The vasotocinergic system in the hypothalamus and limbic region of the budgerigar (*Melopsittacus undulatus*)

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We report a morphological and biochemical analysis on the presence, distribution and quantification of vasotocin in the hypothalamus and limbic region of the budgerigar *Melopsittacus undulatus*, using immunohistochemistry on serial sections and competitive enzyme linked immunoadsorbent assay measurements on tissue extracts.

Analysis of the sections showed large vasotocin-immunoreactive neurons in three main regions of the diencephalon, of both male and female specimens. Vasotocinergic cell bodies were located in the ventral and lateral areas of the hypothalamus, dorsal to the lateral thalamus and medial to the *nucleus geniculatus lateralis*. Immunoreactive neurons were placed also periventricularly, close to the walls of the third ventricle, at the level of the magnocellular paraventricular nucleus. Well evident bundles of immunoreactive fibers were placed ventral to the anterior commissure in the same regions of the hypothalamus and thalamus where vasotocinergic perikarya are localized. Fibers were identified close to the third ventricle, and in the lateral hypothalamic area along the lateral forebrain bundle.

In contrast to what reported for other oscine and non-oscine avian species, we were not able to identify immunopositive neurons in any region above the anterior commissure, or detect relevant differences on the distribution of the vasotocin immmunoreactivity between sexes.

Competitive enzyme linked immunoadsorption assay and image analysis of the extension of immunoreactivity in the tissue sections were consistent with the qualitative observations and indicated that there is no statistically significant dimorphism in the content of vasotocin or in the location and distribution of vasotocinergic elements in the investigated areas of male and female parrot brains.

Key words: vasotocin, diencephalon, limbic system, sexual dimorphism, parrot, *Melopsittacus undulatus*, ADH.

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Arginine-Vasotocin (AVT), the non-mammalian nonapeptide homologue of arginine-vasopressin (AVP), differs from the mammalian form by a single amino acid in the third position. AVT and AVP were originally identified as hormones produced in magnocellular hypothalamic nuclei, released in the neurohypophysis and believed to regulate water reabsorption and hydromineral balance (Ramieri and Panzica, 1989: Morley and Silver, 1992; Leng *et al.*, 1992). The role of AVP in the control of several functions, including those not related to osmoregulation, is well known in mammals. Although the physiological effects of AVT in non-mammalian vertebrates remain partially uncertain (for review see Panzica *et al.*, 1997), previous studies on effects of peptide administration have shown that AVT/AVP modulates a variety of social behaviors in several vertebrate classes including birds (for review see Goodson and Bass, 2001; Panzica *et al.*, 2001).

Avian AVT is directly involved in osmoregulation (Ramieri and Panzica, 1989; Saito *et al.* 2001), in the control of sexual (Castagna *et al.*, 1998; Jurkevich and Grossmann, 2003) and singing behaviors (Voorhuis *et al.*, 1991), and promotes oviduct contraction and oviposition (Sasaki *et al.*, 2000). In the hen, AVT causes contractions of the smooth muscles of the uterus during egg laying (Takahashi *et al.*, 1992). These findings suggest a critical role for this peptide in avian reproductive physiology. In particular, in the quail, AVT-containing neurons in the medial preoptic nucleus (POM) and bed nucleus of the *stria terminalis, pars medialis* (BSTM, Aste *et al.*, 1998), as well as AVT-containing fibers in POM, lateral septum (Viglietti-Panzica *et al.*, 1992, 1994) and in some brainstem regions, show sexual dimorphism (for a review see Panzica *et al.*, 2001). A similar sexual dimorphism of the AVT neuronal system

was also demonstrated in the chicken (Jurkevich *et al.*, 1997), canary (Voorhuis *et al.*, 1988) and Zebra finch (Kimura *et al.*, 1999).

It is clear that AVT is an important constituent of the neuroendocrine system in non-mammalian vertebrates, and information on its brain distribution may contribute to further understand its role and functions. Our investigation is aimed at studying the distribution of AVT in the brain of the budgerigar *Melopsittacus undulatus*, a vocal learning parrot member of the taxum Psittaciformes, a species often used as laboratory model. Both male and female budgerigars are capable of striking lifelong neuroendocrine plasticity independent of seasonal or hormonal factors (Farrabaugh *et al.*, 1992, 1994; Heaton and Brauth, 1999; Banta-Lavenex, 2000; Hile and Striedter, 2000; Hile *et al.*, 2000). Consequently many studies performed in this species were focused on central pathways specialized in vocal learning (Brauth *et al.*, 1994; Streidter, 1994; Durand *et al.*, 1997; Heaton and Brauth 2000a, b; Brauth *et al.*, 2001). Recently Roberts *et al.* (2002), using histochemical markers, have investigated in details the parrot ventral paleostriatal and archistriatal regions. These structures were shown to be chemically comparable and homologue to the mammalian basal forebrain, a region considered important in the regulation of information processing within vocal control system.

