In ovo administration of sex steroids alters vasotocin synthesis in bed nucleus of stria terminalis in female chicken

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The present study was performed to check the effect of in ovo administration of sex steroids on expression of arginine vasotocin (AVT) gene in a sexually dimorphic hypothalamic nucleus, the bed nucleus of stria terminalis (BnST) in chicken. Sex steroids (estradiol benzoate/testosterone propionate; 20 µg/egg) were administered at embryonic day 6 (E6) and embryonic brains were processed for localization of AVT mRNA and immuno reactive (ir)-AVT by in situ hybridization and immunohistochemistry at E18, D7, D21 and D40. Our results indicate that in ovo administration of sex steroids can increase the steady-state level of AVT mRNA in BnST. Sex-steroid administration upregulated the expression/synthesis of AVT transcript/ir-AVT throughout the stages of development studied. In the testosterone propionate-treated group, few neurons showing ir-AVT in BnST were observed at D21 and D40 post-hatch. It is obvious that expression of AVT gene in BnST which is related with reproduction, may be altered following administration of sex steroids in the egg during development (E6). It is suggested that single injection (in ovo) of sex hormone during early embryonic stage(s) may modulate vaso- toxicergic neurons and expression of AVT gene in the developing brain of chicken. Further, administration of sex steroids appears to have an irreversible and long-lasting effect on AVT neurons of sexually dimorphic BnST nuclei, an effect similar to that of sex hormones in mammals during the critical period of differentiation of the brain into male or female type.

ARGinine vasotocin (AVT) is among 16 members of neurohypophyseal peptide family of vertebrates. This principal avian peptide along with mesotocin is homologous to mammalian arginine vasopressin (AVP) and oxytocin (OT), respectively. AVT and AVP were originally identified as neurohypophyseal hormones, synthesized by hypothalamic magnocellular neurons and regulating hydromineral balance and osmoregulation. AVT and AVP are also secreted at the level of the median eminence, probably by numerous nerve terminals within several brain regions, where they regulate the production of adenohypophysseal hormones. Water deprivation not only causes an increase in hypothalamic AVT mRNA by increasing the amount of transcript per neuron, but also by recruitment of clusters of AVT neurons that cannot be identified in basal condition. In chicken administered with hypertonic saline, a similar effect was observed by in situ hybridization.

In contrast to the mammalian system where two neurohormones vasopressin (VP) and OT perform different functions, in the case of birds, AVT takes over both the function of maintaining water balance and oviposition. Since oviposition is a specific function in females, sex difference in physiological regulation and behaviour in relation to the AVT system is not unusual. In situ hybridization studies have demonstrated the presence of AVT mRNA around the third and lateral ventricle as early as embryonic day 6 (E6) (ref. 10). From embryonal day 14 (E14) onwards, the bed nucleus of stria terminalis (BnST) starts exhibiting sexual dimorphism and AVT peptide cannot be located in this region of female chickens. However, AVT transcript can be detected in this region of both sexes (chicken) up to day 35 (D35) post-hatch by in situ hybridization. In ovo administration of estradiol benzoate (EB) at E6 can affect synthesis of AVT both at transcriptional and translational level in BnST and transcript/peptide can be detected by immunohistochemistry and in situ hybridization, even after E14 up to D40.

Initially, in domestic fowl, immunoreactive (ir)-AVT has been demonstrated in the supraoptic nucleus (SON), preoptic area (POA) and paraventricular (PVN) nucleus of the hypothalamus. The ir-AVT fibres have been observed in many brain areas. These fibres are supposed to have their origin in parvocellular neurons located in the hypothalamic and limbic systems. Among all avian species studied so far, AVT neurons in extrahypothalamic locations were observed only in the brains of canary, zebra finch and songbirds, where ir-AVT was localized in BnST close to the lateral septum and dorsal diencephalon. In BnST, ir-AVT was first observed at E4 in embryos of both sexes and sexual dimorphism in the BnST becomes obvious at E14 onwards. This difference may be seen only by immunohistochemistry. However, in situ hybridization studies show no difference in AVT mRNA-containing cells in males and females at E14. After D15 post-hatch sexual dimorphism becomes distinct and neither immunocytochemistry nor in situ hybridization can localize AVT peptide/transcript in BnST of hen at D40 (ref. 17). In quail BnST, both androgen and estrogen receptors are reported to be present. Presumably, these steroids must also be regulating vasotocin synthesis in this region.

