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Some aspects of the hypothalamic and pituitary development, metamorphosis, and reproductive behavior as studied in amphibians☆

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ABSTRACT

In this review article, information about the development of the hypothalamo–hypophyseal axis, endocrine control of metamorphosis, and hormonal and pheromonal involvements in reproductive behavior in some amphibian species is assembled from the works conducted mainly by our research group. The hypothalamic and pituitary development was studied using *Bufo* embryos and larvae. The primordium of the epithelial hypophysis originates at the anterior neural ridge and migrates underneath the brain to form a Rathke's pouch-like structure. The hypothalamo–hypophyseal axis develops under the influence of thyroid hormone (TH). For the morphological and functional development of the median eminence, which is a key structure in the transport of regulatory hormones to the pituitary, contact of the adenohypophysis with the undeveloped median eminence is necessary. For the development of proopiomelanocortin-producing cells, contact of the pituitary primordium with the infundibulum is required. The significance of avascularization in terms of the function of the intermediate lobe of the pituitary was evidenced with transgenic *Xenopus* frogs expressing a vascular endothelial growth factor in melanotropes. Metamorphosis progresses via the interaction of TH, adrenal corticosteroids, and prolactin (PRL). We emphasize that PRL has a dual role: modulation of the speed of metamorphic changes and functional development of organs for adult life. A brief description about a novel type of PRL (1B) that was detected was made. A possible reason why the main hypothalamic factor that stimulates the release of thyrotropin is not thyrotropin-releasing hormone, but corticotropin-releasing factor is considered in light of the fact that amphibians are poikilotherms. As regards the reproductive behavior in amphibians, studies were focused on the courtship behavior of the newt, *Cynops pyrrhogaster*. Male newts exhibit a unique courtship behavior toward sexually developed conspecific females. Hormonal interactions eliciting this behavior and hormonal control of the courtship pheromone secretion are discussed on the basis of our experimental results.

1. Introduction

Most amphibians spend their larval life in an aquatic environment. After the larvae have grown to a certain extent, they undergo metamorphosis. Accompanying this metamorphosis are structural, physiological, biochemical, and behavioral transformations through which aquatic larvae are converted to terrestrial adults. Several amphibian species possess unique characteristics that facilitate an endocrinological analysis of their life cycle phenomena. Using an appropriate species, we are able to study the endocrine control of their growth and metamorphosis (Kikuyama et al., 1993a, 2005).

The advantages of experimental models such as toads and newts (for example, *Bufo japonicus* and *Cynops pyrrhogaster*, respectively) for analyzing the development of endocrine organs is that the embryos are relatively large, making it possible to remove and/or transplant various endocrine organ primordia. A further advantage is that high percentages of embryos survive and develop into tadpoles after diverse surgical interventions (Kawamura and Kikuyama, 1993). Furthermore, the size of the pituitary of adult frogs, such as *Rana catesbeiana*, is large enough to isolate various hormones that have been used for the

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development of homologous radioimmunoassay (RIA) and for the evaluation of their activities in homologous animals (Kikuyama et al., 2005).

Reproductive behaviors of urodele amphibians belonging to the Salamandridae family, wherein fertilization takes place internally, are known to be regulated by various endocrine factors (Kikuyama et al., 2003). Using a specific reproductive behavior pattern as a criterion, it is possible to analyze the mechanism of hormone interactions that are responsible for eliciting these behaviors (Kikuyama et al., 2003). It should also be mentioned that the male reproductive accessory glands of these animals contain courtship pheromones. These attributes make urodele amphibians an excellent model for studies of the effects of hormones on the synthesis and discharge of pheromones (Kikuyama et al., 2013).

In this review, the development of the hypophysis and hypothalamus, endocrine control of metamorphosis, and hormonal and pheromonal involvements in reproductive behavior in amphibians will be introduced. The review was contributed in the context of the Japan Society for Comparative Endocrinology (JSCE) that awarded the first author of this article the Kobayashi Award in 2015. As such the review highlights the contributions of his team to the areas outlined above and it does not aim to exhaustively review the literature. Several excellent reviews (Jenks et al., 1993; Tonon et al., 1993; Shi, 2000; Moore et al., 2005; Eisthen and Polese, 2007; Houck et al., 2008; Kelberman et al., 2009; Denver, 2013; Woodley, 2015; Buchholz, 2017; Frau et al., 2017) complement and further develop various aspects of the present review.

2. Development of hypophysis and hypothalamus

2.1. Origin of the epithelial hypophysis

Since the observations made by Rathke (1838), the epithelial hypophysis (pars distalis, pars intermedia, and pars tuberalis) has long been believed to have its origin in Rathke's pouch (RP), which is formed as a dorsal evagination of the stomodeal ectoderm. In the embryos of the toad, *B. japonicus*, however, we can recognize the hypophyseal primordium existing between the forebrain and the foregut prior to the formation of the stomodeum. Stimulated by this observation, we performed extensive studies to clarify the origin of the epithelial pituitary, tracing back to the developmental stages prior to that of RP formation. For this purpose, we used embryos hatched from albino mutant eggs, as well as wild-type embryos. Through a series of experiments in which wild-type tissue fragments, the cells of which contained a large number of melanin granules, were transplanted to the corresponding sites of albino embryos at the open neurula stage, it was determined that the primordium of the epithelial pituitary is located in the anterior portion of the neural ridge (ANR). In the course of morphogenesis, the ANR detaches from the rostral end of the neural tube, migrates caudally under the forebrain floor, fuses to the rostral end of the foregut, and transiently constitutes an RP-like structure. Subsequently, this structure detaches from the foregut, finally attaching to the infundibulum to form pars distalis, pars intermedia, and pars tuberalis (Fig. 1) (Kawamura and Kikuyama, 1992). When the specimens had reached tadpole stages, an immunohistochemical examination confirmed that the pituitary cells thus developed contained pituitary hormones such as prolactin (PRL), adrenocorticotropic hormone (ACTH), and melanophore-stimulating hormone (MSH).

Prior to our studies, Eagleson et al. (1986) reported that, in *Xenopus*, the radiolabeled ANR transplanted into non-labeled open neurula differentiates into ACTH cells in the anterior pituitary. Using a quail-chick chimera system to identify the developmental fate of the embryonic transplants, Couly and Le Douarin (1985, 1987) provided evidence that at least a subset of the adenohypophyseal cells are derived from the ventral portion of the neural ridge that is comparable to the ANR in amphibians. Our report, however, demonstrated the origin of adenohypophysis by tracing the migrating primordium from its original position to its final one, wherein it develops into the functional pituitary (Kawamura and Kikuyama, 1992). By means of a whole embryo culture technique, we also showed that the adenohypophyseal cells originate from the rostral end of the neural plate in the early rat embryos before the formation of the RP (Kouki et al., 2001). Thus, participation of the neural tissue for the formation of adenohypophysis seems to be a common feature among vertebrates (Kawamura et al., 2002).

