Hormones and Behavior **36,** 53–61 (1999)
Article ID hbeh.1999.1524, available online at http://www.idealibrary.com on IDEAL®

Forebrain Arginine Vasotocin Correlates of Alternative Mating Strategies in Cricket Frogs

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Received August 27, 1998; revised February 24, 1999; accepted February 26, 1999

In cricket frogs, *Acris crepitans,* sexually active males can switch between calling and noncalling (satellite) mating strategies and injections of the neuropeptide arginine vasotocin (AVT) stimulate calling behavior. We report here that this behavioral variation of animals under field conditions is associated with variations in AVT-immunoreactive (AVT-ir) staining in distinct brain nuclei. In both calling and satellite males, one AVT-ir brain region was found in a continuous string of cells between the medial amygdala and the nucleus accumbens (ACC). Satellite males possessed significantly more AVT-ir staining in the brain (cells and fibers) than calling males at the level of the ACC, although not in the medial amygdala. This difference in AVT-ir staining in the ACC can, in part, be explained by differences in the density of staining within the cells and in cell size. In addition, satellite males had significantly higher AVT-ir staining in the fibers medial to the ACC than calling males. Because other studies have demonstrated that AVT stimulates calling behavior, a plausible hypothesis is that calling males are releasing more AVT from neurons in the ACC, depleting reserves within the cells, and that the released AVT elicits calling behavior. AVT immunoreactivity levels are also higher in the ACC in both calling and satellite males than in female cricket frogs, which do not call. Satellite males may therefore have AVT reserves that might allow them to call depending on the social conditions.

Key Words: **arginine vasotocin; nucleus accumbens; mating behavior; anuran; *Acris crepitans;* alternative mating strategies.**

Alternative male phenotypes have recently provided model systems for investigating physiological control of individual variation in behavior. Species with these discrete alternative phenotypes will often use different behavioral strategies to obtain mates; some males defend territories and court females, while others are nonterritorial and may use sneak or satellite strategies for obtaining mates (Gross, 1996). Still others will go through sex changes to increase their reproductive success (e.g., Godwin, 1994).

The sex steroid hormone profiles of these alternative behavioral phenotypes differ either during ontogeny or as adults in the tree lizard *Urosaurus ornatus* (e.g. Hews and Moore, 1996; Hews, Knapp, and Moore, 1994), in a number of teleost species (review by Brantley, Wingfield, and Bass, 1993), as well as in other species (review by Moore, 1991). The neuropeptide arginine vasotocin (AVT) and its mammalian homologue arginine vasopressin (AVP) may also emerge as neurochemicals influencing alternative mating strategies. One species, the marine goby (*Trimma okinawae*), displays rapid reversible sex changes that are correlated with reversible changes in the size of the AVT-immunoreactive (AVT-ir) forebrain cells (Grober and Sunobe, 1996).

Although direct effects of AVT on sex changes have not yet been demonstrated in the marine goby, AVT and AVP influence both mating and aggressive behaviors in several other species. For example, AVP injections increase dominance behavior in golden hamsters, *Mesocricetus auratus* (e.g., Ferris, Melloni, Koppel, Perry, Fuller, and Delville, 1997), and aggression in prairie voles, *Microtus ochrogaster* (Winslow, Hastings, Carter, Harbaugh, and Insel, 1993), but decrease aggression in squirrel monkeys, *Saimiri sciureus* (Winslow and Insel, 1991), and mice, *Mus musculus domesticus* (Roche and Leshner, 1979). The reason for this variation is unknown, but could be due to meth-

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0018-506X/99 $30.00

odological differences or differences in social systems among species.

