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Effect of Hypothalamic Implantation of Testosterone on Photostimulated Testicular Growth in Japanese Quail (*Coturnix coturnix japonica*)

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Summary. Testosterone pellets implanted in the third ventricle inhibited completely photostimulated testicular growth in the Japanese quail (*Coturnix coturnix*). Crowing was induced by intraventricular testosterone in quail with undeveloped testes. Intraventricular cholesterol pellets did not inhibit testicular growth. In these birds crowing occurred normally. When the testosterone implants were located in the nucleus tuberis, photoperiodically induced testicular growth was also prevented. Testosterone implants in several other nuclei did not impair testicular growth. These observations suggest that testosterone is involved in the hypothalamo-gonadal negative feedback mechanism in this species, and the nucleus tuberis is the site of tetsosterone action.

Key words: Tubero-infundibular region — *Coturnix coturnix* — Testosterone sensitivity — Photostimulated testicular growth.

Introduction

Earlier lesion experiments suggested that the aldehyde-fuchsin-positive neurosecretory system is involved in light-induced gonadal growth in the domestic duck (Assenmacher, 1958; Gogan *et al.*, 1963). Several histological observations on this system of passerine birds and Japanese quail (Oksche *et al.*, 1959; Farner *et al.*, 1962; Ishii *et al.*, 1962c; Konishi, 1967) appeared to be consistent with this view. However, recent lesion experiments have demonstrated no relationship between the AF-positive neurosecretory system and gonadal growth, but demonstrated rather that the tubero-infundibular system plays the important role in gonadal growth in the White-crowned Sparrow (*Zonotrichia leucophrys gambelii*), cockerel, and Japanese quail (Wilson, 1967; Graber *et al.*, 1967; Stetson, 1969a, b; Sharp and Follett, 1969). Furthermore, by testosterone implantation experiments, Kordon and Gogan (1964) found that a testosterone implant in the ventromedial nucleus inhibits photoperiodically induced gonadal growth. In addition to this androgen sensitive site, Gogan (1968) found the anterior hypothalamus to be another site sensitive to androgen. More recently, Wilson (1970) found that the tubero-infundibular region is the site of inhibitory action of androgen on photostimulated testicular growth in the Tree Sparrow (*Spizella arborea*). Thus the

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sensitive site(s) to androgen has not yet clearly been determined in the avian hypothalamus.

In the present experiments, testosterone propionate was implanted bilaterally in the hypothalamus of the Japanese quail in order to find the site(s) regulating gonadotropin secretion.

Material and Methods

Male Japanese quail (*Coturnix coturnix*) were obtained from a commercial source at the age of 20 or 21 days and kept under a short daily photoperiod of 8L 16D (lights on from 800 to 1600), which does not induce gonadal growth (Konishi *et al.*, 1965; Follett and Farner, 1966), for about two weeks before the start of the experiment. Implantation of testosterone was undertaken at the age of 32–38 days in birds under a short daily photoperiod. On the day of implantation, the birds were transferred to a long daily photoperiod of 16L 8D (lights on from 800 to 2400) in all experiments, except the second in which a daily photoperiod of 20L 4D was employed. They were kept under these long daily photoperiods for ten days to stimulate testicular growth.

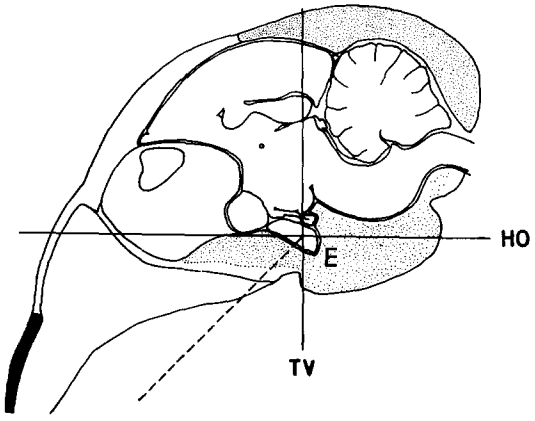

Fig. 1. A paramedial sagittal section through the skull of an adult male quail. The broken line represents the reference plane of the coordinate system, which goes through the center of the earplug and the posterior end of the mouth. *E* center of the earplug; *HO* horizontal zero plane; *TV* transverse zero plane

Crystalline testosterone propionate (TP) and cholesterol (Ch) were mixed in a small amount of ether in certain ratios; the ether was then evaporated. This dried mixture was tamped into the end portion of a stainless steel tube for implantation. Ratios of TP and Ch and the tube sizes are given with the description of each experiment. Cholesterol only was tamped similarly in the tube for control implants.

