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[REVIEW]

Endocrine regulation of reproductive behavior in the newt *Cynops pyrrhogaster*

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ABSTRACT—Hormonal control of the expression of courtship behavior and of secretion of the female-attracting pheromone sodefrin by the male red-bellied newt, *Cynops pyrrhogaster,* together with the hormonal influence on the responsiveness to the pheromone in the female, is reviewed.

Expression of the initial stage of the courtship behavior, *i.e.*, tail vibration by the male in front of the female, is dependent on prolactin (PRL) and androgen. During the courtship, sodefrin seems to be released from the cloaca through the ducts of the abdominal gland. Both content of immunoreactive sodefrin and prepro-sodefrin mRNA levels in the abdominal gland are elevated by a combination of PRL and androgen, indicating that the pheromone synthesis is stimulated by these two hormones. On the other hand, the discharge of sodefrin is accelerated by AVT, its action being mediated by V_1 receptor. In female newts, responsiveness of the vomeronasal epithelium to the pheromone is elevated by a combination of PRL and estrogen. Thus, it can be concluded that PRL, AVT, and sex steroids are key hormones for the reproductive performance in the red-bellied newt. In this article, the significance of the structure of the pheromone molecule as a peptide is also discussed in terms of its species-specificity and its effectiveness in an aquatic environment.

INTRODUCTION

During the breeding season, the red-bellied newt (*Cynops pyrrhogaster*) migrates into the water, and the male exhibits a unique courtship behavior as in the case of several other species of uodeles (Halliday, 1977). The male newt blocks the female's path with his head, and vibrates his tail in front of her (Fig. 1, upper panel). During the tail vibration, the male projects from the cloaca numerous minute tubules that are connected to the abdominal gland. The abdominal gland has been considered to release a female-attracting pheromone which is conveyed to the female's snout by a water stream generated by the tail vibration. After this performance, the male moves forward and the female follows him with her snout in contact with the male's tail (Fig. 1, lower panel). When the male releases spermatophore, the female picks them up with her cloaca.

In general, reproductive events of urodeles including the courtship behavior, are known to be controlled hormonally. Prolactin (PRL) induces migration into the water where courtship and oviposition take place (Chadwick, 1941) and promotes the growth of the tail fin, which is used for directing a water stream toward the female during courtship (Tuchmann-Duplessis, 1949; Vellano *et al.*,1970; Singhas and Dent, 1975; Kikuyama *et al.*, 1986). Moreover, PRL elicits the development of cloacal glands (Kikuyama *et al.*, 1975; Norris *et al.*, 1989). The abdominal gland of the cloaca has been predicted to secrete female-attracting substances (Cedrini and Fasolo, 1970; Malacarne *et al.*, 1984), and the lateral gland in the cloaca is known to secrete substances constituting the spermatophore (Noble, 1954). PRL also brings about the development of the oviduct (Kikuyama *et al.*, 1986), which secretes jelly-like substances to coat the ovum. Involvement of PRL in eliciting courtship behavior in urodeles has also been suggested (Kikuyama *et al.*, 1980; Malacarne *et al.*, 1982). These effects of PRL are often manifested when the hormone is administered together with sex steroids. The fact that plasma concentrations of PRL (Matsuda *et al.*, 1990a) and androgen (Lofts, 1974; Tanaka and Takikawa, 1983) are much higher in males in the breeding season than in the non-breeding season supports the hypothesis that PRL and androgen are important hormones for the performance of courtship behavior in male newts.

