The Parvocellular Vasotocin System of Japanese Quail: A Developmental and Adult Model for the Study of Influences of Gonadal Hormones on Sexually Differentiated and Behaviorally Relevant Neural Circuits

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Vasotocin (VT; the antidiuretic hormone of birds) is synthesized by diencephalic magnocellular neurons projecting to the neurohypophysis. A sexually dimorphic system of VT-immunoreactive (ir) parvocellular elements has been described within the male medial preoptic nucleus (POM) and the nucleus of the stria terminalis, pars medialis (BSTm). VT-ir fibers are present in many diencephalic and extradiencephalic locations, and quantitative morphometric analyses demonstrated their sexually dimorphic distribution in regions involved in the control of different aspects of reproduction. Moreover, systemic or intracerebroventricular injections of VT markedly inhibit the expression of some aspects of male sexual behavior. In adult animals, circulating levels of testosterone (T) have a profound influence on the VT immunoreactivity within BSTm, POM, and lateral septum. Castration markedly decreases the immunoreaction, whereas T-replacement therapy restores a situation similar to the intact birds. We observed no changes in gonadectomized females treated with T. These changes parallel similar changes in male copulatory behavior (not present in castrated male quail, fully expressed in castrated, T-treated males). The restoration by T of the VT immunoreactivity in castrated male quail could be fully mimicked by a treatment with estradiol (E_2), suggesting that the aromatization of T into E_2 may play a key limiting role in both the activation of male sexual behavior and the induction of VT synthesis. This dimorphism has an organizational nature: administration of E_2 to quail embryos (a treatment that abolishes male sexual behavior) results in a dramatic decrease of the VT immunoreactivity in sexually dimorphic regions. Conversely, the inhibition of E_2 synthesis during embryonic life (a treatment that stimulates the expression of male copulatory behavior in treated females exposed in adulthood to T) results in a malelike distribution of VT immunoreactivity. The VT parvocellular system of the Japanese quail can therefore be considered an accurate marker of the sexual differentiation of brain circuits mediating copulatory behavior and could be a very sensitive indicator of the activity of estrogenlike substances on neural circuits. *Key words:* **avian brain, copulatory behavior, development, sexual dimorphism, vasotocin.** *Environ Health Perspect* **110(suppl 3):423–428 (2002).**
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Vasotocin (VT) is a nonmammalian neuropeptide (*1*) originally identified [like its mammalian counterpart, arginine-vasopressin (VP)] as a hormone produced by hypothalamic magnocellular elements (*1*), secreted at the level of the neurohypophysis. VP and VT play a major role in osmoregulation (*2*) and have therefore been called antidiuretic hormones. A few groups of parvocellular VT-immunoreactive (ir) neurons not related to the hypothalamo-neurohypophysial system have also been described in several avian species. One group is located in the mesencephalon around the nucleus rotundus (*3,4*), and another group containing a larger number of neurons is present within the medial part of the avian nucleus of the stria terminalis (BSTm) (Figure 1). This latter group was first identified in oscine birds (*5–8*) and later in galliforms (*4,9–11*).

These parvocellular VT-ir elements have similar characteristics in all avian species investigated so far. The extension of their dendritic arborization is simpler (generally bipolar), and their immunostaining is weaker than that in the magnocellular population. This last characteristic probably accounts for these smaller VT-ir cells not being observed in some older studies. Their visualization actually depends critically on specific aspects of the immunocytochemical technique. A population of parvocellular VP-ir neurons has similarly been clearly identified in the BSTm and in the medial amygdala of mammals but only after colchicine injection (*12*). *In situ* hybridization (ISH) studies of VT or VP mRNA have confirmed the existence of these populations of parvocellular neurons (*4,10,12*).

