ハシブトガラス(Corvus macrorhynchos)副腎における形態計測学的および免疫組織化学的検討

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<tr>
<td>誌名</td>
<td>宇都宮大学農学部学術報告 = Bulletin of the College of Agriculture, Utsunomiya University</td>
</tr>
<tr>
<td>ISSN</td>
<td>05664691</td>
</tr>
</tbody>
</table>
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| 巻/号       | 23巻1号                                                             |
| 掲載ページ   | p. 1-8                                                              |
| 発行年月     | 2012年3月                                                           |
ハシプトガラス (Corvus macrorhynchos) 副腎における形態計測学的および免疫組織化学的検討

A. K. M. Humayun Kober1,2・松田奏尚1・青山真人1・杉田昭栄1

ハシプトガラスの副腎について、形態計測学的および免疫組織化学的検討、すなわち、肉眼的観察、光学顕微鏡による組織解析、皮質と髄質の分布様式的解析、皮質と髄質の割合測定、そしてカテゴールアミン合成に関わる酵素であるチロシンヒドロキシラーゼ (TH) の免疫組織化学的解析を行なった。肉眼的観察の結果、ハシプトガラスの副腎の形状、重量、長さ、幅、厚さは、左右で異なっていた。クロム染色の結果、ハシプトガラスの副腎におけるクロム親和性組織 (副腎髄質) と親和性を持たない組織 (皮質) は、哺乳類のように明確な層を成しておらず、混在した構造を成していた。副腎のおよそ 85％が皮質、14％が髄質、残り 1％は類洞であっただけでなく染色の結果、皮質の組織は比較的小さい (直径約 3.4 μm) 卵形状の核を有し、柱状の細胞が配列した構造を成していた。髄質の組織は、柱状の細胞が様々な大きな集合体となり、副腎組織中に島状に散在していた。髄質の細胞の核は、比較的大きく (直径約 4.0 μm)、円形を呈し、各細胞のほぼ中央に位置していた。免疫組織化学の結果、TH 免疫陽性細胞は、クロム親和性細胞と同様の分布様式を示し、全ての副腎髄質細胞が TH 様性であると考えられた。以上まとめると、ハシプトガラスの副腎は、皮質と髄質の割合が約 6:1 であり、副腎髄質細胞はおそらく全てが TH を合成するもののと考えられた。

キーワード：副腎、皮質と髄質の割合、クロム親和性、チロシン水酸化酵素、ハシプトガラス
Histomorphological and immunohistochemical studies of the adrenal gland of the jungle crow (Corvus macrorhynchos)

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Summary

The adrenal gland is a complex organ that regulates many physiological functions. The morphological and histological study of the avian adrenal gland have been described previously in domestic bird species, but this organ of the jungle crow (Corvus macrorhynchos) has not been examined. In this study, the adrenal gland of the jungle crow was investigated using histomorphological and immunohistochemical methods to determine its gross morphology, general histology, medullary tissue distribution, the cortico-medullary ratio, and the distribution of the tyrosine hydroxylase (TH) catecholamine biosynthetic enzyme. Our analyses showed that the left and right adrenal glands differed significantly in shape, location, weight, length, width and thickness. Dictromate staining showed that the adrenal parenchyma was composed of intermingled cortical (interrenal) and medullary (chromaffin) tissues, with no histological distinction between an outer cortex and inner medulla. Approximately 85% of the adrenal parenchyma was composed of cortical tissue, 14% was medullary tissue, and the remaining 1% was sinusoids. Thus, the jungle crow has an adrenal cortico-medullary ratio of approximately 6:1. Azan staining showed that the cells in the cortical tissue were columnar with comparatively small, oval and eccentric nuclei 3.4 μm in diameter. The medullary tissue formed almost complete meshwork of spotted pattern; it seems as the scattered islets with various sizes. The medullary cells were cylindrical, with comparatively large, rounded or globular, and centrally-placed nuclei of 4.0 μm in diameter. Immunohistochemical staining showed that the TH enzyme was present in chromaffin cells of the adrenal medulla and that almost all of the chromaffin cells were stained.

