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Expression of hypothalamic arginine vasotocin gene in response to water deprivation and sex steroid administration in female Japanese quail

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Summary

Arginine vasotocin (AVT) is a neurohypophyseal hormone involved in reproductive function and control of osmoregulation in birds. In view of the dual function of AVT, the present experiment was designed to observe the effect of water deprivation (WD) and sex steroid [estradiol benzoate (EB) and testosterone propionate (TP)] treatment independently, as well as simultaneously, on the profile/activity of the hypothalamic AVT system. WD resulted in a significant increase in plasma osmolality, sodium ion concentration and AVT concentration, but administration of sex steroids had no significant influence on these parameters. By contrast, the amount of hypothalamic AVT transcript (northern analysis) and the size of immunoreactive vasotocin (ir-AVT) neurons and hybridization signals (in the form of silver grains), representing AVT mRNA in corresponding neurons of paraventricular nuclei (PVN), increased significantly in all the treated groups compared with controls. Our findings indicate that although sex steroid administration has no effect on plasma osmolality and AVT concentration, unlike water deprivation, it may stimulate the profile/activity of AVT neurons of PVN, supporting the possibility of sex steroid receptors on these neurons. It is concluded that in quail, osmotic stress not only upregulates the expression of the AVT gene in existing neurons but also recruits many more neurons to increase the rate of AVT synthesis and secretion, while sex steroids appear to have a stimulatory effect only on the existing number of neurons and only at the level of transcription/translation and hence may influence/modulate hypothalamic AVT gene expression in response to osmotic stress. This study also suggests an interrelationship between reproduction and AVT system/function in birds.

Key words: arginine vasotocin gene, sex steroid, Japanese quail, *Coturnix coturnix japonica*, dehydration, paraventricular nuclei.

Introduction

Arginine vasotocin (AVT) is synthesized in magnocellular diencephalic neurons (Acher et al., 1997) and is released into circulation in a highly coordinated manner, contributing to the peripheral control of oviposition in the hen (Shimada et al., 1986; Shimada and Saito, 1989). A clear-cut sexual dimorphism of the vasotocin system, particularly in a group of immunoreactive AVT (ir-AVT) cells in the bed nucleus of the stria terminalis (BnST; Jurkevich et al., 1997; Aste et al., 1998; Jurkevich and Grossmann, 2003), has been reported in both oscine (canary) and non-oscine (Japanese quail, domestic fowl) birds. In quail, a sexually dimorphic group of ir-AVT neurons is also observed within the boundaries of the medial preoptic nucleus (POM), only in males, by immunocytochemistry (Aste et al., 1998). In addition to a dense network of ir-AVT fibers (Viglietti-Panzica et al., 1994), POM is also characterized by the presence of a dense population of ir-aromatase cells controlled by steroids (Balthazart et al., 1990; Panzica et al., 1996b). There is a marked reduction in ir-AVT fibers in POM and septum during aging and restoration of AVT immunoreactivity following exogenous supplementation of testosterone (T; Panzica et al., 1996a) or estradiol (E; Viglietti-Panzica et al., 2001). The effects of T on the central nervous system are mediated by neural conversion to E by the enzyme aromatase (Balthazart et al., 2003; Soma et al., 2004; Silverin et al., 2004). In castrated birds exposed to short days and in aging birds, ir-AVT fibers in POM disappear and are restored by exogenous administration of T (Viglietti Panzica et al., 1994; Panzica et al., 1996a,b). T-induced alteration in ir-AVT could be caused by changes in secretion/immunostaining of the neuropeptide or by a change in its synthesis. 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. Gonadal steroid hormones and neurohypophyseal peptides are found to act in concert to regulate reproductive

behavior in mammals as well as in non-mammalian vertebrates (Moore, 1992). Recently, a single estrogen responsive element in the distal 1.5 kb portion of the 5.5 kb genomic DNA fragment, 5′ of the AVP coding region, has been found containing the primary positive estrogen responsive sequences for estrogen receptor α and estrogen receptor β that regulate neuronal expression of vasopressin mRNA in rat (Shapiro et al., 2000). However, the effect of sex steroid on magnocellular neurons (which are not reported to be sexually dimorphic) has not yet been reported.

Water deprivation causes an increase in hypothalamic AVT mRNA by increasing the amount of transcript per neuron and by recruitment of clusters of magnocellular AVT neuron that are not identifiable in the basal condition (Chaturvedi et al., 1994). In chickens, a similar effect was observed, by *in situ* hybridization, following saline drinking, hypertonic saline administration or hemorrhage (Chaturvedi et al., 1997; Jaccoby et al., 1997). In view of the effect of osmotic stress and sex steroids on the AVT system, the present experiment was undertaken to study the simultaneous effects of water deprivation and sex steroid administration on the expression of the hypothalamic *AVT* gene and on the magnocellular neurons of paraventricular nuclei (PVN) synthesizing AVT.

