Organizational Effects of Estrogens on Brain Vasotocin and Sexual Behavior in Quail

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ABSTRACT: **Reproductive behavior is sexually differentiated in quail: The male-typical copulatory behavior is never observed in females even after treatment with high doses of testosterone (T). This sex difference in behavioral responsiveness to T is organized during the embryonic period by the exposure of female embryo to estrogens. We showed recently that the sexually dimorphic medial preoptic nucleus (POM), a structure that plays a key role in the activation of male copulatory behavior, is innervated by a dense steroid-sensitive network of vasotocin-immunoreactive (VT-ir) fibers in male quail. This innervation is almost completely absent in the female POM and is not induced by a chronic treatment with T, suggesting that this neurochemical difference could be organizational in nature. This idea was tested by injecting fertilized quail eggs of both sexes on day 9 of incubation with either estradiol benzoate (EB) (25 μg, a treatment that suppresses the capacity to show copulatory behavior in adulthood) or the aromatase inhibitor R76713 (10 μg, a treatment that makes adult females behaviorally responsive to T), or with the solvents as a control (C). At 3 weeks posthatch, all subjects were gonadectomized and later implanted with Silastic capsules filled with T. Two weeks later, all birds were perfused and brain sections were processed for VT immunocytochemistry. Despite the similarity of the adult endocrine conditions of the subjects (all were gonadectomized and treated with T Silastic implants providing the same plasma level of steroid to all subjects), major qualitative differences were observed in the density of VT-ir structures in the POM of the different groups. Dense immunoreactive structures (fibers and a few cells) were observed in the POM of C males but not females; EB males had completely lost this immunoreactivity (and lost the capacity to display copulatory behavior); and, conversely, R76713 females displayed a male-typical VT-ir system in the nucleus (and also high levels of copulatory behavior). Similar changes in immunoreactivity were seen in the nucleus of the stria terminalis and in the lateral septum (VT-ir fibers only in this case) but not in the magnocellular vasotocinergic system. These neurochemical changes closely parallel the effects of the embryonic treatments on male copulatory behavior. The vasotocinergic system of the POM can therefore be considered an accurate marker of the sexual differentiation of brain circuits mediating this behavior.** J Neurobiol 37: 684–699, 1998

Keywords: **preoptic area; bed nucleus of the stria terminalis; lateral septum, sexual differentiation; copulatory behavior; Japanese quail**

Many vertebrate species are characterized by an extensive behavioral dimorphism. Some behaviors, often related to reproduction (mounting and intromission, singing, aggression, territory defense, crouching, and lordosis) are displayed preferentially or exclusively by one sex and rarely or never shown by the other. It was originally thought that these behavioral differences simply reflect the endocrine milieu to which adult subjects are exposed: Androgens would activate one type of behaviors in males and estrogens/progestagens would activate another set in females. It has now become increasingly clear that this simplistic view is not true in many cases and that the behavioral sex dimorphism is often based on the presence of a differential responsiveness of the central nervous system to the activating effects of steroid hormones.

The seminal paper of Phoenix et al. (1959) pro-

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vided a conceptual framework with which these behavioral sex differences can be interpreted. Sex differences in reproductive behavior often result from the early, irreversible action of gonadal steroids that organize the nervous substrate (organizational effects) that will in adulthood control the behaviors. Steroid hormones in adulthood are then often required for the expression of the behaviors (activational effects) in one sex, but at this level the sex difference in the nervous substrate will play a critical limiting role (Goy and McEwen, 1980). These notions that were originally formulated on the basis of experiments investigating lordosis behavior in guinea pigs have been found to have a broad range of application even if the clear-cut separation between organizational and activational effects can sometimes be difficult to ascertain (Arnold and Gorski, 1984). Research carried out on a variety of animal models from different vertebrate classes has identified behavior patterns that are irreversibly differentiated by embryonic and/or neonatal steroids, and consequently can be activated only by androgens or estrogens in one sex only in adulthood (Goy and McEwen, 1980).

In contrast, the search for the neural characteristics that are organized by early steroid action and mediate behavioral sex dimorphism has been relatively frustrating. A number of neuroanatomical, neuroendocrine, and neurochemical sex differences have been identified over the past 20 years in the vertebrate brain, but either these differences are activational in nature (i.e., they disappear when males and females are placed under the same endocrine conditions, suggesting that early steroid action is not involved in their development) or their relationship to the control of behavior remains unclear (see Panzica et al., 1995; Balthazart et al., 1996a; Kawata et al., 1994, for review). The vocal behavior of songbirds appears to be an exception here in that marked anatomical and neurochemical sex dimorphisms have been identified in the brain that are organizational in nature and appear to play a key role in the control of the sexually dimorphic vocal behavior. However, the organizational (endocrine?) mechanisms that control the differentiation of these brain characteristics have eluded investigators so far in this model (Balthazart and Ball, 1995; Arnold, 1996, 1997a,b; Schlinger, 1998).

The extreme form of sex difference in the copulatory behavior of the Japanese quail provides an outstanding experimental model for the analysis of the underlying brain mechanisms. The entire copulatory sequence, including grabbing the female's neck feathers, mounting, and cloacal contact movements, is reliably observed in sexually mature males or in castrated males treated with testosterone (T), but T will not activate these behaviors in females even when provided at doses that are by far higher than the active doses in males (Adkins, 1975; Balthazart et al., 1983). There is therefore a qualitative sex difference in copulatory behavior in quail. This behavioral sex dimorphism has been shown to result from the early exposure of females to estrogens. This conclusion is supported by three independent types of evidence: (a) Injection of estrogens into male eggs completely abolishes the capacity to show copulatory behavior when adult, even in the presence of exogenous T (Adkins, 1979; Schumacher et al., 1989); (b) inhibition of estrogen synthesis in female embryos by injection of an aromatase inhibitor results in adult females that will respond to a T treatment exactly like males (i.e., they will mount and copulate with other females) (Balthazart et al., 1992a); and (c) female embryos actually have much higher levels of circulating estradiol-17β than male embryos during one part of the incubation period (days 9–15) (Schumacher et al., 1988). There is also a well-characterized sensitive period during which the developing brain of quail embryos is highly sensitive to the demasculinizing effects of estrogens: it ends around day 12 of incubation and therefore extensively overlaps with the high levels of endogenous estrogens present in females (Adkins, 1979; Schumacher et al., 1989).

The neural circuit that controls male copulatory behavior in quail has not been completely identified to this date, but it is clearly established that the medial preoptic nucleus (POM) and the bed nucleus of the stria terminalis (BST) are key elements in this network (Panzica et al., 1996b). This conclusion is based on a variety of experiments involving the stereotaxic implantation of T in these nuclei, their electrolytic lesion, and the detection of the expression of the immediate-early genes *fos* and *zenk* in subjects expressing copulatory behavior (Balthazart and Surlemont, 1990; Balthazart et al., 1992b, 1998; Meddle et al., 1997; Ball et al., 1997).

Both POM and the BST contain a dense vasotocinergic system that has been shown in previous studies to be sexually differentiated. Vasotocin-immunoreactive (VT-ir) fibers are present in much higher density in these nuclei in males than in females (Viglietti-Panzica et al., 1997; Aste et al., 1998). VT-ir cell bodies have been observed in POM and BST of males but not in females, and accordingly, a sex difference in VT expression has been confirmed by *in situ* hybridization of VT mRNA in the BST and POM (Aste et al., 1998). The VT innervation of the POM also appears to be steroid sensitive in males: The density of VT-ir fibers in this nucleus decreases in conditions where males experience low levels of circulating T (castration, photoregression, and old age) and are restored to levels typical of sexually mature males by exogenous treatments with T (Viglietti-Panzica et al., 1994; Panzica et al., 1996a).