Thin VT-ir fibers have also been observed in several brain regions outside the hypothalamo-neurohypophysial tract in different avian species. Their distribution has been studied in detail by immunocytochemistry in the brain of several avian species such as the canary (*Serinus canaria*) (*5,13*), the zebra finch (*Taeniopygia guttata*) (*7*), the Junco (*Junco hyemalis*) (*8*), and the Japanese quail (*Coturnix japonica*) (*14*)]. VT-ir fiber endings were observed in the telencephalon (lateral septum, BSTm), in the preoptic region [nucleus preopticus medialis (POM)], in broad areas of the diencephalon, in the mesencephalon [optic tectum, nucleus intercollicularis (ICo), substantia grisea centralis (GCt), area ventralis of Tsai, and substantia nigra], in the pons [raphe nuclei, locus coeruleus (LoC), and tegmentum], and in the medulla (nucleus of the solitary tract). The origins of these thin VT-ir fibers have not been fully identified, but recent results collected in our laboratory (*15*) demonstrate that in quail the VT neurons of the BSTm project to the POM.

Sex Dimorphism of the Vasotocin System in the Avian Brain

A few studies have revealed a robust sexual dimorphism of the parvocellular VT-ir cell groups in the avian BSTm. In galliforms, immunocytochemical studies showed that VT-ir neurons are present in the BSTm of males only and cannot be visualized in females (*10,11*). In the canary this difference is less extreme, and both sexes have VT-ir neurons in the BSTm, but they are present in larger numbers in males than in females (*6,13*). In contrast, no sexual dimorphism was observed at this level in another oscine species, the zebra finch (*7*). In quail, a sexually dimorphic population of scattered VT-ir neurons, which merges caudally with the BSTm, was also observed within the boundaries of the POM, a sexually dimorphic region controlling male reproductive behavior (*16*). These cells are detectable by immunocytochemistry in males only. This sex difference was also confirmed at the level of VT transcripts by ISH techniques (*10,11*).

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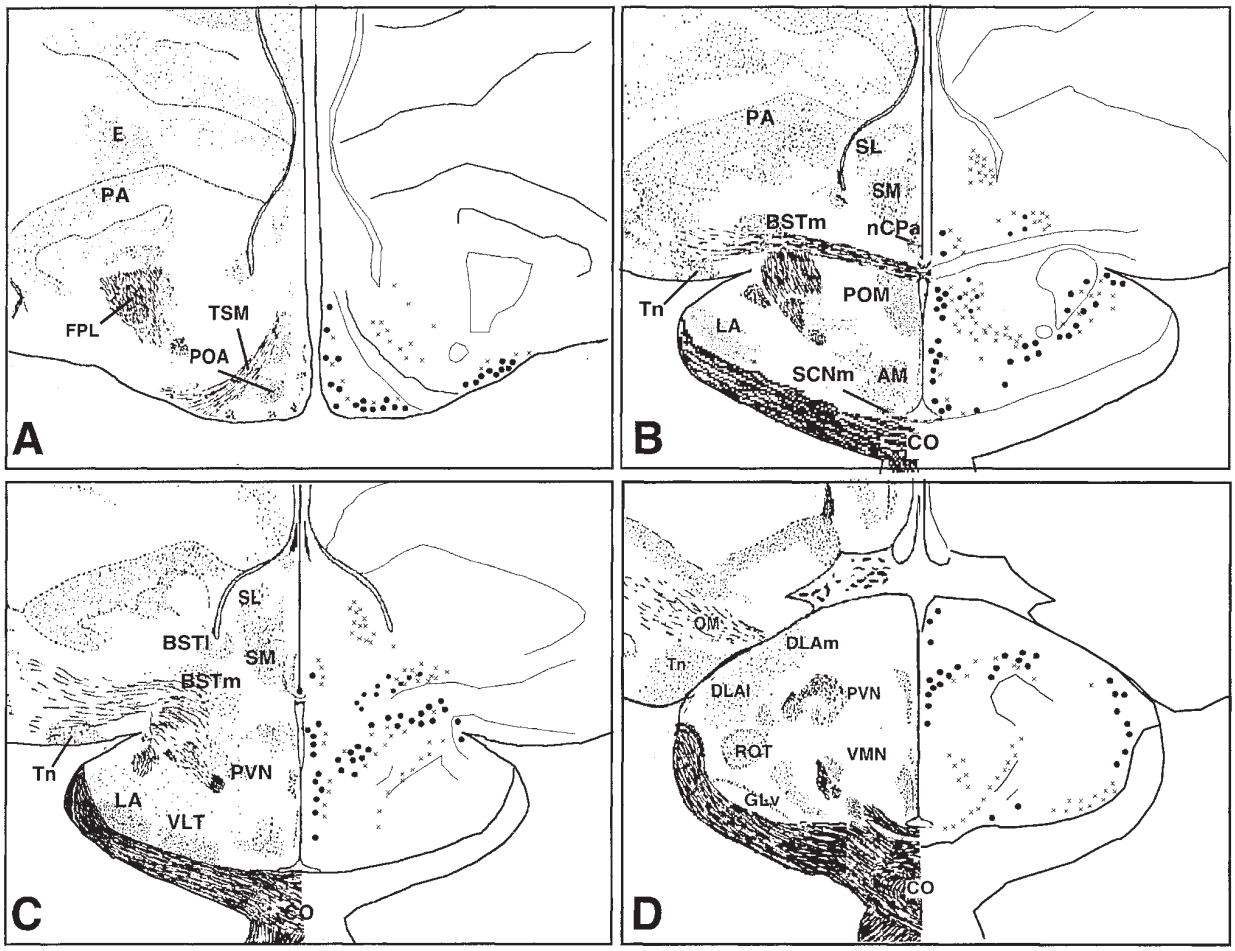

