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Hormones and Behavior 44 (2003) 346–353

Hormones and Behavior

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Involvement of arginine vasotocin in reproductive events in the male newt *Cynops pyrrhogaster*

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Received 16 December 2002; revised 22 April 2003; accepted 23 June 2003

Abstract

Effects of arginine vasotocin (AVT) on reproductive events such as courtship behavior, pheromone release, and spermatophore discharge were investigated in the male newt *Cynops pyrrhogaster*. AVT enhanced the incidence and frequency of androgen-induced courtship behavior. In this case, AVT was likely to act centrally because the behavior was evoked with a much smaller amount of AVT when the hormone was administered intracerebroventricularly than when given intraperitoneally. Involvement of endogenous AVT in spontaneously occurring courtship behavior was also evidenced by the fact that administration of a V1 (vasopressor) receptor antagonist, [d$(CH_2)_5^1$, Tyr(Me)^2, Arg^8-vasopressin] suppressed the expression of the courtship behavior. The water in which AVT-treated males had been kept showed considerable female-attracting activity as compared with the water in which saline-injected males had been kept. Moreover, the content of sodefrin, a female-attracting pheromone in the abdominal gland, was decreased by the intraperitoneal injection of AVT, suggesting that the neurohypophyseal hormone stimulated the release of sodefrin from the abdominal gland into the water. AVT induced contraction of the excised abdominal gland concentration-dependently, and, again, the V1 receptor antagonist suppressed the AVT-induced contraction. Thus, we concluded that AVT induces the pheromone discharge, acting peripherally on a contractile structure of the abdominal gland. AVT was also found to induce spermatophore deposition in the male kept in the absence of the female. Administration of the V1 receptor blocker to the sexually developed males suppressed the spermatophore deposition. All these results indicate the involvement of AVT in reproductive events acting centrally and peripherally.

Keywords: Newt; Reproductive behavior; Courtship; Sodefrin; Spermatophore; Arginine vasotocin (AVT); Vasopressor receptor antagonist

Introduction

During the early stage of courtship behavior of the red-bellied newt, *Cynops pyrrhogaster,* the male attracts the female by sending the surrounding water toward the female's snout by vibrating his tail vigorously. After this performance the male creeps in front of the partner female. She follows the male with her snout, making contact with the tail of the male, and receives spermatophores deposited by the male into her cloacal orifice. Throughout the courtship the male projects from the cloaca numerous minute tubules connected to the abdominal gland. The abdominal gland is known to contain a female-attracting pheromone, sodefrin (Kikuyama et al., 1995), which is considered to be released continuously during courtship for the fulfillment of the sperm transfer. The male courtship behavior is induced by prolactin (PRL) and androgen. Castration and/or hypophysectomy or administration of antiserum against PRL blocks the expression of courtship behavior by the male newts, and supplementation of these animals with androgen and/or PRL allows it to resume (Toyoda et al., 1993, 1996). On the other hand, arginine vasotocin (AVT) is highlighted as an important hormonal factor in the reproduction of the rough-skinned newts, *Taricha granulosa*. The *Taricha* males capture and embrace female partners and clasp them

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doi:10.1016/S0018-506X(03)00156-9

with all four limbs prior to the sperm transfer. AVT, in combination with androgen, induces this clasping behavior in the male (Moore and Zoeller, 1979). Female rough-skinned newts lay eggs several weeks after being inseminated while grasping underwater objects such as sticks, logs, rocks, and aquatic plants. This egg-laying behavior is also induced by AVT and estrogen (Moore et al., 1992). On the basis of the above-cited observations, experiments were conducted to see whether AVT is also involved in the reproductive events in the male of the genus *Cynops*. In this paper, we report that in the *Cynops* newt, AVT is involved in the expression of courtship behavior, the release of sodefrin, and the discharge of spermatophores.

General methods

Animals

Adult males and females obtained in spring or early winter were used. The spring newts were those exhibiting the courtship behavior spontaneously in the field. The winter newts were not sexually active unless they had received an appropriate hormonal treatment. They were kept in tanks in the laboratory and fed *Tubifex* worms daily. When necessary, testes were removed surgically from male newts. Prior to the surgery, injection, or sacrifice, animals were anesthetized 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.

