HORMONES AND BEHAVIOR 9, 141–149 (1977)

Mate Calling Induced by Electrical Stimulation in Freely Moving Leopard Frogs, *Rana pipiens*

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Electrodes were implanted chronically into the preoptic areas of normal or castrated male frogs, *Rana pipiens*, and monopolar monophasic stimulating currents (100 Hz, 0.5-msec duration, and mainly 50–200 μA in intensity) were delivered through the implanted electrodes in freely moving frogs. When the electrodes were placed in the rostral part of the preoptic nucleus, mating calls (mainly trills and sometimes chuckles) were inducible. Fifty microamperes was generally an effective stimulus intensity for induction of calls, and 20 μA was the minimum effective stimulus intensity observed. There was no difference in the threshold to induce calls between pituitary-treated intact and castrated frogs.

Involvement of the preoptic area in sexual behavior has been investigated in various vertebrates, but especially in rats (Vaughan and Fisher, 1962; Van Dis and Larsson, 1971; Merari and Ginton, 1975). In amphibian species, Schmidt (1966, 1968) showed that the mating call, one item in the mating behavioral sequence of frogs, can be evoked by stimulation of the ventral part of the preoptic magnocellular nucleus or adjacent structures (for review, Schmidt, 1973). However, the relatively high-stimulus current intensities required in those experiments, as compared to experiments with other species (in which sexual behavior could be induced by currents as small as 50–200 μA), might have been needed because of inappropriate placement of stimulus electrodes. The frogs in Schmidt's experiment were immobilized, so that evocation of other behaviors by the large currents (e.g., avoidance or escape behaviors) could not be observed. For a more precise localization of the preoptic area related to sexual behavior in this species, we implanted "permanent" electrodes at sites in and near the preoptic nucleus and measured the minimal currents necessary for evocation of sex calling. One aim of these experiments was to determine whether prior alteration of the hormonal state would affect the threshold for electrical evocation of this sexual behavior.

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ISSN 0018-506X

METHODS

Male Northern leopard frogs (*Rana pipiens*) weighing 30–60 g were purchased from MASCO Steinhilber (Fort Atkinson, Wisc.) and were kept in large plastic basins in a room in which the photoperiod (12L, 12D) and temperature (16°C) were controlled. The frogs were force-fed beef liver once a week during the experimental period.

Stimulating electrodes were implanted into 14 frogs anesthetized with MS 222 (immersion in a 0.1% solution), while the head was fixed in a Kopf small-animal stereotaxic apparatus modified for frogs. The electrode was a steel insect pin (00) insulated to the tip with Insl-X. The scalp was incised dorsally in order to expose the skull. A window about 2 mm^2 was made to expose the forebrain, and two holes were also made in the dorsal skull with a dental drill. Stainless steel screws were inserted into the two holes. Then the electrode was lowered according to stereotaxic guiding coordinates through the window and was fixed with dental cement to the skull and to the screws. The electrode was aimed for placement in the medial basal part of the preoptic area. After electrode implantation, the frog was kept individually in a covered translucent plastic box measuring approximately 31 × 17 × 9 cm in a shallow (ca. 1 cm) layer of water in the constant-condition room mentioned above. Electrodes were also implanted into 10 frogs which had been castrated 1 week earlier.

Seven to ten days after implantation, frogs were stimulated electrically through the implanted monopolar electrodes. Negative-going currents (100 Hz, 0.5-msec duration) were delivered through a Grass stimulator (S-4) with a stimulation isolator (SIU-4B); the indifferent electrode was the grounded metal-mesh floor under the frogs. Currents were monitored by use of an oscilloscope (Tektronix 502) across a 1-ohm resistance interposed in series with the stimulation circuit. Current intensity was usually 50, 100, 200, or 300 μA and, sometimes, as high as 400 and 500 μA. In some cases, when there was a response at 50 μA, weaker currents were tried to seek the minimum effective intensity for inducing the response. There were intervals spaced at least 10 min apart between successive stimulation sessions. Currents were applied for 5 min (30-sec on period followed by 30-sec off period, which was repeated five times) in a stimulation session.

