General and Comparative Endocrinology 159 (2008) 107–115

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

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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ARTICLE INFO

Article history:
Received 28 May 2008
Revised 1 August 2008
Accepted 3 August 2008
Available online 14 August 2008

Keywords:
Dopamine
Immunohistochemistry
Native Thai chicken
Reproductive cycle

ABSTRACT

Dopamine (DA) has a pivotal role in avian prolactin (PRL) secretion, acting centrally through D_1 DA receptors to stimulate PRL secretion by operating through vasoactive intestinal peptide (VIP). DA also inhibits PRL secretion by activating D_2 DA receptors at the pituitary level. This study was designed to investigate the distribution of DA neurons in the native Thai chicken, utilizing tyrosine hydroxylase (TH) as a marker for dopaminergic neurons. The differential expression of hypothalamic TH immunoreactive (TH-ir) neurons was also compared across the reproductive cycle. The results revealed that TH-ir neurons and fibers were found throughout the brain of the laying hen and were predominantly located within the diencephalon and mesencephalon. The observed distribution pattern of TH immunoreactivity was consistent with that reported previously in several avian species. However, changes in the number of TH-ir neurons in the nucleus intramedialis (nI) were observed across the reproductive cycle and correlated directly with variations in PRL levels. The population of TH-ir neurons in the nI increased significantly during the egg incubation period, where 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 native 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.

1. Introduction

Two neuroendocrine systems play a pivotal role in the reproductive cycle of birds. One system involves gonadotropin releasing hormone-I (GnRH-I) and the subsequent secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH; Sharp et al., 1998) and the other system involves the prolactin (PRL) releasing factor (PRF), vasoactive intestinal peptide (VIP) and the subsequent secretion of PRL (El Halawani et al., 1997; Chaiseha and El Halawani, 2005). Both systems are influenced by dopamine (DA; Bhatt et al., 2003).

Dopaminergic activity and DA receptor subtype mRNA expression changes according to different physiological states and reproductive behaviors. In bantam hens, dopaminergic activity in the anterior hypothalamus markedly increases in incubating birds when compared with laying or nest-deprived ones (Macnamee and Sharp, 1989). Furthermore, stimulatory D_1 DA receptor mRNA expression has been found to increase in the hypothalamus of incubating turkey hens with hyperprolactinemia and in the pituitary gland of laying hens. However, inhibitory D_2 DA receptor mRNA expression increases in the pituitary gland of photorefractory hens with hypoprolactinemia (Schnell et al., 1999a,b; Chaiseha et al., 2003). Changes in dopaminergic activity during the turkey reproductive cycle paralleled the changes in plasma PRL levels and VIP immunoreactivity, peptide content and mRNA expression within the infundibular nuclear complex (INF; El Halawani et al., 1980, 1984; Mauro et al., 1989; Wong et al., 1991; Chaiseha et al., 2003, 2004).

In birds, dopaminergic neurons are widely dispersed throughout the forebrain, midbrain and hindbrain, and are not located in a single discrete hypothalamic nucleus or region, but instead are dispersed among a variety of hypothalamic regions (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, with GnRH-I neurons in the preoptic area (POA), and with both VIP and GnRH-I terminals in the external layer of the eminentia mediana (median eminence,

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doi:10.1016/j.ygcen.2008.08.002

ME; Contijoch et al., 1992; Fraley and Kuenzel, 1993a; Al-Zailaie and El Halawani, 2000), 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-I/LH–FSH systems.

Recently, DA neurons found in the turkey hypothalamus, including the POA, nucleus mamillaris lateralis (ML) and nucleus premamillaris (PMM), have been proposed as a potential reproductive neuroendocrine circuit that controls reproductive seasonality in turkeys (temperate zone birds; Al-Zailaie et al., 2006; Kang et al., 2007; Thayananuphat et al., 2007a,b). In contrast to the temperate zone seasonal breeding species, the native Thai chicken is a continuously breeding species found in the equatorial zone that produces eggs all year, independent of photoperiodic cues. There are a limited number of studies providing data about the neuroendocrine regulation in this gallinaceous bird from the non-temperate zone. Importantly, there is no study delineating the anatomical distribution and functional aspect of the dopaminergic system in the native Thai chicken.

