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Relation of Mode of Administration of Testosterone to Evocation of Male Sex Behavior in Frogs[1]

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In *Rana pipiens,* mating behavior could be induced readily in intact males by several pituitary implantations, but never in castrates. Systemic testosterone injection (1 mg daily), with or without pituitary implantation, failed to restore mating behavior in castrated frogs. On the other hand, intracranial implantation of testosterone (approximately 60-μg pellets in which testosterone is mixed with cholesterol 1:1) in castrates evoked mating behavior, including mating calls and clasping. The most effective implantation site was the rostral part of the preoptic nucleus. Thus, the rostral part of the preoptic nucleus is the androgen-sensitive site which governs sexual behavior in this species. The relative ineffectiveness of systemic injection of testosterone is discussed.

There is a wealth of published information concerning the stimulatory relationship between testicular androgenic steroids and male reproductive behavior in vertebrates (see reviews in Young, 1961; Davidson, 1972). In Amphibia, however, it has been relatively difficult or often impossible under experimental conditions to evoke male sexual behavior by treatment of adult males with androgens, usually testosterone (Wolf, 1939; Blair, 1946; Schmidt, 1966; Palka and Gorbman, 1973; Obert, 1973). Injection of pituitary hormones, on the other hand, consistently and rapidly evokes male behavior in frogs (Schmidt, 1966; Palka and Gorbman, 1973). Since testosterone in frogs is ineffective in stimulating male behavior, and since the frog testis is known to produce testosterone (see for review: Ozon, 1972), it appears paradoxical that pituitary injections evoke male behavior in intact frogs, but are ineffectual in castrated frogs (Palka and Gorbman, 1973).

In an attempt to resolve this apparent paradox, the requirement of the testis for male frog behavior in a species in which exogenous testicular hormone does not evoke male behavior, we have performed the experiments described here. In these experiments, we have located a limited hypothalamic area where direct implantation of testosterone-containing pellets evokes male sexual-calling behavior, and we have compared the relative sensitivity of a direct application of testosterone to this area with systemic injection of large doses of testosterone.

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METHODS

Animals. Male leopard frogs (*Rana pipiens*) were purchased from Bay Biological Supply, Ltd. (Port Credit, Ontario) or NASCO Steinhilber (Fort Atkinson, Wisconsin) and were kept unfed in large plastic basins with tap water running slowly over the bottom. The basins were in a room in which the photoperiod (12L/12D) and temperature (16°C) were regulated. Frogs were force-fed beef liver once weekly during the experiments.

For use in the experiment, frogs were transferred to transparent covered plastic boxes measuring approximately 31 × 16 × 9 cm, each containing two or three frogs. The boxes were kept under the same conditions mentioned before, except for the actual times of observation of sexual behavior when a female was added to the group.

Observation of sexual behavior. Sexual behavior of the frogs was observed during 30-min periods of continuous playing of a cassette tape of male *Rana pipiens* mating calls. This tape was produced by the repeated recording, from "Voices of the Night" (Cornell University Records, Cornell University Press), of the section representing the call of *Rana pipiens* at a location near Ithaca, New York. The tests were conducted at room temperature (20°C). During the observation period, the total numbers of calls (chuckles and trills) and attempted or consummated claspings were recorded. The observer was shielded from the animals by a neutral-colored screen with a small viewing slit. The use of recorded conspecific mating calls for evocation of male vocalization behavior in *Rana pipiens* has been established as an adequate stimulus by Schmidt (1966, 1968) and by our own earlier experiments (Palka and Gorbman, 1973).

Pituitary implantation. To induce normal sexual behavior in intact *R. pipiens,* pituitaries from *Rana utricularia* (Mexican population of *R. pipiens* complex) suspended in frog saline (one pituitary per day) were injected intraperitoneally for 3 successive days. After three pituitary implantations, almost all intact male frogs exhibited sexual behavior under the conditions of testing.

Castration. Under tricaine methanesulfonate anesthesia (immersion in 0.1% solution), frogs were bilaterally castrated through a single dorsolateral incision. When sacrificed at the end of the experiment, all the experimental animals were examined for possible regenerated testicular tissue. No visible remnants of testicular tissue were found.

Hormone injections. Two weeks after castration, 1.0 mg of testosterone propionate (TP) (Sigma) dissolved in corn oil was injected intraperitoneally. In some of these frogs, daily intraperitoneal pituitary transplants were given for 12 days, beginning on the first day after testosterone injection. Control animals were injected with the oil vehicle alone.