Involvement of androgen in controlling expression of the

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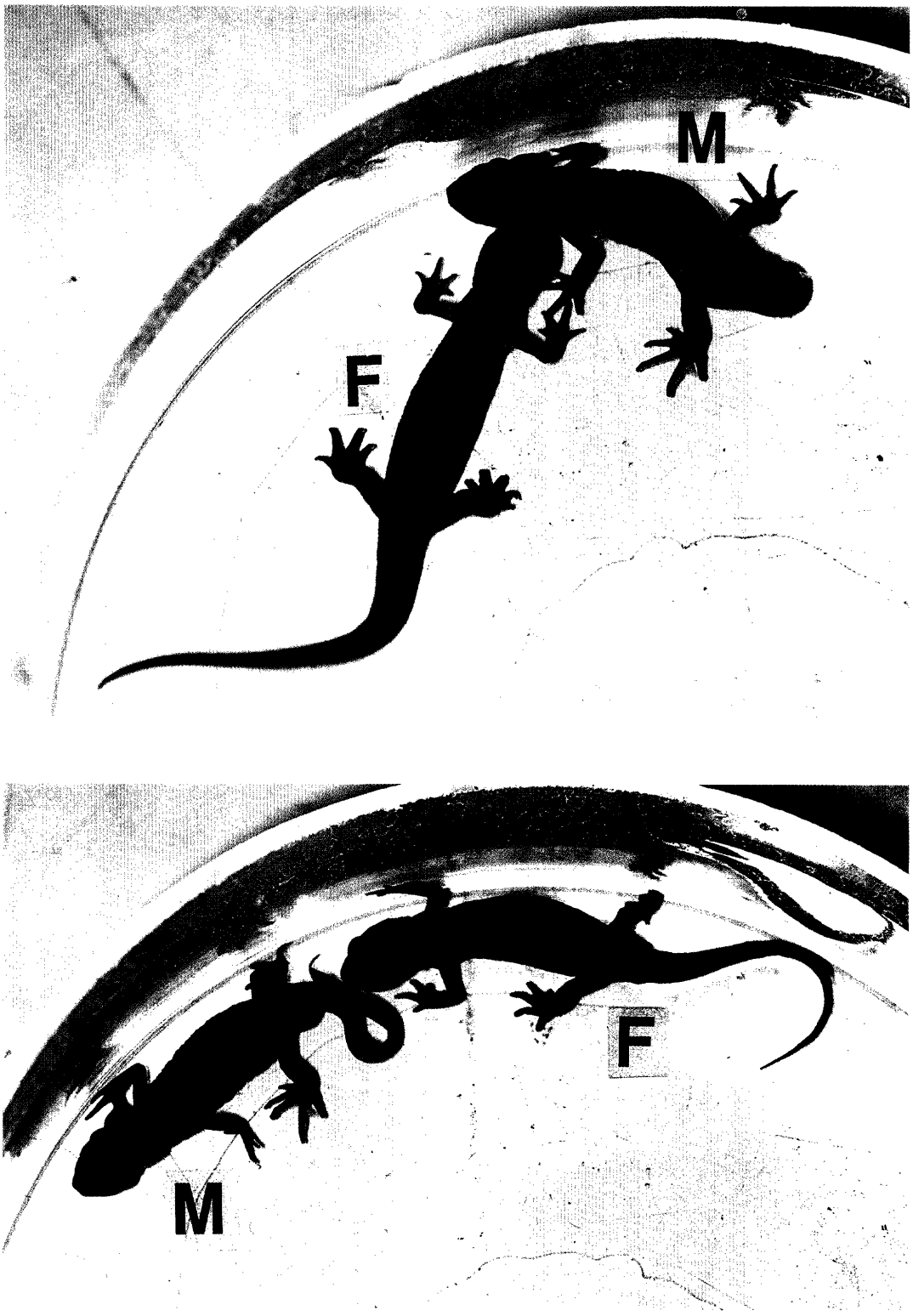

Fig. 1. Courtship behavior of the red-bellied newt, *Cynops pyrrhogaster*. Male (M) vibrates his tail in front of the female (F), sending the water located near his cloaca toward her snout (upper panel). Male parades with his tail undulating and the female follows him with her snout in contact with his tail (lower panel). Then, the male deposits spermatophores from his cloaca and the female picks up them into her cloaca.

male sexual behavior of European and American newts has been suggested by several investigators (Andreoletti *et al.*, 1983; Moore and Zoeller, 1979; Moore and Miller, 1983; Moore, 1987; Moore *et al.*, 1992). Sexual activity of the male declines after castration, and it is restored by androgen supplementation (Andreoletti *et al.,* 1983; Moore, 1987). In Japanese red-bellied newts, androgen induces a significant increase in the mean number of synapses on the somata of Mauthner cells (Matsumoto *et al.*, 1997), which cells are involved in the tail movement (Korn *et al.,* 1990). Furthermore, arginine vasotocin (AVT) has been reported to regulate sexual behavior in the rough-skinned newt, *Taricha granulosa*. In this species, males capture and embrace female partners and clasp them with all four limbs prior to sperm transfer. AVT and androgen induce this clasping behavior in the male (Moore and Zoller, 1979; Moore and Miller, 1983). Female *T. granulosa* lay eggs

several weeks after being inseminated, and this egg-laying behavior is also induced by AVT and estrogen (Moore *et al.*, 1992). Androgen-implanted ovariectomized females injected with AVT exhibit male-like amplectic clasping.

In most urodeles, chemical signals play a major role in sex recognition and courtship behavior (Houck, 1986; Salthe and Mecham, 1974). Several investigators have confirmed the existence of sex pheromones in certain species of urodeles (Twitty, 1955; Malacarne and Vellano, 1987; Malacarne *et al.*,1984; Cedrini and Fasolo, 1971). However, very little work had been carried out to elucidate the biochemical nature of these substances (Belvedere *et al.*, 1988; Andreleotti *et al.*, 1994). We have isolated and characterized a novel female-attracting pheromone, "sodefrin", found in the abdominal glands of male newts *Cynops pyrrhogaster* (Kikuyama *et al.*, 1995).

In this article, the involvement of endocrine factors in the expression of reproductive behavior as well as in the secretion of and response to sodefrin in the red-bellied newt, *C. pyrrhogaster*, will be reviewed.

