

Arginine Vasotocin Facilitation of Advertisement Calling and Call Phonotaxis in Bullfrogs

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Although the neuropeptide arginine vasotocin (AVT) is found in several auditory and vocalization regions of the bullfrog (*Rana catesbeiana*) brain, its functions in these areas are unknown. We examined the effects of AVT injection on two auditory-evoked reproductive behaviors: advertisement calling in male bullfrogs and call phonotaxis in female bullfrogs. AVT (500 µg; intraperitoneal) significantly increased advertisement call frequency and decreased calling latency in male bullfrogs, compared to saline injection. This dose of AVT also significantly decreased the time required for female bullfrogs to reach a call source as well as the latency of females to leave the starting position during call playback. In both males and females, these effects were significant at 0.5 hr and persisted until at least 2 hr after injection. AVT thus facilitated display of two sexually-dimorphic reproductive behaviors in bullfrogs. These effects may be due to direct effects of AVT on auditory processing regions in the bullfrog brain. © 1994 Academic Press, Inc.

Vocalizations play a critical role in the reproductive behaviors of anuran amphibians. In bullfrogs, males give a species-specific advertisement call which likely serves several functions (Wiewandt, 1969; Emlen, 1976; Ryan, 1980). This call attracts female bullfrogs to prospective mates, attracts other males to active choruses, and functions in territory maintenance by males. Female bullfrogs, on the other hand, do not give advertisement calls (Capranica, 1965, 1968). Instead, females are attracted to calling males (call phonotaxis). Hormonal factors which influence calling and phonotaxis behaviors in anurans are relatively unknown.

Castration experiments show that gonadal factors are usually required for display of behaviors such as clasping and calling (Schmidt, 1966; Palka and Gorbman, 1973; Kelley and Pfaff, 1976; Moore, 1978; Wetzel and Kelley, 1983; Deviche and Moore, 1988). Gonadal steroids, however, are not by themselves sufficient (Palka and Gorbman, 1973; Wada, Wingfield, and Gorbman, 1976; Moore, 1978). Various neuropeptides also have robust effects on amphibian reproductive behaviors (Moore, 1987). One of the most potent of these peptides is arginine vasotocin (AVT). Exogenous AVT stimulates amplexic clasping behavior and egg-laying behavior in

the urodele amphibian *Taricha granulosa* (Moore, 1992). In various anuran amphibians, AVT can alter levels of calling (release calling or advertisement calling) and phonotaxis (Diakow, 1978; Raimondi and Diakow, 1981; Schmidt and Kemnitz, 1989; Boyd, 1992; Penna, Capranica, and Somers, 1992). This peptide thus affects both male-typical and female-typical behaviors in amphibians and, in most cases, the effects of AVT differ in males and females.

In the bullfrog, concentrations of immunoreactive AVT in brain are sexually dimorphic and steroid-sensitive (Boyd and Moore, 1992; Boyd, Tyler, and DeVries, 1992; Boyd, 1994). These steroid-AVT interactions are especially prevalent in auditory pathways. We hypothesized that if AVT altered auditory processing in bullfrog brain, auditory-evoked reproductive behaviors might be influenced. In this study, we first examined AVT effects on auditory-evoked advertisement calling in male bullfrogs since another vocalization behavior (tactilely evoked release calling) has been previously shown to be altered by AVT in bullfrogs (Boyd, 1992). Second, we examined call phonotaxis in female bullfrogs since general locomotor activity is stimulated by AVT in female bullfrogs (Boyd, 1991).

METHODS

Adult bullfrogs were obtained from C. Sullivan Co. (Nashville, TN) during the breeding season (June; body weight of males $397 \text{ g} \pm 11 \text{ g SEM}$ and females $406 \text{ g} \pm 15 \text{ g SEM}$). They were maintained in the lab for 1–3 weeks on a 12L:12D controlled photoperiod at 19°C in large fiberglass tanks (60W × 60H × 90L cm) with flow-through filtered well water. They were fed goldfish *ad lib*.

Male Advertisement Call Observation Procedure

Male bullfrogs were placed in individual tanks and tested during the dark phase of the light cycle. Frogs were given an intraperitoneal injection of 500 µg AVT (Sigma) in 0.1 ml saline or a control injection and returned to home tanks. After 30 min, a small speaker (Panasonic) at the top of the tank was used to play a 5-min recording of male bullfrog advertisement calls (recorded without interruption from a single field population of bullfrogs). This recording consisted of about 15-call bouts separated by 3- to 30-sec periods of silence. Both single-frog and multiple-frog bouts were included. Number of advertisement calls given by each experimental male during this 5-min period was recorded. Following 5 min of silence, the 5-min test was repeated. If males gave no calls, then latency to call was recorded as 5 min. Results of both 5-min tests were averaged for individual males to give one value for each male at each test time. Calls given during the silent period (with no recording playback) were not counted. This overall test procedure was repeated at 1 hr and 2 hr after injection but

data from the 1 hr test are not presented because it did not differ significantly from that obtained at 0.5 and 2 hr.