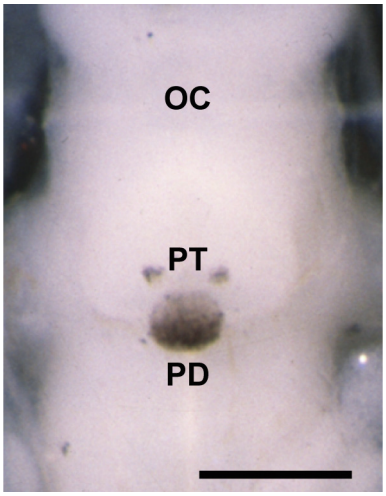

Fig. 1. Ventral view of the brain and pituitary of an albino *Bufo* tadpole of which anterior part of the neural ridge had been removed and transplanted with the counterpart from a wild-type embryo at the open neurula stage. Note that pars distalis (PD) and pars tuberalis (PT) are labeled with melanin granules. OC, optic chiasma. Scale bar = 500 μm.

2.2. Development of the pituitary proopiomelanocortin (POMC) cells

Using a similar experimental procedure as that employed for the identification of the pituitary primordium in *Bufo* embryos, it was demonstrated that the hypothalamus originates from the anterior part of the neural plate adjacent to the ANR (Kawamura and Kikuyama, 1992) and that the olfactory primordia originate from the antero-lateral portion situated on both sides of the ANR (Kawamura and Kikuyama, 1996, 1998). Knowledge about the location of the hypothalamus anlage on the neural plate contributes to the further elucidation of the role of the hypothalamus in the development and differentiation of the adenohypophysis.

In *Bufo* tadpoles from which the central part of the anterior neural plate had been removed at the open neurula stage, the posterior hypothalamus including the infundibulum did not develop. In these specimens, the epithelial hypophysis was formed away from the normal site, without morphological contact with the brain. In the pituitary thus developed, immunoreactive MSH and ACTH cells (i.e., the POMC cells) were lacking, while other types of cells such as PRL, growth hormone (GH), thyroid-stimulating hormone (TSH), and gonadotropic hormone-producing cells were invariably present. Considering that POMC cells originate in the ANR but not in the neural plate, the embryonic brain seems to exert an inductive influence upon the primordial POMC cells (Kawamura and Kikuyama, 1995). If the pituitary primordium situated underneath the brain at the tail-bud stage was isolated from the brain and transplanted ectopically, POMC cells developed (Kikuyama et al., 1993b; Kawamura and Kikuyama, 1995). This implies that the inductive influence from/via the posterior hypothalamus on the hypophyseal primordium is exerted between the open neurula and the tail-bud stages.

In general, both larval and adult amphibians change their skin color depending on the background color of their surrounding environment. *B. japonicus*, however, is unique in that the larvae are devoid of the

ability to respond to the background color; the melanin granules in their dermal melanophores are in a state of constant dispersion independent of the background color to which they are subjected. Pharmacological and histochemical studies revealed that MSH is being released without inhibition presumably because of the lack of innervation of dopamine neurons (Kouki et al., 1998a).

In the hypothalamus of the postmetamorphic toad, dopaminergic cell bodies, as revealed by staining with an antibody against tyrosine hydroxylase, are localized in three clusters: the preoptic recess organ (PRO), the paraventricular organ (PVO), and the infundibular nucleus (IN). The PRO can be divided into two sections: the rostral PRO (rPRO) and caudal PRO (cPRO). In the larval hypothalamus, the tyrosine hydroxylase-positive PVO, IN, and cPRO, but not the rPRO, are recognizable. It is near the end of metamorphosis that rPRO becomes tyrosine hydroxylase-positive, presumably under the influence of thyroid hormone (TH) (Kikuyama et al., 1979), and that the intermediate lobe is innervated with tyrosine hydroxylase-positive neurons. This prompted us to confirm rPRO is the inhibitory center for MSH secretion. Embryos from which the primordium of the rPRO situated at the boundary between the anterior end of the neural plate and the neural ridge had been removed were kept until the end of metamorphosis. Their body color was invariably black irrespective of the background color, and tyrosine hydroxylase-immunoreactive neurons were absent in the intermediate lobe. Thus, it was concluded that rPRO is the center for background adaptation, at least in *B. japonicus* (Kouki et al., 1998b). In *Xenopus*, however, suprachiasmatic nucleus has been nominated as the inhibitory control center of melanotropes (Tuinhof et al., 1994).

As mentioned above, the anterior lobe and intermediate lobe of the pituitary share the same embryonic origin. Their characteristics, however, diverge both morphologically and functionally during development. The anterior lobe is highly vascularized, whereas the intermediate lobe is essentially non-vascularized, but highly innervated. It should also be pointed out that some of the anterior lobe cells (corticotropes) and most of the intermediate lobe cells (melanotropes) synthesize a common precursor protein called POMC but that corticotropes and melanotropes generate different hormones as final products, i.e., ACTH and α-MSH, respectively. As a step to elucidate the significance of vascularization and avascularization, attempts were made to generate transgenic *Xenopus* frogs expressing vascular endothelial growth factor-A (VEGF-A), specifically in the melanotropes. The intermediate lobes of the transgenic frogs were distinctly vascularized but poorly innervated. The experimentally induced vascularization in the intermediate lobe, as examined after metamorphosis, resulted in a decrease in the expression of both α-MSH and prohormone convertase 2, a cleavage enzyme required for generating α-MSH. In the transgenic specimens, the plasma concentrations of α-MSH were low and the responsiveness of dermal melanophores was incomplete when they were placed on a black background, demonstrating that angiogenesis induced in the intermediate lobe may cause both functional and structural alterations in this organ (Tanaka et al., 2013).

2.3. Development of the hypophysiotropic system in the hypothalamus

It has been well established that the hypothalamus exerts its influence on the function of the anterior lobe of the pituitary by transporting various hypothalamic factors through the median eminence (ME) to the pituitary portal vessels. In amphibians, morphological development of the ME (i.e., thickening of the median floor and embedding of capillaries in the neural tissue) occurs as metamorphosis progresses (Etkin, 1965). The factors inducing the development of the ME were revealed to be TH and contact of the epithelial pituitary with the ME. In *Bufo americanus* tadpoles thyroidectomized at the external gill stage, development of the ME was never attained unless they were treated with TH. In the specimens from which the pituitary primordium was removed at the tail-bud stage, the ME never developed even if they were supplemented with TH. Interestingly, however, in the hypophysectomized and TH-treated tadpoles, a pile-up of neurosecretory materials was observed in the infundibulum at about one third of the distance from the posterior end independently of the materials in the posterior lobe (Etkin et al., 1965). According to immunohistochemical studies with *B. japonicus* larvae, the pile-up was revealed to be corticotropin-releasing factor (CRF) and arginine vasotocin (AVT) (Kawamura and Kikuyama, 1993). Thus, it was concluded that the epithelial pituitary situated in contact with the ME is a prerequisite for the development of the ME, and for the extension of neurons containing hypophysiotropic peptides such as CRF and AVT.

