General and Comparative Endocrinology 224 (2015) 96–103

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Possible hormonal interaction for eliciting courtship behavior in the male newt, *Cynops pyrrhogaster*

Fumiyo Toyoda [a,*], Itaru Hasunuma [b], Tomoaki Nakada [c], Shogo Haraguchi [d], Kazuyoshi Tsutsui [d], Sakae Kikuyama [b,d]

[a] Department of Neurophysiology, Nara Medical University, Nara 634-8521, Japan
[b] Department of Biology, Faculty of Science, Toho University, Chiba 274-8510, Japan
[c] Department of Comparative and Behavioral Medicine, Nippon Veterinary and Life Science University, Tokyo 180-8602, Japan
[d] Department of Biology, Waseda University, and Center for Medical Life Science of Waseda University, Tokyo 162-8480, Japan

ARTICLE INFO

Article history:
Received 9 January 2015
Revised 7 June 2015
Accepted 29 June 2015
Available online 2 July 2015

Keywords:
Newt
Courtship behavior
Hormones

ABSTRACT

Reproductive behavior in amphibians, as in other vertebrate animals, is under the control of multiple hormonal substances. Prolactin (PRL), arginine vasotocin (AVT), androgen, and 7α-hydroxypregnenolone (7α-OH PREG), four such substances with hormonal activity, are known to be involved in the expression of the tail vibration behavior which is the initial step of courtship performed by the male newt, *Cynops pyrrhogaster*. As current information on the interaction(s) between these hormones in terms of eliciting tail vibration behavior is limited, we have investigated whether the decline of expression of tail vibration behavior due to suppression of the activity of any one of these hormones can be restored by supplying any one of the other three hormones exogenously. Expression of the behavior was determined in terms of incidence (% of test animals exhibiting the behavior) and frequency (number of times that the behavior was repeated during the test period). Neither PRL nor androgen restored the decline in the incidence and frequency of the tail vibration behavior caused by the suppression of the activity of any one of other three hormones. AVT completely restored both the anti-PRL antibody-induced and flutamide (an androgen receptor antagonist)-induced, but not ketoconazole (an inhibitor of the steroidogenic CYP enzymes)-induced decline in the incidence and frequency of the tail vibration behavior. The neurosteroid, 7α-OH PREG, failed to restore flutamide-induced decline in the incidence and frequency of the behavior. However, it was able to restore both anti-PRL antibody-induced and AVT receptor antagonist-induced decline in the incidence, but not in the frequency of the behavior. In another experiment designed to see the activity of hormones enhancing the frequency of the tail vibration behavior, AVT was revealed to be more potent than 7α-OH PREG. The role of each hormonal substance in determining the expression of the tail vibration behavior was discussed based on the results.

1. Introduction

In terms of reproductive systems, newts are unique in that fertilization takes place internally, but in the absence of specific copulatory organs. To achieve successful sperm transfer, the male exhibits a courtship behavior which is characteristic to each species (Salthe and Mecham, 1974). During the breeding season, the male red-bellied newt, *Cynops pyrrhogaster*, attracts a female partner by releasing a specific sex pheromone, sodefrin (Kikuyama et al., 1995), into the surrounding water, sending it toward the chosen female's snout by vibrating his tail vigorously. The male newt performs this behavior repeatedly, following which he parades in front of the female, who then follows him. The male ultimately deposits spermatophores, and the female picks them up with her cloacal orifice (Kikuyama et al., 2003).

Extensive studies have been carried out using intact, castrated, and hypophysectomized male red-bellied newts, both in the breeding and non-breeding season, with the aim of elucidating the hormonal factors involved in the expression of this courtship behavior (Toyoda and Kikuyama, 2000; Kikuyama et al., 2003). Earlier work on the male red-bellied newt suggested that both prolactin (PRL) and androgen are required to maintain male courtship behavior for extended periods of time (Kikuyama et al., 1980; Toyoda et al., 1993). Endogenous PRL was found to act centrally

* Corresponding author at: Department of Neurophysiology, Nara Medical University, Shijo-chou 840, Kashihara, Nara 634-8521, Japan.
E-mail address: tfumiyo@naramed-u.ac.jp (F. Toyoda).

http://dx.doi.org/10.1016/j.ygcen.2015.06.016
0016-6480/

to bring about the tail-vibrating behavior (Toyoda et al., 1996, 2005). Subsequent studies revealed that arginine vasotocin (AVT) enhances the expression of the courtship behavior (Toyoda et al., 2003), and more recently a neurosteroid, 7α-hydroxypregnenolone (7α-OH PREG) has been added to the list of hormones involved in eliciting the courtship behavior (Toyoda et al., 2012).

To date, however, information about the interaction of these hormones is scanty. Haraguchi et al. (2010) observed that hypophysectomy decreases the synthesis of 7α-OH PREG in the brain and that the administration of PRL, but not gonadotropin, to the hypophysectomized newts causes a dose-dependent increase in 7α-OH PREG synthesis. These authors also showed the presence of the PRL receptor in the preoptic neurons expressing cytochrome $P450_{7\alpha}$ (CYP7B), a steroidogenic enzyme that catalyzes the formation of 7α-OH PREG. It is therefore likely that at least one of the roles of PRL in eliciting male courtship behavior is to stimulate 7α-OH PREG synthesis.