Key words: Adrenal gland, cortico-medullary ratio, chromaffin tissue, tyrosine hydroxylase, jungle crow

1. Introduction

The avian adrenal gland is a complex organ responsible for the production of a wide variety of hormones. It also regulates many physiological functions, including stress and immune responses, inflammation, carbohydrate metabolism, protein catabolism, blood electrolyte levels, and behavior. Avian adrenal glands are small, yellowish, ovoid or triangular glands, located bilaterally at the anterior pole of the metanephric kidney, just anterior to the postcaval vein. The weight, length, width and thickness of adrenal gland can vary between species, and are also influenced by breed, age, health and environment. The adrenal medulla secretes catecholamines in response to stress, and synthesis of these compounds is controlled by the rate-limiting enzyme, tyrosine hydroxylase (TH). In the chicken, immunohistochemical analysis has shown that all adrenal medullary cells are TH immunoreactive.

The structure and function of the adrenal gland of various bird species have been described previously, for example, chicken, pigeon, goose, quail, duck, and African ostrich. However, most of these studies focused on domesticated bird species, and the adrenal gland of the jungle crow (Corvus macrorhynchos) has not been examined. The structure of the avian adrenal gland is also known to change with the physiological status of the bird. The jungle crow is a wild species and inhabits a very different environment to those of domesticated birds. The histomorphological characteristics of their adrenal glands may reflect variations in the level of production of the catecholamines that are involved in stress or physiological responses to the environment. Catecholamines in the adrenal gland are synthesized by TH; however, to the best of our knowledge, the cellular localization of TH in the adrenal gland of the jungle crow has not been determined. Here, we investigated the cytophysiology of the adrenal gland of the jungle crow to determine its histomorphology and also immunohistochemical distribution of TH.

2. Materials and methods

Collection of experimental birds

Seventeen healthy adult jungle crows (9 males, 8 females)
weighing 600-800 g were used for this study. The birds were caught in the city of Niza, in Saitama prefecture, Japan and at the Experimental Farm of Utsunomiya University located in the city of Moka, Japan. All the crows were cared for in accordance with the guidelines for the care and use of laboratory animals at Utsunomiya University. The catching of crows was permitted by Saitama (Permit for the Catching of Wild Animals No. 0010) and Tochigi prefectures (Permit No. 0010). The birds were identified as adults on the basis of the rudimentary size of the bursa of Fabricius and the black color of the upper palate of the beak. All procedures involving animals were carried out in accordance with the Animal Protection Regulations of Japan.

Tissue processing and morphometric measurements of the adrenal gland

For Azan staining and immunohistochemical study, 12 crows (6 of each sex) were sacrificed using an overdose of pentobarbital sodium (30 mg/kg body weight) (Dainippon Pharmaceutical, Osaka, Japan). The birds were perfused transcardially with Ringer’s solution and Zamboni’s solution (consisted of 2 % paraformaldehyde, 0.2 % picric acid, 0.1 M phosphate buffer with pH 7.4), then the paired adrenal glands were removed. The adrenal glands were weighed on an electronic balance; the length of each adrenal gland was measured through the longitudinal axis using slide calipers, width was measured at the thickest portion of the gland, and the maximum thickness was measured dorsoventrally. The adrenal glands were then post-fixed with Zamboni’s solution for 1 week at 4°C. Tissue blocks were prepared and processed for paraffin wax embedding. Serial sections were cut at a thickness of 5 µm in the coronal plane using a microtome (Sakura Sledge Microtome IVS-400; Sakura Seiki, Tokyo, Japan) and mounted on glass slides. Randomly selected sections were stained with Azan stain 23). All sections were observed under a light microscope (Olympus digital camera DP12; Olympus Corporation, Tokyo, Japan).