Female Call Phonotaxis Observation Procedure

Phonotaxis behavior was tested in an empty 2.7 × 2.7-m room with a grid of 81 squares (approximately 30 × 30 cm each) marked on the floor. Movements of females across this grid were observed under dim red lights through a window. Females were injected with AVT or saline (as above) and placed under an opaque bucket in a corner of the room opposite a small speaker (4.5 × 6 cm Panasonic speaker placed 10 cm above the floor to act as a point source resembling a calling male). Bullfrogs were left undisturbed for 30 min and then the speaker was activated and the bucket removed. The speaker was used to play a 15 min field-recording of male bullfrog advertisement calls which consisted of single-male and multiple-male call bouts separated by 3- to 30-sec periods of silence. Movements of individual females were analyzed to yield latency to leave the starting square, elapsed time from leaving the starting square to reaching the same square as the speaker, and number of grid lines crossed during the 15 min test period (as a measure of general activity). Females who never left the starting square were assigned a latency of 15 min and those who never reached the speaker were assigned an elapsed time of 15 min. Frogs were replaced under the bucket at the end of the test period and left undisturbed in silence until the procedure was repeated at 1 and 2 hr after injection (data from 1 hr test not shown). All females were gravid at the time of the experiment but the dose of AVT did not stimulate oviposition in any animal.

RESULTS

AVT significantly facilitated the display of two auditory-evoked behaviors in bullfrogs. First, the frequency of advertisement calls given by male bullfrogs, in response to taped calls, was significantly increased and latency to call was significantly decreased, compared to saline-injected controls (Table 1). All AVT-injected males gave at least 6 calls/min (maximum of 20 calls/min) while many saline-injected males did not call at all (saline-injected males calling at 0.5, 1 and 2 hours was 43, 43, and 29%, respectively). Even among the saline-injected males which did call, the mean call frequency was only 2.4 calls/min (± 0.43 SEM). All males typically ceased calling within one minute after the end of the test tape. Spontaneous mate calls were never heard.

Second, AVT facilitated display of call phonotaxis in female bullfrogs (Table 1). Latency to approach the call source was significantly shorter in AVT-injected females than in saline-injected controls. Time to reach the call source (after movement had begun) was also shorter in AVT-treated bullfrogs. Display of successful phonotaxis was quite rare in saline-

TABLE 1
Effects of AVT (500 μg ip) or Saline Injection on Auditory-Evoked Behaviors in Male and Female Bullfrogs

	Saline injection	AVT injection ^a
Males ($N = 7/\text{group}$)		
Advertisement calls/min		
0.5 hr	1.0 \pm 0.6	14.4 \pm 1.5
2 hr	0.2 \pm 0.1	12.6 \pm 1.1
Latency to call (min)		
0.5 hr	4.74 \pm 0.1	1.19 \pm 0.3
2 hr	4.90 \pm 0.1	1.36 \pm 0.3
Females ($N = 5/\text{group}$)		
Latency to approach call source (min)		
0.5 hr	10.61 \pm 0.4	4.69 \pm 1.2
2 hr	11.42 \pm 1.0	5.66 \pm 0.8
Time to reach call source (min)		
0.5 hr	10.25 \pm 2.6	1.67 \pm 0.3
2 hr	15.00 \pm 0.0	0.83 \pm 0.2

^a Saline and AVT-treated groups differ significantly on all parameters (Mann-Whitney U test; $P < 0.01$). Data presented are mean \pm SEM.

injected females. At 0.5 hr after saline injection, only two of five females reached the call source. At 1 hr, only one female reached the call source and none reached the source at 2 hr after saline injection. All AVT-injected females, on the other hand, successfully reached the speaker on all three trials. In general, responsive females first remained motionless in the starting position until suddenly directing their attention (turning head and body) toward the speaker. Most then moved directly (diagonally) across the room to the speaker and climbed on it, although a few followed the room walls. Female bullfrogs approached the speaker most frequently by "walking" on all four legs with abdomen off the ground but some frogs did "hop" in the more traditional fashion.

General activity of AVT-injected females was significantly greater than that of saline-injected females (number of grid lines crossed by AVT-treated females was 15.7 ± 0.4 while number of lines crossed by saline-treated animals was 10.1 ± 1.3 ; $P < 0.01$, Mann-Whitney U test). AVT nonetheless appeared to directly stimulate phonotaxis behavior rather than having a nonspecific effect on locomotion. The minimum number of lines that had to be crossed to reach the speaker was 15 and most AVT-treated frogs reached the speaker with that minimum (range in AVT-treated females = 15–19 lines). Most saline-injected females, on the other hand, either never left the starting position (0 lines crossed) or moved to an adjacent room corner and remained there (8 lines crossed). In the three instances where saline-injected frogs successfully reached the speaker, they

did more wandering and took a less direct route than AVT-injected frogs (successful AVT-treated frogs: 15.7 ± 0.4 lines crossed versus successful saline-treated frogs: 21.7 ± 2.8 lines crossed; $P < 0.05$, Mann-Whitney U test).