This variation in effects of AVT on aggressive behavior may or may not occur in anurans. AVT consistently stimulates mate calling in a number of anuran species: cricket frogs, *Acris crepitans* (Marler, Chu, and Wilczynski, 1995; Chu, Marler, and Wilczynski, 1998); green treefrogs, *Hyla cinerea* (Penna, Capranica, and Somers, 1992); bullfrogs, *Rana catesbeiana* (Boyd, 1994a); Great Plains toads, *Bufo cognatus* (Propper and Dixon, 1997); and gray treefrogs, *Hyla versicolor* (Semsar, Klomberg, and Marler, 1998). In the cricket frog, AVT does not increase aggression; instead it either decreases the aggressive nature of the calls, or, perhaps more likely, the changes in aggression may simply be a side effect of increased calling behavior to attract females (Marler *et al.*, 1995; Chu *et al.*, 1998). In a second species, the gray treefrog, AVT-injected males were able to take over calling sites from uninjected males, probably through changes in advertisement calls (Semsar *et al.*, 1998). Whether the uninjected resident males perceived the AVT-injected intruders as being more aggressive or perhaps better able to attract females is unclear. These behavioral studies suggest that AVT has a significant impact on acoustic communication in anurans [AVT also influences acoustic communication in birds, e.g., Maney, Goode, and Wingfield (1997)]. In addition, AVT-ir cells and fibers and AVT receptors are found throughout anuran brain areas associated with vocal communication (review by Boyd, 1997).

Based on the effects of AVT/AVP across a wide variety of species, and in particular because of the effect of AVT on calling behavior previously described, we investigated the association between the alternative mating strategies adopted by male cricket frogs which consisted of either calling or noncalling behaviors and the levels of AVT immunoreactivity in their brains. Males of some anuran species can call to attract females ("calling males") or alternatively they can silently situate themselves near a calling male and intercept females approaching a calling male ["satellite males," e.g., Wells (1977)]. Cricket frogs were used in this study because males displaying the different behavioral strategies are easily identifiable and calling behavior has been studied in detail in this species (Nevo and Capranica, 1985; Perrill and Sheperd, 1989; Wagner, 1989a,b,c, 1992; Ryan and Wilczynski, 1991; Ryan, Perrill, and Wilczynski 1992; Ryan, Warkentin, McClelland, and Wilczynski, 1995). Apart from the difference in calling behavior, satellite males can also be identified because they situate themselves within approximately 20 cm of the calling male and adopt a characteristic low posture (Perrill and Magier, 1988; Wagner, 1992). Individual males can switch between strategies in response to social interactions and no significant differences in size have been found between male cricket frogs displaying the different behavioral strategies (Perrill and Magier, 1988; Wagner, 1992). For example, males may switch within minutes from calling to satellite behavior in response to playbacks of low-frequency calls, which are characteristic of larger males (Wagner, 1992). Anurans may therefore provide a nice contrast with other model systems of alternative mating strategies incorporating acoustic communication because they show more plasticity than other model systems such as the plainfin midshipman [*Porichthys notatus* (Bass, Bodnar, and McKibben, 1997)].

We identified "satellite" and "calling" males in the field, captured them while they were displaying these behaviors in the field, and then fixed the brains for immunocytochemistry. This approach allowed us to examine associations between forebrain levels of AVT and alternative mating strategies.

METHODS AND MATERIALS

Males and females were collected in April 1992 in a natural breeding chorus in Central Texas near Bastrop. Calling and satellite male cricket frogs were first identified through their behavior. Satellite males adopt a low posture, position themselves approximately 20 cm away from a calling male, remain motionless, and do not call (Perrill and Magier, 1988; Wagner, 1992). Some males will abandon calling in response to playbacks of male conspecifics (Wagner, 1992), but do not adopt the low position. Thus only males that fit the criteria listed above were identified as satellite males in this study. This assessment took 5–20 min. Animals were treated in accordance with the recommendations of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals.*

The fixation procedure was initiated within 5–10 min of capture to avoid stress effects. Frogs were decapitated, part of the brain was exposed, and the head was fixed in 5% acrolein in a 0.1 M phosphate buffer (pH 7.2) for a minimum of 2 h. Brains were removed from the skull, postfixed in a fresh acrolein solution for another 2 h, and then stored in 30% sucrose in phosphate buffer at 4°C. Brains were embedded in butter and cooled to 4°C, and 75-μm sections were cut with a vibratome filled with Tris buffer at