Among mammals, in rat, BnST is an important integrative centre involved in the control of sexual behaviour. Presence of aromatase in rat BnST suggests that not only androgens, but estrogens also must be involved in...
regulation of AVT in BnST. In birds, limbic areas (septum, nucleus of stria terminalis) play an important role in controlling sexually differentiated and sex steroid-dependent reproductive behaviour, male copulatory behaviour. The embryonic injection of EB resulted in complete loss of copulatory behaviour in males. In view of the effect of sex steroids on the AVT system, the present experiments were conducted to study the effect of testosterone and estradiol administration in chicken egg on the expression of AVT gene. We have seen that by in ovo administration of sex steroids, ir-AVT can be detected even after E14 and after D15 post-hatch in female subjects. Since gonadal function begins around E6 in chicken, we selected E6 for administering sex steroids in the developing egg.

Eighty fertilized eggs of white leghorn hens were obtained from a local poultry farm and were set in an incubator (37.5°C, 60% relative humidity). At E6, the eggs were brought out and wiped with 70% ethanol prior to injection. A hole was drilled through the shell and 0.1 ml of test material [EB (Sigma) and testosterone (Nicholas Piramal)] at a dose of 20 µg/egg was injected into the amniotic fluid using a 25-G needle and the hole was sealed with wax. Twenty-five eggs received testosterone as test material, another 25 eggs received EB and 30 control eggs received sesame oil. Only female subjects were processed for study at all the stages (except E15 and D40, when both male and female controls were processed for comparison). After injection, the eggs were kept back into the incubator. The eggs were turned manually four times a day. The first set (six embryos of each group) of embryos were sacrificed at E18. Embryos were anaesthetized using an injection of nembutol sodium (Abbott, IL, USA), which was given through the same hole used to inject test material (1.0 mg/0.1 ml/egg). Embryos exposed after gently removing the shell were fixed on a dissecting tray and the heart was exposed for whole body perfusion using 0.02 M PBS and Zamboni fixative by perfusion pump at the speed of 1 ml/min. Similarly, birds at D2, D14, D21 and D40 post-hatch were anaesthetized by nembutol sodium (3–4 mg/100 g body wt) and perfused through the heart. The brain was removed and post-fixed in fresh Zamboni fixative (for ~4–6 h). For cryoprotection, brains were transferred to 25% sucrose solution in PBS, till they sank to the bottom (~24 h) at 4°C. Brains were frozen using tissue freezing medium (TBS; Durham, NC, USA) and cut at 18 µm in a cryostat. For IHC, sections were washed in 0.02 M PBS and rinsed in PBS until the fixative was washed out completely, treated with 0.6% hydrogen peroxide (Merck) in PBS for 30 min, incubated in 5% normal goat serum (Sigma, USA) containing 0.2% triton × 100 (Sigma; USA) for 30 min followed by incubation for 36 h at 4°C in a 1:2000 solution of rabbit anti-AVT serum (gifted by D. A. Fisher, USA) in PBS containing 0.2% triton × 100, 1% normal goat serum and 0.1% sodium azide. Sections were thoroughly washed and incubated in goat-anti rabbit biotinylated IgG (Vector, USA) 1:500 in PBS containing 0.2% triton × 100 for 90 min at room temperature, rinsed in PBS (4 × 15 min) and incubated for 90 min with avidin biotin peroxidase conjugate (ABC–HRP; Vector, 1:1000) solution in PBS containing 0.2% triton × 100 and 1% crystalline bovine serum albumen (Sigma, USA). For immunodetection, 3,3’ dianimobenzidene (DAB; Sigma) in 0.05 M tris buffer (pH 7.6) with 0.005% hydrogen peroxide was used. Sections were subsequently rinsed in PBS and distilled water, air-dried, dehydrated and cover-slipped using DPX mountant (Qualigens, Glaxo, India).