Intracranial hormone implantation. Intracranial implantation was also

done 2 weeks after castration. For this purpose, a 1:1 mixture of TP and cholesterol was tamped into the detached barrel of a 22-gauge stainless steel hypodermic injection needle. The hormone-filled needle was fixed into a stereotaxic apparatus. The anesthetized frog was placed in the apparatus, clamping the snout and holding the upper orbital ridges firmly with two grooved bars. After removing a flap of temporal bone, the needle containing the hormone mixture was lowered into the desired brain area, and the mixture was extruded as a small pellet with a piston of appropriate diameter; the needle was removed, and the skin was sutured. The pellet was about 60 μg in average weight. For control animals, a cholesterol pellet was similarly placed. For precise stereotaxic location of the hormone implant, it was necessary to adapt and extend for *R. pipiens* the brain atlas prepared by Kemali and Braitenberg (1969) for *Rana esculenta*.

At the end of the experiment, frogs were killed by decapitation, and the brains were fixed in Bouin's fixative, embedded in paraffin, sectioned serially at 10 μm, and stained with toluidine blue O. Precise anatomical sites of the pellets in each animal were determined by study of the serial sections.

RESULTS

Injection Experiments

As expected from earlier experiments (Palka and Gorbman, 1973), three daily injections of whole pituitary tissue uniformly evoked mating-call responses and clasping behavior in intact frogs, and castration completely abolished this behavior (Fig. 1). Two weeks after castration, 1.0 mg of testosterone propionate was injected intraperitoneally into 10 frogs. Five of these castrated testosterone-injected frogs received, in addition, daily pituitary transplants for 12 days. In neither group of treated castrates was any recovery of reproductive behavior observed during the injection period (Fig. 1). Ten frogs treated in the same way, except that oil vehicle was given instead of testosterone in oil, showed no reproductive behavior responses to sex-call stimuli.

Implantation Experiments

All animals used in this experiment were castrated 2 weeks before testosterone implantation. Testosterone propionate pellets were implanted into a total of 34 frogs mainly in the forebrain, using stereotaxic coordinates. Of these pellet implants, nine were bilateral, and the others were medial implants. Cholesterol pellets of the same size were implanted into eight additional control frogs. Precise locations of the implanted pellets, verified by study of serial sections, are summarized in Fig. 2. Effective sites were located mostly in the rostral part of the nucleus

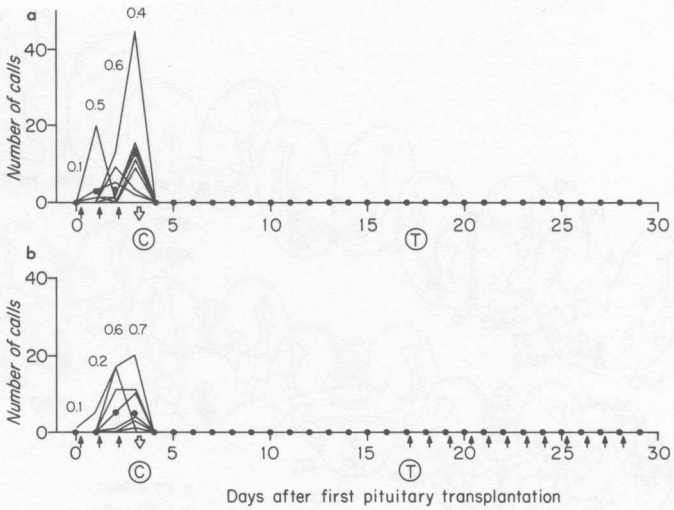

FIG. 1. Frequencies of mating-call behavior in 20 individual male frogs (separate lines) observed during daily 30-min test sessions for 30 days. Mean values are represented by small black circles. In a are shown the values for 10 frogs given single subcutaneous pituitary implants daily for 3 days, Days 1, 2, and 3 (small upward-pointing arrows). They were castrated Ⓒ on Day 4 (thicker downward-pointing arrow) and were given a 1.0-mg intraperitoneal injection of testosterone propionate or vehicle on Day 17 Ⓣ. In b are shown the records of 10 frogs treated in the same way, except that single daily pituitary implants (small arrow) were given in addition on Days 17–29. Numbers written above the records indicate the mean number of clasping attempts per frog observed on each of several days when they occurred.

preopticus, fairly close to the midline. In several instances, bilateral implants which contacted the lateral ventricles induced mating calls. The behavior response was never observed in frogs bearing cholesterol pellet implants.