The present study was aimed to localize AVT-immunoreactive (-ir) neurons and fibers in the forebrain of *Melopsittacus undulatus* through immunohistochemical staining, and to study possible sexual differences in vasotocin distribution and content by image analyses and competitive enzyme immunoassay.

Materials and Methods

Animals

The budgerigars (*Melopsittacus undulatus*) belonged to a colony kept at the Faculty of Veterinary Medicine of the University of Padua. They were housed in couples in separated cages with 14 hours of light and 10 h of dark, given free access to food and water and allowed to breed. For the present study we used a series of 38 budgerigars (9 males and 11 females for immunohistochemistry and 9 males and 9 females for competitive enzyme immunoassay). All animals were non-breeding young adults.

Animals were treated according to Italian and European regulations concerning animal welfare, in agreement with the ethical standards of the University of Padua and with permission from the Italian Ministry of Health.

Immunohistochemistry

For immunohistochemical studies the animals were sacrificed by an intraperitoneal overdose of barbiturates (Penthotal®, Abbott, Latina, Italy), and subsequently perfused through the heart with 200 mL of heparin in phosphate buffered saline 0.01 M, pH 7.4 (PBS) followed by 500 ml of a solution of 4% paraformaldehyde in PBS as fixative. The brains were then carefully removed and postfixed in freshly prepared fixative for 16-20 hours at 4°C. After several washes in PBS, the brains were cryoprotected in a solution of 30% sucrose in PBS, frozen in isopentane at -25/-30°C and stored at -80°C.

Serial coronal 20 µm thick sections cut with a cryostat were collected, mounted on gelatinized slides and stored at -20°C. Immunohistochemical protocols followed a consolidated laboratory procedure (Fabris *et al.*, 2004). After rinsing in 0.01 M PBS (pH 7.2-7.4), the sections were incubated in 1% hydrogen peroxide in 0.01 M PBS for 10 min to inhibit endogenous peroxide activity. Successively, they were pre-incubated in 4% normal swine serum in PBS with 1% bovine serum albumin (BSA fraction V, Sigma, Milan, Italy) and 0.4% Triton-X100 for 20 min at room temperature (RT) to lower unspecific tissue reactions and then incubated with: a) primary polyclonal anti-AVP antisera raised in rabbit (Incstar, Stillwater, Minnesota, USA) diluted 1:500 in a solution of PBS with 20% sodium azide, 1% BSA, 0.4% Triton-X100 and 1% normal serum; or b) specific anti-vasotocin antisera (AVT and AVT-R, kind gift of Dr. Sabine Blaehser, University of Giessen, Germany, see Goossens *et al.*, 1977 for characterization), raised in rabbit against synthetic arginine-vasotocin, diluted 1:5000. Incubation was carried out for 1 h at RT and then at 4°C overnight. After washing in PBS with 0.25% BSA and 0.02% Triton-X100, the sections were incubated for 1 h in biotinylated swine anti-rabbit immunoglobulins (Dakopatts, Copenhagen, Denmark) diluted 1:200 in PBS with 0.02% Triton-X100 added. The sections were then washed in PBS and 0.25% BSA and incubated for 1 h in streptavidin-biotin-horseradish peroxidase complex (Dakopatts, Copenhagen, Denmark) diluted 1:125 in PBS and 1% BSA. After washing in PBS and in

0.05 M Tris-HCl pH 7.6 (Tris), peroxidase activity was revealed with a solution containing 0.04% diaminobenzidine (DAB) and 0.03% hydrogen peroxide in Tris buffer for 20 min. After some brief sequential washes in buffers the sections were dehydrated and cover-slipped with balsam.

Controls

Cross-reactivity of the antibodies with oxytocin has been factory tested by addition of 100 μg/mL oxytocin to the primary antisera, resulting in less than 1% cross-reactivity.