To control the specificity of immune reaction, sections were incubated with 5% normal goat serum. In control sections, no immune positive signal was detected. For morphometric measurements, brain sections containing ir-AVT neurons in the BnST were selected from each experimental group. Immunostained cells/neurons were counted manually under ordinary light microscope (Weswox Optik model-TR II 66, Ambala Cantt., India).

The nomenclature of the brain structures and stereotaxic planes of the sections were adjusted in reference to the chicken brain atlas of Kuenzel and Masson (1988). Student’s ‘t’-test was used to assess comparisons between group means.

For in situ hybridization, sections were washed in PBS and dehydrated in graded ethanol series and air-dried. AVT cDNA was labelled 32p dCTP by random priming method (megaprime DNA labelling system, Amersham, Braunshweig, Germany) and separated from unincorporated nucleotides by using sephadex G-50 columns (Nick columns, Pharmacia, Friedeburg, Germany). The probe was diluted with hybridization buffer (50% formamid, 5 × Denhardt’s solution, 10% dextran sulphate, 0.75 M NaCl, 25 mM PIPES, 25 mM EDTA, 0.2% (w/v) sodium dodecyl sulphate and 250 µg/ml herring sperm DNA) to give final counts of approx. 2500 cpm/µl. Then 40 µl of AVT probe was applied on each section and hybridization was carried out for about 16 h at 52°C in a moist chamber. Washing was carried out in 4X sodium chlo-ride–sodium citrate buffer (SSC) for 3 × 10 min and 2X SSC for 3 × 10 min at room temperature. Final washing was done in 70% ethanol and then sections were dried in vacuum for 2 h. Thereafter, sections were covered with photographic emulsion (LM-1, Amersham) diluted 1:1 with distilled water. After exposure for 7–10 days at 4°C, slides with coated emulsion were developed using Ilford Phenisol and subsequently fixed. Slides were lightly counterstained with toluidine blue, and after air-drying were cover-slipped with Entellan (Merck). Sections were viewed with a Nikon Epiphot microscope equipped with dark field condenser. To check the specificity of hybridization signals, subsequent controls were treated with RNAase A (50 µg/ml, 0.5 M NaCl, 0.01 M Tris-HCl, 1 mM EDTA, pH 7.5; Boehringer, Mannheim, Germany) for 10 min at 37°C before prehybridization.
On the basis of in situ hybridization and immunohistochemical observations, it becomes clear that in ovo administration of sex steroids increases vasotocin synthesis in BnST neurons. In control condition AVT mRNA observed at E_{18} and D_{7} decreases gradually, with almost no signals at D_{40}. However, in steroid-treated embryo AVT mRNA can be demonstrated up to D_{40} post-hatch in females irrespective of male or female sex steroid administration (Figure 1). No immunostaining was seen in the BnST of control females throughout the study. However, among the treated groups, all the subjects (females) which received sex steroids at E_{6} showed ir-AVT in BnST neurons at E_{18}, D_{7}, D_{21} and D_{40}. The number of ir-AVT neurons as well as intensity of immunostaining which was not different among testosterone propionate (TP) and EB-treated groups at E_{18} and D_{7}, exhibited a significant increase in the EB-treated group compared to the TP-treated group at D_{21} and D_{40} (Figures 2 and 3, Table 1). On the other hand, by in situ hybridization, silver grains representing AVT mRNA can be demonstrated up to D_{40} post-hatch in females (Figure 1). Further, under basal condition, ir-AVT neurons were seen only in males and were completely lacking in females (Figure 4).