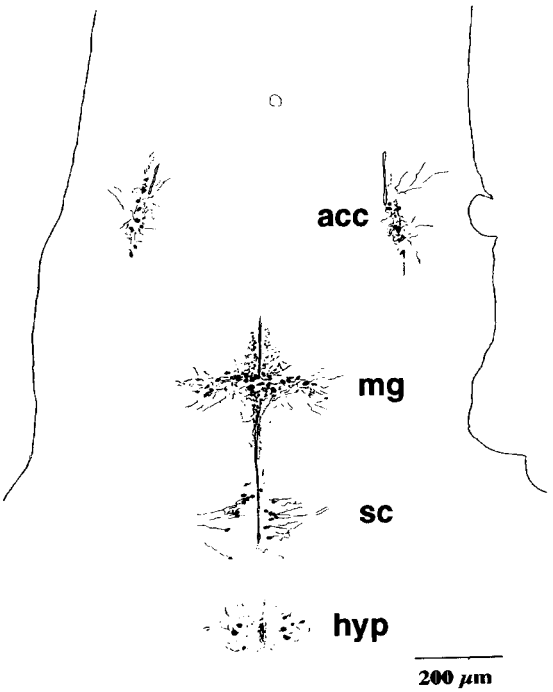

FIG. 1. Camera lucida drawing (top, rostral) of the distribution of AVT-ir cells and fibers through a horizontal section of male cricket frog brain showing the nucleus accumbens (acc), the magnocellular preoptic region (mg), the hypothalamus (hyp), and the suprachiasmatic nucleus (sc).

0°C. Half of the brains were sectioned horizontally and half were sectioned coronally.

Immunocytochemistry was performed as previously described using rabbit anti-vasopressin serum (ICN, 1:2000) in a triple-bridge PAP procedure with DAB as the chromagen [for a check of the specificity of the antibody see Boyd, Tyler, and De Vries (1992)]. Although anti-vasopressin serum was used, we will interpret AVP immunoreactivity as representing AVT immunoreactivity as in Boyd *et al.* (1992).

The brains sectioned coronally were used to help identify brain areas. Quantitative data were taken solely from the horizontal sections because only one to two cells were visible in each frontal section of the nucleus accumbens. The areas quantified included the nucleus accumbens (ACC), medial amygdala, magnocellular preoptic region, hypothalamus, and suprachiasmatic nucleus (see Results for descriptions of the cell populations). The magnocellular preoptic region, the hypothalamus, the suprachiasmatic nucleus, and the nucleus accumbens were measured in the section containing AVT-ir cells in all four areas (Fig. 1). If the nucleus accumbens was not present in this section then it was measured in the section closest to it. The medial amygdala was measured in a section with AVT-ir cells also present in the magnocellular preoptic region.

Slides were coded so that the researcher was blind to the animal's group. In addition, a second researcher repeated the measurements and obtained the same qualitative results, although the data are not presented here. Density of cells and fibers was measured using the image analysis software Image 1.44 developed by Dr. Rasbaud at NIH (e.g., Shipley, Luna, and McLean, 1989; Bamshad, Novak, and De Vries, 1994). Camera and light settings were kept constant across all brain sections. All density measurements were made on brain sections run in the same assay. The image of the brain was digitized into pixels with gray values ranging from 0 to 255. A set point was then established such that AVT-ir cells and fibers appeared in one color and the background in another color, resulting in a binary picture of the image. The number of pixels displaying cells and fibers was divided by the total number of pixels to obtain an estimate of AVT immunoreactivity in a 2.00-mm^2 box placed within each brain nucleus where AVT neurons were present. Thus cell and fiber density is presented as the percentage coverage in an area. Separate readings were taken on each side of the brain and averaged for each animal. There were five animals per group; however, in one female the suprachiasmatic nucleus could not be measured and in one calling male and one satellite male the hypothalamus could not be measured. Groups were compared using parametric ANOVA followed by post hoc Newman–Keuls tests, unless stated otherwise.

