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Mammalian nonapeptides activate territorial behavior in an amphibian

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ABSTRACT

Arginine vasopressin (VP) and oxytocin (OT) are two nonapeptides present in mammals and execute a wide array of physiological and behavioral functions. In amphibians arginine vasotocin (VT) is hypothesized as a homologous nonapeptide for VP and also performs physiological and behavioral tasks. Studies have demonstrated that the structural and functional relationships between VP, OT, and VT receptor families are similar; however, little behavioral data has complimented these studies. The objective of this investigation was to determine if the mammalian nonapeptides VP and OT would activate behavioral manifestations naturally activated by VT. Frogs are particularly attractive for such an investigation because it is well documented that VT activates advertisement calling and territorial behavior. This investigation was a large sample size field study that utilized the territorial frog, *Eleutherodactylus coqui*. Fieldwork occurred on the Islands of Puerto Rico and Hawai'i and focused on territorial (calling) and non-territorial (silent) males. Frogs were administered exogenous injections of VP, OT, VT (positive control), or saline (control) in the field, placed back in their original locations, and were observed for behaviors. Exogenous injections of VP and OT significantly activated silent males to emit advertisement calls and exhibit territorial behavior. Additionally, silent males moved into new areas prior to calling whereas territorial males remained in their own territories. Control (saline) males displayed normal behaviors. This is the first study to demonstrate that mammalian nonapeptides activate calling and territorial behaviors in frogs and corroborates the close evolutionary relationships within the nonapeptide family.

1. Introduction

Nonapeptides are an archaic family of conserved peptides that have evolved for > 700 million years. Functional properties of nonapeptides and their phylogenetic dispersion within vertebrate clades are wide-ranging and diverse [1], and yet the biochemical differences among the nonapeptides are remarkably minor [2]. Vasopressin- and oxytocin-related nonapeptides are present in representatives of both protostomian and deuterostomian lineages [3–8]. This suggests that this signaling system originated very early in metazoan evolution. Due to the structural and positional similarities of the vasopressin and oxytocin genes it is hypothesized that they originated from the duplication of a common ancestral gene, likely following the radiation of the jawless fish about 500 million years ago [3,6,9–10]. This genetic event is certainly plausible since gene duplication is a common evolutionary pathway toward the adaptation of genes to new functions [11].

Arginine vasopressin (VP) and oxytocin (OT) are two nonapeptides that are present in mammals and execute a wide array of physiological and behavioral functions [12]. In other classes of vertebrates these two neuropeptide systems are not present but homologous neuropeptide systems are existent. Instead of VP, arginine vasotocin (VT) occurs in birds, reptiles, amphibians, and fish [2,3]. Co-evolving with these peptides were their respective receptors and signaling pathways that are responsible for conducting molecular and cellular functions.

Functionally, nonapeptides execute an assortment of physiological and behavioral tasks. Traditionally, VP is known as an antidiuretic hormone for its role in water retention [13], vasoconstriction [14], and water homeostasis [15]. Meanwhile OT is classically recognized for its physiological role during parturition and lactation [16–18]. Intriguingly, a large volume of literature has been generated on the behavioral outcomes of nonapeptides. While substantial variation ensues between taxa, all linages of vertebrates are characterized by having specific behaviors governed by these peptides that can include: aggression, agonistic behavior, pair-bond formation, vocalizations, gregariousness, cooperation, and paternal and/or maternal care [for reviews see: [1,3,19–22]].

A number of investigations (and reviews) on nonapeptides have concentrated on the functional aspects of nonapeptide systems [23–27]. While the majority of these studies have utilized mammals as their model organism some have employed amphibians [28,29] and fish [30,31]. For example, it was discovered replacing the fish gene for isotocin (teleost homolog of OT) with the mammalian gene for OT in

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transgenic rats did not adversely affect their physiology [31]. Further, it was demonstrated that the mammalian nonapeptides, OT and VP, can modulate social behavior in fish [32]. This could indicate that receptor mechanisms and signaling factors mediating the physiological regulation of nonapeptides are possibly conserved between mammals and fish. Investigations in both newts and frogs disclosed that both mesotocin and vasotocin 1a nonapeptide receptors are present [24,28,29]. In amphibians, the VT system activates indispensable social and reproductive behaviors [33–37] and it is firmly established that in male frogs VT activates territorial and reproductive behaviors [38]. In fact, previous research in the Puerto Rican coquí frog, *Eleutherodactylus coqui*, has demonstrated that VT will activate advertisement vocalizations [37]. These are signals used in the establishment and maintenance of territories as well as the attraction of mates. This investigation also found that exogenously activated, non-territorial frogs moved into and established new territories and commenced advertisement calling [39].

Clearly, nonapeptides perform a key role in the expression of social behaviors and that distinctive classes of nonapeptides exist in different clades of vertebrates. The objectives of this investigation were to 1) determine if the closely related non-amphibian nonapeptides, VP and OT, would activate territorial behavior in male Puerto Rican coquí frogs, *E. coqui*; 2) determine if non-territorial frogs move into new territories and commence advertisement calling, and 3) to elucidate, if VP and/or OT activate additional and/or alternative social or reproductive behaviors. Due to functional and structural components of nonapeptide receptor-ligand binding properties it is hypothesized that VP and OT will activate advertisement calling and territorial behaviors in *E. coqui*.

