FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 60, No. 2, 2022
pp. 146–155

ORIGINAL PAPER

VIA MEDICA

Distribution of neuropeptide Y in the brain of the male native Thai chicken

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Abstract

Introduction. Neuropeptide Y (NPY), a 36 amino acid neurotransmitter/neuromodulator, is involved in food intake and parental cares in birds. NPY is associated with the regulation of the reproductive system in the female native Thai chickens. However, the role of NPY in the male native Thai chicken has not been studied. Therefore, the objective of this study was to investigate the distributions of NPY immunoreactive (-ir) neurons and fibers in the brain of the male native Thai chickens.

Material and methods. The distribution of NPY-ir neurons and fibers in the hen brain was elucidated utilizing immunohistochemical technique.

Results. The distributions of NPY-ir neurons and fibers were located throughout the brain, predominantly in the hypothalamus. The numbers of NPY-ir neurons within the nucleus paraventricularis magnocellularis (PVN) were significantly higher than those of the nucleus septalis lateralis (SL), nucleus supraopticus (SOv), and nucleus inferioris hypothalami and nucleus infundibuli hypothalami (IH-IN). In addition, the numbers of NPY-ir neurons within the SL, SOv, and IH-IN were significantly higher than those of the tractus septomesencephalicus and nucleus dorsolateralis anterior thalami.

Conclusions. These results indicated, for the first time, that the distributions of NPY-ir neurons and fibers in the brain of the male native Thai chickens were markedly observed in the hypothalamus, especially within the PVN, implicating that the NPYergic system within the PVN might be related to the regulation of feeding behavior and parental cares in this equatorial species. *(Folia Histochemica et Cytobiologica 2022, Vol. 60, No. 2, 146–155)*

Keywords: birds; brain; hypothalamus; male; native Thai chicken; neuropetide Y; IHC

Introduction

Neuropeptide Y (NPY), a 36 amino acid neurotransmitter/neuromodulator, was first isolated and characterized from the porcine brain [1, 2]. Amino acid sequences of NPY are different by a single residue between mammalian and avian species. NPY has been found abundantly distributed in the central nervous systems of both birds and mammals [3, 4]. There are numerous reports regarding the physiological functions of NPY in birds such as food intake regulation [5–7], sexual maturation [8, 9], body temperature and heat stress regulation [10–12], insulin secretion [13], gonadotropin-releasing hormone (GnRH) secretion [14, 15], parental hyperphagia [16], and food restriction [17, 18].

In birds, NPY is one of the most potent orexigenic regulators of food intake [5, 19–22]. Food deprivation enhances expression of NPY mRNA [23], neuron activity [24], and NPY contents in the nucleus paraventricularis magnocellularis (paraventricular nucleus; PVN) [25–27]. Up to date, it has been reported that

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Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0016
ISSN 0239-8508, e-ISSN 1897-5631

NPY is involved in the regulation of the reproductive cycle and maternal behaviors in female native Thai chickens [28, 29].

The anatomical distributions of NPY-immunoreactive (-ir) neurons and fibers in the brain of several avian species have been investigated including chicken [13, 14, 30, 31], quail [30, 32], pigeon [33, 34], black-capped chickadee, blue tit, great tit, dark-eyed junco [35], collared dove [36], domestic duck [37], and female native Thai chicken [28, 29]. The distributions of NPY neurons and fibers have been mapped in several avian species and were found located in several regions of the brain such as the septalis lateralis (SL), nucleus suprachiasmaticus, pars medialis (SCNm), nucleus periventricularis hypothalami (PHN), regio lateralis hypothalami (LHy), PVN, nucleus rotundus (ROT), nucleus inferioris hypothalami (IH), and nucleus infundibuli hypothalami (IN) [13, 14, 31–37]. Similarly, in the female native Thai chickens, NPY-ir neurons and fibers are extensively distributed throughout the brain. The numbers of NPY-ir neurons within the PVN are low in non-egg laying and egg-laying stages and then markedly increase during incubating eggs and rearing chick stages and changes in body weight are found in the opposite direction of changes in the numbers of NPY-ir neurons across the reproductive stages [28]. Moreover, it has been suggested that the numbers of NPY-ir neurons within the PVN play a pivotal role in the transition from incubating to rearing behavior in the female native Thai chickens [29].

