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EFFECT OF TESTOSTERONE ON THE DISTRIBUTION OF VASOTOCIN IMMUNOREACTIVITY IN THE BRAIN OF THE ZEBRA FINCH, *TAENIOPYGIA GUTTATA CASTANOTIS*

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Summary

The distribution of vasopressin or vasotocin immunoreactive cells and fibers in the lateral septum and the bed nucleus of the stria terminalis are sexually dimorphic in many vertebrates including several species of birds examined to date. We examined the vasotocin-like immunoreactivity in the zebra finch brain. Male birds had a higher level of immunoreactive staining in some telencephalic and diencephalic regions. The density of immunostaining increased in the testosterone-treated females to levels typically seen in males. The sexual dimorphism and testosterone dependence of the vasotocin-like immunoreactivity are similar to that found in the canary. Thus this pattern of vasotocin localization and testosterone dependence may be a general feature in brains of passerine songbirds.

Key Words: zebra finch, brain, vasotocin, testosterone, sexual dimorphism, immunohistochemistry, bed nucleus of stria terminalis, lateral sperm

An avian analogue of mammalian vasopressin is known as vasotocin (VT). As with vasopressin, the central actions of VT are involved in reproductive and adaptive behaviors, memory processes and the control of salt balance (1-6). VT is synthesized in neurosecretory cells within the preoptic, supraoptic, and paraventricular regions of the avian hypothalamus and is axonally transported to and stored within the axon terminals of the neural lobe of the pituitary, from which it is released into the systemic circulation. Numerous studies have demonstrated that limbic brain circuits containing vasopressin

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exhibit considerable sexual dimorphism (Reviewed in 7). These circuits include the bed nucleus of the stria terminalis, the medial amygdaloid nucleus, and a large plexus of vasopressin-immunoreactive fibers in the lateral septum (LS). Released directly into the brain interstitial fluid, vasopressin derived from limbic structures may account for neuromodulator- and/or neurotransmitter-like effects (8), including regulation of sex-specific behavior (9, 10).

In several species of mammals studied to date, the distribution of vasopressin immunoreactivity in the brain is sexually dimorphic and testosterone sensitive (11-13). Moreover, sexually dimorphic VT-immunoreactive (VT-ir) cells and fibers located in extra-hypothalamic regions have been described in amphibians (14, 15) and in reptiles (16).

Studies of the canary brain showed that the VT system is sexually dimorphic and testosterone dependent (17, 18) as is the vasopressin system in the mammalian brain. Voorhuis and de Kloet (19), however, reported that the distribution of immunoreactive vasotocin in the brain of another avian species, the zebra finch, was not sexually dimorphic, and administration of testosterone to female zebra finches did not affect the degree of immunoreactivity. In this report, we describe attempts to duplicate these results in the zebra finch, but found contrary results.

Materials and Methods

Adult zebra finches were obtained from local pet suppliers and kept in an aviary at Sophia University under a constant 16-hour day and 8-hour night cycle with temperature between 23 and 29 °C, humidity between 60 and 70 %. Under this condition, birds could breed throughout the year. Female birds were subcutaneously implanted with a Silastic tubing (inner diameter 0.76 mm, outer diameter 1.64 mm, length 5.0 mm) filled with testosterone (T; n=4) or cholesterol (C; n=8). Male (n=4) birds did not receive the implantation.

Four weeks after implantation blood sera were collected and assayed for plasma T level by radioimmunoassay. Assays were carried out in 10 µl plasma samples in duplicate without any extraction. The assay protocol was two incubation double-antibody method with intraassay variations of 5.7 %. To avoid an interassay variation, we assayed all samples in a single run.

Birds were deeply anesthetized with sodium pentobarbital and perfused intracardially with 60 ml 0.01 M phosphate buffered saline (PBS) of pH 7.4 containing heparin (50 U/ml). After PBS perfusion, 40 ml 0.1 M PBS with 4 % paraformaldehyde containing 0.2 % picric acid (pH 6.0) was used for fixation. Finally, 60 ml 0.1 M borate buffered solution with 4 % paraformaldehyde (pH 9.0) was applied. The brain was removed and immersed into the second fixative for 16 hr and washed with 0.1 M PBS. The brain was embedded into fresh egg yolk and then immersed sequentially into the 0.1 M PBS containing 10 % and 20 % sucrose for cryoprotection. The block was frozen and 25 µm sections were cut by

