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RAPID COMMUNICATION

Concentration of Gonadotropin-Releasing Hormone in the Brain During Metamorphosis in the Lamprey, *Petromyzon marinus*

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ABSTRACT The concentration of lamprey gonadotropin-releasing hormone (lamprey GnRH) and relative amounts of a second GnRH-like form from brains of larvae (ammocoetes) and individuals during the seven stages of lamprey metamorphosis were measured using a lamprey GnRH radioimmunoassay following elution on a reverse-phase high performance liquid chromatography (HPLC) system. Lamprey GnRH was not detectable in ammocoetes and was present in small quantities in only a few samples of metamorphosing individuals in stages 1–5 (0.475–0.667 ng/brain) but was more conspicuous at stage 6 (0.873 ± 0.308) and had increased significantly (7.8 ± 0.371) by the final stage (7). The second, GnRH-like hormone eluted at a different time on HPLC compared to lamprey GnRH and was detected by the lamprey GnRH antiserum. This second GnRH-like hormone appeared at stage 4 and was present, at a progressively increased relative concentration, in almost all samples to the end of metamorphosis where the level of 5.15 ± 0.625 ng/brain was significantly lower than that of lamprey GnRH. The brain weight increased by the final two stages but concentration of the two hormones/brain sample weight revealed, for both hormones, a trend of increased levels as metamorphosis progressed and significant differences in concentration between stages 6 and 7. The timing of increased concentration of GnRH in the brain of metamorphosing lampreys is discussed with reference to the acceleration of gonad maturation during and immediately after metamorphosis.

Lamprey metamorphosis is a programmed and highly synchronized process which involves dramatic changes in both external and internal features (Youson, '80, '85, '88; Potter et al., '82). During this interval of the lamprey life cycle, the filter-feeding, rather sedentary larva (ammocoete) transforms into an adult which, in parasitic species, is capable of macrophagous feeding on the tissues and blood of host fish (Hardisty and Potter, '71a,b). There have been numerous descriptions of organ transformation and development during lamprey metamorphosis (for review see Youson, '88), yet there is still no definitive explanation of the factors which initiate and control the complex series of events. There are also only a few reports on the measurements of physiological parameters during this phase of the lamprey life cycle, mainly due to the inability to obtain large numbers of animals at each of the various stages of metamorphosis.

One explanation of the relevance of lamprey metamorphosis might be that it is an essential step towards assuring the continuation of the species. There is also a strong belief that neoteny and paedomorphosis do not exist in lampreys (Vladykov, '85) and even at the time of initiation of metamorphosis, the gonads of larval lampreys are immature (Hardisty, '71). Although sexual maturation begins at different times in the various lamprey species (Hardisty et al., '86), they all have in common the fact that it accelerates in postmetamorphic life. In nonparasitic species the final intervals of gonad maturation are initiated immediately after metamorphosis, whereas in parasitic species the same phases of gonad maturation are spread out over a feeding period of several months to up to 2 years. Despite this variation, in both life history types there is at least some growth in the gonad following metamorpho-

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sis. Therefore, it might be expected that the gonad is either stimulated during metamorphosis or immediately after the completion of the event.

Gonadotropin-releasing hormone (GnRH) from the brains of adult lampreys, *Petromyzon marinus,* has been characterized (Sherwood et al., '86) and physiological (Sower et al., '87; Sower, '89) and immunocytochemical (King et al., '88) evidence imply that the hormone regulates a pituitary-gonadal axis. However, lamprey gonadotropin(s) have not been identified. Recently, it has been shown through radioimmunoassay and high performance liquid chromatography (HPLC) analysis that there is a relationship between concentration of brain GnRH and gonad development in *P. marinus* during their upstream, spawning migration (Fahien and Sower, '90). It was the objective of the present study to examine GnRH concentration in the brains of lampreys during late larval life and during the various stages of their metamorphosis to establish the time at which this organ might exert an influence on the gonad.

