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Impact of estrogen and photoperiod on arginine vasotocin and VT3 receptor expression in the shell gland of quail

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1. ABSTRACT

Role of estrogen and photoperiod is well-established in avian reproduction. In addition, the distribution and the expression of arginine vasotocin (AVT) and its receptor VT3 to ensure reproductive/ breeding conditions in Japanese quail influenced by them has been the main focus of this review. To consider this aspect the mRNA expression of VT3 receptor and its ligand AVT in the shell gland has been emphasized. In birds, AVT performs a dual role as an anti-diuretic hormone and the functions accomplished by oxytocin in mammals. The physiological actions of AVT in birds are mediated through its diverse receptor subtypes VT1, VT2, VT3 and VT4. Dynamic alteration of VT3 expression during different reproductive and photo-sexual conditions of quail can be modulated by estrogen. In addition to the endocrine effect of AVT, the shell gland is complemented by its paracrine action via its receptors. Evidences indicate that the expression of shell gland AVT modulated by estrogen appears to play a priming role by modulating the availability of VT3 receptor for the required action of neurohypophysial AVT during oviposition.

2. INTRODUCTION

Birds offer excellent opportunities for behavioral and neuroendocrine experiments and the studies conducted at this interface have historically placed avian model on the cutting edge of research. Reproductive cycles in birds result from various interactions of external and internal factors. These factors serve as synchronizers and stimulate higher brain centre and consequently neuroendocrine system for preparing the organism physiologically to reproduce in time. The general function of the avian reproductive system relies on hypothalamic synthesis and secretion of releasing hormones. The contributions of arginine vasotocin (AVT) to various aspects of avian physiology and behaviour have been known so far. Too often we neglect to consider the potential impact of season, time of day, sex or reproductive status on the specific functions of AVT. Therefore, in this review possible attempts have been made to consider role of AVT and its receptor in reproductive physiology of quail and also intended to draw attention to the diverse roles for its receptor in the neuro-endocrine

system involved during various light schedules or photoperiodic conditions.

The neurohypophysial hormones arginine vasopressin (AVP) and oxytocin were first observed by Oliver and Schafer in 1895 (1). Arginine vasotocin (AVT) is the non-mammalian homologue of AVP and was first synthesized as an AVP analogue (2). Henry Dale was the first who in 1906 demonstrated the oxytocic properties of pituitary extract on the mammalian uterus. AVT is the basic neurohypophysial peptide present in all vertebrates except mammals and is characterized by the nine amino acid structure including a disulphide bridge linking cystines in positions 1 and 6 (3). AVT and mesotocin (MT) are synthesized by hypothalamic magnocellular neurons of supra-optic (SON) and paraventricular nuclei (PVN) then they are released primarily from nerve terminals into the posterior pituitary gland from where they are released in the peripheral circulation. There are at least 14 additional neurohypophysial hormones that appear in non-mammalian vertebrates (4). The most primitive vertebrate cyclostomes possess single neurohypophysial hormones AVT while all vertebrate species possess two neurohypophysial hormones one vasopressin like and another oxytocin-like. A number of studies suggest that AVT has a dual function in birds including Japanese quail, in water conservation acting as ADH on kidney and during oviposition as an oxytocic hormone on the shell gland, thus involved in reproductive behavior.

AVT has several important regulatory functions in non-mammalian vertebrates including birds (5). In birds, AVT regulates fluid balance, blood pressure and the stress response (6, 7, 8, 9, 10, 11). Interestingly, AVT and its gene transcripts are also expressed in different peripheral tissues of chickens including shell gland suggesting the possible paracrine role of AVT in avian physiology (12). In the ovary, the amount of AVT varies during oviposition cycle (13). In addition, AVT has well-documented effects on various reproductive behaviors, stimulatory effects on shell gland contractility and oviposition (14, 15, 16, 17, 18). Numerous reports indicate that AVT is involved in oviposition in laying hens. The plasma levels of AVT increase at the time of oviposition. AVT has oxytocic activity and the injection of AVT can induce premature oviposition in laying hens (19). In this review, we summarize the present knowledge of the oxytocic-like receptor (VT3) system gained in the different fields of research, thereby focusing mainly on the experiments performed to study the effect of sex steroid (estrogen) and its antagonist (tamoxifen) on the expression of AVT and VT3 in the shell gland of Japanese quail (*Coturnix coturnix japonica,* order Galliformes) during different reproductive / photoperiodic conditions. The diverse effects of AVT are mediated by the expression of its different receptor subtypes in different tissues which further strengthens the multifarious role of this peptide.

3. TYPES OF AVT RECEPTORS

The neurohypophysial hormones exert their biological effects by binding to the cell surface receptors that together represent a super -family of G protein coupled receptors (20). Four avian vasotocin receptors have been identified to date, all of them identified in chickens. These have been named as VT1, VT2, VT3 and VT4 likely to correspond to mammalian V1, V1b, Oxytocin receptor and V1a receptor respectively. Their nomenclature reflects the chronological order in which they were described. The four AVT receptors have been cloned from chicken (*Gallus gallus*) (21, 22). All these receptors are members of G-protein coupled receptor super family, consisting of seven hydrophobic trans-membrane alpha helices joined by intracellular and extracellular loops, an extracellular N-terminal domain and a cytoplasmic C-terminal domain (23).