a cryostat microtome (SLEE, MTE type, LONDON). The free-floating sections were incubated in 0.1 M PBS containing 0.3 % Triton X-100 (PBS-T) and normal nonimmunized goat serum at 37 °C to increase permeability of immunoreagents and to reduce nonspecific staining. The sections were washed with PBS and then incubated with antiserum raised against VT in a rabbit (a gift from Dr. Urano at Hokkaido University) diluted into PBS-T (1:1000) for 16 hr at 37 °C. The antibody is highly specific for vasotocin and its cross-reactivity to mesotocin is 0.3 % (20-22). Some sections were processed with the identical procedure, except for the use of primary antiserum or primary antiserum which was pre-absorbed with 10 μg/ml arg-VT, both of which blocked immunostaining and served as controls. Sections were processed by the avidin-biotin-peroxidase complex method (The ABC kit; Vector Labs) and visualized by diaminobenzidine tetrahydrochloride (Dojin) and H_2O_2. The sections were then mounted onto chromium-alum gelatin-coated glass slides and dried in air. Alternative sections of the brain were stained with cresyl violet acetate. The number of vasotocin-like immunoreactive cells in the bed nucleus of the stria terminalis was counted from a slide glass which contained the most caudal part of the anterior commissure. In our preparations VT-ir cells were clearly identifiable since they contained grains of the DAB reaction product distributed within the cell bodies.

Results and Discussion

The plasma T level of C-treated females (0.84 ± 0.10 nmol/l) was about half of that reported by Voorhuis and de Kloet (1.87 ± 0.55 nmol/l). The T levels of our T-treated females (12.98 ± 5.91 nmol/l) were similar to their data (14.35 ± 1.53 nmol/l) (Fig. 1). The T levels of our T-treated females were significantly higher than those of our C-treated females ($p=0.027$ by Duncan's multiple range test). The plasma T levels of males (4.31 ± 2.02 nmol/l) were about 5 times that of C-treated females, but the difference was not significant due to the large within group variation.

As reported by Voorhuis and de Kloet (19), we also found a large number of (VT-ir) cells and fibers in the brain. A large cluster of VT-ir cells was found in paraventricular nucleus, supraoptic nucleus, ventro-lateral part of hypothalamus, anterior part of preoptic area, and suprachiasmatic nucleus. VT-ir innervation is also found in the medial posterior hypothalamic nucleus (the avian equivalent of the mammalian ventromedial nucleus), and in the stratum cellulare internum. A few VT-ir fibers were scattered in neostriatum caudale of some birds, both male and female. VT-ir fibers in paleostriatum augmentum run along anterior commissure and Tractus occipitomesencephalicus toward some regions. We also found VT-ir fibers inside and in the surroundings of a vocal control nucleus, robust nucleus of archistriatum, and the nucleus intercollicularis. Thus, our results generally confirmed the report of Voorhuis and de Kloet.