Formal-dichromate staining and calculation of adrenal cortico-medullary ratio

Five adult jungle crows (3 males, 2 females) were used to evaluate the relative proportions of the cortex and medulla and to examine the patterns of medullary tissue in the adrenal gland. After collection, the adrenal glands were incubated in a chrome staining solution composed of a 10:1 mixture of 5% potassium dichromate and 5% potassium chromate for 48 hours 46. Only the adrenal medulla demonstrates the chromaffin reaction. Thereafter, the glands were incubated for 24 hours in 10% formalin and for 24 hours in 30% sucrose. Finally, 30 µm coronal serial sections were cut using a freezing microtome and mounted on gelatin-coated glass slides. The relative proportions of the different tissue components were evaluated in each selected section that included the whole adrenal gland. Cross sections of the adrenal gland were photographed (Olympus digital camera DP12), scanned and printed, and then traced on paper. Medullary tissue was marked as black areas on the trace, sinusoids were marked as black circles, and the cortical tissue was left white. The total area of the cross section, and the area of medullary tissue and sinusoids were measured using ImageJ software (National Institutes of Health, Bethesda, MD). The cortical area was then estimated by subtracting the combined area of the medullary tissue and sinusoids from the total area. The values for the proportions of each area (cortical, medullary and sinusoids) were averaged from two sections for each bird, and expressed as means ± standard error (SE).

Measurement of diameter of the cell nuclei of adrenal gland

Six adult jungle crows (3 males, 3 females) were used to measure the diameters of the cell nuclei in the cortical and medullary tissues. The diameter of the cell nuclei were quantified from 100× photographs (Olympus digital camera DP12) of Azan stained sections using ImageJ software. The diameters were calculated by averaging the values from 50 randomly selected cortical and medullary cell nuclei from each bird. Values are expressed as means ± SE.

Immunohistochemical staining

Simple immunohistochemical staining was performed using the avidin-biotin complex (ABC) method 12) in seven birds (4 males, 3 females). Briefly, after dewaxing, rehydrated sections were treated with 3% hydrogen peroxide (H₂O₂) in methanol for 30 minutes at room temperature to block endogenous peroxidase activity, followed by rinsing for 15 minutes in 0.01 M phosphate buffered saline (PBS) at pH 7.4. Background staining was prevented by incubating in 2% normal goat serum and 2% bovine albumin. The sections were incubated at 40C for 24 hours in rabbit anti-bovine tyrosine hydroxylase (TH) polyclonal antibodies (LS-C 232; Lifespan Biosciences Inc., WA) diluted to 1:500-1000 with PBS (pH 7.4) containing 2% normal goat serum and 2% bovine albumin. Subsequently, the sections were rinsed 3 times in PBS for 15 minutes. The sections were incubated for 20 minutes in biotinylated goat anti-rabbit IgG (BA-1000; Vector Laboratories, Inc., CA) diluted 1:500 in PBS. Thereafter, the sections were incubated for 20 minutes in ABC solution (PK-4000; Vector Laboratories, Inc., CA). The immunoreactions were visualized using a freshly prepared solution of 3,3’-diaminobenzidine tetrahydrochloride (DAB, Dojin Laboratories, Kumamoto, Japan) (50 mL of 0.05 M Tris-HCl buffer pH 7.6 containing 10 mg DAB, 0.15 g ammonium nickel and 0.03% H₂O₂). Lastly, all sections were dehydrated through an alcohol series and mounted. The sections were observed using a light microscope (Olympus BX51). Some sections were incubated without primary antibody as negative controls. We did not find any expression in the control sections.
**Adrenal gland histology**

The adrenal glands of the jungle crow are small, brownish-yellow to grayish-yellow organs located next to the kidneys and are associated with the gonads (Fig. 1). The right adrenal gland (Fig.1a) often had a triangular shape with rounded angles, and was wider and larger than the left adrenal gland (Fig.1b). It also adhered in part to the anterior cranial region of the right kidney, and was located close to the anterior end of the right testis. The left adrenal gland had a more elongated shape and was situated on the medial border of the left kidney.

### Adrenal gland quantitative histology

<table>
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<tr>
<th>Parameters</th>
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<th>Female (n = 6)</th>
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<td>Width (cm)</td>
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<tr>
<td>Thickness (cm)</td>
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Asterisk (*) indicates significant difference (Paired t-test, *P < 0.05) compared to the left adrenal gland.