Hormones and arginine vasopressin (AVP) receptor antagonists

Human chorionic gonadotropin (HCG) was purchased from Teikoku Hormone Mfg. Co. (Tokyo, Japan). Ovine PRL and testosterone propionate (TP) were purchased from Sigma Chemical Co. AVT, mesotocin (MT), V1 (vasopressor) receptor antagonist [$d(CH_2)_5^1$, $Tyr(Me)^2$, Arg^8-vasopressin], and V2 (antidiuretic) receptor antagonist [$d(CH_2)_5^1$, D-lle^2, lle^4, Arg^8, Ala-$NH_2{}^9$-vasopressin] were products of Bachem (CA, USA). TP was dissolved in a minute volume of ethanol and then suspended in 0.6% NaCl. Pituitary hormones and arginine vasopressin receptor antagonists were dissolved in a 0.6% NaCl solution.

Injection

Intraperitoneal (IP) injection of the appropriate amount of hormones and AVP receptor antagonists in 0.05 ml of vehicle were performed by using a tuberculin syringe. Intracerebroventricular (ICV) injection of AVT and receptor antagonists of AVP were performed according to the method described by other investigators (Moore and Miller, 1983; Salek et al., 2002) with a little modification. Briefly, a glass micropipette (tip diameter = 50 μm) was filled with each solution and then connected to a microsyringe which was inserted by the aid of a micromanipulator into the third ventricle to a depth of approximately 1 mm through a small hole drilled (drill bit diameter = 0.5mm) in parietal bone posterior to bregma. Ten seconds after inserting the micropipette, 1 μl of AVT or AVP receptor antagonists solutions were infused over a 5 s period, and then 20 s later, the micropipette was removed. The hole was filled with acrylic resin (Shofu, Kyoto). As a pilot experiment, the accuracy of the injection was confirmed by visually inspecting the brains of the newts injected with 1 μl of 0.15% methylene blue dissolved in saline. In both IP and ICV injection, control animals received the vehicles only. Immediately after injections, the animals were returned to water. Behavioral tests were commenced 4 h after injection.

Observation of male courtship behavior

Each test male was paired with a winter female, which had attained sexual maturity after treatment with PRL (1 IU) and HCG (25 IU) for 7–10 successive days, or a sexually developed spring female (Toyoda et al., 1994). In every test, the female partner was renewed and never used repeatedly. The incidence and frequency of courtship behavior, i.e., vibration of the tail in front of the partner, were monitored for 1 h. The incidence was expressed as the percentage of animals that exhibited this behavior, whereas the frequency was expressed as the mean number of times that this behavior was recorded per test animal over the test period (1 h) (Toyoda et al., 1993).

Contraction of the abdominal gland

Strips of the abdominal gland (5 mm long $\times$ 3 mm wide) were vertically suspended in a bath containing 5 ml of Ringer solution and hung on an isotonic transducer (TD-112S, Nihon Kohden, Tokyo). All preparations were initially equilibrated for 30 min. In order to study the effect of AVT on the contraction of the abdominal gland, we replaced the bath solution with AVT solution (10^{-8}–10^{-6} M) or vehicle. Contraction was measured using an amplifier (AA-601H, Nihon Kohden, Tokyo). In another set of experiments, V1 or V2 receptor antagonist (10^{-6} M) was added to the AVT (10^{-7} M) solution, and contraction of the abdominal gland was recorded. At the end of the experiment, all the gland strips were subjected to 10^{-5}M acetylcholine to ascertain the contractile potential of the preparation.

Deposition of spermatophores

The males treated with various doses of AVT or V1 receptor antagonist or saline were kept separately in each container. Eight hours later, the number of spermatophores deposited in the container by each male was counted.

Statistical analyses

Statistical analyses of incidence and frequency of the courtship behavior were performed using two-tailed Fisher's exact probability test (Siegel, 1956) and Kruskal-Wallis one-way ANOVA followed by the Mann-Whitney U-test, respectively (Siegel, 1956). In the preference test, the percentage of the time spent by the snout portion of the test female in each sector was compared by using Friedman two-way analysis of variance (ANOVA) followed by the Wilcoxon matched-pairs signed-rank test (Siegel, 1956). Other experimental data were analyzed by Kruskal-Wallis one-way ANOVA followed by the Mann-Whitney U-test.