The various parameters of observed sex behavior of the castrated frogs bearing testosterone implants in the rostral part of the nucleus preopticus are summarized in Table 1. The earliest responses to playing the taped *R. pipiens* calls were largely trills. In a few instances, trills were associated with chuckles and clasping behavior (see also Fig. 3). In intact animals given pituitary transplants, chuckles were heard relatively more frequently in response to taped *R. pipiens* calls. Responses of the hormone pellet-implanted frogs were usually comparatively less vigorous, except in a few cases. This could reflect, in part, the absence of exposure of other parts of the nervous system to hormone. The time of the first expression of evoked sex behavior varied considerably among individuals, but 3–6 days after pellet-implantation was the most common time. The shortest latency was 1 day. This animal had bilateral hormone pellet implants, with each pellet in contact with the lateral ventricles.

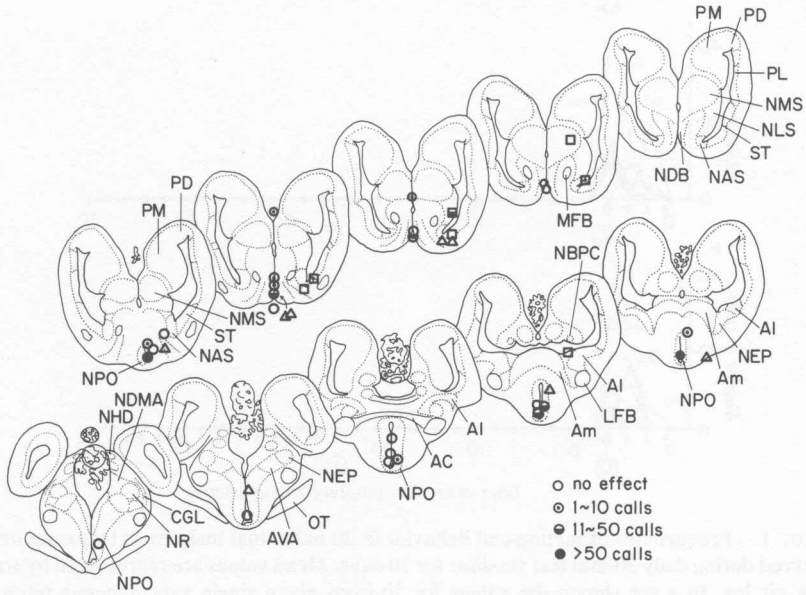

FIG. 2. Locations of implanted testosterone pellets shown in tracings of serial cross-sections of forebrains of leopard frogs. Each site represents approximate midpoint of a pellet; actual sizes of pellets were greater than the symbols drawn. Open symbol = no response; symbol with dot = 1–10 calls; lower half of symbol filled = 11–50 calls; solid symbol = more than 50 calls during the 2-week observation period. Circles represent single medial implants, squares represent bilateral implants, and triangles indicate placement of cholesterol implants. AC, anterior commissure; A1, amygdala pars lateralis; Am, amygdala pars medialis; AVA, area ventralis anterior thalami; CGL, corpus geniculatum laterale; LFB, lateral forebrain bundle; MFB, medial forebrain bundle; NAS, nucleus accumbens septi; NBPC, bed nucleus of the pallial commissure; NDB, nucleus of the diagonal band of Broca; NDMA, nucleus dorsomedialis anterior thalami; NEP, nucleus entopeduncularis; NHP, nucleus habenulari dorsalis; NLS, nucleus lateralis septi; NMS, nucleus medialis septi; NPO, nucleus preopticus; NR, nucleus rotundus; OT, optic tract; PD, pallium dorsale; PL, pallium laterale; PM, pallium mediale; ST, striatum.

Two sets of bilateral hormone implants in the nucleus isthmic region, which is thought to be the sensory correlation center, were not effective. Cholesterol implants also were not effective, even when the implanted site was the same as that of the effective testosterone implants (Fig. 2).

DISCUSSION

It is of interest that the older literature reviewed by Dodd (1960) contains a number of reports of induction of breeding behavior in male amphibians by injections of crude testicular extracts or suspensions and cessation of such behavior after castration. On the other hand, pure androgenic steroids, usually testosterone or its propionate, have most

TABLE 1
Observed Behavior of Frogs in which Testosterone Propionate or Cholesterol Pellet Implants Were Located in the Rostral Nucleus Preopticus