Materials and methods

Animals

Sexually immature, 3-week-old, female Japanese quail (*Coturnix coturnix japonica* L.) maintained under short days (8 h:16 h L: D) were provided with commercial ration and water *ad libitum*. After recording body mass, birds were randomly divided into six groups (N=10 per group): (1) control (C), provided water *ad libitum*; (2) water deprived for 2 days (WD); (3) injected with estradiol benzoate (Sigma, Deisenhofen, Germany; 1 mg day^{-1}) for 16 days (EB); (4) injected with estradiol benzoate for 16 days and deprived of water for the last 2 days of injection (EB+WD); (5) injected with testosterone propionate (Eifelfango, Bad Neuenahr, Ahrweiler, Germany; 1 mg day^{-1}) for 16 days (TP) and (6) injected with testosterone propionate for 16 days and deprived of water for the last 2 days of injection (TP+WD). Each group was maintained in individual cages. All injections were given intraperitoneally. At the end of the study, birds of all the groups were of the same age and were sacrificed on the same day after receiving the prescribed treatment of estradiol/testosterone and/or 2 days of water deprivation. Five birds from each group were sacrificed by decapitation at the end of the experiment (on the 17th day), and the hypothalami isolated were used for northern blot analysis. The remaining five birds from each group were used for immunohistochemical and *in situ* hybridization studies.

Northern blot analysis

Brains were quickly removed, and hypothalami were isolated, snap frozen in dry ice and stored at –70°C until used for northern blot analysis. Total RNA was isolated using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the method described by Chomczynski and Sacchi (1987). 20 μg of total RNA was separated on 1.4% w/v agarose denaturing formaldehyde gel in morpholino-propane sulfonic acid (MOPS) buffer, pH 8.0, and subsequently blotted overnight by capillary transfer onto nylon membranes (Hybond N$^+$; Amersham, Braunschweig, Germany), followed by UV crosslinking (150 mJ; gene linker, Bio-Rad, München, Germany). The AVT-specific probe (a 260 bp cDNA directed towards the distal 3′ glycopeptide part of the chicken *AVT* gene; Hamann et al., 1992) was labeled with [^{32}P]dCTP by the random priming method (megaprime DNA labeling system; Amersham) according to Feinberg and Vogelstein (1983) and separated from unincorporated nucleotides by using Sephadex G50 columns (Nick columns; Pharmacia, Freiburg, Germany). Hybridization proceeded overnight at 42°C in 50% formaldehyde-containing buffer according to Wahl et al. (1979). Approximately 5×10^6 c.p.m. were used per filter in 6 ml of hybridization buffer without dextran sulfate. Exposure time was 48 h at –70°C using one intensifying screen. After stripping of the AVT cDNA, the filter was rehybridized with the housekeeping gene *GAPDH* to show the loading of RNA samples in each lane. Autoradiographs showing *AVT* (700 bp) and *GAPDH* (1.4 kb) gene expression were sequentially exposed to storage phosphor screens (Bio-Rad), and densitometric bands corresponding to AVT and GAPDH were calculated. The volume of the AVT band was divided by the volume of the GAPDH band and multiplied by 100 to determine the % hypothalamic AVT mRNA.

Immunohistochemistry (IHC)

Quail were anaesthetized using pentobarbital sodium (3–4 mg per 100 g body mass), and a blood sample (~1.5 ml) was obtained from the wing vein in a heparinized syringe. Whole-body perfusion was done through the heart using 0.02 mol l^{-1} phosphate-buffered saline (PBS) and Zamboni fixative (Stefanini et al., 1967) by perfusion pump at a speed of 2–3 ml min^{-1}. Fixed brain tissue was processed for cryostat sectioning and subsequent IHC. For cryoprotection, brains were transferred to 25% sucrose solution in PBS at 4°C until the brain sank to the bottom (~24 h). Brains were frozen using tissue-freezing medium and cut at 18 μm in a cryostat. For IHC, sections were washed several times in 0.02 mol l^{-1} PBS until fixative was washed out completely, treated with 0.6% hydrogen peroxide (Sigma) in PBS for 30 min, incubated in 5% normal goat serum (DAKO, Hamburg, Germany) containing 0.2% Triton X-100 for 30 min, followed by incubation for 36 h at 4°C in a 1:5000 solution of rabbit anti-AVT serum in PBS containing 0.2% Triton X-100, 1% normal goat serum and 0.1% sodium azide. The AVT antiserum was kindly provided by Dr D. Gray (Max Planck Institute for Physiological and Clinical Research, Bad Nauheim, Germany). Sections were thoroughly washed and incubated in goat anti-rabbit biotinylated IgG (DAKO; 1:500 in PBS containing 0.2% Triton X-100) for 90 min at room temperature, rinsed in PBS (four times for 15 min each) and

incubated for 90 min with avidin biotin peroxidase conjugate (ABC–HRP; DAKO; 1:1000) solution in PBS containing 0.2% Triton X-100 and 1% crystalline bovine serum albumen (Sigma). For immunodetection, 3,3′ diaminobenzidene (DAB; Sigma) in 0.05 mol l^{-1} Tris buffer (pH 7.6) with 0.005% hydrogen peroxide was used. Sections were subsequently rinsed in PBS and distilled water, air-dried and coverslipped using entellan (Merck, Darmstadt, Germany). To control the specificity of the immune reaction, sections were incubated with 5% normal goat serum instead of AVT antiserum. In control sections, no immune-positive signal was detected (Fig. 7).