Native Thai chicken (*Gallus domesticus*), an equatorial, tropical, non-seasonally breeding species, has been domesticated without genetic selection. It expresses strong maternal behaviors which are inherited from the ancestor, the wild jungle fowl in Southeast Asia [38–41]. It is well documented that the neuroendocrine regulation of the reproductive cycle and maternal behaviors is associated with GnRH, vasoactive intestinal peptide (VIP), dopamine, mesotocin (MT), and NPY in the female native Thai chickens [28, 29, 42–45]. As aforementioned, the neuroendocrine regulation of reproductive behaviors has been extensively studied in the female native Thai chickens. However, there are limited data regarding the neuroendocrine regulation of reproductive activities in males. It has been reported that changes in the numbers of VIP-ir neurons within the IH-IN are observed across the reproductive stages and mirrored directly with circulating PRL and testosterone levels in the male native Thai chickens [46]. The distributions of MT-ir neurons and fibers have also been reported, suggesting that MT-ir neurons within the nucleus supraopticus, pars ventralis (SOv) and nucleus preopticus medialis might play an important role in the reproductive activities and/or parental behaviors in the native Thai roosters [47]. Presently, the distribution of TH immunoreactivity within the nucleus intramedialis and nucleus mamillaris lateralis might be involved in the reproductive activities of the male native Thai chickens [48]. It has been well documented that male birds exhibit parental behaviors such as nest building, brooding, protection, and feeding of the young in many species [42, 49]. Interestingly, Ramakrishnan *et al.* [16] reported that NPY plays various physiological functions involving courtship and nest building interactions in breeding doves. To date, there has been no report regarding the physiological role(s) of the NPYergic system in the male native Thai chicken. Thus, the objective of this study was to investigate the localization of the NPY neuronal groups in the brain of the male native Thai chickens, enabling further studies of neuroendocrinology related to behaviors. The findings of the distributions of NPY-ir neurons and fibers might be related to the regulation of food intake and/or paternal behaviors in the male native Thai chickens.

Material and methods

Experimental animals. Male native Thai chickens (Gallus domesticus), 12 months of age, were used. They were reared and housed together with mature females (1 male: 8 females) in floor pens equipped with basket nests under natural daylight (approximately 12 h of light and 12 h of darkness; 12L: 12D). Feed and water were provided ad libitum. The animal protocols used adhered to the guidelines approved by the Suranaree University of Technology Animal Care and Use Committee, Thailand.

Experimental design. To determine the distributions of NPY-ir neurons and fibers in the brain of the male native Thai chicken, 6 mature males were used. The brains of mature roosters were fixed by pressure perfusion with a freshly prepared 4% paraformaldehyde (Code No. 416780010, Lot No. A0331790, Acros Organics, Inc., New Jersey, NJ, USA) in 650 mL of 0.1 M phosphate-buffered saline (PBS; pH 7.4). Tissue sectioning was performed in the coronal plane at a thickness of 16 μm utilizing a cryostat and further processing by immunohistochemistry (IHC) technique according to a previously described method [28, 48].

Tissue preparation and immunohistochemistry. To elucidate the distributions of NPY-ir neurons and fibers, brain tissue sections were prepared and IHC was performed as previously described [28]. The primary and secondary antibodies used for detecting NPY immunoreactivity were primary rabbit polyclonal antibody directed against NPY