VT1, the first cloned neurohypophysial hormone receptor is expressed in both the shell gland and brain (24). The effects of AVT on shell gland contractility appear to be mediated by two AVT receptors, the VT1 and VT3 subtypes (24, 25). The shell gland is the part of oviduct in birds where the shell formation takes place. The shell gland exhibits well developed musculature 'tunica muscularis' consisting of longitudinal and circular muscles on the outer region. The inner region 'tunica mucosa' is considerably thicker, which is next to tunica muscularis (fig1). VT1 is expressed in the tunica mucosa of the shell gland in chicken and not observed in the tunica muscularis. Confinement of the VT1 receptor to the tunica mucosa (Fig.2) suggests its role in induction of uterine contraction limited to a paracrine mechanism possibly working through prostaglandins. In brain, the expression of the VT1 receptor has been found to have a limited distribution in the white throated sparrow than zebra finches but was highly concentrated in nucleus pretectalis of both species. These receptors are concentrated in many areas where neurohypophyseal peptides were already demonstrated to affect social behavior in birds and mammals, for example, the Lateral Septum and the hypothalamus (26). The V1-type receptor, which is widely distributed in the CNS of birds, amphibians, and fish, is one of the most important receptors involved in the expression of social and reproductive behaviors (27). The central administration of VT or their antagonists has been shown to affect social behavior in songbirds (28). So the expression of VT1 in the brain suggests a possible role in influencing sexual, maternal or social behaviour. Functional characterization revealed that VT1 receptor binds VT-like hormones with greater potency than OT-like hormones (24). Phylogenetic analysis indicates that this receptor clusters with the mammalian V2 receptors and may be closely related to them (29).

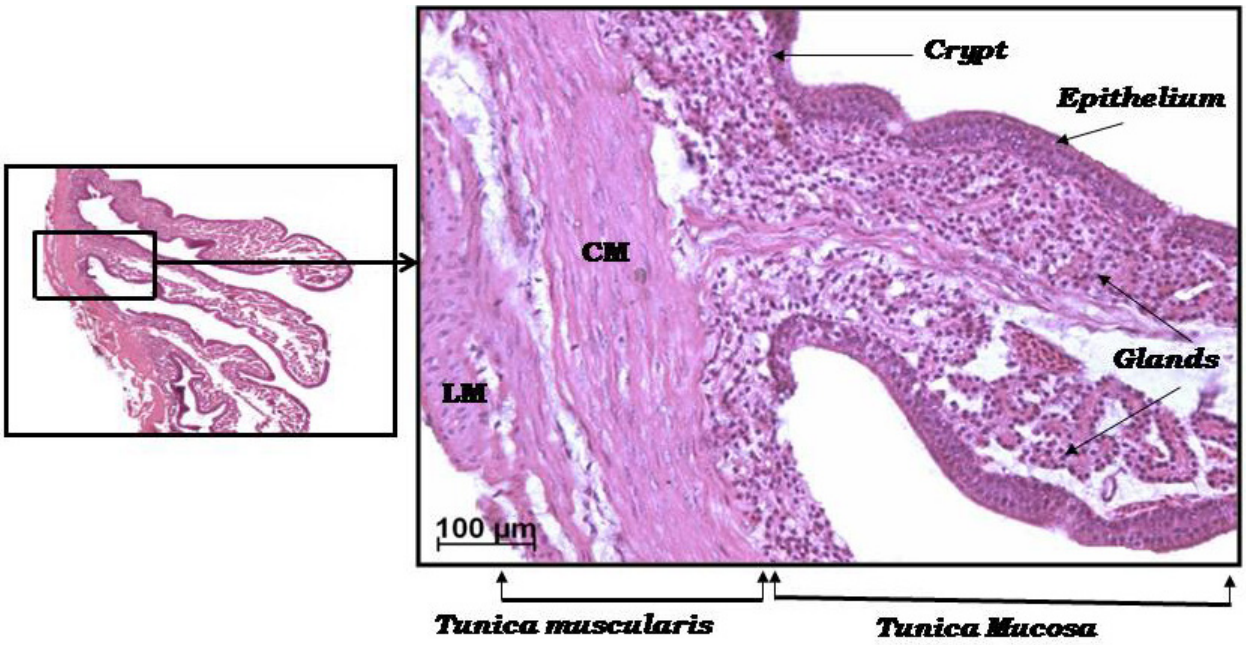

Figure 1. Photomicrograph showing the Hematoxylin and eosin stained transverse section of the shell gland in the low and high magnification. The wall of shell gland is composed of well-developed longitudinal (LM) and circular muscle (CM) layers, with mucosa arranged in numerous leaf-shaped folds lined with luminal/secretory epithelium surrounding large number of secretory glands. Deep invaginations i.e. crypt can be seen between the folds.