Morphometric measurements

Brain sections containing ir-AVT neurons in the PVN were selected from each experimental and control group of quail. Immunostained cells/neurons were counted manually under an ordinary light microscope (Weswox Optik model TR Hl 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). Area/size of these neurons was measured by occulomicrometer (length × width). Student's *t*-tests were used to assess comparisons between group means.

In situ *hybridization (ISH)*

Fixed brain tissue was processed for cryostat sectioning and subsequent ISH. Brain sections were washed in PBS, dehydrated in a graded ethanol series and air-dried. The probe was labeled by the random priming method as described for northern analysis but using [^{33}P]dCTP. The probe was diluted with hybridization buffer [50% formamide, 5× Denhardt's solution, 10% dextran sulfate, 0.75 mol l^{-1} NaCl, 25 mmol l^{-1} PIPES, 25 mmol l^{-1} EDTA, 0.2% (w/v) sodium dodecyl sulfate (SDS) and 250 μg ml^{-1} herring sperm DNA] to give final counts of ~2500 c.p.m. μl^{-1}. 40 μl of AVT probe was applied to each section, and hybridization was carried out for ~16 h at 52°C in a moist chamber. Washing was carried out in 4× sodium chloride–sodium citrate buffer (SSC) for 3×10 min and 2× SSC for 3×10 min at room temperature. Final washing was done in 70% ethanol and then sections were dried under vacuum for 2 h. 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, lightly counterstained with toluidine blue, and after air drying were coverslipped with Entellan (Merck). Sections were viewed with a Nikon Epiphot microscope equipped with a dark-field condenser. Hybridization signals, in the form of silver grains observed over the neurons under dark field, represent AVT mRNA. To check the specificity of hybridization signals, subsequent controls were treated with RNase A (50 μg ml^{-1}; Boehringer, Mannheim, Germany; 0.5 mol l^{-1} NaCl, 0.01 mol l^{-1} Tris-HCl, pH 7.5, 1 mmol l^{-1} EDTA) for 10 min at 37°C before the prehybridization (Fig. 7).

Plasma analysis

Plasma samples were extracted and processed for radioimmunoassay (RIA) for ir-AVT by the method of Gray and Simon (1983). AVT was extracted from plasma with two volumes of acetone and two volumes of petroleum ether. The extract was dried under vacuum in a speed vac concentrator (Savant Instruments Inc., New York, USA). The dried extract was dissolved in assay buffer (0.1 mol l^{-1} Tris-HCl, pH 7.4, 2% BSA and 0.2% neomycin) and stored at –20°C until assayed. RIA was performed in duplicate using synthetic AVT as a standard (Sigma). Plasma osmolality was measured by vapor pressure osmometry (Wescor, model 5500; Logan, UT, USA). Plasma levels of sodium and potassium were analyzed using a sodium/potassium analyzer (Ciba Corning Diagnostics, Sudbury, UK).

Statistics

For statistical analysis of data, analysis of variance (ANOVA) followed by Newman–Keul's multiple range test was applied. For estimating northern blot data, Student's *t*-test was employed to compare between control and experimental groups. Student's *t*-test was also employed to make individual comparisons between sex steroid and sex steroid coupled with water deprivation groups.