10.5603/FHC.a2022.0016
ISSN 0239-8508, e-ISSN 1897-5631

(Catalog No. 22940, Lot No. 812001, ImmunoStar, Inc., Hudson, WI, USA) and Cy™3-conjugated AffiniPure donkey anti-rabbit IgG (Code No. 711-165-152, Lot No. 98888, Jackson ImmunoResearch Laboratories, West Grove, PA, USA), respectively. The specificity of the primary antibody used has been validated in previous studies [50–52] and has been employed to localize NPY-ir neurons in birds [28, 29, 35]. To rule out non-specific labeling, the IHC was carried out with the same protocol omitting the primary antiserum [28]. Briefly, tissue sections were thawed to room temperature before use. They were treated with PBS (pH 7.4) for 30 min at room temperature. After PBS removal, the sections were then incubated with 60 μL of primary antibody at 1:1000 dilution with PBS containing 1% bovine serum albumin (Catalog No. 268130100, Lot No. A0324472, Acros Organics, Inc.) and 0.3% Triton-X 100 (Catalog No. 215680010, Acros Organics, Inc.) at 4°C overnight in a moist chamber. The slides were washed three times with PBS (pH 7.4) for 5 min each. After washing, 60 μL of secondary antibody at 1:500 dilution was applied to the sections under dark conditions. The slides were further incubated in a moist, dark, chamber at room temperature for 1 h, washed with PBS (pH 7.4) 3 times for 5 min each, and then mounted with DPX mountant (Product No. 06522, Lot No. BCBH3443V, Sigma-Aldrich, Inc., Steinheim, Germany). Microscopic images of brain sections were visualized and further analyzed.

Image analysis. Microscopic images of the brain sections were visualized under a fluorescence microscope (Nikon ECLIPSE 80i, Tokyo, Japan) fitted with a cooled digital color camera (Nikon DS-Fi1, Tokyo, Japan). The images were captured and stored by NIS-Elements Documentation software (Nikon, Tokyo, Japan). NPY-ir neurons and fibers in each individual area of the brain were visualized and analyzed. The numbers of NPY-ir neurons of six adjacent sections for each rooster (6 roosters per area) were counted manually to determine changes in the numbers of NPY-ir neurons within the individual areas (SL, SOv, tractus septomesencephalicus (TSM), PVN, nucleus dorsolateralis anterior thalami, pars magnocellularis (DLAmc), and IH-IN). To avoid double-counting neurons with cell bodies that appeared on two adjacent sections, sections were viewed under 400× magnification, and only NPY-ir neurons with detectable nuclei or showing the appropriate shape (round and smooth-edged) were included in the analysis. The mean values were compared across the hypothalamic areas [53]. The mean values were then compared across the individual areas [28]. Atlas of the chick brain [13] and the chicken hypothalamus [54] were used for the identification of specific brain regions of NPY immunoreactivity.

Statistical analysis. Significant differences among the numbers of NPY-ir neurons within the SL, SOv, TSM, PVN, DLAmc, and IH-IN (mean ± SEM) were compared by employing a one-way analysis of variance (ANOVA). Significant differences between each individual hypothalamic area were computed utilizing the Tukey's HSD test. $P < 0.05$ was considered statistically significant. All statistical tests were analyzed using SPSS for Windows software (version 17.0, SPSS Inc., Chicago, IL, USA).

Results

The results of this study revealed that the distributions of NPY-ir neurons and fibers were located throughout the brain of the native Thai roosters. NPY-ir neurons and fibers were predominantly located within the hypothalamus. NPY-ir fibers were extensively distributed in the diencephalon and very dense fibers were observed in the internal and external layers of the eminentia mediana (median eminence, ME; Fig. 1).

NPY immunoreactivity were extensively located along the ventriculus tertius (third ventricle; V III), also distributed in a discrete region lying close to the V III through the hypothalamus within the diencephalon (Figs. 2–4). The distributions of NPY-ir neurons and fibers were found within the SL (Fig. 2A), SOv (Fig. 2B), TSM (Fig. 2C), PVN (Fig. 2D), DLAmc (Fig. 2E), and IH-IN (Fig. 2F). Higher magnification of NPY-ir neurons within the PVN and SOv was illustrated (Fig. 3). Moreover, the numbers of NPY-ir neurons in six areas including the SL, SOv, TSM, PVN, DLAmc, and IH-IN were compared (Fig. 4). The numbers of NPY-ir neurons were significantly higher ($P < 0.05$) within the PVN (10.21 ± 0.67 cells) when compared with the SL (3.83 ± 0.24 cells), SOv (3.83 ± 0.22 cells), and IH-IN (3.63 ± 0.26 cells). The numbers of NPY-ir neurons within the SL, SOv, and IH-IN were significantly higher than those of the TSM (1.92 ± 0.12 cells) and DLAmc (1.92 ± 0.38 cells).