The stereotaxic coordinates were determined in order to place the tip of the tube into the desired position in the brain. Preliminarily a lateral X-ray exposure of the quail fixed to the stereotaxic apparatus was taken. On the film, the horizontal zero plane was made by drawing a line at 45° angle to the line which went through the center of the earplugs and the posterior end of the mouth. The sagittal zero plane was parallel to the ventricle and the transverse zero plane was at right angles to the first two planes through the center of the earplugs (Fig. 1). For the implantation of the pellets, a quail was anesthetized with Nembutal and fixed to the stereotaxic apparatus. After making a sagittal cut in the dorsal cranial skin, an opening with a diameter of about 2 mm was made with a dental drill at the position on the skull, which seemed to be directly over the desired position in the brain. Then the tip of the tube attached to the stereotaxic apparatus was lowered to the opening of the skull, and

antero-posterior and lateral X-ray exposures were made. The position of the tube was adjusted stereotaxically on the basis of the coordinate mentioned above so that the tip of the tube was placed directly over the desired position in the brain. Then the tube inserted into the brain. In the first and second experiments, the mixture (TP:Ch = 1:3 by weight) in the tube (20-gauge, inside diameter 0.7 mm) was ejected out as a pellet by a closely fitting stylet either into the third ventricle near the median eminence or bilaterally in the hypothalamic regions 1 mm lateral to the third ventricle. In the third and fourth experiments, the tube (28-gauge, inside diameter 0.2 mm) containing the mixture (TP:Ch = 1:1) was fixed to the skull with dental cement so that the tip of the tube was placed either in the adenohypophysis or bilaterally in the hypothalamic regions 0.3 to 0.5 mm lateral to the third ventricle. After the implantation, X-ray photographs were taken again to provide a record of the implantation sites.

To ascertain possible leakage of testosterone from the brain into the systemic circulation, the size of the cloacal gland, whose development is dependent on androgen (Sachs, 1967, 1969), was measured and compared with that of quail receiving testosterone pellets subcutaneously.

Body weight, cloacal gland, and crowing of each bird were recorded beginning from a week before the start of the experiment to autopsy. The maximum and minimum diameters of cloacal gland were measured by a scale and the development of the cloacal gland was expressed in term of relative area (mm^2/100 g body weight) according to Sachs (1967). Crowing was checked for 30 minutes in the morning, because quail crow frequently a few hours soon after the lights are turned on than thereafter (Arimatsu, personal communication).

After photostimulation for ten days, experimental birds were killed by decapitation. The hypothalamic region was dissected out after the tubes were removed with the skull from the brain. The hypothalamus was fixed in Bouin's solution, embedded in paraffin, sectioned serially at 10 μ, and stained with paraldehyde fuchsin and toluidine blue O (Asai *et al.*, 1969). The testes, adrenals, thyroids, and adenohypophysis were weighed at autopsy and fixed in Bouin's solution. The paraffin-embedded tissues were cut at 6 μ and stained with Delafield's hematoxylin and eosin.

The sites of the implants in the hypothalamus were determined by reference to the atlas of the domestic fowl described by van Tienhoven and Juhász (1962) and their terminology was principally used in the present study.

Results

Experiment 1. Implantation of Testosterone Pellet (TP:Ch = 1:3) in the Third Ventricle

When testosterone pellets were implanted in the third ventricle, the testes of none of the eight quail developed even under a long daily photoperiod (Table 1). The weight of testes was almost the same as that of the nonphotostimulated birds. The seminiferous tubules contained only spermatogonia (Fig. 2c). This state of the testes of the testosterone-implanted birds was similar to that of the nonphotostimulated control birds (Fig. 2b). The state of testes of six birds receiving testosterone subcutaneously were the same as those of the intact (Fig. 2a) and the cholesterol-implanted birds (Fig. 2d).

It has been demonstrated that the cloacal gland protrudes from the body surface in response to a long daily photoperiod or to androgen (Sachs, 1967, 1969). This phenomenon also occurred in the present experiments (Fig. 3a). Birds with testosterone pellets in the third ventricle showed no development of the cloacal gland, whereas cholesterol-implanted birds did (Fig. 3b). Birds with subcutaneous implants of testosterone had large cloacal glands (Fig. 3b). The adenohypophysis of the birds implanted with testosterone in the third ventricle weighed significantly less than that of cholesterol-implanted control birds (Table 1).