The present findings indicate that sex steroids play a significant role in the regulation/maintenance of vasotocin producing sexually dimorphic areas of avian brain, a phenomenon (sexual dimorphism) established early during ontogeny. The ir-AVT which was completely lost after E_{14} in control females, was present even up to D_{40} following sex-steroid administration at E_{6}. Thus sex-steroid administration upregulated the expression/synthesis of AVT transcript/ir-AVT throughout the stages of

**Figure 1.** Effect of in ovo administration of testosterone propionate (TP) and estradiol benzoate (EB) on BnST of female chicken showing in situ hybridization under dark field at day 7 (a–c), day 21 (d–f) and day 40 (g–i) post-hatch. The condition of E_{18} (not shown here) was similar to D_{7}. Arrowheads represent AVT mRNA. C, Control; LS, Lateral septum, BnST, Bed nucleus of stria terminalis. Scale bar is equivalent to 100 µm.
development studied. In TP-treated group, few neurons showing ir-AVT in BnST were observed at D21 and D40 post-hatch, while estradiol treatment showed more ir-AVT in neurons of BnST at D21 and D40 suggesting differential influence of sex steroids on AVT gene expression at later stages of development (Figure 2). In general, while the steady state level of AVT mRNA falls down in both TP- and EB-treated groups at D21 post-hatch; the underlying reasons are not completely understood.

Physiologically, steroid hormones produce three types of effects, i.e. long-term effects (for many days or weeks or for lifetime), short-term effects (for lesser time) or

Table 1. Effect of in ovo administration of sex steroids on AVT neurons of BnST at different stages of development

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>TP</th>
<th>EB</th>
<th>AVT mRNA (in situ hybridization)</th>
<th>Control</th>
<th>TP</th>
<th>EB</th>
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<tr>
<td>E18</td>
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+, Intensity of immunostaining of AVT neurons; *, Intensity of positive hybridization signals on AVT neurons; –, No activity; +/*, Minimum and +++/***, Maximum activity/signal. TP, Testosterone propionate and EB, Estradiol benzoate.

Figure 2. Effect of in ovo administration of sex steroids on ir-AVT in BnST of female chicken. a–d, Following testosterone administration at embryonal day 18 (E18), D7, D21 and D40 post-hatch, respectively, and e–h, Estradiol administration at E18, D7, D21 and D40, respectively. Scale bar is equivalent to 100 µm.

Figure 3. Effect of sex steroid administration on ir-AVT neurons in BnST of female chick at different stages of development. Values presented are mean ± SEM, *P < 0.01, significance of difference from other group.

Figure 4. Photomicrograph of chicken brain showing immunohistochemistry under bright field with ir-AVT in BnST of female and male chicken at E18 (a, b) and D40 post-hatch (c, d). Scale bar is equivalent to 100 µm.
rapid effects which are also called immediate effects (for seconds or minutes). Sex steroids can act at the genetic level by lowering or over expressing genes and can alter the sequence of biochemical events. The sex steroids can act on neuronal cells in several ways, causing increase or change in cell body size or shape, change in nuclear size, change in neuronal enzyme content, neuropeptide and neurotransmitter production, growth of dendrite processes, modification and alteration of efferent and afferent circuits, etc.\textsuperscript{26}. Variation in cell number takes place generally on the basis of three different mechanisms or modes of action. Either the steroid hormone stimulates neurogenesis in one sex or it could regulate processes influencing the differentiation of neurons or could prevent neuronal death\textsuperscript{27}. Gonadal steroid implants are reported to restore the behavioural effect of AVT injection. One hypothesis is that gonadal steroids affect AVT target neuron by altering AVT receptor concentration or binding affinity.\textsuperscript{28}