1. Hormonal control of courtship behavior

Effects of PRL, gonadotropic hormone (GTH) and sex steroids on the initial stage of male courtship behavior (tail vibration in front of the female) have been studied (Toyoda *et al.*, 1993). Treatment of sexually non-responsive or hypophysectomized newts with a combination of PRL of bovine, ovine or frog origin and GTH (human chorionic gonadotoropin or frog luteinizing hormone and follicle-stimulating hormone) elevated both the incidence and frequency of the behavior when treated males were paired with females treated similarly. The effect of PRL or GTH alone was less marked than that of PRL plus GTH. In hypophysectomized males, the combination of PRL and GTH significantly increased both the incidence and frequency of the behavior. PRL alone was not effective, and the effect of GTH alone was less pronounced than that in the intact animal receiving GTH injections. The effect of GTH was nullified by castration. In the castrated male testosterone administered together with PRL was a potent inducer of the behavior. This indicates that GTH is indirectly involved in the manifestation of tail vibration by stimulating the secretion of androgen by the testis.

According to Zerani *et al.* (1992), estradiol levels in male newts (*T. carnifex*) reach their maximum at the beginning of courtship, and aromatase activity in the brain increases at this stage. They proposed that estradiol converted from testosterone is an important factor for inducing courtship behavior in the male of this species. However, this interpretation does not fit, at least, with the data on *C. pyrrhogaster* males in which the expression of courtship behavior was enhanced by dihydrotestosterone as well as testosterone but not by estradiol. It should also be mentioned that ovariectomized *C. pyrrhogaster* females exhibited male-like behavior when testosterone, but not estradiol in combination with PRL, was administered (Toyoda and Kikuyama, 1995). It is known that androgenization elicits male-like behavior in the female of other classes of vertebrates (Kelley and Pfaff, 1976). This fact suggests that the neuronal system, which is involved in the expression of male behavior, is not completely altered during the course of sex differentiation. However, the site of action of androgen to elicit courtship behavior in urodeles is not definite. In the rough-skinned newt, *Taricha granulosa*, androgen receptor-immunoreactivity was demonstrated in various regions of the central nervous system including the olfactory bulbs, preoptic area, hypothalamus, and motor nuclei of the medulla oblongata (Davis and Moore, 1996).

Involvement of endogenous PRL in the tail vibration behavior has also been demonstrated (Toyoda *et al.*, 1996). Male red-bellied newts, which had been exhibiting the tail vibration behavior in the field, were taken to the laboratory together with female partners. The male newts were treated with either antiserum against newt PRL or preimmune serum. Within 24 hr of the first injection of antiserum, both the incidence and frequency of courtship behavior declined markedly as compared with those in the preimmune serum-injected newts. The anti-PRL serum-induced decline became even more conspicuous thereafter. Cessation of antiserum injection and administration of PRL restored the expression of courtship behavior (Fig. 2). Malacarne *et al.* (1982) provided indirect evidence that endogenous PRL is involved in the manifestation of courtship behavior in the crested newt (*T. critatus*). They demonstrated that administration of a dopamine agonist (bromocriptine) to the sexually developed newt led to the cessation of courtship behavior, presumably by suppressing the release of PRL, as demonstrated by Matsuda *et al.* (1990b).

At present, the site of action of PRL in terms of eliciting the courtship behavior is not clear. In the newt *T. cristatus*, the courtship behavior center is presumed to be located in the preoptic area, as lesions to this area abolish sexual behavior (Malacarne and Giacoma, 1980). According to Muccioli *et al.* (1990), PRL-binding sites in the brain of Xenopus are located in the hypothalamus and choroid plexus. These investigators assumed that PRL-binding at the level of the choroid plexus may play a role in transporting the hormone across the blood-cerebrospinal fluid barrier. Although there is little information about the PRL-binding sites in the urodele brain, it is probable that PRL acts on the hypothalamus to elicit sexual behavior by entering into the brain through the choroid plexus. Recently, PRL receptor cDNAs were cloned from *C. ensicauda* (Kato *et al.*, 1997) and *C. pyrrhogaster* (Yamamoto *et al.*, 1998). They are expected to become a useful tool for the identification of the initial site of action of PRL that elicits courtship behavior.