### Adrenal gland quantitative histology

The relative proportions of cortical and medullary tissues in each sex are given in Table 2; as there was no statistical difference between the sexes the pooled overall mean is also shown. The mean percentages of cortical tissue and medullary tissue were 84.7 ± 0.3%, 0.9%, respectively (Table 2) and the remaining 0.9 ± 0.3% was sinusoids. Thus, the ratio of cortical to medullary tissue was approximately 6:1.

### Distribution of TH

The distribution of TH in the adrenal medullary cells of the jungle crow was examined immunohistochemically. Most chromaffin cells showed TH positive staining, and individual cells with darkly stained cytoplasm and unstained nuclei were seen (Fig. 4 a, b). Many medullary cells were strongly TH positive; these cells were distributed throughout the gland as either solitary cells or in large clusters (Fig. 4 a). The cell clusters formed islets, isolated islets, large islets and elongated islets (Fig. 4 a, b). The cortical cells in the adrenal gland did not show TH staining (Fig. 4 a, b).
Anatomical location and gross morphology of the adrenal glands (non-perfused) of the jungle crow. (a) Glands are shown in situ. The adrenal glands are located at the anterior poles of the kidneys. The right adrenal gland (arrowhead) is partly adherent to the anterior cranial pole of the right kidney, and the left gland (arrow) is located on the medial border of the left kidney, close to the gonads. (b) Comparative appearance of the adrenal glands. The right adrenal gland (A) is generally triangular with rounded corners, and the left adrenal gland (B) is elongated. Scale bar in panel a or b indicates 1 cm or 5 mm, respectively.

Table 2. Relative proportions (mean ± SE) of cortical and medullary tissues in the adrenal gland of the jungle crow

<table>
<thead>
<tr>
<th>Overall cortex mean (%) (n = 5)</th>
<th>Overall medulla mean (%) (n = 5)</th>
<th>Sex</th>
<th>Overall CM ratio</th>
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<td></td>
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<td>Cortex (%)</td>
<td>Medulla (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>84.0 ± 1.2</td>
<td>15.0 ± 1.3</td>
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<td></td>
<td></td>
<td>Female (n = 2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortex (%)</td>
<td>Medulla (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>85.8 ± 0.8</td>
<td>13.0 ± 0.1</td>
</tr>
</tbody>
</table>

CM: cortico-medullary ratio
Fig. 3
Photomicrographs of sections of the adrenal gland of the jungle crow. Sections were stained with Azan. (a) Prominent eosinophilic cortical cells (light staining) and basophilic medullary cells (darker staining) arranged in islets, together with blood vessels. The cortical (arrow) and medullary (arrowhead) tissues are intermingled. (b) Nuclear orientation distinguishes cortical tissue from medullary tissue. The cortical cells are columnar, with comparatively small, oval and eccentrically located nuclei. Medullary cells are cylindrical, with comparatively large, rounded or globular and centrally-placed nuclei. C: adrenal capsule, S: strands of cortical cells, M: group of medullary cells. Scale bar in panel a or b indicates 100 μm or 10 μm, respectively.

Fig. 4
Photomicrographs of sections of the adrenal gland of the jungle crow. Sections were stained immunohistochemically using an anti-TH antibody and visualized with DAB. (a) Positive staining for TH was present in almost all medullary cells, which are distributed throughout the adrenal gland. (b) The TH positive cells (arrow) have dark cytoplasm and unstained nuclei. * in respective panel indicates the cortex. Scale bar in panel a or b indicates 400 μm or 50 μm, respectively.
4. Discussion

In this study, we examined histomorphological and immunohistochemical features of the adrenal glands of the jungle crow. In this species, the positioning of the adrenal glands is similar to that described for the chicken by Wells and Wight. The adrenal glands of the chicken are situated on each side of a median line just anterior of the bifurcation of the caudal vena cava, close to the gonads and the anterior division of the kidneys. The left adrenal gland of the jungle crow was found here to be elongated in shape, whereas the right adrenal gland had a more compact, triangular appearance. This dimorphism has also been described in African and thickness between the right and left adrenal glands in the jungle crow. In a previous study on the chicken, we identified biometrical differences may be species-specific. The biological significance of the asymmetry in the left and right adrenal glands in birds is not clear. In female birds, only the left ovary is developed. However, the asymmetrical development in the gonadal gland in female birds does not seem to be responsible for the asymmetry in the adrenal glands, because the asymmetry in the adrenal glands were seen also in male birds, which have a pair of testis in the similar size.