The aim of our study was to increase our understanding of the interaction between PRL, AVT, androgen, and 7α-OH PREG in terms of eliciting the male courtship behavior. To this end, we investigated whether the decline in the expression of this behavior due to suppression of the activity of one of these hormones can be restored by supplying exogenously any one of the other three hormones. On the basis of the results, we hypothesized as follows. If the declined behavior thus caused by suppressing the activity of a given hormone is rescued by the administration of any one of other hormones, the latter hormone is involved in the downstream of the pathway of hormonal action exerted by the former. If the latter fails to rescue the decline, it is involved in the upstream of the pathway or each hormone in an independent pathway. To evaluate the activity of each hormone in restoring the courtship behavior, we paid particular attention to whether the relevant hormone was effective in increasing the incidence (the number of animals which exhibited the behavior per total number of test animals) and/or the frequency (the number of times the behavior was repeated during the test period). On the basis of these criteria, we estimated whether a given hormone contributes to increase the incidence, the frequency or both of the behavior.

2. Materials and methods

2.1. Animals

Adult male and female newts (*C. pyrrhogaster*) obtained in the spring (April and May) and in the winter (January) were used as experimental animals. The "spring" newts were sexually active, exhibiting spontaneous courtship behavior in the field at the time of capture. The "winter" newts were sexually inactive, showing no courtship behavior unless they received the appropriate hormonal treatment (Toyoda et al., 1993).

The male and female newts were kept in separate tanks in the laboratory and fed daily with *Tubifex* worms. Prior to the injection of the chosen hormone or compound, the animals were anaesthetized in 0.1% *m*-aminobenzoic acid ethylester methanesulfonate (Sigma, St. Louis, MO, USA). All experimental procedures were approved by the Animal Care and Use Committee of Nara Medical University.

2.2. Reagents

Ovine PRL was purchased from Sigma Chemical Co. Human chorionic gonadotropin (HCG) was purchased from Teikoku Hormone Mfg. Co. (Tokyo, Japan). AVT and V1a (vasopressor) receptor antagonist [$d(CH_2)^1_5$, $Tyr(Me)^2$, Arg^8-vasopressin] were products of Bachem (Torrance, CA, USA). 7α-OH PREG was purchased from Steraloids (Newport, RI, USA). Ketoconazole, an inhibitor of the steroidogenic CYP enzymes, flutamide, an androgen receptor antagonist, and testosterone propionate (TP) were products of Sigma Chemical Co. Dimethyl sulfoxide (DMSO) was obtained from Wako Pure Chemical (Osaka, Japan). Normal rabbit immunoglobulin G (IgG) was purchased from Dako A/S (Glostrup, Denmark). Antibody against a synthetic oligopeptide (SLLYKTEGKNTYSEC) that matched to a portion of the extracellular domain of the newt PRL receptor was generated in a rabbit (Hasunuma et al., 2005).

2.3. Injections

PRL, AVT, the PRL receptor antibody, the V1a receptor antagonist and HCG were dissolved in saline. Ketoconazole, 7α-OH PREG, TP, and flutamide were dissolved in 99.0% DSMO. PRL receptor antibody, V1a receptor antagonist, 7α-OH PREG, ketoconazole, flutamide, and their vehicles were intracerebroventricularly injected according to the method of Toyoda et al. (2003). Briefly, a glass micropipette (tip diameter = 50 μm) filled with the chosen solution and connected to a microsyringe was inserted with the aid of micromanipulator 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, 1 μl of the solution was infused over a 5-s period; 20 s thereafter, the micropipette was removed. The hole was then filled with acrylic resin (Shofu, Kyoto, Japan). PRL, AVT, TP, HCG and flutamide in a volume of 0.05 ml were also injected intraperitoneally.

Immediately after the injection, the animals were returned to water. The effective amount of each substance in solution, with the exception of flutamide, had been determined in earlier studies (Toyoda et al., 1993, 2003, 2005, 2012).

For flutamide, we determined its effective dose in the present experiment. Injection of flutamide was performed either intracerebroventricularly or intraperitoneally. In another experiment, TP was administered intraperitoneally immediately after intracerebroventricular (ICV) injection of flutamide in order to confirm that the exogenously supplied androgen counteracts flutamide to restore the tail vibration behavior.

In a series of experiments conducted to see whether the decline of expression of the tail vibration behavior caused by a substance suppressing the activity of any one of the hormones can be restored by supplementing one of the other hormones, PRL, AVT or TP was injected intraperitoneally immediately after the ICV injection of the substance. In the case of 7α-OH PREG to be supplemented, which was administered intracerebroventricularly, its injection was performed 1 h after the injection of a substance that is to suppress the behavior. When ketoconazole was used for suppressing the behavior, the hormone supplementation was performed 4 h after ketoconazole injection since it's effect becomes conspicuous 6 h after the ICV injection (Toyoda et al., 2012)

The final experiment was conducted to compare the activity of enhancing the frequency of the tail vibration behavior between AVT and 7α-OH PREG. To this end, sexually inactive male newts (winter newts) were injected intraperitoneally with 1 IU PRL and 5 μg TP daily for 7 days. Likewise, winter female newts received 1 IU PRL and 25 IU HCG (Toyoda et al., 1993). Twenty-four hours after the last injection, the male newts received an ICV injection of AVT, 7α-OH PREG, or their respective vehicles.

2.4. Observation of male courtship behavior

Each test male newt was paired with a sexually mature female captured in the field unless stated otherwise. The behavioral test