DISCUSSION

Although vocalizations play an important role in reproduction in both male and female bullfrogs, the behavioral response of frogs to conspecific vocalizations is sexually dimorphic. When male bullfrogs hear advertisement calls given by other males, they respond by calling as well. Female bullfrogs, on the other hand, never give mate calls—instead, they show a positive phonotactic response and move toward the source of the calls. In bullfrogs, AVT facilitates the display of both behavioral responses to auditory stimulation.

This is the first report of any hormone or neurotransmitter altering advertisement calling in bullfrogs. The neuropeptide AVT thus facilitates display of two vocalization behaviors in male bullfrogs—advertisement calls and release calls (Boyd, 1992). This peptide has been previously shown to stimulate mate calls in the treefrog *Hyla cinerea*, cricket frog *Acris crepitans*, and the toad *Bufo americanus* (Schmidt and Kemnitz, 1989; Penna *et al.*, 1992; Marler, unpublished observation), but it inhibits release calls in the leopard frog *Rana pipiens* (Raimondi and Diakow, 1981). AVT may have been at least partly responsible for stimulation of mate calling in early studies with *R. pipiens*, *H. versicolor*, and *H. cinerea* where whole pituitaries were injected or the magnocellular preoptic area was electrically stimulated (Schmidt, 1966; Palka and Gorbman, 1973; Wada and Gorbman, 1977a, 1977b). Neurohypophysial peptide influence on vocalizations may be a general vertebrate phenomenon since an AVT analog alters singing in canaries (Voorhuis, DeKloet and DeWied, 1991) and AVT, vasopressin and oxytocin can alter vocalizations in mice and rats (Delanoy, Dunn, and Tintner, 1978; Insel and Winslow, 1991; Witt and Insel, 1991).

Gonadal steroids are likely required for AVT stimulation of advertisement calling in male bullfrogs. This experiment was done during the breeding season, when androgen levels are at their highest (Licht, McCreery, Barnes, and Pang, 1983; Mendonca, Licht, Ryan, and Barnes, 1985; Boyd, 1992). In other anurans, castration is followed by a disappearance of mate calling (Schmidt, 1966; Palka and Gorbman, 1973; Wetzel and Kelley, 1983; Penna *et al.*, 1992). Androgen replacement can sometimes restore calling behavior (Wada and Gorbman, 1977b; Wetzel and Kelley, 1983; Penna *et al.*, 1992) and sometimes not (Schmidt, 1966; Palka and Gorbman, 1973). Failures may have resulted when some other necessary factor—such as AVT—was also missing. Androgens may serve to maintain AVT receptors (Boyd and Moore, 1991), AVT concentrations

(Boyd, 1994), neural structures controlling vocalizations (Schmidt, 1982, 1983; Takami and Urano, 1984; Wetzell, Haerter, and Kelley, 1985; Kelley, Fenstermaker, Hannigan, and Shih, 1988) or the larynx itself (Sassoon and Kelley, 1986; Sassoon, Segil, and Kelley, 1986).

Mate-call phonotaxis is a proceptive reproductive behavior shown by gravid females of many anuran species around the time of ovulation. In this study, we induced phonotaxis in gravid females with AVT treatment alone. Induction of robust phonotaxis in female American toads (*B. americanus*), on the other hand, requires combined treatment with progesterone, prostaglandin $F_{2\alpha}$ and AVT (Schmidt, 1984, 1985). Phonotaxis can also be stimulated by human chorionic gonadotropin in *Xenopus*, toads (*B. americanus* and *B. woodhousei fowleri*) and treefrogs (*H. cinerea*) (Schmidt, 1969, 1984; Picker, 1983). Some of these differences are likely due to differences in the initial hormonal state of the females as well as species differences in social systems.

These behavioral effects of AVT in male and female bullfrogs are likely occurring through a central nervous system action. Intracerebroventricular injections of AVT are significantly more effective at altering behaviors in rough skinned newts and bullfrog tadpoles, compared to intraperitoneal injections (Moore and Miller, 1983; Boyd, 1991). In the bullfrog, AVT cells and fibers are found throughout brain areas which control vocalizations and locomotion, including the pretrigeminal nucleus, amygdala, preoptic area and lateral subtoral tegmentum (Boyd and Moore, 1992; Boyd *et al.*, 1992).

It would be most parsimonious to hypothesize that AVT altered behaviors in both sexes of bullfrogs through a common mechanism. Both male mate calling and female phonotaxis behaviors were evoked by the same stimulus—a recording of male bullfrog calls. AVT-immunoreactive fibers are found throughout the bullfrog auditory system and AVT may thus have affected bullfrog behaviors by altering auditory processing of the mate-call stimulus. Cells sensitive to auditory stimuli are present in many brain areas, including the pretrigeminal nucleus and anterior preoptic nucleus (Urano and Gorbman, 1981; Aitken and Capranica, 1984). Both AVT and gonadal steroids affect auditory sensitivity in amphibians (Narins and Capranica, 1976; Urano and Gorbman, 1981; Yovanof and Feng, 1983; Aitken and Capranica, 1984; Hillery, 1984; Penna *et al.*, 1992). Steroids and AVT may therefore interact within auditory regions of the bullfrog brain and ultimately alter display of sex-typical vocalization or locomotor behavior.

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