There are therefore many experimental situations in which the VT structures of POM and/or BST vary with the expression of male copulatory behavior, which suggests the existence of causal links between the peptide and the behavior. This notion is further reinforced by a set of recent experiments demonstrating that the peripheral or intracerebroventricular injection of VT or of a specific V1-receptor antagonist markedly affects the appetitive and consummatory aspects of male sexual behavior in castrated T-treated male quail (Castagna et al., 1998).

The sex difference in VT structures of POM and BST may be responsible for the sex difference in copulatory behavior. Indirect evidence suggests that the VT system of these brain areas is indeed sexually differentiated in the organizational sense. The dimorphism is also present in the lateral septum and is present even after treatment of ovariectomized females with T (Viglietti-Panzica et al., 1992). The effect of T treatment on the VT innervation of the POM or BST has not been studied in females, however. The present study was therefore carried out to investigate two related questions—namely, (a) whether the VT structures of POM and BST in adult female quail can be enhanced by adult treatment with T; and (b) if this is not the case, whether the embryonic hormones that mediate the sexual differentiation of male copulatory behavior also organize the sex difference in VT structures of POM and/or BST.

MATERIALS AND METHODS

Subjects and Endocrine Treatments

This experiment was carried out on male and female Japanese quail (*Coturnix japonica*) that were hatched in the laboratory from eggs that had been bought from a local breeder in Belgium (Dujardin Farms, Liernu). Eggs were set in the incubator (38°C and 50–60% relative humidity) and turned twice every day throughout the incubation period. On day 9 of incubation (day 0 being the day when eggs were set in the incubator), eggs were injected with either 25 μg estradiol benzoate (EB) (Sigma, St. Louis, MO) dissolved in 50 μL sesame oil or 10 μg of the aromatase inhibitor R76713 (6-[(4-chlorophenyl)(1$\underline{H}$-1,2,4-triazol-1-yl)methyl]-1-methyl-1$\underline{H}$-benzotriazole; Janssen Pharmaceutica, Beerse, Belgium) or racemic vorozole™ (Wouters 1989; De Coster, 1990) dissolved at a dose of 500 μg/mL in saline (0.9%) containing 20% polyethyleneglycol (PEG400; UCB7908, Leuven, Belgium). Control birds were always injected with the corresponding volumes of solvent (oil or PEG/saline). Because no effect of these two solvents on sexual behavior could be detected, these two groups of control subjects were pooled in all subsequent analyses. The combination of the sex and embryonic treatments of the subjects therefore defined six experimental groups: control males and females, males and females injected with EB, and males and females injected with R76713. Injections were administered with a 25-gauge needle inserted approximately 5 mm into the albumen of the egg (small end, opposite to the air chamber). Before each injection, needles were sterilized in a flame and needle holes in the shells were sealed with melted paraffin. Solutions were always sterilized prior to injection by heating them in boiling water (EB) or passing them through a 0.45-μm filter (R76713).

After hatching on day 17 of incubation, chicks were raised in heterosexual groups until the beginning of the experiment at 7 weeks of age. Throughout their life in the laboratory, birds were kept under long days (16:8 h light/dark cycle; lights on at 0600 h) under controlled temperature (38–40°C for the first 2 weeks of life, 28–30°C for the next 2 weeks, and ±22°C for the rest of their life). Water and food were available *ad libitum*.

All subjects were gonadectomized at 3 weeks of age under total anesthesia (Hypnodil™; Janssen Pharmaceutica, Belgium; 15 mg/kg). Both testes were removed through a unilateral incision on the left side. Only the left ovary of females was taken away. The right one is not developed and does not regenerate even after removal of the left gonad (Gibson et al., 1975). At the age of 7 weeks, birds were isolated in individual cages and implanted with two 20-mm-long Silastic capsules filled with crystalline testosterone [Fluka Chemika-Biochemika, Buchs, Switzerland; Dow Corning Silastic Tubing 602-265; 1.57 mm inner diameter (i.d.), 2.41 mm outer diameter (o.d.); Dow Corning, Midland, MI]. Implants were always cleaned and preincubated for at least 12 h in a 0.9% saline solution at 41°C to initiate steroid diffusion through the tube wall. This endocrine treatment has been shown previously to establish in both males and females similar circulating levels of plasma testosterone that are in the physiological range for sexually mature males (Balthazart et al., 1983, 1986; Schumacher and Balthazart, 1986).

Birds were sacrificed at the completion of the behavioral tests, 2–3 weeks after the beginning of T treatment. At that time, completeness of castration and presence of the T implants were checked.

Behavioral Tests

The male-typical sexual behavior of all subjects was recorded during three 5-min-long presentations to an adult sexually mature female that took place 10–14 days after the beginning of the T treatment. Birds were tested in an arena (50 × 60 cm) following a standard procedure that has been previously described (Schumacher and Balthazart, 1984). Briefly, the stimulus female was introduced in the arena containing the experimental subject for 5 min and the frequencies and latencies for the following behavior patterns were recorded: strut, neck grab, mount attempt (MA; counted only when a bird which is showing a neck grab, raises one leg, and puts it over the back of the test female), mount (M), and cloacal contact movements (CCM) (see

Adkins and Adler, 1972; Hutchison, 1978, for a detailed description).

Morphology

Birds were weighed to the nearest gram on several occasions: at 3 weeks of age before gonadectomy, just before the start of T treatment (7 weeks old), and before sacrifice. Their cloacal gland, an androgen-dependent (Sachs, 1967), sexually differentiated (Adkins and Adler, 1972; Adkins, 1975; Balthazart et al., 1983) structure, was also measured with a caliper (greatest length × greatest width = cloacal gland area) just before placing the T implants and at sacrifice.

Vasotocin Immunocytochemistry

During the week following the end of the behavior tests (birds were then 9–10 weeks old), all subjects were injected with 100 μL of heparin solution (20 mg/mL, i.e., ±3300 IU/mL; Sigma) and deeply anesthetized with Hypnodil™ (Janssen Pharmaceutica) (50 mg/kg body weight). They were perfused through the heart with saline solution (9 g/L; 0.15 *M*) until the return blood was clear, and then with 400 mL of Somogyi fixative (4% paraformaldehyde, 0.2% glutaraldehyde, and 15% of a saturated solution of picric acid in 0.1 *M* phosphate buffer, pH 7.35). Brains were dissected out of the skull, postfixed for 1 h in the same fixative without glutaraldehyde, rinsed in 0.1 *M* phosphate buffer, and placed overnight in a 20% sucrose solution in 0.1 *M* phosphate buffer. The next day, they were frozen on powdered dry ice and stored in a freezer at −75°C. The completeness of castration/ovariectomy and the presence of the Silastic implants were checked at that time.

Selected brains ($n = 5$ in each group) were used for immunocytochemical studies based on the behavior displayed by the subjects so that only subjects that are fully representative of their endocrine conditions were included. Brains were cut in the coronal plane with a cryostat at 30 μm thickness and every third section (one section every 90 μm) was stained for vasotocin by immunocytochemistry. Alternate sections were stained by immunocytochemistry for aromatase; results of quantitative analyses of these sections have been reported elsewhere (Balthazart et al., 1996a). These sections were also used in the present study to precisely identify the areas where quantitative data on vasotocin innervation were recorded—namely, the POM and the BST.