Vasotocin in birds is reported to control sexual behaviour\textsuperscript{29}, aggressive behaviour\textsuperscript{30} and vocalization\textsuperscript{29–31} in males. In females, it is mainly involved in regulating oviposition. To a large extent, these behaviours are dependent and regulated by sex steroids. Evidently, a sex steroid-sensitive and sexually dimorphic nucleus is found in the preoptic area of Japanese quail and is found to regulate male copulatory behaviour. This medial preoptic nucleus is significantly larger in adult male than in adult female Japanese quail\textsuperscript{31}. Immunoreactive aromatase elements are found to be clustered within the sexually dimorphic medial preoptic nucleus (POM), BnST, nucleus accumbens, and ventromedial and tuberal hypothalamus\textsuperscript{32}, which further support the notion that testosterone-dependent aromatase system is directly innervated by a testosterone-dependent peptidergic system. In rats, the effects of testosterone on the expression of vasopressin in the parvocellular system has been shown to depend to a large extent on the conversion of this steroid into estrogen by aromatase\textsuperscript{33}. Sex steroid-induced changes in AVP/AVT immunoreactivity could be caused by changes in the secretion/half-life of the neuropeptide or by changes in its synthesis\textsuperscript{34}. Most of the steroid-induced regulation takes place at the transcriptional or pre-translational level\textsuperscript{35,36}. Steroids are known to stabilize mRNA; hence, both transcription and stabilization of AVT mRNA could result in the observations reported above. In rats, it has been clearly demonstrated that changes in AVP immunocytochemistry are paralleled by changes in AVP mRNA concentration/distribution, suggesting that most of the steroid-induced regulation takes place at the transcriptional level. There are discrepancies in the number of neurons containing AVT transcript and those containing ir-AVT, suggesting that steroids may also act at post-transcriptional level\textsuperscript{37}. Further, neuroanatomical observations have revealed the existence of widespread interactions between estrogen-synthesizing neurons and vasotocinergic fibres. Recently, it has been found that a single estrogen responsive element in the distal 1.5 kb portion of the 5.5 kb fragment contains the primary positive estrogen responsive sequences for estrogen receptor \( \alpha \) and estrogen receptor \( \beta \) which regulate neuronal expression of vasopressin mRNA in rat\textsuperscript{18}.

In quail, ir-AVT in POM and BnST varies with sexual behaviour, being low in castrated males and males exposed to short days, and high in testosterone-treated and in males exposed to long days\textsuperscript{19}. On the basis of the present findings, it has been suggested that expression of AVT gene and localization of ir-AVT in sexually dimorphic BnST may be modulated by \textit{in ovo} administration of sex steroids. Also, the vasotocinergic system of developing brain is sensitive to sex steroid even in early embryonic conditions, and is comparable to the effects of sex steroids in differentiation of mammalian brain into male or female type.
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Arterial oxygen saturation under hypoxic environment of high-altitude associates with routine physical activities of natives


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Per cent oxygen saturation of arterial haemoglobin levels (SaO₂), a measure of hypoxemia has been analysed in the permanent residents of Ladakh. The population recognized as high-altitude controls (HAC) and high-altitude monks (HAM), resided at the same altitude of 3600 m but differed in their routines. SaO₂ of HAM had 3.08% higher SaO₂ than the HAC, with mean SaO₂ of 91.8 ± 6.1% and 89.0 ± 2.6%, respectively. Furthermore, the younger HAM also revealed an elevation of 4.55% SaO₂ compared to the HAC, with mean SaO₂ of 91.8 ± 6.1% and 89.0 ± 2.6%, respectively. Therefore, the younger HAM also revealed an elevation of 4.55% SaO₂ compared to the HAC.

HIGH-ALTITUDE (HA) environment is characterized by hypobaric hypoxia. A low barometric pressure at high altitudes causes reduction in the partial pressure of oxygen in inspired air. Accordingly, the oxygen saturation of haemoglobin is reduced in HA-living organisms. The resulting hypoxic condition stresses the metabolic processes of an individual for want of oxygen. Nevertheless, the aerobic physiological processes must be maintained to avoid mountain disorders. The native highlanders (HLs) have a long history of exposure and opportunity for natural selection. Therefore, they show adaptive phenotypes with respect to physiological parameters such as oxygen saturation, haemoglobin concentration, hypoxic ventilatory rate, and blood pressure. Contrary to this, sojourners show variation in these parameters and experience various levels of physical discomfort at HA primarily due to significant fall in arterial blood oxygen saturation. This results in somewhat reduced level of performance in individuals when faced with tasks demanding high levels of physical abilities. The

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