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Arginine Vasotocin: Structure-Activity Relationships and Influence on Gonadal Growth and Function

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SYNOPSIS When AVT (arginine vasotocin) was given neonatally during the period when the brain is undergoing sexual differentiation, increased growth of the reproductive organs was observed in adulthood. Injection of AVT after this neonatal period in immature animals led to diminished growth of the accessory organs and in some cases the gonads themselves. The hypertrophic response of the *in situ* ovary in adult mice following unilateral ovariectomy (UO) was inhibited in a dose-related manner by a single intraperitoneal injection of freshly prepared AVT. Much less AVT was required for this response when injected into the third ventricle. After intraperitoneal injection, arginine vasopressin (AVP), lysine vasopressin (LVP), and 4-leucine vasotocin (4-leu-AVT) also inhibited compensatory ovarian hypertrophy whereas oxytocin did not. The commonality in the structure of these antigonadotrophic peptides include a closed ring and a basic amino acid in position 8. After opening the disulfide bond of these nonapeptides with mercaptoethanol, a single injection of the reduced AVT, AVP, LVP, or 4-leu-AVT into UO mice causes exaggerated hypertrophy of the remaining ovary. When added with leuteinizing hormone-releasing hormone (LRH) to culture medium containing hemipituitaries from castrated estrogen-progesterone primed female rats, AVT significantly increased the release of radioimmunoassayable LH above that due to LRH alone. AVT might interact at all levels of the hypothalamo-hypophyseal-gonadal axis.

Nine neurohypophyseal principles subserving water balance and oxytocic functions have been tentatively characterized among vertebrates (Sawyer, 1968). Arginine vasotocin (AVT) is the most ubiquitous, occurring in all major vertebrate groups from cyclostomes to mammals and is the probable ancestor of all the other active compounds (Heller, 1963). Arginine vasopressin (AVP) and oxytocin were apparently evolved and retained early in phylogeny. With the emergence of the class mammalia and newly acquired unique modes of fetal retention and postnatal suckling, AVP and oxytocin were pressed to further sub-specialized functions dictated by the needs of the organism. From the standpoint of evolutionary practicality, the appearance of single amino acid substitutions in either position 3 or 8 of the AVT molecule produced the desired functional specificity. Until recent evidence proved otherwise, it was assumed that AVT had been forfeited *in toto* by mammals in favor of the two new peptides, vasopressin and oxytocin. However, in a few laboratories, a tenacious search for the active pineal antigonadotrophic principle has focused on several low molecular weight peptides isolated from pineal tissue. Milcu and collaborators (1963) discovered a polypeptide with pressor and oxytocic activity in extracts of bovine pineal glands. The biological and chromatographic characteristics were similar to those of AVT. Biochemical identification of AVT has been performed by Cheesman (1970) and radioimmunoassay data indicate that the bovine pineal gland contains 8.9 μU/mg tissue (Rosenbloom and Fisher, 1974). Human and cat cerebrospinal fluid contains AVT (Pavel, 1970; Pavel and Coculescu, 1972) which Pavel speculates is derived from the

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pineal gland by ependymosecretion (Pavel, 1971). The *raison d'etre* for AVT in mammals remains obscure although a role in reproduction has been suggested (Pavel and Petrescu, 1966; Vaughan *et al.*, 1974*a*; 1974*b*). The influence of AVT in known reproductive model systems in laboratory rodents will be the theme of this presentation.

PRENATAL AND NEONATAL ADMINISTRATION OF AVT

Regarding the potential influence of the pineal gland in establishing a possible feedback circuit with the hypothalamo-hypophyseal-gonadal axis either during ontogeny or during the neonatal "critical period" of sexual differentiation, precious little information is available. In the mouse, pineal ablation on the day of birth effected significant increases in the weight of the prostate, ovaries, testes and thyroid (Lombard des Gouttes 1967), although these observations were not confirmed by a similar experiment in the rat (Kincl and Benagiano, 1967). Melatonin administered to rats 2 days after birth resulted in elevated testicular weights at 80 days of age (Vaughan, 1970). Since Pavel (1973) has recently purported that injection of melatonin in adult animals depletes pineal AVT by 50%, the aforementioned effect of melatonin could potentially be due to the release of AVT from the pineal or neurohypophysis of the newborn (Visolyi and Perks, 1969; Perks and Visolyi, 1973). Thus, the question of whether administration of AVT prenatally or neonatally might mimic the effects of neonatal melatonin injection was considered.