Specific methods and results

Effect of neurohypophyseal hormones on courtship behavior

In order to see whether administered neurohypophyseal hormones have any effect on the courtship behavior, winter male newts were castrated for 30 days and then pretreated with 10 μg TP or saline intraperitoneally for the following 14 days. On the day after the last injection, the saline-injected animals received a single intraperitoneal injection of 50 μg AVT ($n = 18$), MT ($n = 10$), or saline ($n = 20$). Similarly, the TP-treated animals were injected with AVT ($n = 18$), MT ($n = 10$), or saline ($n = 20$). Four hours later, each male was paired with a female and the incidence and frequency of the courtship behavior were monitored. The castrated newts receiving saline did not show courtship behavior, and this situation was not altered by AVT or MT (data not shown). On the other hand, TP-primed castrated newts exhibited courtship behavior, with an incidence of 50% and a frequency of 1.15 ($\pm$ 0.31) per test. In the castrated newts treated similarly with androgen, AVT administration significantly enhanced both incidence (to 88.9%; $P < 0.05$) and frequency (to 4.89 $\pm$ 0.93; $P < 0.01$), but MT did not significantly increased the incidence (to 60%) or frequency (to 1.40 $\pm$ 0.40).

In another series of experiments, the effectiveness of intracranially and intraperitoneally administered AVT was compared. Castrated and androgen-primed male newts received a single ICV or IP injection of isotonic saline or various doses of AVT. Both ICV and IP injections of AVT increased the incidence and frequency of the courtship behavior dose dependently. In the case of the ICV injection, the minimum effective dose of AVT was 0.1 μg (Fig. 1A), whereas it was 50 μg when the hormone was injected intraperitoneally (Fig. 1B). In order to ascertain whether endogenous AVT is involved in the spontaneously occurring courtship behavior, male newts exhibiting courtship behavior in the field in spring were captured, kept in the labolatory for 24 h, and then injected intraperitoneally with 25 μg of a V1 receptor antagonist ($n = 10$), 25 μg of a V2 receptor antagonist ($n = 10$), or saline ($n = 10$). Four hours after the injection, each male was paired with a female captured similarly in the field, and courtship behavior was monitored for 1 h. The saline-injected newts exhibited courtship behavior with incidence (80%) and frequency (2.00 $\pm$ 0.67). V2 receptor antagonist did not significantly affect the incidence (to 80%) and frequency (to 1.80 $\pm$ 0.42). On the other hand, the V1 receptor antagonist significantly reduced in incidence (to 20%; $P < 0.05$ vs saline or V2 receptor antagonist-injected group) and frequency (to 0.30 $\pm$ 0.21; $P < 0.05$ vs saline or V2 receptor antagonist-injected group) of courtship.

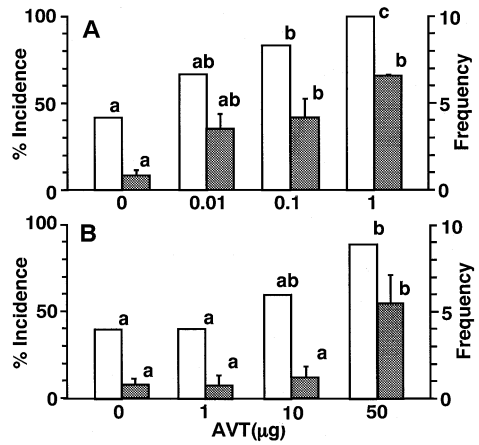

Fig. 1. Effect of various doses of AVT administered intracerebroventricularly or intraperitoneally on incidence and frequency of courtship behavior in androgen-primed castrated males. (A) ICV injection; (B) IP injection. Each ICV and IP group consisted of 12 and 10 animals, respectively. Each column and vertical bar represent the mean and SEM, respectively. Values with the same superscript do not differ significantly from each other ($P > 0.05$).