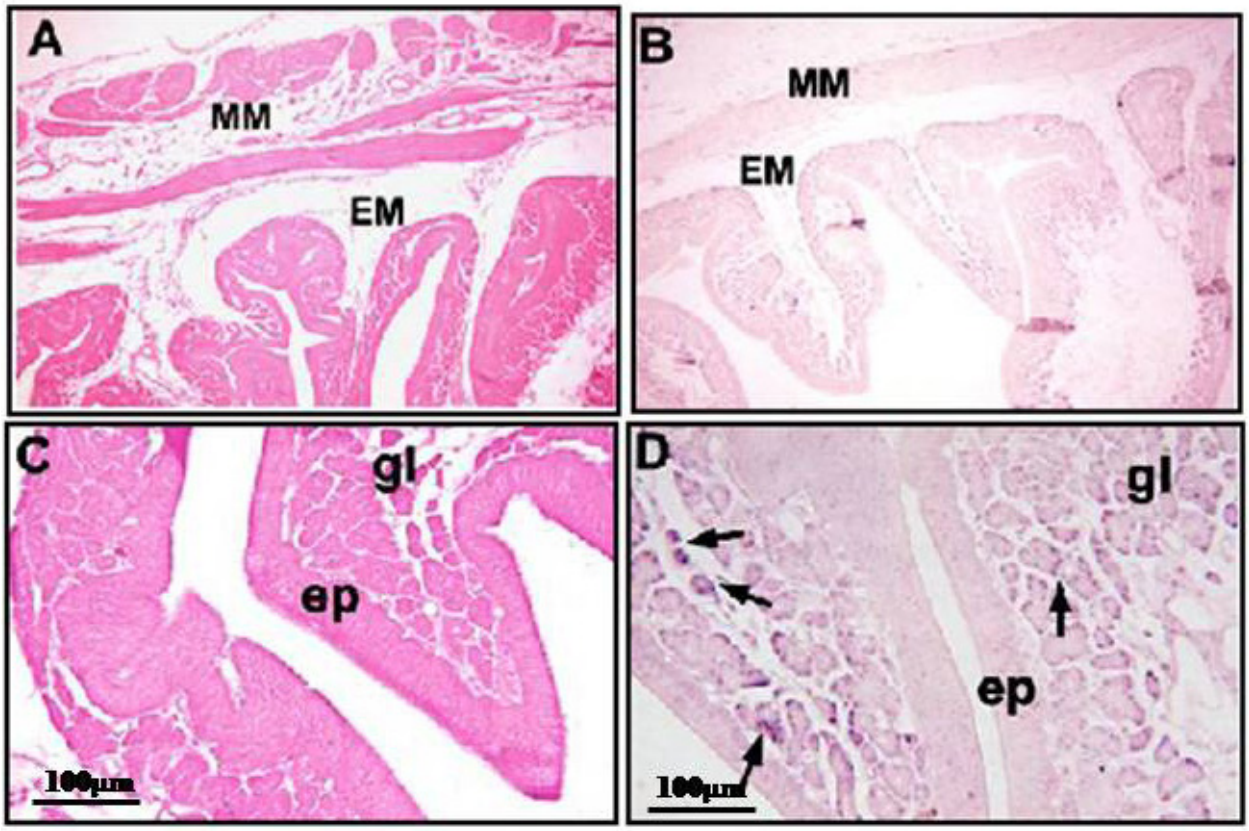

Figure 2. *In-situ* hybridization RT-PCR localization of VT1 receptor expression in the endometrium of the chicken shell gland. Upper panel shows the low magnification of shell gland while lower panel shows its higher magnification. A and C are the control sections of the shell gland while B and D show the RT-PCR localization. Arrows indicate RT-PCR of VT1 expression in the endometrium. Abbreviations MM represents the myometrium, EM= Endometrium, ep= epithelial layer, gl= secretory glands.

VT2, the second vasotocin receptor subtype is thought to be homologous to the mammalian V1b receptor (30). Like the V1b, it is expressed in high level in pituitary corticotrophs where it forms a heterodimer with CRH receptor ((31, 32). VT2 receptor has not been observed in the avian brain neither by Northern blot analysis or immunohistochemistry (31) where as V1b has been found in hippocampus, hypothalamus and amygdala in rodents (33). Its primary function is to mediate the effect of AVT through adreno-corticotropic hormones during stress.

VT3, the third avian neurohypophysial hormone receptor, shares a high sequence identity with mammalian OT receptor and is therefore referred as "OT-like". It appears to be oxytocin-like because of its expression in tunica muscularis and playing an important role in contraction of the chicken shell gland which is the characteristic of oviposition (25). Other studies have shown that tunica muscularis responds to AVT even after tunica mucosa has been stripped away suggesting direct effect of AVT on tunica muscularis (34, 35). Only two South American marsupials (*Didelphis marsupialis* and *Philander opossum*) express oxytocin exclusively (36). Furthermore, some marsupials have both oxytocin and mesotocin receptors present in uterus and both hormones play a role in stimulating uterine contraction ((37, 38). The function of mesotocin is not clear. The expression and regulation of VT3 have been characterized in quail shell gland by photoperiod, estrogen and age but not been investigated in brain (39, 40, 41).

VT4, the most recently cloned fourth receptor of AVT was found to be expressed in chicken brain and pituitary (22). It has identity with mammalian V1a receptor (Gen-bank ACCN [ABV] 24997). Vasotocin (VT) and corticotrophin releasing hormone (CRH) act via four vasotocin receptors (VTs) and two CRH receptors in mediating neuroendocrine functions in stress.

The results of some studies suggested that receptor distribution varies according to species. The neural distribution of neurohypophyseal peptide binding has been investigated in song bird species by using radio-labelled vasopressin and synthetic ligands (42, 43, 44). Whereas VT1 receptor mRNA was more widespread in zebra finch brain, VT3 receptor mRNA was more prevalent in sparrow brain (26). In addition to brain, VT1 (fig2) and VT3 receptors have been localized in chicken shell gland mediating shell gland contractions during egg-laying (25).

Japanese quail is a photoperiodic avian species and a very good research model for studying reproductive physiology including mechanisms of egg-laying. Initially a wild locally migratory species, the Japanese quail has been domesticated for game and egg production. The Japanese quail breeds throughout the year if maintained under long photoperiod. Interestingly, this species also exhibits all the known phenomenon of photoperiodism i.e. photorefractoriness (relative) as well as scotorefractoriness if maintained continuously under long and short photoperiod (45, 46). On the other hand, if maintained under natural day length Japanese quail behaves like a typical temperate zone photoperiodic species showing seasonal gonadal with displays annual gonad recrudescence. With increasing day length during spring, gonads attain maximum growth during summer and undergoes regression in post reproductive phase during still long (longer than spring days) but decreasing day length of the autumn period (45, 46). Hence, this species was studied to investigate the role of AVT and its receptors during different reproductive conditions. The present review includes the study carried on : 1) age dependent variation in the expression of arginine vasotocin (AVT) and its receptor (VT3) in the shell gland 2) the effect of estrogen and its antagonist tamoxifen on the expression of AVT and its receptor VT3 in the shell gland of sexually immature and mature quail, 3) the effect of estrogen and tamoxifen on the shell gland AVT and VT3 of Japanese quail during different photoperiodic conditions such as a) scotosensitive and scotorefractory, and b) photosensitive and photorefractory condition.

