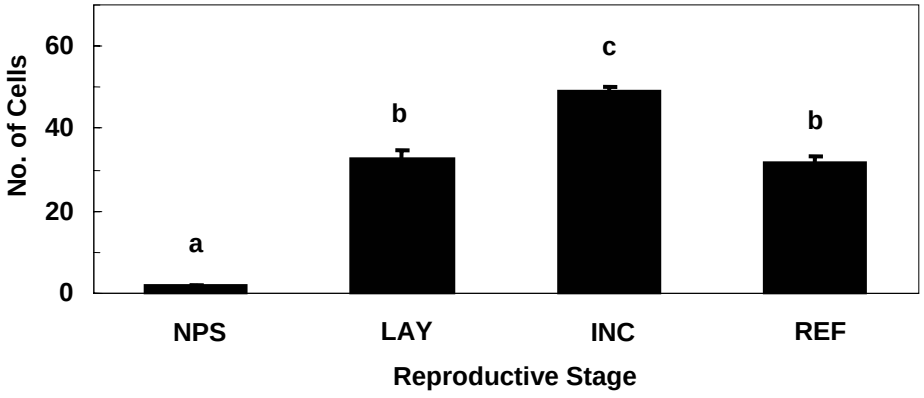
a
b
c
a
NL
LAY
INC
R
Reproductive Stage

B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b
c
b
NPS
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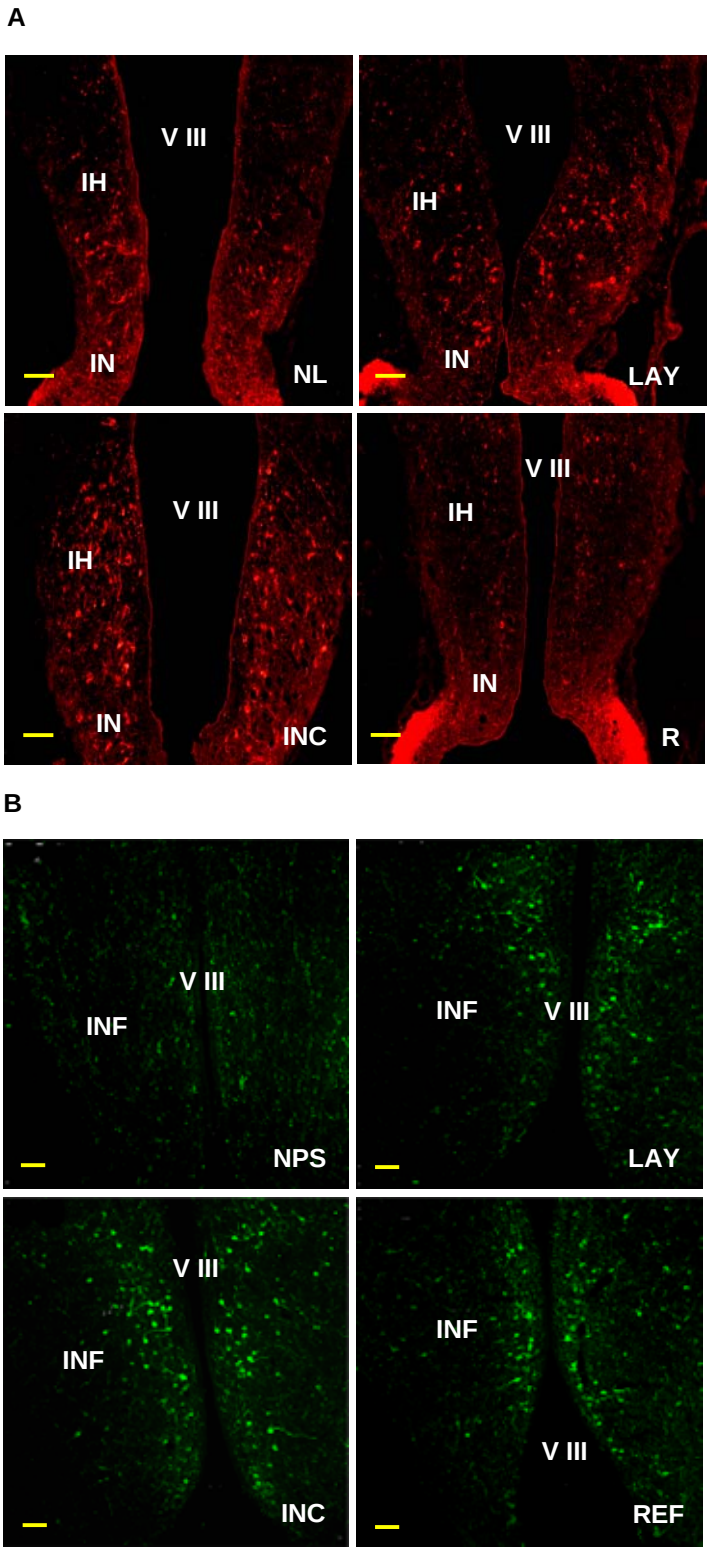
INC
REF
Reproductive Stage

Fig. 3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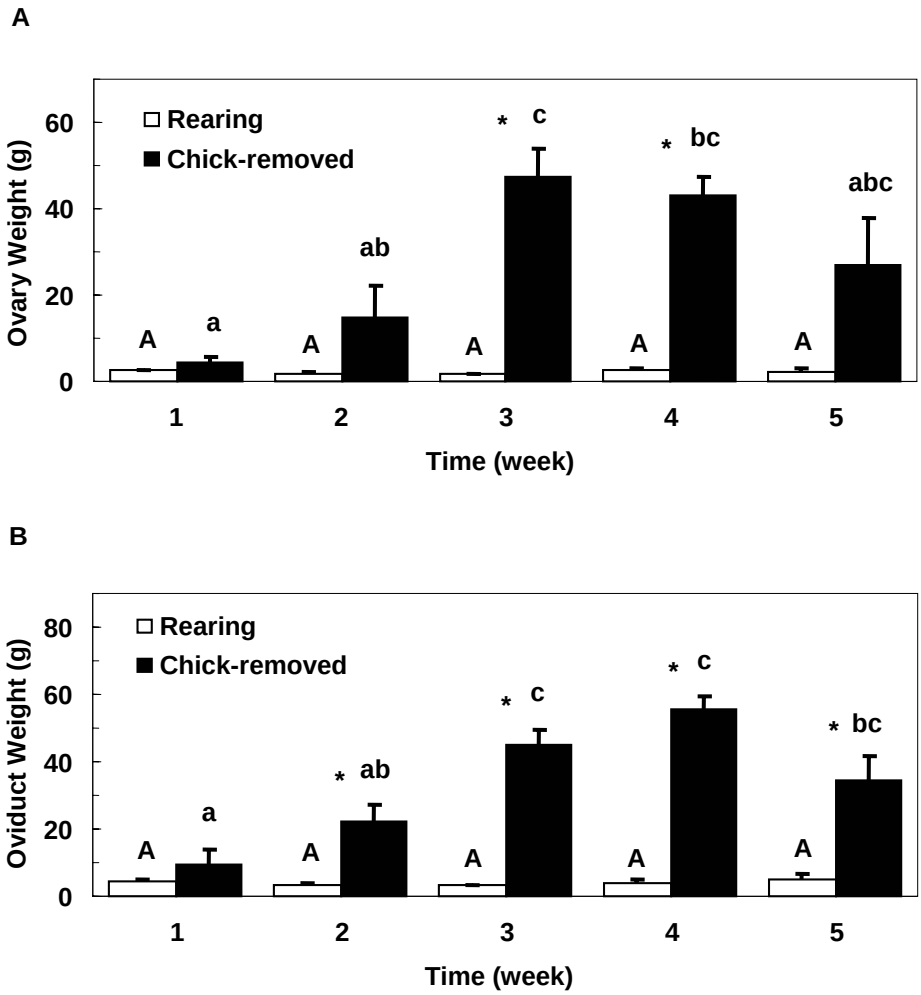
REF

Fig. 4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Chick-removed
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A
abc
1
2
3
4
5
Time (week)
B
Oviduct Weight (g)
Rearing
Chick-removed
0
20
40
60
80
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1
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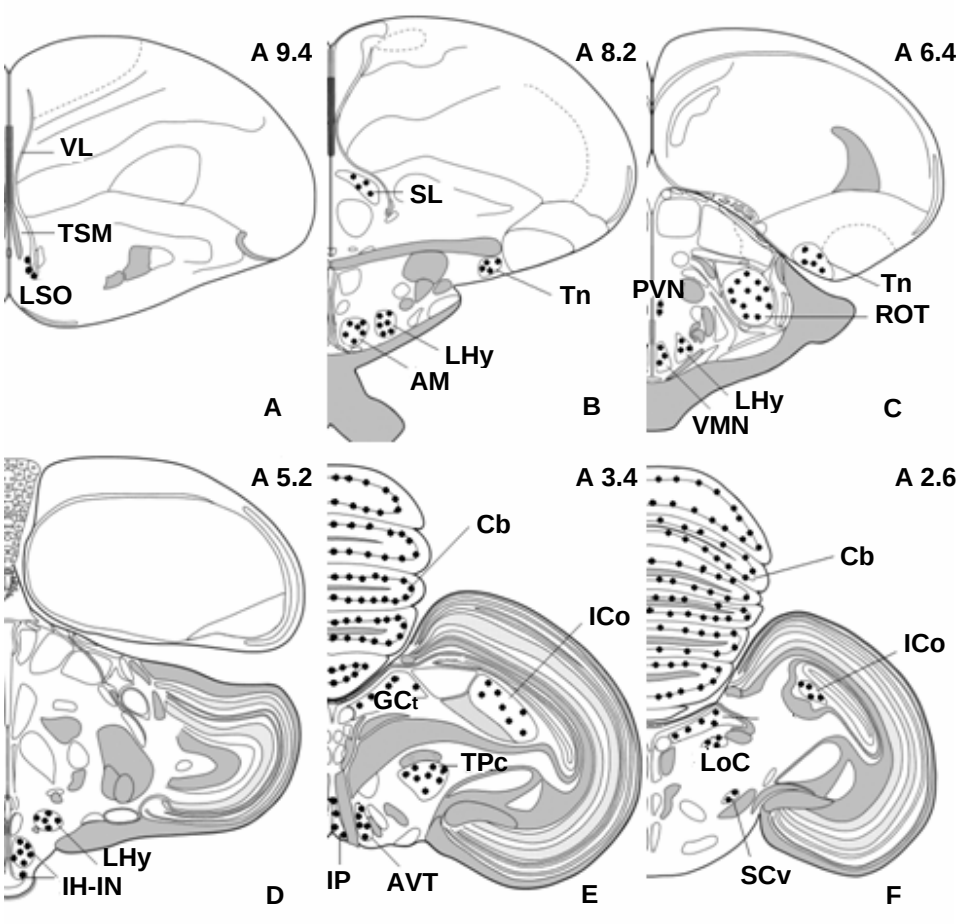
5
Time (week)

Fig. 5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VMN
C
A 5.2
LHy
IH-IN
D
A 3.4
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AVT
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A 2.6
Cb
ICo
LoC
SCv
F