As indices of sexual activity, latency for the first observable behavioral event after onset of the first stimulation and the number of calls made during a stimulation period (i.e., 5 min of on and off periods) and after the stimulation period of 5 min were noted.

These frogs were then injected intraperitoneally, on successive days, with three or six single pituitaries, taken from *R. utricularia* and given suspended in frog saline. After the period of pituitary treatment, the electrical stimulation program described above was repeated.

Frogs were killed by decapitation. The electrode and dental cement were removed, and the brains were fixed in Bouin's fixative. They were embedded in paraffiin, sectioned at 10 μm, and stained with toluidine blue 0. The positions of the electrode tips were determined by examination of these preparations. Usually, tracks of electrodes and precise loci of the tips were easily recognized.

RESULTS

Electrical stimulation induced mating calls (mainly mating trills) in freely moving frogs in which implanted monopolar electrodes were appropriately positioned in the preoptic area. After onset of stimulation, frogs turned slightly, inflated their lungs, filled their vocal sacs, and called. Induced calls were long and undisturbed vocalizations (trills), when stimulating currents were in particular ranges. The increase of current intensity above this range resulted in irregularities of calls, escape behavior, avoidance behavior, and/or motor seizures. The responses were all stimulus related; there was no spontaneous calling during control sessions in which currents were not delivered.

Figure 1 summarizes the anatomical placements of effectively and ineffectively implanted electrode tips in intact and castrated frogs. Minimum currents adequate to induce callings were determined in intact and castrated frogs without pituitary treatment. All of the effective sites were located in the rostral extreme of the preoptic nucleus. Electrodes in other sites induced neither calling responses nor other sexual behavior before escape behavior or motor seizures also occurred. No obvious differences were found before and after pituitary treatment in intact frogs, nor between pituitary-treated intact and castrated frogs (Table 1), with respect to numbers of calls or latency to first call after electrical stimulation. In these groups, there was a tendency for larger currents to evoke more calls (linear regression is statistically significant in these groups, $P < 0.05$, using the least-squares method; see example in Fig. 2) and to do it with a shorter latency. During the current-on period, calls were repeated; trills were completed when stimulus currents were turned off during a call. During the off period, calls continued to be given, especially at higher stimulus intensities. During the 5-min poststimulation period, frogs continued to call, especially at higher stimulus intensities. Two to three hundred microamperes delivered to the anterior preoptic region seemed to be the maximum current intensity which evoked sex calls without involvement of other motor behavioral centers through current spread.

In a few instances, electrically induced trills (extended type of vocalization) were accompanied by a small number of chuckles (brief vocalization). Moreover, stimulation induced a clasping-like behavior. In the presence of females and frog pituitary-treated intact males, trills and