In the telencephalon, the greatest density of NPY-ir fibers was distributed within the nucleus commissurae pallii (nCPa, Fig. 5A), nucleus suprachiasmaticus, pars medialis (SCNm, Fig. 5B), and commissura pallii (CPa, Fig. 5C). These fibers were large and found closely to the midline on both sides of the V III. A modest density of NPY-ir fibers was found within the PHN (Fig. 5D). In the diencephalon, a few of NPY-ir fibers were found within the LHy (Fig. 5E), nucleus rotundus (ROT, Fig. 5F), and densely packed fibers were present in the internal and external layers of ME (Fig. 5G).

Discussion

The results of this study demonstrated the distributions of NPY immunoreactivity in the brain of the male native Thai chickens. NPY-ir neurons and

10.5603/FHC.a2022.0016
ISSN 0239-8508, e-ISSN 1897-5631

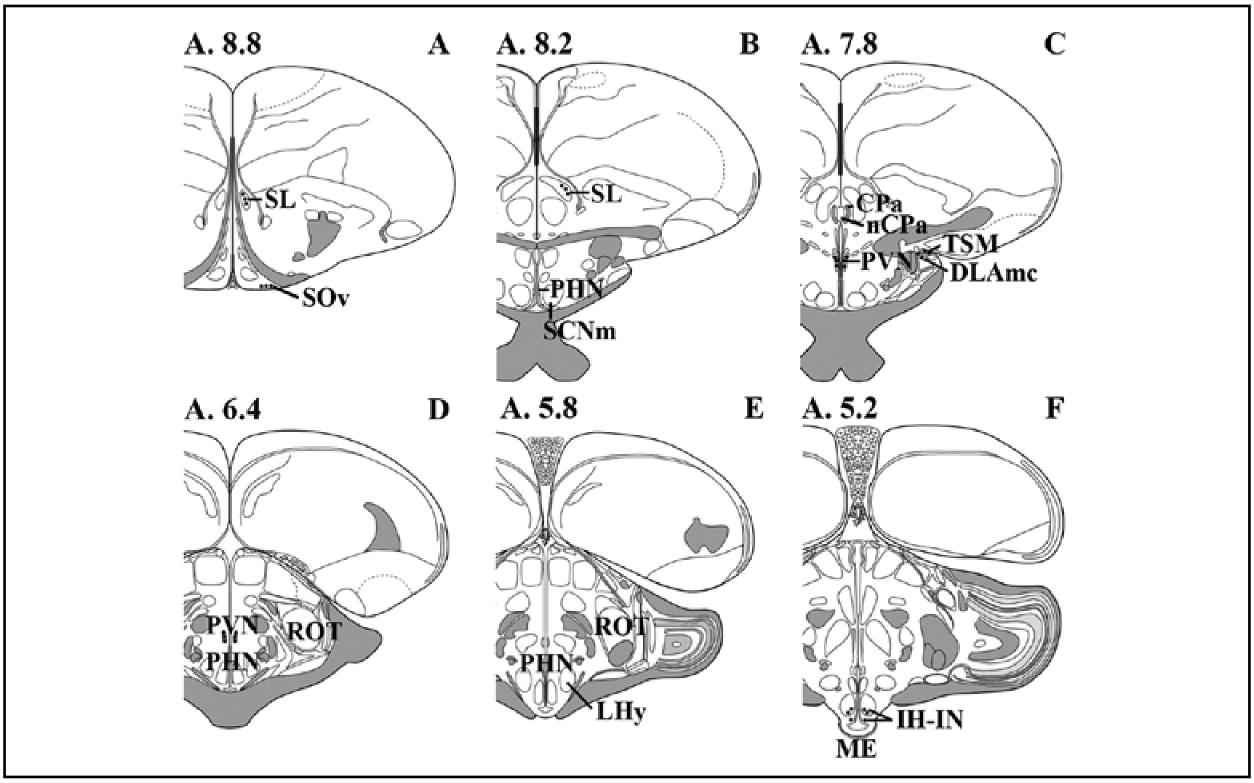

Figure 1. Schematic diagrams of coronal sections illustrating the areas of the chick brain showing the distributions of NPY-ir neurons (black dots) and fibers throughout the brain of the male native Thai chicken. Coronal illustrations were redrawn from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988). The following abbreviations are used in the Figure legends: CPa — commissura pallii; DLAmc — nucleus dorsolateralis anterior thalami, pars magnocellularis; IH — nucleus inferioris hypothalami; IN — nucleus infundibuli hypothalami; LHy — regio lateralis hypothalami; ME — eminentia mediana (median eminence); nCPa — nucleus commissurae pallii; SL — nucleus septalis lateralis; PHN — nucleus periventricularis hypothalami; PVN — nucleus paraventricularis magnocellularis; ROT — nucleus rotundus; SCNm — nucleus suprachiasmaticus; SOv — nucleus supraopticus, pars ventralis; TSM — tractus septomesencephalicus; VIII — third ventricle.