It is worth mentioning that in amphibians, CRF and AVT are the main releasing factors for TSH (Ito et al., 2004; Okada et al., 2005) and ACTH (Okada et al., 2016), respectively, and that TSH and ACTH accelerate metamorphosis by stimulating the release of TH and adrenal corticoids, respectively. This issue will be one of the themes of the following section.

Earlier studies on the development of the gonadotropin-releasing hormone (GnRH)-neuronal system in mammalian and avian species indicated that GnRH-positive cells are detectable in the nasal region prior to their appearance in the brain and that the distribution of the main cell population containing GnRH shifts from the olfactory region to the brain (Schwanzel-Fukuda and Pfaff, 1989; Murakami et al., 1991). This feature was also confirmed in the newt, *C. pyrrhogaster*. In addition, it was demonstrated that GnRH neurons in the brain originate from the nasal region. Removal of both sides of the olfactory placode in the newt larvae resulted in the absence of immunoreactive GnRH cells in the nasal and brain regions as examined at larval and postmetamorphic stages. Unilateral placodectomy resulted in the absence of GnRH cells in the brain and nasal regions only of the operated side (Murakami et al., 1992).

3. Endocrine control of metamorphosis

3.1. Involvement of CRF, TSH, and TH in metamorphosis

As for the pituitary protein hormones, we have obtained and characterized GH (Kobayashi et al., 1989; Yamamoto et al., 2000a) and PRL (Yamamoto and Kikuyama, 1981; Yamamoto et al., 1986a; Matsuda et al., 1990; Yasuda et al., 1991) from some species of amphibians including the bullfrog, *R. catesbeiana*. Luteinizing hormone and follicle-stimulating hormone were also obtained from the bullfrog and were characterized by a group at Gunma University (Takahashi and Hanaoka, 1981; Hayashi et al., 1992a,b). These amphibian pituitary hormones and their RIA systems were often used for the study of growth (Kikuyama et al., 2005) and metamorphosis (Kikuyama et al., 1993a) as well as reproduction (Polzonetti-Magni et al., 2004). The last protein hormone in the amphibian anterior pituitary left to be isolated was TSH. Thus, attempts were made to isolate TSH from the bullfrog pituitary. The TSH preparation obtained was 2.5–4.0 times as potent as porcine or bovine TSH in stimulating the *in vitro* release of thyroxine (T_4) from the thyroid glands taken from hypophysectomized bullfrog tadpoles (Sakai et al., 1987, 1991). Since only a minute amount of this preparation was obtained, the first 10 amino acid residues of the N-terminal sequence of its β-subunit could be elucidated (Sakai, 1992). The sequence, however, coincided precisely with the amino acid sequence of the comparable region deduced from the nucleotide sequence of bullfrog TSHβ cDNA (Okada et al., 2000). Using the TSH β-subunit cDNA as a probe, we measured changes in mRNA expression in larval bullfrog pituitaries. The TSHβ mRNA levels increased progressively throughout prometamorphic stages, reaching their maximum at the end of prometamorphosis. The maximum level was maintained throughout early- and mid-climax and declined thereafter (Okada et al., 2000). A bullfrog TSHβ antiserum was generated by employing a C-terminal peptide (106–129) synthesized based on the amino acid sequence deduced from the bullfrog TSHβ cDNA. The antiserum and antigen

peptide were used for the development of a specific RIA for the measurement of bullfrog TSH (Okada et al., 2004).

Studies endeavoring to identify the hypothalamic factor(s) that stimulates the release of TSH from the amphibian pituitary had been under way for quite a long time. However, no definite answer was obtained due to the lack of a suitable RIA for amphibian TSH. Initially, thyrotropin-releasing hormone (TRH) was considered to be the hypothalamic hormone inducing the release of TSH in amphibians. In the meantime, GnRH and CRF emerged as possible TSH-releasing factors (see Denver, 1996; Shi, 2000). Our group has thus tried to elucidate the contributions of the various TSH-stimulating factors.

Firstly, we studied the effects of TRH, mammalian GnRH (mGnRH), and ovine CRF (oCRF) on the release of immunoassayable TSH by the dispersed anterior pituitary cells of bullfrog larvae and adults. Both TRH and mGnRH moderately stimulated the release of TSH from the adult pituitary cells but not from the larval cells, whereas oCRF markedly stimulated the release of TSH from both larval and adult pituitary cells (Okada et al., 2004). Subsequently, the cDNA encoding a bullfrog CRF (fCRF) precursor was isolated from a cDNA library of the bullfrog hypothalamus (Ito et al., 2004). The amino acid sequence of fCRF showed 83% and 100% identities with oCRF and CRF isolated from the brain of the European green frog, *Rana esculenta* (Okada et al., 2005), respectively. Both oCRF and synthetic fCRF exhibited an equipotent activity in stimulating the release of TSH from the dispersed pituitary cells of adult bullfrogs in an *in vitro* system. Culture of pituitary cells in the presence of the hypothalamic extract (HE) and α-helical $CRF_{9–41}$, a CRF receptor (CRFR) antagonist, revealed that the antagonist suppressed the HE-inducible TSH release by 50%, suggesting that endogenous CRF contributes as a TSH-releasing factor, and that a TSH-releasing substance(s) other than CRF exists in the hypothalamus (Ito et al., 2004). In fact, frog (*Rana ridibunda*) vasoactive intestinal polypeptide (fVIP) and pituitary adenylate cyclase-activating polypeptide (fPACAP) were revealed to have considerable TSH-releasing activities (Okada et al., 2007a).

The actions of CRF are known to be mediated mostly by two types of receptors: type 1 CRFR (CRFR1) and type 2 CRFR (CRFR2). We have obtained the results indicating that fCRF acts through CRFR2 to stimulate the release of TSH. The fCRF-induced release of TSH from the dispersed pituitary cells of the bullfrog was completely blocked by a general CRF receptor antagonist (astressin) or a CRFR2-specific antagonist (antisauvagine-30), whereas a CRFR1-specific antagonist (antalarmin) had no effect on the release of TSH (Okada et al., 2007b). The two types of full-length CRFR cDNAs were obtained from a bullfrog hypothalamic library (Ito et al., 2006). The tissue distribution of the mRNAs encoding the two CRFRs was studied. CRFR1 mRNA was detected almost exclusively in the brain and pituitary, whereas CRFR2 mRNA was recognized in peripheral tissues as well as in the brain and pituitary. *In situ* RT-PCR using bullfrog CRFR2 primers combined with immunohistochemistry using an antiserum against TSHβ confirmed that CRFR2 mRNA is expressed in the bullfrog TSH cells (Okada et al., 2009).