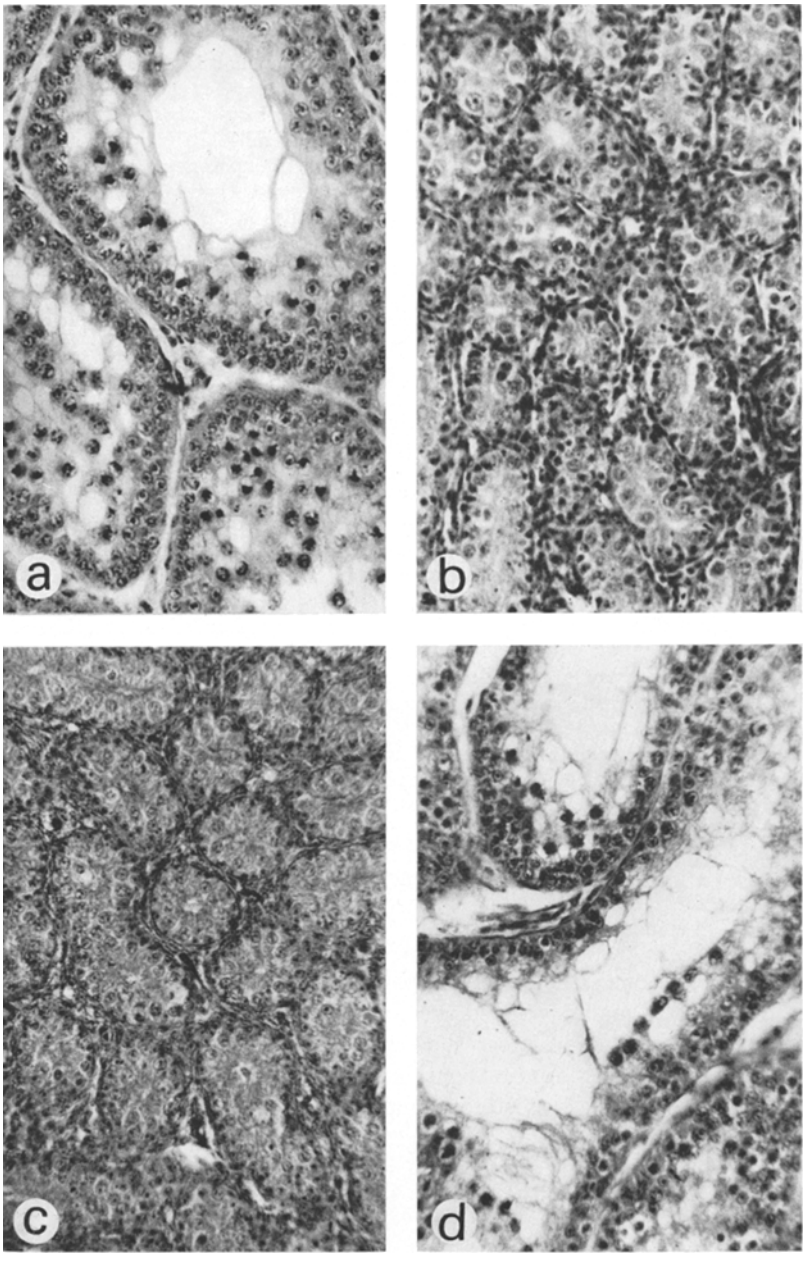

Fig. 2a–d. Micrographs of the testes. a, intact photostimulated bird; b, intact nonphotostimulated bird; c, photostimulated bird with an intraventricular testosterone implant; d, photo stimulated bird with an intraventricular cholesterol implant. ×240

Table 1. *Effect of implantation of the testosterone pellet (TP:Ch = 1:3) in the hypothalamic regions on photostimulated testicular growth and the weights of other endocrine organs*