The aim of this study was to identify DA neuronal groups that may be associated with the reproductive regulatory system in the native Thai chicken. Therefore, using the rate-limiting enzyme tyrosine hydroxylase (TH) as a marker, the distribution of dopaminergic neurons throughout the brain was determined by immunohistochemistry. In addition, the correlation between changes in DA neuronal group(s) in the hypothalamic area and reproductive stage was investigated by determining changes in numbers of TH immunoreactive (TH-ir) neurons in the hypothalamus of chickens at different reproductive states. 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*) ranging between 16 and 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 h of light and 12 h of dark; 12L:12D). Feed and water were provided *ad libitum*. Birds were divided into four 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 confirmed by post-mortem examination. Birds were sacrificed according to their reproductive stages ($n = 5$). Briefly, Group NL hens had never laid eggs, Group L hens were in their first laying cycle and had been laying for 7 days, Group B hens had stopped laying and had been exhibiting incubating behavior for 10 days, and Group R hens had been rearing chicks for 14 days. Blood samples were withdrawn from a brachial vein to analyze plasma PRL levels as an aid to confirming reproductive condition. Laying hens were used to study the localization of TH-ir neurons and fibers throughout the brain. Changes in the number of TH-ir neurons in individual brain areas in hens in different 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 brachial vein of 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). The assay of plasma PRL levels in native Thai chickens was validated. Pooled plasma samples of native Thai chickens produced a dose–response curve that paralleled a chicken PRL standard curve. Plasma samples were determined in duplicate within a single assay. The intra-assay coefficient of variation was 5.0%.

2.3. Processing of tissues for immunohistochemistry

After collecting a blood sample, each bird was intravenously injected with 3 ml of heparin (Baxter Healthcare Corporation, Deerfield, IL, USA; 1000 U/ml) and then euthanized with pentobarbital sodium (Nembutal, Ceva Sante Animale, Libourne, France; 2 ml/kg). The head was removed and immediately fixed by pressure-perfusion via the carotid arteries. Reagents included 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 3–5 min, followed by freshly prepared 4% paraformaldehyde (pH 7.4) for 30 min according to a previously described method (Al-Zailaie et al., 2006). The brain with the pituitary gland attached was then removed from the skull and for cryo-protection was placed in 20% sucrose in PBS at 4 °C for 48 h or until saturated. The brain was then frozen in powdered dry ice for 1 h, and stored at −35 °C until sectioned. Frozen brains were sectioned in the coronal plane at a thickness of 16 μm using a cryostat (Leica CM1850, Leica Instruments GMbH, Nussioch, Germany). Sections were mounted onto gelatin-subbed slides with two sections per slide and stored desiccated at −20 °C until further processed for immunohistochemistry.

2.4. Immunohistochemistry

The methods used to determine TH distribution throughout the brain of the laying hen and within individual brain regions of hens in different reproductive stages has been previously described (Al-Zailaie et al., 2006). Briefly, tissue sections from different areas throughout the brains of laying hens ($n = 5$), as well as four adjacent sections from each hypothalamic area of the nucleus anterior medialis hypothalami (AM), nucleus paraventricularis magnocellularis (PVN), nucleus intramedialis (nI) and ML of hens ($n = 5$) in each reproductive stage, were placed in PBS for 30 min at room temperature. Each section was then 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 °C in a moisture chamber for 24 h. The next day, after removal of excess antibody, the sections were then washed three 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 to each section. The sections were further incubated in a moist dark chamber at room temperature for 1 h. The slides were then rinsed with PBS to stop the reaction, washed again three 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 4×, 10×, 20× and 40× magnification. Images were captured with a digital camera (Olympus DP70, Tokyo, Japan), and stored by DP70-BSW software (Olympus, Tokyo, Japan). The number of TH-ir neurons in four adjacent brain sections of the AM, PVN, nI and ML of hens in each reproductive stage ($n = 5$) were counted