In brain areas where significant differences were found, AVT-ir staining density measurements were also made of cell size, density of staining within individual cells, and number of cells in the same sections used for measuring surface area covered by AVT cells and fibers (see above). Cell numbers were counted on each side of the brain and averaged for each animal. For each animal, 10 of the most heavily stained cells were traced and the surface area and optical density of each cell were measured with NIH Image 1.44. Optical density was calibrated using a Kodak Density Step Wedge. The average cell surface area and optical density were determined for each subject.

RESULTS

Distribution of Cells and Fibers

Well-defined populations of AVT-ir cells were identified in several areas. The pattern of AVT-ir staining

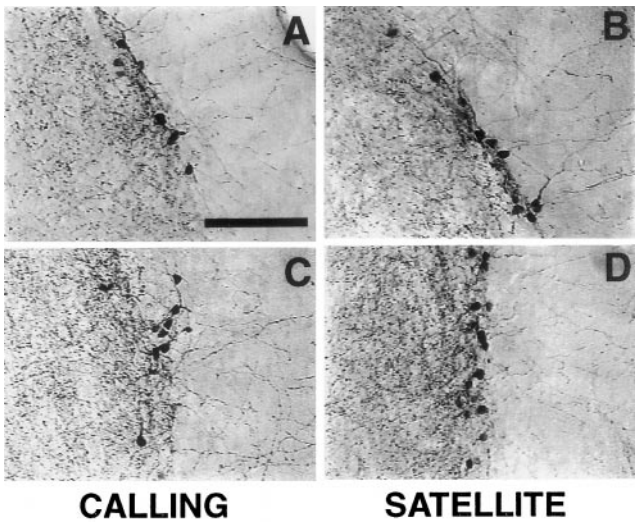

FIG. 2. Horizontal sections (right, lateral) showing AVT-ir cells and fibers in the nucleus accumbens of calling (A, C) and satellite (B, D) males of four representative male cricket frogs. Bar = 100 μm.

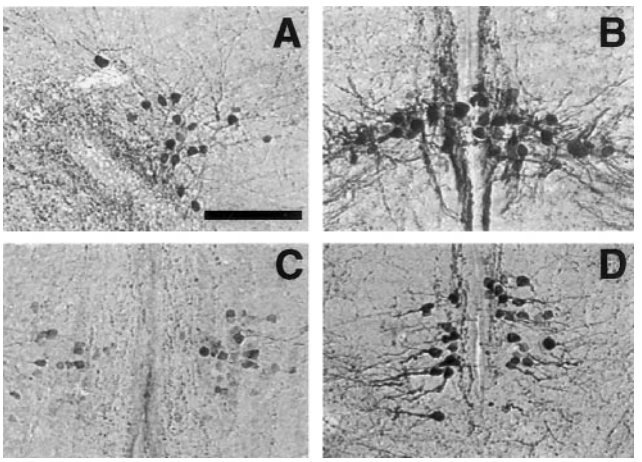

FIG. 3. Horizontal sections (right, lateral) showing AVT-ir cells and fibers in the medial amygdala (A), magnocellular preoptic region (B), hypothalamus (C), and suprachiasmatic nucleus (D) in a calling male. Bar = 100 μm.