AVT is another important endocrine factor for the expression of courtship behavior in urodeles. It augments both the incidence and frequency of courtship behavior in red-bellied newts. Furthermore, AVT induces spermatophore deposition even in the solitary males exhibiting no courtship. Intraperitoneal administration of a V_1 (vasopressor) antagonist but not that of a V_2 (antidiuretic) receptor antagonist to male red-bellied newts exhibiting a spontaneous courtship behavior significantly reduced both the incidence and frequency of courtship and the number of spermatophores deposited, indi-

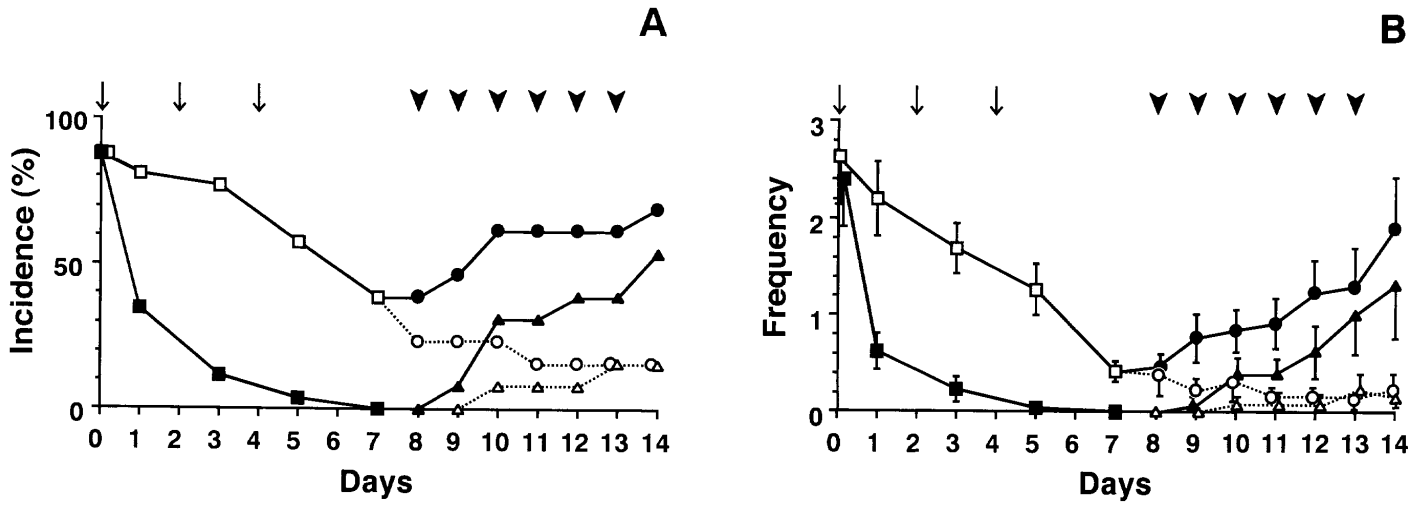

Fig. 2. Effect of anti-PRL serum and PRL on the incidence (A) and frequency (B) of male courtship behavior in sexually developed newts. The incidence is expressed as the percentage of animals that exhibited the behavior, and the frequency is expressed as the mean number of times that the behavior was recorded. Animals were injected with either anti-PRL serum (20 μl, ■) or preimmune serum (20 μl, □) on the day indicated by an arrow. A daily injection of ovine (1 IU) PRL was started 4 days after the last injection of anti-PRL or preimmune serum. The arrowheads indicate the days of PRL or saline injection. ●, preimmune-serum pretreatment and PRL treatment; ○, preimmune-serum pretreatment and saline injection. ▲, anti-PRL serum pretreatment and PRL treatment; △, anti-PRL serum pretreatment and saline injection. Vertical bars in B represent standard errors of the mean.

cating that endogenous AVT is involved in the expression of these reproductive events. It is highly probable that AVT acts centrally to enhance the expression of courtship behavior in *C. pyrrhogaster*. According to our unpublished data, 5 μg of AVT was not effective, and a dose of 50 μg was required for the enhancement of frequency and incidence of courtship behavior when AVT was administered intraperitoneally. However, spermatophore deposition was elicited by as little as 1 μg of AVT (Kikuyama *et al.*, 1999). According to Moore and Zoeller (1979), courtship clasping in *Taricha* males could be induced by a systemic injection of AVT at a dose of 100 μg but not at 10 μg or less. They also showed that this behavior could be induced by a lower dose of AVT when administered intracerebroventricularly than when given intraperitoneally.

2. Hormonal control of the secretion of and response to a female-attracting pheromone sodefrin

2-1. Isolation and characterization of sodefrin

The water in which male red-bellied newts treated with PRL and GTH had been kept was revealed to attract female newts treated similarly with hormones but not females injected with saline (Toyoda *et al.*, 1994). This finding indicates that the male-conditioned water contained a female-attracting substance and that the responsiveness of female newts to the attractant was dependent on PRL and GTH. Castration nullified the effect of PRL and GTH on the secretion of the female-attracting substance. In the castrated animals, however, a combination of PRL and androgen was effective in enhancing the secretion of the attractant. Thus, it is evident that the effect of GTH is mediated through the gonads, androgen being the directly acting substance.