Vasotocin was visualized on free-floating sections by a classical peroxidase–antiperoxidase immunocytochemical technique as previously described (Balthazart et al., 1997). Briefly, sections were collected in 0.01 *M* phosphate buffer saline at pH 7.2 [phosphate-buffered saline (PBS)], rinsed three times in PBS for 5 min, and incubated successively for 20 min at room temperature in PBS containing 0.35% H_2O_2 (inhibition of endogenous peroxidase), for 30 min at room temperature in PBS containing 0.1% Triton X-100 (PBST) and 5% normal goat serum, and overnight at 4°C in the primary polyclonal vasotocin antibody (kindly provided by Dr. D. G.Gray, Max Planck Institute, Bad Nauheim, Germany) (diluted 1:2000 in PBST). The specificity of this anti-VT antibody was tested and described elsewhere (Gray and Simon, 1983; Viglietti-Panzica et al., 1994). On the next day, sections were incubated for 2 h at room temperature in goat anti-rabbit secondary antibody diluted 1:400 in PBST and 2.5 h at room temperature in a peroxidase–antiperoxidase complex diluted 1:400 in PBST (both were kindly provided by Dr. F. Vandesande, University of Leuven, Belgium). The peroxidase activity was finally revealed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) as chromogen (4 mg/10 mL). Sections were rinsed several times in PBST between each step. Sections were then mounted on microscope slides and coverslipped with a gelatin-based mounting medium. Brains were always stained in groups of six (one from each experiment group) so that between-assays variance could not be at the origin of systematic group differences.

Quantitative Analysis of Vasotocinergic Structures

In each subject, the density of VT-ir structures was quantified by computerized image analysis in three distinct brain areas: the POM, the BST, and the lateral septum (SL). The density of VT-ir structures was analyzed on a PowerPC 7100 Macintosh equipped with a digitizing board (Pixel Buffer; Perceptics, Knoxville, TN) connected to a Leitz Othorlux I microscope through a CCD videocamera (C4200; Hamamatsu Italy, Milano, Italy). The software used was NIH Image, version 1.55 VDM (a public domain program written by Wayne Rasband, NIH, Bethesda, Maryland, and modified by Perceptics).

The POM was studied at four successive levels in the rostro-caudal axis that were standardized in different individuals based on the first section that contained the anterior commissure (section labeled "CA"). The other sections were located 90, 180, and 270 μm, respectively, rostral to this section (sections "CA-1," "CA-2," and "CA-3," respectively). For each level, two images were aligned and digitized with a ×6.3 objective: one from the VT-immunostained section and one from the adjacent aromatase-immunostained section. The extension of the POM for each considered section was calculated by manually tracing an area of interest following its boundary detected on the ARO-stained section; the software automatically measured the number of pixels within this area. This area of interest was superimposed on the VT-immunostained sections, and positive structures (cells and fibers) in the area of interest were separated from the background by an interactive method of thresholding using high and low thresholding levels. All objects falling within the designated threshold range were automatically counted. Structure density was expressed as a ratio of the number of pixels within the threshold range versus the total number of pixels in the area of interest. This ratio was considered to be the fractional area covered by immunoreactive structures. For each animal, we considered in the statistical analyses the fractional area values of each single level, as well as the average of fractional area values recorded for the four POM levels.

The VT-ir system (fibers and a few cells) of the BST was analyzed at a single level immediately posterior to the anterior commissure, where the structure assumes a characteristic V shape centered on the third ventricle. The area of interest was determined by the same method as described for the POM using adjacent sections stained for ARO.

The SL was analyzed at a standardized caudal level, posterior to the pallial commissure, where a high density of VT-ir fibers is reliably observed. This is the location where the highest density of VT-ir fibers was detected in a previous study (Aste et al., 1997). The area containing VT-ir fibers in the septum cannot be defined based on an independent markers as done for POM and BST. We therefore measured the fractional area covered by immunoreactive fibers in the entire septum including the medial part. The fractional area values were then calculated as for the POM and the BST.

All structures were localized and named according to the stereotaxic atlases of the chick (Kuenzel and VanTienhoven, 1982; Kuenzel and Masson, 1988; Breazile and Kuenzel, 1993) and quail (Baylé et al., 1974) brains with modifications according to Panzica and collaborators (1991) for the POM and Aste and collaborators (1998) for the BST.

Data Analysis

Morphological data, behavioral frequencies, and fractional areas covered by VT-immunoreactive fibers were analyzed by one- or two-way analyses of variance (ANOVA) followed, when appropriate, by the Fisher protected least significant difference (PLSD) test to compare individual means. Percentages of birds showing a given behavior were analyzed by nonparametric statistics (chi-square and Fisher's exact probability test). Effects were considered significant at $p \leq .05$. Probabilities reported are two-tailed.

RESULTS

Morphology and Behavior

A total of 115 subjects were submitted to the tests assessing the sexual differentiation of male copulatory behavior. In general, the sexual behavior of these experimental subjects confirmed the results of previous studies by our as well as other laboratories (e.g., Adkins, 1979; Adkins-Regan, 1983; Schumacher et al., 1989; Aste et al., 1991; Balthazart et al., 1992a). Most control males were sexually active (21 of 24 showed CCM at least once) [Fig. 1(A)], while control females did not show this behavior (0 of 11). All subjects that had been treated with estrogens in the egg (10 males and 20 females) were sexually inactive (CCM not observed) despite the fact that they were exposed in adulthood to behaviorally effective doses of T. Finally, most females treated in egg with the aromatase inhibitor exhibited male-typical copulatory behavior that was indistinguishable from the behavior shown by untreated sexually mature males (18 of 21 performed at least one full copulatory sequence including CCM). R76 injections had no apparent effect on the behavior of males (26 of 29 showed CCM during at least one test). The differences in the percentage of active birds in the six experimental groups were therefore highly significant ($\chi^2 = 82.91$; $p < .0001$).

To optimize the probability of detecting brain–behavior correlates of the embryonic treatments, five subjects that fully conformed to the expected pattern of behavior were selected in each group for histological analyses. A summary of the behavioral and morphological data collected on these subjects is shown in Figure 1(B–D).

Analysis of the cloacal gland areas measured at the end of the experiment in the six subgroups of five subjects by two-way ANOVA with the two sexes as one factor and the three treatments as the other confirmed the presence of overall sex differences [$F(1, 24) = 17.110$; $p = .0004$] and of significant treatment effects [$F(2, 24) = 10.576$; $p = .0005$]. There was, however, no interaction between these two factors [$F(2, 24) = 0.116$; $p = .8910$]. Fisher PLSD tests indicated that the cloacal gland area of females was smaller than in males in control birds and in birds treated with EB in egg, and that EB decreased the gland size in both males and females [Fig. 1(B)].

The same model of analysis also indicated that the body weight measured at the end of the experiment was larger in females than in males [$F(1, 24) = 4.552$; $p = .0433$] and affected by the embryonic treatments [$F(12, 24) = 3.458$; $p = .0479$], but there was no interaction between these two variables [$F(2, 24 = 1.592$; $p = .2243$]. Two of the post hoc Fisher PLSD tests only indicated significant differences between groups: they concerned the comparison of males treated with EB with control males and females treated with EB [males EB: 210 ± 6 g (mean ± standard error [S.E.]); other groups had mean weight ranging between 238 and 262 with an S.E. between 6 and 20 g].