in the forebrain regions investigated was largely comparable to that described in previous studies of anuran amphibians (Boyd *et al.*, 1992; Gonzalez and Smeets, 1992a, b; Mathieson, 1996; Marin, Smeets, and Gonzalez, 1998). One AVT-ir cell population appeared to form a continuous caudal-to-rostral distribution from the medial amygdala to the ACC (Figs. 1–3), based on cytoarchitectonic divisions and nomenclature of anuran forebrain organization described by Marin *et al.* (1998). The most rostral population of cells was coextensive with the ACC (Figs. 1, 2), and the coronal sections showed this population to travel just ventral to the lateral ventricles. These cells appeared as a long string of cells in the horizontal sections (Figs. 1, 2). The AVT-ir cells in the ACC appeared to be continuous with the second group of cells and fibers that were concentrated in the medial amygdala [Fig. 3A; nomenclature of Marin *et al.* (1998); this area is termed the lateral amygdala by Bruce and Neary (1995); see Marin *et al.* (1998) for a comparison of nomenclature of other authors]. These cells were clumped in this area, often forming a triangular shape. AVT-ir staining was high in the magnocellular preoptic region and low in the suprachiasmatic nucleus and hypothalamus (Figs. 3, 4).

AVT-ir cells and fibers were observed scattered throughout other areas. For example, AVT-ir fibers were identified at the level of the accessory olfactory bulb. AVT-ir cells were also observed scattered throughout the striatum.

Differences among Satellite Males, Calling Males, and Females

No differences were found in the density of AVT-ir cells and fibers among satellite males, calling males, and females in the medial amygdala [Fig. 4; $F(2, 12) = 1.84$, $P = 0.20$], magnocellular preoptic region [F(2,

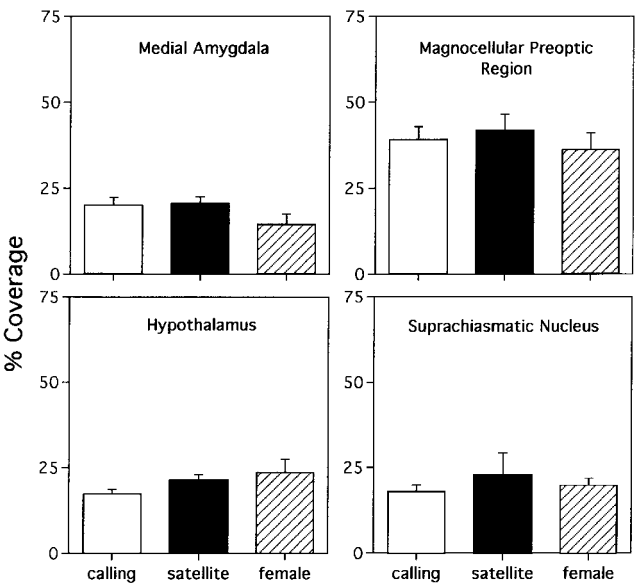

FIG. 4. Percentage coverage as a measure of AVT-ir staining in the medial amygdala, magnocellular preoptic region, hypothalamus, and suprachiasmatic nucleus in calling males, satellite males, and female cricket frogs. Data are presented as means ± SE. There are no significant differences.

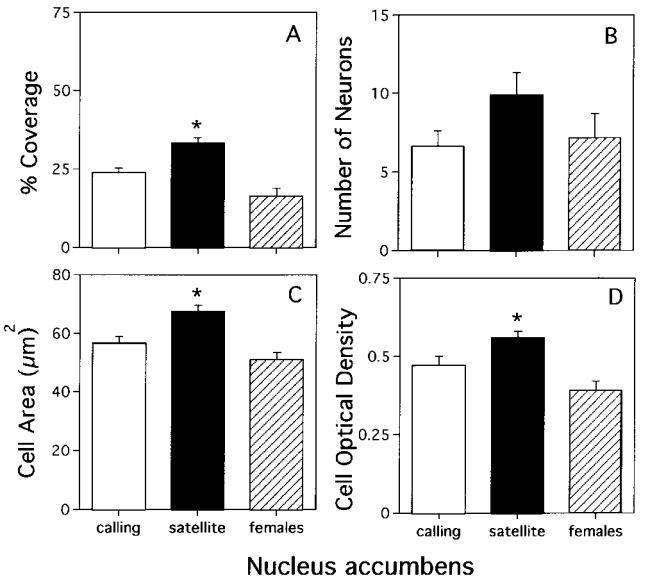

FIG. 5. Percentage coverage as a measure of AVT-ir staining (A), number of neurons (B), cell size (C), and cell optical density (D) per section of the nucleus accumbens in calling males, satellite males, and female cricket frogs. Data are presented as means ± SE. * $P < 0.05$.