Site of implantation	Number of quail	Body weight (g)	Organ weight (mg/100 g BW)				% increase of cloacal protrusion
			Testes	Adrenals	Thyroids	Adenohypophysis	
Nonphotostimulated control	8	91 ± 3.8[a]	31 ± 8.3	7.95 ± 0.61	6.78 ± 0.70	1.01 ± 0.03	−1.3 ± 4.6
Photostimulated control	8	94 ± 2.6	462 ± 63.3	7.71 ± 0.55	6.38 ± 0.45	1.42 ± 0.05	205.0 ± 20.6
Third ventricle (Ch control)	6	92 ± 1.7	375 ± 38.0	8.22 ± 0.43	6.99 ± 0.87	1.66 ± 0.12	214.0 ± 36.4
Subcutaneous	6	90 ± 2.8	597 ± 82.7	9.54 ± 1.05	8.11 ± 1.24	1.59 ± 0.08	283.9 ± 50.7
Third ventricle	8	93 ± 1.9	32 ± 7.2[b]	7.72 ± 0.47	7.54 ± 1.45	1.15 ± 0.03[b]	5.9 ± 8.1[b]
Lateral division of nucleus paraventricularis magnocellularis[c]	3	98 ± 1.5	772 ± 44.5	12.21 ± 1.28[b]	6.42 ± 0.89	1.78 ± 0.25	339.0 ± 46.0
Nucleus hypothalamicus lateralis[c]	5	97 ± 2.4	586 ± 25.8	10.01 ± 1.20	6.93 ± 0.94	2.01 ± 0.27	318.7 ± 33.3
Nucleus ovoidalis[c]	4	94 ± 2.7	713 ± 243.5	10.26 ± 1.51	6.81 ± 1.46	1.94 ± 1.68	258.6 ± 26.9
Outside of hypothalamus[c]	2	100 ± 0	460 ± 102.5	8.62 ± 0.08	6.31 ± 0.39	1.87 ± 0.19	269.2 ± 29.5

[a] Mean ± standard error.
[b] Highly significant ($p < 0.01$) compared with Ch control group.
[c] Photoperiod of 20L 4D was employed in these cases, and 16L 8D in other cases.

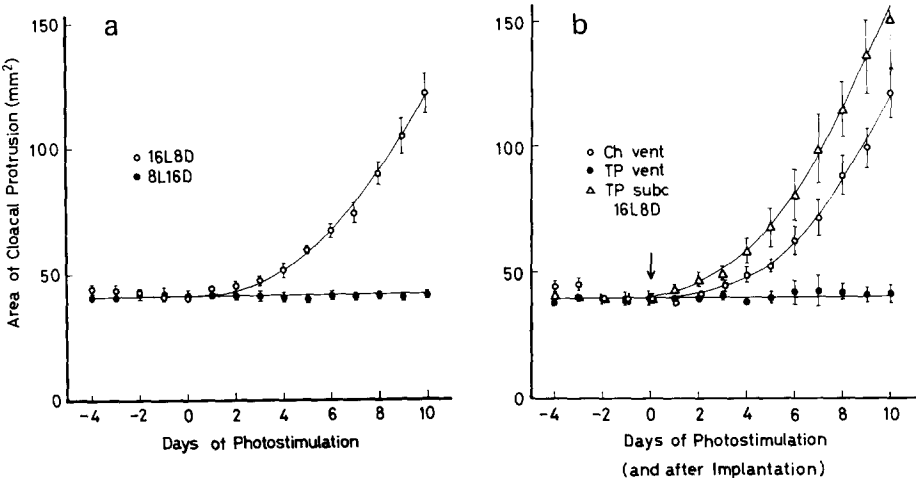

Fig. 3a and b. Development of the cloacal protrusion due to enlargement of the cloacal gland. a, intact groups; b, experimental groups. The arrow indicates the day of implantation. *vent* ventricular; *subc.* subcutaneous

Table 2. *Effect of implantation of the testosterone pellet (TP:Ch = 1:3) in the third ventricle on crowing*

Group	Number of quail crowing	Days between the implantation and first crowing
Nonphotostimulated control	0/8	—
Photostimulated control	7/8	8.9 ± 0.3[a]
Ch ventricular implant, photostimulated	4/6	8.3 ± 1.0
TP subcutaneous implant, photostimulated	5/6	5.8 ± 1.0
TP ventricular implant, photostimulated	7/8	4.0 ± 0.5

[a] Mean $\pm$ standard error.

This may be due to the lack of the development of the gonadotrophs, because it has been shown that a weight increase of the adenohypophysis in the photostimulated quail is due to an increase in number of gonadotrophs (Tixier-Vidal *et al.*, 1968). The adrenals and thyroids showed no significant change in size following implantation of testosterone pellet in the third ventricle.