Figure 1. Rostrocaudal distribution of VT-ir cell bodies (●) or fibers (×) within the septodiencephalic region of the Japanese quail brain. AM, nucleus anterior hypothalami; BSTl, bed nucleus of the stria terminalis, pars lateralis; BSTm, bed nucleus of the stria terminalis, pars medialis; CO, optic chiasma; DLAl, nucleus dorsolateralis thalami, pars lateralis; DLAm, nucleus dorsolateralis thalami, pars medialis; E, ectostriatum; FPL, fasciculus prosencephalicus lateralis; GLv, nucleus geniculatus lateralis, pars ventralis; LA, nucleus lateralis thalami; nCPa, nucleus of the pallial commissure; OM, tractus occipitomesencephalicus; PA, paleostriatum augmentatum; POA, nucleus preopticus anterior; POM, nucleus preopticus medialis; PVN, nucleus paraventricularis; ROT, nucleus rotundus; SCNm, nucleus suprachiasmaticus pars medialis; SL, septum lateralis; SM, septum medialis; Tn, nucleus taeniae; TSM, tractum septomesencephalicum; VLT, nucleus ventrolateralis thalami; VMN, nucleus ventromedialis.

VT-ir fibers also show a sexually dimorphic distribution in parts of the avian brain. The lateral septum of males contains a denser VT-ir innervation than the female septum in Japanese quail (*17*), domestic fowl (*11*), and the canary (*6*). However, this sex dimorphism was not reported for other oscine species such as the zebra finch (*7*). Male and female Japanese quail contain different densities of VT-ir fibers in the POM (*17*).

We have recently examined quantitatively the distribution of VT-ir fibers in the entire Japanese quail brain (*14*). The sex dimorphism in the distribution of parvocellular VT-ir perikarya appears to be restricted to the cell group extending throughout the BSTm and POM. Both immunocytochemistry and ISH (*4*) failed to demonstrate labeled neurons in the avian equivalent of the medial amygdala (nucleus taeniae and parts of the archistriatum), whereas in mammals this nucleus contains an important cluster of sexually dimorphic VP-producing neurons (*18*). VT-ir fibers are distributed in large portions of the diencephalon and brain stem. In the telencephalon, they are present only within the limbic system. In many places [e.g., the lateral septum, the POM, the ventromedial nucleus (VMN), the GCt, the ICo, and the LoC], these fibers are present in significantly higher densities in males than in females.

Interestingly, the regions that are characterized by a sexually dimorphic distribution of VT-ir fibers are frequently associated with the control of reproduction and contain dense populations of estrogen receptor–positive neurons. In the lateral septum, we have recently demonstrated the existence of a sexually dimorphic population of gonadotropin-releasing hormone neurons that are specifically innervated, in a sexually dimorphic manner, by VT-ir and vasointestinal polypeptide–ir fibers (*19*). Moreover, estrogen receptors are extremely abundant in the lateral septum. The POM also contains a high number of estrogen receptor–containing neurons, as well as one of the major clusters of aromatase-producing elements (*16*). In previous studies on male quail, we have demonstrated close relationships between VT-ir fibers and aromatase cell bodies not only at the level of POM but also within the BSTm, the VMN, and the GCt (*20,21*). The ICo is an important brain center controlling vocalizations in galliforms and nonoscine birds (*22–24*). Many of these vocalizations are sexually dimorphic and under the control of androgens (*25,26*). Moreover, a large