12) = 0.39, $P = 0.69$], hypothalamus [$F(2, 10) = 1.24$, $P = 0.33$], or suprachiasmatic nucleus [$F(2, 11) = 0.31$, $P = 0.74$] in the horizontal sections. There were, however, significant differences in the ACC among the three groups [Fig. 5A; $F(2, 12) = 19.41$, $P = 0.0002$; analysis also repeated with Kruskal–Wallis: $H = 11.58$, $P = 0.003$]. Post hoc comparisons revealed that satellite males had significantly more AVT-ir staining than both calling males (Figs. 2 and 5A; $P = 0.02$) and females (Fig. 5A; $P = 0.0002$) in the ACC. Calling males also had a higher level of staining than females ($P = 0.05$).

Some of these differences in density of AVT-ir cells and fibers in the ACC can be explained through an analysis of individual cells (Figs. 5B–5D). There was a significant difference among the three groups in cell size [$F(2, 12) = 13.89$, $P = 0.0008$], with satellite males having larger cells than calling males ($P = 0.006$) and females ($P = 0.0008$). No significant difference, however, was found between calling males and females, although there was a nonsignificant trend ($P = 0.09$). There was also a significant difference among the three groups in density of staining within cells [$F(2, 12) = 12.97$, $P = 0.001$]. Satellite males had more cell AVT-ir staining than calling males ($P = 0.01$) and females ($P = 0.001$). Calling males also had significantly higher levels of staining than females ($P = 0.05$). While cell size and staining within cells differed among the three groups, no significant differences were found in number of cells [Kruskal–Wallis ANOVA, $H(2, 15) = 3.28$, $P = 0.19$].

Differences in overall AVT-ir staining in the ACC may also be caused, in part, by a difference in fiber AVT-ir staining. We measured this indirectly by measuring the fibers just medial to the cells in the ACC (Fig. 2). There were significant differences among all three groups (Fig. 6; $F(2, 12) = 5.08$, $P = 0.03$]. Satellite males had significantly more AVT-ir staining than calling males ($P = 0.02$), although only a nonsignificant trend was found between satellite males and females ($P = 0.06$). There was no significant difference in AVT-ir staining between calling males and females.

DISCUSSION

Previous studies examining how AVT influences mating and aggressive behavior in anurans have clearly demonstrated that AVT increases calling behavior, but there has been no evidence statistically correlating natural variations in calling behavior with AVT levels (Penna *et al.*, 1992; Boyd, 1994a; Marler *et al.*, 1995; Propper and Dixon, 1997; Semsar *et al.*, 1998; Chu *et al.*, 1998). We found, under field conditions, that males calling to attract females had significantly less AVT-ir staining in the ACC than noncalling satellite males which obtain mates by intercepting females approaching a calling male. Thus there is an association between alternative reproductive strategies and AVT immunoreactivity in the ACC. In this same species we previously found that AVT increases the probability that male cricket frogs will call while in

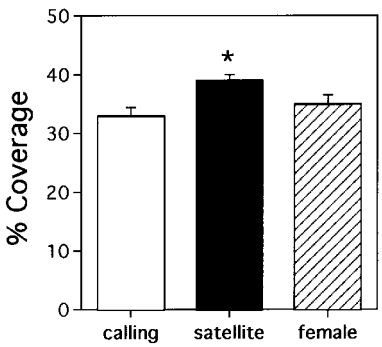

FIG. 6. Percentage staining as a measure of density of AVT-ir fibers medial to the nucleus accumbens in calling males, satellite males, and female cricket frogs. Data are presented as means ± SE. * $P < 0.05$.

a chorus, during and after a simulated agonistic encounter, as well as earlier during the encounter (Marler *et al.*, 1995; Chu *et al.*, 1998). In other words, AVT may motivate males to call more vigorously across a variety of social conditions, again strengthening the link between AVT levels and calling behavior. The distribution of AVT in the brain also suggests that AVT may be influencing motivation. While the function of the ACC in amphibians is not known, it is associated with motivation related to sexual behavior in mammals (review by Robbins and Everitt, 1996; Packard, Cornell, and Alexander, 1997). These studies clearly support the hypothesis that one underlying cause of variation in calling behavior is differences in the neuropeptide AVT.