All mice were derived from Swiss-Webster stock (Hilltop Lab Animals) and were maintained under photoperiodic conditions of 12L:12D. Virgin mice were impregnated by proven males and the day of mating recorded. Cages were checked twice daily for the appearance of new litters. On days 1 through 5 after birth, pups received a single daily subcutaneous injection of diluent or of 0.02, 0.2 or 2 μg AVT (compliments of Dr. Roderich Walter). All animals were weaned at 21 days of age and males and females from each litter were necropsied at 30, 60 and 90 days of age. Testis weights were significantly elevated at 30 and 60 days in groups receiving 0.02 and 2.0 μg AVT (Fig. 1). In general, accessory organs (seminal vesicles and coagulating glands) and ventral prostate weights were also elevated at 30 and 60 days in all groups receiving AVT. By 90 days of age, the size of the gonads in the control animals were equivalent to those of the AVT-treated mice. In AVT-treated female mice, ovaries were significantly heavier than control animals only at the 90-day time point (Fig. 1).

The observation that neonatal administration of AVT can produce the same precocious gonadal growth spurt as seen after neonatal injection of melatonin in the male rodent evokes consideration of Pavel's hypothesis that the effects of melatonin are due to the release of pineal AVT. The gonads and accessory organs of the control male animals eventually did attain the same size as those of the AVT-treated animals indicating a possible early maturation of the hypothalamo-hypophyseal-gonadal axis after AVT injection. Further experiments are needed, however, to confirm this.

In timed-pregnant female mice, daily administration of 2 μg AVT resulted in delayed parturition and death of seven out of eight litters by the morning following delivery. One litter consisting of four runted newborns expired 48 hours postpartum (Table 1). The causative factors involved are not clearly understood. As AVT is structurally similar to oxytocin, it might be speculated that some interaction at uterine receptor sites would be inevitable (Rudinger and Krejci, 1968). Thus, the occupation of uterine receptor sites by AVT, which possesses only 25% oxytocic activity (Berde and Boissonnas, 1968) might prolong and/or delay parturition. Although AVT has not been demonstrated to cross the placental barrier, the increased input of AVT from the maternal side might have upset the delicate mechanism by which control over the volume and circulation of amniotic fluid is maintained. AVT has been