number of estrogen receptors are present in the medial subdivision of ICo (*27*), and its neurons respond to an increase in plasma testosterone (T) in a sexually dimorphic manner (*28*). Finally, the LoC is the origin of the majority of noradrenergic fibers of the brain and therefore potentially affects the activity of a large number of brain areas. The sexually dimorphic VT innervation of this nucleus may therefore potentially regulate the noradrenergic networks and influence many physiological and behavioral functional systems (*29–34*). These anatomical data thus clearly support the notion that VT may be implicated in the control of sexually differentiated reproductive processes.

Effects of Gonadal Steroids in Adult Birds

The sex differences in the vasotocinergic innervation reported in song birds seem to be related only to the presence of higher plasma levels of T in males. In canaries, treatment of females with T enhances the VT immunoreactivity in the lateral septum to a male-typical level (*6*). This suggests that the sex dimorphism observed in adult birds reflects only a differential activation by T in males and females. Conflicting results have been obtained in zebra finches. One study reported an absence of sex difference in the number of VT-ir cells or fibers in the septum and BSTm of this species (*7*), whereas significant differences were observed in another study in several telencephalic and diencephalic regions (*35*). However, these sex differences were eliminated by treatment of adult females with T, suggesting an activational origin in this songbird species (*35*). Additional studies would be needed to clarify this discrepancy, but it seems clear already that species differences will be found in the expression of VT and its control by steroids within birds. For example, contrary to what has been reported in oscine species, in quail the treatment of females with exogenous T usually fails to induce a density of vasotocinergic structures similar to that observed in males (*17,36*).

Several studies have investigated changes in VT innervation in males as a function of physiological and experimental manipulations of circulating levels of T. It is well known that many species of birds are highly photoperiodic, and their circulating T levels fall to very low values when males are exposed to short days (during the fall and the winter in natural environments). In these conditions, the amount of VT-ir fibers in the lateral septum, BSTm, and POM, as well as the number of cells in the BSTm, dramatically decreases both in oscines [canary (*37*); junco (*38*)] and in nonoscine birds [quail (*17,39*)].

In male quail the amount of VT-ir fibers in lateral septum and POM is strongly reduced or completely disappears in gonadectomized birds, whereas T treatment of castrated males restores the innervation to a density that is typically seen in sexually mature males (*17,40*). The T dependence of the VT-ir circuitries is also reflected by the drop in the density of immunoreactive fibers in the POM and lateral septum observed during aging (when circulating T levels spontaneously decrease to very low values) and by the restoration of VT innervation that was observed in aged male quail treated with exogenous T (*39*). Castration also completely eliminates neurons expressing VT mRNA in the sexually dimorphic parvocellular VT cell groups, located in the POM and in the anterior and posterior part of the BSTm. These effects are completely reversed by a 3-week treatment with exogenous T. These changes are observed only at the level of the sexually dimorphic neurons of the limbic and preoptic regions and not at the level of the magnocellular neurons located in the paraventricular and supraoptic nuclei or at the level of the hypothalamo-neurohypophysial tract (*17,39–42*).

T activates sexual behavior mainly through its aromatization into an estrogen [e.g., 17β-estradiol (E_2)], but nonaromatizable androgens such 5α-dihydrotestosterone (DHT) synergize with estrogens to activate the behavior (*43–47*). In parallel, the restoration by T of the VT immunoreactivity in the POM, BSTm, and lateral septum of castrated male quail can be fully mimicked by a treatment with E_2. The androgen DHT had absolutely no effect on the VT immunoreactivity in these conditions, and at the doses used, DHT did not synergize with E_2 to enhance the density of VT-ir structures (*42*). The steroid sensitivity of the VT-ir structures in the male quail brain thus brings additional support to the idea that this neuropeptide is more or less directly involved in the control of male sexual behavior.