Fig. 6

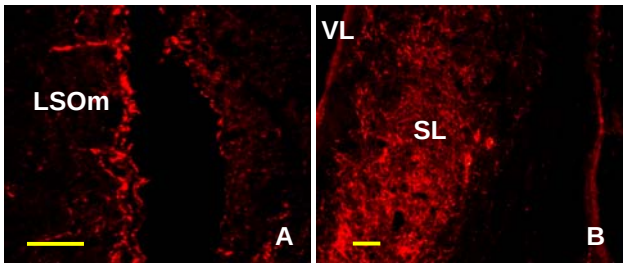


Fig. 7

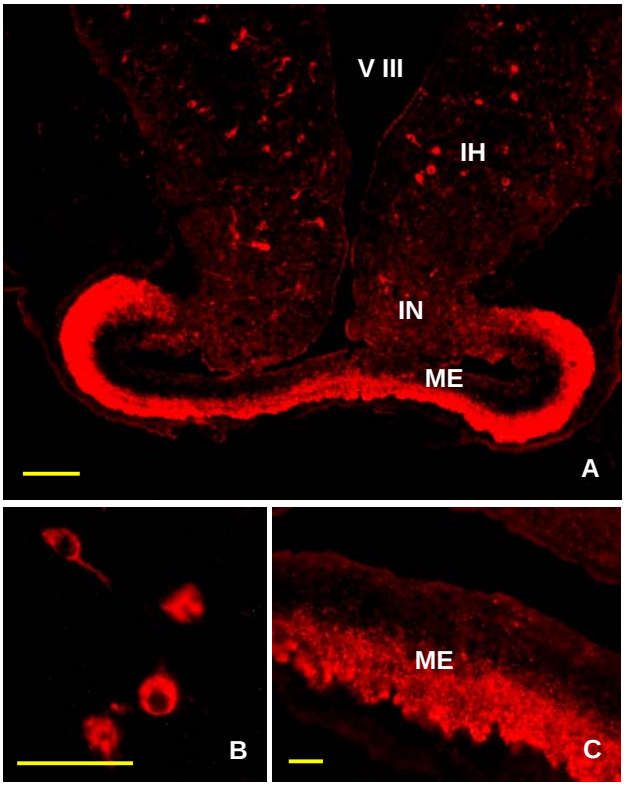


Fig. 8

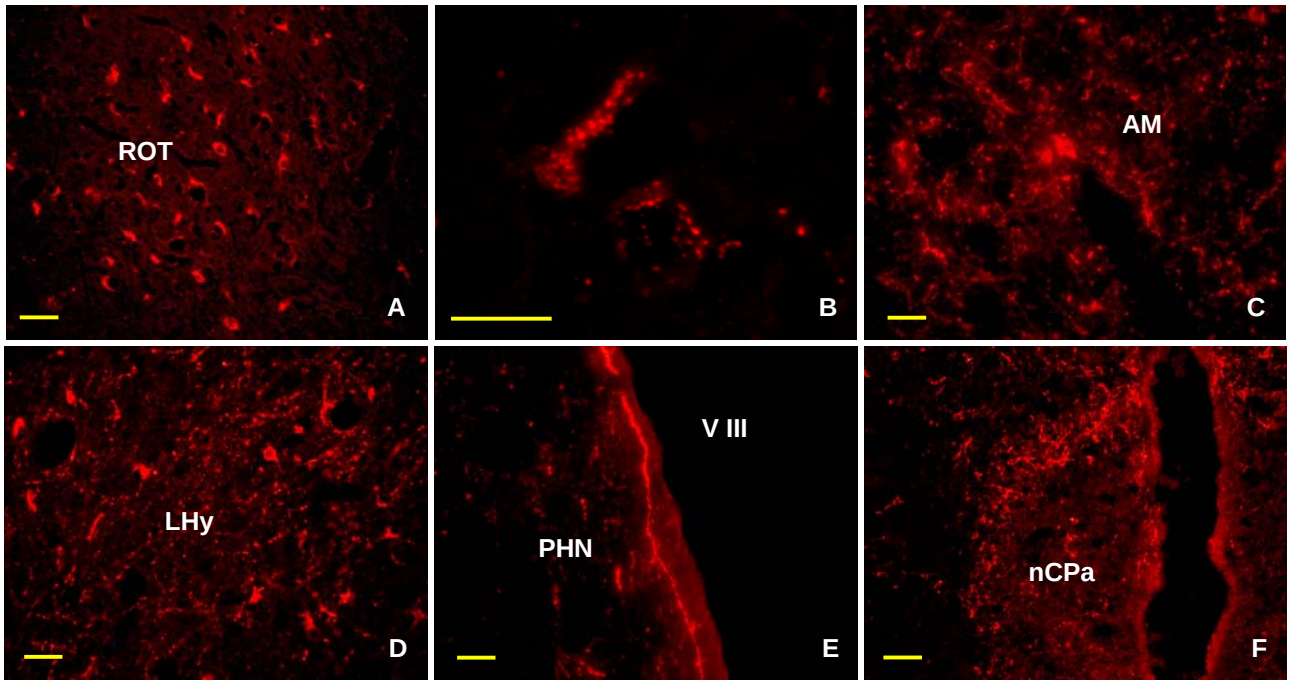


Fig. 9

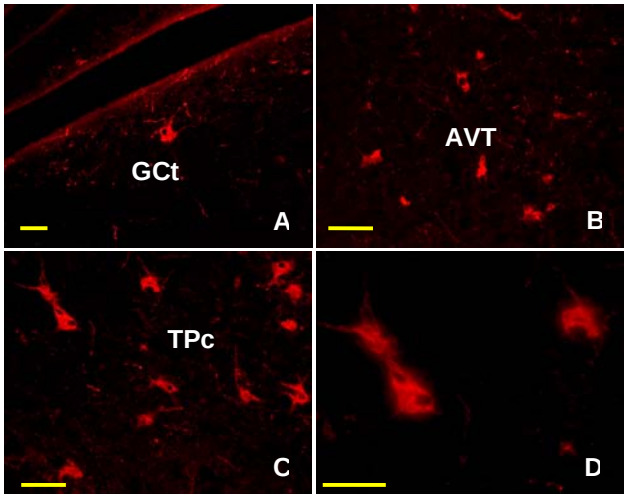
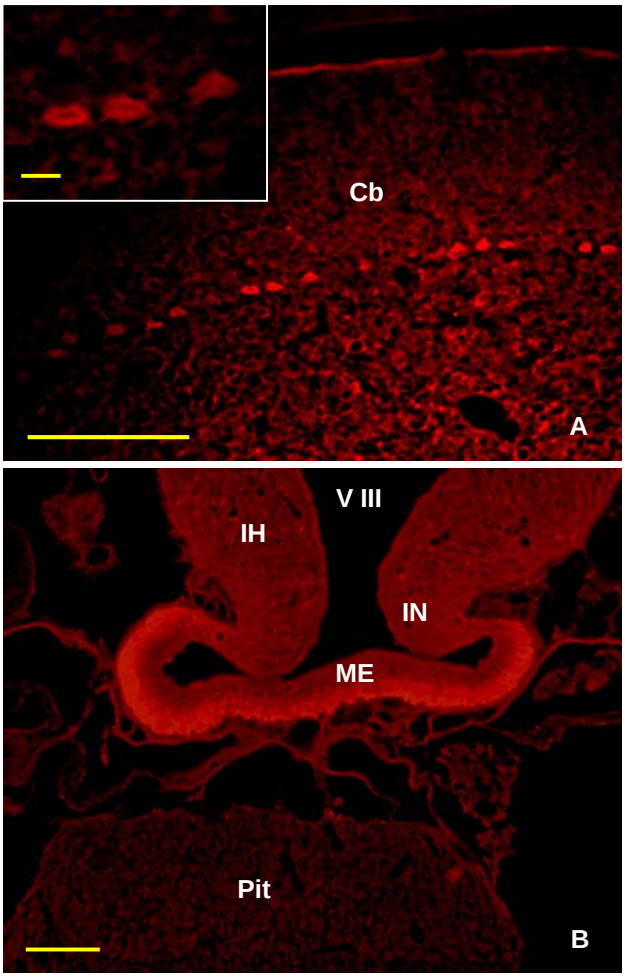


Fig. 10



4. Copy of the manuscript #3:

Sartsoongnoen N, Kosonsiriluk S, Prakobsaeng N, Songserm T, El Halawani ME, **Chaiseha Y** (2007). The dopaminergic system in the brain of the native Thai chicken, *Gallus domesticus*: Localization and differential expression across the reproductive cycle. **General and Comparative Endocrinology** (Submitted; **Impact factor** = 2.487).

The Dopaminergic System in the Brain of the Native Thai Chicken, *Gallus domesticus*: Localization and Differential Expression across the Reproductive Cycle

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Running Title: Dopamine and Reproduction in the Native Thai Chicken

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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 level. The population of Th-ir neurons in 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.

Key words: Dopamine; Immunocytochemistry; Native Thai chicken; Reproductive Cycle

1. 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 DAergic 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 DAergic 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 Meities, 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 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 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, 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).

In birds, it has been shown that DAergic activity and receptor mRNA expression are changed according to 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, 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 DAergic 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). DAergic 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 DAergic 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., 2000), collared dove (den Boer-Visser and Dubbeldam, 2002), turkey (Al-Zailaie and El Halawani, 2000), and canary (Appeltants et al., 2001). In birds, DAergic neurons are widely dispersed throughout the forebrain, midbrain, and hindbrain. DAergic 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, 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 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 DAergic 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 DAergic neurons throughout its brain utilizing tyrosine hydroxylase (TH, the rate limiting enzyme for DA synthesis) antibody as a marker for DAergic 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 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.

2. Materials and Methods

2.1. 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 immunoreactive (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.

2.2. Prolactin Hormone Assay

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). Plasma PRL was determined in duplicate within a single assay.

2.3. 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 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.

2.4. 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, nucleus intramedialis (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).

2.5. 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 DAergic system across the reproductive cycle four adjacent brain sections (eight microscopic fields) 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.

2.6. 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).

3. Results

3.1. Changes in number of TH-ir neurons in the hypothalamus of the native Thai chicken across the reproductive cycle

Plasma PRL levels were determined across the reproductive cycle of the native Thai chicken (n = 5, Fig. 1A). Plasma PRL levels were low in NL (23.33±2.42 ng/ml), gradually augmented in L (45.15±8.39 ng/ml), significantly higher in B (240.13±35.10 ng/ml, $P<0.05$), and declined dramatically to the same level of NL in R (27.63±2.17 ng/ml).

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 1). 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. 1B and 2). The number of TH-ir neurons was low in NL (31.60±2.43 cells) and slightly increased in L (38.10±3.57 cells). When the hens began to incubate eggs, the number of TH-ir neurons markedly increased to the highest level (48.70±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 L to R (35.00±2.14 cells). A high density of TH-ir neurons was also observed in the AM (Table 1). 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± 5.29 vs R = 41.50±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 1, Fig. 3).

3.2. Distribution of TH-ir neurons and fibers throughout the brain of the native Thai chicken

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. 4).

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. 5A). 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. 5B) and the organum septi laterale (lateral septum organ; LSO).

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. 6A) and nucleus suprachiasmaticus, pars medialis (SCNm, Fig. 6B). 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. 6C and E). 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. 6D and F). 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. 6G). 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. 7A and B). 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. 7C and D). 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. 7E). In the dorsal zone of the hypothalamus, TH-ir neurons were noted within the substantia grisea centralis (GCt, Fig. 7F). 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).

Mesencephalon

Dense, heavily-stained TH-ir neurons and fibers were found in the area ventralis (AVT, Fig. 8A), 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. 8B) 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).

Rhombencephalon

Diffuse TH-ir neurons and fibers were found in the locus ceruleus (LoC, Fig. 8C) 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. 8D) but the intensity of the TH immunoreactivity was markedly low.

4. 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 VIII. 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, 2008). 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 McCarlson, 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 DAergic 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 DAergic neurons located in the PVN and ML might be possibly influencing gonadal maturation (Kuenzel, 2002). 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 DAergic 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 (Frakey 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 DAergic 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 Pickut, 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 DAergic 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 DAergic 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; Moon 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 DAergic 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 DAergic 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 Balthazrt, 1993). However, the distribution of DA-ir neurons has been reported in the AVT, TPc, LoC, BCA, VeM and SCv of the chicken (Moon et al., 1994). Thus, it might be possible that TH immunoreactivity observed in these areas of the native Thai chicken could be DAergic 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 DAergic 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 occuring in VIP release and ensuing PRL secretion.

Acknowledgements

The authors would like to acknowledge the financial support from the Thailand Research Fund (TRF) through the Royal Golden Jubilee (RGJ) Ph.D. program (Grant No. PHD/0226/2545 to NS and YC), the TRF (Grant No. RSA4780001 to YC). The authors are grateful to University Farm of Suranaree University of Technology for providing the chicken barns.

References

Absil, P., Foidart, A., Hemmings, H.C., Jr., Steinbusch, H.W., Ball, G.F., Balthazart, J., 2001. Distribution of DARPP-32 immunoreactive structures in the quail brain: Anatomical relationship with dopamine and aromatase. J. Chem. Neuroanat. 21, 23-39.

Al Kahtane, A., Chaiseha, Y., El Halawani, M.E., 2003. Dopaminergic regulation of avian prolactin gene transcription. J. Mol. Endocrinol. 31, 185-196.

Al-Zailaie, K.A., 2003. Neuroanatomical relationship between hypothalamic dopamine and vasoactive intestinal peptide in the regulation of PRL: Immunocytochemical and tract-tracing studies. PhD Thesis, University of Minnesota.

Al-Zailaie, K.A., El Halawani, M.E., 2000. Neuroanatomical relationship between immunoreactive dopamine and vasoactive intestinal peptide neurons in the turkey hypothalamus. Poult. Sci. 79 (suppl 1), 50.

Al-Zailaie, K.A., Kang, S.W., Youngren, O.M., Thayananuphat, A., Bakken, T., Chaiseha, Y., Millam, J.R., Proudman, J.A., El Halawani, M.E., 2006. Identification of dopamine, gonadotrophin-releasing hormone-I, and vasoactive intestinal peptide neurons activated by electrical stimulation to the medial preoptic area of the turkey hypothalamus: A potential reproductive neuroendocrine circuit. J. Neuroendocrinol. 18, 514-525.

Appeltants, D., Ball, G.F., Balthazart, J., 2001. The distribution of tyrosine hydroxylase in the canary brain: Demonstration of a specific and sexually dimorphic catecholaminergic innervation of the telencephalic song control nuclei. Cell Tissue Res. 304, 237-259.

Bailhache, T., Balthazart, J., 1993. The catecholaminergic system of the quail brain: Immunocytochemical studies of dopamine β-hydroxylase and tyrosine hydroxylase. J. Comp. Neurol. 329, 230-256.

Balthazart, J., Foidart, A., Baillien, M., Harada, N., Ball, G.F., 1998. Anatomical relationships between aromatase and tyrosine hydroxylase in the quail brain: Double-label immunocytochemical studies. J. Comp. Neurol. 391, 214-226.

Balthazart, J., Foidart, A., Sante, P., Hendrick, J.C., 1992. Effects of alpha-methyl-para-tyrosine on monoamine levels in the Japanese quail: Sex differences and testosterone effects. Brain Res. Bull. 28, 275-288.

Barclay, S.R., Harding, C.F., 1990. Differential modulation of monoamine levels and turnover rates by estrogen and/or androgen in hypothalamic and vocal control nuclei of male zebra finches. Brain Res. 523, 251-262.

Bhatt, R., Youngren, O.M., Kang, S.W., El Halawani, M.E., 2003. Dopamine infusion into the third ventricle increases gene expression of hypothalamic vasoactive intestinal peptide and pituitary prolactin and luteinizing hormone beta subunit in the turkey. Gen. Comp. Endocrinol. 130, 41-47.

Ben-Jonathan, N., Arbogast, L.A., Hyde, J.F., 1989. Neuroendocrine regulation of prolactin release. Prog. Neurobiol. 33, 399-447.

Ben-Jonathan, N., Hnasko, R., 2001. Dopamine as a prolactin (PRL) inhibitor. Endocr. Rev. 22, 724-763.

Bentivoglio, M., Morelli, M., 2005. The organization and circuits of mesencephalic dopaminergic neurons and the distribution of dopamine receptors in the brain. In: Bjorklund, A., Hokfelt, T. (Eds.), Handbook of chemical neuroanatomy. Elsevier, Amsterdam, pp. 1-108.

Berk, M.L., 1991. Distribution and hypothalamic projection of tyrosine-hydroxylase containing neurons of the nucleus of the solitary tract in the pigeon. J. Comp. Neurol. 312, 391-403.

Bern, H.A., Nicoll, C.S., 1968. The comparative endocrinology of prolactin. Recent Prog. Horm. Res. 24, 681-720.

Bharati, I.S., Goodson, J.L., 2006. Fos responses of dopamine neurons to sociosexual stimuli in male zebra finches. Neuroscience 143, 661-670.

Bottjer, S.W., 1993. The distribution of tyrosine hydroxylase immunoreactivity in the brains of male and female zebra finches. J. Neurobiol. 24, 51-69.

Breitenbach, R.P., Meyer, R.K., 1959. Pituitary prolactin levels in laying, incubating and brooding pheasants (*Phasianus colchicus*). Proc. Soc. Exp. Biol. Med. 101, 16-19.

Burke, W.H., Dennison, P.T., 1980. Prolactin and luteinizing hormone level in female turkeys (*Meleagris gallopavo*) during a photoinduced reproductive cycle and broodiness. Gen. Comp. Endocrinol. 41, 92-100.

Chadwick, A., Bolton, N.J., Hall, T.R., 1978. The effect of hypothalamic extract on prolactin secretion by chicken pituitary glands in vivo and in vitro. Gen. Comp. Endocrinol. 34, 70.

Chaiseha, Y., El Halawani, M.E., 1999. Expression of vasoactive intestinal peptide/peptide histidine isoleucine in several hypothalamic areas during the turkey reproductive cycle: Relationship to prolactin secretion. Neuroendocrinology 70, 402-412.

Chaiseha, Y., El Halawani, M.E., 2005. Neuroendocrinology of the female turkey reproductive cycle. J. Poult. Sci. 42, 87-100.

Chaiseha, Y., Tong, Z., Youngren, O.M., El Halawani, M.E., 1998. Transcriptional changes in hypothalamic vasoactive intestinal peptide during a photo-induced reproductive cycle in the turkey. J. Mol. Endocrinol. 21, 267-275.

Chaiseha, Y., Youngren, O.M., Al-Zailaie, K.A., El Halawani, M.E., 2003. Expression of D_1 and D_2 dopamine receptors in the hypothalamus and pituitary during the turkey reproductive cycle: Colocalization with vasoactive intestinal peptide. Neuroendocrinology 77, 105-118.

Chaiseha, Y., Youngren, O.M., El Halawani, M.E., 1997. Dopamine receptors influence vasoactive intestinal peptide release from turkey hypothalamic explants. Neuroendocrinology 65, 423-429.

Chaiseha, Y., Youngren, O.M., El Halawani, M.E., 2004. Expression of vasoactive intestinal peptide receptor messenger RNA in the hypothalamus and pituitary throughout the turkey reproductive cycle. Biol. Reprod. 70, 593-599.

Charlier, T.D., Ball, G.F., Balthazart, J., 2005. Sexual behavior activates the expression of the immediate early genes c-fos and Zenk (egr-1) in catecholaminergic neurons of male Japanese quail. Neuroscience 131, 13-30.

Civelli, O., Bunzow, J., Albert, P., Van Tol, H., Grandy, D., 1991. Molecular biology of the dopamine D_2 receptor. NIDA Res. Monogr. 111, 45-53.

Contijoch, A.M., Gonzalez, C., Singh, H.N., Malamed, S., Troncoso, S., Advis, J.P., 1992. Dopaminergic regulation of luteinizing hormone-releasing hormone release at the median eminence level: Immunocytochemical and physiological evidence in hens. Neuroendocrinology 55, 290-300.