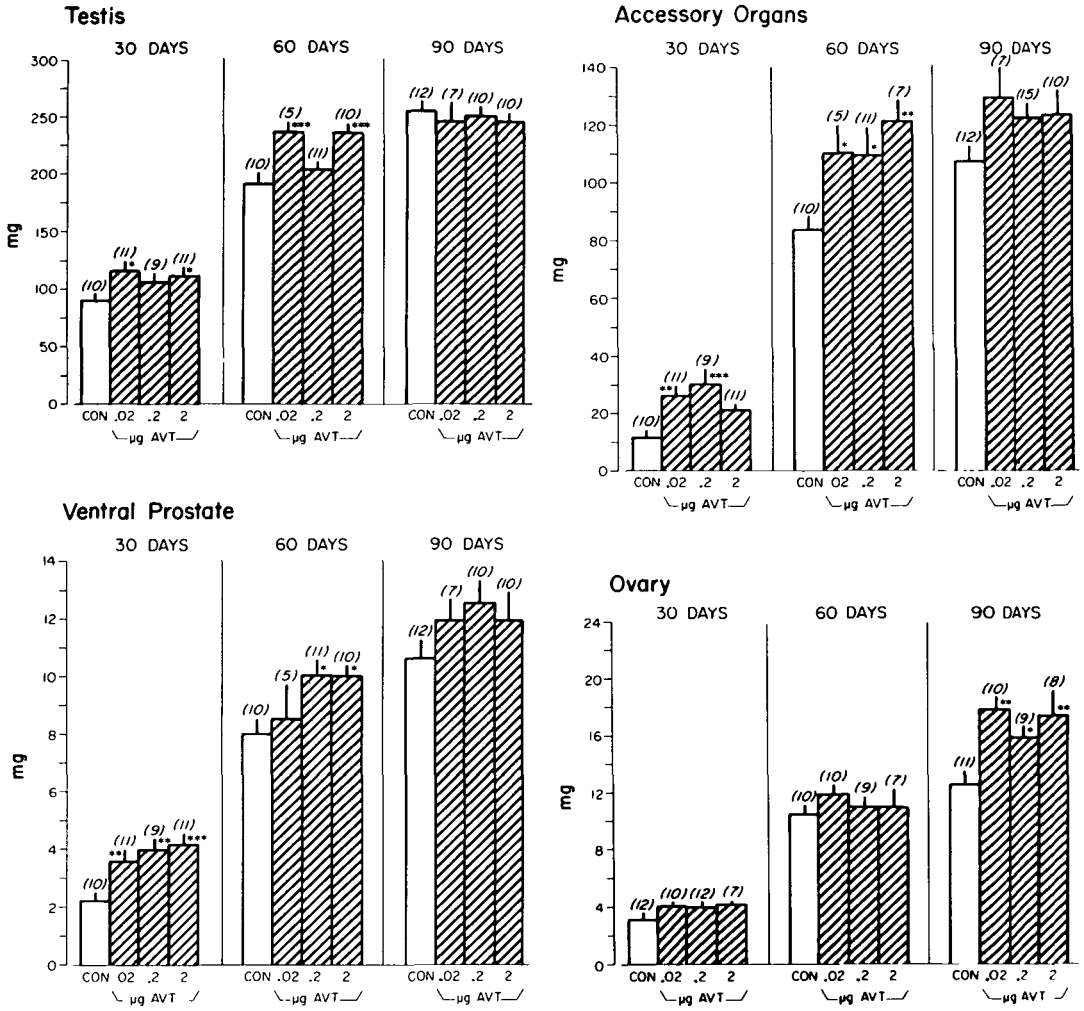

FIG. 1. Reproductive organ weights in Swiss-Webster mice at 30, 60 and 90 days of age. Each mouse received a daily subcutaneous injection of diluent or AVT (0.02, 0.2 or 2 µg) on postnatal days 1 through 5. Numbers in parentheses indicate the number of mice per group. Accessory organs indicates the combined weight of the seminal vesicles and coagulating glands in male mice. Standard errors are indicated. One-way analysis of variance was used to compare AVT groups to respective controls (CON). $*P<0.05$, $**P<0.01$ and $***P<0.001$ vs. CON. Sample size is indicated by numbers in parentheses.

chemically identified in the *pars nervosae* of fetal seals (*Callorhinus ursinus*) and occurs conjointly with AVP and oxytocin in the guinea pig neurohypophysis. Using the isolated amnionic membranes from gravid guinea pigs, Visolyi and Perks (1974) were able to slow or completely reverse fetal-maternal flow of fluid through the amnion using AVT or AVP but not oxytocin. Thus, although AVT can produce effects very early in life, the precise role of AVT during development is unknown.

INFLUENCE OF AVT IN IMMATURE RODENTS

In immature rodents, ovulation induced by pregnant mare's serum (PMS) has been used to demonstrate the suppressive influence of purified fractions isolated from the pineal gland. Three classes of compounds indigenous to the pineal are inhibitory in this model system: 1) indoles, 2) low molecular weight polypeptides and 3) proteins. Of the indoles, melatonin has been the most thoroughly tested for its in-

TABLE 1. *Effect of daily intraperitoneal injection of 2 μg arginine vasotocin (AVT) on pregnancy in the mouse.*

Group	No. mice pregnant	Pregnancy length (days)	Pups/litter surviving	Implantation sites
Saline	8	19.3 ±0.3†	9.4 ±0.7	12.2 ±0.8
AVT	8	20.5* ±0.5	0	12.6 ±0.6

*$P<0.05$
† SEM values

hibitory role on ovulation in the PMS-primed immature rat. Administration of melatonin at the time of the LH surge (52-56 hours post-PMS) has been shown by several investigators to suppress ovulation (Longenecker and Gallo, 1971; Reiter, 1973; Pomerantz and Sorrentino, 1973) and to prevent the rise in serum LH (Reiter and Sorrentino, 1971).