MATERIALS AND METHODS

Animals

Larval lampreys (ammocoetes) of *P. marinus* were collected in May 1989 by electrofishing several streams in New Brunswick, Canada. They were transported back to the laboratory and were housed in fiberglass aquaria containing 5–10 cm of river silt and recycled, dechlorinated water at 17–20°C. The animals were kept on a light:dark cycle which paralleled their natural environment and were fed yeast and Liquifry for Live Bearers (Hagen, Montreal, P.Q.) three times a week. In the second week of July the ammocoetes were examined for external signs of the beginning of metamorphosis (stage 1). Metamorphosing individuals were separated from nonmetamorphosing ammocoetes and were housed in aquaria as described above, except that the animals were not fed, for this is a nontrophic phase of their life cycle. At specific intervals over the next 4 months (Potter et al., '78), 12 animals at each of the seven stages of metamorphosis (Youson and Potter, '79) were removed and anaesthetized in tricaine methanesulfonate (MS222). In the middle of August when all metamorphosis would have been initiated (Potter et al., '78), 12 ammocoetes of metamorphosing size, at least 2.0 g and 110 mm, were anaesthetized. The ammocoetes and metamorphosing individuals at each stage (1, earliest; 7, latest) were killed by decapitation. Brains were extirpated, immediately frozen in liquid nitrogen, and stored at −70°C.

Tissue extraction

Brain tissue was extracted according to the method of Yu et al. ('87) and described in Fahien and Sower ('90) with the following modifications. Three brains were pooled to give a total of four samples in ammocoetes and in each of the seven stages of metamorphosis. Samples were weighed and homogenized in 2 ml of 2.0 M ice-cold acetic acid with a Polytron using two 5-second bursts at speeds 5 and three 5-second bursts at speed 8. The homogenates were then centrifuged, dried overnight on a refrigerated vacuum centrifuge, reconstituted in 250 μl of millipore-filtered water, and further centrifuged.

Individual extracts of 20 μl were injected into a 10 μl loop of an HPLC system which consisted of a Perkin and Elmer series 100 pump with a Pecosphere 3 CR C18 (0.46 × 8.3 cm) reverse-phase column (see Fahien and Sower, '90). The isocratic mobile phase consisting of 7.40 g ammonium acetate and 3.04 g citric acid in 1 L of 19% acetonitrile/water (final pH was adjusted to 4.6 with phosphoric acid) (Stopa et al., '88) gave a retention time of 5 minutes at a flow rate of 2 ml/min. The second GnRH-like form gave a retention time of 2.5 minutes. Twenty 600 μl fractions were collected for each sample at a rate of 600 μl/18 seconds. HPLC water was injected onto the column after every sample (as a blank control), and 20 fractions were collected. At the end of the samples, a lamprey GnRH standard (0.5 mg/ml) was injected, and 20 fractions were collected. Duplicate, 100 μl aliquots of the peak fractions from each sample, blank control, and standard fractions were assayed for lamprey GnRH by radioimmunoassay. In some samples, a second GnRH-like molecule eluted at the same place as has been noted in extraction of GnRH in brains of adult lampreys. This fraction was also assayed using the lamprey GnRH radioimmunoassay.

Radioimmunoassay

Radioimmunoassay for lamprey GnRH was carried out in the manner as previously described (Stopa et al., '88). Lamprey GnRH antisera (J.A.K. 1467 and 21-134) were used at final dilutions of 1:50,000 and 1:100,000. Antisera generated against lamprey GnRH (J.A.K. 1467) demonstrated less than 0.02% cross-reactivity with mammal GnRH (J.A.K. and R.M., unpublished).