Contreras, F., Fouillioux, C., Bolívar, A., Simonovis, N., Hernández-Hernández, R., Armas-Hernandez, M.J., Velasco, M., 2002. Dopamine, hypertension and obesity. J. Hum. Hypertens. 16, 13-17.

Divac, I., Thibault, J., Skageberg, G., Palacios, J.M., Dietl, M.M., 1994. Dopaminergic innervation of the brain in pigeons. The presumed 'prefrontal cortex'. Acta. Neurobiol. Exp. (Wars). 54, 227-234.

den Boer-Visser, A.M., Dubbeldam, J.L., 2002. The distribution of dopamine, substance P, vasoative intestinal polypeptide and neuropeptide Y immunoreactivity in the brain of the collared dove, *Streptopelia decaocto*. J. Chem. Neuroanat. 23, 1-27.

Dominguez, J.M., Hull, E.M., 2005. Dopamine, the medial preoptic area, and male sexual behavior. Physiol. Behav. 86, 356-368.

Durstewitz, D., Kröner, S., Hemmings, H.C., Jr., Güntürkün, O., 1998. The dopaminergic innervation of the pigeon telencephalon: Distribution of DARPP-32 and co-occurrence with glutamate decarboxylase and tyrosine hydroxylase. Neuroscience 83, 763-779.

El Halawani, M.E., Burke, W.H., Dennison, P.T., 1980. Effect of nest-deprivation on serum prolactin level in nesting female turkeys. Biol. Reprod. 23, 118-123.

El Halawani, M.E., Burke, W.H., Millam, J.R., Fehrer, S.C., Hargis, B.M., 1984. Regulation of prolactin and its role in gallinaceous bird reproduction. J. Exp. Zool. 232, 521-529.

El Halawani, M.E., Fehrer, S.C., Hargis, B.M., Porter, T.E., 1988. Incubation behavior in the domestic turkey: Physiological correlates. CRC Crit. Rev. Poult. Biol. 1, 285-314.

El Halawani, M.E., Youngren, O.M., Chaiseha, Y., 2000. Neuroendocrinology of PRL regulation in the domestic turkey: In Dawson, A., Chaturvedi, C.M. (Eds.), Avian Endocrinology. Narosa Publishing House, New Delhi, pp. 233-244.

El Halawani, M.E., Youngren, O.M., Pitts, G.R., 1997. Vasoactive intestinal peptide as the avian prolactin-releasing factor. In: Harvey, S., Etches, R.J. (Eds.), Perspectives in Avian Endocrinology. Journal of Endocrinology Ltd, Bristol, pp. 403-416.

El Halawani, M.E., Youngren, O.M., Silsby, J.L., Phillips, R.E., 1991. Involvement of dopamine in prolactin release induced by electrical stimulation of the hypothalamus of the female turkey (*Meleagris gallopavo*). Gen. Comp. Endocrinol. 84, 360-364.

Fraley, G.S., Kuenzel, W.J., 1993a. Immunocytochemical and histochemical analyses of gonadotrophin releasing hormone, tyrosine hydroxylase, and cytochrome oxicase reactivity within the hypothalamus of chicks showing early sexual maturation. Histochemistry 99, 221-229.

Fraley, G.S., Kuenzel, W.J., 1993b. Precocious puberty in chicks (*Gallus domesticus*) induced by central injections of neuropeptide Y. Life Sci. 52, 1649-1656.

Gellert, R.J., Ganong, W.F., 1960. Precocious puberty in rats with hypothalamic lesions. Acta. Endocrinol. 33, 569-576.

Goldsmith, A.R., Williams, D.M., 1980. Incubation in mallards (*Anas platyrhnchos*): Changes in plasma levels of prolactin and luteinizing hormone. J. Endocrinol. 86, 371-379.

Hall, T.R., Chadwick, A., 1984. Dopaminergic inhibition of prolactin release from pituitary glands of the domestic fowl incubated *in vitro*. J. Endocrinol. 103, 63-69.

Hof, P.R., Dietl, M.M., Charnay, Y., Martin, J.L., Bouras, C., Palacios, J.M., Magistretti, P.J., 1991. Vasoactive intestinal peptide binding sites and fibers in the brain of the pigeon *Columbia livia*: An autoradiographic and immunohistochemical study. J. Comp. Neurol. 305, 393-411.

Hull, E.M., Du, J., Lorrain, D.S., Matuszewich, L., 1995. Extracellular dopamine in the medial preoptic area: Implications for sexual motivation and hormonal control of copulation. J. Neurosci. 15, 7465-7471.

Kang, S.W., Thayananuphat, A., Bakken, T., El Halawani, M.E., 2007. Dopamine-melatonin neurons in the avian hypothalamus controlling seasonal reproduction. Neuroscience 30, 223-233.

Kiss, J.Z., Peczely, P., 1987. Distribution of tyrosine-hydroxylase (TH) immunoreactive neurons in the diencephalon of the pigeon (*Columba livia domestica*). J. Comp. Neurol. 257, 333-446.

Knapp, T.R., Fehrer, S.C., Silsby, J.L., Porter, T.E., Behnke, E.J., El Halawani, M.E., 1988. Gonodal steroid modulation of basal and vasoactive intestinal peptide-stimulated prolactin release by turkey anterior pituitary cells. Gen. Comp. Endocrinol. 72, 226-236.

Knigge, K.M., Piekut, D.T., 1985. Distribution of CRF- and tyrosine hydroxylase-immunoreactive neurons in the brainstem of the domestic fowl (*Gallus domesticus*). Peptides 6, 97-101.

Kragt, C.L., Meites, J., 1965. Stimulation of pigeon pituitary prolactin release by pigeon hypothalamic extract in vitro. Endocrinology. 76, 1169-1176.

Kosonsiriluk, S., 2008. Biological studies of the reproductive cycle and the effects of photoperiod upon the reproductive system in the female native Thai chicken. PhD Thesis. Suranaree University of Technology.

Kosonsiriluk, S., Sartsoongnoen, N., Prakobsaeng, N., Rozenboim, I., El Halawani, M.E., Chaiseha, Y., 2007. Prolactin and luteinizing hormone profiles during the reproductive cycle in the native Thai chicken. Poult. Sci. 86 (suppl 1), 650.

Kuenzel, W.J., 2000. Central nervous system regulation of gonadal development in the avian male. Poult. Sci. 79, 1679-1688.

Kuenzel, W.J., Kirtinitis, J., Saidel, W.S., 1992. Comparison of tyrosine hydroxylase (TH) vs. dopamine (DA) specific antibody procedures for mapping DA-containing perikaya throughout the chick brain. Soc. Neurosci. Abstr. 18, 329.

Kuenzel, W.J., Masson, M., 1988. A stereotaxic atlas of the brain of chick (*Gallus domesticus*). Johns Hopkins University Press, Baltimore.

Kuenzel, W.J., Mccune, S.K., Talbot, R.T., Sharp, P.J., Hill, J.M., 1997. Sites of gene expression for vasoactive intestinal polypeptide throughout the brain of the chick (*Gallus domesticus*). J. Comp. Neurol. 381, 101-118.

Kuenzel, W.J., van Tienhoven, A., 1982. Nomenclature and location of avian hypothalamic nuclei and associated circumventricular organs. J. Comp. Neurol. 206, 293-313.

Lamberts, S.W., Macleod, R.M., 1990. Regulation of prolactin secretion at the level of the lactotroph. Physiol. Rev. 70, 279-318.

Lea, R.W., Clark, J.A., Tsutsui, K., 2001. Changes in central steroid receptor expression, steroid synthesis, and dopaminergic activity related to the reproductive cycle of the ring dove. Microsc. Res. Tech. 55, 12-26.

Lehman, M.N., Durham, D.M., Jansen, H.T., Adrian, B., Goodman, R.L., 1996. Dopaminergic A14/A15 neurons are activated during estradiol negative feedback in anestrous, but not breeding season, ewes. Endocrinology 137, 4443-4450.

Levant, B., McCarson, K.E., 2001. D_3 dopamine receptors in rat spinal cord: Implications for sensory and motor function. Neurosci. Lett. 303, 9-12.

Lookingland, K.J., Moore, K.E., 2005. Functional neuroanatomy of hypothalamic dopaminergic neuroendocrine systems. In: Bjorklund, A., Hokfelt, T. (Eds.), Handbook of Chemical Neuroanatomy. Elsevier, Amsterdam, pp. 435-497.

Macnamee, M.C., Sharp, P.J., 1989. The functional activity of hypothalamic dopamine in broody bantam hens. J. Endocrinol. 121, 67-74.

Mauro, L.J., Elde, R.P., Youngren, O.M., Phillips, R.E., El Halawani, M.E., 1989. Alterations in hypothalamic vasoactive intestinal peptide-like immunoreactivity are associated with reproduction and prolactin release in the female turkey (*Meleagris gallopavo*). Endocrinology 125, 1795-1804.

Mauro, L.J., Youngren, O.M., Proudman, J.A., Phillips, R.E., El Halawani, M.E., 1992. Effects of reproductive status, ovariectomy, and photoperiod on vasoactive intestinal peptide in the female turkey hypothalamus. Gen. Comp. Endocrinol. 87, 481-493.

Mello, C.V., Pinaud, R., Ribeiro, S., 1998a. Noradrenergic system of the zebra finch brain: Immunocytochemical study of dopamine-β-hydroxylase. J. Comp. Neurol. 400, 207-228.

Mello, C.V., Vates, G.E., Okuhata, S., Nottebohm, F., 1998b. Descending auditory pathways in the adult male zebra finch (*Taeniopygi guttata*). J. comp. Neurol. 395, 137-160.

Moons, L., D'Hondt, E., Pijcke, K., Vandesande, F., 1995. Noradrenergic system in the chicken brain: Immunocytochemical study with antibodies to noradrenaline and dopamine-β-hydroxylase. J. Comp. Neurol. 360, 331-348.

Moons, L., van Gils, J., Ghijsels, E., Vandesande, F., 1994. Immunocytochemical localization of L-dopa and dopamine in the brain of the chicken (*Gallus domesticus*). J. Comp. Neurol. 346, 97-118.

Neill, J.D., 1988. Prolactin secretion and its control. In: Knobil, E., Neill, J.D. (Eds.), The Physiology of Reproduction. Raven Press, New York, pp. 1379-1390.

Nicoll, C.S., 1977. Aspects in the neural control of prolactin secretion. In: Martini, L., Ganong, W.F. (Eds.), Frontiers in Neuroendocrinology. Oxford University Press, London, pp. 1-8.

Nicoll, C.S., Swearingen, K.C., 1970. Preliminary observations on prolactin and growth hormone turnover in rat adenohypophyses *in vitro*. In: Martini, L., Motta, M., Fraschini, F. (Eds.), The Hypothalamus. Academic Press, New York, pp. 449-462.

Ottinger, M.A., Schumacher, M., Clarke, R.N., Duchala, C.S., Turek. R., Balthazart, J., 1986. Comparison of monoamine concentrations in the brains of adult male and female Japanese quail. Poult. Sci. 65, 1413-1420.

Porter, T.E., Silsby, J.L., Behnke, E.J., Knapp, T.R., El Halawani, M.E., 1991. Ovarian steroid production *in vitro* during gonadal regression in the turkey. I. Changes associated with incubation behavior. Biol. Reprod. 45, 581-586.

Proudman, J.A., Siopes, T.D., Vandesande, F., Berghman, L.R., 2001. Thyroid hormone and prolactin profiles in male and female turkeys following photostimulation: Validation of an ELISA for turkey prolactin. Poult. Sci. 80 (suppl 1), 469.

Reiner, A., Karten, H.J., Solina, A.R., 1983. Substance P: Localization within paleostriatal tegmental pathways in the pigeon. Neuroscience 9, 61-85.

Reiner, A., Karle, E.J., Anderson, K.D., Medina, L., 1994. Catecholaminergic perikaya and fibers in the avian nervous system. In: Smeets, W.J., Reiner, A. (Eds.), Phylogeny and Development of Catecholamine Systems in CNS of Vertebrates. Cambridge University Press, Cambridge, pp. 135-181.

Riddle, O., Bates, R.W., Lahr, E.L., 1935. Prolactin induces broodiness in fowl. Am. J. Physiol. 111, 352-360.

Roberts, T.F., Cookson, K.K., Heaton, K.J., Hall, W.S., Brauth, S.E., 2001. Distribution of tyrosine hydroxylase-containing neurons and fibers in the brain of the budgerigar (*Melopsittacus undulatus*): General patterns and labeling in vocal control nuclei. J. Comp. Neurol. 429, 436-454.

Schnell, S.A., You, S.K., El Halawani, M.E., 1999a. D_1 and D_2 dopamine receptor messenger ribonucleic acid in brain and pituitary during the reproductive cycle of the turkey hen. Biol. Reprod. 60, 1378-1383.

Schnell, S.A., You, S.K., Foster, D.N., El Halawani, M.E., 1999b. Molecular cloning and tissue distribution of an avian D_2 dopamine receptor mRNA from the domestic turkey (*Meleagris gallipavo*). J. Comp. Neurol. 407, 543-554.

Sharp, P.J., Dawson, A., Lea, R.W., 1998. Control of luteinizing hormone and prolactin secretion in birds. Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol. 119, 275-282.

Sharp, P.J., Macnamee, M.C., Sterling, R.J., Lea, R.W., Pedersen, H.C., 1988. Relationships between prolactin, LH and broody behavior in bantam hens. J. Endocrinol. 118, 279-286.

Swanson, R., Sawchenko, P.E., 1983. Hypothalamic integration: Organization of the paraventricular and supraoptic nuclei. Annu. Rev. Neurosci. 6, 269-324.

Tixier-Vidal, A., Bayle, J.D., 1996. Assenmacher I: Etude cytologique ultrastructurale de l'hyphpyse de pigeon après autograffe ectopique: Absence de stimulation des cellules à prolactine. C. R. Acad. Sci. (Paris). 262, 675-678.

Thayananuphat, A., Kang, S.W., Bakken, T., Millam, J.R., El Halawani, M.E., 2007a. Rhythmic dependent light induction of gonadotrophin-releasing hormone-I expression and activation of dopaminergic neurones within the premammillary nucleus of the turkey hypothalamus. J. Neuroendocrinol. 19, 399-406.

Thayananuphat, A., Kang, S.W., Bakken, T., Millam, J.R., El Halawani, M.E., 2007b. Rhythm-dependent light induction of the c-fos gene in the turkey hypothalamus. J. Neuroendocrinol. 19, 407-417.

van den Pol, A.N., Herbst, R.S., Powell, J.F., 1984. Tyrosine hydroxylase-immunoreactive neurons of the hypothalamus: A light and electron microscopic study. Neuroscience 13, 1117-1156.

van Dijken, H., Dijk, J., Voom, P., Holstege, J.C., 1996. Localization of dopamine D_2 receptor in rat spinal cord identified with immunocytochemistry and in situ hybridization. Eur. J. Neurosci. 8, 621-628.

von Bartheld, C.S., Bothwell, M., 1992. Development and distribution of noradrenergic and cholinergic neurons and their trophic phenotypes in the avian ceruleus complex and midbrain tegmentum. J. Comp. Neurol. 320, 479-500.

Weindl, A., 1973. Neuroendocrine aspects of circumventricular organs. In: Ganong, W.F., Martini, L. (Eds.), Frontiers in Neuroendocrinology. Oxford University Press, London, pp. 3-32.

Wong, E.A., Ferrin, N.H., Silsby, J.L., El Halawani, M.E., 1991. Cloning of a turkey prolactin cDNA: Expression of prolactin mRNA throughout the reproductive cycle of the domestic turkey (*Meleagris gallopavo*). Gen. Comp. Endocrinol. 83, 18-26.