Pavel and Petrescu (1966) demonstrated that either a partially purified pineal extract or synthetic AVT inhibited the stimulatory action of PMS on mice uteri and ovaries. A similar experiment from our laboratory corroborated the results of Pavel and Petrescu. Female 25-day-old Swiss-Webster mice were injected with 10 IU PMS and subsequently received a subcutaneous dose of 2 μg AVT daily for 3 days. On the fourth day, the ovaries and uteri were weighed and the results are illustrated in Table 2. AVT in conjunction with PMS significantly attenuated growth of the ovaries compared to PMS treatment alone. In another experiment, female 25-day-old Swiss-Webster mice were injected at 0 hours with 10 IU PMS and at 48 hours with 1 IU human chorionic gonadotrophin (HCG). The mice received daily subcutaneous injections of 2 μg AVT at 0, 24, 48 and 72 hours and were autopsied 96 hours post-PMS treatment. The ovarian weight of PMS-HCG treated mice was 9.82±0.84 mg vs. 4.79±0.33 mg for PMS-HCG-AVT injected animals ($p < 0.001$). The results of a similar experiment were recently reported by Cheesman and Forsham (1974) in which 2, 10 or 50 μg of AVT failed to inhibit ovulation in PMS-HCG treated mice. The negative results obtained by these investigators with AVT are difficult to interpret. It can only be assumed that the AVT was of recent origin, freshly prepared and injection occurred at the 48 hour timepoint although this was not specifically stated.

Using immature rats, Smith and co-workers (1972) injected 5 to 6 μg AVT at 28 and 53 hours into PMS-primed rats. AVT reduced both the mean number of ova shed and the mean ovarian weight. Recent experiments executed in our laboratory confirmed the decreased ovarian weight after AVT and our radioimmunoassay data indicate a desynchronization in the appearance of the immunoreactive gonadotrophin peaks compared to PMS controls Vaughan, 1975). At least one other pineal peptide, A_1, chromatographically distinct from AVT also inhibited the number of animals ovulating and the total number of ova shed in the same experiment. At present, it is not definitely known if the peptide isolated by Ebels and co-workers (1973) is identical to the A_1 or the A_3 peptides (Vaughan *et al.*, 1974*a*) but comparison of the R_f values under similar chromatographic conditions tend to negate this possibility. It would thus appear that at least

TABLE 2. *Effect of arginine vasotocin on PMS-induced hypertrophy of the ovaries and uterus in immature Swiss-Webster mice.*

Group	No.	BW (g)	Ovaries (mg)	Uterus (mg)
Controls	11	16.2 ±0.2†	4.75 ±.26	27.0 ±1.8
AVT	11	15.8 ±0.4	3.39* ±.21	25.7 ±3.3
PMS	13	16.8 ±0.4	10.38* +0.59	82.7* ±4.4
PMS+AVT	13	16.5 ±0.5	7.14* ±0.48	75.7 ±3.5

*$P<0.001$ vs. related control group
† SEM values

three distinct peptides from the pineal gland are capable of inhibiting this model system.

Three other compounds deserve mention at this time. Gonadotrophin-inhibiting substance (GIS) isolated by Ota and coworkers (1971) is a potent inhibitor of PMS-induced ovulation either when given only at the time of PMS administration or only during the period of the LH surge (Johnson and Vaughan, unpublished data). This high molecular weight compound is isolated from human urine and is thought to be of pineal origin since in the rat, pinealectomy prevents its appearance in the urine. Similarly, Chazov and collaborators (1972) have isolated a pineal fraction dubbed "anovulin" which is described as an albumino-peptide. This fraction inhibited ovulation in PMS-LH treated immature rats. Using the model of PMS-HCG primed immature mice, an inhibitory pineal globular protein with an approximate molecular weight of 100,000 has been described by Cheesman and Forsham (1974).