In order to study the effectiveness between ICV and IP administration of V1 receptor antagonist in suppressing spontaneously occurring male courtship behavior, male newts were captured in spring, kept for 24 h, and injected with various doses of V1 receptor antagonist either intracranially or intraperitoneally. Four hours after injection, each male was paired with a female captured similarly in the field and courtship was monitored. As shown in Fig.2A, in the case of ICV injection, 0.25 and 0.05 μg V1 receptor antagonist were required for decreasing the incidence and frequency of courtship behavior, respectively. In the case of the IP injection, the minimum effective dose of V1 receptor antagonist to decrease the incidence and frequency was considered to lie in the range of 5–25 μg (Fig.2B).

Effect of neurohypophyseal hormones on discharge of female-attracting substance

In order to ascertain whether neurohypophyseal hormones stimulate the release of female-attracting substance(s) into the water, three groups of newts obtained in

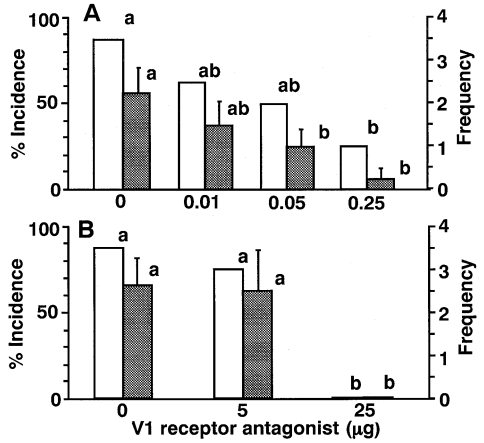

Fig. 2. Effect of intracranially or intraperitoneally administered V1 receptor antagonist on incidence and frequency in spontaneously occurring courtship behavior. (A) ICV injection; (B) IP injection. Each group consisted of eight animals. Each column and vertical bar represents the mean and SEM, respectively. Values with the same superscript do not differ significantly from each other ($P > 0.05$).

early winter, each comprising eight males, were injected with 5 μg AVT or MT or saline. Each animal was kept separately in 500 ml water for 2 days. The water thus conditioned by each male was used for the preference test. According to the method described elsewhere (Toyoda et al., 1994). Briefly, a plastic container ($\sim$ 37 cm) filled with tap water (3000 ml) was divided into three sectors into which three blocks of sponges of the same size (5.6 $\times$ 7.3 $\times$ 3.4 cm), one containing 100 ml of male-conditioned water, and the remaining two containing tap water, were placed. Each test female was introduced into a round fence ($\sim$ 15 cm) made of stainless steel mesh that had been placed in the center of the container. Thirty seconds later, the fence was taken away carefully, the position of the snout of the test animal was traced, and the time spent by the snout in each sector was recorded by a video apparatus for 10 min. One hundred ml of 10$\times$ diluted water in which the saline-injected newts had been kept did not significantly attract the test females, whereas in the case of the water in which AVT-injected newts had been kept, the 100$\times$ diluted water was still effective in attracting female newts ($P < 0.05$ vs tap water). This dilution of water in which MT-injected newts had been kept was ineffective, but the 30-times diluted water was able to attract the female newts ($P < 0.05$ or $P < 0.01$ vs tap water) (Fig. 3).