Further specificity controls were performed by replacing the primary antibody with normal swine serum and by pre-adsorbing the primary antisera with an excess of AVT (50 μg/mL diluted antiserum) for 24 h. Under these conditions there was no immunostaining at all.

Tissue extraction and competitive enzyme-linked immunoadsorbent assay (ELISA)

Animals were sacrificed as described before and the brains were quickly removed after decapitation. Each dissected forebrain was homogenized in 5 mL of 0.1 M CH_3COOH with a tightly fitting glass-glass homogenizer. Homogenates were boiled for 10 min and centrifuged at 15,000 g for 20 min. Supernatants were passed through disposable Sep-Pak Classic C-18 cartridges (Waters, Milford, MA) for peptide extraction, and eluants dried in a Speed-Vac concentrator. Dried samples, were subjected to a commercial ELISA kit (Peninsula Labs, San Carlos, CA) designed to detect vasotocin. The sensitivity of the method was 2 pg/well. Intra and inter-assay coefficients of variation were <5% and <14%, respectively. Tissue content of vasotocin was expressed as pg *per* mg of wet tissue. The protein content of each sample was assayed using the Bradford method (Bio-Rad, Hercules, CA). Tissue concentration of AVT in each dissected specimen was measured in duplicate.

Brain topography

One series of sections of each brain was Nissl-stained and employed for topographical reference. Brain nomenclature and stereotaxic planes of the section are based on previously published papers on *Melopsittacus undulatus* (Cozzi *et al.*, 1997; Roberts *et al.*, 2002), as well as according to the new avian brain nomenclature (Reiner *et al.*, 2004).

Photography and computer analysis

The sections were observed and photographed with an Olympus AHBT3 VANOX microscope equipped with an Olympus Camedia digital camera. Immunostained sections were analyzed using Neurolucida® software (Microbrightfield, Colchester, VT, USA) to define nuclear areas as well as distribution and number of immunoreactive neurons and fibers.

Data obtained were compared between sexes and analyzed statistically using Student's *t* test. Differences were considered significant if $p \leq 0.05$.

Results

Anti-AVP, anti-AVT and anti-AVT-R antibodies marked the same populations of neurons with no difference other than those concerning the background and occasional variations between series of slides. Sections incubated with pre-adsorbed antisera, or in which the primary antiserum was substituted with swine normal serum, showed no immunostaining at all. Sections incubated with primary antisera showed several positive perikarya and a wide array of immunoreactive nerve fibers in the diencephalon and limbic region of budgerigars.

General topography of neurons and distribution of fibers

Groups of AVT-ir neurons were present ventrally to the anterior commissure, close to the borders of the third ventricle and in the lateral and ventral hypothalamic and thalamic subregions. Positive cell clusters consisted of numerous round bipolar or multipolar perikarya surrounded by very dense plexus of beaded AVT-ir fibers. We observed no stained neurons in the region above the anterior commissure, presumptive location of BSTM (Roberts *et al.*, 2002).

Periventricular region

In the rostralmost immunostained sections, vasotocinergic perikarya were located very close to the wall of the third ventricle, in the periventricular diencephalic area, forming a linear dorsal-ventral mass of positive elements along the ventricular rim (Figure 1). Heavily stained immunoreactive neurons were also distributed slightly lateral to the ventricular walls, and some of them were located within the nucleus corresponding to the nucleus (n.) *paraventricularis magnocellularis* (Panzica *et al.*, 1999b).