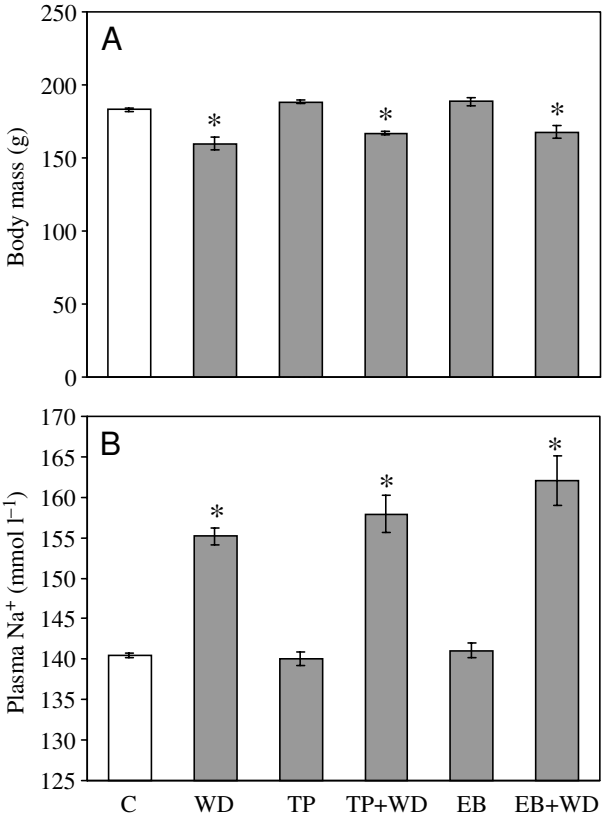

Fig. 1. Effect of dehydration and sex steroid administration on (A) body mass and (B) plasma sodium ion concentration of Japanese quail. Values presented are means ± S.E.M. (N=10). *P<0.001 (significance of difference from control).

Results

Body mass and Plasma analysis

Body mass decreased significantly in WD, EB+WD and TP+WD quails but no difference was observed in the steroid-treated birds compared with controls (Fig. 1A). There was a significant increase in plasma Na^+ concentration following water deprivation, in EB+WD and in TP+WD quail compared with controls but no differences were observed after sex steroid administration (Fig. 1B). Plasma osmolality and AVT concentration also did not vary following sex steroid administration, but dehydration (water deprivation) alone and sex steroid administration coupled with dehydration increased the values of these parameters (Fig. 2A,B).

Northern blot analysis

An AVT mRNA transcript corresponding to 700 bp was detected in the hypothalamus of quail. After normalization with the housekeeping gene *GAPDH*, % increase in *AVT* gene expression was calculated. Levels were lower in controls when compared with water-deprived and steroid-treated animals. Although increased hypothalamic AVT transcript was detected in all the treated groups, it was highly significant (P<0.001) in animals treated with sex steroids coupled with water deprivation (Fig. 3).

In situ *hybridization*

Following *in situ* hybridization studies under dark field, silver grains representing steady-state levels of AVT mRNA/hybridization signals were localized over neurons that are immediately lateral to the third ventricle (3V). Compared with controls, an increase in the intensity and density of silver grains over AVT neurons can be visualized (not quantified) in the PVN region of steroid-treated animals. But, a highly remarkable increase in the density of silver grains representing AVT transcript is apparent in water-deprived quail and in quail subjected to sex steroid administration and water deprivation simultaneously (Fig. 4).

Immunohistochemistry

A significant increase in the number and area of ir-AVT neurons as well as in the intensity of immunostaining was seen in the PVN of WD, EB, EB+WD and TP+WD quail compared with controls (Fig. 5). Although TP-treated quail appeared to have more ir-AVT neurons compared with the control (Fig. 6), the difference was not statistically significant (Fig. 5).

Discussion

The present results confirm our earlier reports that, in quail, osmotic stress increases the quantity of AVT mRNA

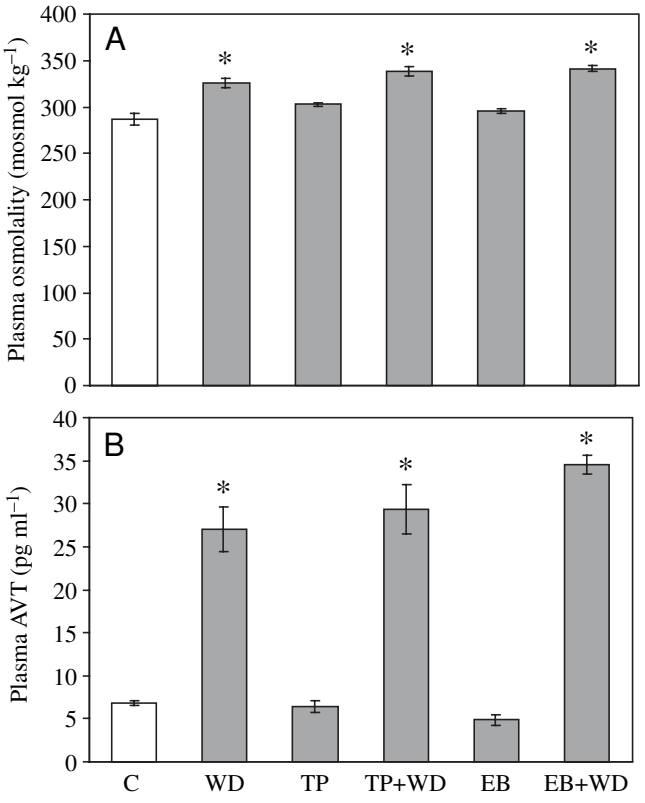

Fig. 2. Effect of dehydration and sex steroid administration on (A) plasma osmolality and (B) plasma AVT concentration of Japanese quail. Values presented are means ± S.E.M. (N=10). *P<0.001 (significance of difference from control).