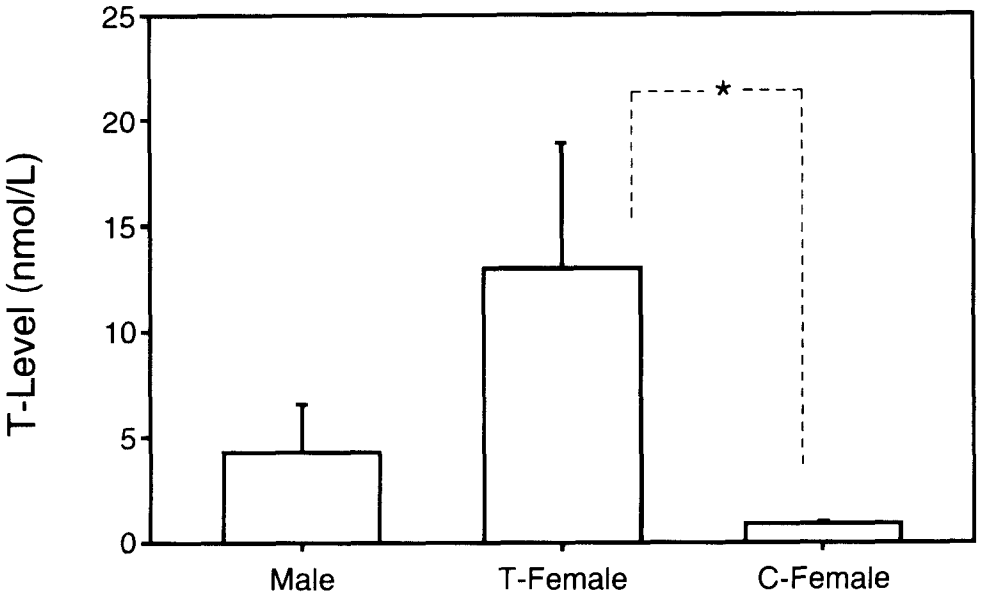

Fig. 1

Plasma testosterone level (mean ± SEM) of male zebra finches (n=4), T-treated female zebra finches (n=4), and C-treated female zebra finches (n=8). Asterisks (*) indicate significant comparison at $p<0.05$ level using Duncan's multiple range test.

However, we found that the distribution of VT-ir was sexually dimorphic in the lateral septum (LS) and the bed nucleus of the stria terminalis (BST). At the most caudal part of the anterior commissure, more VT-ir cells and fibers were labeled in BST of males than in C-treated females (Fig. 2A, C). Implantation of T in female zebra finches resulted in a much-increased VT immunoreactivity in BST (Fig. 2B). In C-treated females (Fig. 2C), the VT-ir system remained comparable to those of normal females (data not shown). The number of VT-ir cells was counted in BST for statistical analysis. The immunohistochemical data of 4 of the 8 cholesterol-implanted females were accidentally lost. We used the remaining 4 data for statistical analysis. The average number of VT-ir cells was 33.3 ± 5.3 (S.E.M.) in the males, 49.5 ± 7.0 in the T-treated females, and 11.5 ± 1.4 in the C-treated females. The cell number of males and C-treated females ($p=0.012$), C-treated females and T-treated females ($p=0.001$) were significantly different as analyzed by Duncan's multiple range test (Fig. 3). In LS, VT-ir fibers were labeled and qualitatively showed a tendency to be testosterone dependent as were the VT-ir cells and fibers in BST (data not shown).

We have examined the effect of T on the distribution of VT-ir in the zebra finch brain. In contrast to the earlier study of Voorhuis and de Kloet reporting the lack of sexual dimorphism and T dependence in VT-ir distribution in the zebra finch brain, we found that the VT-ir distribution in LS and BST are sexually dimorphic and T dependent.

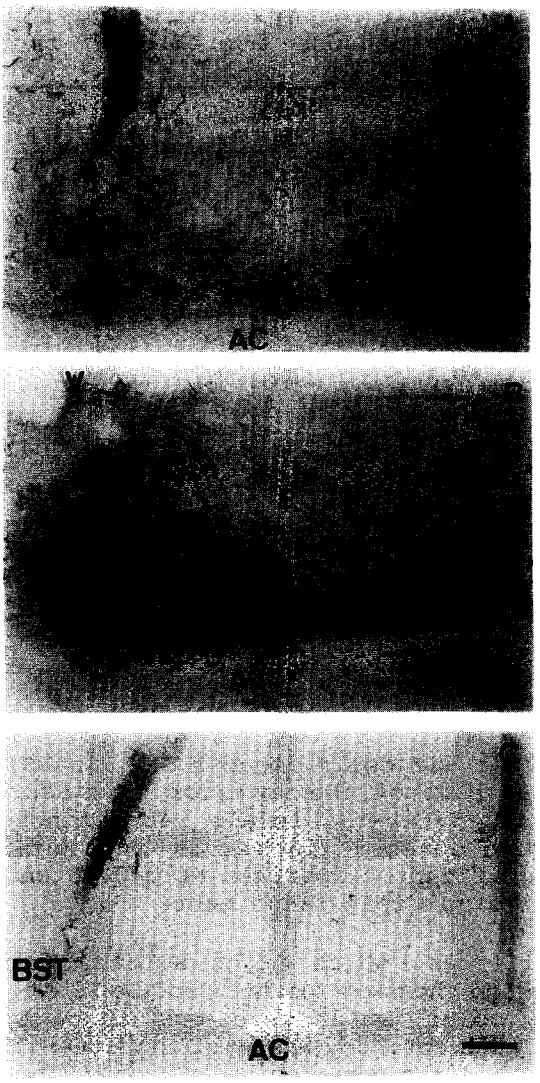

Fig. 2

Photomicrographs of vasotocin-like immunoreactivity in BST at the level of the caudal part of the anterior commissure. (A) Male zebra finch, (B) T-treated female zebra finch, (C) C-treated female zebra finch. AC: anterior commissure. V: lateral ventricle. BST: bed nucleus stria terminalis. Bar: 100 μm.