The female-attracting substance seems to be secreted by or through the abdominal gland, which is known to develop in response to PRL and androgen, since the female-attracting activity of the male-conditioned water was markedly reduced when the abdominal gland was surgically removed (Toyoda *et al.*, 1994). On the basis of this observation, an attempt was made to isolate and characterize the substance(s) possessing this female-attracting activity (Kikuyama *et al.*, 1995). Female-attracting pheromone activity was monitored by a preference test (Toyoda *et al.*, 1994). An aqueous extract of the abdominal glands of sexually developed red-bellied newts exhibited a considerable female-attracting activity. When the extract was placed in a sponge block in a container filled with 3000 ml of water, the minimum effective amount required to attract a sexually mature female was the equivalent of 0.1% of the abdominal gland content.

The active substance in the abdominal gland was revealed to be soluble in water but not in organic solvents. When an aqueous extract of the abdominal glands was subjected to gel-filtration (G-100) column chromatography, the female-attracting activity emerged in a fraction with a relative molecular mass of less than 5000. When this fraction was incubated with pronase, its female-attracting activity was completely lost, indicating the active substance to be a peptide. After two purification cycles of reverse-phase high-performance liquid chromatography (HPLC), the active peptide was isolated from the gel filtration fraction. The final product was revealed to be a decapeptide with the amino acid sequence Ser-Ile-Pro-Ser-Lys-Asp-Ala-Leu-Leu-Lys. COOH-terminal analysis by carboxypeptidase-P digestion revealed that the C-terminal residue was a free Lys residue. The relative molecular mass of 1071.2 estimated from fast atom bombardment mass spectrometry corresponded with that calculated from the amino acid sequence. The peptide showed no sequence homology with any known peptide and was designated sodefrin, which derived from the ancient Japanese word "sodefuri", meaning "soliciting" (Kikuyama *et al.*, 1995).

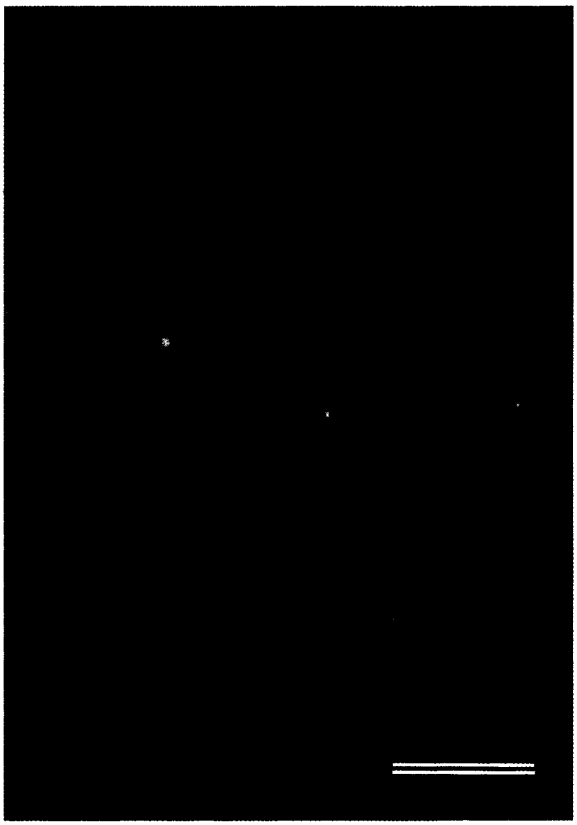

Fig. 3. Immunofluorescence micrograph showing the localization of sodefrin in the abdominal gland of a red-bellied newt. The sodefrin antibody labels the epithelial cells red with rhodamine. Nuclear DNA is stained blue with 4',6-diamino-2-phenylindole. Bar represents 50 μm.

Fig. 4. Immunoelectron micrograph showing the localization of sodefrin in the epithelial cells of the abdominal gland of a red-bellied newt. Immunogold particles indicating the presence of sodefrin are observed within the secretory granules. Bar represents 500 nm.

Ten nanograms of native sodefrin absorbed by a sponge block was enough to attract sexually developed female but not male newts in a container filled with 3000 ml of water (Kikuyama *et al.*, 1995; Toyoda *et al.*, 1995). Synthetic sodefrin exhibited female-attracting activity similar to that of the native material, with a minimum effective concentration within the range of 0.1–1.0 pM.

Frozen sections of abdominal glands immunolabelled with a fluorescent antibody against sodefrin showed that the epithelial cells were positive for sodefrin (Fig. 3). An immunoelectron microscopic study of the abdominal gland using sodefrin antiserum and goat anti-rabbit IgG labeled with gold particles as a second antibody showed the particles to be localized mainly within the secretory granules (Fig. 4, Toyoda *et al.*, 1995). This clearly indicates that sodefrin is secreted by the epithelial cells of the abdominal gland.