In amphibians, the lack of a TSH RIA prevented the collection of direct evidence for TH-mediated negative feedback. As mentioned above, we have developed a homologous RIA for bullfrog TSH (Okada et al., 2004). Using this RIA system, we demonstrated that at least TH-mediated negative feedback on the pituitary TSH release is operating in prometamorphic and climactic larvae, as well as in juvenile and adult frogs (Kaneko et al., 2005). In this experiment, we studied the effect of TH on the release of TSH from the enzymatically dispersed anterior pituitary cells cultured in the absence or presence of CRF of frog origin (Okada et al., 2005). TH, especially triiodothyronine (T_3), suppressed the release of TSH that was enhanced by CRF in a concentration-dependent manner, but the basal release of TSH was minimally affected.

RIA data presented by various investigators indicate that plasma TH levels in the larvae of various species, such as *R. catesbeiana* (Regards et al., 1978; Mondou and Kaltenbach, 1979; Suzuki and Suzuki, 1981), *Xenopus laevis* (Leloup and Buscaglia, 1977), *Rana clamitans* (Weil, 1986), *Eurycea bislineata* (Alberch et al., 1986), *Ambystoma tigrinum* (Larras-Regard et al., 1981), *Ambystoma gracile* (Eagleson and McKeown, 1978), and *Hynobius nigrescens* (Suzuki, 1987), become elevated as metamorphosis progresses, reach maximum levels during the early stage of metamorphic climax, and decline toward the end of metamorphosis. It should be pointed out that, although the average TH levels are high in metamorphically-climactic larvae, the values fluctuate widely among individuals. We hypothesized that the release of TH is pulsatile as a result of negative feedback of TH on the pituitary and/or hypothalamus (Kikuyama et al., 1993a).

3.2. Involvement of AVT, ACTH, and corticoids in metamorphosis

In the 1950s, several researchers reported that adrenal steroids such as deoxycorticosterone acetate, cortisol, and cortisone accelerated metamorphic changes in various species of amphibian larvae when administered together with TH (Frieden and Naile, 1955; Kaltenbach, 1958; Kobayashi, 1958; Dodd and Dodd, 1976). However, this phenomenon had not been extensively studied for more than 20 years. Thus, experiments were undertaken to analyze the interaction between TH and corticosteroids in tadpoles. Firstly, it was confirmed that endogenous corticoids are involved in the acceleration of TH-induced tail resorption. *B. japonicus* tadpoles, which were in a state of metamorphic stasis via treatment with thiourea, were treated with TH. The metamorphic changes caused by the exogenous TH were slowed by the administration of Amphenone B (an inhibitor of corticoid synthesis) indicating the participation of endogenous corticoids in metamorphosis (Kikuyama et al., 1982). In order to ascertain whether corticosteroids act directly on the TH target organs or indirectly on other sites such as the hypothalamus, pituitary gland, and thyroid gland, we tested various steroid hormones including corticosterone and aldosterone, both of which were known as the main corticoids in *Rana* and *Bufo* species (Carstensen et al., 1961; Crabbé, 1961), for their ability to accelerate the TH-induced shrinkage of tail segments from *B. japonicus* tadpoles *in vitro*. Among the steroid hormones tested, aldosterone and corticosterone exhibited potent activity in accelerating the TH-induced shrinkage of tail segments. Gonadal steroid hormones such as estradiol, testosterone, and progesterone did not exert synergistic action with TH. In the absence of TH, none of the test substances induced tail resorption (Kikuyama et al., 1983). Interestingly, both actions of T_4 and T_3 were enhanced by the corticoids (Kikuyama et al., 1982, 1983).

Regarding the action of corticoids, two mechanisms were proposed. Our group demonstrated that both aldosterone and corticosterone increased the T_3 nuclear binding capacity in the tail fin of bullfrog larvae cultured in the presence of radiolabeled T_3. The increase in T_3 binding was blocked by cycloheximide (an inhibitor of protein synthesis) and actinomycin D (an inhibitor of RNA synthesis) (Suzuki and Kikuyama, 1983). A similar result was obtained in the tail slices of toad (*B. japonicus*) tadpoles (Niki et al., 1981). In later years, it was demonstrated that dexamethasone (Dex, a synthetic corticoid) elevated the expression of TH receptor β (TRβ) mRNA in the tail of *Xenopus* larvae (Iwamuro and Tata, 1995). In this case, the effect of Dex alone was moderate, but it became remarkable when the corticoid was combined with T_3. These findings suggest that corticosteroids elevate the responsiveness to TH by upregulating TRs in the target tissue.

Another mechanism of action of the synergism between TH and corticosteroids is that the latter hormones increase type II iodothyronine 5′-deiodinase, and simultaneously decreases type III iodothyronine 5-deiodinase, indicating that corticoids are involved in the conversion of T_4 to the more biologically active T_3 (Galton, 1990). More recently, it was demonstrated that T_3 plus corticosterone synergistically upregulated the TRβ mRNA in *Xenopus* tail explants, tail and brain *in vivo*, and tissue culture cells, and that the same combination of hormones enhanced the expression of type II iodothyronine 5′-deiodinase mRNA in the tail both *in vitro* and *in vivo* (Bonett et al., 2010),

indicating that both mechanisms of action proposed earlier are prevailing in the tissues of metamorphosing larvae.

Plasma concentrations of aldosterone and corticosterone were determined in the larvae of amphibian species such as *R. catesbeiana* (Jaffe, 1981; Krug et al., 1983; Kikuyama et al., 1986a), *X. laevis* (Jaudet and Hatey, 1984), and *Ambystoma tigrinum* (Carr and Norris, 1988). Most of the data indicated a marked elevation of these hormones concurrently with the elevation of TH levels. It is notable that both plasma levels of corticosterone and aldosterone in bullfrog larvae simultaneously started to rise at the beginning of climax, reached maximum levels at mid-climax, and declined at the end of climax (Jaffe, 1981; Kikuyama et al., 1986a). In amphibians, as in mammals, ACTH is generally believed to be the main factor for stimulating the secretion of corticosteroids (Büchmann et al., 1972; Vaudry et al., 1977; Delarue et al., 1979). The effect of ACTH on the release of aldosterone was confirmed in hypophysectomized bullfrog larvae (Kikuyama et al., 1986a). In this experiment, it was noticed that TH supplementation enhanced the ACTH-inducible release of the corticosteroid. As for the mRNA of POMC, the precursor protein for ACTH, elevation of its levels in the pituitaries of bullfrog larvae was revealed to start by the mid-prometamorphic stage, and reach its maximum at mid-climax (Aida et al., 1999).

CRF was first purified from the ovine hypothalamus as a peptide that possessed potent ACTH-releasing activity (Vale et al., 1981). Subsequently, arginine vasopressin (AVP) was shown to act synergistically with CRF to stimulate the release of ACTH in various mammalian species, although the activity of AVP alone was generally lower than that of CRF. In some species of teleosts, the stimulatory effect of AVT (an AVP ortholog) for the release of ACTH was reported (Fryer et al., 1985; Baker et al., 1996). In amphibians, CRF and AVP exhibited almost equipotent activity in stimulating the release of ACTH from the marsh frog (*R. ridibunda*) pituitary *in vitro* (Tonon et al., 1986). In duck (Castro et al., 1986) and chicken (Mikhailova et al., 2007; Madison et al., 2008), a synergistic action of CRF and AVT on corticotropes has also been shown.