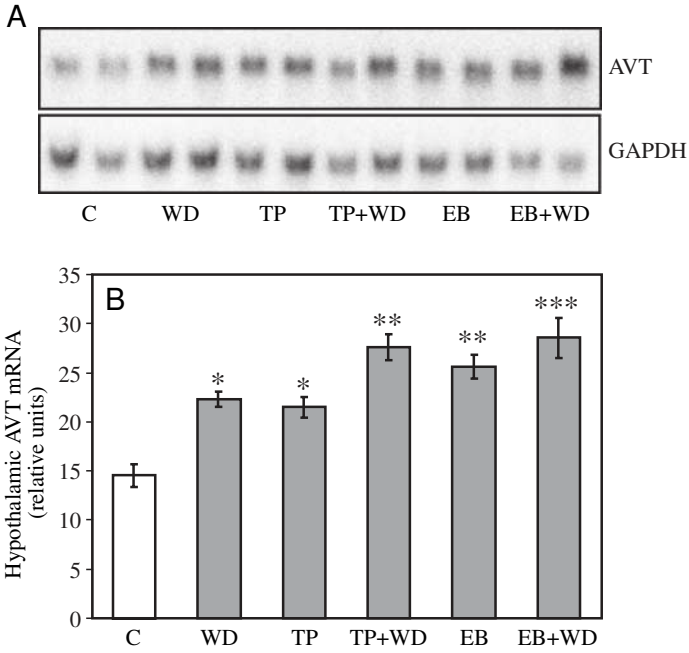

Fig. 3. (A) Northern blot hybridization of hypothalamic RNA probed with fowl *AVT* cDNA and *GAPDH*. Each lane was loaded with 20 μg of total cellular RNA. C, control; WD, water deprived; TP, testosterone propionate treated; TP+WD, testosterone propionate treated + water deprived for last 2 days of injection; EB, estradiol benzoate treated; EB+WD, estradiol benzoate treated + water deprived for last 2 days of injection. (B) Hypothalamic AVT mRNA. Values are means ± S.E.M. (N=10). *P<0.05; **P<0.01; ***P<0.001 (significance of difference from control).

(Chaturvedi et al., 2000), as in the hypothalamus of chickens (Mühlbauer et al., 1992). The results are consistent with *in situ* hybridization studies that also demonstrate that dehydration causes an increase in the amount of AVT mRNA per neuron as well as in the number of neurons in the PVN/hypothalamus that express the *AVT* gene (Fig. 4). A significant increase ($P<0.05$) in the size and number of ir-AVT neurons following estradiol administration and % increase in the AVT mRNA in