The 21-2134 antibody demonstrated less than 0.001% cross-reactivity with mammal, chicken I, chicken II, and salmon GnRH (Calvin et al., '89). The lower limit of detection was 9.8 pg/0.1 ml based on the confidence limits of the estimate of the zero standard distinguished from least concentration of standard. The range of antibody binding was 36–43%. Synthetic lamprey GnRH was iodinated using a modification of the chloramine-T method and purified as described by Stopa et al. ('88). The second GnRH-like form was detected by both lamprey GnRH antibodies and is also recognized by a mammalian GnRH antibody (1245). In a previous study on adult lampreys (Sherwood et al., '86), a mammalian GnRH antibody (R-42) with specificity for the entire molecule, and not fragmented or extended molecules, was used to show that this second GnRH-like form in lampreys is a decapeptide with the same terminal residues as other GnRH molecules. The mammalian GnRH antibody 1245 is similar to R-42 and is able to recognize the second lamprey GnRH in adult lampreys (Sower, unpublished observations). The similarity of the second GnRH-like form to lamprey GnRH permitted us to quantitate fractions of the former with the lamprey GnRH radioimmunoassay.

Statistical analysis

Data for hormone concentrations of stages 5 through 7 were analyzed by a Tukey HSD test (Zar, '74) after preliminary analysis of variance. In all tests, the level of significance for differing samples was $P < 0.001$.

RESULTS AND DISCUSSION

Synthetic lamprey GnRH eluted at a retention time of 5 minutes (Fig. 1A). In this HPLC system, synthetic standards of mammal, chicken II, and salmon elute at fraction numbers 7, 26, and 35 (data not shown). Brain samples showed two separate and distinct peaks of GnRH as shown from one sample from stage 7 (Fig. 1B). The weights of pooled brain samples were consistent in larvae and over stages 1–5 of metamorphosis (20–32 mg) but were higher (46–54 mg) in stages 6 and 7. Data were calculated as concentrations per brain. Lamprey GnRH was detected in only one of the four samples of stage 1 and stage 3 but no samples in ammocoetes and in stages 2 and 4 contained detectable levels of hormone (Fig. 2). Two of four samples possessed hormone at stage 5 and all fractions contained lamprey GnRH in the final two stages. However, hormone concentration in the brain at stage 6 was not substantially differ-

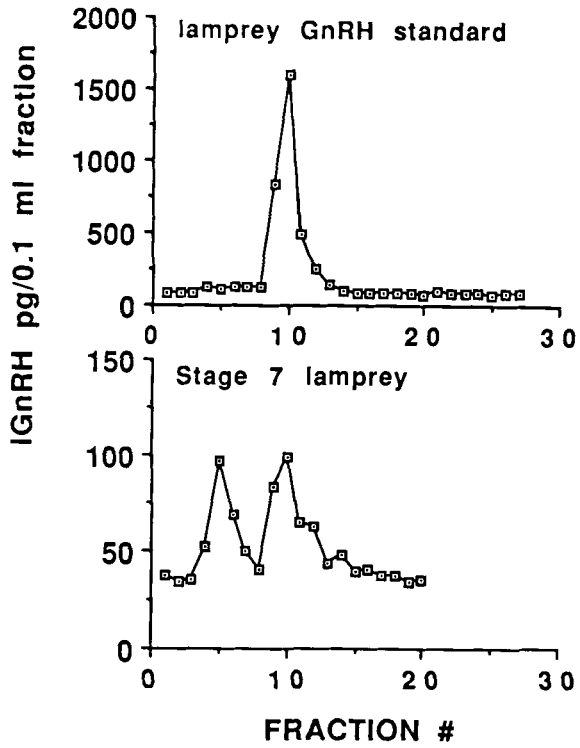

Fig. 1. **(Top)** HPLC of synthetic lamprey GnRH. **(Bottom)** One brain sample (stage 7) showing two separate and distinct peaks of GnRH following HPLC elution and radioimmunoassay.