fibers were extensively located throughout the brain, predominantly in the hypothalamus and lying close to the midline on both sides of the V III of the hypothalamus. The distributions of NPY-ir neurons and fibers were found within the SL, SOv, TSM, PVN, DLAmc, and IH-IN. Moreover, the numbers of NPY-ir neurons within the PVN were significantly higher than those of the SL, SOv, and IH-IN. The numbers of NPY-ir neurons within the TSM and DLAmc were significantly lower when compared with those of the SL, SOv, and IH-IN. Dense clusters of NPY-ir fibers were innervated within the nCPa, SCNm, CPa, and very dense fibers were observed in the internal and external layers of ME. A modest density of NPY-ir fibers was found within the PHN. A few labelled fibers were observed within the LHy and ROT as well. These present findings suggested that the NPYergic system within the PVN might be associated with the physiological function(s) of reproductive activities in the male native Thai chickens.

The anatomical distributions of NPY-ir neurons and fibers in this present study are in accordance with previous studies that indicated the distributions of NPY neurons and fibers throughout the avian brain including chicken [13, 14, 31], quail [32], pigeon [33, 34], collared dove [36], black-capped chickadee, blue tit, great tit, dark-eyed junco [35], domestic duck [37], and female native Thai chickens [28, 29].

In this study, NPY immunoreactivity was distributed within the SL, SOv, TSM, PVN, DLAmc, and IH-IN. The prominent groups of NPY immunoreactivity were found within the PVN. These results are in accordance with previous reports in the female native Thai chickens as well [28, 29] and other species such as domestic chicken [13, 31], quail [32], pigeon [34], and collared dove [36]. In the female native Thai chickens, changes in the numbers of NPY-ir neurons within the PVN are mirrored directly with bodyweight across the reproductive stages. The highest accumulation of NPY-ir neurons is found during incubating eggs and rearing chick stages when the hens are naturally fasting [28, 29]. A modest density of NPY-ir neurons and fibers was observed within the SL, SOv, and IH-IN, and these results are in good agreement with the