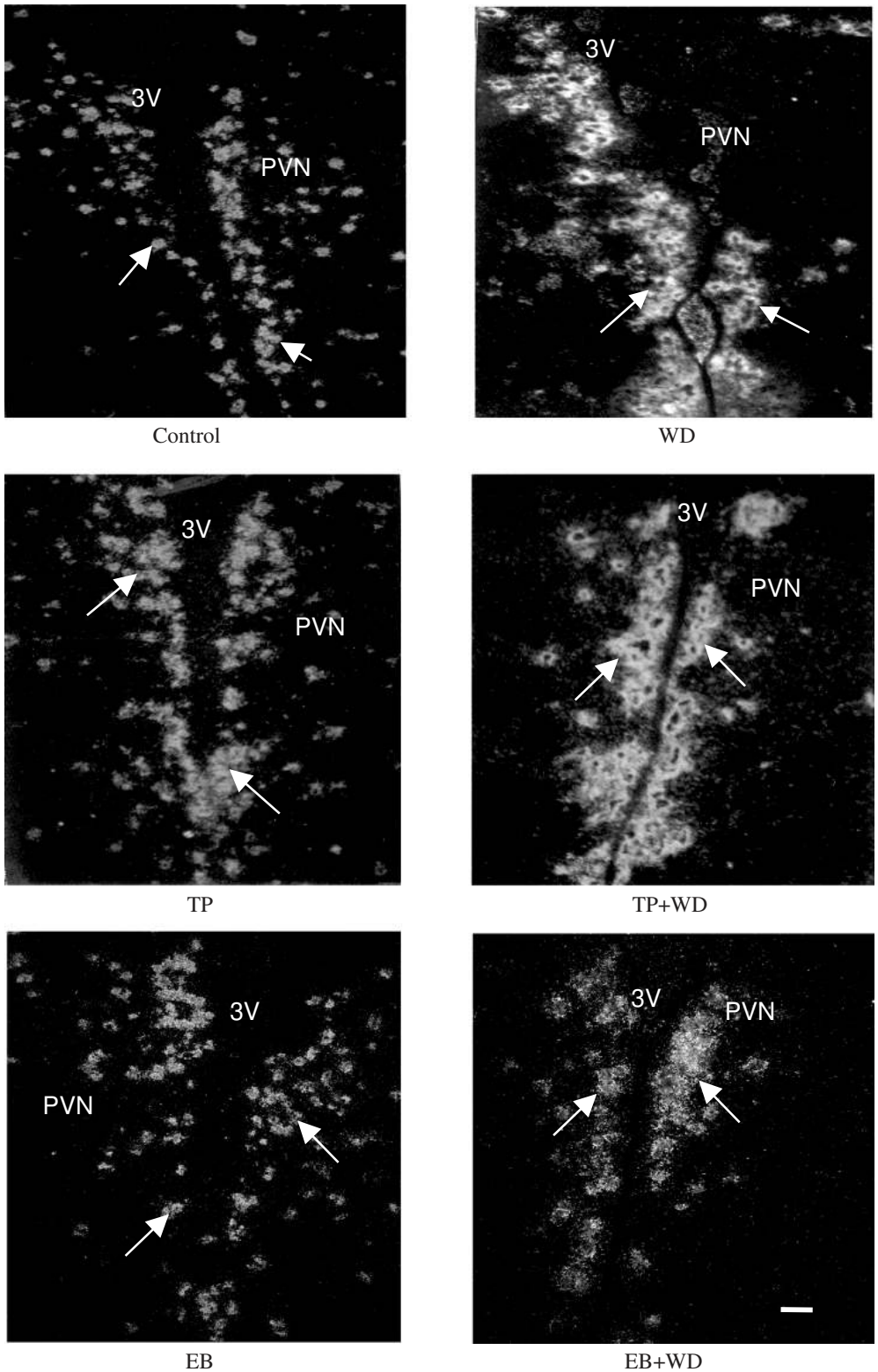

Fig. 4. Photomicrographs of quail brain showing *in situ* hybridization of AVT mRNA (arrows), under dark field, in paraventricular nuclei on either side of the third ventricle (3V). Abbreviations as in Fig. 3. Scale bar, 150 μm.

the hypothalamus as well as mRNA copies in AVT neurons following estradiol/testosterone administration suggest the involvement of sex steroids in the upregulation of hypothalamic AVT gene expression in quail (Figs 3, 4). The AVT transcript, immunostaining and number of ir-AVT cell bodies increased further in hypothalamic neurons when sex-steroid-administered quail were simultaneously subjected to water deprivation. A few empty neuronal cells were also visible in the EB+WD group (represented by asterisk in Fig. 6) and it is quite possible that, due to increased demand, the neuropeptide is immediately released into the circulation (Figs 3, 5, 6).

Physiologically, steroid hormones produce three different effect periods. Either these hormones produce long-term effects (for many days or weeks or for life), short-term effects (for a lesser time) or rapid effects, which are also called immediate effects (for seconds or minutes). Sex steroids can act at the genetic level by lowering or overexpressing genes and can alter the sequence of biochemical events. The sex steroids can act on neuronal cells in several ways, causing an increase or change in cell body size or shape alteration, changes in nuclear size, changes in neuronal enzyme content, neuropeptide and neurotransmitter production, growth of dendrite processes, modification and alteration of efferent and afferent circuits, etc (Panzica et al., 1997). The variation in cell number generally occurs on the basis of three different mechanisms: (1) the steroid hormone stimulates neurogenesis in one sex, (2) it could regulate processes influencing the differentiation of neurons or (3) could prevent neuronal death (Arnold and Schlinger, 1993). Gonadal steroid implants are reported to restore the behavioral effect of AVT injection. One hypothesis also suggests that gonadal steroids affect the AVT target neuron by altering AVT receptor concentration or binding affinity (Moore, 1992).

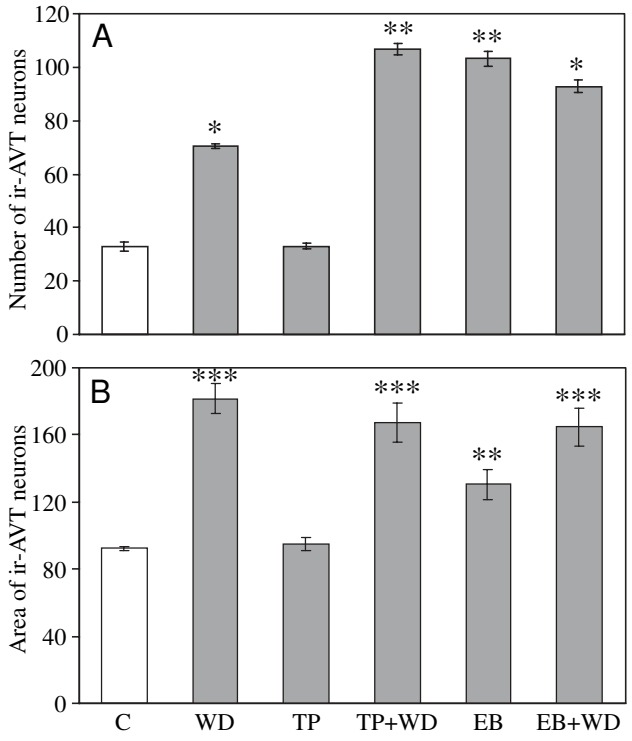

Fig. 5. Effect of dehydration and sex steroid administration on (A) number and (B) area of ir-AVT cell bodies in the paraventricular nucleus (PVN) region of the hypothalamus of Japanese quail. Values are means ± S.E.M. (N=10).*P<0.05; **P<0.01; ***P<0.001 (significance of difference from control). Abbreviations as in Fig. 3.