Organizational Effects of Steroids

The immunocytochemical and ISH studies described above clearly indicate that, in birds, a high level of VT expression is invariably present when subjects are studied in physiological conditions in which they express male-typical reproductive behavior. Conversely, sexually inactive subjects show a very low level of VT expression. In quail specifically, this correlation could be confirmed in a wide variety of experiments comparing VT expression in females (which do not show male typical behavior) and in males (which show these behaviors), in castrated males that are sexually inactive, in castrates treated with exogenous T (which restores sexual activity), in old sexually inactive males, and in similar males treated with exogenous T.

Studies performed in quail also clearly indicate that the sex difference in VT expression in the POM, BSTm, and septum is not simply the result of a differential activation by T. Although VT immunoreactivity and VT mRNA are suppressed by castration and enhanced by a treatment with exogenous T in males, the same treatment has no effect in females; ovariectomized subjects treated with T still do not show substantial levels of VT-ir structures in the POM, BSTm, and septum (*17,36*). These results suggest that the sexually dimorphic vasotocinergic innervation of these structures in adult quail is not controlled only by the endocrine condition in adulthood and that this sex difference could be organizational in nature.

Similarly, the sex differences in the expression of copulatory behavior in quail are not exclusively controlled by the different hormonal milieus in adulthood. Castration of males suppresses the expression of the male-typical copulatory sequence, and T treatment restores these behaviors. However, the same endocrine treatment cannot activate these behaviors in females. These behavioral sex differences in response to T are the result of a differential exposure to estrogens during embryonic development. The high levels of estrogens that are typical of female embryos (*48*) irreversibly suppress their capacity to display male-typical copulatory behaviors even in the presence of high levels of T in adulthood (*49*). The females are "demasculinized." Male embryos are not exposed to such high levels of estrogens and therefore retain their behavioral potential. These irreversible changes in the behavioral phenotype can be mimicked by injections of exogenous hormones or hormone inhibitors during the critical period of sensitivity. Male embryos injected with estrogens before day 12 of incubation thus will be completely demasculinized (*50,51*), whereas females treated during the early part of their embryonic development with an inhibitor of aromatase (the enzyme that catalyzes the production of estrogens) will be protected from the demasculinization of their reproductive behavior and will show a male behavioral phenotype in response to T in adulthood (*52*). It is thus possible in quail to completely reverse the behavioral phenotype by manipulations of the estrogenic exposure of the embryos (*53*). Parallel changes were observed in the vasotocinergic innervation of the three brain nuclei that were studied in detail: the POM, BSTm, and lateral septum of birds subjected during

embryonic development to endocrine treatments that irreversibly affect the behavioral phenotype (*36*). In this experiment, quail embryos were injected early during incubation with either the estrogen estradiol benzoate or with an aromatase inhibitor (R76713). After hatching, all subjects were gonadectomized and treated in adulthood with doses of exogenous T that are sufficient to activate sexual behavior and produce a maximal increase in the density of VT-ir structures in the brain. Males that had been treated *in ovo* with estradiol benzoate had completely lost the VT-ir structures in the POM, BSTm, and septum (Figure 2) and had, in parallel, lost the capacity to display copulatory behavior, as previously shown in many experimental studies (*50,51,54*). Conversely, aromatase-inhibitor–treated females displayed a male-typical VT-ir innervation of these three brain regions after T treatment and also displayed high levels of malelike copulatory behavior, as previously reported (*52*). The magnocellular vasotocinergic system was apparently not affected by these embryonic manipulations of the hormonal milieu, and in particular, no change in the density of the VT-ir material could be detected at the level of the hypothalamo-hypophysial tract (*36*).

These results clearly demonstrate that, in quail, the vasotocinergic innervation of the POM, lateral septum, and BSTm and their sensitivity to T in adulthood are organized during embryonic development. Exposure to high levels of estrogens results in a female phenotype as far as these vasotocinergic inputs are concerned; the (relative) absence of estrogens in the embryos leads to the male phenotype. The sexual dimorphism observed in the adults is truly organizational in its nature, even if the presence of T is required for this dimorphism to be fully expressed during adult life. Similar findings also have been recently reported in domestic fowls (*55*).