4. AGE-DEPENDENT VARIATIONS IN THE EXPRESSION OF AVT AND ITS OXYTOCIC-LIKE RECEPTOR (VT3R) IN THE SHELL GLAND OF JAPANESE QUAIL

The majority of studies on the shell gland with reference to development or reproductive performance have been carried out in domestic fowl and relatively little is known in Japanese quail (47, 48, 49). Therefore, studies have been done to examine the age dependent development of the quail shell gland under natural day length conditions. To assess the expression of AVT and VT3 in the shell gland, Japanese quail of different age groups (3, 4, 5, 6, 8 and 12 weeks old and that of 18 months age) were maintained in the month of February under natural day length. It was found that body weight, GSI, oviduct weight, length of shell gland mucosal folds and plasma estradiol increases with increasing age up to 12 weeks of age followed by a decline in the old birds (50). Immunohistochemical studies do not reveal the presence of AVT immunoreactivity (AVT-ir) in the shell gland sections of 4 week old birds. However, AVT-ir was observed in both layers of the shell gland in sexually active 12 week old quail as well as in abundant amount in its ovary. In old Japanese quail, AVT-ir is not found in the ovary but was observed in the shell gland myometrium although the amount is significantly less compared to actively laying 12 weeks old quail. Similarly by *in situ* hybridization study, VT3 transcripts were not detected in the shell gland of sexually immature 4 weeks old quail, while abundant

expression was observed in both the myometrium and endometrium of sexually active as well as old Japanese quail (41). It has been clearly demonstrated that changes in AVT immunohistochemistry are paralleled by changes in VT3 cDNA distribution suggesting the endogenous steroid induced regulation taking place in natural daylength. The endocrinology of reproductive aging in avian species has been described primarily in poultry birds, which show major changes in hormones and reproductive performance. During aging, the decline of neuroendocrine and behavioral components of reproduction ultimately leads to reproductive failure (48, 51). An increase in the expression of immunoreactive AVT in the myometrium has been observed when quail is in egg laying condition along with significant increase in hypothalamic, plasma and shell gland AVT during full breeding condition. During this condition since the plasma estradiol increases so does the estrogen receptor alpha in the shell gland thereby exhibiting its action on shell gland. Further, low expression of AVT-ir has been observed in non-breeding specific phase relation of 8 h quail along with a significant decrease in hypothalamic, plasma and shell gland AVT with the suppression of gonads thereby stopping the egg-laying behaviour (52).

Taken together it can be concluded that in addition to a gradual increase in the growth and activity of ovary and shell gland, AVT and VT3 increases with increasing age in quail followed by a decline in old (aged) quail. These age dependent changes are the result of variation (increase) in endogenous estrogen, which not only influences the activity of shell gland but also alters the expression of AVT-ir and its receptor VT3 in it (41, 50). This clearly explains that AVT and VT3 are expressed in a series of concomitant physiological and behavioral changes primarily based on endogenous mechanisms stimulated by estrogens. In the following section we concentrate on two questions a) what would be the AVT and VT3 expression pattern if sex steroid was given exogenously and b) how this system is organized to fulfill this pattern when changes are induced by changing the photoperiodic conditions. To answer these questions next study was undertaken as follows.

5. ESTROGEN-INDUCED EFFECT ON THE EXPRESSION OF AVT AND ITS RECEPTOR VT3 IN THE SHELL GLAND OF JAPANESE QUAIL

Estradiol benzoate treatment in sexually immature quail increases the body weight, oviduct weight, mucosal fold length as well as total protein in plasma and shell gland significantly, while its antagonist tamoxifen administration in sexually mature quail leads to a significant decrease only on the oviduct weight and protein content in the shell gland. It could not produce a significant effect on body weight and mucosal old length.

Sex steroid estrogen provides important stimuli triggering the onset of sexual maturity in animals. This function is particularly evident as AVT immunostaining was not detected in the ovary of sexually immature quail. However, ovary of sexually mature quail exhibits the presence of AVT-ir in the stromal region between the follicles but it was again not detected in the ovary of tamoxifen treated quail. Further, no AVT-ir was observed in the shell gland of sexually immature quail while it was observed in the myometrium only following estrogen administration. However, immunoreactivity was present in both the tunica muscularis and mucosa of sexually mature quail. Following ten days of tamoxifen treatment AVT-ir was not observed in the myometrium, but traces were seen in the endometrial region. *In-situ* hybridization signals for VT3 receptors were not detected in sexually immature and tamoxifen-treated sexually mature quail but were clearly observed in the myometrium as well as endometrium of estradiol treated sexually immature as well as sexually mature quail (38).

The increase in AVT and VT3R observed after estrogen administration in sexually immature quail is thus likely to reflect proliferation and differentiation of the shell gland, hence preparing it for egg laying. The variable decrease in the expression of AVT and VT3 reflects the morphological status of shell gland thereby decreasing the egg-laying after tamoxifen administration in sexually mature quail. The findings point to the important role of steroids not only in the expression of AVT and VT3, but in addition, indicate significant increases in the ovarian and shell gland activity (mucosal fold length, proliferation of epithelial lining and differentiation of tubular glands, AVT-ir and VT3 mRNA) of quail after attaining sexual maturity. In general, the presence of AVT-ir and the expression of VT3 transcripts in the shell gland of sexually immature quail following estrogen administration as well as in the sexually mature quail and its absence in the immature as well as tamoxifen-treated mature quail suggests that estrogen influences the local synthesis of AVT as well as its receptor (VT3) in quail in addition to its well characterized effect on shell gland/uterus differentiation and activity (39).

The conclusion derived from these results after estrogen administration in sexually immature quail and comparing it with sexually active quail, it becomes clear that in the quail having hypertrophied shell gland, either directly through estrogen administration or through functional HPG axis, there is up regulation in the expression of AVT-ir and VT3 receptor transcripts in the shell gland. Thus, the underlying basis of this observed up regulation of AVT and VT3 appears to be due to increase in estrogen level.