AVT can also inhibit growth of the gonads or accessory organs when given to immature animals unchallenged with exogenous gonadotrophin (Vaughan *et al.*, 1974*a*; 1974*b*). In Swiss-Webster mice, wild house mice, and hamsters, 2 μg AVT given each day for 3 days significantly retarded growth of gonadal dependent organs in males (Vaughan *et al.*, 1974*a*). Treatment of mice as young as 17 days of age for 3 days with AVT reduced testis weight when measured 2 weeks later (Vaughan *et al.*, 1974*b*). It can thus be seen that while neonatal treatment with AVT from days 1 to 5 resulted in enhanced gonadal and accessory organ weight in later life, treatment outside the critical period as early as 17 days of age has the opposite effect. Whether this effect is due to a direct influence of AVT on the hypothalamo-hypophyseal-gonadal axis either before or after CNS sexual differentiation has yet to be determined.

INFLUENCE OF AVT IN THE MATURE ANIMAL

Compensatory ovarian hypertrophy (COH) with its attendant rise in serum FSH (Benson *et al.*, 1969) following unilateral overiectomy (UO) is a model often used to measure the potency of antigonadotrophic compounds. The hypertrophic response of the *in situ* ovary after UO can be inhibited by two classes of pineal constituents, indoles (Vaughan *et al.*, 1972) and polypeptides (Ebels *et al.*, 1973; Pavel *et al.*, 1973; Bensinger *et al.*, 1973). As this inhibition is uniformly reproducible, this response has been utilized to trace antigonadotrophic fractions through diverse extraction procedures from bovine (Bensinger *et al.*, 1973) and sheep (Ebels et al., 1973) pineal glands. One pineal peptide, arginine vasotocin (AVT), has received particular attention as it is available commercially and relatively pure. Administered as a single intraperitoneal injection at the time of UO, AVT inhibited COH in a dose-related fashion when the mice were necropsied 8 days later (Fig. 2). Similar results using the in-

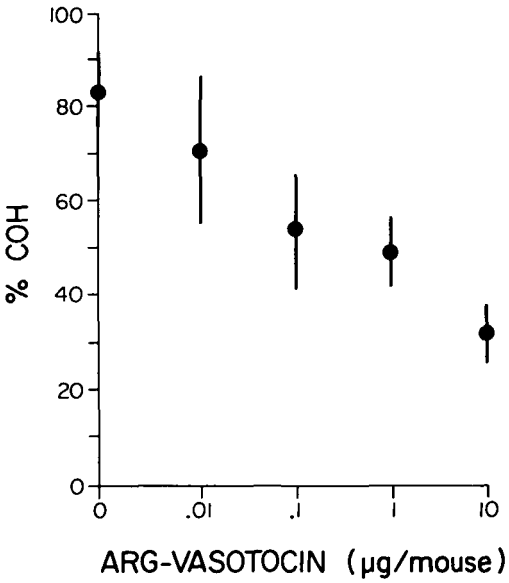

FIG. 2. AVT dose-response inhibition of compensatory ovarian hypertrophy (COH) after unilateral ovariectomy in 6-week-old Swiss-Webster mice. A single injection of vehicle or AVT (0.01, 0.1, 1.0 or 10 μg) was administered at the time of the operation to groups of 10 mice. 100% COH would indicate a doubling in weight of the ovary removed at autopsy compared to the one removed at the time of unilateral ovariectomy. Standard errors are indicated. A one-way analysis of variance indicated that significant inhibition of COH was obtained beginning with the 0.1 μg dose ($P<0.05$).

traperitoneal route of injection have been attained by Pavel *et al.* (1973, 1974) although injection of only 0.5 μU into the third ventricle was as effective as 25 μU administered intraperitoneally. Using an AVT preparation with pressor potency equal to 120 U/mg, we have repeated Pavel's experiment. A single intraventricular (IVT) injection of 2 ng/2 μl completely inhibited COH as measured 7 days later (Control hypertrophy = 34.0 ± 1.4% vs. AVT hypertrophy = −8.1 ± 9.7%; $p < .001$). Intraocular injection of 2 ng was completely ineffective. Thus, the cerebrospinal fluid might be the normal route by which pineal AVT could gain access to the hypothalamus. AVT bioactivity is found in the CSF of the cat and the human (Pavel, 1970; Pavel and Coculescu, 1972). However, possible secretion into the bloodstream first cannot be discounted until more sophisticated methods of monitoring AVT become available.