2-2. Possible existence of sodefrin-like pheromone in *Cynops ensicauda*

If sodefrin acts as a courtship pheromone, it would be expected to exert a species-specific action and thus contribute to reproductive isolation. In order to observe the effect of sodefrin on a congeneric species of newt, we chose the sword-tailed newt, *C. ensicauda*, to use as a model. *C. ensicauda* females were not attracted to sodefrin, but they were attracted to a water extract of abdominal glands from males of their own species (Kikuyama *et al.*, 1995). On the other hand, *C. pyrrhogaster* females responding to sodefrin were not sensitive to the water extract of the abdominal glands from *C. ensicauda* males. These results indicate that the female-attracting substances differ between these two species of the genus *Cynops*. Belvedere *et al.* (1988) performed a series of behavioral tests where female newts in a Y-maze apparatus were allowed to choose water flows coming from aquaria under different experimental conditions. They observed that different species of Italian newts belonging to the genus *Triturus* showed preference for water coming from aquaria with homospecific pairs actively engaged in courtship. This is in good accord with our result.

In order to ascertain whether the abdominal gland of *C. ensicauda* possesses molecules that immunoreact with the sodefrin antiserum, Yamamoto *et al.* (1996) subjected an aqueous extract of the abdominal gland to a sodefrin RIA. This extract showed no cross-reactivity in this RIA system, whereas a water extract from the abdominal gland of *C. pyrrhogaster* showed an inhibition curve parallel to the sodefrin standard (Yamamoto *et al.*, 1996).

To further our understanding of female-attracting peptide pheromones at a molecular level, Iwata *et al.* (1999) prepared and examined sodefrin precursor cDNA. A sodefrin precursor cDNA isolated from a cDNA library constructed from *C. pyrrhogaster* abdominal gland mRNA was found to contain 1364 bps with an open reading frame of 567 bps and to encode a sodefrin precursor protein of 189 amino acids residues (Fig. 5A). The precursor protein included a predicted signal peptide and, in a region close to the C-terminus, the

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                                                  TGGCAGGTGAACAGGTGCAGAGACTCCATCACCCTATTCCTTACTCTCCTAGCACC
ATGAGGGCCATCCTTGCAGCTGTCGTCCTGCTCCAGGCACTGATAACTGGAGATTGCCTATTATGCGAGCAGTGT
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 F  A  L  Q  T  S  S  C  S  G  I  F  T  Q  C  S  P  D  V  T  H  C  V  A  G
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 W  S  Y  F  P  I  R  R  T  H  V  A  P  S  M  E  L  P  P  G  R  L  R  L  G
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 R  (S) (I) (P) (S) (K) (D) (A) (L) (L) (K)  I  S  A  *
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 F  C  N  G  G  D  V  Q  V  P  P  P  D  D  T  P  N  G  C  G  S  D  Q  S  A
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 N  A  C  T  A  P  G  H  L  R  V  T  V  R  S  T  T  S  I  R  E  K  R  R  E
CGACTGAATGTGTTCAGTGTTCTGGCAAGCAGAAGGCGTGTGGCACCTTCTAAGGAACTGCCTCTAGGCGTGATA
 R  L  N  V  F  S  V  L  A  S  R  R  R  V  A  P  S  K  E  L  P  L  G  V  I
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TAATTTACACA
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Fig. 5. Nucleotide and deduced amino acid sequences of the cDNA clone encoding sodefrin (A) and sodefrin-like peptide (silefrin) (B). The predicted amino acids are shown below the nucleotide sequence. The amino acids comprising sodefrin and silefrin molecules are circled. The asterisk indicates the termination codon, and the polyadenylation signal (AATAAA) is underlined.

sodefrin molecule. Northern blot analysis of sodefrin mRNA in the abdominal gland revealed the size to be about 1.5 kb and sodefrin mRNA to be expressed exclusively in the abdominal gland. However, sodefrin seems to be generated in a way different from commonly observed processing of peptide hormone precursors, since the sodefrin sequence is not sandwiched by two pairs of dibasic amino acids. Synthetic sodefrin C-terminally extended with isoleucine, serine, and alanine (C-terminal portion of sodefrin precursor consisting of 13 amino acid residues) does not attract the females, indicating that these three amino residues must be removed from the C-terminus of this molecule for acquisition of biological activity.

A cDNA clone encoding sodefrin precursor-like protein was isolated from a *C. ensicauda* abdominal gland cDNA library (Fig. 5). Its nucleotide sequence showed 93% homology with *C. pyrrhogaster* cDNA. The deduced amino acid sequence showed 82% homology with the *C. pyrrhogaster* molecule. The sodefrin-like peptide from *C. ensicauda* has two substitutions (Leu for Pro at position 3, Gln for Leu at position 8) compared with sodefrin. The [Leu^3, Gln^8]-sodefrin attracts *C. ensicauda* females but not *C. pyrrhogaster* females. Likewise, sodefrin attracts only *C. pyrrhogaster* females (Iwata *et al.*, 1999). These differences in the structure and female-attracting properties of the pheromone in these two species of newt appear to be part of the mechanism which is responsible for reproductive isolation. Recently, we isolated the [Leu^3, Gln^8]-sodefrin from the aqueous extract of the abdominal glands of C. ensicauda. Both the native and synthetic peptide were revealed to have an equivalent activity in attracting conspecific females. This peptide was designated silefrin, a combination of the first three N-terminal amino acids, SIL and -efrin, derived from sodefrin (Yamamoto *et. al.*, 2000).