Xu, M., Proudman, J.A., Pitts, G.R., Wong, E.A., Foster, D.N., El Halawani, M.E., 1996. Vasoactive intestinal peptide stimulates prolactin mRNA expression in turkey pituitary cells: Effects of dopaminergic drugs. Proc. Soc. Exp. Biol. Med. 212, 52-62.

Youngren, O.M., Chaiseha, Y., El Halawani, M.E., 1998. Regulation of prolactin secretion by dopamine and vasoactive intestinal peptide at the level of the pituitary in the turkey. Neuroendocrinology 68, 319-325.

Youngren, O.M., Chaiseha, Y., Phillips, R.E., El Halawani, M.E., 1996a. Vasoactive intestinal peptide concentrations in turkey hypophysial portal blood differ across the reproductive cycle. Gen. Comp. Endocrinol. 103, 323-330.

Youngren, O.M., Chaiseha, Y., Al-Zailaie, K., Whiting, S., Kang, S.W., El Halawani, M.E., 2002. Regulation of prolactin secretion by dopamine at the level of the hypothalamus in the turkey. Neuroendocrinology 75, 185-192.

Youngren, O.M., El Halawani, M.E., Silsby, J.L., Phillips, R.E., 1991. Intracranial prolactin perfusion induces incubation behavior in turkey hens. Biol. Reprod. 44, 425-431.

Youngren, O.M., Pitts, G.R., Phillips, R.E., El Halawani, M.E., 1995. The stimulatory and inhibitory effects of dopamine on prolactin secretion in the turkey. Gen. Comp. Endocrinol. 98, 111-117.

Youngren, O.M., Pitts, G.R., Phillips, R.E., El Halawani, M.E., 1996b. Dopaminergic control of prolactin secretion in the turkey. Gen. Comp. Endocrinol. 104, 225-230.

Table and figure legends

Table 1. The number of TH-ir neurons within individual hypothalamic areas (AM, PVN, nI, and ML) in the native Thai chicken at different reproductive stages (NL=non-egg laying, L=laying, B=incubating, and R=chick rearing). Values represent the mean±SEM (n=5). Values with different superscripts are significantly different ($P<0.05$) within each group.

Fig. 1. (A) Plasma PRL concentrations and (B) numbers of TH-ir neurons in the nI in the native Thai chicken at different reproductive stages (NL= non-egg laying, L=laying,

B=incubating, and R=chick rearing). Values are presented as the mean±SEM (n=5). Values with different superscripts are significantly different ($P<0.05$).

Fig. 2. Photomicrographs illustrating the expression of TH-ir neurons in the nI during different reproductive stages (NL=non-egg laying, L=laying, B=incubating, and R=chick rearing). *Bar* =100 μm. For abbreviations, see Fig. 4.

Fig. 3. Photomicrographs illustrating the expression of TH-ir neurons in the ML during different reproductive stages (NL=non-egg laying, L=laying, B=incubating, and R=chick rearing). *Bar* =100 μm. For abbreviations, see Fig. 4.

Fig. 4. 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 sterotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988). The following abbreviations are used in the figure legends: 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; ML, nucleus mamillaris lateralis; nDBC, nucleus decussationis brachiorum conjunctivorum; nI, nucleus intramedialis; nVm, nucleus mesencephalicus nervi trigemini; NIII, nervus oculomotorius; OM, tractus occipitomesencephalicus; 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. 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 lateral ventricle (VL). *Bar*= 50 μm. For abbreviations, see Fig. 4.

Fig. 6. 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 Fig. 4.

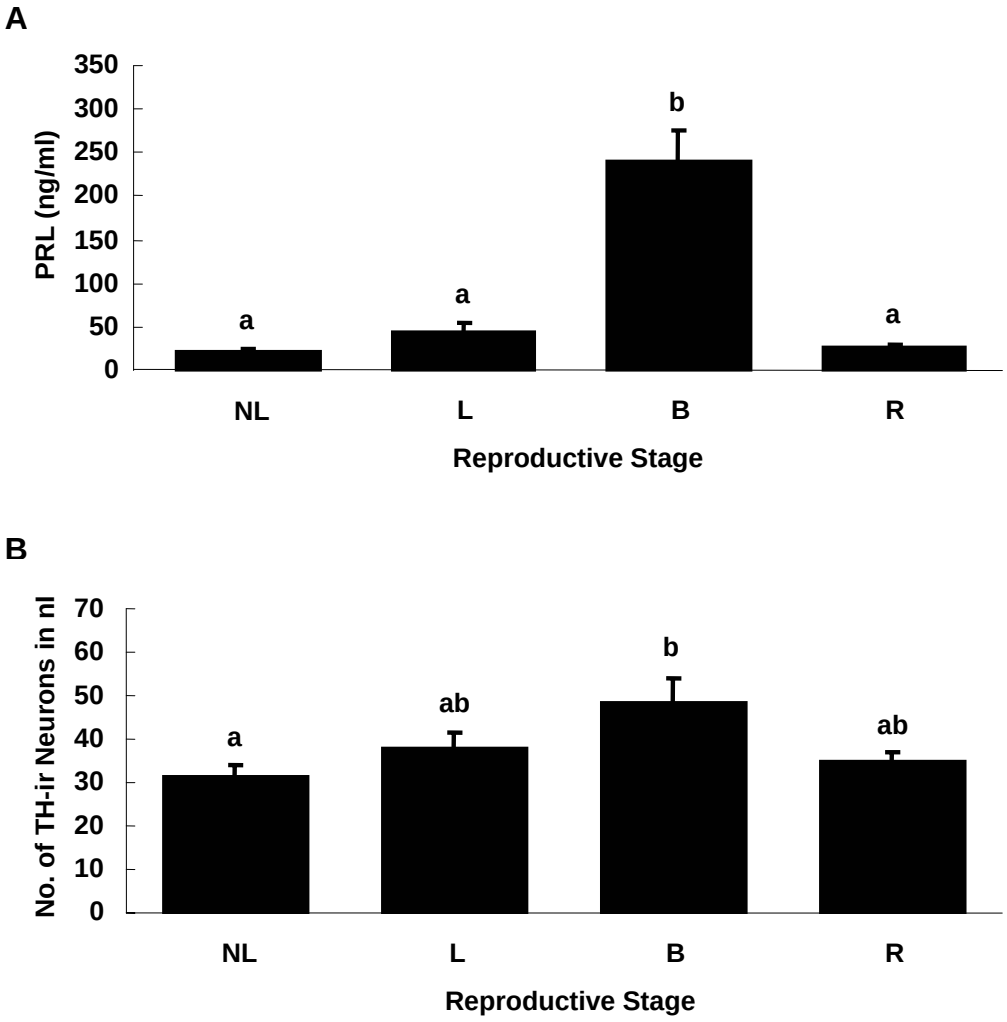
Fig. 7. Photomicrographs showing TH-ir neurons and fibers in the caudal hypothalamus. (A) A compact group of TH-ir neurons in nI, located on both sides of the fused ventricle. (B) Large, ovoid, and intensely labeled TH-ir neurons are found in the nI. (C) In the tuberal hypothalamus, TH-ir cells are observed 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 Fig. 4.

Fig. 8. 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 Fig. 4.

Table 1. The number of TH-ir neurons within individual hypothalamic areas (AM, PVN, nI, and ML) in the native Thai chicken at different reproductive stages (NL=non-egg laying, L=laying, B=incubating, and R=chick rearing). Values represent the mean±SEM (n=5). Values with different superscripts are significantly different ($P<0.05$) within each group.

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}

Fig. 1


A
PRL (ng/ml)
350
300
250
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100
50
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a
a
b
a
NL
L
B
R
Reproductive Stage
B
No. of TH-ir Neurons in nl
70
60
50
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30
20
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0
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ab
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ab
NL
L
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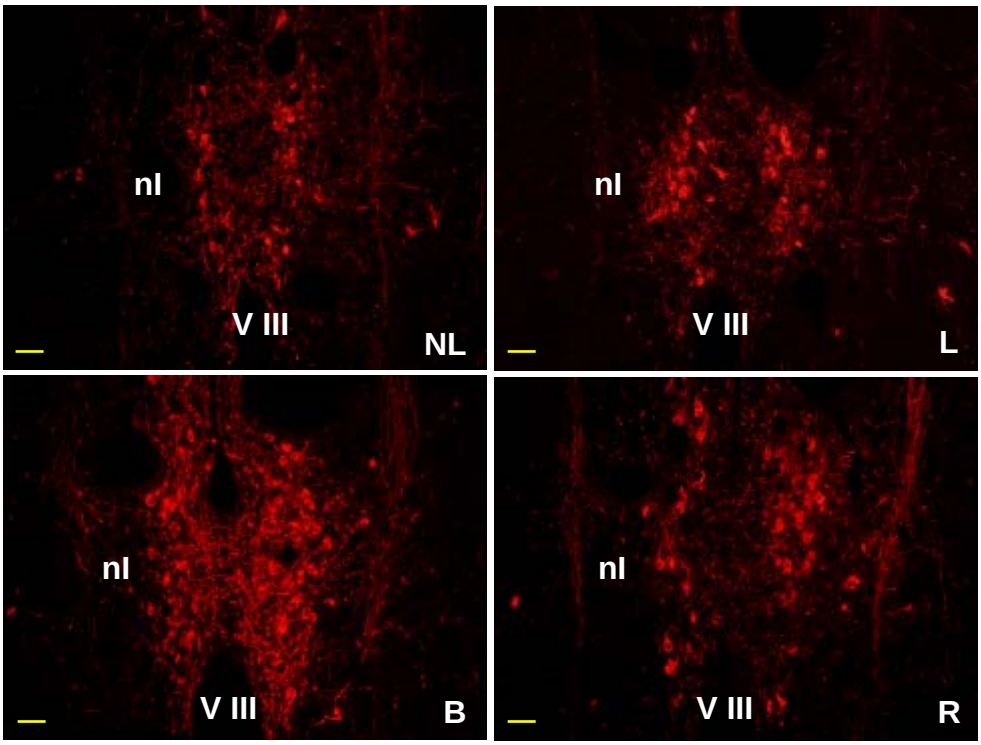
Reproductive Stage

Fig. 2



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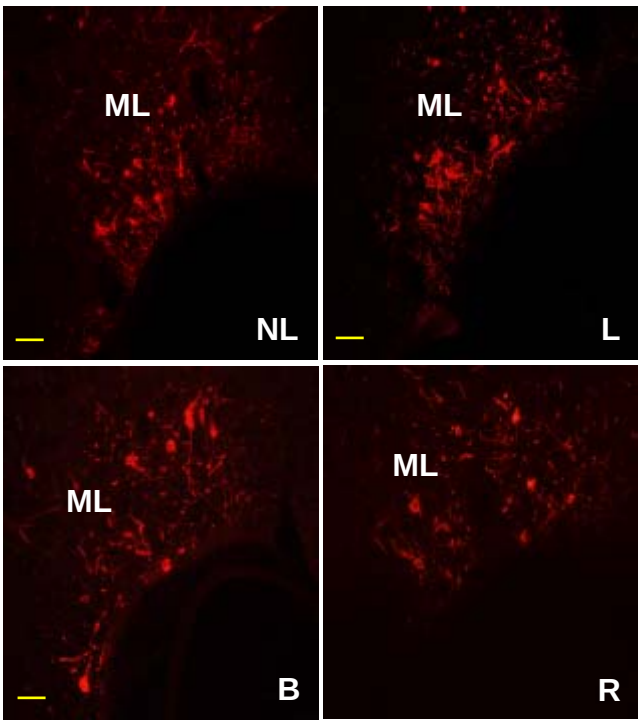

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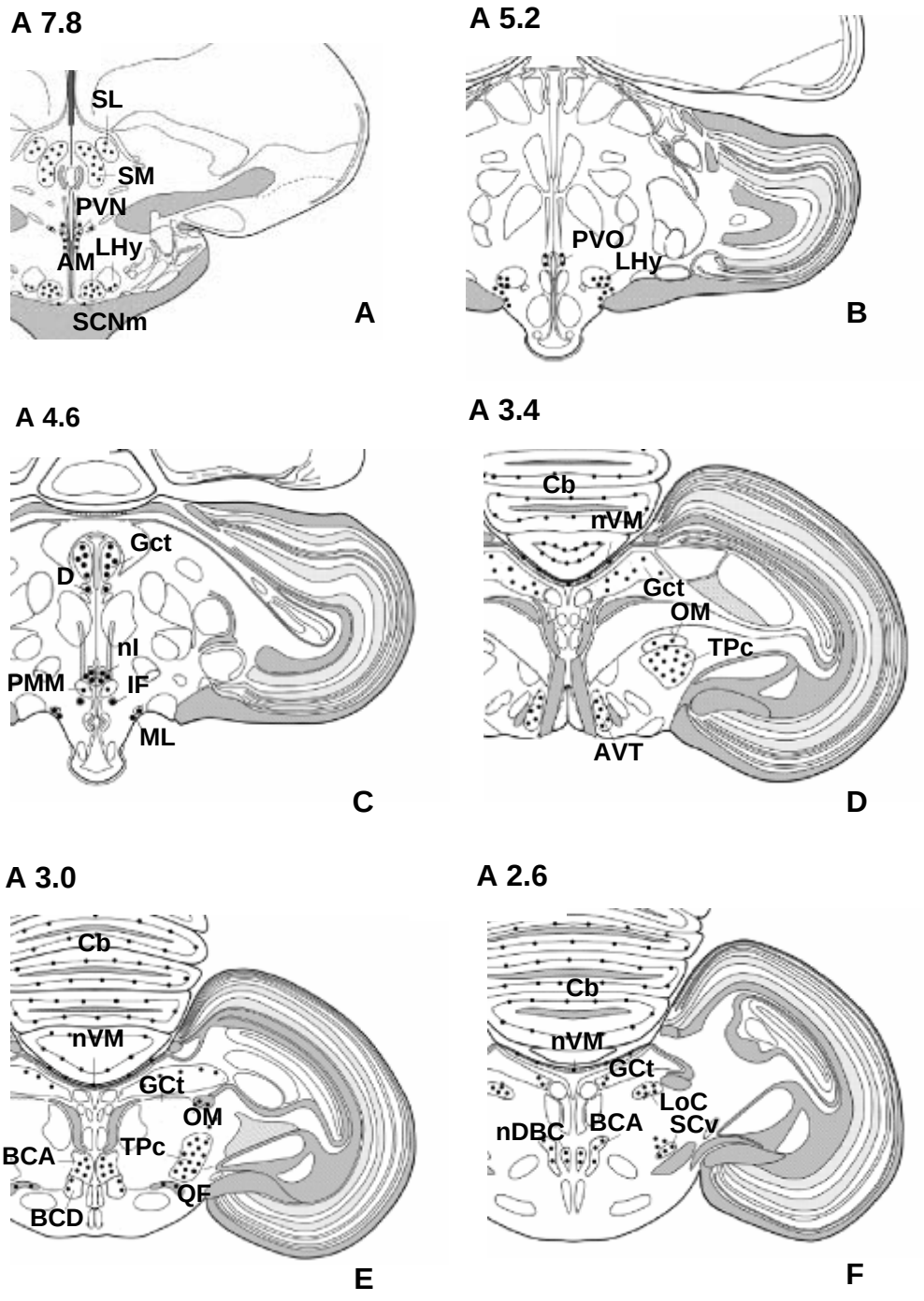
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Fig. 5