The mechanism(s) underlying the development of this irreversible sex difference has not yet been identified. Early exposure to estrogens could quite possibly lead in females to the specific loss of a population of VT-expressing cells that is at the origin of the vasotocinergic innervation of these brain areas. Alternatively, estrogens could alter only in an irreversible manner the phenotype of these cells so that they become unable to express the neuropeptide. This process should take place during the early postnatal development because VT-ir cells have been detected in the female BSTm day 1 posthatching, but they are no longer visible at day 10 (*56,57*). This mechanism could be identified only by an analysis of the effects of early estrogens on the survival of immunocytochemically defined cell populations.

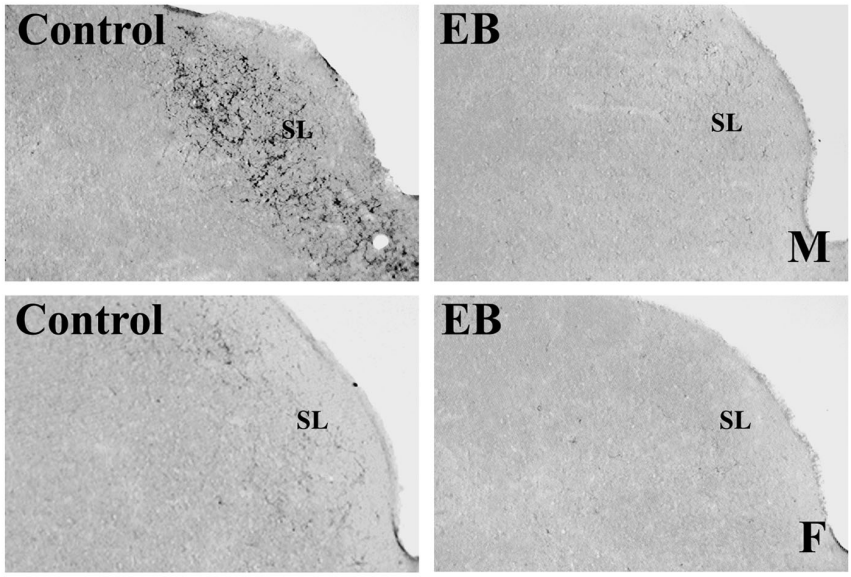

Figure 2. Photomicrographs illustrating the decrease of the VT-ir fibers in the lateral septum (SL) of male (M) and female (F) quail that were treated during embryonic life with estradiol benzoate (EB) in comparison with birds that were injected with the solvent at the same time (control). All birds were gonadectomized at 4 weeks of age and treated with exogenous T in adulthood.

Behavioral Effects of Vasotocin in Birds

Although VT was originally named based on its action in the control of osmoregulation and blood pressure, this neuropeptide is also implicated in the control of several aspects of avian reproduction such as oviposition by causing oviduct contractions (*58–60*), and in the regulation of male sexual behavior (*61–64*), as well as male vocalizations (*65*). VT or VP in fact exert a broad range of effects on a variety of social behaviors in all classes of vertebrates (*66,67*). However, huge interspecific variation exists in the types of behaviors that are affected by VT/VP and in the direction of the observed effects. Whereas the neuropeptide facilitates most behaviors of males of a large number of species, similar behaviors are inhibited in other species. These inhibited behaviors include aggression in some songbirds (but not in others), sexual and vocal behavior in Japanese quail, and production of courtship sounds in fish (*66*). In most cases, the sources of this variation have not been identified. In songbirds, VT has been postulated to facilitate aggression in colonial species but inhibit it in territorial species (*66*), but this idea is based on a very small number of species, and more studies would be needed to assess the validity of this generalization.

In quail specifically, the role played by the organized sex difference in VT innervation of the POM and BSTm remains at present unclear. Embryonic estrogens apparently suppress in parallel the capacity to display male typical copulatory behavior in response to T and the T-dependent vasotocinergic innervation of the brain. On the other hand, an active copulatory behavior has been shown to be associated with a dense expression of VT in the brain (*17,36*). These anatomical and physiological data therefore suggest that the T-induced changes in VT activity are part of the biochemical cascade of neurochemical events initiated by T through which this steroid activates male sexual behavior. Females in this scenario would fail to show this behavior because they lack the dense VT innervation.