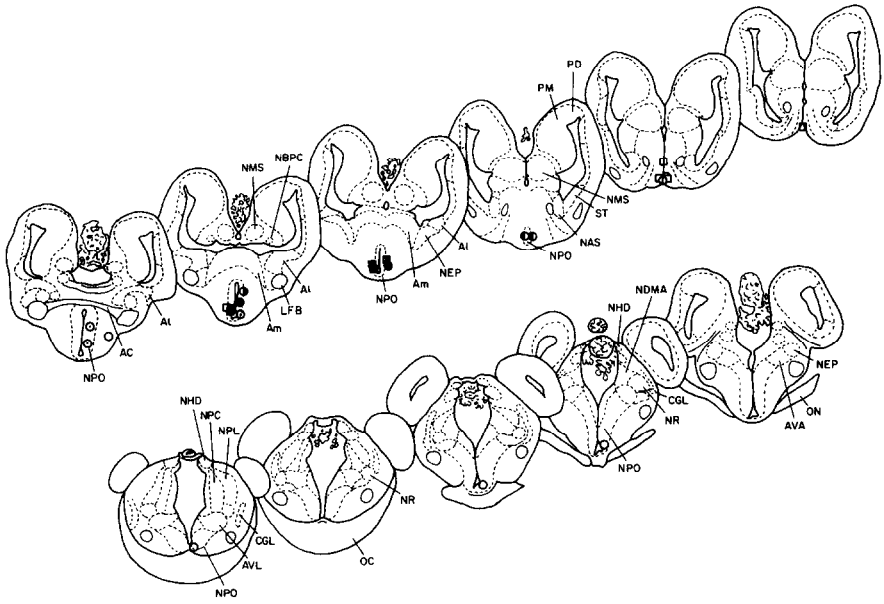

FIG. 1. Sites of electrode-tip placements in the forebrains of frogs, *Rana pipiens*. Circles indicate the electrodes implanted into intact frogs, and squares indicate those placed into castrated frogs given no further treatment. Minimum currents required to induce calls are designated as follows: Darkened symbols = 50 μA; symbols with right half-darkened = 100 μA; symbols with left half-darkened = 200 μA; symbols with dots = 300 μA; blank symbols = no calls, but escape behavior or motor seizures occurred. Each drawing represents an antero–posterior level at successive 0.2-min intervals. Abbreviations: AC, anterior commissure; Al, amygdala, pars lateralis; Am, amygdala, pars medialis; AVA, area ventralis anterior thalami; AVL, area ventralis lateralis thalami; CGL, corpus geniculatum laterale; LFB, lateral forebrain bundle; NAS, nucleus accumbens septi; NBPC, bed nucleus of pallial commissure; NDMA, nucleus dorsomedialis anterior thalami; NEP, nucleus entopeduncularis; NHD, nucleus habenularis dorsalis; NMS, nucleus medialis septi; NPC, nucleus posterocentralis thalami; NOP, nucleus preopticus; OC, optic chiasma; ON, optic nerve; PM, pallium mediale; PD, pallium dorsale.

clasping are observed in response to the auditory stimulation supplied by playing of repeated recorded mating calls of the same species (Palka and Gorbman, 1973; Wada and Gorbman, 1976). Under such auditory-stimulus test conditions, clasping is usually simultaneous with calls in the form of trills. At the time of electrical stimulation, some of the frogs leaped forward while they vocalized a trill. Since the observation boxes did not contain a female frog, or another object of similar dimension, it is unclear whether this behavior is in any way equivalent or related to true clasping behavior.

Electrically induced calling responses were usually consistent in number and latency time to the first call in each of the six stimulation

TABLE 1
Electrically Induced Calls and Latency to First Call during Stimulation Sessions in Frogs Bearing Electrodes in the Rostral Preoptic Nucleus

	N	Current Intensity (μA)	Calls				Latency to first call (sec)
			Stimulation period (A)		Poststimulation period (B)	A + B	
			On	Off			
Intact	5	50	3.2 ± 2.30[a]	1.4 ± 1.40	0	4.6 ± 4.12	19.0 ± 2.00 (2)[b]
		100	7.0 ± 2.41	5.2 ± 2.44	2.4 ± 1.47	14.6 ± 6.16	22.5 ± 9.31
		200 (4)[c]	9.5 ± 2.40	9.8 ± 2.50	6.0 ± 1.35	25.3 ± 5.72	11.0 ± 3.09
Castrated	5	50	2.6 ± 1.25	2.2 ± 1.96	1.2 ± 0.97	6.0 ± 3.69	31.7 ± 13.30 (3)[b]
		100	6.8 ± 1.24	7.0 ± 1.58	3.2 ± 1.24	17.0 ± 3.46	21.2 ± 6.52
		200 (3)[c]	8.7 ± 3.71	7.7 ± 2.60	2.3 ± 1.45	18.7 ± 5.49	21.7 ± 11.89

[a] Number of calls includes both trills and chuckles. Trills are prolonged vocalizations several seconds in duration. Chuckles are brief vocalizations approximately 1 sec in duration. While trills are more common in sexually active male *Rana pipiens*, both kinds of calls are part of normal sex calling in this species. Values are expressed as mean ± SE.