10.5603/FHC.a2022.0016
ISSN 0239-8508, e-ISSN 1897-5631

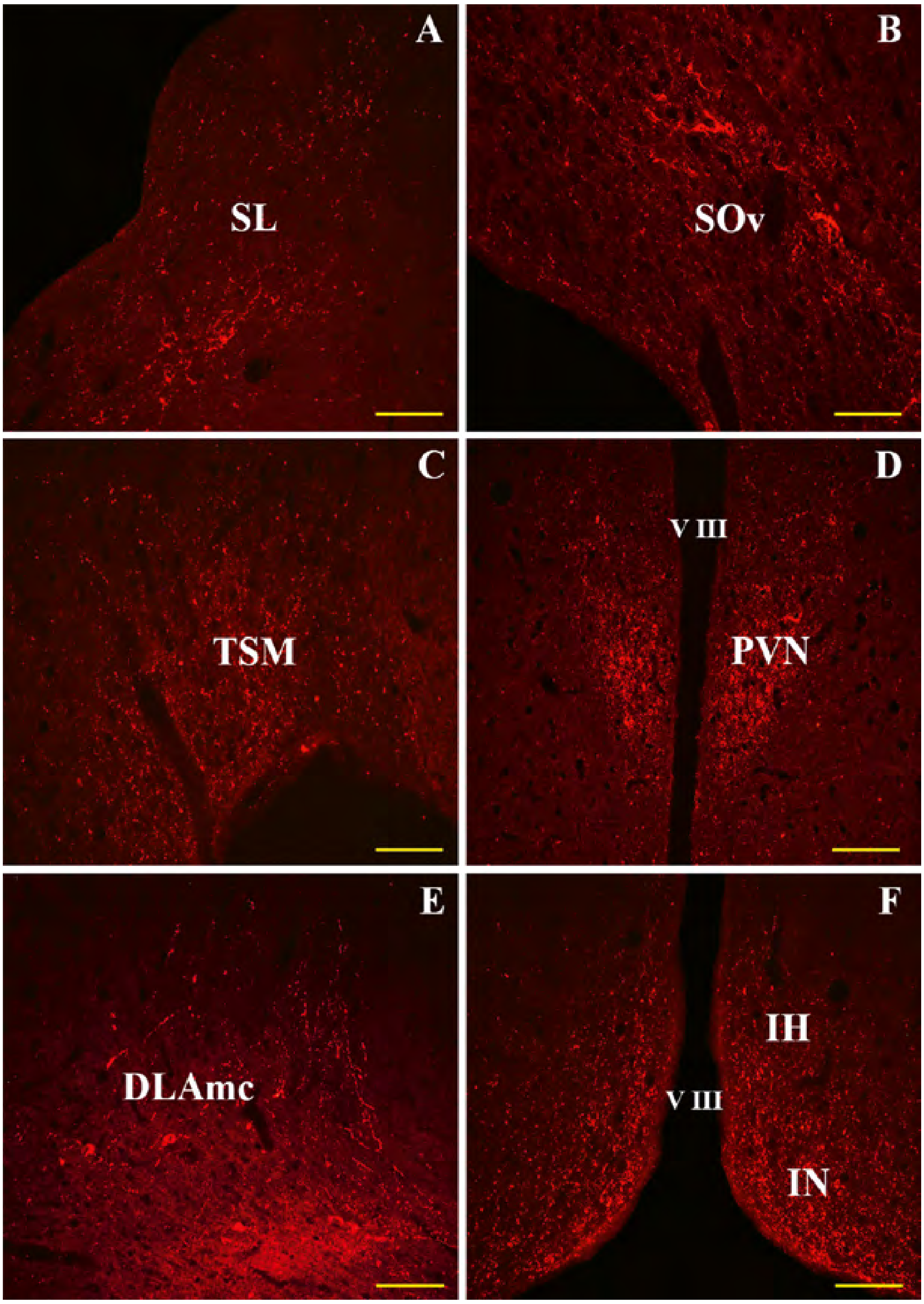

Figure 2. Photomicrographs illustrating the distributions of NPY-ir neurons and fibers within the (**A**) SL, (**B**) SOv, (**C**) TSM, (**D**) PVN, (**E**) DLAmc, and (**F**) IH-IN of the male native Thai chicken. Scale bar = 100 μm. See Fig. 1 for a description of the abbreviations.

previous report on the fasted native Thai chickens. It has been reported that NPY-ir neurons and fibers are found within the SL, but the neuron of NPY is not detected within the SOv [28, 29]. In other avian species, NPY mRNA was found within the SL in chicken [55]. Interestingly, NPY-ir neurons and fibers within the SL were not observed in pigeons [33], collared doves [34], and domestic chickens [31]. Thus, it is possible that the occurrence of NPY neurons in those areas is sex- and species-specific. In this study, a few of NPY-ir neurons and fibers were distributed within the TSM and DLAmc as the previous report in the female native Thai chickens [28, 29]. Thus, there are no differences in the distribution of NPY-ir neurons between male and female brains. In breeding birds, the numbers of NPY-ir neurons and NPY mRNA