Effect of AVT on the sodefrin content in the abdominal gland

Five and six male newts captured in early winter and kept in the laboratory for 1 month received an intraperitoneal injection of 5 μg AVT and saline for 2 successive days, respectively. Twenty-four hours after the second injection, the males were sacrificed, and their abdominal glands were then dissected out. The sodefrin content of each abdominal gland was measured by radioimmunoassay (RIA), according to the method described by Yamamoto et al. (1996). Briefly, RIA was performed using a double antibody method. The sodefrin antibody diluted 1:1000 exhibited the ability to specifically bind 32% of the added radioligand in the absence of any unlabeled ligand, when 100 μl diluted antiserum and 100 μl labeled sodefrin (20,000 cpm) were added to each incubation tube containing 300 μl 1% BSA-PBS. The reference standard and test samples were serially diluted with 1% BSA-PBS and added to each assay tube containing 200 μl 1% BSA-PBS, in 100-μl volumes. The sodefrin antiserum was diluted 1:200 (final dilution 1:1000) with 0.05 M EDTA-PBS containing 1% normal rabbit serum. One hundred microliters of diluted antiserum and 100 μl labeled sodefrin diluted with 1% BSA-PBS were added to each tube. After 16 h, incubation, the immune complexes were precipitated by the addition of 200 μl of 1:100 dilution of goat anti-rabbit IgG serum with 0.05 M EDTA-PBS containing 3.2% polyethylene glycol 6000. The radioactivity in each precipitate was counted. The average count in tubes containing antiserum and labeled sodefrin, but no unlabeled sodefrin, was designated 100% and the counts in other tubes were expressed as a fraction of this. The sensitivity of the RIA averaged 30.5 $\pm$ 3.4 pg. The intra- and interassay coefficients of variation were 1.4 and 4.5%, respectively. Throughout the experiment, no sign of courtship behavior was observed in any animal. The immunoassayable sodefrin content in the abdominal gland was significantly lower ($P < 0.05$) in AVT-injected newts (1.61 $\pm$ 0.51 μg/gland) than in the saline-injected ones (4.12 $\pm$ 0.69 μg/gland).

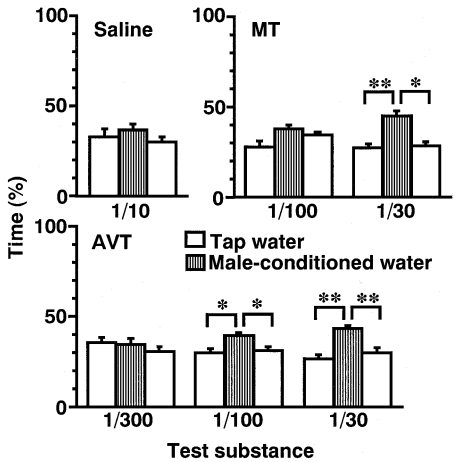

Fig. 3. Effect of neurohypophyseal hormones on the release of female-attracting substance by the male newts. Test substances were samples of water in which male newts that had received saline, MT, or AVT had been kept. Three sponge blocks, one containing a test substance the other two containing tap water, were placed in the container, where the test animal was released. Each column and vertical bar represents the mean percentage of time spent by the test animals ($n = 8$) in each sector containing a test substance and SEM, respectively, *$P < 0.05$, **$P < 0.01$ vs, tap water.

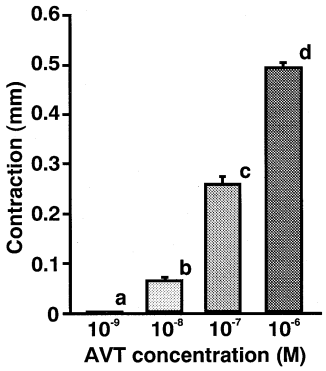

Fig. 4. AVT-induced contraction of the abdominal gland. Average responses (± SEM, $n = 8$) to the various concentrations of AVT. Each column and vertical bar represent the mean and SEM. Means with the same superscript do not differ significantly from each other ($P > 0.05$).

Effect of AVT on the contraction of the abdominal gland

In another series of experiments, we studied the effect of AVT on the contraction of the abdominal gland on the assumption that AVT acts on the contractile element around the tubular structure of the gland. Male newts obtained in early winter were sacrificed by decapitation and the abdominal gland was excised and subjected to AVT, V1 receptor antagonist, or V2 receptor antagonist. AVT induced the contraction of the gland concentration dependently (Fig. 4). The V1 receptor antagonist but not the V2 receptor significantly suppressed the AVT-induced contraction ($P < 0.05$) (Fig. 5).