How do we interpret what the differences in AVT immunoreactivity levels mean? In rodents, relative differences found using AVP staining densities are identical to those found using radioimmunoassays of AVP concentrations (e.g., Everts, de Ruiter, and Koolhaas, 1997). This suggests that the AVT staining density differences we observed in the cricket frog ACC reflect differences in AVT levels in this area. A criticism that arises with the use of immunoreactivity to measure neuropeptide differences is the difficulty in interpreting whether the differences in immunoreactive levels are caused by a difference in synthesis and/or release of the neuropeptide. The advantage, however, of this method is that we can obtain a snapshot of the relative AVT levels and distribution in the brain when an animal is expressing a behavior in its natural habitat. We can also hypothesize which scenarios could explain the results obtained.

In this study, one reasonable scenario is that calling males are releasing more AVT than satellite males while they are expressing the different mating behaviors (or satellite males are inhibiting the normal release of AVT). Several lines of evidence support this scenario. First, AVT injections cause males to call more frequently and earlier in agonistic encounters, not only in cricket frogs (Marler *et al.*, 1995; Chu *et al.*, 1998), but also in a number of other anuran species (Penna *et al.*, 1992; Boyd, 1994a; Propper and Dixon, 1997; Semsar *et al.*, 1998). Second, males were collected while they were displaying the behavioral characteristics of calling or not calling (brains were fixed within minutes of capture). Because males are predicted to release more AVT while they are calling, calling should cause a decrease in brain AVT reserves. In the monogamous prairie vole, *Microtus ochragaster*, behavioral changes (e.g., increased mate guarding behavior) that can be caused by increased AVP levels are also associated with a decrease in AVP-ir staining in the lateral septum and the lateral habenular nucleus (Bamshad *et al.*, 1993, 1994; Winslow *et al.*, 1993). Third, AVT injected directly into the brain of gray treefrogs can induce calling behavior (Marler, unpublished data), suggesting that AVT can influence behavior via central effects. We therefore propose that calling males release more AVT from neurons in the ACC than satellite males, depleting reserves within the cells. The released AVT then elicits calling behavior. Another potential scenario is that the brains of the calling males produce less AVT, but are more sensitive to AVT. These hypotheses remain to be tested.

Sexual dimorphisms exist in the extrahypothalamic regions (including the medial amygdala and bed nucleus of the stria terminalis) in numerous species, with AVT/AVP levels typically lower in females than in males [e.g., birds: Jurkevich, Barth, Aste, Panzica, and Grossman (1996); monkeys: Wang, Moody, Newman, and Insel (1997); lizards: Stoll and Voorn (1985)]. Our data are consistent with the sex differences previously found in other species. This sexual dimorphism has also been found in bullfrogs, *Rana catesbeiana* (Boyd, 1994a), and in the roughskin newt, *Taricha granulosa* (Boyd and Moore, 1992). How does variation in these extrahypothalamic regions in male cricket frogs fit into this scenario? Both female and satellite male cricket frogs do not call, yet AVT levels are higher in satellite males than calling males and AVT levels are lower in females than calling males. One interpretation is that satellite males retain the ability to call (via stores of adequate amounts of AVT) in cricket frogs, whereas females do not. Another possibility is that AVT may be released in females for purposes other than calling behavior (review by Boyd, 1997).