Except for a single case, quail with intraventricular testosterone pellets crowed like mature birds during the earlier days (2nd to 6th) of photostimulation. The intact birds and the cholesterol-implanted birds crowed during the later days (6th to 10th) of photostimulation (Table 2). In the latter case, the cloacal gland was considerably enlarged (Fig. 3b), suggesting androgen secretion. Therefore, crowing seems to have been induced by endogenous androgen in this case.

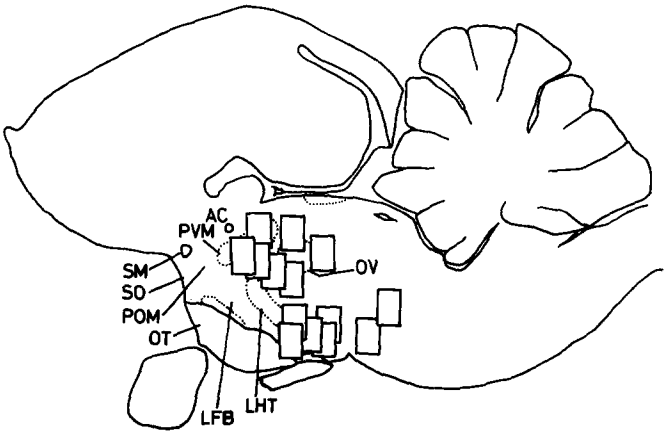

Fig. 4. The sites of testosterone pellets in the hypothalamic regions 1 mm lateral to the third ventricle. *AC* anterior commissure; *LFB* lateral forebrain bundle; *LHT* n. hypothalamicus lateralis; *OT* optic tract; *OV* n. ovoidalis; *POM* n. preopticus medialis; *PVM* n. paraventricularis magnocellularis; *SM* tr. septomesencephalicus; *SO* n. supraopticus

Experiment 2. Bilateral Implantation of Testosterone Pellet (TP:Ch = 1:3) in the Hypothalamic Regions 1 mm Lateral to the Third Ventricle

In this experiment, no implants inhibited photo-induced testicular growth (20L 4D) (Table 1). Three implants were placed in the lateral division of the nucleus paraventricularis magnocellularis, five implants in the nucleus hypothalamicus lateralis, four implants in other hypothalamic regions near the nucleus ovoidalis, and two implants were placed outside of the hypothalamus near the nucleus ruber (Fig. 4). Weight of the testes and the development of the cloacal gland of these birds tended to be more advanced than that of the intact photostimulated (16L 8D) birds in Experiment 1 (Table 1). A photoperiod of 20L 4D was not undertaken for intact controls. The increase in testicular weight in this experiment, compared with the intact photostimulated birds in Experiment 1, could be due to longer photoperiod of 20L 4D. The adrenals and thyroids were statistically not different in size, compared with the intact photostimulated birds, except that the weights of the adrenals increased in birds with testosterone pellets implanted in the lateral division of the nucleus paraventricularis magnocellularis. The reason for this increase is unknown.

Experiment 3. Implantation of Testosterone (TP:Ch = 1:1) in the Adenohypophysis

There were four birds with testosterone implants in the adenohypophysis and one with cholesterol implant. In no case was there any inhibition of testicular growth (Table 3). The development of the cloacal gland and crowing were similar to that seen in intact photostimulated birds.

Table 3. *Effect of implantation of the testosterone (TP:Ch = 1:1) in the adenohypophysis and the hypothalamic regions on photostimulated testicular growth and the weights of other endocrine organs*