The total frequencies of MA and CCM observed in the six subgroups of five subjects during the three behavioral tests are shown in Figure 1(C,D). These behavioral frequencies were significantly affected by the sex of the birds [MA: $F(1, 24) = 18.597$; $p = .0002$; CCM: $F(1, 24) = 12.896$; $p = .0015$] and by the experimental treatments [MA: $F(2, 24) = 38.369$; $p < .0001$; CCM: $F(2, 24) = 23.062$; $p < .0001$], but there was also a very significant interaction between these two factors [MA: $F(2, 24) = 10.403$; $p = .0006$; CCM: $F(2, 24) = 7.888$; $p = .0023$]. Fisher PLSD

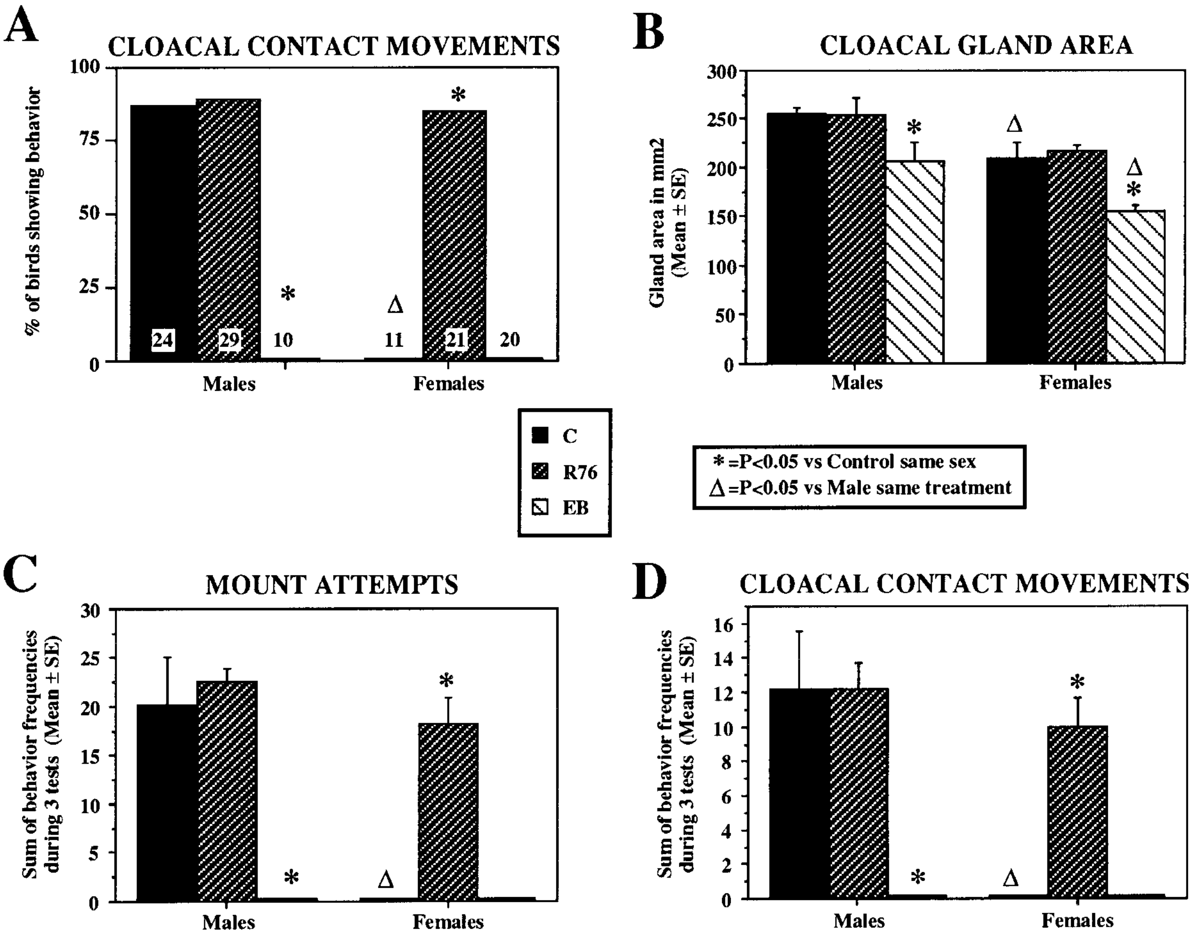

Figure 1 Effects of embryonic treatments with estradiol benzoate (EB) (25 μg on day 9 of incubation) or with the aromatase inhibitor R76713 (R76, racemic vorozole™; 10 μg on day 9 of incubation) on the male typical copulatory behavior and cloacal gland area of male and female Japanese quail. In adulthood, all subjects were gonadectomized and treated with a same amount of exogenous testosterone. One group of control subjects treated only with solvents during embryonic life is available for both sexes. (A) Percentage of birds in the six experimental groups who showed cloacal contact movements at least once during the three 5-min behavior tests performed in adulthood. The number of subjects that were tested in each group is indicated at the bottom of the corresponding bar. (B) Mean cloacal gland area in the five subjects of each experimental group that were selected for immunocytochemical analyses. (C,D) Mean frequency of mounts attempts (C) and of cloacal contact movements (D) observed during the sum of three 5-min behavior tests in the five subjects of each experimental group that were selected for immunocytochemical analyses. Data were compared by chi-square tests (A) or two-way ANOVA (B–D), followed when appropriate by Fisher's exact probability tests (A) or Fisher's PLSD tests (B–D), whose results are indicated above corresponding bars as indicated in the insert ($^*p < .05$ compared to control birds of the same sex; $\triangle p < .05$ compared to males submitted to the same treatment).

tests identified the origins of these overall effects: EB injections had markedly depressed sexual behavior in males, while R76 injections strongly increased the behavior in females [see Fig. 1(C,D) for details of the post hoc comparisons of groups 2 × 2). In summary, these embryonic treatments with estrogens or with an aromatase inhibitor had fully sex-reversed the behavioral phenotype of the males and females, respectively.

Vasotocin Innervation

In control males, VT-ir fibers were found to be distributed in the same brain areas as previously described (Viglietti-Panzica et al., 1992, 1994; Aste et al., 1998; Panzica et al., 1997). In particular, a high amount of positive fibers were located in brain nuclei that were shown previously to receive a sexually dimorphic innervation such as the POM, BST, and