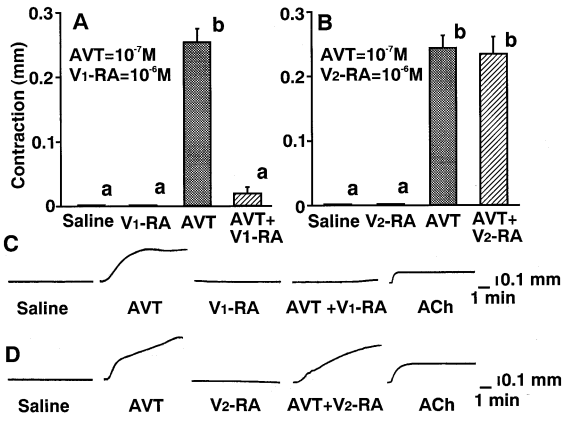

Fig. 5. Effect of AVT and its receptor antagonists on the contraction of the abdominal gland. (A) Each bar shows the average response (±SEM, $n = 8$) to saline or to AVT with or without the V1 receptor antagonist. (B) Each bar shows the average response (±SEM, $n = 8$) to saline or to AVT with or without the V2 receptor antagonist. Each column and vertical bar represent the mean and SEM. Means with the same superscript do not differ significantly from each other ($P > 0.05$). (C) Representative profiles of contractile responses to saline or AVT with or without the V1 receptor antagonist. (D) Representative profiles of contractile responses to saline or AVT with or without the V2 receptor antagonist. The responsiveness to acetyl choline (Ach 10^{-5} M) was checked after removal of test substances.

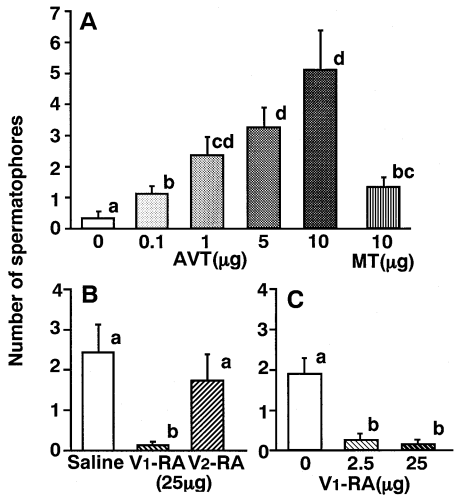

Fig. 6. Effect of neurohypophyseal hormones and AVT receptor antagonists on the deposition of spermatophores. Male newts captured in winter were injected intraperitoneally with AVT ($n = 8$–11) or MT ($n = 9$)(A). Courting males captured in spring were injected with V1 receptor antagonist (V1-RA), or V2 receptor antagonist (V2-RA) ($n = 10$)(B), and with two different doses of V1-RA or saline ($n = 8$)(C). Each column and vertical bar represents the mean number of spermatophores deposited and SEM. Means with the same superscript do not differ significantly from each other ($P > 0.05$).

Effect of neurohypophyseal hormones on spermatophore deposition

In order to study the effect of AVT on spermatophore deposition, we captured male newts in early winter, kept them for 1 month in the laboratory, and then gave them an intraperitoneal injection of 0.1–10 μg of AVT, 10 μg MT, or saline on two successive days. Second, male newts exhibiting spontaneous courtship behavior in the field were captured, kept in the laboratory for 24 h, and given an injection of V1 or V2 receptor antagonist or saline. After the injection, each male newt was isolated in a container. The number of spermatophores deposited in the container during 8 h after the injections was recorded. During the experiment the male newts were kept in isolation and never exhibited any sign of courtship behavior. As shown in Fig. 6A, AVT caused spermatophore deposition in a dose-dependent manner. The activity of MT was approximately one-hundredth of the activity of AVT. The V1, but not the V2, receptor antagonist significantly suppressed the deposition of spermatophores ($P < 0.05$) (Fig. 6B), the minimum effective dose of V1 receptor antagonist being less than 2.5 μg (Fig. 6C).

Discussion

AVT and its mammalian homologue AVP exert behavioral effects in a variety of vertebrate species. In mammals, AVP affects sexual behaviors including intromission, ejac-

ulation (Bohus, 1977), and lordosis (Albers and Rawls, 1989; Södersten et al., 1983). In birds, AVT modifies sexual behaviors (Kihlström and Danninge, 1972) and singing (Goodson, 1998; Goodson and Adkins-Regan, 1999; Maney et al., 1997; Voorhuis et al., 1991). In amphibians, AVT also modifies vocalization (Boyd, 1994a; Marler et al., 1995; Penna et al., 1992; Propper and Dixon, 1997; Semsar et al., 1998), and elicits amplectic clasping behavior (Moore and Miller, 1983; Moore and Zoeller, 1979) and egg-laying behavior (Moore et al., 1992). In fish, AVT enhances courtship behaviors (Pickford and Strecker, 1977; Salek et al., 2002; Semsar et al., 2001).