Reduction of the disulfide bond in the cyclic moiety of either arginine vasopressin (AVP) or oxytocin significantly reduces their capacity to perform their specialized functions (Rudinger and Krejci, 1968). To test if the antigonadotrophic properties of AVT might also be lost after reduction with mercaptoethanol (ME), the following experiment was designed. Female 6-week-old virgin mice were unilaterally ovariectomized and injected with a single intraperitoneal dose of 2 μg of AVT or AVT which had been reduced by mercaptoethanol or dithiothreitol (DTT). Reduction of the AVT was accomplished by incubation with 5 mM ME or 16 mM DTT for 3 hours at 37C. Ten days following unilateral ovariectomy, the remaining ovary in mice treated with reduced AVT was hypertrophied more than those receiving a control injection ($p<.001$) whereas growth of the remaining ovary in mice treated with unreduced AVT was inhibited. Subsequently, several natural and synthetic analogs of AVT were tested on the COH model in both their reduced and unreduced form. The experimental design was similar to the initial experiment with arginine vasopressin (AVP), oxytocin, 4-leucine vasotocin (4-leu-AVT), and lysine vasopressin (LVP) being incubated for 3 hours at 37C with 5 mM ME. Injection of ME or DTT alone into UO control animals had no effect on COH. The results are illustrated in Figure 3. AVT, AVP, 4-leucine vasotocin and LVP in the unreduced form inhibited COH, indicating that the presence of a closed ring plus a basic amino acid in position 8 of the sidechain are necessary requirements for antigonadotrophic activity. When the ring was opened, AVT, AVP and LVP significantly enhanced hypertrophy of the remaining ovary as did the tripeptide Proline-Arginine-Glycinamide (Pro-Arg-Gly (NH_2)) indicating that the specificity for progonadotrophic activity involves a basic sidechain with a basic amino acid in position 8 and an open ring. It cannot be completely discounted that the sidechain tripeptide Pro-Arg-Gly (NH_2) might have broken off from the main molecule during the incubation procedure and thus accounts for the progonadotrophic activity seen in the COH model. The tripeptide was chosen for study because a previous report by de la Lastra and co-workers (1973) indicated progonadotrophic activity in the proestrus-chlorpromazine blocked female rat. It is interesting to note that the terminal tripeptide of LRH contains these same three amino acids, though arranged in a different sequence, Arg-Pro-Glycinamide. It is not known if AVT can competitively inhibit LRH receptor sites at the level of the pituitary or stimulate LH release depending on its molecular configuration, although this is an intriguing possibility which warrants further experimentation. Alternatively, AVT may act at the hypothalamic level to govern LRH synthesis and/or release. Another possibility is that AVT inhibits the gonads directly.

To determine if AVT might compete for receptor sites, at the pituitary level and thus inhibit gonadotrophin release, the following experiment was designed. Pituitaries of castrated adult female Sprague-Dawley rats pretreated 3 days previously with 50 μg estradiol benzoate and 25 mg progesterone were quickly removed, hemisected and weighed. Following a 1-hour preincubation, the old Krebs-Ringer bicarbonate (KRB) medium was