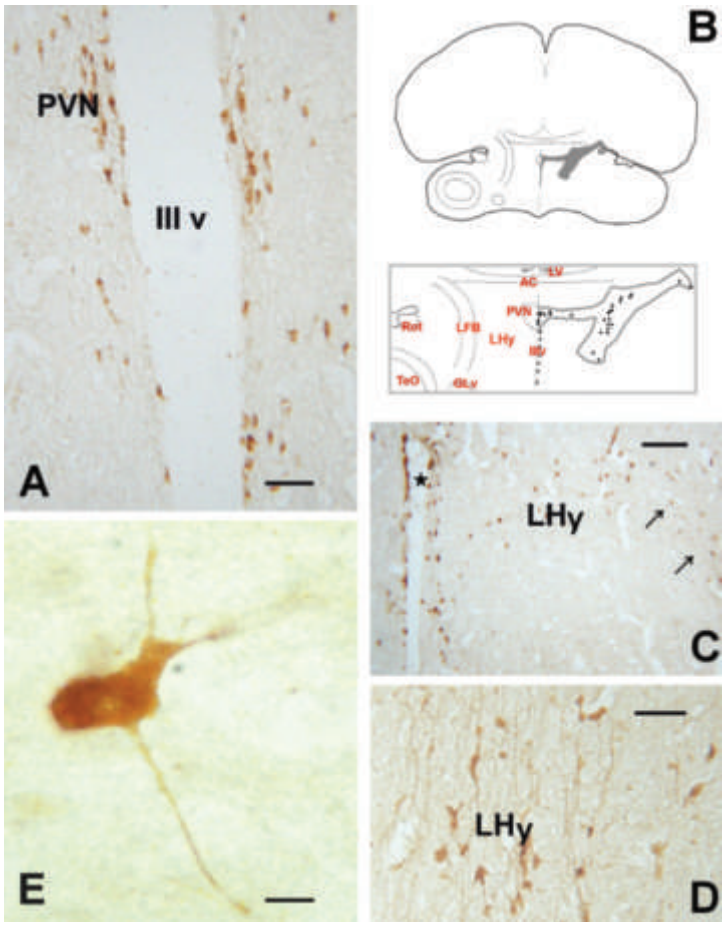

Figure 1. A. Paraventricular nucleus (PVN) and adjacent areas of the hypothalamus. IIIv, third ventricle. Scale bar = 200 μm. B. Drawings showing nuclear subdivision (dots, upper left side) and area considered for Neurolucida® cell counts (upper right side, gray shade) in a significant section of the hypothalamus of the budgerigar. Lower drawings show an enlargement of the same region; AC, anterior commissure; LFB, lateral forebrain bundle; GLv, n. geniculatus lateralis, pars ventralis; LHy, lateral hypothalamus; LV, lateral ventricles; PVN, paraventricular nucleus; Rot, n. rotundus; TeO, tectum opticum; IIIv, third ventricle. C. Lateral hypothalamus (LHy) and adjacent subregions. Arrows point towards fibers entering the lateral forebrain bundle. The asterisk is in the roof of the third ventricle. Scale bar = 1 mm. D. AVT-ir multipolar neurons in the lateral hypothalamus. Scale bar = 250 μm. E. Ramified multipolar AVT-ir neuron in the lateral hypothalamus. Scale bar = 20 μm.

Lateral and ventral regions

In rostral sections of the periventricular diencephalons, we found vasotocinergic neurons at the level of the *n. preopticus medialis* (POM) and ventromedially to the *n. geniculatus lateralis pars ventralis* (GLv), in an area corresponding to the supraoptic nucleus.

More caudally, a large cluster of AVT-ir perikarya were located in the dorsal part of the lateral hypothalamus, extending to the lateral thalamic areas till the borders of the *n. rotundus* (Figure 1 B-E). Other vasotocinergic cell bodies were observed intermingled within the fibers of the lateral forebrain bundle, in the lateral hypothalamus, in the *n. ventrolateralis thalami* and, ventro-medially, in the GLv.

Several AVT-ir beaded fibers were evident below the anterior commissure, lateral to the ventricular border and reaching the more lateral regions of the diencephalon, till the lateral thalamic areas and the border of the *n. rotundus*.

We observed a large and diffuse network of vasotocin immunoreactive nerve fibers along the rims of the lateral forebrain bundle apparently traveling from the dorso-lateral to the ventro-medial areas of the diencephalon following the same immunoreactive extension of the AVT-ir cell bodies.

Dorsal regions and basal forebrain

We observed few immunoreactive fibers but no perikarya dorsal to the anterior commissure, at the level of the septal region.

Differences between sexes

Perikarya and fibers were located in the same positions in both sexes. Cell counts, performed with Neurolucida®, showed a slight but not statistically significant prevalence of vasotocinergic cell-bodies in females as compared to males (data not shown).

Vasotocin concentration in hypothalamic tissues

Data from competitive enzyme immunoassay (Figure 2) indicated that the content of AVT in tissue homogenates is higher in females (125 pg/mg wet tissue) than in males (107 pg/mg wet tissue). However the difference of AVT content between the two groups was not statistically significant ($p > 0.05$). Values obtained showed a high degree of intragroup variability.