Substance implanted	Individual frog number	Total number of calls[a]	Number of clasping trials	Latency to first behavior (days)	Duration of positive responses (days)
Testosterone	5	19	0	10	3
Propionate	6	8	0	3	2
and	26	32 (5)	0	6	6
cholesterol	35	29	0	5	7
(1:1)	37	8	0	4	2
	38	428 (86)	4	4	9
	44	6	0	4	3
	45	0	0	—	0
	46	0	0	—	0
	50	0	0	—	0
	52	72 (7)	0	4	5
	56	11	0	9	2
		Mean 51.1		5.3 (9)	3.3
		SE 34.77		0.85	0.85
Cholesterol	9	0	0	—	0
	10	0	0	—	0
	64	0	0	—	0
	66	0	0	—	0

[a] Calls observed during daily 30-min periods over a period include both trills and chuckles. In some instances, chuckles are shown in parentheses separately.

often proven generally ineffective in evoking male sex behavior in amphibians (Wolf, 1939; Palka and Gorbman, 1973 for *R. pipiens;* Blair, 1946 for *Bufo;* Schmidt, 1966 for *Hyla* and *Rana;* Obert, 1973 for *Bombina variegata*). Pituitary treatment of male *R. pipiens* provokes an increase in plasma levels of testosterone (Wada *et al.,* 1976), indicating that at least this part of the pituitary–gonad mechanism regulating sex behavior in most vertebrates is orthodox in its functional expression in the frog. Furthermore, Kelley *et al.* (1975) have shown in another frog, *Xenopus laevis,* that injected radioactive testosterone localizes in cells of the ventral preoptic nucleus.

Despite the ineffectiveness of testosterone, pituitary preparations and, particularly, crude frog pituitary material readily evoke male vocalization and clasping behavior (Dodd, 1960; Schmidt, 1966). Yet, pituitary preparations do not affect sex behavior in castrated males (Palka and Gorbman, 1973).

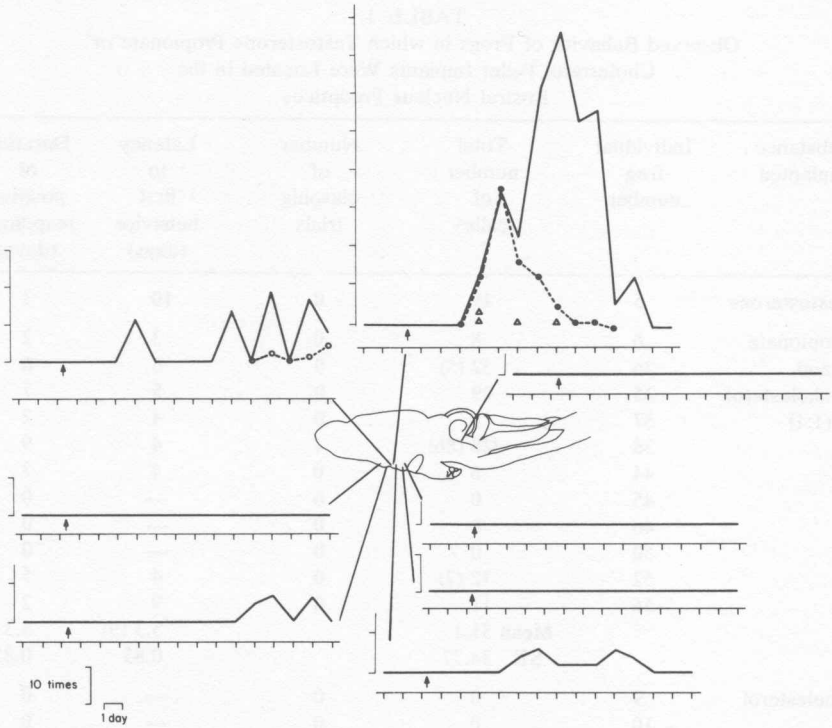

FIG. 3. Typical patterns of frequency of mating behavior observed in eight different frogs after testosterone implantation into each of several areas of the frog brain. Solid arrows indicate the day of hormone implantation. Solid lines represent mating-call trills, broken lines represent chuckles, and △s represent numbers of clasping attempts observed during a 30-min observation period on a particular day. Abscissa = days. Ordinate = numbers of calls or clasping attempts.

Thus, it is clear that some functional feature of the testis, aside from or in addition to testosterone, is necessary for male behavior in frogs, and this function can be stimulated by a pituitary gland factor. The ineffectiveness of systemic testosterone suggests that the amphibian testis may produce an androgen other than testosterone, yet several workers have found that testosterone is a normal product of steroidogenesis in amphibian testes (Dale and Dorfman, 1967; Ozon, 1969; Tamaoki *et al.*, 1970; Rao, 1969). Furthermore, Palka and Gorbman (1973) have shown that androstenedione, androsterone, dihydrotestosterone, as well as several estrogens, progestogens, and corticoids are similarly ineffectual.