As mentioned above, fCRF acts as the main TSH-releasing factor in the bullfrog, contributing to the promotion of metamorphosis through the pituitary-thyroid axis. In order to clarify the roles of CRF and AVT in regulating the release of ACTH from the bullfrog pituitary, we developed a homologous time-resolved fluoroimmunoassay system for bullfrog ACTH. Using this assay system, the stimulatory effects of AVT and fCRF, either separately or in combination, on the release of ACTH from the bullfrog pituitary were investigated *in vitro*. Both AVT and fCRF stimulated the release of immunoassayable ACTH from dispersed cells of the anterior pituitary in a concentration-dependent manner. AVT exhibited far more potent ACTH-releasing activity than CRF. When both secretagogues were applied in combination, however, they exerted a marked synergistic action. In an attempt to identify the receptor for AVT in the ACTH cells, cDNAs encoding the bullfrog AVT V1a-type and V1b-type receptors were cloned. The expression of mRNAs for V1a- and V1b-type receptors in the anterior lobe of the pituitary was examined via reverse transcriptase-PCR using specific primers. The anterior pituitary predominantly expressed AVT V1b-type receptor mRNA, but expression of AVT V1a-type receptor mRNA was low. Abundant signals for V1b-type receptor mRNA in the ACTH cells were detected by dual-label *in situ* hybridization (Okada et al., 2016). The results indicate that, in the bullfrog, AVT is the major ACTH-releasing factor acting through the AVT V1b-type receptor, and that CRF acts synergistically with AVT to enhance the release of ACTH.

It should be added that AVT possesses a corticosteroid-releasing activity acting directly on the adrenal tissue (Larcher et al.,1989; Kloas and Hanke, 1990; Iwamuro et al.,1991) through V2 and/or oxytocin-like receptors (Larcher et al., 1992).

3.3. Role of PRL in metamorphosis

In amphibians, PRL emerged as a hormone that regulates larval growth. Etkin and Lehrer (1960) investigated the growth rate of *R. pipiens* larvae in which the pituitary primordium was transplanted to the tail during the embryonic stage. The growth rate of the tadpoles from which pituitary primordium was removed, but not transplanted ectopically, was significantly reduced. The growth rate of the specimens with autografted pituitaries exhibited a growth rate similar to that of intact larvae at an early stage of development, and exceeded that of the normal tadpoles at later stages. In mammals, PRL, among the anterior lobe hormones, is known as the only hormone that is released autonomously when the pituitary is disconnected from the hypothalamus. Using the mode of secretion of PRL in mammals as an analogy, they assumed that the growth-promoting substance is PRL. Later, the growth-promoting effect of PRL was confirmed in bullfrog larvae using mammalian PRL (Berman et al., 1964). Another important finding with regard to the action of PRL in amphibian larvae is that PRL antagonizes the action of TH, and thus suppresses metamorphic changes. Administration of mammalian PRL to metamorphosing larvae was shown to block tail resorption (Bern et al., 1967; Etkin and Gona, 1967).

Secretion of anterior pituitary hormones is regulated by hypothalamic hormones that are conveyed to the ME and transported to the pituitary via the hypophyseal portal system. As mentioned in Section 2, development of the ME in amphibian larvae occurs around the onset of metamorphic climax. Provided that PRL secretion in amphibians is primarily under the inhibitory control of the hypothalamus as in mammals, the amount of inhibiting factor(s) supplied to the anterior pituitary is expected to be low at early larval stages and increased as the development of the ME proceeds at later stages. On the basis of this view, Etkin (1970) hypothesized that PRL levels are high during premetamorphosis and early prometamorphosis, and the levels gradually decline as metamorphosis progresses. Higher PRL levels during the earlier stages would facilitate the larval growth, and declined PRL levels during later stages would be advantageous for TH to induce metamorphic changes. Although this hypothesis was widely accepted, it remained to be demonstrated until the plasma PRL levels in the larvae could be measured by an RIA specific for amphibian PRL. For this purpose, attempts were made to purify PRL from the bullfrog pituitary. As a result, we obtained highly purified bullfrog PRL (Yamamoto and Kikuyama, 1981), which was utilized for antibody production (Yamamoto and Kikuyama, 1982a) and later, for determination of its amino acid sequence (Yasuda et al., 1991). A sensitive and specific RIA for the bullfrog PRL was developed employing the bullfrog PRL and its antiserum thus obtained (Yamamoto and Kikuyama, 1982a). Using this RIA system, plasma PRL levels in tadpoles at various metamorphic stages were measured. In contrast to Etkin's hypothesis, plasma PRL levels were relatively low during premetamorphosis, and remained low during prometamorphosis. During the early climax period, a slight elevation of PRL levels and a marked elevation at late climax occurred, followed by a slight decline at the end of metamorphosis (Yamamoto and Kikuyama, 1982a). PRL concentrations in the pituitaries of climax tadpoles were higher than the values obtained from preclimax tadpoles (Yamamoto et al., 1986b). Subsequently, pituitary PRL mRNA levels in metamorphosing bullfrog tadpoles were measured using bullfrog PRL cDNA as a probe. PRL mRNA levels rose at mid-climax, showing a pattern of changes similar to the plasma PRL concentration and pituitary PRL content (Takahashi et al., 1990). Similar changes of pituitary PRL mRNA levels have been confirmed in *X. laevis* larvae (Buckbinder and Brown, 1993). In addition, we found that an almost identical pattern of changes in the PRL receptor (PRLR) mRNA levels takes place in the tail fin and kidney of the bullfrog tadpoles (Hasunuma et al., 2004).

Immunoneutralization of bullfrog larvae with antiserum against homologous PRL caused a precocious resorption of their tail (Yamamoto and Kikuyama, 1982b) and slowing of lung maturation by suppressing the increase in pulmonary surfactant that occurs during

late climax (Oguchi et al., 1994). Considering that the tail is a larval organ and the lung is an adult organ, PRL seems to play a dual role: modulation of metamorphic speed and contribution to the development of organs necessary for adult life. For metamorphosing larvae, regulation of the speed of metamorphic changes by PRL, counteracting the action of TH, seems to be of vital importance. For instance, an excessively rapid resorption of the larval organs such as the tail may result in the loss of decomposed substances generated by TH that should be used as materials for construction of organs for adult life, and as an energy source for larvae fasting during the climax period. The molecular mechanism of interaction of PRL and TH is described elsewhere (Shi, 2000).

In order to understand the mechanisms involved in the hypersecretion of PRL occurring during late climax, pituitary stalk transection or pituitary auto-transplantation were performed in bullfrog tadpoles at stage XXII (two stages prior to the stage where PRL levels in the intact animals reach their maximum). Metamorphosis of these animals progressed normally, but their PRL levels remained low as measured at stage XXIV. It was concluded that hypothalamic stimulation, rather than release from hypothalamic inhibition, is necessary for the PRL surge during the late climax stage (Kawamura et al., 1986).