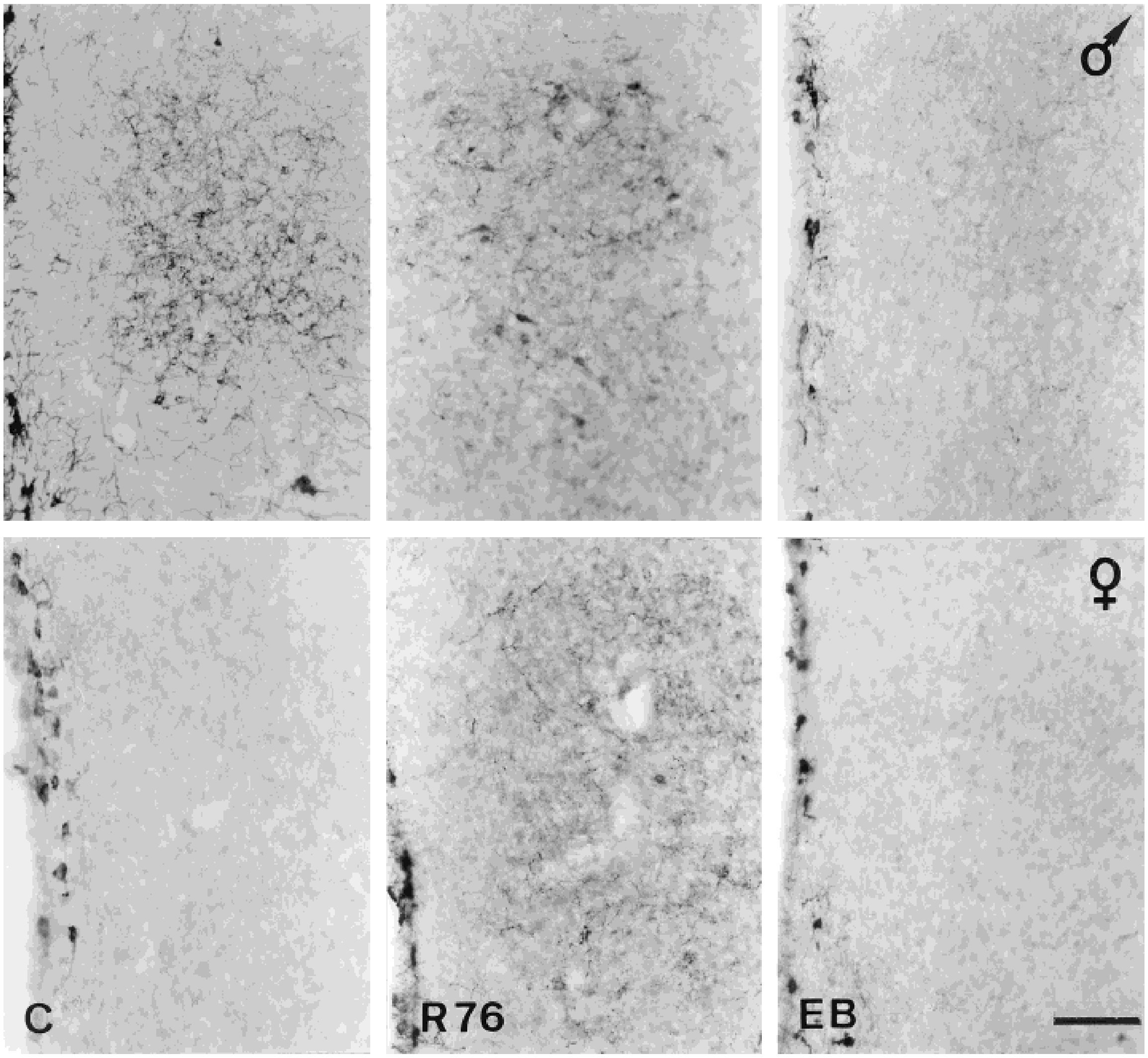

Figure 2 Effects of embryonic treatments with estradiol benzoate (EB) or an aromatase inhibitor (R76) on the vasotocin-immunoreactive structures of the medial preoptic nucleus (POM). Sections obtained at similar rostro-caudal levels in the nucleus are shown for control males and females (C), males and females injected *in ovo* with R76713 (R76), and males and females injected *in ovo* with EB (B). The third ventricle is on the left side in each panel. Magnification bar = 100 μm.

SL. A few immunoreactive cells could also be detected in the POM and BST. The present study focused exclusively on these brain areas. The injection of EB into the embryos markedly decreased the density of the immunoreactive structures in all areas considered, and conversely, the injection of R76 into female embryos led to the appearance of significant numbers of positive structures in areas that were devoid of them in control females (Figs. 2–4).

These qualitative observations were confirmed by the quantitative analyses. The fractional area covered by VT-ir structures measured in the POM at four successive rostro-caudal levels were first analyzed by a two-way ANOVA with the six experimental groups as one independent factor and the four levels as a repeated factor. This analysis indicated the presence of very significant differences between groups [*F*(5, 18) = 715.381; p = .0001] and an effect of the position in the nucleus [*F*(3, 54) = 3.636; p = .0184] but no interaction between groups and position [*F*(15, 54 = 1.193; p = .33052. The four values of the fractional area observed in each subject were therefore averaged and then analyzed by two-way ANOVA with the sex (two levels) and treatments (three levels) of the birds as two independent factors. This revealed the existence of significant differences related to the sex of the subjects [*F*(1, 23) = 20.874; $p < .0001$], their treatment [*F*(2, 23) = 22.988; $p < .0001$], and the interaction between the two factors [*F*(2, 23) = 10.126; p = .0207) (Fig. 5, top). The area covered

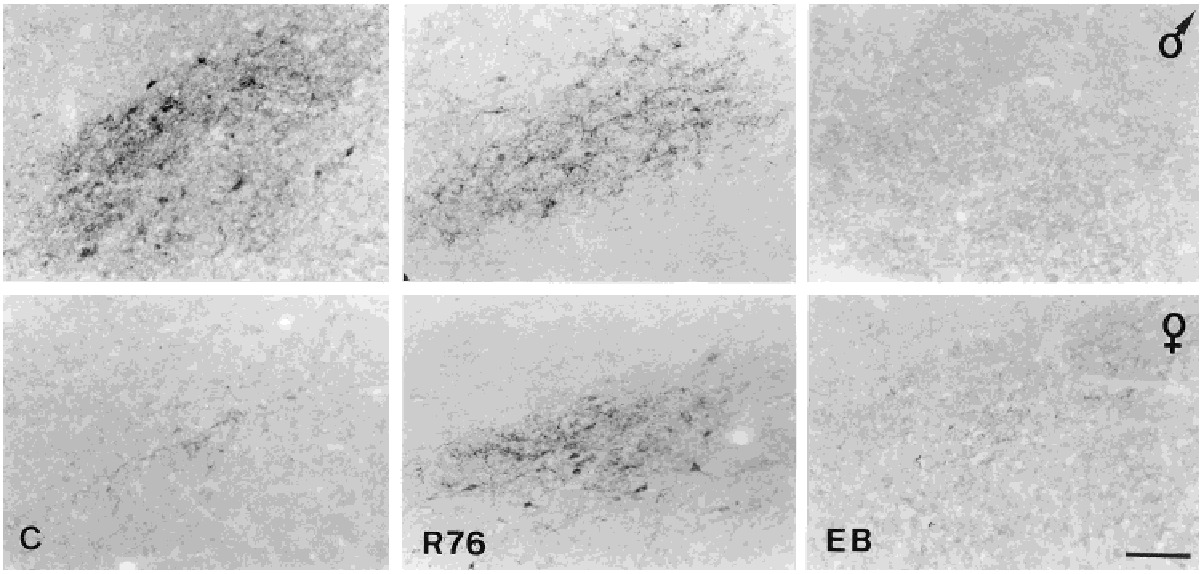

Figure 3 Effects of embryonic treatments with estradiol benzoate (EB) or an aromatase inhibitor (R76713) on the vasotocin-immunoreactive structures of the bed nucleus of the stria terminalis (BST). Sections obtained at similar rostro-caudal levels in the nucleus are shown for control males and females (C), males and females injected *in ovo* with R76713 (R76), and males and females injected *in ovo* with EB. The third ventricle is on the left side in each panel. Magnification bar = 100 μm.

by VT-ir structures in the POM was different in control males and females despite the fact that all birds were treated in adulthood with testosterone, and it was decreased by the EB treatment in males (Fisher's PLSD tests, $p < .05$). In both sexes, the fractional area values were also significantly larger in birds treated with R76 in egg than in birds treated with EB. These fractional area values in R76-treated females were also significantly much higher than in control females, as can be easily observed in Figure 2.

Nearly identical effects were observed on the VT-ir structures of the BST that were affected by the sex [$F(1, 22) = 27.700$; $p < .0001$] and treatment of the subjects [$F(2, 22 = 52.579$; $p < .0001$] as well as by the interaction between the two factors [$F(2, 22 = 15.030$; $p < .0001$]. The post hoc Fisher's PLSD tests identified the same differences between pairs of groups (Fig. 5, middle). Identical overall effects were also seen in the septum [sex effect: $F(1, 23) = 34.464$; $p < .0001$; treatment effect: $F(2, 23) = 61.261$; $p < .0001$; interaction: $F(2, 23) = 15.242$; $p < .0001$], but in this case, a significant difference was also present in the percentage of area covered by VT-ir fibers in males and females that had been treated in egg with R76 (Fig 5, bottom).

In agreement with our previous work focusing on sex differences, qualitative observation of the magnocellular VT-ir cell bodies in the supraoptic and in the paraventricular nuclei did not suggest the presence of sex- or treatment-related differences in these nuclei (Fig. 6 shows this absence of change in the supraoptic region). Therefore, no detailed quantitative analysis was carried out on these cell groups.

DISCUSSION

The present experiment confirmed that the presence or absence of embryonic estrogens is a necessary and sufficient endocrine stimulus to fully sex-reverse copulatory behavior (and to a large extent cloacal gland growth) in Japanese quail (see Balthazart et al., 1996a, for a previous description and discussion of these effects). It also showed that the sex difference in vasotocin innervation of the POM/BST/SL (lighter in females than in males) does not only result from a lack of activation by T in females, suggesting that the difference is organizational in nature. This conclusion was then confirmed by demonstrating that male embryos treated with EB on day 9 of incubation have a female phenotype as far as the VT immunoreactivity in these nuclei is concerned, while treatment of female embryos with the aromatase inhibitor R76713 produces adult subjects that have a vasotocinergic innervation very similar to that seen in males.