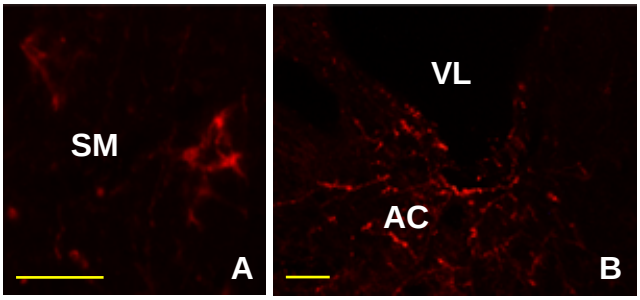
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Fig. 6

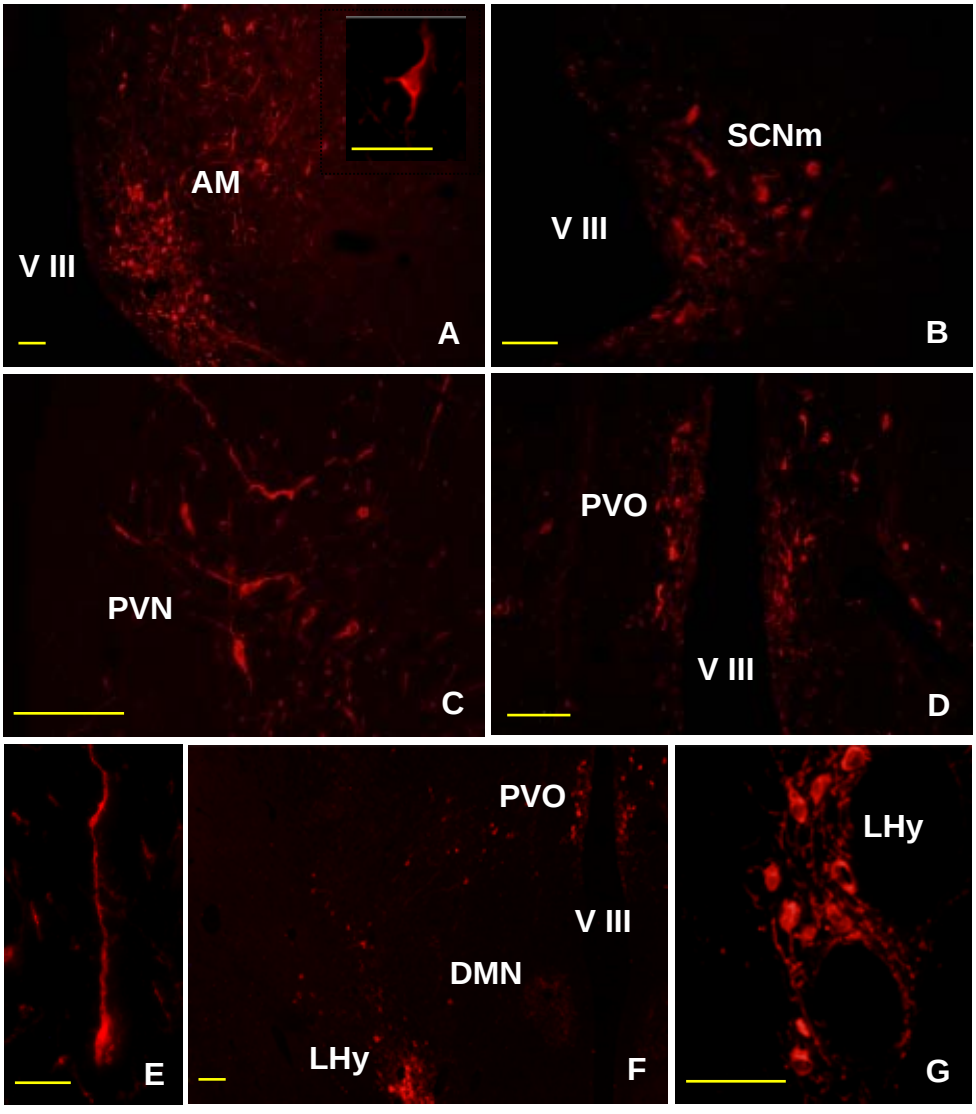
AM
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Fig. 7

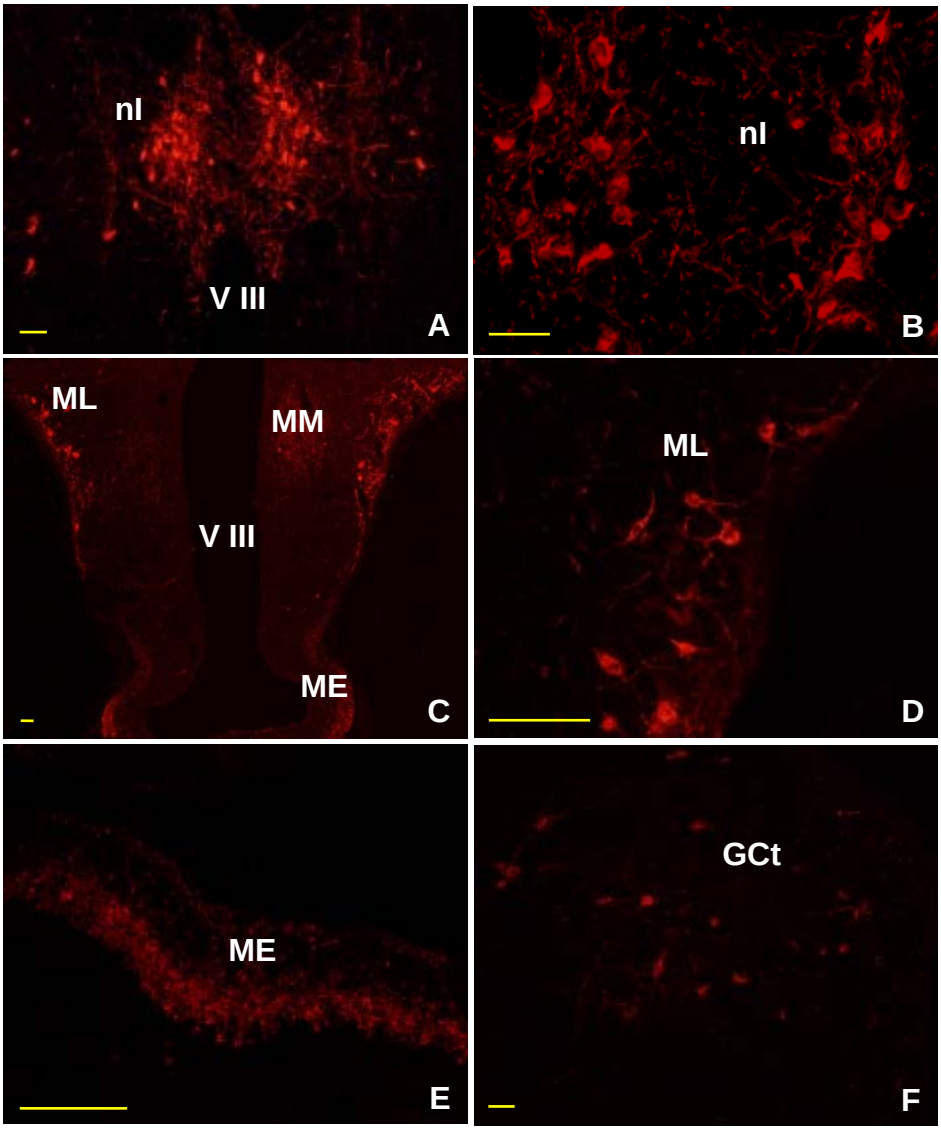
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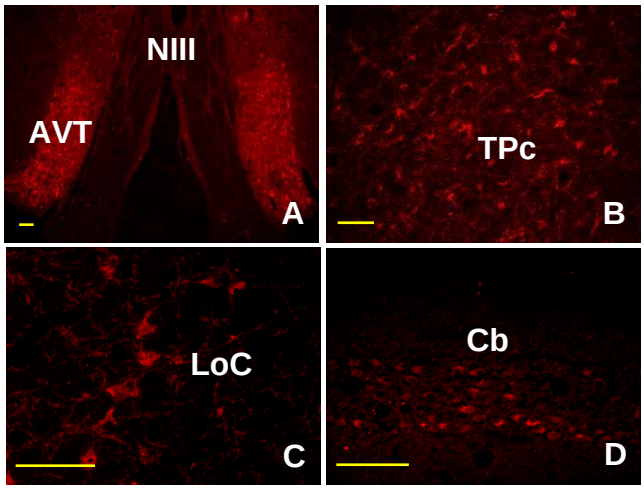
Fig. 8.


NIII
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Sartsoongnoen N, Kosonsiriluk S, Prakobsaeng N, Songserm T, El Halawani ME, **Chaiseha Y** (2007). Localization and differential expression of gonadotropin releasing hormone across the reproductive cycle of the native Thai chicken. **General and Comparative Endocrinology** (Submitted; **Impact factor** = 2.487).

Localization and Differential Expression of Gonadotropin Releasing Hormone across the Reproductive Cycle of the Native Thai Chicken

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Running title: GnRH-Containing Neurons in Native Thai chicken

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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.

Key words: Birds; GnRH; Hypothalamus; Immunohistochemistry; Native Thai chicken

1. 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 is designed to investigate the distribution of GnRH-I-ir neurons

throughout the brain. In addition, the changes in number of GnRH-I-ir neurons are 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.

2. Materials and methods

2.1. 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.

2.2. Experimental procedures

2.2.1. Experiment 1: The distribution of GnRH-I immunoreactivity throughout the brain of the laying native Thai hen

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.

2.2.2. Experiment 2: Changes in GnRH-ir neurons in the nCPa area of the native Thai chicken across the reproductive cycle

To determine the changes in number of GnRH-I-ir neurons within the 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.

2.3. 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°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°C until further processed for immunohistochemistry.

2.4. 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°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™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).

2.5. 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.

2.6. Statistical analysis

Significant differences (mean±SEM) in plasma PRL levels in the native Thai chicken, the number of VIP-ir neurons per section among reproductive stages of the native Thai chicken and turkey, and the ovary and oviduct weights of rearing and chick-removed native Thai hens were compared by one way analysis of variance (ANOVA) with 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).

3. Results

3.1. The distribution of GnRH-I immunoreactivity throughout the brain of the laying native Thai hen

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. 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. 1A). At this level, a small amount of GnRH-I-ir neurons were found extending from the V III into the POM (Fig. 2A). With in the POM, the oval shape with a monopolar process neurons containing GnRH-I immunoreactivity were observed (Fig. 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. 2C). Very dense GnRH-I-ir fibers were observed in and around the OVLT (Fig. 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. 2D, 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.

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. 4A) and PVN (Fig. 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. 5A). All perikaya immunostained in the LHy were bipolar, fusiform in shape, and less immunoreactive fibers were found coexisted (Fig. 5B). Other groups of GnRH-I-ir neurons were seen more lateral in the nucleus ventrolateralis thalami (VLT; Fig. 5C), as well as to the tip of the TSM in the nucleus dorsolateralis anterior thalami and pars magnocellularis (DLAmc; Fig. 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. 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. 6B). Moving in caudal hypothalamus, very intense GnRH-I-ir fibers were innervated in the external layer of the ME (Fig. 7A, 7B). Small numbers of GnRH-I-ir fibers were found in the nucleus inferioris hypothalami (IH) and nucleus infundibuli hypothalami (IN; Fig. 7C). There

were no GnRH-I-ir neuron observed in the IH-IN area, and also no immunostaining observed in the pituitary (Pit; Fig. 7A).

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. 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. 8B, 8D). At this level of the brain, a number of GnRH-I-ir fibers was found within the organum subseptale (supseptal organ, SSO; Fig. 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. 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. 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.

3.2. Changes in GnRH-ir neurons in the nCPa area of the native Thai chicken across the reproductive cycle

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. 10, 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.

4. 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, but not other areas, 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 prepotic 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 contras 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.

Acknowledgements

The authors would like to acknowledge the financial support from the Thailand Research Fund (TRF) through the Royal Golden Jubilee (RGJ) Ph.D. program (Grant No. PHD/0226/2545 to NS and YC), the TRF (Grant No. RSA4780001 to YC). The authors are grateful to University Farm of Suranaree University of Technology for providing the chicken barns.

References

Adkins-Regan, E., Watson, J.T., 1990. Sexual dimorphism in the avian brain is not limited to the song system of songbirds: A morphometric analysis of the brain of the quail (*Coturnix japonica*). Brain Res. 514: 320-326.

Advis, J.P., Contijoch, A.M., Johnson, A.L., 1985. Discrete hypothalamic distribution of luteinizing hormone-releasing hormone (LHRH) content and of LHRH-degrading activity in laying and nonlaying hens. Biol. Reprod. 32: 820-827.

Al-Zailaie, K.A., 2003. Neuroanatomical relationship between hypothalamic dopamine and vasoactive intestinal peptide in the regulation of PRL: Immunocytochemical and tract-tracing studies. Ph.D. Dissertation, University of Minnesota, Minnesota.

Al-Zailaie, K.A., Kang, S.W., Youngren, O.M., Thayananuphat, A., Bakken, T., Chaiseha, Y., Millam, J.R., Proudman, J.A., El Halawani, M.E., 2006. Identification of dopamine, gonadotrophin-releasing hormone-I, and vasoactive intestinal peptide neurons activated by electrical stimulation to the medial preoptic area of the turkey hypothalamus: A potential reproductive neuroendocrine circuit. J. Neuroendocrinol. 18: 514-525.

Barry, J., 1979. Immunofluorescence study of the preoptico-terminal LRH tract in the female squirrel monkey during the estrous cycle. Cell Tissue Res. 198: 1-13.

Ben-Jonathan, N., Mical, R.S., Porter, J.C., 1974. Transport of LRF from CSF to hypophysial portal and systemic blood and the release of LH. Endocrinology 95: 18-25.

Blahser, S., King, J.A., Kuenzel, W.J., 1989. Testing of arg-8-gonadotropin releasing hormone-directed antisera by immunological and immunocytochemical methods for use in comparative studies. Histochemistry 93: 39-48.

Blahser, S., Oksche, A., Farner, D.S., 1986. Projection of fibers immunoreactive to an antiserum against gonadoliberin (LHRH) into the pineal stalk of the white-crowned sparrow, *Zonotrichia leucophrys gambelii*. Cell Tissue Res. 244: 193-196.

Bluhm, C.K., Schwabl, H., Schwabl, I., Perera, A., Follett, B.K., Goldsmith, A.R., Gwinner, E., 1991. Variations in hypothalamic gonadotrophin releasing hormone content, plasma and pituitary LH and *in vitro* testosterone release in a long distance migratory bird, the garden warbler (*Sylvia borin*), under constant photoperiods. J. Endocrinol. 128: 339-345.

Bons, N., Kerdelhue, B., Assenmacher, I., 1978. Immunocytochemical identification of an LHRH-producing system originating in the preoptic nucleus of the duck. Cell Tissue Res. 188: 99-106.

Cho, R.N., Hahn, T.P., MacDougall-Shackleton, S., Ball, G.F., 1998. Seasonal variation in brain GnRH in free-living breeding and photorefractory house finches (*Carpodacus mexicanus*). Gen. Comp. Endocrinol. 109: 244-250.

Davies, D.T., Follett, B.K., 1975. The neuroendocrine control of gonadotrophin release in Japanese quail. I. The role of the tuberal hypothalamus. Proc. R. Soc. Lond. B 191: 285-301.

Dawson, A., Follett, B.K., Goldsmith, A.R., Nicholls, T.J., 1985. Hypothalamic gonadotrophin-releasing hormone and pituitary and plasma FSH and prolactin during

photorefractoriness in intact and thyroidectomized starlings (*Sturnus vulgaris*). J. Endocrinol. 105: 71-77.