[b] Number in parentheses indicates the number of frogs used for this calculation after eliminating frogs at the lower stimulus level that did not respond.

[c] Number in parentheses indicates the number of frogs used for this calculation after eliminating the frogs at the higher stimulus level which showed avoidance behavior or motor seizures at this intensity.

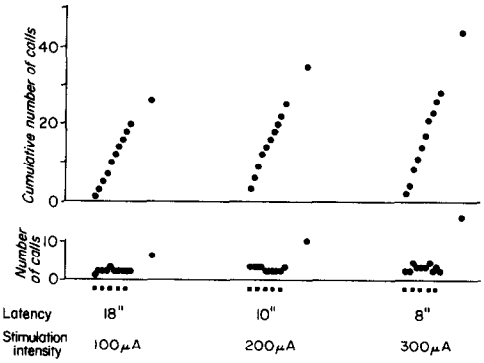

FIG. 2. One example of the patterns of responses to electrical stimulations in a pituitary-injected leopard frog in which there was an electrode tip in the rostral part of the nucleus preopticus. Each darkened circle represents the number of calls counted during an on or off period of a stimulation session or during a 5-min poststimulation period. Darkened squares represent 30-sec periods of the stimulation session, each of which was separated from the next stimulation by a 30-sec pause. Latency means lag time to the first call after beginning the first set of five 30-sec stimulations.

sessions. In one instance in a pituitary-injected intact frog, a stimulus current of only 20 μA evoked a mean number of 7.3 $\pm$ 1.23 calls (means $\pm$ SE) during the six test sessions, and the mean latency to the first call in each session was 16.5 $\pm$ 2.74 sec. This 20-μA stimulus was also the minimum effective current intensity for the entire series of experiments.

DISCUSSION

The present experiments show that relatively small electrical stimulation currents are adequate for induction of mating-call vocalization when the electrodes are appropriately placed in the rostral part of the preoptic nucleus. In the same species, Schmidt (1968, 1974) applied higher stimulus intensities to a more postero-dorsal part of the preoptic nucleus (200 Hz, 0.3 msec, 700 μA; or 100 Hz, 0.5 msec, 300–900 μA), and he concluded that a stimulus, at least 10 times stronger, was needed to evoke mating calls than to evoke release calls, and that the responsive area in the brain for electrical evocation of mating calls is the ventral magnocellular neuronal region of the preoptic nucleus (also see Schmidt, 1973). In the experiments described here, in which stimulation was applied to freely moving *R. pipiens* males, 20 μA was found to be the minimal effective intensity of stimuli used to evoke mating calls; the generally effective range of stimulus intensity was 50–200 μA, if the electrodes were placed in the rostral part of the preoptic nucleus. Stimulation to the more caudal regions of the preoptic nucleus required somewhat higher stimulus inten-

sities and induced avoidance behavior or motor seizures along with the vocalizations. Schmidt's (1968, 1974) experiments utilized frogs that were immobilized, as well as motor denervated. We would presume, from the conditions found adequate in our experiments, that the higher current intensities used by Schmidt (1968, 1974) may have made it difficult to decide the precise localization of the sex vocalization area in the preoptic nucleus. Using such reasoning, we can reconcile the basis for Schmidt's identification of an area in the preoptic nucleus posterior to the one identified in our experiments.

The area in which electrical stimulation induced sexual calls in *R. pipiens,* in the experiments described here, is closely comparable to the region of the preoptic nucleus which concentrates labeled testosterone in *Xenopus laevis* (Kelley, Pfaff, and Morrell, 1975; Morrell, Kelley, and Pfaff, 1975). The androgen-concentrating cells were found by these investigators in the anterior preoptic area, always rostral to the preoptic magnocellular nuclei. Recently, we have also found that testosterone pellets implanted in the rostral part of the nucleus preopticus induce sexual behavior in *R. pipiens* (Wada and Gorbman, 1976).