The effects of steroids on the VT-ir structures appear to be anatomically specific in that no major qualitative changes were observed here between the

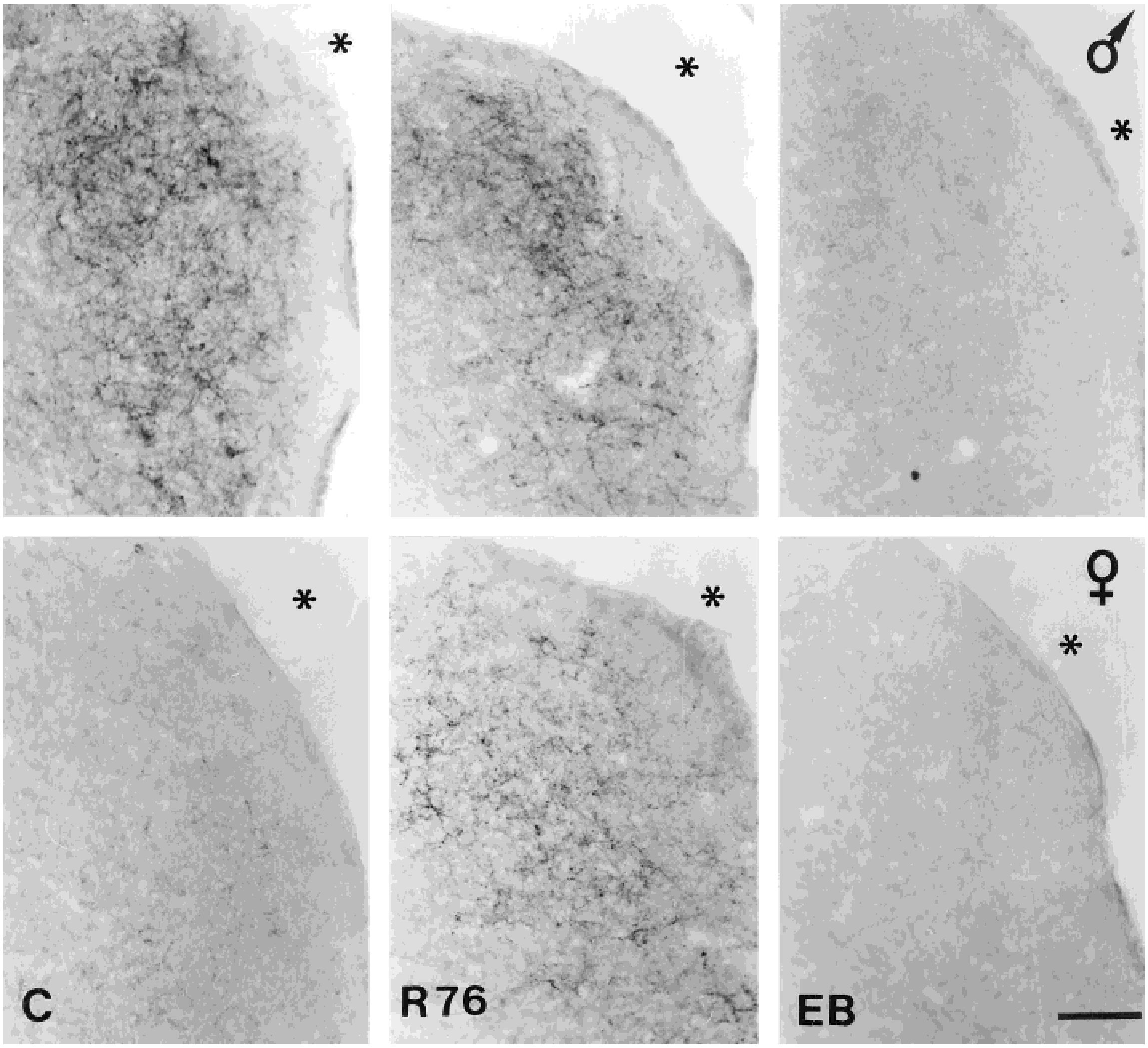

Figure 4 Effects of embryonic treatments with estradiol benzoate (EB) or an aromatase inhibitor (R76713) on the vasotocinergic innervation of the lateral septum. Sections obtained at similar rostro-caudal levels in the nucleus are shown for control males and females (C), males and females injected *in ovo* with R76713 (R76), and males and females injected *in ovo* with EB. The asterisk indicates the lateral ventricle in each panel. Magnification bar = 100 μm.

six experimental groups in the immunostaining of the VT-ir neurons in the supraoptic and paraventricular nuclei. This further extends previous findings of our laboratory showing that the changes in VT-ir structures associated with sex or endocrine differences concern mostly the brain areas innervated by thin fibers that are presumably not originating in the magnocellular systems (Viglietti-Panzica et al., 1994; Panzica et al., 1997).

Previous experiments had shown that the POM and BST of quail contain a sexually differentiated VT-ir system, but the endocrine control of this sex difference had not been investigated (Viglietti-Panzica et al., 1994). It was known that in another brain area, the lateral septum, the sex difference is still present if birds of both sexes are placed in the same endocrine condition (i.e., treated with a same dose of T) (Viglietti-Panzica et al., 1992). We now demonstrate that this conclusion can be extended to the POM and BST, and therefore, that presumably the sex difference affecting all these VT-ir structures is organizational in nature. The corresponding neural network should therefore be substantially different in males and in females, and it could be speculated that these sex differences come about through the effects of embryonic hormones. Because VT-ir structures have been found to be anatomically associated to the areas that control copulatory behavior, and because behavioral experiments have shown that VT actually participates to the control of male copulatory behavior, it was

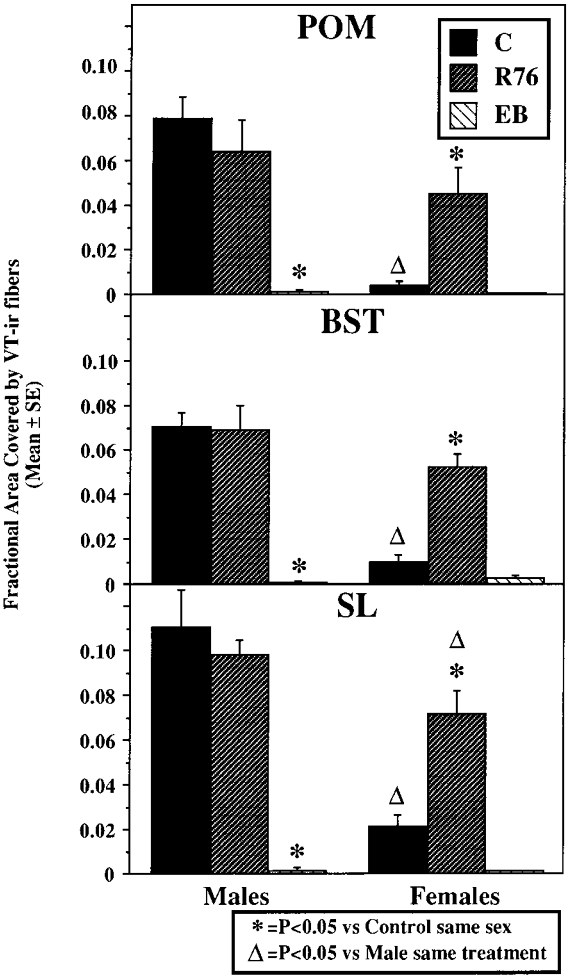

Figure 5 Effects of embryonic treatments with estradiol benzoate (EB) (25 μg on day 9 of incubation) or with the aromatase inhibitor R76713 (R76, racemic vorozole™; 10 μg on day 9 of incubation) on the fractional area of the medial preoptic nucleus (POM), bed nucleus of the stria terminalis (BST), and lateral septum (SL) covered by vasotocin-immunoreactive structures. Data were compared by two-way ANOVA followed by Fisher's PLSD tests whose results are indicated above corresponding bars as indicated in the insert (*$p < .05$ compared to control birds of the same sex; $\triangle p < .05$ compared to males submitted to the same treatment).

decided to test whether the specific treatments that differentiate this behavior (presence or absence of estrogens before day 12 of egg incubation) also differentiate the VT-ir systems; a fully positive answer was obtained to this question.