As for the hypothalamic factor for the stimulation of PRL release from the amphibian pituitary, TRH has been regarded as the most potent PRL-releasing substance contained in the amphibian hypothalamus. Earlier works indicated that the tripeptide simulates the release of PRL from the pituitary *in vitro* (Clemons et al., 1979; Hall and Chadwick, 1984; Seki and Kikuyama, 1986) and *in vivo* (Kühn et al., 1985). An acid extract of bullfrog hypothalami had potent stimulating activity on the release of PRL from the bullfrog pituitary *in vitro*. This activity was markedly reduced by coincubation with the IgG fraction obtained from the antiserum against TRH (Seki et al., 1988). Isolation and characterization of the most potent PRL-releasing substance contained in the bullfrog hypothalamus were performed, and the substance was determined to be TRH (Nakajima et al., 1993).

We have recently cloned cDNAs for three types of bullfrog TRH receptors: TRHR1, TRHR2, and TRHR3. Analyses with semi-quantitative reverse transcription-PCR and *in situ* hybridization revealed that TRHR3 mRNA is expressed in the anterior lobe, and that the signals reside mostly in the PRL cells. It was also noted that the expression levels of TRHR3 mRNA in the anterior pituitary as well as in the PRL cells of tadpoles elevate as metamorphosis progresses (Nakano et al., 2018). Thus, TRH was revealed to be the major PRL-releasing factor in the hypothalamus, acting on the PRL cells through TRHR3.

Hypothalamic factors that regulate the release of major pituitary hormones involved in metamorphosis and their receptors expressed on the relevant pituitary cells are shown in Fig. 2.

4. Endocrine control of reproductive behavior and courtship pheromone secretion

4.1. Hormones eliciting courtship behavior in Cynops newts

During the breeding season, the male red-bellied newt, *C. pyrrhogaster*, approaches a female and confirms that she is sexually mature, by way of a specific pheromone emitted through her cloaca. Then the male blocks the female's snout with his neck, generates a water current toward her snout by vibrating his tail vigorously sending a female-attracting pheromone (Fig. 3). The male newt performs this behavior repeatedly, after which the male parades in front of the female partner, who then follows him. The male ultimately deposits spermatophores, and the female picks them up with her cloacal orifice (Kikuyama et al., 2003). A congeneric species, the sword-tailed newt (*Cynops ensicauda*) is known to show courtship behaviors similar to those of the red-bellied newt.

In order to elucidate the hormonal mechanisms involved in the expression of this courtship behavior, extensive studies have been carried out using intact, castrated, and hypophysectomized newts obtained during both the breeding and non-breeding seasons (Kikuyama et al., 2003; Toyoda et al., 2015). The results of behavioral tests were evaluated by the percentage of animals that exhibited tail-vibration toward the sexually developed female partner (defined as incidence), and by the mean number of times of this behavior performed per test animal over the test period (defined as frequency). Earlier study on the male red-bellied newt suggested that both PRL and androgen are required for maintaining the courtship behavior for extended periods of time (Toyoda et al., 1993). It was evidenced that endogenous PRL in the male acts to bring about the tail-vibrating behavior, since administration of an antiserum against newt PRL to sexually active male newts suppressed the expression of tail vibration behavior (Toyoda et al., 1996).

Intracerebroventricular injection of male newts with antiserum against newt PRL or anti-newt PRLR antibody was far more effective than intraperitoneal injection of the antiserum or antibody in blocking tail vibration behavior, indicating that PRL acts centrally to elicit the courtship behavior of male newts (Toyoda et al., 2005). In this case, PRL seems to be of pituitary origin, since the male newts that never exhibit tail vibration behavior after the removal of their pituitary come to show the behavior by systemic injections of PRL and gonadotropin (Toyoda et al, 1993). In the newt, PRLR immunoreactivity and PRLR mRNA were detected in the epithelial cells of choroid plexus of the brain. Thus, it has been assumed that the choroid plexus may serve for transporting PRL from the blood into the cerebrospinal fluid through the PRLRs (Hasunuma et al., 2005). As for androgen, intracerebroventricular injection of male newts with flutamide (an androgen receptor antagonist) suppressed spontaneously occurring tail vibration behavior more effectively than intraperitoneal injection of the antagonist, indicating that androgen also acts centrally to elicit courtship behavior (Toyoda et al., 2015).

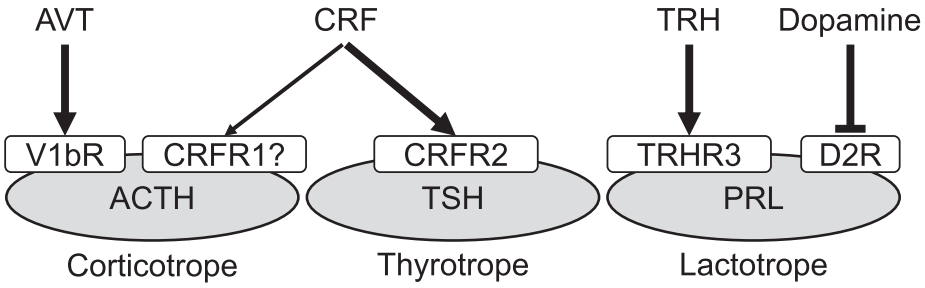

Fig. 2. Hypothalamic regulation of the release of major pituitary hormones that are involved in the process of amphibian metamorphosis. AVT stimulates the release of ACTH via V1b-type AVT receptor (V1bR). Although ACTH-releasing activity of CRF by itself is far lower than that of AVT, CRF acts on the corticotropes synergistically with AVT to enhance the ACTH release. Presence of type 1 CRF (CRFR1) receptor on corticotropes has not been confirmed in amphibians but in other classes of vertebrates. CRF potently stimulates the release of TSH through type 2 CRF receptor (CRFR2). The release of PRL is stimulated via type 3 TRH receptor (TRHR3) and suppressed by dopamine through D2 dopamine receptor (D2R).

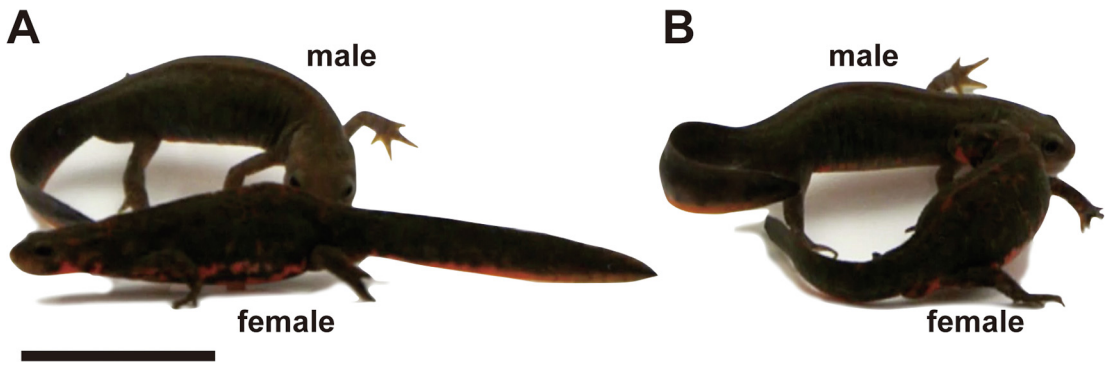

Fig. 3. Photographs of the behavior performed by sexually developed male and female *Cynops* newts. Prior to the courtship, the male newt confirms that the female partner is sexually developed one by means of a pheromone (imorin) emitted through her cloaca (A). Subsequently, the male blocks her path with his neck in contact with female's snout and vibrates his tail sending a pheromone (sodefrin) emitted through his cloaca toward her nostril (B). Scale bar = 30 mm.