This notion was tested by injecting sexually mature male quail, which had been previously castrated and treated with standardized amounts of exogenous T, with either VT or a VT receptor antagonist specific for the V1 receptor subtype. VT and its receptor antagonist were injected systemically or intracerebroventricularly. Effects of these treatments on appetitive and consummatory aspects of male sexual behavior were separately assessed in these studies because previous work in mammals and quail suggested that these two components of male sexual behavior may be controlled by neural mechanisms that are at least partly different (*67–72*).

In contradiction with the prediction (i.e., VT should activate male sexual behavior), these pharmacological studies consistently indicated, during 4 independent experiments, that VT exerts an inhibitory action on both the appetitive and consummatory aspects of male copulatory behavior

as well as on crowing, a T-dependent vocalization (*64*). It is therefore difficult to reconcile the anatomical and behavioral data that suggest respectively that VT is needed for but also inhibits the expression of male-typical behaviors. Possible explanations have been suggested (*36,64,73*) and are now awaiting experimental testing.

Conclusions

The sexual dimorphism provided by the vasotocinergic parvocellular cells of the BSTm and the innervation of the POM and lateral septum in the brain of galliforms, as well as its sensitivity to T, appears as a very suitable model system to investigate effects of gonadal hormones and related substances on neural circuits. VT neurons represent a population of brain cells with a clearly identifiable neurochemical phenotype, undergoing a profound and irreversible sexual differentiation during ontogenetic development. This model of sex differences displays unique features: it is sensitive to the level of androgen in adulthood but differentiates during early stages of ontogeny under the influence of estrogens in parallel with the differentiation of sexual behavior.

The quail model therefore appears particularly valuable for studies of the molecular processes of sexual differentiation with a cellular level of anatomical resolution. The exquisite sensitivity of the VT parvocellular system to estrogens in the egg and the irreversible nature of these effects suggest that these neural circuits can thus be useful to monitor exposure to environmental estrogens. Some of these compounds (e.g., bisphenol A or ethinylestradiol) are known to alter sexual behavior or reproduction in mammals as well as in birds (*74*). Halldin et al. (*75*) described a significant reduction of sexual behavior in quail exposed to the environmental pollutant ethinylestradiol during embryonic development. It therefore seems of interest to investigate alterations of neural circuits that could influence these behavioral (and physiological) changes. In this respect, the parvocellular sexually dimorphic VT system of quail could represent an optimal model to investigate the interactions of behaviorally active environmental estrogens with a specific cerebral circuit devoted to the control of reproduction and sexual behavior.

These features may provide unique opportunities to identify and track down in natural settings the possible exposure of a wild animal population to increased levels of estrogenic compounds. The adult activation by T and its estrogenic metabolites of the VT-ir system and of male-typical copulatory behavior appears to be reversible and could therefore be used as a marker of exposure to xenoestrogens that would be essentially synchronized with the period of analysis. In contrast, we have clearly established that the embryonic exposure of a quail embryo to increased levels of estrogens will permanently modify the expression of VT in the male brain and will also irreversibly inhibit the expression of male copulatory behavior. These neurochemical and behavioral modifications should thus, in theory, allow investigators to demonstrate the existence of an environmental contamination by estrogenic compounds that could have taken place months before investigations are conducted. The changes of the VT system and of behavior therefore constitute permanent markers (at least as long as the animals survive) of endocrine events that may no longer be present in the environment but that could have deeply affected the reproductive capacities of many species.

It is true that the Japanese quail is not a widely distributed species in natural environments, and feral populations are found only in very specific locations, including East Asia and remote parts of the Hawaiian islands. However, many related species such as the bobwhite quail (*Colinus virginianus*) are broadly distributed and could potentially serve the same role as Japanese quail in the wild. We already know that the activational effects of steroids on the brain VT system that have been described here also occur in other avian species, such as the domestic chicken (*76*), and several passerine species such as the junco and the canary (*6,38*). Biological markers of estrogenic exposure are therefore already present in the wild, and introducing quail is not necessary to have a constant monitoring of these compounds. However, whether the permanent organizational effects of estrogens also affect VT and sexual behavior in these other species remains to be established. This is known to be the case in the chicken (*55*), but generalization to more distant species should not be made at this time without additional experiments.

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