10.5603/FHC.a2022.0016
ISSN 0239-8508, e-ISSN 1897-5631

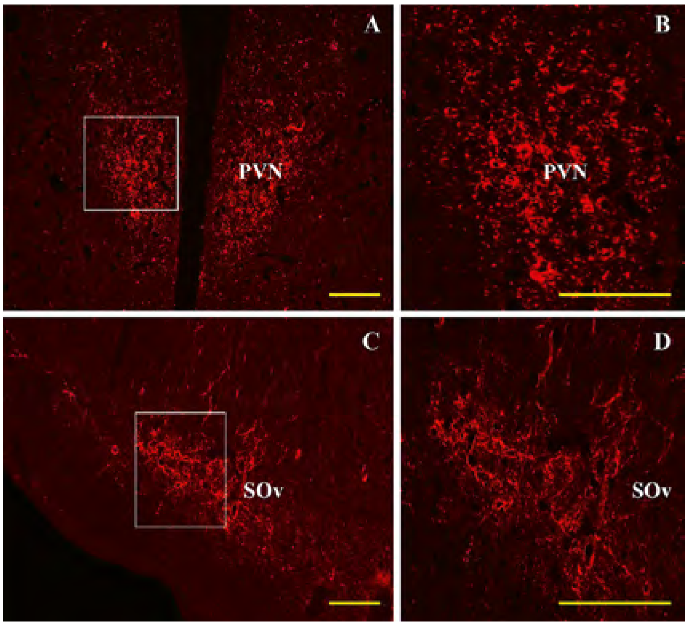

Figure 3. The distributions of NPY-ir neurons and fibers within the (**A**) PVN and (**C**) SOv of the male native Thai chicken. Rectangles indicate areas from which higher magnification photomicrographs were taken in the (**B**) PVN and (**D**) SOv. Scale bar = 50 μm. See Fig. 1 for a description of the abbreviations.

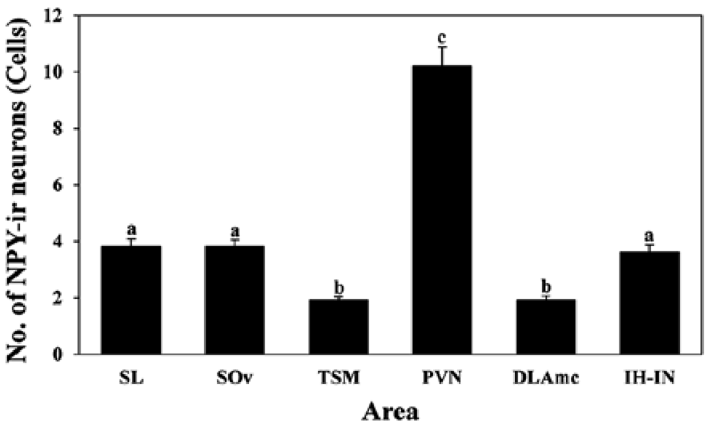

Figure 4. The numbers of NPY-ir neurons within the SL, SOv, TSM, PVN, DLAmc, and IH-IN of the male native Thai chicken. Significant differences between values (mean ± SEM) in each hypothalamic nuclei are indicated by different letters ($P < 0.05$).

levels in the hypothalamus are high in breeding doves during the period of parental hyperphagia after hatching when compared with the late incubation period when food intake remains unchanged [16]. Likewise, in a seasonal breeding photoperiodic bird, food restriction negatively affects levels of the hypothalamic contents of NPY immunoreactivity in the male house finches [18].

Dense clusters of NPY-ir fibers in this study were innervated within the nCPa, SCNm, CPa, and in the internal and external layers of the ME. A modest density of NPY-ir fibers was found within the PHN. Small numbers of NPY-ir fibers were also found within the LHy and ROT. These results are in accordance with previous reports in laying and fasted native Thai chickens [28, 29]. Interestingly, Sartsoongnoen *et al.* [28] did not find the distributions of NPY-ir fibers within the LHy in laying and fasted hens. It has been reported that NPY mRNA levels in the LHy involved in the regulation of food intake and body weight changes in the domestic chicken [56, 57]. However, there was some specific species difference in which NPY-ir fibers were not observed within the PHN of the quail brain [32]. Thus, it is possible that differences in the distributions of NPY-ir neurons and fibers also depend upon the species. Youngren *et al.* [58]

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Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0016
ISSN 0239-8508, e-ISSN 1897-5631