Site of implantation	Number of quail	Body weight (g)	Organ weight (mg/100 g BW)				% increase of cloacal protrusion
			Testes	Adrenals	Thyroids	Adenohypophysis	
Adenohypophysis (control)	1	82	368	10.65	13.93	1.79	223.3
Adenohypophysis	4	89 ± 2.3[a]	371 ± 62.2	9.17 ± 0.97	5.85 ± 1.15	1.43 ± 0.06	176.9 ± 26.2
Control (Ch)	10	87 ± 2.7	366 ± 38.6	9.57 ± 0.75	7.18 ± 0.81	1.50 ± 0.05	216.0 ± 28.2
N. supraopticus	4	93 ± 2.3	310 ± 36.1	9.83 ± 1.43	7.02 ± 1.02	1.31 ± 0.18	136.6 ± 33.6
N. preopticus medialis	3	94 ± 2.6	297 ± 11.1	8.04 ± 0.65	4.98 ± 0.76	1.19 ± 0.24	139.0 ± 35.9
N. paraventricularis magnocellularis–n. hypothalamicus anterior medialis	3	87 ± 3.9	449 ± 52.0	8.03 ± 0.70	8.65 ± 2.28	1.80 ± 0.08	202.1 ± 57.7
N. hypothalamicus posterior medialis	4	95 ± 5.6	310 ± 24.3	9.19 ± 0.94	7.22 ± 0.51	1.51 ± 0.04	138.8 ± 39.1
N. tuberis	7	91 ± 4.0	56 ± 12.4[b]	8.68 ± 1.15	6.57 ± 0.53	1.15 ± 0.10[b]	−5.7 ± 5.2[b]
Posterior portion of n. tuberis	3	92 ± 3.5	146 ± 15.0[c]	10.83 ± 1.46	7.71 ± 0.76	1.23 ± 0.14	17.9 ± 14.6[b]
Periphery of n. tuberis	5	91 ± 2.2	274 ± 37.7	10.24 ± 0.71	7.24 ± 1.32	1.30 ± 0.02	78.0 ± 22.5[b]
N. mammillaris medialis	2	86 ± 0	352 ± 61.5	8.13 ± 0.64	6.34 ± 1.43	1.16 ± 0.05[b]	244.0 ± 61.6
Stratum cellulare internus-stratum cellulare externum	5	103 ± 1.3[b]	412 ± 67.2	8.39 ± 0.63	5.30 ± 0.40	1.57 ± 0.10	223.9 ± 29.5

[a] Mean ± standard error.
[b] Highly significant ($p < 0.01$) compared with Ch control group.
[c] Significant ($0.01 < p < 0.02$) compared with Ch control group.

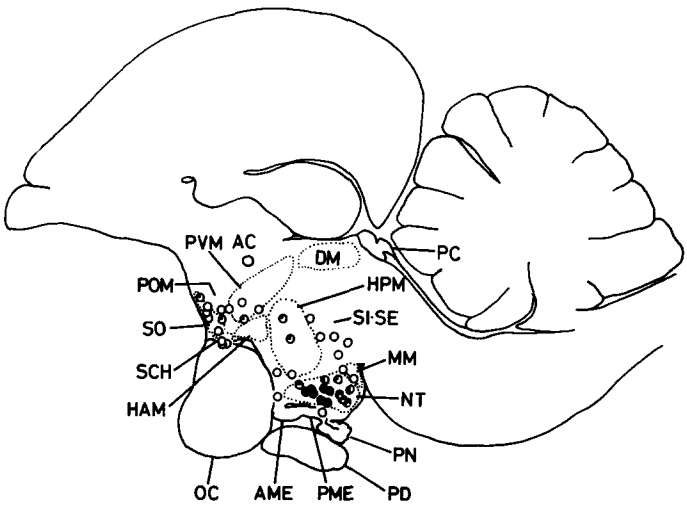

Fig. 5. The sites of testosterone implants in the hypothalamic regions 0.3–0.5 mm lateral to the third ventricle. The sites and the nuclei are projected on the mid-sagittal plane. A right half of a circle represents the degree of inhibition on testicular growth: ○ no inhibition: ◑ partial inhibition (testes 100–200 mg); ● complete inhibition (testes < 100 mg). A left half represents the degree of inhibition on development of cloacal gland: ○ no inhibition; ◖ partial inhibition; ● complete inhibition. *AC* anterior commissure; *AME* anterior median eminence; *DM* n. dorsomedialis; *HAM*, n. hypothalamicus anterior medialis; *HPM* n. hypothalamicus posterior medialis; *MM* n. mammillaris medialis; *NT* n. tuberis; *OC* optic chiasma; *PC* posterior commissure; *PD* pars distalis; *PME* posterior median eminence; *PN* pars nervosa; *POM* n. preopticus medialis; *PVM* n. paraventricularis magnocellularis; *SCH* n. suprachiasmaticus; *SE* stratum cellulare externum; *SI* stratum cellulare internum; *SO* n. supraopticus

Experiment 4. Bilateral Implantation of Testosterone (TP:Ch = 1:1) in the Hypothalamic Regions 0.3 to 0.5 mm Lateral to the Third Ventricle

In this experiment, testosterone implants were placed more medially than in the Experiment 2. Of the 41 bilateral implants, seven in the nucleus tuberis distinctly inhibited testicular development (Table 3, Figs. 5 and 6e). These birds showed neither development of the cloacal gland nor crowing. Three implants in the posterior portion of the nucleus tuberis resulted in reduced testicular development, and the cloacal gland showed little or no enlargement (Table 3). The implants at the periphery of the nucleus tuberis (Figs. 5 and 6d) gave variable effects on the testicular development and the cloacal gland (Table 3). Implants in the other hypothalamic nuclei (Figs. 5 and 6a–c, f) did not impair testicular development. Sites of the implants and their results are shown in Table 3 and Figure 5.