Studies on several avian species including the domestic fowl (23), the Japanese quail (24, 25) and the canary (18) demonstrated that there is a sexually dimorphic VT-like immunoreactivity in the brain of these species. In the male quail, castration and aging reduce the number of visible VT-ir innervation and testosterone replacement restores the VT-like immunoreactivity (24). However, testosterone

was ineffective in increasing VT-like immunoreactivity in the female quail (25). In the female canary, as in our present data, treatment with testosterone increased the number of VT-ir cells and fibers.

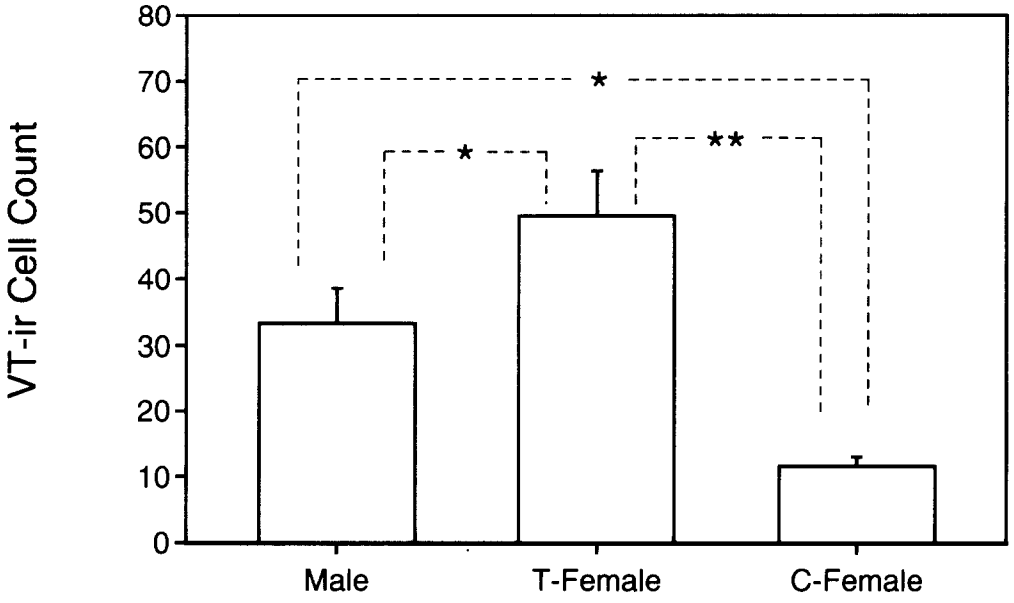

Fig. 3

The number of VT-ir cells (mean ± SEM) in BST at the level of the caudal part of the anterior commissure: Male zebra finches (n=4), T-treated female zebra finches (n=4), and C-treated female zebra finches (n=4). Asterisks (*, **) indicate significant comparison at $p<0.05$ and $p<0.01$ level using Duncan's multiple range test.

Several possibilities, which might account for the discrepancy between our data and those of Voorhuis and De Kloet, may be discussed. First, the antibodies, which were used in each experiment, were different. But each antibody was well characterized (20-22, 26) and specifically recognized vasotocin. Second, the conditions under which the zebra finches were kept were different. Voorhuis and de Kloet kept zebra finches in natural conditions at Utrecht in the Netherlands. We kept them in controlled, constant temperature/humidity conditions under which they could breed throughout the year. These conditions might have influenced the physiological state of the birds (27, 28). If this were the case, however, they should have seen seasonal dependence of the VT-ir distributions, but they reported the contrary. Third, since the baseline level of testosterone in females was twice as high in Voorhuis and de Kloet than in our samples, female zebra finches in their samples might already have had a T-level sufficient to increase VT-ir in LS and BST to its maximum possible level (ceiling effect). We think this to be the most possible explanation. If this should be the case, castration of male zebra finches or hypophysectomy in female zebra finches might change the density of VT-ir in LS and BST as seen in other vertebrates (24, 29, 30). Further studies are needed to discern

the relationship between circulating testosterone and VT-ir distribution in the brain of songbirds.

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