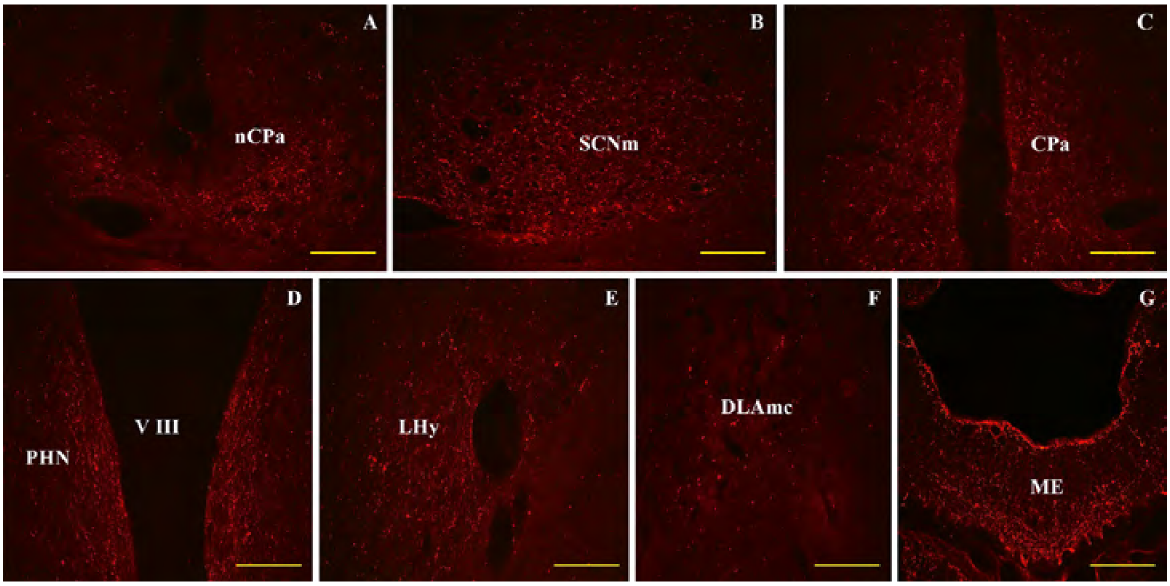

Figure 5. Photomicrographs illustrating the distributions of NPY-ir fibers within the (**A**) nCPa, (**B**) SCNm, (**C**) CPa, (**D**) PHN, (**E**) LHy, (**F**) DLAmc, and (**G**) ME. V III, ventriculus tertius (third ventricle). Scale bar = 100 μm. See Fig. 1 for a description of the abbreviations.

reported that variations in VIP immunoreactivity and VIP mRNA steady-state levels occurring within the hypothalamus, VIP peptide contents in the ME, and plasma VIP levels in hypophysial portal blood are correlated with changes in the circulating PRL levels throughout the turkey reproductive cycle [59–61]. Given their widespread distributions in the previous study [48], DA immunoreactivity in the hypothalamus whose axons and fibers project to the ME, demonstrates that DA neurons and their fibers are found intermingled with VIP neurons in the INF, GnRH neurons in the preoptic area, and with both VIP and GnRH terminals in the external layer of the ME [62, 63]. In this present study, NPY-ir immunoreactivity was also extensively located in the hypothalamus and lying close to the midline on both sides of the V III. In addition, the NPY-ir fibers were distributed extensively in the ME. Thus, it is possible that the discovery of a large population of NPY immunoreactivity in the hypothalamus whose axons and fibers project to the ME led to the hypothesis that NPY participates in the regulation of pituitary functions, probably *via* PRL regulation, involving reproductive activities and food intake regulation [64–66].

In conclusion, these present findings revealed the distributions of NPY immunoreactivity throughout the brain of male native Thai chickens. The greatest density of NPY-ir neurons and fibers was found within the PVN, suggesting that NPY neurons in this nucleus may be involved with the physiological function(s) of feeding and reproductive activities in this equatorial species.

Acknowledgments

This work was supported by (i) Suranaree University of Technology (SUT), (ii) Thailand Science Research and Innovation (TSRI), and (iii) National Science, Research and Innovation Fund (NSRF) (Project code 90464; Contract number Full-time 61/23/2564 to B. Kamkrathok). The authors thank Ms. Intu-orn Ayamuang for conducting some part of the immunohistochemistry technique. The authors would like to thank the University Farm of Suranaree University of Technology for providing the chicken barns.

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Submitted: 3 March, 2022
Accepted after reviews: 26 May, 2022
Available as AoP: 6 June, 2022