Cholesterol implants in the various sites (preoptic area, nucleus tuberis, nucleus hypothalamicus anterior medialis, and posterior hypothalamus) did not inhibit the development of the testes and of the cloacal gland (Table 3) induced by long daily photoperiods. Crowing was also normal.

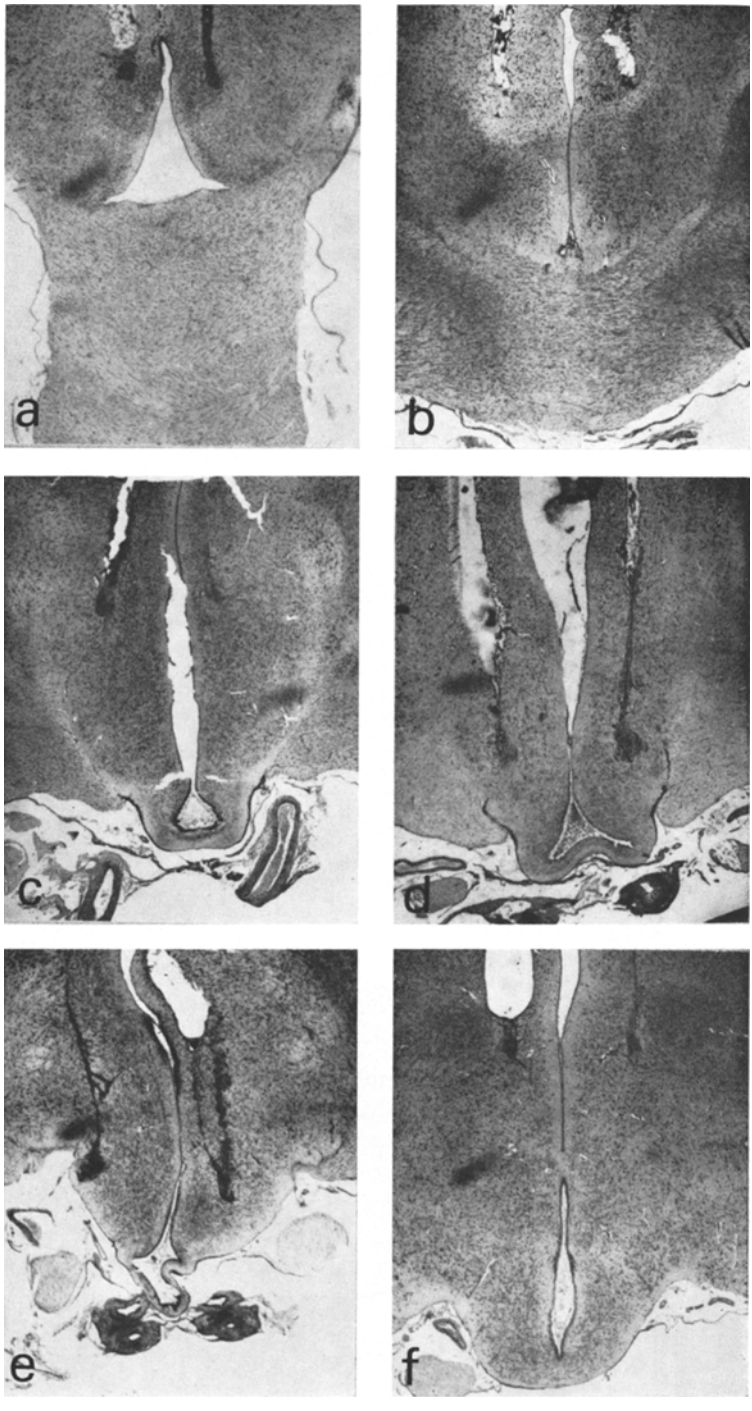

Fig. 6a–f. Micrographs of the hypothalamus of the quail bearing testosterone implants. a, implant in the preoptic area; b, implant in the nucleus paraventricularis magnocellularis; c, implant in the nucleus hypothalamicus posterior medialis; d, implant in the periphery of the nucleus tuberis; e, implant in the nucleus tuberis; f, implant in the posterior hypothalamus. $\times 16$

The adrenals and thyroids showed no significant change in size as a result of implantation of testosterone in any hypothalamic site.