The present study suggests a significant upregulation of the *AVT* gene in the magnocellular neurons of PVN of Japanese quail following 2 days of water deprivation. Although sex steroid administration over a period of 16 days also induced some increases in number of ir-AVT neurons and an increase in the steady-state level of AVT mRNA transcript in these neurons as well as in total hypothalamic RNA, the effect was remarkably augmented when the two conditions (water deprivation and sex steroid administration) were applied simultaneously. Increased localization of the steady-state level of AVT mRNA transcript and ir-AVT in the neurons of PVN and a simultaneous increase in the concentration of plasma AVT following water deprivation suggest that the osmotic stress stimulates transcription of the *AVT* gene in hypothalamic neurons and causes release of AVT in the peripheral circulation, thus affecting synthesis (transcription and translation) in the hypothalamus as well as secretion of AVT from neurohypophysis. Furthermore, since the number of neurons expressing ir-AVT in the TP-treated group was not statistically different from the control group, but the amount of AVT transcript in the hypothalamus and the intensity of hybridization signals (not quantified) in individual neurons was higher in these quail, it appears that testosterone treatment upregulates *AVT* gene expression (transcription only) in the existing neurons. However, estradiol administration induced significant increase in the number of neurons expressing ir-AVT in the PVN region, suggesting a differential response of sex steroids on the AVT system in birds.

Comparing the two effects, i.e. water deprivation and TP administration, on AVT neurons, it is obvious that, although both conditions upregulate AVT gene expression in the hypothalamus (northern analysis) and also in existing (basal) numbers of ir-AVT neurons of PVN, an additional number of neurons (which were dormant in basal condition) is recruited for *AVT* gene expression in only the osmotically stimulated condition (but not in TP-treated quail). Another point of difference is that, unlike in WD quail, localization of ir-AVT/intensity of immunostaining and plasma AVT concentration was not increased in the TP-treated quail. Taken together, these observations suggest that TP upregulates the transcription of AVT in PVN neurons (as well as in other vasotocinergic regions), an effect that appears to be mediated through testosterone receptors present on these AVT neurons, but possibly has no role at the translational level. On the other hand, the water-deprivation-induced effect on the AVT system (at both a transcriptional and translational level) is due to/mediated through an increase in plasma osmolality (not observed in the TP-treated group). A direct relationship exists