The distribution of medullary tissue in the jungle crow adrenal gland was similar to that described for the Florida Bobwhite (Colinus virginianus floridanus), which has a meshwork of medullary cell islets. In the jungle crow, the medullary cells were cylindrical with large, round and centrally-placed nuclei, and showed basophilic staining. These observations resemble those reported in the chicken by Hodges, who also suggested that the adrenal medulla cells had a polygonal shape, with large, round and centrally-placed nuclei. The basophilic staining of the cytoplasm is due to the presence of numerous small basophilic granules. The medullary cell nuclei in the jungle crow were 4.0 ± 0.0 μm in diameter; by contrast, medullary cell nuclei have a diameter of 8 μm in ostrich chicks, and of 6.7 μm in domestic hens. Medullary tissue in the jungle crow was composed of several rounded and distorted cords and distributed as spots. This pattern differed from that found by Hodges in the chicken, in which the medullary cells form a meshwork of clumps and irregular cell masses. Therefore, the results of the current study suggest that there are species-specific differences in the arrangements of adrenal medullary cells.

The entire adrenal gland of the jungle crow was encircled by a capsule of connective-tissue that contained many blood vessels. A similar structure was identified in the chicken. The cortical and medullary tissues of the jungle crow were completely intermingled throughout the whole adrenal gland in a similar fashion to other avian species. However, the cortical cells occurred as irregular masses of cells; by contrast, the cortex of the chicken consists of solid and irregular cords of cortical cells. Thus, as for medullary cells, our results indicate that there are species-specific differences in the arrangements of cortical cells in the adrenal glands among birds.

The parenchyma of the jungle crow adrenal gland was composed of two main tissue types, the cortex and medulla, that were not present as distinct zones but rather were intermixed. In the jungle crow, as in the chicken, cortical cells were columnar in shape, with small, round, eccentrically-placed nuclei, and showed eosinophilic staining. However, the diameter of the nuclei in the jungle crow (3.4 ± 0.0 μm) was smaller than in ostrich chicks (5 - 7 μm) or the chicken (5 μm).

To the best of our knowledge, this is the first study to determine the relative proportions of cortical and medullary tissues in the adrenal glands of a wild avian species. The cortical and medullary tissues composed approximately 85% and 14%, respectively, of the jungle crow adrenal gland. These proportions differ from those reported for the chicken, 60% and 39 - 40%, respectively, i.e., a ratio of approximately 6:1. Undoubtedly, most of the variation in the proportions of cortex and medulla can be attributed to species, age, sex, breed, health and environment. The functional significance of these different ratios is uncertain. Possibly, in its usual forest environment, the jungle crow might have need of an increased regulatory capability of electrolytes or water, and might mediate this control via adrenal cortical hormones. The cortex, for example, produces many hormones (glucocorticoids and mineralocorticoids) that function in metabolic pathways for conserving water. Therefore, the relatively large cortex of the jungle fowl may represent an adaptation to its particular habitat.

The distribution of TH, the rate limiting enzyme in the conversion of tyrosine to dihydroxyphenylalanine (L-DOPA), was restricted to adrenal medullary cells. Our analysis indicated that the majority of medullary cells was TH positive. Similar results have been reported by Kober et al. and Ohmori et al. in the chicken. TH immunostaining was limited to the cytoplasm of the medullary cells in both jungle fowl and the chicken.
the adrenal glands of the jungle crow conforms to the general avian pattern, it also shows species-specific characteristics. In particular, the proportions of cortical and medullary tissues differed significantly from domesticated species, such as the chicken. The function of this difference remains to be elucidated. Our immunohistochemical analysis confirmed the localization of the catecholamine biosynthesizing enzyme, TH, in the adrenal medulla.

Acknowledgment

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture, Sports and Technology of Japan (No.083281).

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