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Involvement of the neurosteroid 7α-hydroxypregnenolone in the courtship behavior of the male newt *Cynops pyrrhogaster*

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ABSTRACT

Reproductive behavior in amphibians, as in other vertebrate animals, is controlled by multiple hormones. A neurosteroid, 7α-hydroxypregnenolone, has recently been found to enhance locomotor activity in the male newt, *Cynops pyrrhogaster*. Here, we show that this neurosteroid is also involved in enhancing the expression of courtship behavior. Intracerebroventricular (ICV) injection of 7α-hydroxypregnenolone enhanced courtship behavior dose-dependently in the sexually undeveloped males that had been pretreated with prolactin and gonadotropin, which is known to bring the males to a sexually developed state. But, unlike the case in the locomotion activity, 7α-hydroxypregnenolone did not elicit the behavior in males receiving no prior injections of these hormones. ICV administration of ketoconazole, an inhibitor of the steroidogenic enzyme cytochrome P450s, suppressed the spontaneously occurring courtship behavior in sexually active males. Supplementation with 7α-hydroxypregnenolone reversed the effect of ketoconazole in these animals. It was also demonstrated that the effect of the neurosteroid on the courtship behavior was blocked by a dopamine D2-like, but not by a D1-like, receptor antagonist. These results indicate that endogenous 7α-hydroxypregnenolone enhances the expression of the male courtship behavior through a dopaminergic system mediated by a D2-like receptor in the brain.

Introduction

During the breeding season, the male red-bellied newt, *Cynops pyrrhogaster*, attracts a female partner using the female-attracting pheromone sodefrin (Kikuyama et al., 1995). This pheromone is secreted from the male's abdominal gland into the surrounding water and, through vigorous vibrations of the tail, the water is directed towards the female's snout. The male then creeps in front of the targeted female, who starts to follow the male, with her snout making contact with his tail. The male then discharges spermatophores, which are captured by the female's cloacal orifice and transported into the cloacal cavity (Kikuyama et al., 2003). Long-term research conducted by our group has demonstrated that multiple hormones, including androgen (Toyoda et al., 1993), prolactin (PRL) (Toyoda et al., 1993, 1996, 2005), gonadotropin (GTH) (Toyoda et al., 1993), and arginine vasotocin (AVT) (Toyoda et al., 2003), are involved either directly or indirectly in the expression of this behavior by the breeding red-bellied male newts. This is exemplified by the large increase in plasma concentrations of PRL (Matsuda et al., 1990), GTH (Tanaka et al., 1980), and androgen (Tanaka and Takikawa, 1983) in male newts during the breeding season in comparison to the non-breeding season.

Various regions of the vertebrate brain are able to synthesize bioactive steroids from cholesterol (for reviews, see Baulieu, 1997; Compagnone and Mellon, 2000; Do Rego et al., 2009; Mellon and Vaudry, 2001; Tsutsui et al., 1999, 2003). Studies in mammals and various birds have revealed that these 'brain-born' steroids, termed neurosteroids, regulate various aspects of behavior activities, including aggressiveness, anxiety, sexual behavior, and locomotion (Matsunaga et al., 2004; Meieran et al., 2004; Melchior and Ritzmann, 1994; Tsutsui et al., 2008). In amphibians, Matsunaga et al. (2004) reported that a neurosteroid, 7α-hydroxypregnenolone, is present in abundance in the brain of the red-bellied newt. The synthesis of this neurosteroid was observed to reach a maximum level in the male newt during the spring breeding period concomitant with increases in their locomotor activity. Moreover, intracerebroventricular (ICV) injection of 7α-hydroxypregnenolone to non-breeding males caused an acute elevation of locomotor activity, an action shown to be mediated by dopamine via a dopamine D2-like receptor.

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During the breeding season, the sexually developed male red-bellied newt moves around more actively than the female, searching for a sexually mature female. Once a potential mate has been located, the male starts to vibrate his tail vigorously. The aim of the study reported here was to ascertain whether 7α-hydroxypregnenolone is involved in the expression of this first stage of courtship behavior.

Material and methods

Animals

Adult male and female newts (*C. pyrrhogaster*) were captured in the field during the spring (April and May) and summer (July) and brought into the laboratory for use in our experiments. During the spring, we focused on capturing newts which were spontaneously exhibiting courtship behavior in the field. Those captured during the summer were not displaying courtship behavior. It has been shown that newts are not sexually active during the summer unless they receive an appropriate hormonal treatment (Toyoda et al., 1993). The groups of male and female newts were kept separately in tanks in the laboratory and fed daily with *Tubifex* worms. Prior to injection or sacrifice, the animals were anesthetized with 0.1% *m*-aminobenzoic acid ethylester methanesulfonate (Sigma Chemical Co., St. Louis, MO, USA). All experimental procedures were approved by the Animal Care and Use Committee of Nara Medical University.