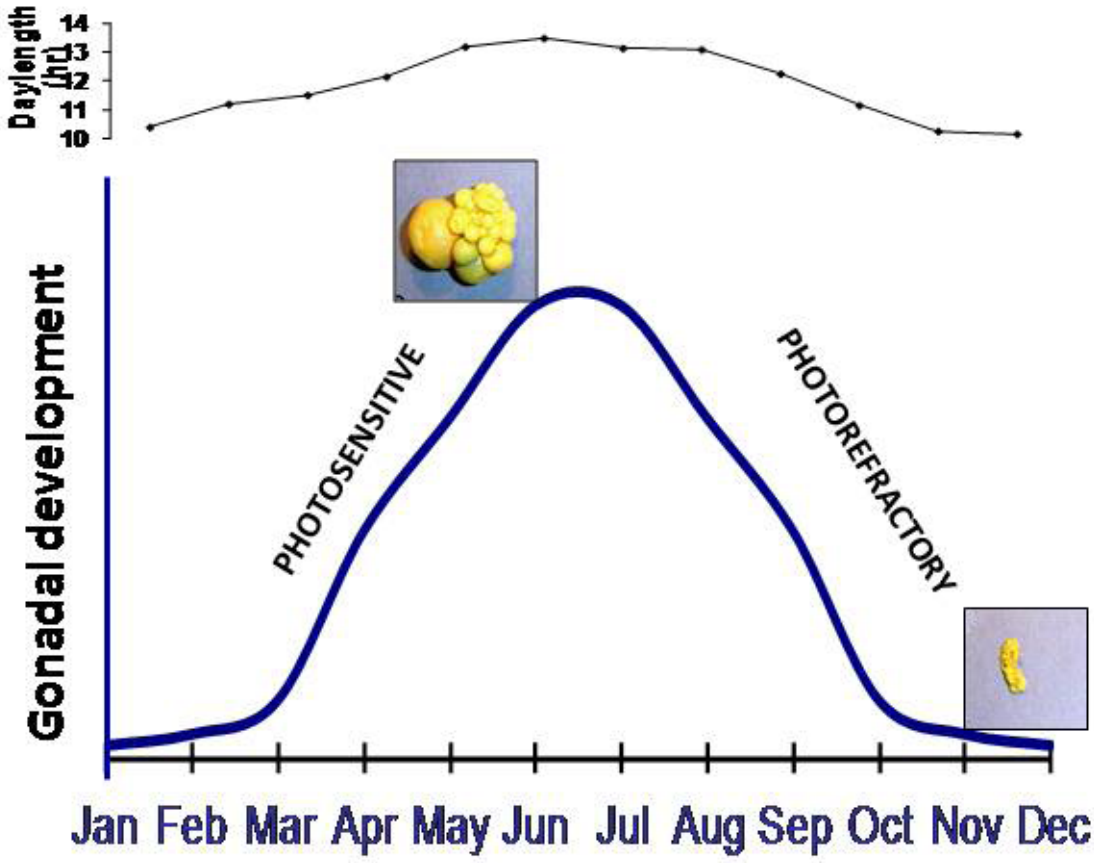

Figure 3. Annual variations in the development of *Coturnix coturnix japonica* ovary under natural condition. Upper panel shows variation in annual day length at Varanasi.

To further strengthen the observation and to investigate the role of estrogen photoperiodic studies have been done. The expression of AVT and its receptor have also been monitored in the shell gland of quail whose reproductive condition/gonadal status was modulated by artificial photoperiod.

6. PHOTOPERIOD-INDUCED RESPONSE ON THE EXPRESSION OF AVT AND ITS RECEPTOR VT3 IN THE SHELL GLAND OF JAPANESE QUAIL

Reproduction involves precisely timed neural and endocrine events which occur within a well-defined schedule and seems to be synchronized with external and internal factors such as photoperiod, availability of food and favorable conditions, neural inputs and hormonal balance etc. (52). Effects of all the external and/or internal factors regulating reproduction are funneled through hypothalamo-hypophyseal-gonadal axis. Since, stimulation of reproductive system during both short day length and long day length occurs in a different manner and yet to be defined so it was thought worthwhile to investigate the factors which may modulate gonadal axis to execute photo-responses. Like any typical photoperiodic bird species, initially Japanese quail are sensitive to the gonado-inhibitory effect of short days. These Japanese quail are scotosensitive. If exposure to short days is continued for longer period, short days are no longer inhibitory leading to spontaneous gonadal growth and birds are said to be scotorefractory (46). Japanese quail behave like a typical long day breeding photoperiodic species when maintained in nature. The annual gonadal cycle consists of two periods of different reproductive physiological states: photosensitivity, during which birds are able to respond immediately to a change in photoperiod, and photorefractoriness, during which birds show no immediate response to a change in photoperiod (fig3). Thus, this single species exhibits various forms of avian photoperiodism. The reproductively active condition of egg-laying occurs in both scotorefractory and photosensitive condition and gonadal inactivity/cessation of egg-laying can be involved in scotosensitive and photorefractory condition. To find out the correlation of AVT and VT3 with reproductive performance further photoperiodic

studies under short day length and long day length were performed in quail.

6.1. Under short daylength photoperiodic condition: the effect of estrogen and tamoxifen on the shell gland AVT and VT3 of scotosensitive and scotorefractory japanese quail

Sexually mature six week old female Japanese quail, when transferred to short photoperiod (6L: 18D) for 9 weeks, underwent ovarian regression exhibiting the phenomenon of scotosensitivity. These scotosensitive non-laying quail were injected with estradiol benzoate for 15 days. Another group of birds were continued under short days for an additional 4-6 weeks, when at the age of 19 weeks egg-laying began indicating attainment of scotorefractoriness. These scotorefractory quail were then administered with tamoxifen. Following estradiol administration in scotosensitive quail all the parameters show activity of ovary and shell gland including an increase in body weight but tamoxifen treatment exhibited mixed effect when given in scotorefractory quail. The gonado-somatic index, oviduct weight and mucosal fold length increased in sexually active scotorefractory condition compared to sexually quiescent scotosensitive quail. On the other hand, length of mucosal folds increased not only in the estrogen but also in the tamoxifen treated quail compared to their respective controls. It was observed that following estrogen administration, both the AVT-ir and VT3 transcript levels increased in the shell gland of scotosensitive quail and decreased following tamoxifen treatment in the scotorefractory birds (40). It is supported by the evidence from earlier reports that the reproductive response depend on the previous photoperiodic experience of bird and the influence of gonadal steroids and/or day length on AVT and its receptor gene expression (54,55). Further, as expected, both AVT-ir and VT3 gene expression was up-regulated on steroid treatment and down regulated in tamoxifen-treated quail, strengthening the suggestion that sex steroid regulates AVT system in the shell gland (40).