Against this background of experience which *appears* to indicate that testosterone is not the stimulant of amphibian male behavior, our finding that testosterone precisely placed in the frog's preoptic nucleus is such a stimulant must be properly interpreted. One possibility might be that

testosterone works synergistically with a pituitary hormone in evoking male behavior. This possibility is denied, however, by our data (Fig. 1) which show that pituitary treatment plus testosterone are ineffective in castrated *R. pipiens*. Since pituitary treatment of *R. pipiens* does increase plasma testosterone levels (Wada *et al.*, 1976), it appears that, under some circumstances (pituitary stimulation of endogenous testosterone secretion or direct implantation into the hypothalamus), testosterone is related to stimulated sexual behavior, but it does not have this action when injected systemically at several dosage levels. This leads to a second possibility: that the testis secretes something that promotes transfer of testosterone into the sex behavior centers of the frog brain. No data exist at this time to confirm or deny this possibility. A supporting bit of evidence can be found in one experiment by Palka and Gorbman (1973) in which it was found that implantation of several halved testes, taken from previously pituitary-stimulated frogs, evoked clasping behavior in castrated male *R. pipiens*. Thus, the testis contains all of the factors necessary for evocation of the behavior, even if they are several in number. If two testicular factors are required, and if the action of testosterone pellets in the preoptic nucleus indicates that this hormone is one of them, then the function of the second factor may be to make the preoptic nucleus accessible to the steroid hormone.

A second point which seems to be settled by our data is the locus of at least one hormone-sensitive regulatory center for male reproductive calling behavior in *R. pipiens* in the rostral ventral preoptic nucleus. This effective area is comparable to an area in which testosterone-concentrating cells have been reported by Kelley *et al.* (1973, 1975) and Morrell *et al.* (1975) in the *Xenopus* brain. In *Xenopus*, autoradiography of [H^3]testosterone showed that such androgen-concentrating cells are near the third ventricle and are distributed from the anterior border of the optic chiasma to just rostral to the preoptic recess; this distribution of labeled cells did not appear to include the more dorsal magnocellular group, described as neurosecretory cells. In *R. pipiens*, aldehyde fuchsin-positive preoptic nuclear neurosecretory magnocellular cells appear to be caudal and dorsal to the sex-calling regulatory area and just anterior to the point where the optic tracts emerge and extend dorso–caudally. Thus, the androgen-concentrating and androgen-sensitive cells do not seem to be part of the magnocellular component of the nucleus preopticus, postulated by Schmidt (1968, 1973) for *Hyla* and *Rana* to be involved in male sex-calling behavior. Moreover, recent unpublished work on electrical stimulation in free-moving frogs has indicated that the effective area for induction of male mating behavior with small currents is the rostral part of the medial basal preoptic nucleus (Wada and Gorbman, unpublished). Several medial hormone implants placed rostral to the

preoptic nucleus or in contact with the lateral ventricles had slight male sex-behavioral effects. In these cases, testosterone might have diffused to the effective region via the ventricular cerebrospinal fluid.

The involvement of the preoptic area in control of vertebrate sexual behavior is well documented. In male rats, testosterone implants are effective in activating copulatory behavior when placed in the preoptic and anterior hypothalamic areas (Davidson, 1966; Lisk, 1967; Johnston and Davidson, 1972). Also, testosterone implanted into the preoptic area induces copulatory behavior in capons (Barfield, 1969) and courtship behavior in ring doves (Hutchison, 1967). Latencies of behavioral evocation observed in such species, 6.9 ± 0.8 days in rats (Johnston and Davidson, 1972) or a minimum of 4 days in capons (Barfield, 1969), are basically similar to the latencies found in the present study.

Comparisons of the latencies of behavioral responses after pituitary implants versus latencies after intrahypothalamic implants of testosterone (Fig. 1 compared to Fig. 3 and Table 1) show that the latter procedure was much slower, despite the fact that it placed the hormone in direct contact with the presumed responding cells. Furthermore, as noted earlier, the evoked behavior was less vigorous and less prolonged after hypothalamic testosterone implantation than after whole pituitary treatment. It is possible, in explanation of this finding, that, although the ventral preoptic nuclear area is the principal male sex behavior-regulating center, it does not contain all of the nervous elements involved in this function. Kelley *et al.* (1975), for example, though finding the greatest binding of [^{3}H]testosterone in the ventral preoptic nucleus of *Xenopus*, also described binding of this hormone in the "ventral infundibular nucleus" and in two regions of the medulla.

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