Dawson, A., Talbot, R.T., Dunn, I.C., Sharp, P.J., 2002. Changes in basal hypothalamic chicken gonadotropin-releasing hormone-I and vasoactive intestinal polypeptide associated with a photo-induced cycle in gonadal maturation and prolactin secretion in intact and thyroidectomized starlings (*Sturnus vulgaris*). J. Neuroendocrinol. 14: 533-539.

Dellmann, H.D., 1964. On the structure of the vascular organ of the terminal lamina in poultry. Anat. Anz. 115: 174-83.

Dunn, I.C., Beattie, K.K., Maney, D., Sang, H.M., Talbot, R.T., Wilson, P.W., Sharp, P.J., 1996. Regulation of chicken gonadotropin-releasing hormone-I mRNA in incubating, nest-deprived and laying bantam hens. Neuroendocrinology 63: 504-513.

Dunn, I.C., Sharp, P.J., 1999. Photo-induction of hypothalamic gonadotrophin releasing hormone-I mRNA in the domestic chicken: A role for oestrogen?. J. Neuroendocrinol. 11: 371-375.

Ehrlich, D., Mark, R., 1984. Topography of primary visual centres in the brain of the chick, *Gallus gallus*. J. Comp. Neurol .223: 611-25.

El Halawani, M.E., Silsby, J.L., Foster, L.K., Rozenboim, I., Foster, D.N., 1993. Ovarian involvement in the suppression of luteinizing hormone in the incubating turkey (*Meleagris gallopavo*). Neuroendocrinology 58: 35-41.

Foster, R.G., Follett, B.K., Lythgoe, J.N., 1985. Rhodopsin-like sensitivity of extra-retinal photoreceptors mediating the photoperiodic response in quail. Nature 313: 50-52.

Foster, R.G., Panzica, G.C., Parry, D.M., Viglietti-Panzica, C., 1988. Immunocytochemical studies on the LHRH system of the Japanese quail: Influence by photoperiod and aspects of sexual differentiation. Cell Tissue Res. 253: 327-335.

Foster, R.G., Plowman, G., Goldsmith, A.R., Follett, B.K., 1987. Immunocytochemical demonstration of marked changes in the luteinizing hormone releasing hormone system of photosensitive and photorefractory European starlings. J. Endocrinol. 115: 211-220.

Goldsmith, A.R., Ivings, W.E., Pearce-Kelly, A.S., Parry, D.M., Plowman, G., Nicholls, T.J., Follett, B.K., 1989. Photoperiodic control of the development of the LHRH neurosecretory system of European starlings (*Sturnus vulgaris*) during puberty and the onset of photorefractoriness. J. Endocrinol. 122: 255-268.

Goldsmith, P.C., Thind, K.K., Sone, T., Kim, E.J., Boggan, J.E., 1990. Localization of the neuroendocrine gonadotropins-releasing hormone neurons in the monkey hypothalamus by retrograde tracing and immunocytochemistry. J. Neuroendocrinol. 2: 157-168.

Guemene, D., Williams, J.B., 1999. LH responses to chicken luteinizing hormone-releasing hormone I and II in laying, incubating, and out of lay turkey hens. Domest. Anim. Endocrinol. 17: 1-15.

Hahn, T.P., Ball, G.F., 1995. Changes in brain GnRH associated with photorefractoriness in house sparrows (*Passer domesticus*). Gen. Comp. Endocrinol. 99: 349-363.

Hirunagi, K., Kiyoshi, K., Adachi, A., Hasegawa, M., Shizufumi, E., Korf, H.W., 1994. Electron-microscopic investigations of vasoactive intestinal peptide (VIP)-like immunoreactive terminal formations in the lateral septum of the pigeon (*Columbia livia*). Cell Tissue Res. 278: 415-418.

Hirunagi, K., Rommel, E., Oksche, A., Korf, H.W., 1993. Vasoactive intestinal peptide-immunoreactive cerebrospinal fluid-contacting neurons in the reptilian lateral septum/nucleus accumbens. Cell Tissue Res. 274: 79-90.

Joseph, S.A., Sorrentino, S., Sundberg, D.K., 1975. Releasing hormone, LRF and TRF, in the cerebrospinal fluid of the third ventricle. In Brain-endocrine interaction. II. The ventricular system in neuroendocrine mechanism, pp 306-312. Eds. Knigge, K.M., Scott, D.E., Kobayashi, H., and Ishii, S. Karger, Basel.

Jozsa, R., Mess, B., 1982. Immunohistochemical localization of the luteinizing hormone releasing hormone (LHRH)-containing structures in the central nervous system of the domestic fowl. Cell Tissue Res. 227: 451-458.

Kang, S.W., Thayananuphat, A., Rozenboim, I., Millam, J.R., Proudman, J.A., El Halawani, M.E., 2006. Expression of hypothalamic GnRH-I mRNA in the female turkey at different reproductive states and following photostimulation. Gen. Comp. Endocrinol. 146: 86-94.

King, J.A., Millar, R.P., 1982. Structure of chicken hypothalamic luteinizing hormone-releasing hormone. I. Structural determination on partially purified material. J. Biol. Chem. 257: 10722-10728.

Kiyoshi, K., Kondoh, M., Hirunagi, K., Korf, H.W., 1998. Confocal laser scanning and electron-microscopic analyses of the relationship between VIP-like and GnRH-like immunoreactive neurons in the lateral septal-preoptic area of the pigeon. Cell Tissue Res. 293: 39-46.

Korf, H.W., Fahrenkrug, J., 1984. Ependymal and neuronal specializations in the lateral ventricle of the Pekin duck, *Anas platyrhynchos*. Cell Tissue Res. 236: 217-227.

Krishnan, K.A., Proudman, J.A., Bahr, J.M., 1992. Purification and characterization of chicken follicle-stimulating hormone. Comp. Biochem. Physiol. B 102: 67-75.

Krishnan, K.A., Proudman, J.A., Bolt, D.J., Bahr, J.M., 1993. Development of an homologous radioimmunoassay for chicken follicle-stimulating hormone and measurement of plasma FSH during the ovulatory cycle. Comp. Biochem. Physiol. Comp. Physiol. 105: 729-734.

Kuenzel, W.J., Blahser, S., 1991. The distribution of gonadotropin-releasing hormone (GnRH) neurons and fibers throughout the chick brain (*Gallus domesticus*). Cell Tissue Res. 264: 481-495.

Kuenzel, W.J., Blahser, S., 1994. Vasoactive intestinal peptide (VIP)-containing neurons: Distribution throughout the brain of the chick (*Gallus domesticus*) with focus upon the lateral septal organ. Cell Tissue Res. 275: 91-107.

Kuenzel, W.J., Golden, C.D., 2006. Distribution and change in number of gonadotropin-releasing hormone-1 neurons following activation of the photoneuroendocrine system in the chick, *Gallus gallus*. Cell Tissue Res. 325: 501-512.

Kuenzel, W.J., Masson, M., 1988. A stereotaxic atlas of the brain of chick (*Gallus domesticus*). Johns Hopkins University Press, Baltimore and London.

Kuenzel, W.J., van Tienhoven, A., 1982. Nomenclature and location of avian hypothalamic nuclei and associated circumventricular organs. J. Comp. Neurol. 206: 293-313.

Marsh, R.H., MacDougall-Shackleton, S.A., Hahn, T.P., 2002. Photorefractoriness and seasonal changes in the brain response to changes in day length in American goldfinches (*Carduelis tristis*). Can. J. Zool. 80: 2100-2107.

McNaughton, F.J., Dawson, A., Goldsmith, A.R., 1995. A comparison of the responses to gonadotrophin-releasing hormone of adult and juvenile, and photosensitive and photorefractory European starlings, *Sturnus vulgaris.* Gen. Comp. Endocrinol. 97: 135-144.

McNeill, T., Kozlowski, G.P., Abel, J.H., Zimmerman, E.A., 1976. Neurosecretory pathways in the mallard duck (*Anas platyrhynchos*) brain: Localization by aldehyde fuchsin and immunoperoxidase techniques for neurophysin (NP) and gonadotrophin releasing hormone (Gn-RH). Endocrinology 99: 1323-1332.

Mcneilly, A.S., Glasier, A., Jonassen, J., Howie, P.W., 1982. Evidence for direct inhibition of ovarian function by prolactin. J. Reprod. Fertil. 65: 559-569.

Mikami, S., Yamada, S., Hasegawa, Y., Miyamoto, K., 1988. Localization of avian LHRH-immunoreactive neurones in the hypothalamus of the domestic fowl, (*Gallus domesticus*) and the Japanese quail, (*Coturnix japonica*). Cell Tissue Res. 251: 51-58.

Millam, J.R., Craig-Veit, C.B., Adams, T.E., Adams, B.M., 1989. Avian gonadotrophin-releasing hormones I and II in the brain and other tissues in turkey hens. Comp. Biochem. Physiol. 94: 771-776.

Millam, J.R., Craig-Veit, C.B., Faris, P.L., 1995. Concentration of chicken gonadotropin-releasing hormones I and II in microdissected areas of turkey hen brain during the reproductive cycle. Domest. Anim. Endocrinol. 12: 1-11.

Millam, J.R., Faris, P.L., Youngren, O.M., El Halawani, M.E., Hartman, B.K., 1993. Immunohistochemical localization of chicken gonadotrophin-releasing hormones I and II (cGnRH-I and II) in turkey hen brain. J. Comp. Neurol. 333: 68-82.

Millam, J.R., Ottinger, M.A., Craig-Veit, C.B., Fan, Y., Chaiseha, Y., El Halawani, M.E., 1998. Multiple forms of GnRH are released from perifused medial basal hypothalamic/preoptic area (MBH/POA) explants in birds. Gen. Comp. Endocrinol. 111: 95-101.

Millar, R.P., King, J.A., 1984. Structure-activity relations of LHRH in birds. J. Exp. Zool. 232: 425-430.

Millar, R.P., Milton, R.C., Follett, B.K., King, J.A., 1986. Receptor binding and gonadotropin-releasing activity of a novel chicken gonadotropin-releasing hormone ([His5, Trp7, Tyr8]GnRH) and a D-Arg6 analog. Endocrinology 119: 224-231.

Miyamoto, K., Hasegawa, Y., Minegishi, T., Nomura, M., Takahashi, Y., Igarashi, M., Kangawa, K., Matsuo, H., 1982. Isolation and characterization of chicken hypothalamic luteinizing hormone-releasing hormone. Biochem. Biophys. Res. Commun. 107: 820-827.

Norgren, R.B. Jr., Gao, C., 1994. LHRH neuronal subtypes have multiple origins in chickens. Dev. Biol. 165: 735-738.

Oliver, J., Bayle, J.D., 1982. Brain photoreceptors for the photo-induced testicular response in birds. Experientia 38: 1021-1029.

Panzica, G.C., Viglietti-Panzica, C., Balthazart, J., 1996. The sexually dimorphic medial preoptic nucleus of quail: A key brain area mediating steroid action on male sexual behavior. Front. Neuroendocrinol. 17: 51-125.

Panzica, G.C., Viglietti-Panzica, C., Sanchez, F., Sante, P., Balthazart, J., 1991. Effects of testosterone on a selected neuronal population within the preoptic sexually dimorphic nucleus of the Japanese quail. J. Comp. Neurol. 303: 443-456.

Parry, D.M., Goldsmith, A.R., Millar, R.P., Glennie, L.M., 1997. Immunocytochemical localization of GnRH precursor in the hypothalamus of European starlings during sexual maturation and photorefractoriness. J. Neuroendocrinol. 9: 235-243.

Peczely, P., 1989. The role of gonadotropin releasing hormone (GnRH) in the regulation of gonadal functions of birds. Review article. Acta Biol. Hung. 40: 161-193.

Perera, A.D., Follett, B.K., 1992. Photoperiodic induction in vitro: The dynamics of gonadotropin-releasing hormone release from hypothalamic explants of the Japanese quail. Endocrinology 131: 2898-2908.

Porter, J.C., Ben-Jonathan, N., Oliver, C., Eskay, R.L., 1975. Secretion of releasing hormones and their transport from CSF to hypophysial portal blood In Brain-endocrine interaction. II. The ventricular system in neuroendocrine mechanism, pp 295-305. Eds. Knigge, K.M., Scott, D.E., Kobayashi, H., and Ishii, S. Karger, Basel.

Rothfeld, J.M., Gross, D.S., 1985. Gonadotropin-releasing hormone within the organum vasculosum lamina terminalis in the ovariectomized, estrogen/progesterone-treated rat: A quantitative immunocytochemical study using image analysis. Brain Res. 338: 309-315.

Rozenboim, I., Silsby, J.L., Tabibzadeh, C., Pitts, G.R., Youngren, O.M., El Halawani, M.E., 1993. Hypothalamic and posterior pituitary content of vasoactive intestinal peptide and gonadotrophin releasing hormones I and II in the turkey hen. Biol. Reprod. 49: 622-626.

Saldanha, C.J., Deviche, P.J., Silver, R., 1994. Increased VIP and decreased GnRH expression in photorefractory dark-eyed juncos (*Junco hyemalis*). Gen. Comp. Endocrinol. 93: 128-136.

Shalev, E., Leung, P.C., 2003. Gonadotropin-releasing hormone and reproductive medicine. J. Obstet. Gynaecol. Can. 25: 98-113.

Sharp, P.J., Talbot, R.T., Main, G.M., Dunn, I.C., Fraser, H.M., Huskisson, N.S., 1990. Physiological roles of chicken LHRH-I and -II in the control of gonadotrophin release in the domestic chicken. J. Endocrinol. 124: 291-299.

Sherwood, N.M., Wingfield, J.C., Ball, G.F., Dufty, A.M., 1988. Identity of gonadotropin-releasing hormone in passerine birds: Cmparison of GnRH in song sparrow (*Melospiza melodia*) and starling (*Sturnus vulgaris*) with five vertebrate GnRHs. Gen. Comp. Endocrinol. 69: 341-351.

Shivers, B.D., Harlan, R.E., Morrell, J.I., Pfaff, D.W., 1983. Immunocytochemical localization of luteinizing hormone-releasing hormone in male and female rat brains. Quantitative studies on the effect of gonadal steroids. Neuroendocrinology 36: 1-12.

Silver, R., Ramos, C., Machuca, H., Silverin, B., 1992. Immunocytochemical distribution of GnRH in the brain of adult and post hatching great tit (*Parus major*) and ring dove (*Streptopelia roseogrisea*). Ornis. Scand. 23: 222-232.

Silverman, A.J., Livne, I., Witkin, J.W., 1994. The gonadotropins releasing hormone (GnRH) neuronal system: Immunocytochemistry and in situ hybridization. In The physiology of reproduction, pp 1638-1709. Eds. Knobil, E., and Neill, J.D. Raven, New York.

Sterling, R.J., Sharp, P.J., 1982. The localization of LH-RH neurons in the diencephalon of the domestic hen. Cell Tissue Res. 222: 283-298.

Stevenson, T.J., Macdougall-Shackleton, S.A., 2005. Season- and age-related variation in neural cGnRH-I and cGnRH-II immunoreactivity in house sparrows (*Passer domesticus*). Gen. Comp. Endocrinol. 143: 33-39.

Sun, Y.M., Dunn, I.C., Baines, E., Talbot, R.T., Illing, N., Millar, R.P., Sharp, P.J., 2001. Distribution and regulation by oestrogen of fully processed and variant transcripts of gonadotropin releasing hormone I and gonadotropin releasing hormone receptor mRNAs in the male chicken. J. Neuroendocrinol. 13: 37-49.

Teruyama, R., Beck, M.M., 2000. Changes in immunoreactivity to anti-cGnRH-I and -II are associated with photostimulated sexual status in male quail. Cell Tissue Res. 300: 413-426.