Reagents

Human chorionic gonadotropin (HCG) and ovine PRL were purchased from Teikoku Hormone Mfg. Co. (Tokyo, Japan) and Sigma Chemical Co., respectively. These hormones were dissolved in isotonic saline before being injected into the experimental animals. 7α-hydroxypregnenolone was purchased from Steraloids (Newport, RI, USA), mammalian dopamine D1-like receptor antagonist (SCH23390) and mammalian dopamine D2-like receptor antagonist (sulpiride) were obtained from Tocris (Ellisville, MO, USA), and ketoconazole, an inhibitor of the cytochrome P450 steroidogenic enzymes, and testosterone propionate (TP) were purchased from Sigma Chemical Co. Prior to use, these reagents were dissolved in a vehicle, dimethyl sulfoxide (DMSO; Wako, Pure Chemical, Osaka, Japan).

Injections

ICV injections of 7α-hydroxypregnenolone, ketoconazole, SCH23390, sulpiride, and their vehicle (DMSO) were performed according to the method described by Toyoda et al. (2003). Briefly, a glass micropipette (tip diameter = 50 μm) filled with 1 μl of the chosen solution and connected to a microsyringe was inserted, with the help of a micromanipulator to facilitate proper placement, into the third ventricle to a depth of approximately 1 mm through a small hole drilled (drill bit diameter = 0.5 mm) in the parietal bone posterior to the bregma. Ten seconds after the micropipette had been inserted, the contents of the micropipette were infused over a 5-second period, and 20 s later, the micropipette was removed. The hole was filled with acrylic resin (Shofu, Kyoto, Japan). Immediately after the injection, the animals were returned to water. The accuracy of the injection was confirmed in a preliminary experiment by visually inspecting the brains of the newts injected with 1 μl of 0.15% methylene blue dissolved in isotonic saline.

Observation of male courtship behavior

Each test male was paired with a sexually developed female captured in the field or with a "summer" female which had achieved sexual maturity following intraperitoneal (IP) injections of PRL (1 IU) and HCG (25 IU) on 7–10 successive days. The first behavioral test was conducted 2 h after the last injection of hormones and/or drugs, unless stated otherwise. New test animals were used for each test, and no animal was ever used repeatedly. The incidence and frequency of the targeted courtship behavior, i.e., vibration of the tail in front of the female partner, were monitored for 1 h using the method described by Toyoda et al. (1993). Incidence and frequency were expressed as the percentage of animals exhibiting the behavior and the mean number of times the behavior was recorded per test animal over the 1-h test period, respectively.

Statistical analyses

The incidence of tail vibration behavior was statistically analyzed using Fisher's two-tailed exact tests (Siegel, 1956). Other experimental data were analyzed using the Kruskal–Wallis one-way analysis of variance (ANOVA) followed by the Mann–Whitney *U* test. A *P* value of <0.05 was considered to be significant.

Results

Effect of 7α-hydroxypregnenolone on the expression of courtship behavior

To determine the effect of 7α-hydroxypregnenolone on the expression of courtship behavior, we observed the tail vibration behavior of "summer" male newts which had received IP injections of PRL (1 IU) and HCG (25 IU) every other day for 1 week followed by ICV injections of various doses of 7α-hydroxypregnenolone. Control animals received the vehicle only. Primed male newts receiving ICV injections of 7α-hydroxypregnenolone showed an increased incidence and frequency of the tail vibration behavior that was dose-dependent (Fig. 1A). The minimum effective amount was 10 ng.

In "summer" males that had not been pretreated with PRL and HCG, the ICV injections of 7α-hydroxypregnenolone barely elicited the tail-vibrating behavior, even at a dose of 100 ng (Fig. 1B).

Effect of ketoconazole on the expression of courtship behavior

In order to determine whether endogenous 7α-hydroxypregnenolone is involved in eliciting the courtship behavior, we tested the effect of ketoconazole, an inhibitor of cytochrome P450s, on male newts captured in spring that were spontaneously exhibiting the courtship behavior in the field. Twenty-four hours after capture, "spring" male newts received an ICV injection of 2 μg of ketoconazole. Control animals received the vehicle only. Every 2 h until 8 h after injection, each male was paired with a test female captured similarly in the field, and courtship behavior was monitored. The suppressive effect of ketoconazole on the expression of the behavior became conspicuous 6 and 8 h after the treatment (Fig. 2A).

In another set of experiment, effect of ICV injection of various doses of ketoconazole on the courtship behavior of "spring" males captured in the field was examined. Eight hours after the injection, the behavioral test of each male was performed by introducing a test female captured similarly in the field, and courtship behavior was monitored. ICV injection of ketoconazole decreased both the incidence and frequency of the tail-vibrating behavior. The minimum effective dose of ketoconazole was 2 μg (Fig. 2B).

The ability of 7α-hydroxypregnenolone to restore the courtship behavior suppressed by ketoconazole was tested by injecting the male newts that showed suppressed tail-vibrating behavior due to a 2-μg ICV injection of ketoconazole with 100 ng 7α-hydroxypregnenolone at 6 h after the ICV injection of 2 μg ketoconazole. After an elapse of 2 h following ICV administration of the neurosteroid or its vehicle, each male was subjected to the behavioral test. As shown in Fig. 3, the group of newts that had shown suppressed tail-vibrating behavior following injection with 2 μg ketoconazole resumed the tail-vibrating behavior following the subsequent administration of 7α-hydroxypregnenolone. Injection of