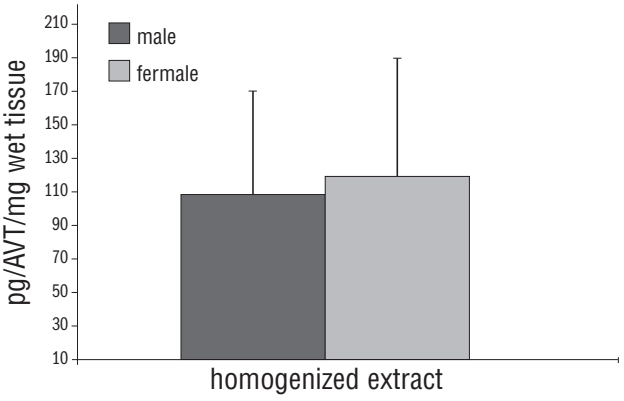

Figure 2. Concentrations of AVT in the hypothalamus of males (n = 9) and females (n = 9) budgerigars. Values (pg/mg wet tissue) are expressed as mean ± SD.

Discussion

The distribution of AVT in the budgerigar brain mainly follows a pattern similar to that observed in oscine birds (Voorhuis *et al.*, 1988; Panzica *et al.*, 1999b). However, the topography of the AVT system in the brain of *Melopsittacus undulatus* shows peculiar differences if compared with other birds. Although we have seen the typical periventricular clusters of immunoreactive neurons that are generally interpreted as equivalent of the mammalian paraventricular and periventricular magnocellular neurons (for review see Panzica *et al.*, 1997, 2001), contrary to what described in other oscine and non-oscine birds (Viglietti-Panzica *et al.*, 1994; Jurkevich *et al.*, 1997; Aste *et al.*, 1998; Panzica *et al.*, 1999b), we have not found positive elements in presumptive location of BSTM. This finding is in contrast to a previous study on *Melopsittacus undulatus* (Roberts *et al.*, 2002) reporting the existence of a few AVT-ir elements in this region. The avian BSTM, as described in the quail (Aste *et al.*, 1998) and domestic fowl (Jurkevich and Grossmann, 2003), has been considered as homologous of the medial part of the bed nucleus of the *stria terminalis* of mammals, a limbic nucleus functionally related to mating behavior in rodents. It has been demonstrated to be sexually dimorphic (for review see Panzica *et al.*, 2001) and, in the quail, it contains, in addition, aromatase-immunoreactive neurons (for a review see Absil *et al.*, 2001). The sexually dimorphic vasotocinergic system formed by the BSTM, POM and lateral septum is, in male quail and junco, sensitive to circulating testosterone levels, both for peptide and for mRNA expression (Viglietti-Panzica *et al.*, 2001, Panzica *et al.*, 1999a, Plumari *et al.*, 2004). No data are till now available to suggest that this is also the case of budgerigar.

To further study a possible sexual dimorphism in the AVT content in *Melopsittacus*, we decided to measure AVT in brain extracts by the use of a competitive ELISA assay. The data obtained in our laboratory have not shown any statistically significant sexual dimorphism in the AVT content. A sexual dimorphism in the distribution of vasotocin has been shown by immunohistochemistry and in situ hybridization in other avian species (for review see Panzica *et al.*, 2001, Jurkevich and Grossmann, 2003), but the hormonal content in the hypothalamus has been described only in a few species. AVT concentration in the hen has been studied in the supraoptic and paraventicular nuclei in relation to oviposition (Sasaki *et al.*, 1998), but we are aware of no studies performed in birds comparing hypothalamic AVT content between sexes. Other studies performed on the budgerigar (Roberts *et al.*, 2002) reported no indications of sexual dimorphism in the AVT-system.

In conclusion, present results indicate that the vasotocinergic system in the brain of the budgerigar does not include the bed nucleus of the *stria terminalis* and the septal nuclei, and, apparently, is not sexually dimorphic. Partial explanations for these discrepancies can be related to the physiological status of the animals. It is possible that the budgerigar AVT system may show changes in the expression of the vasotocin mRNA according to different physiological conditions, such as breeding cycle, oviposition, or altered water balance. In other avian model, it has been demonstrated that these are conditions affecting AVT content and expression (Saito and Grossman, 1998; Chaturvedi *et al.*, 2000; Panzica *et al.*, 1999a; Viglietti-Panzica *et al.* 2001). Further studies will elucidate if sexual differences in AVT content may become evident in budgerigar under specific physiological and behavioral circumstances.

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