2-3. Hormonal control of sodefrin secretion.

A radioimmunoassay (RIA) has been developed to measure sodefrin, and was used to investigate the effects of PRL, androgen, and PRL plus androgen on the sodefrin content in hypophysectomized and castrated animals. A combination of PRL and androgen is known to stimulate the structural development of the abdominal gland markedly (Kikuyama *et al.*, 1975). Treatment of hypophysectomized and castrated male newts with androgen but not prolactin significantly increased the sodefrin content of the abdominal glands. A combination of both hormones produced a synergistic effect resulting in a further increase in the sodefrin content (Fig. 6) (Yamamoto *et al.*, 1996). This observation is consistent with the finding that the treatment of *C. pyrrhogaster* males with PRL plus androgen enhances the release of female attractant into the water (Toyoda *et al.*, 1994). Further evidence that PRL and androgen stimulate sodefrin synthesis comes from Northern blot analysis using sodefrin precursor cDNA (Iwata *et al.*, 1999) as a probe. Sodefrin mRNA levels were elevated moderately by the treatment with either PRL or androgen and markedly by the combined administration of PRL and androgen (Fig. 6, Iwata *et al.*, 2000). Since androgen receptors reside in the nuclei of the epithelial cells of the abdominal gland (Matsumoto

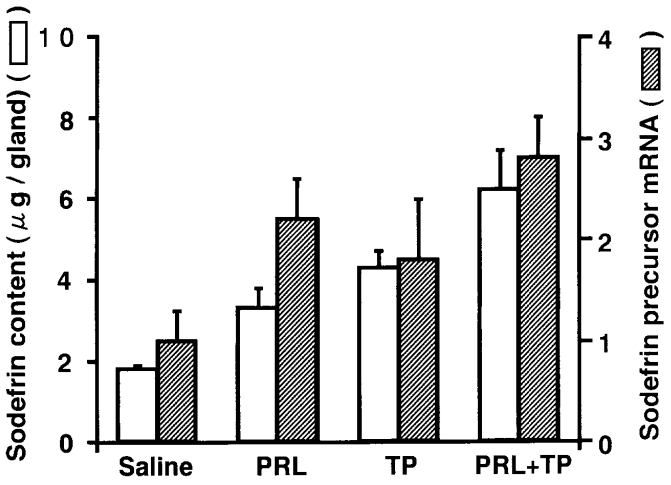

Fig. 6. Effects of PRL and /or testosterone propionate (TP) on the sodefrin content and sodefrin precursor mRNA level in the abdominal glands of male newts. Data for sodefrin content were obtained from hypophysectomized and castrated specimens receving saline, PRL (1 IU), TP (5 μg) or a combination of both hormones on alternate days for 20 days. Data for sodefrin mRNA were obtained from hypophysectomized newts injected with saline, PRL (1 IU), TP (5 μg) or a combination of PRL and TP every other day for 8 days. Each densitometry datum was normalized by that for actin mRNA.

et al., 1996) and PRL receptor mRNAs have been expressed in the gland (unpublished data), these two hormones are considered to be major factors for enhancement of the pheromone production.

Recently, we found that AVT caused a decrease in the content of sodefrin in the abdominal gland, suggesting that it induces the discharge of sodefrin. Administration of a V_1 receptor antagonist, but not that of a V_2 receptor one, suppressed the decrease in sodefrin content of abdominal glands in sexually developed intact males (Toyoda *et al.*, 1997). Therefore, AVT is considered to act as an inducer of sodefrin discharge through the V_1 (vasopressor) receptor. The existence of an actin-like protein in a structure around the ducts of the abdominal gland suggests that AVT acts on that contractile structure to induce the discharge of the sodefrin through the ducts (Kikuyama *et al.*, 1999).

2-4. Hormonal control of olfactory response to sodefrin

Sodefrin is considered to act through the olfactory organ of female newts since attraction to this substance was abolished by bilateral nostril plugging or nerve transection between the nasal cavity and the olfactory bulb (Toyoda *et al.*, 1995; 1999).