Previously we have demonstrated that PRL in addition to androgen is a prerequisite for eliciting courtship behavior in the male newt *Cynops pyrrhogaster* (Toyoda et al, 1993, 1996). The present study clearly showed that AVT is another important factor for the expression of courtship behavior in this species. Moreover, AVT was revealed to be involved in the discharge of sodefrin and deposition of spermatophores.

We confirmed that injection of AVT enhanced the courtship behavior in androgen-primed male newts. Androgen may act by maintaining the behavioral response to AVT as considered to be the case in *Taricha granulosa* (Moore and Zoeller, 1979; Zoeller and Moore, 1982; Moore and Miller, 1983). Male *T. granulosa* initiate amplexus at the onset of courtship, detecting a sexually developed female using visual and olfactory cues (female sex pheromones) (Thompson et al., 1999). According to Thompson and Moore (2000), AVT seems to act on sensory pathways to modulate responsiveness of neurons to behaviorally relevant sensory stimuli.

There was no evidence that MT, another neurohypophysial hormone in amphibians, can activate male courtship behavior. In this experiment, MT was not effective in enhancing courtship behavior. The effects of ICV and IP injection of AVT were compared in order to see whether AVT acts centrally or peripherally to induce the courtship behavior. Both of ICV and IP injection of AVT increased the incidence and frequency of the behavior dose dependently. In the case of ICV injection, the minimum effective dose of AVT was 0.1 μg, whereas it was 50 μg when injected intraperitoneally, indicating that AVT acts centrally to enhance the expression of courtship behavior. Moore and Zoeller (1979) found that systemically administered AVT stimulates courtship clasping in *T. granulosa* males at a dose of 100 μg but not at 10 μg or less. Moore and Miller (1983) found that this behavior could be induced by a lower dose of AVT when the hormone was administered intracerebroventricularly than when it was given intraperitoneally. They also showed that a potent antagonist of AVP, as well as an antiserum against AVT, blocked clasping behavior when administered intracranially. We also noticed that the V1 receptor antagonist but not the V2 receptor diminished the occurrence of spontaneous courtship behavior. In the case of ICV injection, the minimum effective dose of the V1 receptor antagonist was 0.05–0.25 μg, whereas it was 25 μg when injected intraperitoneally, indicating that endogenous AVT acts centrally to enhance the expression of courtship behavior and that it acts through vasopressor rather than antidiuretic receptors. The fact that intraperitoneally administered AVT was effective in enhancing courtship behavior indicates the possible passage of AVT through the blood–brain barrier by a peptide transport system (Banks et al., 1987). It is of interest to note that the receptors for AVT are found in the brain areas with a leaky blood–brain barrier or close to the circumventricular organs (Hernando et al., 2001; Ostrowski et al., 1994).

In this experiment, marked effects of AVT and its antagonist on courtship behavior were observed 4 h after the treatment. This is in agreement with the results with other amphibian species in that the effects of the neurohypophyseal hormone on the reproductive behaviors persisted for a few hours or became prominent after a few hours (Diakow, 1978; Moore and Miller, 1983; Moore and Zoeller, 1979; Penna et al., 1992; Propper and Dixon, 1997). In birds and mammals, on the other hand, behavioral effects by AVT or AVP became evident mostly within 10–30 min and no longer persisted (Albers and Rawls, 1989; Goodson, 1998; Kihlström and Danninge, 1972; Södersten et al., 1983). This discrepancy may partly be attributable to the fact that amphibians are poikilothermal, whereas birds and mammals are homeothermal.