The phenotype of the VT-ir system in the adult quail brain therefore results from the interaction of embryonic estrogens that impose a female-typical innervation with adult androgens that activate the system provided the embryo has not been exposed to high levels of estrogens. We showed previously that the preoptic aromatase in male quail is also affected by steroid treatments. The enzyme activity, number, and size of aromatase-immunoreactive cells and amount of aromatase mRNA in the POM are decreased after castration and restored to intact levels by treatment with exogenous T (Schumacher and Balthazart, 1986; Foidart et al., 1994; Aste et al., 1994, 1998; Balthazart et al., 1996b; Harada et al., 1992). The preoptic aromatase also appears to be sexually differentiated (Schumacher and Balthazart, 1986), but the ontogeny of this sex difference is not clearly linked to embryonic effects of estrogens as demonstrated here for VT. In particular, we failed to identify clear-cut effects of embryonic treatments with EB or R76713 on the number of aromatase-immunoreactive cells visualized in the adjacent sections collected in this study and that were used to locate the cytoarchitectonic boundaries of the POM and BST (Balthazart et al., 1996a). Therefore, to date, the VT-ir system of the POM/BST/SL represents the neurochemical system of the quail brain that is the most sensitive to early effects of estrogens. It is also the only neurochemical system that is affected by steroids both during ontogeny and in adulthood in parallel with the behavioral phenotype.

A similar interaction between early and adult hormones was identified in the control of the vasopressin (VP) system in rats (de Vries and Al-Shamma, 1990; de Vries et al., 1992, 1994a,b). There are, however, major differences in the anatomical organization of the VT/VP systems and differences in the endocrine control of sexual differentiation between rodents and galliforms. Most studies on rats have focused on the innervation of the lateral septum by VP and on the population of vasopressin-immunoreactive (VP-ir) neurons in the BST and medial amygdala. These VP-ir structures are in general more developed in males than in females, and treatment of adult females with exogenous T usually fails to suppress these sex differences (some increase in VP-ir innervation of the septum is observed in T-treated females, but it never reaches a male-typical level) (de Vries and Al-Shamma, 1990). These sex differences have been shown to result, at least in part, from the early postnatal organizational effects of T in males (Wang et al., 1993). Castration of male newborns before day 7 significantly decreases the number of VP-ir neurons in the BST and medial amygdala and depresses the VP-ir innervation of the septum, even if these males are treated with exogenous T in adulthood. Conversely, the treatment with T of neonatal females (7 days postpartum) increases the density of VP-ir fibers in the septum, but this treatment fails to increase the number of immunoreactive neurons in the BST and medial amygdala, presumably because the treatment

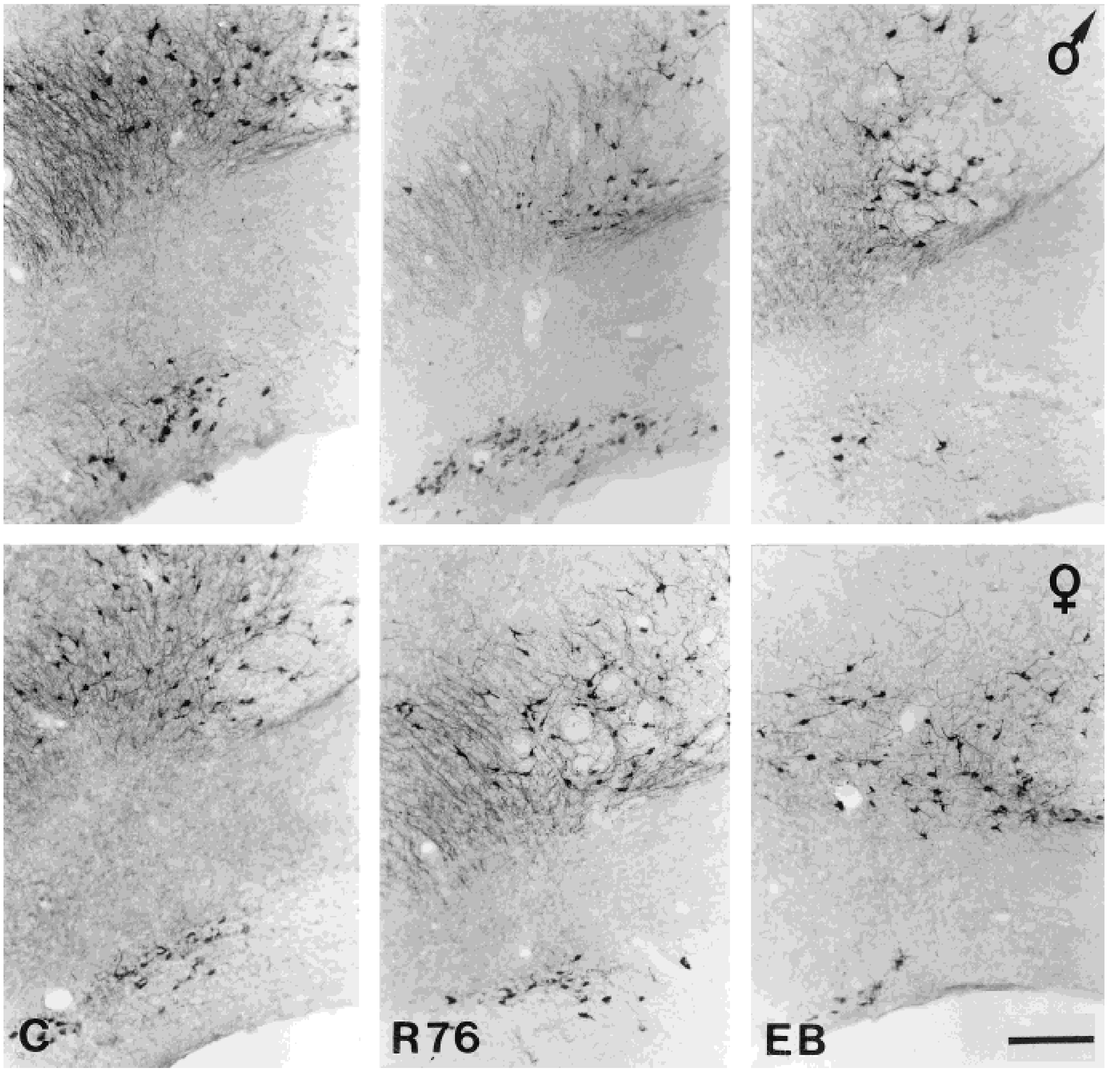

Figure 6 Effects of embryonic treatments with estradiol benzoate (EB) or an aromatase inhibitor (R76713) on the magnocellular vasotocinergic system in the surpraoptic region. Sections obtained at similar rostro-caudal levels in the area are shown for control males and females (C), males and females injected *in ovo* with R76713 (R76), and males and females injected *in ovo* with EB. No obvious difference in the density of immunoreactive cells or fibers can be detected. Magnification bar = 200 μm.

was applied too late when the differentiation of the neuronal cells is well under way.