6.2. Under long daylength photoperiodic condition: the effect of estrogen and tamoxifen on the shell gland AVT and VT3 of photorefractory and photosensitive japanese quail

Sexually mature female Japanese quail were maintained under long-day length condition (16L: 8D) for three months and then transferred to an intermediate day length (13.5.L: 10.5.D) for four weeks result in the development of relative photorefractoriness and cessation of egg laying. The photorefractory birds were administered with estradiol benzoate. Another set of long day birds continued to be exposed to long day length condition (16L: 8D) for an additional four weeks, to maintain photosensitivity / egg laying. These sexually active quail were administered with tamoxifen.

Estradiol treatment in photorefractory bird results in significant increase in body weight, shell gland weight, length of mucosal folds, total protein (shell gland) and alkaline phosphatase in plasma and shell gland. In contrast, tamoxifen treatment reduces these parameters that regained photosensitivity. AVT-ir, which was not detected in the photorefractory quail ovary, was observed following estrogen treatment. On the other hand, abundant immuno-staining seen in photosensitive quail was not observed in tamoxifen treated quail (50). The distribution of AVT-ir appears more widespread throughout the myometrium in estrogen treated photorefractory quail compared to photorefractory and much reduced following tamoxifen treatment compared to photosensitive quail. *In-situ* hybridization studies revealed an increase in the distribution of myometrial receptor VT3 transcripts in estradiol treated photorefractory quail and decrease in tamoxifen treated photosensitive quail. These findings indicate that the body mass, activity and length of mucosal folds of the shell gland of photosensitive quail are higher compared to that of photorefractory quail. Treatment of photorefractory birds with estrogen increases and that of photosensitive birds treated with estrogen antagonist decreases AVT-ir and VT3R receptor gene transcript in the shell gland myometrium of Japanese quail (56).

Combining all the findings of the above mentioned experiments it is obvious that under physiological conditions, the expression of shell gland AVT and VT3 runs parallel with reproductive activity in Japanese quail suggesting interrelation of sex steroid and shell gland AVT-VT3 system. A role of sex steroid in the up-regulation of transcription of AVT gene in the hypothalamus / PVN neurons has also been reported in birds in response to stress (54). Further, activity of AVT synthesizing hypothalamic neurosecretory neurons is also reported to vary seasonally. A significant and gradual increase in the number of *ir*-AVT neurons was observed from quiescent to breeding phase, which decreased during regressive phase of the annual gonadal cycle (57). This indicates a functional interaction / correlation between sex steroid and AVT synthesizing neurons. Up-regulation of this system is also observed in sexually mature 12 week old quail maintained under natural day length (NDL), photosensitive long day (LD) quail and scotorefractory short day (SD) quail; all with fully active gonad and in the actively laying condition. Moreover, estradiol induced an increase and tamoxifen induced a decrease of these functions in sexually inactive and active quail, respectively. These observations further support the suggestion that sex hormone influences shell gland AVT and VT3 expression. Obviously tamoxifen, a synthetic estrogen antagonist that inhibits the transcription of the target genes by binding to estrogen receptor also blocks estrogen induced transcription of shell gland AVT and VT3. In addition to the well

documented release of AVT from neurohypophysis during oviposition, herein reviewed studies for the first time provides experimental evidence that oxytocic role of AVT is induced via VT3 receptors localized predominantly in the myometrium of shell gland, which appears to be regulated, by both estradiol and AVT.

In addition to shell gland, ovarian tissue also contains AVT-ir in the thecal and granulosa cells as well as in the inter-follicular region, but only in the sexually active condition (50). As suggested for mammals, in quail also, the presence of AVT may be involved in the contraction of follicular wall at the time of ovulation. The presence of immunoreactive-AVT varies with follicular growth. The immunostaining of AVT was not detected in quail ovary during sexually inactive condition (sexually immature, old and photorefractory birds). Moreover, even on estrogen administration AVT immunostaining was not observed in the ovary of immature quail possibly because sex steroid normally does not increase ovarian activity / function, unlike the activity of its target organ shell gland.

VT3R have been observed to express them in the outer tunica muscularis layer of shell gland while another receptor VT1R is found in inner tunica mucosa layer of chicken. We are able to confirm the presence of both the receptors with expression in sexually mature state of shell gland in chicken. It is of interest to note that the shell gland which stains strongly for the AVT were generally always expressing the receptors. The effect of receptor ligand binding was of up-regulation in that the administration of estrogen made the detection of VT3R by *in situ* hybridization more feasible. Comparing the results it has been observed that AVT and VT3 concentrations can differ as they are ligands and receptors respectively and the protein and mRNA mismatches do occur. Based on an *in vitro* fowl oxytocic assay, it has been reported that chicken uterus / shell gland is most sensitive to the neurohypophysial peptides (rank order of oxytocic potencies: AVT=VP>OT) immediately after oviposition compared to 2 h before or 5 h after oviposition (35). An earlier RT-PCR study from our laboratory also indicated that hypothalamic AVT and shell gland receptor VT1R gene expression increases at the time of egg-laying (0h) but is low at 2h before and 2h after oviposition. However, the shell gland AVT is increased 2h before egg laying followed by a decline attaining low value at the 0 hr, which remained unaltered thereafter. Increases in the number of AVT-ir neurons and mRNA copies in magnocellular neurons of paraventricular region at the time of oviposition (0 h) supports the involvement of AVT through the up-regulation of hypothalamic AVT gene expression in the regulation of oviposition in chicken. The increased level of shell gland receptor VT1 at the time of oviposition facilitates the sensitivity of avian uterus to AVT for its oxytocic action although indirectly via prostaglandin (PG) during expulsion of egg (54). It seems that local synthesis / paracrine release of AVT in the shell gland from endometrium (secretory cells) stimulates local PG synthesis, which in turn is responsible for the act of egg laying / smooth muscle contraction. It is suggested that increased gene expression of shell gland AVT, 2h prior to egg laying results in paracrine effect to initiate PG secretion from surrounding tissues. This mechanism is further enhanced by a surge in circulating AVT level (endocrine secretion from hypothalamus and posterior pituitary) and an increase in the AVT receptor VT3 density in the shell gland at the time of egg-laying. It appears that initiation / preparation of shell gland contraction is mediated through local AVT synthesis (proximate factor) and endocrine synthesis and secretion plays a final role in the expulsion of egg as an ultimate factor / cause. Increased AVT gene expression in the hypothalamus is necessary for egg-laying, it varies between two poultry species (quail and chicken). While in quail this increases just before egg laying, in the chicken it starts increasing approximately 2 hours before oviposition (54).