Discussion

When testosterone implants were placed in the third ventricle of quail, both testicular growth and enlargement of the cloacal gland were completely inhibited. This indicates that the testosterone implants inhibited gonadotropin secretion. There are at least three possible explanations for the inhibition: (1) Diffusion from the third ventricle to the sensitive site(s) in the brain may inhibit gonadotropin-releasing factor (GRF) production. (2) The neurons that produce GRF may extend their fibers to the ventricle in addition to the median eminence. The endings adjacent to the ventricle could receive and convey information concerning concentration of androgen in the ventricle to the perikarya, thus resulting in the inhibition of the formation of GRL and/or its release into the portal vessels. (3) The ependymal cells of the median eminence absorb GRF from the ventricular fluid and send it to the portal vessels through their processes (for review see Kobayashi *et al.*, 1970). The presence of GRF in the ventricular fluid has been demonstrated by the fact that the adenohypophysis implanted in the third ventricle of rats showed hypertrophied basophil cells (Szenthágothai *et al.*, 1968). It is possible, therefore, that testosterone implanted in the third ventricle may inhibit GRF absorption by the ependymal cells, resulting in inhibition of gonadotropin release. There seems to be no possibility that testosterone implanted in the third ventricle affected the hypothalamic neurons producing GRF *via* the systemic circulation, because birds with testosterone implants in the third ventricle did not show any enlargement of the cloacal gland. Testosterone appears to be unable to inhibit directly gonadotropin release from the adenohypophysis, because testosterone implants in the adenohypophysis gave no effect on testicular growth.

In the present experiments, testosterone implants in the nucleus tuberis inhibited testicular growth. This finding is in agreement with the results obtained with lesion experiments by Wilson (1967) and Stetson (1969) in the White-crowned Sparrow, by Graber *et al.* (1967) in the cockerel, and Sharp and Follett (1969) and Stetson (1969b) in the Japanese quail, and also with the results obtained by androgen implantation in mammals by Davidson and Sawyer (1961) and Lisk (1962) and in the Tree Sparrow by Wilson (1970). In the domestic duck, however, Gogan (1968) reported that the ventromedial nucleus is one of the androgen-sensitive sites. She is of opinion that this nucleus corresponds to the site sensitive to sex steroids in mammals (Lisk, 1960, 1962; Ramirez *et al.*, 1964) and to the site controlling LH secretion in the cockerel (Graber *et al.*, 1967). Considering all of these observations, it is likely that the ventromedial nucleus defined by Gogan corresponds to the nucleus tuberis of other birds or includes at least a part of the nucleus tuberis. In any case, at least the ventral sensitive sites in the duck, Tree Sparrow, and quail appear to be identical. The nucleus tuberis neurons of the quail could regulate the production of GRF by other neurons and its release, or they could themselves be the source of GRF. Since Follett (1970) has demonstrated *in vitro* that the basal hypothalamus, including the nucleus tuberis, of the quail shows gonadotropin-releasing activity, it is possible that the nucleus

tuberis itself synthetizes GRF. Accordingly, inhibition of gonadotropin release by testosterone implantation in the nucleus tuberis is due to inhibition of GRF production by testosterone in the neurons of the nucleus tuberis.

The cells of the nucleus paraventricularis magnocellularis, nucleus hypothalamicus posterior medialis, nucleus hypothalamicus lateralis, the preoptic area, and the posterior hypothalamus do not seem to be involved in regulation of gonadotropin secretion, because testosterone implanted in these nuclei was not effective in inhibition of testicular growth.

Gogan (1968) reported that androgen implanted in the anterior hypothalamus partially inhibited testicular growth in the domestic duck. In the present experiments, when testosterone was implanted in the preoptic area, the nucleus hypothalamicus anterior medialis, and the nucleus hypothalamicus posterior medialis, five birds showed delayed development of the cloacal gland, indicating partial inhibition of androgen secretion although the testicular weights of these birds were not inhibited. At the present time, it is not known whether these regions are entirely independent of the nucleus tuberis or whether they have some fiber connection with this nucleus.

In the birds implanted with testosterone in the third ventricle, crowing was induced earlier than it occurred in intact birds under a long daily photoperiod. This shows that testosterone in the third ventricle stimulated directly the neural elements that control the crowing, but not through the systemic blood flow. At present, the site(s) of action of testosterone in inducing crowing is not known.

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