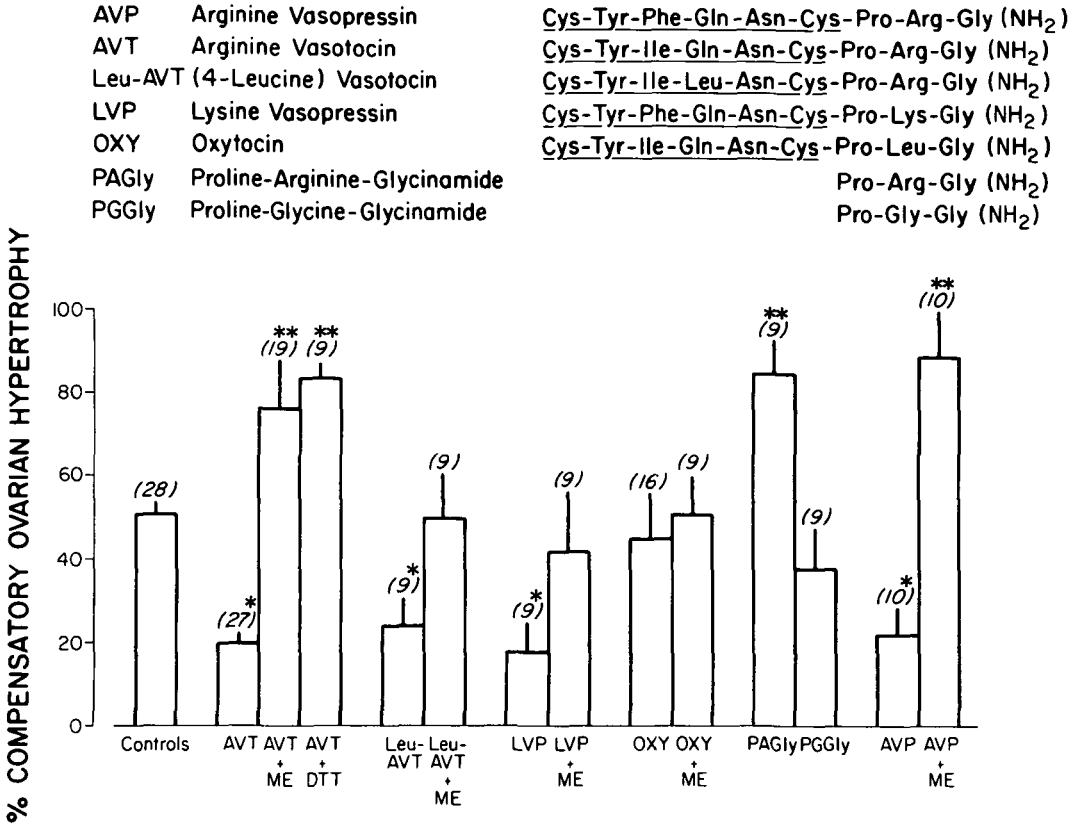

FIG. 3. Structure-activity relationship for peptide effects on COH in the mouse. Each compound was given as a single 2 μg intraperitoneal dose at the time of unilateral ovariectomy. Controls received either diluent alone or mercaptoethanol (ME) or dithiothreitol (DTT). $*P<0.05$, $**P<0.01$ compared to controls. Standard errors are indicated and sample size is indicated by the numbers in parentheses.

discarded and 2 ml of fresh culture medium added. In Experiment 1, LRH (Beckman, 10 ng/ml) or LRH + AVT (Schwarz-Mann, 2.5 μg/ml) was added to vials containing corresponding pituitary halves and incubated for 5 hours at 37C in a Dubnoff shaker aerated with 95% O_2 and 5% CO_2. At the end of the experiment, the incubation fluid was drawn off and analyzed by radioimmunoassay for LH and prolactin. Results were analyzed by the paired t-test. AVT significantly augmented the accumulation of LH in the culture medium of pituitaries also receiving LRH (Fig. 4). Using the same protocol as described above, corresponding pituitary halves were preincubated for 1 hour with AVT or with KRB medium only. The preincubation fluid was discarded and fresh medium containing only LRH was added to both halves. Prior incubation with AVT was not sufficient to cause the elevated LH response as observed when AVT was present during the whole incubation period (Fig. 4). A third experiment demonstrated the response of pituitaries to LRH in comparison to control hemipituitaries incubated in KRB alone (Fig. 4). These results indicate that AVT was not antigonadotrophic but rather progonadotrophic at the level of the pituitary. Another hypothesis can be cautiously proffered for this seemingly progonadotrophic response. Incubation for 5 hours at 37C under aerobic conditions could possibly cause modification of the structure of the AVT molecule thereby exposing portions of the molecule with progonadotrophic activity. This could involve opening of the ring simulating the results in the COH model