We may speculate, in evaluating the results of site-limited androgen implants and localized electrical stimulation, that the preoptic area relates to most of the elements in male sexual behavior. Furthermore, it is probably the androgen-sensitive site in this species if we accept the results of testosterone implantation and the findings by others of autoradiographic localization of labeled testosterone in this hypothalamic region. However, it is generally agreed that the mechanisms controlling clasping behavior and other male sex behaviors are complex (see Hutchison, 1967), and simplistic interpretations probably should be avoided until further experimental evidence is at hand.

The preoptic area is generally considered to be an important or even primary center for sex behaviors in many classes of vertebrates. In rats, electrical stimulation directed to this area induces stimulus-bound copulation (Vaughan and Fischer, 1962; Van Dis and Larsson, 1971; Merari and Ginton, 1975), and testosterone implantation into the preoptic area restores copulatory behavior in castrates (Johnston and Davidson, 1972). Some elements of sexual behavior have been elicited by stimulation of the preoptic area in other mammals as well (MacLean and Ploog, 1962; Robinson and Mishkin, 1966). Reproductive behavior has been released by electrical stimulation of the preoptic area of unanesthetized freely moving pigeons (Åkerman, 1966). Bauer and Demski (1974) showed that vertical banding, a sexual display in the green sunfish, was induced by electrical stimulation applied to the preoptic area. In addition, testosterone-concentrating neurons are found in relatively large num-

bers in the preoptic areas of several classes of vertebrates (for review, see Morrell *et al.*, 1975) in which autoradiographic studies have been completed.

As noted above, the evocation of elements of sexual behavior by implantation of testosterone into the preoptic area and the localization of radiotestosterone in the same area indicate that endocrine modulation of sex behavior is exerted at this anatomic locus. However, the nature of such modulation remains difficult to define. One possibility is that the hormone might alter the sensitivity or minimum stimulus thresholds of nervous elements in the preoptic region. However, in the experiments described here, there was no apparent difference in electrical stimulation threshold for evoked mate calling between intact pituitary-treated and castrated frogs, at least in the 50- to 200-μA stimulus range. Mate calling can be evoked readily in pituitary implant-treated frogs by playing tapes of mate calls of the same species (Palka and Gorbman, 1973; Wada and Gorbman, 1976). Since, in frogs, such an auditory stimulus can be made an effective evocator of mate-calling behavior by pituitary treatment, we sought, at first, to distinguish a possible difference in threshold for electrical evocation of mate calling between pituitary-treated intact and castrated frogs. A possible explanation of the negative results is that the electrical stimulation of the preoptic area was too intense to discriminate a minor difference between the two groups. It is also possible that the sex hormone-responsive nervous units that modulate relative sensitivity to exogenous sex behavior-evocative stimuli are located at a site or sites other than the preoptic area. Kelley *et al.* (1975) found radiotestosterone concentrated in at least three areas other than the preoptic nucleus. Schmidt (1968), as we, found no difference in preoptic induction of mating calls before and after castration in *R. pipiens*. In the rat, castration does not affect mating approach and mounting behavior when the animal is stimulated intracranially (Van Dis and Larsson, 1971). However, there are steroid-sensitive cells in the preoptic area the firing rates of which are changed with altered steroid levels in the blood stream of the rat (Lincoln, 1969; Yagi, 1973). We hope that continued definition of the functional changes of neuronal units in varying endocrine environments will lead to better understanding of the mechanisms of regulation of mate calling and other sex behaviors by hormones in vertebrates.

ACKNOWLEDGMENT

This study was aided by a grant (No. NS 11343) from the National Institutes of Health, United States Public Health Service.

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