Compared to the uterine OT receptors in mammals, little molecular information is available regarding shell gland AVT receptor in chicken. Unlike mammalian system, out of the two receptor subtypes VT1 and VT3 reported in chicken, VT1 is found to be localized in the endometrium only (based on *in-situ* RT-PCR), VT3 is present in both endometrium and myometrium, but is predominantly localized in the latter region. Although VT1 is reported to vary with the time of oviposition, it is believed to be involved in shell gland contraction indirectly, possibly through prostaglandins. On the other hand, VT3 appears to be OT-like receptor playing a major role in mediating myometrial contraction because of the following reasons:

1. In sequence homology, this receptor has high homology with mammalian OT receptor.

2. The report that myometrium responds to AVT even after endometrium has been stripped away further suggests direct action of AVT on myometrium through VT3 receptors for muscular contraction required during egg-laying / expulsion of the egg (58, 35).

3. The VT3 is predominantly localized in the myometrium, and its expression runs parallel with reproductive / egg laying activity not only in the control and different physiological conditions (viz. photosensitive and scotorefractory) but also in the experimental condition following estrogen administration in sexually quiescent / inactive quail.

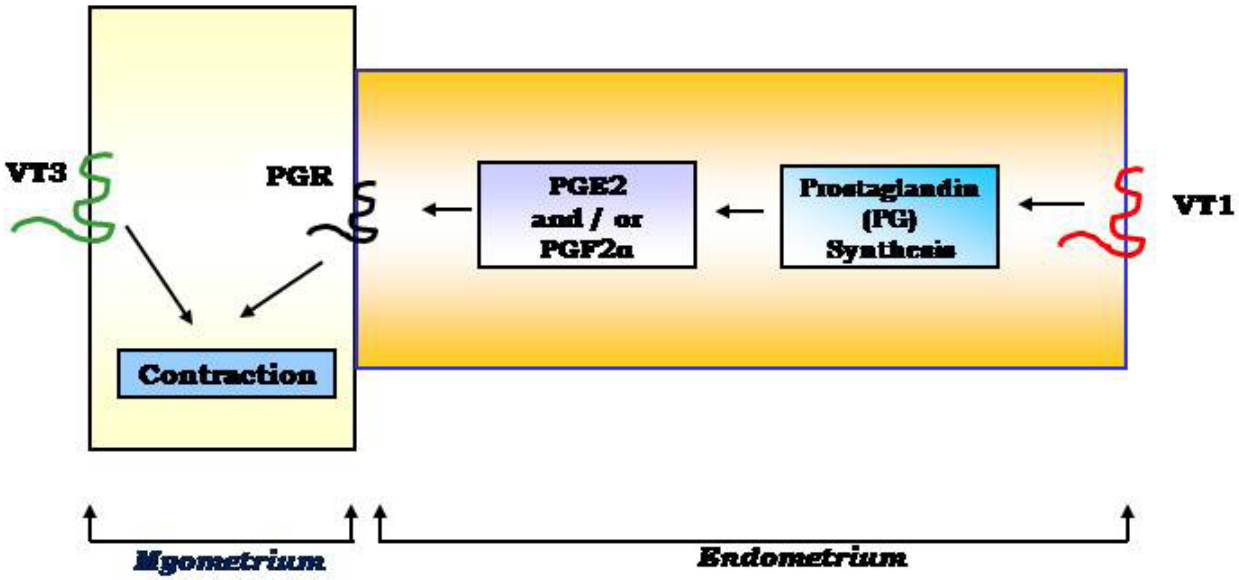

Figure 4. Suggested Model of mechanisms by which arginine vasotocin (AVT) regulates shell gland contractility during oviposition via vasotocin receptors VT1 & VT3. We hypothesize that in addition to direct stimulation of myometrial contraction via VT3 receptors (localized predominantly in myometrium); AVT also activates endometrial prostaglandin synthesis via VT1 receptors. Through a paracrine mechanism endometrial prostaglandin also stimulates myometrial contraction.

Since VT1 is restricted to tunica mucosa; VT3 is localized mainly in tunica muscularis in quail. This may explain specific/different role of tunica mucosa vasotocin receptor (VT1) and tunica muscularis receptor (VT3) in birds. Comparable to mammalian distributions of OTR it has been reported that endometrial OTRs increase during luteolysis and myometrial OTRs during parturition (59, 60). Since sex steroids (in particular progesterone and estrogen) play pivotal roles in the above process it is also possible that these hormones participate in the regulation of OT receptor expression/concentration; generally an up-regulation of OTR mRNA or the number of OTR binding sites. Based on the only information available in birds regarding localization of VT1 and VT3 receptors their direct as well as indirect roles during shell gland contraction is suggested as in fig4.

In fowl, it has been already reported that estrogen treatment increases the sensitivity of uterine muscles to AVT, indicating that the sex steroid can up-regulate the AVT receptor (35). Our findings for the first time provide molecular basis for this effect, since up-regulation of shell gland AVT and VT3 receptor occurs in reproductively active quail, not only in basal / natural breeding condition, but also during the experimentally induced breeding condition (photosensitive/ scotorefractory phases). Up-regulation of the expression of shell gland AVT and VT3 genes following estrogen and down regulation by tamoxifen in reproductively inactive and active quail, respectively, strengthens estrogen-regulated expression of VT3 receptors. However, it is yet to be shown whether this up-regulation is a direct or indirect (through AVT) effect. Combined findings of all the experiments described in this review are presented diagrammatically in the fig5.