Previous studies revealed that AVT enhances courtship behavior (Toyoda et al., 2003). Interestingly, colocalization of PRLR immunoreactivity and AVT immunoreactivity were demonstrated in the magnocellular preoptic nucleus (Hasunuma et al., 2005). More recently, a neurosteroid, 7α-hydroxypregnenolone (7α-OH PREG), known as a neuronal activator to stimulate locomotion activity in the breeding newts (Matsunaga et al., 2004), was added to the list of hormones involved in eliciting courtship behavior. Our experiments with male red-bellied newts demonstrated that 7α-OH PREG enhances the expression of courtship behavior through a dopminergic system mediated by D2 dopamine receptor in the brain (Toyoda et al., 2012). It was also revealed that preoptic neurons expressing cytochrome-P450$_{7\alpha}$ that catalyzes the synthesis of 7α-OH PREG were equipped with PRLR (Haraguchi et al., 2010). Thus, the interaction of the above mentioned four substances (androgen, PRL, AVT, and 7α-OH PREG) was analyzed by examining whether the cessation of the behavior caused by the deprivation of any one of the actions of the four substances is restored by supplementation with any one of the remaining three substances. It was revealed that PRL and androgen are likely to participate mainly in the activation of the AVT system, and that PRL may also contribute to the increase of incidence of the behavior through the activation of 7α-OH PREG-producing neurons. AVT by itself enhances the frequency of the tail-vibrating behavior, and also contributes to the increase of the incidence through the activation of 7α-OH PREG neurons (Toyoda et al., 2015).

In adult *Cynops*, the male is equipped with a broader tail with a well-developed fin as compared with the female. This tail structure in the male is considered to be suitable for sending the pheromone-containing water effectively toward the female partner. We have shown that PRL increases the tail height markedly and that estrogen suppresses the action of PRL (Kikuyama et al., 1986b).

In urodeles, anuran larvae and fish, Mauthner cells located at the level of VIII cranial nerve root in the medulla oblongata are known to be large in size and involved in tail movement (Faber and Korn, 1978). In the red-bellied newts, nuclei and cell bodies of Mauthner cells are larger in sexually responsive males than in sexually non-responsive males or sexually responsive females. Hypophysectomy in sexually responsive males resulted in decreases in nuclear and cell volumes, which were restored by the supplementation with PRL and gonadotropins (Matsumoto et al., 1995). Moreover, quantitative electron microscopic analysis revealed that the number of synapses on the somata of Mauthner cells is increased by a combination of androgen and PRL (Matsumoto et al., 1997). Thus, it is presumed that the hormonally inducible development of Mauthner cell function is important for the tail vibration exhibited during the initial phase of courtship behavior.

4.2. Courtship pheromones and hormonal control of their secretion

It had long been postulated that urodele males that exhibit courtship behavior emit courtship pheromones to attract conspecific females (Houck, 1986). Based on the observation that the water in which sexually active male *C. pyrrhogaster* specimens had been kept exhibited an attracting activity toward sexually developed females (Toyoda et al., 1994), the first amphibian pheromone possessing potent female-attracting activity was isolated from the abdominal gland. This pheromone, designated sodefrin, was a peptide composed of 10 amino acid residues (SIPSKDALLK). In fact, sodefrin was the first peptide pheromone identified in a vertebrate (Kikuyama et al., 1995). RIA and immunohistochemistry using antiserum against sodefrin revealed that a combination of PRL and androgen enhances the synthesis of sodefrin in the abdominal gland (Yamamoto et al., 1996). Immunoelectron-microscopic study revealed that the pheromone resides within the secretory granules in the epithelial cells of the abdominal gland (Kikuyama et al., 1997). Sequence analysis of cDNA encoding sodefrin that was obtained from an abdominal gland cDNA library revealed that sodefrin is generated from a precursor protein composed of 198 amino acid residues and that the sodefrin molecule is to be flanked by monobasic amino acid residues sandwiching the sodefrin sequence (Iwata et al., 1999). Presence in the abdominal gland of proteolytic enzymes that are capable of generating sodefrin was demonstrated using crude abdominal gland extracts and synthetic substrates. Activity of the enzymes presumed to belong to the serine protease family was higher in the extract of the abdominal glands of sexually developed males than in that of sexually undeveloped ones (Nakada et al., 2007a).

In relation to the sodefrin precursor protein, an interesting finding is that proteins secreted by the mental gland in the terrestrially courting lungless salamanders (Plethodontidae) function as courtship pheromones and that they have sequence similarity to the sodefrin precursor protein, but lack the sodefrin sequence (Houck et al., 2008). Consequently, these proteins were termed Sodefrin Precursor-like Factors (SPF) (Palmer et al., 2007). Subsequently, the cloacal gland in the aquatically courting newt species (*Lissotriton helveticus*) was shown to secrete the SPF pheromone as well (Van Bocxlaer et al., 2015). Furthermore, analyses of SPF-family transcripts in cloacal glands of Salamandridae revealed that sodefrin precursor transcripts contain an extra 62-base insert as compared to SPF transcripts and that the insertion causes a frameshift in protein translation, generating the sodefrin sequence and enzymatic cleavage sites for sodefrin. This frameshift mutation is assumed to have occurred in an ancestral *Cynops* lineage approximately 34.3–26.5 million years ago (Janssenswillen et al., 2015).

The possible existence of a sodefrin-like peptide in the abdominal gland of the cloaca of the sword-tailed newt, *C. ensicauda* male was also shown by the analysis of a cDNA clone encoding sodefrin precursor-like protein (Iwata et al., 1999). Subsequently, a decapeptide

(SILSKDAQLK) was isolated from the abdominal glands of sword-tailed newts. The peptide was different from that of sodefrin by two amino acid residues, and this peptide was designated silefrin (Yamamoto et al., 2000b). Both native and synthetic silefrin exerted a potent attracting activity toward conspecific females (Iwata et al., 1999; Yamamoto et al., 2000b). Using Northern blot analysis, Iwata et al. (2000) demonstrated that the expression of silefrin precursor mRNA is markedly enhanced by treating hypophysectomized males with a combination of PRL and androgen. The presence of receptors for androgen and PRL in the abdominal gland was reported in red-bellied newts (Matsumoto et al., 1996) and sword-tailed newts (Matsukawa et al., 2004), respectively.