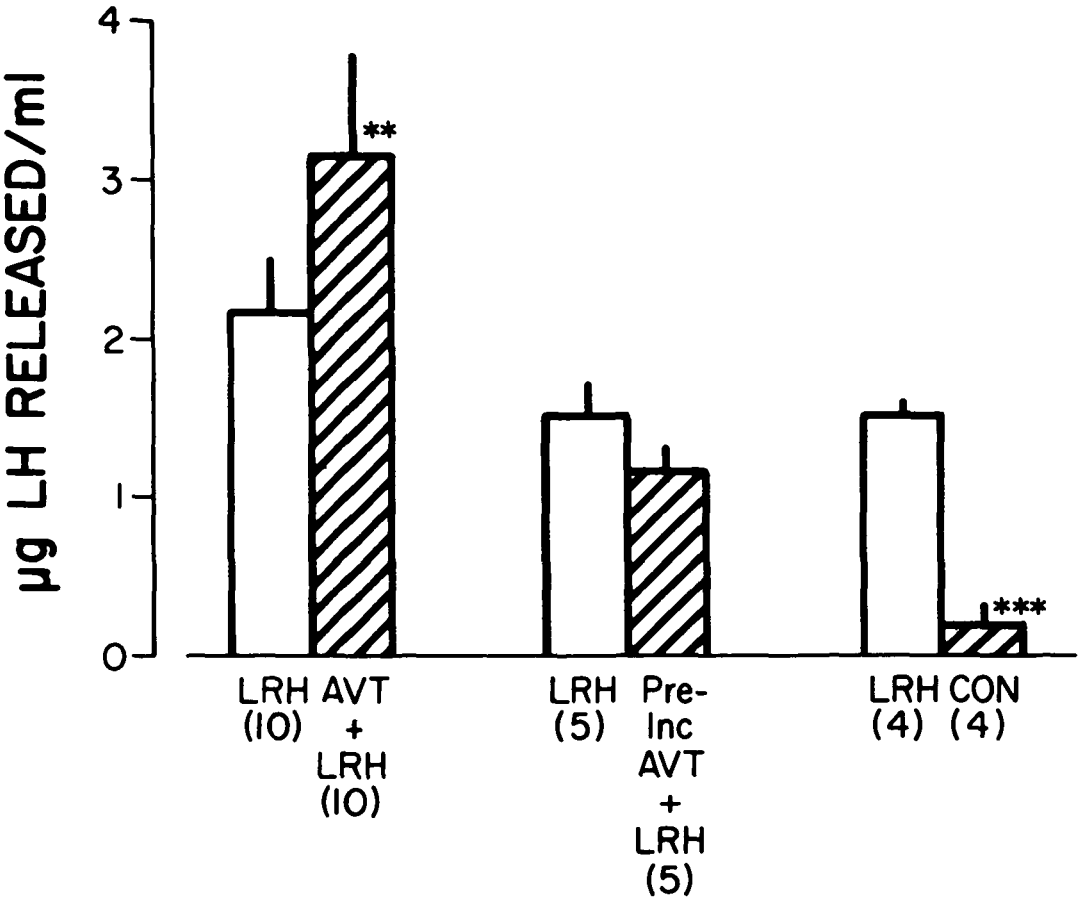

FIG. 4. Enhancement of pituitary LH release by LRH in the presence of AVT. Numbers in parentheses indicate the number of incubated hemipituitaries from adult castrated estrogen-progesterone primed female rats. Preincubation of the hemipituitaries with AVT for one hr did not affect the subsequent response to LRH. Standard errors are indicated. $^{**}P<0.01$, $^{***}P<0.001$ by paired t-test vs. respective LRH group. Sample size is indicated by the numbers in parentheses.

after incubation with mercaptoethanol or dithiothreitol. Other possible explanations include liberation of the terminal tripeptide, dimerization of molecules, or progonadotrophic contaminants of the synthetic AVT. Further experiments are needed to establish the dose-response nature and the structure-activity relationships for the increased release of gonadotrophins by AVT and other natural and synthetic analogs. Furthermore, there is evidence that the terminal tripeptide of both arginine vasopressin and AVT (Pro-Arg-Gly-NH_2) can potentiate the activity of FSH and LH on rat ovaries (de la Lastra, 1973). Thus, the effects of pineal peptides and related compounds must be investigated at all levels of the hypothalamo-hypophyseal-gonadal axis.

SUMMARY

The data presented here in conjunction with those in the literature indicate that exogenously administered AVT could possibly act at all levels of the hypothalamo-hypophyseal-gonadal axis. The observation that structural modification of the AVT and other similar nonapeptides by disulfide bond splitting can cause opposite effects on the reproductive system is intriguing. Further experiments, however, are necessary to determine the physiological importance of these structure-activity

relationships of endogenously secreted AVT. Whether this compound is secreted directly into the CSF as indicated by Pavel or circulates via the bloodstream to act directly on the gonads awaits a sensitive micro-determination of AVT in the blood.

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