It is summarized that the dynamics of vasotocin receptor expression can be modulated by stimulation not only with the endogenous estrogen but also by exogenous administration of this sex hormone in the sexually immature, non-breeding / sexually quiescent condition. AVT-*ir*, VT3 transcript and activity of shell gland vary with the age, reproductive phase and photoperiodic condition of the Japanese quail. In general, estradiol benzoate administration increases and its antagonist tamoxifen decreases, AVT and VT3 gene expression, not only in the sexually immature and mature quail but also in the sexually quiescent (Scotosensitive and Photorefractory) and sexually active (Scotorefractory and Photosensitive) condition respectively. The results suggest that, in addition to its established role in the regulation of accessory sex organs, estrogens may also modulate AVT system (AVT and VT3) in the shell gland. Further, in addition to endocrine effect of hypothalamic AVT on the myometrial contraction, shell gland AVT is also involved in oviposition through its paracrine effect(s). Present experimental findings also suggest that, local AVT prepares the shell gland in advance, for the egg laying, possibly by increasing the expression of VT3 receptors and this regulation is estrogen dependent. Further, based on the functional similarities of Estrogen-AVT-

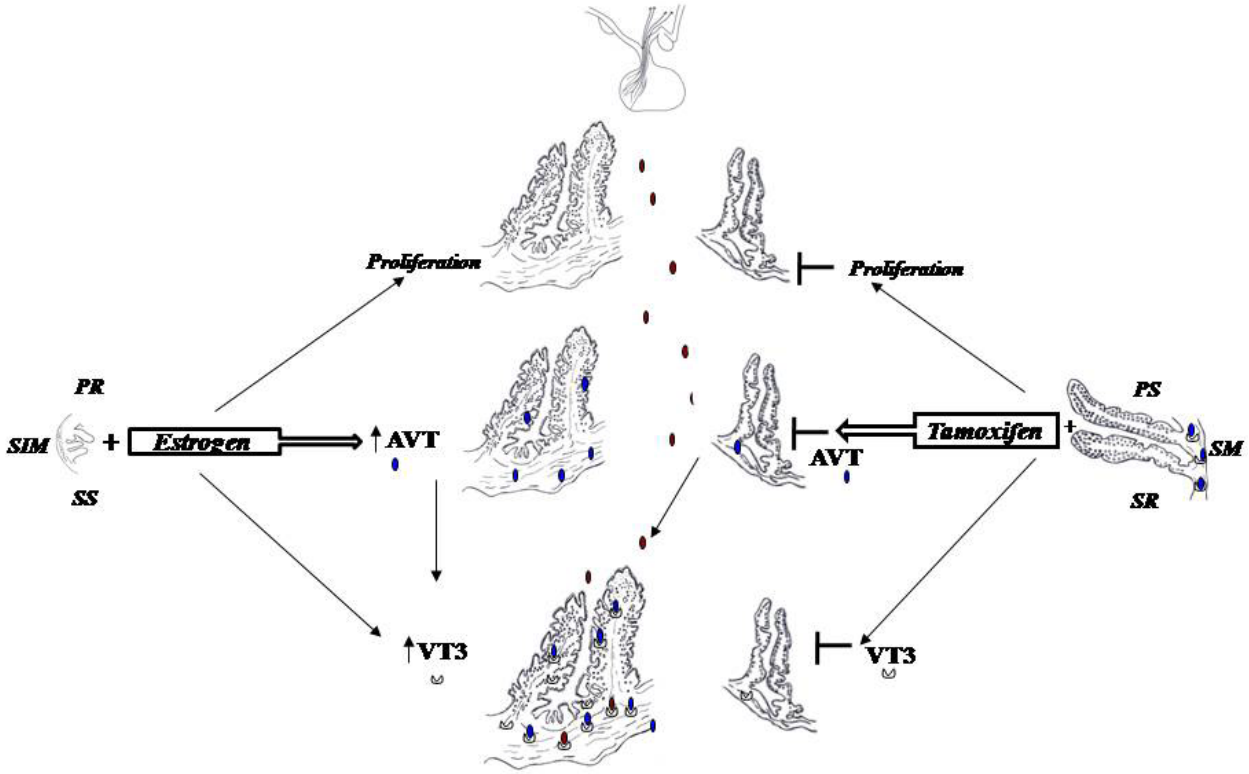

Figure 5. Diagrammatic representation of the summarized effects of 1. estrogen on the inactive shell gland of sexually immature (SIM), scotosensitive (SS) and photorefractory (PR) quail leading to its proliferation as well as up-regulation of the expression of ir-AVT and its receptor VT3 2. estrogen antagonist Tamoxifen on the active shell gland of sexually mature (SM), scotorefractory (SR) and photosensitive (PS) quail leading to down-regulation of the above effects.

VT3 and Estrogen-OT-OTR systems it may also be suggested that mechanisms for regulating avian uterus during oviposition may have been conserved evolutionarily in mammals.

7. CONCLUSIONS

AVT is the neurohypophyseal peptide in quail directly regulating the functions of shell gland via its oxytocic-like receptor VT3 subtype. The VT3 receptor and its genes are important components in the contractility of reproductive smooth muscle cells of the shell gland of quail. They are clearly regulated in a manner compatible with and instrumental in the reproductive physiology of quail. Thus the VT3 is an excellent indicator for the molecular mechanisms involved in the control of egg-laying in birds. The expression of AVT and its specific VT3 receptor appears to be intrinsically linked to the reproductive function in quail, evident in a response to the changing differentiation-dependent levels of the sex steroid. The effect of ligand (AVT) and receptor (VT3) binding was of up-regulation, in that administration of estrogen made the detection of AVT and VT3 receptor more feasible. Given their age and the selective pressure upon reproductive hormone systems under different photoperiodic conditions, finally it becomes clear that AVT and VT3 receptor genes are regulated by estrogen. These novel mechanisms are going to be of much more general importance in reproductive physiology and will provide valuable insight into other aspects of sex steroid function. Further studies are required to unravel the roles of VT1 and VT3 in the shell gland and the mechanism by which they are regulated and interact.

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