The olfactory system of urodeles consists of two morphologically distinct epithelia, namely, the main olfactory epithelium and the vomeronasal epithelium (Eisthen, 1992). In *C. pyrrhogaster*, the main chamber of the nasal cavity is lined with sensory and non-sensory epithelia. The sensory epithelium consists of both ciliated and microvillar cells. Lateral to the main chamber of the nasal cavity there is a diverticulum, which is lined with vomeronasal epithelium. The sensory epithelium of this region contains only microvillar cells (Jones *et al.*, 1994). The axons of olfactory receptor cells terminate in

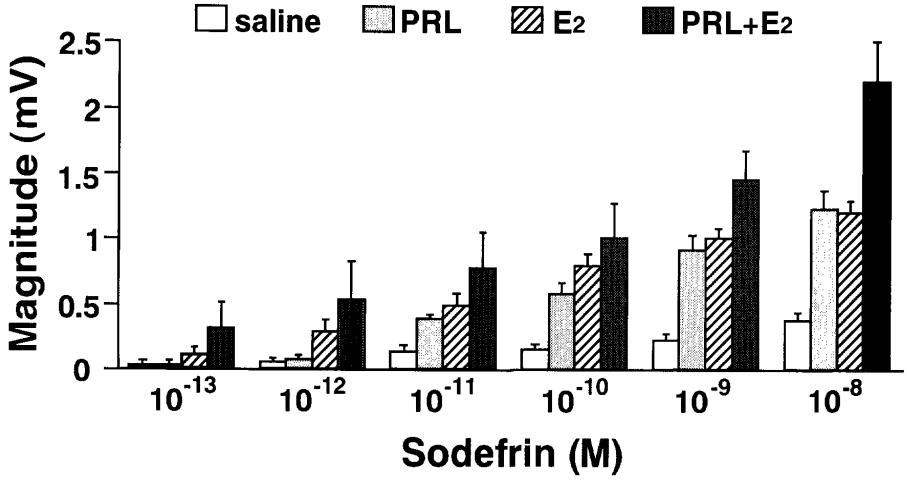

Fig. 7. Effects of PRL and/or estradiol on EOG responses to various concentrations of sodefrin in ovariectomized newts. Test females received daily injections of 1 IU PRL and/or 5 μg estradiol (E_2) for two weeks starting one week after ovariectomy. The EOG response was recorded in the central region of the vomeronasal epithelium.

the main olfactory bulb at the rostral portion of the telencephalon, whereas the axons of the vomeronasal receptor cells project to the accessory olfactory bulb located dorsocaudally to the main olfactory bulb (Toyoda *et al.*, 1999).

Electrophysiological studies revealed that sodefrin evoked a marked electro-olfactogram (EOG) response when applied to the vomeronasal region (Toyoda *et al.*, 1999; Toyoda and Kikuyama, 2000). In sexually developed female newts, the threshold concentration of sodefrin required for the induction of EOG response was 0.1 pM. This concentration was close to the minimum effective concentration (0.1–1.0 pM) required to attract female newts (Kikuyama *et al.*, 1995). Interestingly, the vomeronasal epithelium of sexually undeveloped females scarcely responded to sodefrin. Treatment of sexual undeveloped females with prolactin and gonadotropin restored responsiveness to the pheromone (Toyoda *et al.*, 1999). Likewise, a combination of PRL and estrogen markedly enhanced EOG responses in the ovariectomized female newts. The EOG response to the pheromone was also enhanced moderately by treatment with either PRL or estrogen alone (Fig. 7). A slight but significant elevation was observed in castrated males receiving PRL plus estrogen or estrogen alone. Thus, it was concluded that the main site of action of sodefrin resides in the vomeronasal epithelium and that sensitivity to sodefrin is under the control of PRL and estrogen. Existence of sexual dimorphism in olfactory responsiveness to the hormones and/or the pheromone was noted.

Concluding remarks

In this article, we decribed that PRL, sex steroids, and AVT are important endocrine factors that regulate the expression of male reproductive events, such as courtship behavior and spermatophore deposition, in the male newt *Cynops pyrrhogaster*. Both site and mechanisms of action of these hormones remain to be clarified.

We discovered the presence of a female-attracting peptide pheromone (sodefrin) that is secreted by the abdominal gland of the male red-bellied newt. Sodefrin is the first amphibian pheromone to be identified, and the first peptide pheromone identified in a vertebrate. Considering that in most urodeles reproduction takes place in an aquatic environment, a non-volatile but water-soluble peptide seems to be an appropriate sex-pheromone. Analysis of cloned sodefrin cDNA revealed that it encodes a molecule larger than sodefrin molecule indicating the existence of a pheromone precursor molecule. A congeneric species, *C. ensicauda*, has a sodefrin-like peptide (silefrin) with two amino acid substitutions compared with sodefrin. Both sodefrin and silefrin attract only conspecific females. This raises the possibility that urodele species belonging to the same genus have species-specific female-attracting pheromones. An inter-species difference in the structure of the pheromonal peptide may be significant in ensuring reproductive isolation. Peptide molecules are ideal as species-specific reproductive pheromones, since many variant forms can be generated by the modification of the nucleotide sequence of the pheromone gene.

Sodefrin was demonstrated to act on the vomeronasal epithelium of the female. The fact that the responsiveness to sodefrin is dependent on prolactin and estrogen suggests that these hormones may have some influence upon the expression of sodefrin receptor molecules. Identification and characterization of the sodefrin receptor are definitely needed to shed further light on the mechanism of chemical communication during reproduction in urodeles.

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