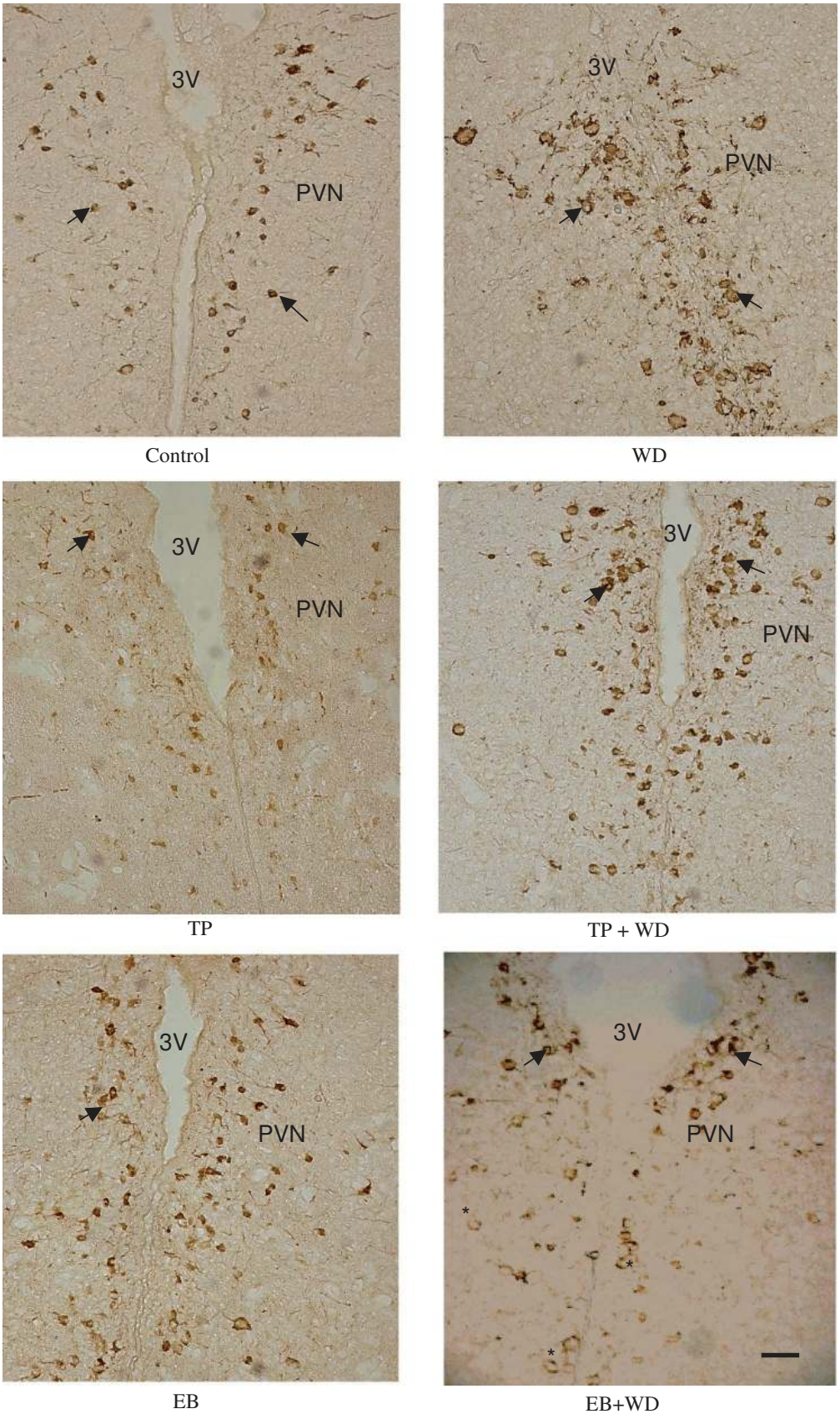

Fig. 6. Immunohistochemistry of quail brain showing localization of ir-AVT (arrows) in paraventricular nuclei (PVN) on either side of the third ventricle (3V). Scale bar, 150 µm. Asterisks show empty neuronal cells. Abbreviations as in Fig. 3.

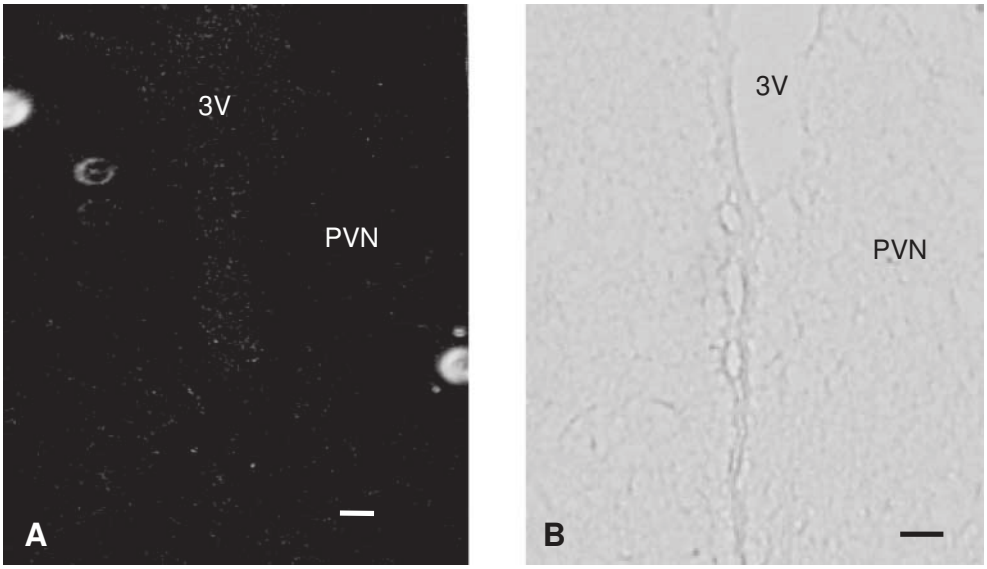

Fig. 7. Photomicrographs of quail brain showing negative control for (A) *in situ* hybridization. Brain sections were treated with RNase A prior to hybridization. (B) Brain section was not treated with AVT antiserum and served as a negative control for immunohistochemistry. Scale bars, 150 μm. PVN, paraventricular nuclei; 3V, third ventricle.

between increase in osmolality and AVT secretion following upregulation of *AVT* gene expression. On the other hand, when comparing estradiol-treated and control group birds, a highly significant increase in *AVT* gene expression as well as in the number of ir-AVT neurons and intensity of immunostaining is seen in the same region. These findings further suggest the differential role of sex steroid/breeding status in upregulating transcription of the *AVT* gene in the hypothalamus/PVN neurons and hence appears to show a stimulatory role on the activity of the hypothalamo–neurohypophyseal axis (AVT system) of birds in response to osmotic stress.

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