Discharge of the male pheromone, sodefrin is likely to be elicited by AVT through the AVT V1-type receptor. Administration of AVT to the male newts captured in the pre-reproductive period and kept in solitude decreased immunoassayable sodefrin content in the abdominal gland without any sign of courtship behavior (Toyoda et al., 2003). The sexually developed male newts received an injection of V1- or V2-type receptor antagonist in advance and were paired with females. The sodefrin content of the abdominal gland was significantly higher in the V1-type receptor antagonist-injected group than in the V2-type receptor antagonist-injected group, as measured 24 h after injection (Kikuyama et al., 1999).

As for the responsiveness of the female newts to sodefrin, bilateral nostril plugging completely abolished the attracting activity of the pheromone, indicating that sodefrin acts on the olfactory organ (Toyoda et al., 1995). The olfactory system of urodeles consists of two morphologically distinct epithelia, namely the main olfactory epithelium and the vomeronasal epithelium (Eisthen, 1992). Electrophysiological analyses by means of electro-olfactogram (EOG) recordings revealed that sodefrin elicits marked dose-dependent responses on the epithelium of the lateral nasal sinus (LNS) region, a putative vomeronasal organ (VNO) (Jones et al., 1994), of the sexually responsive females. In ovariectomized females, treatment with PRL and estradiol markedly enhanced the EOG response (Toyoda and Kikuyama, 2000). It was concluded that the main site of action of sodefrin resides in the LNS, and that responsiveness to the pheromone is under the control of PRL and estrogen. This was also confirmed at the cellular level. Sodefrin elicited a marked elevation of intracellular concentrations of Ca^{2+} ($[Ca^{2+}]_i$) in a small population of dissociated LNS epithelial cells from sexually developed females. The population of cells exhibiting sodefrin-induced elevation of $[Ca^{2+}]_i$ increased in a concentration-dependent manner. In the cells from hypophysectomized and ovariectomized females, the sodefrin-inducible increase of $[Ca^{2+}]_i$ did not occur, whereas the cells from the operated females supplemented with PRL and estradiol exhibited a high incidence of $[Ca^{2+}]_i$ responses to the pheromone (Iwata et al., 2013). It has been assumed that sodefrin is detected by a certain population of microvillus neurons of the VNO expressing vomeronasal type-2 receptors coupled with one of the G-protein α-subunits, $G\alpha_o$ (Nakada et al., 2014).

Finally, it should be noted that a female pheromone with amino acid sequence AEF, which is emitted by sexually responsive newts and attracts males who are ready to commence the tail vibration behavior toward the females, was recently isolated from the oviduct. This female pheromone, designated imorin, was confirmed to be expressed in the ciliary cells of the proximal portion of the oviduct in sexually developed females, but not in sexually undeveloped females (Nakada et al., 2017).

5. Conclusion and perspective

Evidence suggests that the adenohypophysis originates from the neuroectoderm, indicating that RP is an intermediate structure of the adenohypophyseal primordium. It is worth mentioning that a subset of the neural cells in the cerebral ganglion and dorsal strand cells in an ascidian species (*Halocynthia roretzi*) belonging to the protochordates contained PRL-immunoreactive granules (Terakado et al., 1997), and that a different subset of cells contained POMC-immunopositive substances (Kawahara et al., 2002), as well as prohormone convertase-like substances (Kawahara et al., 2003). This implies that progenitors of vertebrate adenohypophyseal cells consist of neural cells.

Using *Bufo* embryos, we have demonstrated that an inductive influence from/via the posterior hypothalamus is prerequisite for the development of POMC cells, whereas other pituitary cells develops independent of the posterior hypothalamus (Kawamura and Kikuyama, 1995). Prior to the contact with the posterior hypothalamus, the pituitary primordium fuses to the foregut to form RP-like structure (Kawamura and Kikuyama, 1992). We have obtained a result suggesting that the foregut endodermal cells play an essential role in the differentiation of adenohypophyseal cells, provided that the development of GH-immunoreactive cells is regarded as a criterion (Furuya et al., 1997).

It is generally accepted that metamorphosis in amphibians is under the control of PRL, TH, and adrenal corticoids and that the release of TH and corticosteroids is stimulated by TSH and ACTH, respectively. Interestingly, the major TSH-releasing hormone in amphibians is CRF, whereas in mammals it is TRH, indicating that a shift in the role of hypothalamic releasing factors occurred during evolution. In this regard, the fact that TSH secretion in amphibians is controlled by CRF and not by TRH may be reasonable. There are indirect evidences that in amphibians, the secretion of TRH is enhanced when they are subjected to a cold temperature (Takahashi et al., 2001; Tonosaki et al., 2004; Mosconi et al., 2002; Yazawa et al., 1999). If the release of TSH in amphibian larvae were stimulated by TRH, they could be forced to metamorphose in winter. This situation would pose problems for the juvenile frogs, which are devoid of any metabolic system for generating heat.

Very recently we have obtained evidence that a novel type of PRL (PRL1B) exists in the bullfrog pituitary (Okada et al., 2019). Since PRL1B is detected exclusively in the larval pituitary and the expression levels of PRL1B mRNA in the pituitary are relatively high during pre- and pro-metamorphosis, and decline abruptly at the onset of metamorphic climax, clarification of the role(s) of PRL1B in larval growth and metamorphosis is awaited.

Recently, an interesting finding has been reported by Van Bocxlaer et al. (2016). According to them, *C. pyrrhogaster* males release not only the peptide pheromone sodefrin but also the SPF family proteins during courtship and that the proteins function as courtship pheromones. For the following reasons, however, it can be said that sodefrin is the major pheromone that is contained in the abdominal gland and released into the water. One reason is that among the fractions obtained at each step for isolation of the female-attracting substance from the abdominal gland of the red-bellied newts, sodefrin was invariably contained in the fraction exhibiting the most potent activity (Kikuyama et al., 1995). Another reason is that in an experiment with an anti-sodefrin antibody-coupled affinity column, the adsorbed fraction of a male-conditioned water was found to retain more potent female-attracting activity than the unadsorbed fraction (Yamamoto et al., unpublished).

We have observed that two congeneric species of male newts possess decapeptide pheromones for courtship. Each pheromone is slightly different in terms of amino acid sequence and attracts only conspecific females, leading them to reproductive isolation. Considering that their pheromones are peptides, a slight mutation of pheromone precursor genes might lead to differentiation of species (Kikuyama et al., 2013). In fact, we isolated [Val^8] sodefrin in addition to sodefrin from the abdominal gland of a population of the red-bellied newts that inhabit a limited area of the central region of the main island of Japan. The region-specific sodefrin variant was revealed to exhibit a potent activity only toward the females belonging to the same population (Nakada et al., 2007b).

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Declarations of interest

None.

Appendix A. Supplementary data

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