Therefore, the endocrine signal that differentiates reproductive behavior also differentiates, in parallel, parts of the VT/VP-ir systems in both quail and rats. There are, however, substantial species differences in the anatomical organization of these structures that could have important consequences for the control of behavior. In rats, the VP-ir innervation of the medial preoptic area has not yet been shown to be sexually differentiated, and it is supposed to originate exclusively in the suprachiasmatic nucleus with no contribution of the BST and medial amygdala (de Vries et al., 1985). The highly dimorphic VP-ir innervation of the lateral septum is thus thought to derive from the BST and medial amygdala cell groups (de Vries et al., 1985), and these cell populations are more numerous in males than in females (de Vries et al., 1994a, 1995). In contrast, we have shown in quail that the POM is characterized by a sexually dimorphic VT-ir innervation, but its origin is still impossible to specify. In quail, no VT-ir neurons and no neurons expressing the VT mRNA have been detected in the archistriatum and, in particular, in the nucleus taeniae that are thought to be the homolog structures to the mammalian amygdala (Zeier and Karten, 1971; Thompson et al., 1998). It can therefore be hypothesized that the innervation by VT of the sexually dimorphic POM originates in the BST where a large population of VT-ir and VT gene-expressing cells is located (Aste et al., 1998). This notion is supported by the presence of sparse connections between these nuclei (Balthazart et al., 1994; Balthazart and Absil, 1997), but the formal proof of this anatomical connection should be obtained by retrograde tracing combined with VT immunocytochemistry. Alternatively, recent studies using optimized immunocytochemical conditions revealed the presence of a sparse population of small VT-ir neurons within the caudal part of the POM (Aste et al., 1998). Therefore, these neurons could also participate in the VT-ir innervation of the nucleus.

In both quail and in a number of mammalian species, the anatomical localization of VT/VP-ir systems and their modulation by steroids both in adulthood and during development therefore suggest that this peptide may be intimately related to the control of various aspects of reproductive behavior and to its sexual dimorphism. A number of studies on a variety of vertebrate species have indeed demonstrated that VP or VT has a diversity of actions on behaviors ranging from the spawning reflex in some species of fishes to the mating call of amphibians and the flank-marking response in mammals (de Wied et al., 1988; Moore, 1992). Many of these studies suggest that VT or VP increases the occurrence frequency of the behaviors, but some inhibitory effects have also been demonstrated (e.g., inhibition of lordosis in female rat, of the release call in *Rana pipiens*, of spontaneous locomotion in *Rana catesbiana* (Södersten et al., 1983; Diakow, 1978; see Moore, 1992, for review). However, to our knowledge, no study has identified specific effects of VP in mammals on the expression of male copulatory behavior.

Unfortunately, little experimental work has been carried out in birds. One early study showed that the injection of VT in intact sexually mature pigeons or cocks produces a short-term increase in the frequency of copulatory acts (Kihlström and Danninge, 1972); more recently, it was shown that VT injections stimulate singing in female white-crowned sparrows, *Zonotrichia leucophrys gambelii* (Maney et al., 1997). Other studies in a variety of songbird species have, however, shown that the injection of exogenous VT can either decrease or increase song production, but that the direction of the observed effect may depend on the species considered or the season when subjects are studied (Goodson et al., 1996; Goodson, 1998; Voorhuis et al., 1991). One detailed set of studies recently came to the conclusion that in male quail, VT exerts a powerful inhibitory effect on both appetitive and consummatory components of male sexual behavior as well as on the crowing vocalization. These studies also demonstrated that the effect of VT is centrally mediated and can be dissociated from the general stress reaction induced by systemic injections of the peptide. Finally, it was shown that blockade of the V1 receptors by a powerful V1 antagonist leads to a significant stimulation of all these behavioral responses, indicating that endogenous VT is likely to exert tonic inhibitory effects on these behaviors (Castagna et al., 1998).

It is obviously difficult to make direct comparisons between these different studies because they were carried out in different species under very different experimental conditions (different seasons, endocrine conditions, etc.), they concerned different aspects of reproductive behavior that are not necessarily homologous, and the anatomical organization of the VT/VP system appears to be species specific to some extent. The identification of inhibitory effects of VT on sexual behavior in male quail was, however, somewhat unexpected because previous anatomical studies and the experiment described here invariably demonstrate that VT-immunoreactive fibers in the POM, BST, and SL are denser in physiological conditions where male copulatory behavior is expressed (males versus females, T-treated males versus castrates, females injected with R76713 in the egg versus control female) (Viglietti-Panzica et al., 1992, 1994; Panzica et al., 1996a; Aste et al., 1997). Based on these anatomical/physiological data and on previous literature on the effects of VT/VP on behavior in other species, we therefore hypothesized that the increase in VT production is part of a cascade of biochemical events triggered by T in the brain that results in the activation of male sexual behavior. The behavioral studies in quail apparently make this interpretation impossible. It is unlikely that increases in the density of VT-ir fibers after T treatment (or in males compared to females or in females treated with R76713 compared to controls) reflects a blockade of the peptide release resulting in its accumulation in fibers and terminals, because in mammals and in a limited set of studies available in quail and chicken, it has been shown that the changes in VT/VP immunoreactivity are paralleled by changes in the concentration of the VT/VP mRNA (G. C. Panzica, M. Pessati, J. Balthazart, and R. Grossmann, unpublished results), suggesting alterations of the synthesis rate (see Castagna et al., 1998, for further discussions).

We therefore suggest that the apparent contradiction between anatomical and behavioral data indicates that the increase in VT immunoreactivity should not be considered one of the central consequences of the T action that leads to the activation of male sexual behavior, but rather the development of a mechanism that is implicated in the maintenance of behavioral homeostasis. When the male sexual behavior is activated (e.g., by high levels of circulating T and by the embryonic treatment with R76713 of females), a negative control mechanism may need to be established to organize the distribution of behavioral occurrences on a short-term basis. This mechanism is obviously not needed in castrates or females that are behaviorally inactive. A steroid-sensitive peptidergic innervation would provide adequate support for such a control that could be directly sensitive to environmental stimuli. On a long-term basis, changes in steroid levels would establish the anatomical substrate of this behavioral control (control of the synthesis of VT and possibly of the growth of VT-containing fibers), while on a short-term basis, environmental and social stimuli could regulate the release of VT from its terminals and in this way switch off behavior for limited periods of time. Additional work is obviously required to test this hypothesis, including the identification of the brain areas (POM, BST, septum, etc.) where VT exerts its behavioral effects and their specific nature.

Alternatively, one should also leave open the possibility that the changes in VT innervation described here are not the cause but rather the consequence of the differences in behavior that were induced by the embryonic manipulations. Because behavior tests were, by necessity, carried out before birds were killed and processed through the immunocytochemical studies, one could in theory imagine that the fact of performing copulatory behavior enhances VT immunostaining. The higher density of innervation seen in control males and in birds of both sexes treated with R76713 would then simply be a consequence of the behavior (e.g., behavior increases synthesis or depletes the neurotransmitters stores and causes active resynthesis). However, the perfusion of birds was performed 4–6 days after the end of the behavior tests, which makes this idea relatively unlikely. This scenario would require fairly long-lasting neurochemical effects of the behavioral performance that may not be compatible with the dynamic nature of a postulated environmental control of neurochemistry. It must be reminded, however, that after castration of rats, VP immunoreactivity disappears only gradually over a period of 2–3 months in the projections of the BST and medial amygdala (De Vries et al., 1984). Therefore, the possibility of relatively slow changes that would be driven by behavior should not be dismissed in the absence of additional data.

In conclusion, the data collected in the present study demonstrate that embryonic treatment with estrogens or with an aromatase inhibitor induces a full sex reversal of the behavioral phenotype in male and female quail, respectively, that is closely paralleled by a complete sex reversal of the distribution of vasotocinergic structures of the POM, BST, and septum. These data therefore add to the multidimensional array of correlations that link VT/VP to the control of reproductive behavior. However, at present it is impossible to ascertain the specific role played by the peptide in the control of the behavioral sex dimorphism.

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