Teruyama, R., Beck, M.M., 2001. Double immunocytochemistry of vasoactive intestinal peptide and cGnRH-I in male quail: Photoperiodic effects. Cell Tissue Res. 303: 403-414.

Ulloa-Aguirre, A., Timossi, C., 2000. Biochemical and functional aspects of gonadotrophin-releasing hormone and gonadotrophins. Reprod. Biomed. Online 1: 48-62.

van Gils, J.A., Absil, P., Grauwels, L., Moons, L., Vandesande, F., Balthazart, J., 1993. Distribution of luteinizing hormone-releasing hormones I and II (LHRH-I and -II) in the quail and chicken brain as demonstrated with antibodies directed against synthetic peptides. J. Comp. Neurol. 334: 304-323.

Viglietti-Panzica, C., Panzica, G.C., Fiori, M.G., Calcagni, M., Anselmetti, G.C., Balthazart, J., 1986. A sexually dimorphic nucleus in the quail preoptic area. Neurosci. Lett. 64: 129-34.

Wingfield, J.C., Crim, J.W., Mattocks, P.W., Farner, D.S., 1979. Responses of photosensitive and photorefractory male white-crowned sparrows (*Zonotrichia leucophrys gambelii*) to synthetic mammalian luteinizing hormone releasing hormone (syn-LHRH). Biol. Reprod. 21: 801-806.

You, S., Foster, L.K., Silsby, J.L., El Halawani, M.E., Foster, D.N., 1995. Sequence analysis of the turkey LH beta subunit and its regulation by gonadotrophin-releasing hormone and prolactin in cultured pituitary cells. J. Mol. Endocrinol. 14: 117-129.

Table and figure legends

Table 1 Abbreviations of brain areas. Nomenclature and abbreviations are from a stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988).

Fig. 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 1.

Fig. 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 1.

Fig. 3. Photomicrographs illustrating the distribution of GnRH-I-ir fibers in the OVLT (**A**) and POP (**B**). Bar=50 μm. For abbreviations, see Table1.

Fig. 4. Photomicrographs illustrating the distribution of GnRH-I-ir neurons in the AM (A) and PVN (B). Bar=50 μm. For abbreviations, see Table 1.

Fig. 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 1.

Fig. 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 1.

Fig. 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 1.

Fig. 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 1.

Fig. 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 1.

Fig. 10. Photomicrographs illustrating the expression of GnRH-I-ir neurons in the nCPa during different reproductive stages (NL=non-egg laying, L=egg laying, B=incubating eggs, and R= rearing chicks). Bar=100 μm. For abbreviations, see Table 1.

Fig. 11. Changes in number of GnRH-I-ir neurons in the nCPa of the native Thai chicken at different reproductive stages (NL=non-egg laying, L=egg laying, B=incubating eggs, and R=rearing chicks). Values (number of GnRH-I-ir neurons/section) are presented as the mean±SEM (n=6). Values with different superscripts are significantly different ($P<0.05$).

Table 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
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. 1

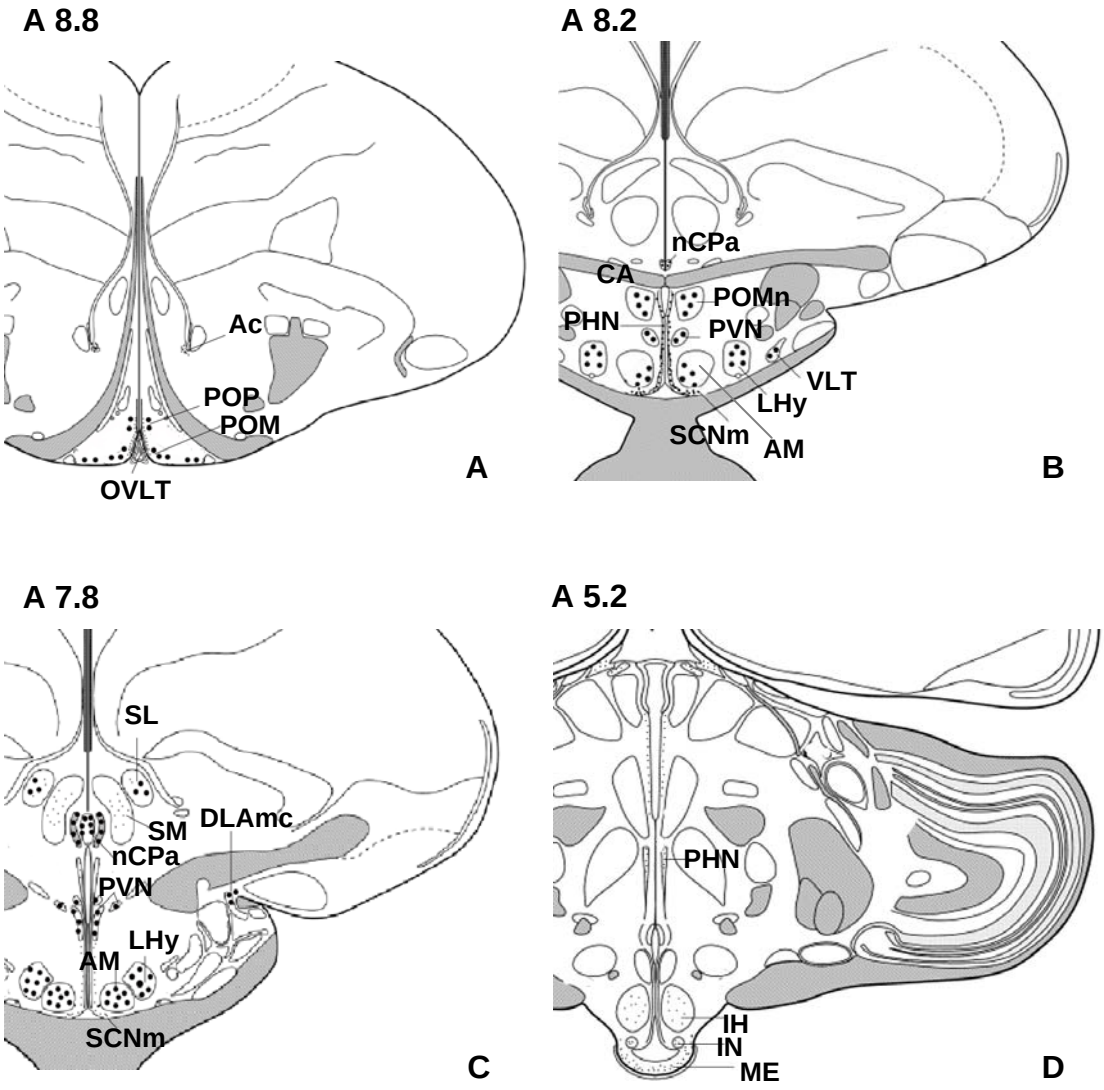
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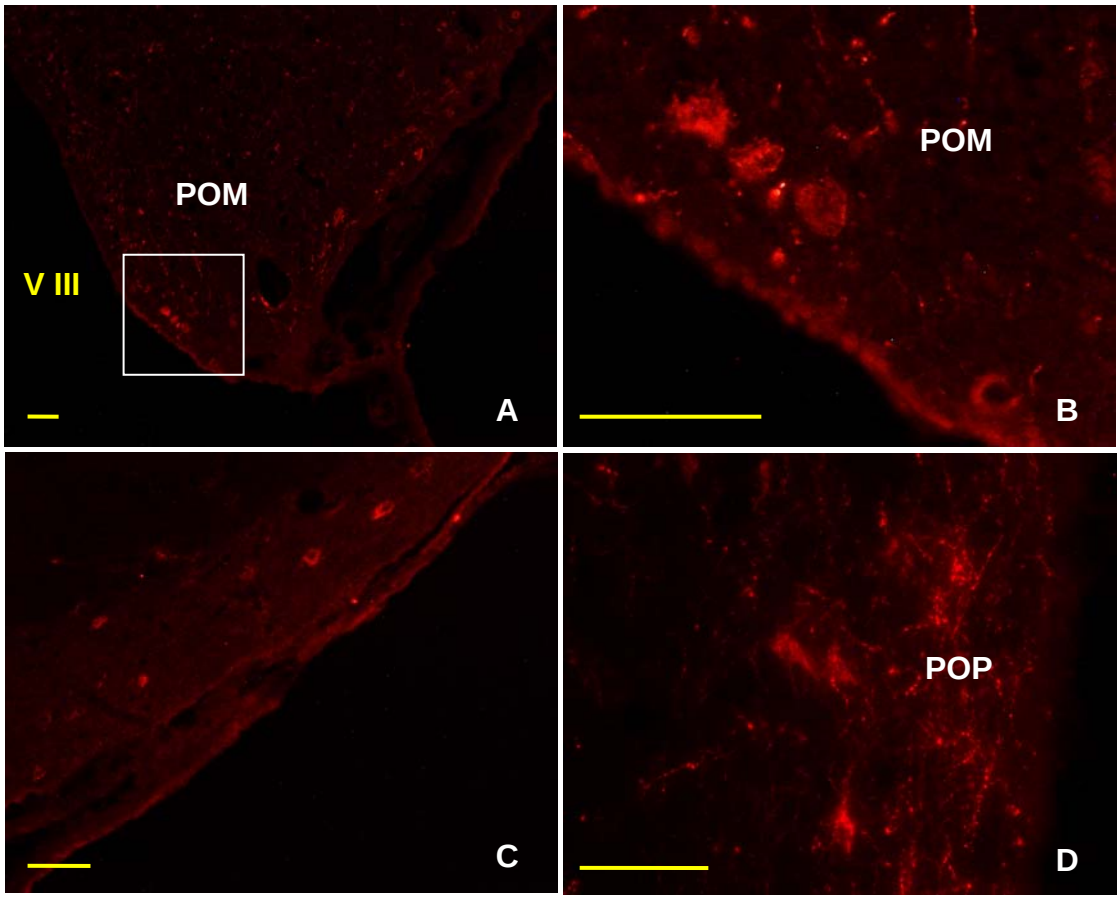
Fig. 2



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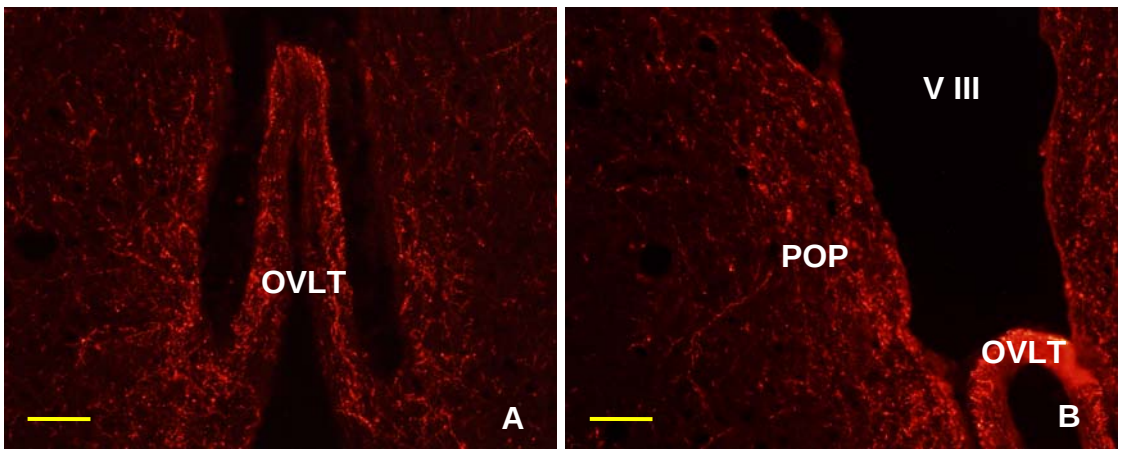

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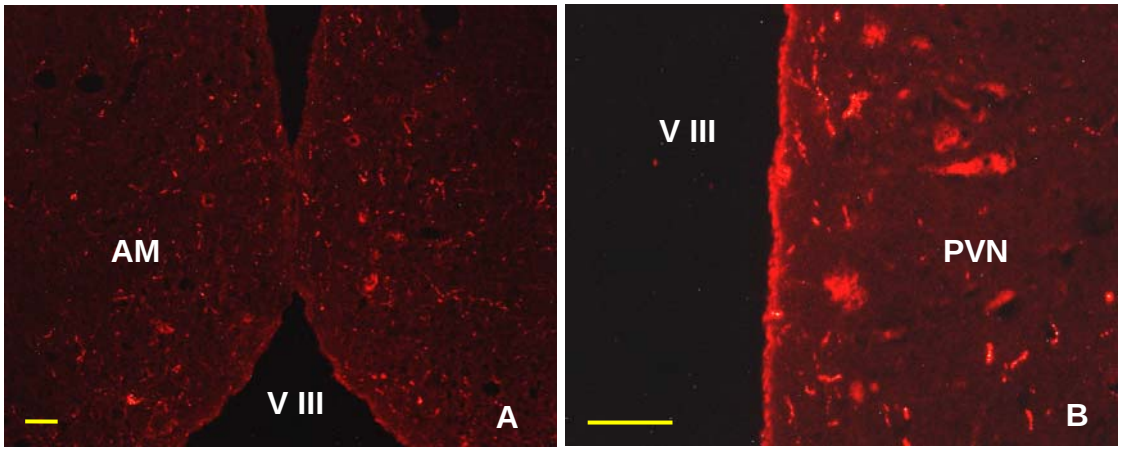

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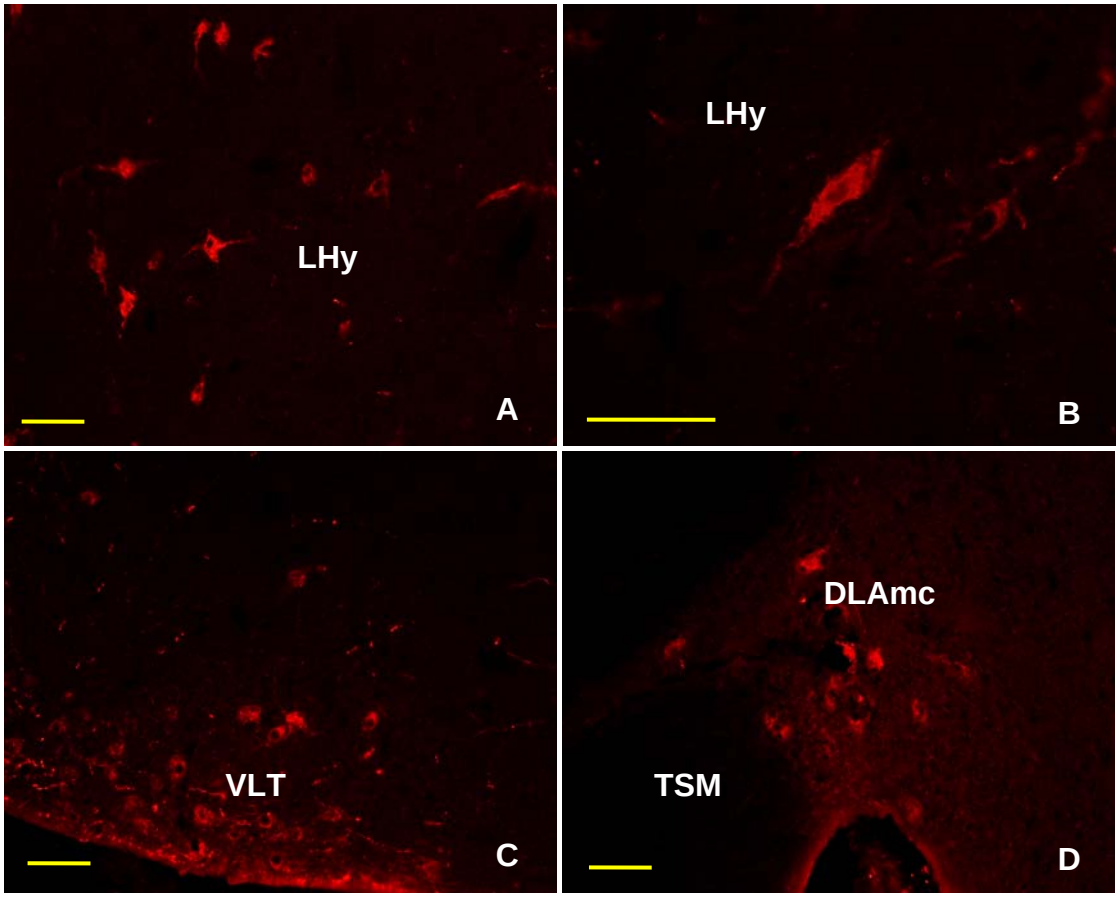