2. Materials and methods

2.1. Field site locations

Field work was carried out at two locations: 1) the Caribbean National Forest located in the Luquillo Mountains of northeastern Puerto Rico, approximately 1 km east of the El Verde Field Station (350–400 m) of the University of Puerto Rico at Rio Piedras; and 2) the Waiakea Field Research Area (500 m) of the University of Hawai'i at Hilo, 924 Stainback Rd., Hilo, HI. Permits were obtained from the Departmento de Recursos Naturales y Ambientales of Puerto Rico and from the Department of Land and Natural Resources, Division of Forestry and Wildlife of the State of Hawaii for work on *E. coqui*. Care of all animals was conducted in accordance with the regulations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committees at New York University and the University of Hawaii. Experiments were conducted throughout the year that coincided with *E. coqui* continuous year-around breeding season. No differences of reproductive behavior have been noted during various times of the year [39–42]. At both locations, choruses typically begin around sunset, approximately 6–7 pm [either AST or HST] depending on season of the year, and lasted well into the night; although most males ceased calling between 2400 and 0100 h. *E. coqui* is endemic to the Island of Puerto Rico; populations of Hawaiian *E. coqui* are the result of accidental release on the Island of Hawai'i [43]. No differences in any category of results were found between Puerto Rican and Hawaiian frogs.

2.2. Male identification and individual marking

Prior to capture, territorial and silent males were observed for several minutes to ensure behavioral/hierarchical status. Males that were detected to produce advertisement calls to advertise (and defend) an area were assigned as territorial males. Males within one meter (distance explained later) of a territorial male that did not produce any vocalizations and did not physically contact the territorial male were assigned as silent ("satellite-like") males. Silent males were always captured in the territory of a calling male and observed for several minutes to ensure silent status, i.e., no calling or other territorial characteristics. After determining the social status of the male, the frog was captured and injected with a drug or saline (control). To identify and observe individual males the dorsum of the frog was marked with a non-toxic fluorescent powder (Pearl Ex Pigments; Rupert, Gibbon, Spider, Inc., Healdsburg, CA) that would produce excellent illumination when exposed to a UV flashlight. Following injections, all vocalizations, behavior, movement, and respected time were recorded. Frogs were injected shortly after sunset 7–8 pm [local AST or HST] depending on the season of the year and were observed throughout the remainder of the evening until the time when the majority of frogs ceased calling (~2400–0100 h).

2.3. Pharmaceutical administrations

All pharmaceutical treatments were conducted in the field and specific treatments (drugs) were blind to the observer. Frogs (calling and silent) were located with the aid of a flashlight and captured by hand with the assistance of a headlamp. Once captured all males were quickly measured for snout-vent length (SVL) and then injected intraperitoneal (IP) with a respected drug or control (randomly selected). Frogs were injected within the first hour and half after sunset. All treated frogs (experimental and controls) were at least 10 m away from other treated frogs. In addition, no territory had both territorial and silent treated individuals and no frogs were every used more than once. Following injection, experimental and control groups, all frogs were placed back in the exact location that they were captured.

2.4. Nonapeptides

Territorial and silent males were IP injected with either 50 μg VP, OT, or VT (Sigma Chemical Co., St. Louis, MO)/100 μl amphibian Ringer's saline cocktail or 100 μl of amphibian Ringer's saline (control). The dosage of administrations were determined from a previous VT study on *E. coqui* [39] and a preliminary study (10–100 μg VP and 10–100 μg OT in 100 μl of amphibian Ringer's saline) that indicated 50 μg of VP and OT was the lowest dose that resulted in the most consistent response of vocalizations and behavioral changes. Furthermore, it has been demonstrated that all three of the peptides cross the blood-brain barrier when given systemically (IP) [44–46] and that the average variance in adult male frog size was < 5% (SVL = 32.6 mm ± 0.4).

2.5. Control groups

Two groups of controls were included in the experiments. Positive controls consisted of VT-injected territorial and silent males due to the fact that it has been demonstrated that this nonapeptide activates territorial behaviors in male *E. coqui* [39] and is a closely related neuropeptide to both VP and OT [3,6,9–10]. Controls (negative) consisted of saline injections into both territorial and silent males.

2.6. Pharmaceutical and control groups

Experimental groups: Fifty territorial and 50 silent males were IP injected with 50 μg of VP/100 μl amphibian Ringer's saline cocktail. For the OT assemblages, another 50 territorial and 50 silent males comprised another set of experimental groups and were injected with 50 μg of OT/100 μl amphibian Ringer's saline cocktail. No individual frog received more than one drug treatment and all frogs were placed back in the exact location that they were captured.

Control frogs: Fifty territorial and 50 silent were injected with 100 μl of amphibian Ringer's saline solution and placed back in the exact location where they were captured. The positive control group consisted of 50 territorial and 50 silent males that were injected with