In mammals testosterone (T) influences many of the same behaviors as AVP (Nelson, 1995) and the two may act in concert to shape the sexually dimorphic behaviors described above (Wang and De Vries, 1995; Delville, Mansour, and Ferris, 1996; Albers, Liou, and Ferris, 1988). In anuran males, higher T levels are associated with mating and calling behavior and are thought to be necessary but not sufficient to induce calling behavior (review by Houck and Woodley, 1995; Emerson and Hess, 1996; Marler and Ryan, 1996; Solis and Penna, 1997; but see Mendonca, Licht, Ryan, and Barnes, 1985). Castrated *H. cinerea* males will exhibit AVT-induced calling behavior only when given T implants (Penna *et al.*, 1992). In addition, castration decreases AVT concentrations in the amygdala of adult male bullfrogs (Boyd, 1994b). AVT therefore can also interact with T and influence sexually dimorphic

behavior such as calling behavior. Why would both AVT/AVP and T influence these behaviors? AVT/AVP may be more involved in activating and perhaps fine tuning rapid responses to social encounters (e.g., Semsar *et al.*, 1998), whereas T may be more important for organizing neurochemical systems for the breeding season so that the neurochemical systems respond to changes in the social environment.

Hormonal differences have been found in other species displaying alternative phenotypes, although the emphasis has been on sex steroid hormones. There is considerable variation among species with alternative strategies (reviews by Moore, 1991; Moore, Hews, and Knapp, 1998). In some species these phenotypes are developmentally fixed. In other species individuals can switch between strategies either with (as in sex changing fish) or without (as in some anurans) any morphological changes. Moore and colleagues have proposed that irreversible changes in alternative phenotypes may be determined by organizational effects of hormones, whereas reversible switches during adulthood between alternative phenotypes may be more likely to be controlled by activational effects of hormones (Moore, 1991; Moore *et al.*, 1998).

Neuropeptides may complement sex steroid hormone effects such that T could have organizational effects on these neuropeptide systems in fixed alternative strategies, but neuropeptides, i.e., AVT, may be particularly important in rapid reversible changes such as in the marine goby (Grober and Sunobe, 1996) and the cricket frog. Both of these species can display rapid changes between behavioral phenotypes (see introduction) which have been associated with differences in the AVT neurochemical system. AVT/AVP can cause behavioral changes within minutes (e.g., Ferris and Potegal, 1988; Albers *et al.*, 1988; Marler, unpublished data) in contrast to T, which can take days to weeks to cause behavioral changes. Another teleost fish, the plainfin midshipman (*Porichthys notatus*), does not show rapid changes between phenotypes, but instead displays developmentally fixed, alternative reproductive strategies. In contrast to the cricket frog and the marine goby, the alternative reproductive phenotypes of the plainfin midshipman do not have any differences in AVT-ir cells that cannot be explained primarily by differences in body size or that are at least likely to be set prior to maturation (Foran and Bass, 1998). Based on previous studies and on the central nervous system differences we find in male cricket frogs we propose that AVT/AVP may play an important role in influencing reversible changes and may be particularly important for reversible behavioral changes that occur rapidly in response to changing social conditions. The AVT differences we find in the ACC of cricket frog males may be one neural correlate of fine-tuning in response to social conditions, reflecting the adjustment of calling behavior that is one of the fundamental differences in the behaviors of satellite and calling males.

ACKNOWLEDGMENTS

We thank Ying Tang and Patricia Martin for technical assistance, Joanne Chu for help in collecting frogs, Mike Mooseker for use of equipment in his laboratory, and William Bement, Janet Bester, Jim Bull, Joanne Chu, Kurt Klomberg, Blinda McCelland, and Mike Ryan for discussion and comments on the manuscript. We also thank Richard Gill and the subsequent owners of Gill Ranch and L. Stengl for permission to work on their property. This research was supported by NIMH R03 MH53392 and NIMH NRSA F32 MH10204 (C.A.M.), NIH R29 HD24653 (S.K.B.), and NIMH RO1 MH 45350 (W.W.).

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