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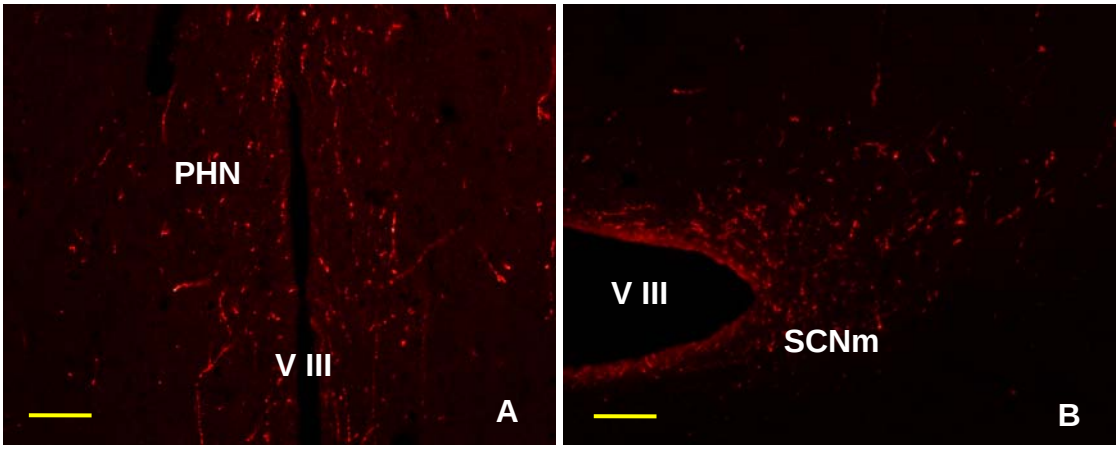

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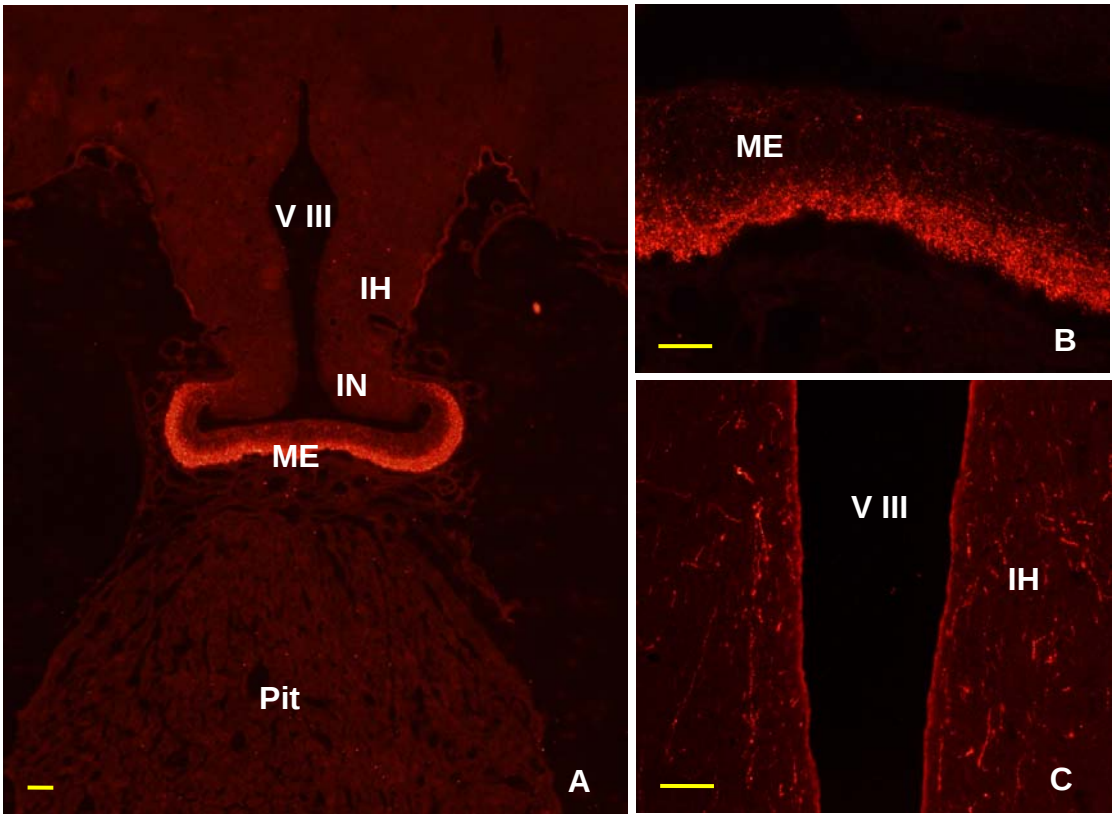

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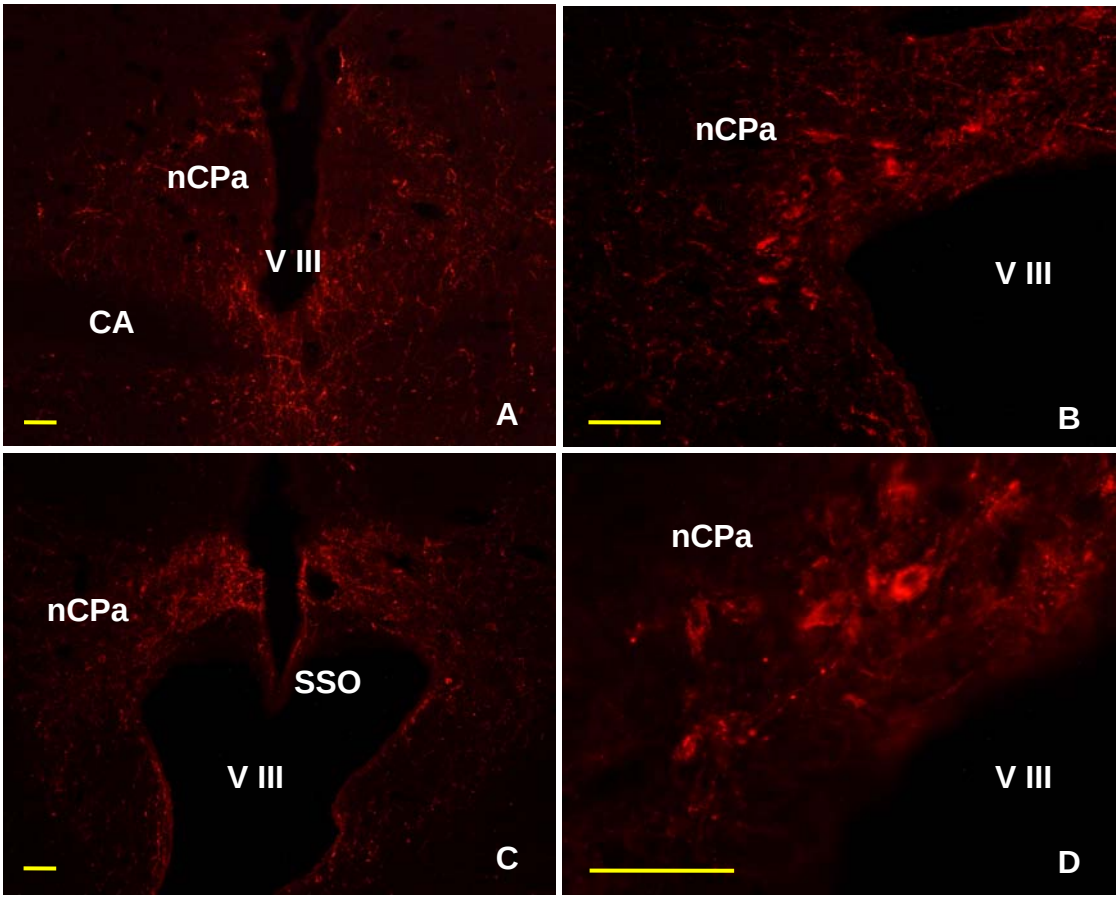
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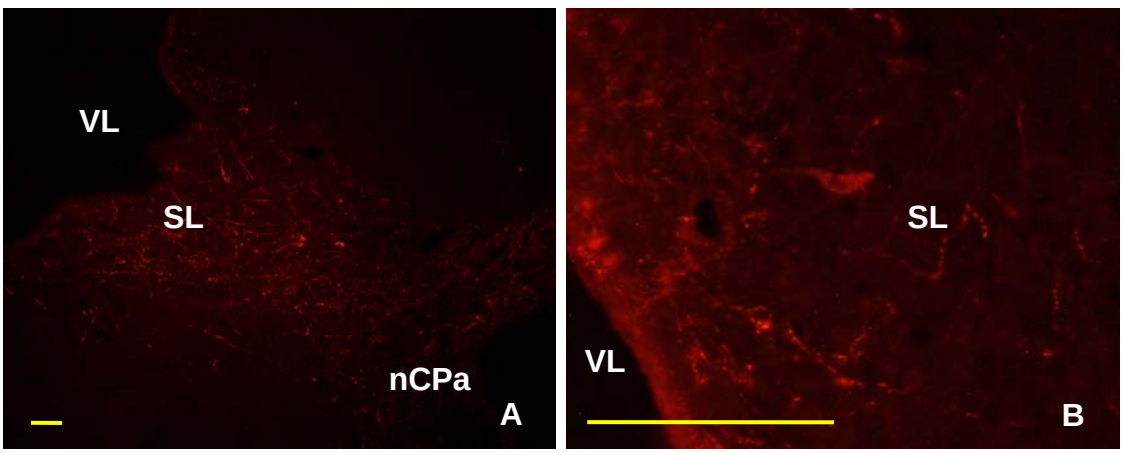
Fig. 9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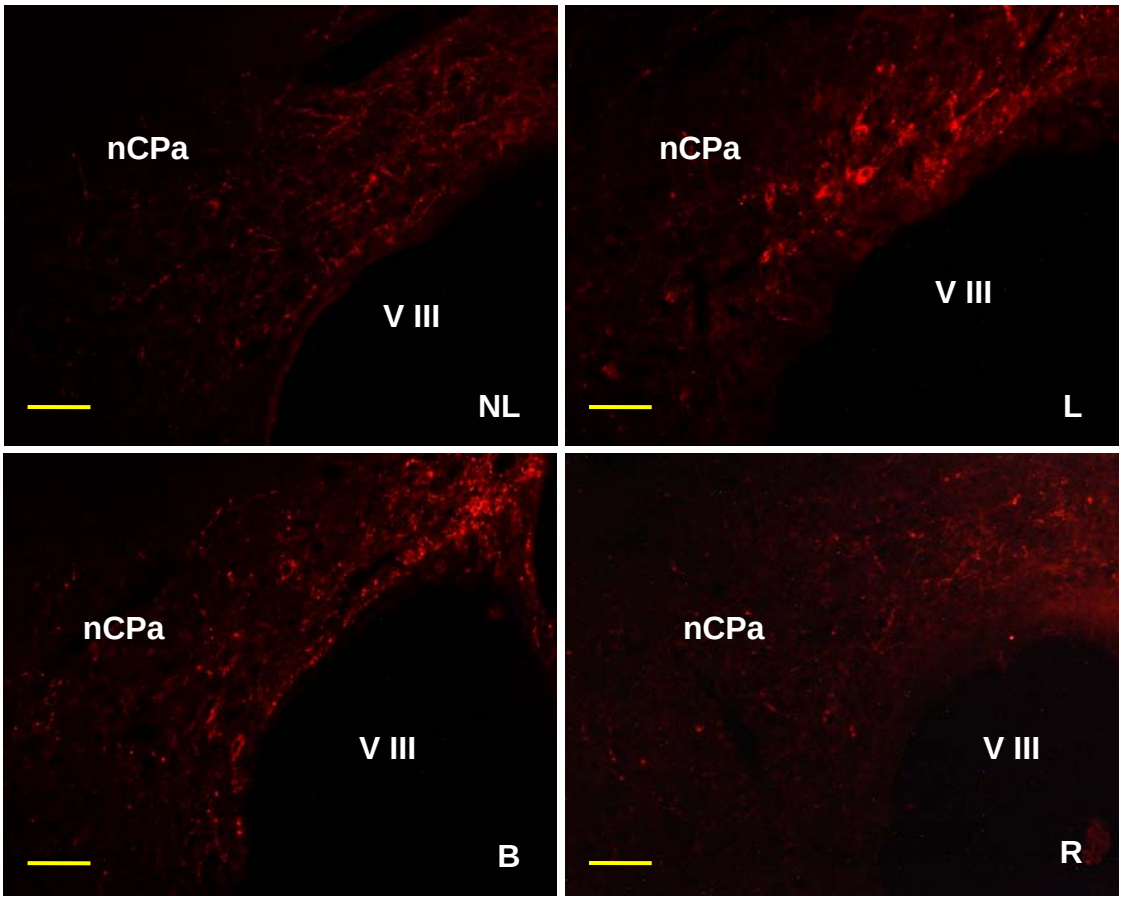

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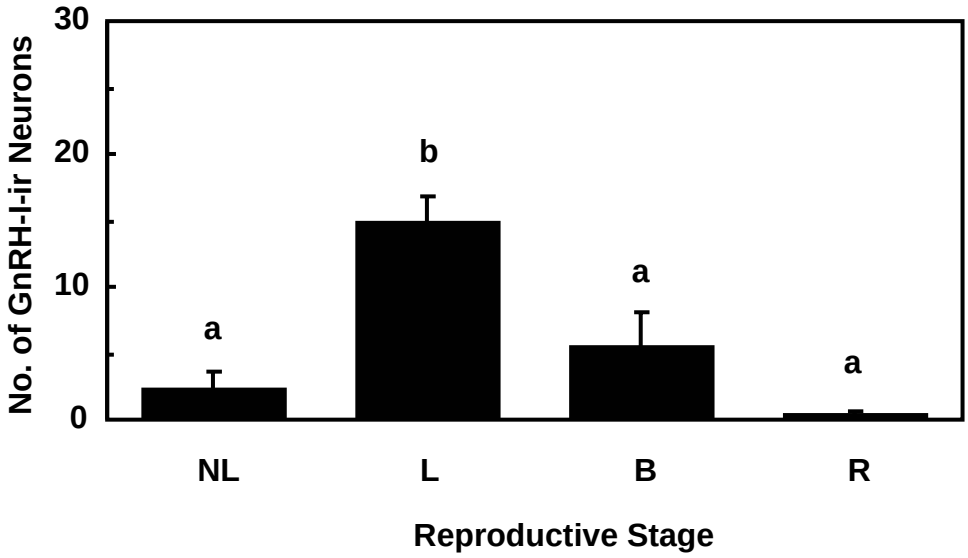
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