Earlier immunohistochemical studies revealed elaborate systems of AVT and MT neuronal elements in the brain of a variety of anurans (Boyd et al., 1992; Boyd, 1994a, 1994b; Carr and Norris, 1990; Conway and Gaimer, 1987; González et al., 1995; González and Smeets, 1992a, 1992b; Jokura and Urano, 1987; Lamacz et al., 1989; Mathieson, 1996; Smeets and González, 2001; Tonon et al., 1986; Van Vossel-Daeminck et al., 1981) and of urodeles (Fasolo and Gaudino, 1981; González and Smeets, 1992a; Moore et al., 2000; Smeets, and González, 2001). Apart from a well-developed hypothalamo–hypophysial system, the antibodies demonstrated the existence of extrahypothalamic AVT- and MT-immunoreactive cell groups as well as extensive extrahypothalamic networks of immunoreactive fibers. The wide distribution of AVT- and MT-immunoreactive fibers throughout the brains of amphibians suggests that these two neuropeptidergic systems are involved not only in hypothalamo–hypophysial interactions, but also in a variety of other brain functions. Sexual dimorphism in the AVT system is present in some amphibians (Boyd and Moore, 1992; Boyd et al., 1992; Boyd, 1994a; 1994b, 1997; Moore et al., 2000). In *T. granulosa*, Moore et al. (2000) demonstrated that there were significantly greater numbers of AVT immunoreactive cells in the bed nucleus of the stria terminalis, the nucleus amygdalae dorsalis, and the anterior preoptic area in males than in females. These AVT neurons might be involved in male-specific behaviors.

In the present experiment, we found that AVT induced spermatophore deposition and possible discharge of sode-

frin. Systemic injection of AVT induced a decrease in the sodefrin content and induced spermatophore deposition without the occurrence of courtship behavior. The water in which AVT-injected newts had been kept was more effective in attracting females than that in which saline-injected males had been kept. The AVT-induced reduction in the sodefrin content in the abdominal gland strongly suggests that AVT caused the discharge of sodefrin. AVT injection also induced the spermatophore deposition dose dependently. MT seemed to be less effective in inducing spermatophore deposition than AVT. The fact that blockade of V1 receptor but not of V2 receptor diminished the number of spermatophores deposited in the container indicates that endogenous AVT participates in this phenomenon as well as in courtship and that it acts through vasopressor rather than antidiuretic receptors. Ten micrograms of AVT was insufficient to elicit courtship behavior and a dose of 50 μg was required when the hormone was administered systemically, whereas spermatophore deposition was significantly enhanced by 0.1 μg of AVT. These data suggest that AVT acts at different sites when eliciting courtship behavior and enhancing spermatophore deposition and possibly the discharge of sodefrin. It is highly probable that AVT acts peripherally to promote spermatophore deposition and sodefrin release, whereas AVT acts centrally to elicit courtship behavior. *Cynops* males possess three kinds of glands around the cloaca, namely, lateral, pelvic, and abdominal. The first two are thought to secrete substances which constitute the sac of the spermatophore (Noble, 1954), whereas the abdominal gland is considered to be a possible source of courtship pheromones (Malacarne et al., 1984; Toyoda et al., 1994). In fact, a female-attracting peptide, sodefrin, has been identified in the abdominal gland of *C. pyrrhogaster* as the first amphibian courtship pheromone (Kikuyama et al., 1995). In this experiment, we confirmed that AVT induced the contraction of the excised abdominal gland via the V1 receptor. Although pelvic and lateral glands are difficult to isolate to test the responsiveness to AVT, it is highly probable that AVT acts on the contractile elements in three kinds of glands to elicit contraction and consequently induces the discharge of spermatophores and sodefrin.

V1 receptor is known to consist of two subtypes, V1a and V1b receptors. Presence of both receptors and their mRNA has been demonstrated in the brain and peripheral organs of mammals (Hernando et al., 2001; Lolait, et al., 1995; McDougall et al., 2000; Ostrowski et al., 1994; Szot et al., 1994). The compound [d$(CH_2)_5^1$, Tyr(Me)^2, Arg^8-vasopressin] that we used as V1-receptor antagonist is a specific V1a receptor antagonist (Manning et al., 1995). Although we did not employ a specific V1b receptor antagonist, all the actions of AVT observed in the present experiments are likely to be mediated through V1a receptor, because the data obtained indicate all the actions were almost completely abolished by the V1a receptor antagonist.

Acknowledgments

Part of this work was supported by grants-in-aid from the Japan Society for the Promotion of Science (JSPS) to F.T. and S.K., and by research grants from Asahi Glass Research Foundation and Uehara Memorial Research Foundation to S.K.

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