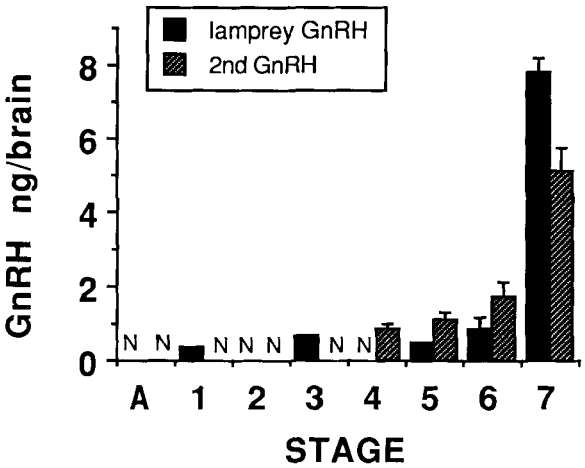

Fig. 2. Concentration (ng/brain) of two forms of GnRH in brain extracts from ammocoetes (A) and from individuals at seven (1–7) stages of metamorphosis. Data are means of four pooled samples (three brains in each pool) ± 1 S.E. (vertical bars). N, not detectable.

ent from that of earlier stages (Fig. 2). The increase in concentration of lamprey GnRH from 0.873 ± 0.308 ng/brain in stage 6 to 7.8 ± 0.371 ng/brain at stage 7 was significantly different ($P < 0.001$). The second form of GnRH-like molecule occurred in three out of the four samples in both stages 4 and 5 (relative amounts of 0.886 ± 0.113 and 1.113 ± 0.177 ng/brain, respectively) and all samples in the last two stages (Fig. 2). As with lamprey GnRH, this molecule showed a significant increase ($P < 0.001$) in concentration between stages 6 (1.72 ± 0.413 ng/brain) and 7 (5.15 ± 0.625 ng/brain).

This present study has used HPLC separation and radioimmunoassay to show that in the parasitic lamprey, *P. marinus,* brain concentrations of lamprey GnRH begin to increase during the late stages of metamorphosis. Specifically, between stages 5 and 6 there is an increased frequency of detectable hormone and it is significantly elevated by stage 7. These data indicate that there have been either marked morphological changes in the brain or that existing cells within the brain have been stimulated to synthesize the hormone. There have been no extensive investigations of changes in the lamprey brain using samples of animals during metamorphosis (Youson, '80). The significance of an apparent increase in brain weight in late metamorphic stages is not known. The extirpation of intact lamprey brains from surrounding tissues is difficult, but we used a consistent procedure throughout the experimental period. Moreover, the increased brain weight cannot be accounted for by differences in animal size due to growth during metamorphosis. Lamprey metamorphosis is a nontrophic phase and is marked by a decline in body length and weight (Potter et al., '82). Comparisons between larval and adult brains suggest that major changes are confined to the visual and trigeminal systems (Rovainen, '79) and that most regions show an enlargement and maturation of neurons which have been present since they first appeared in the embryo (Rovainen, '82). DNA synthesis is rather limited in neurons of most regions of the brain during metamorphosis (Rovainen, '79, '82). Therefore, it is most likely that existing cells in certain regions of the brain either begin synthesizing hormone for the first time or they increase their capacity to synthesize GnRH. The release rates of GnRH from the neurons are completely unknown.

We have no explanation for the small concentrations of GnRH in one sample of each of stages 1 and 3 other than the fact that there is probably individual variation and that existing cells have the potential for synthesis of the hormone. The variation may also reflect some sexual dimorphism, for we made no effort to distinguish between sexes in this small, but rare, sample. Crim et al. ('79) demonstrated a few cells in the preoptico-neurohypophyseal region of ammocoetes which were immunoreactive to an antiserum against mammalian luteinizing hormone releasing hormone (LHRH) but in recently- metamorphosed adults, LHRH-immunoreactive cells were more abundant in this region. Immunohistochemistry has also been used to show that lamprey GnRH is restricted to the preoptico-neurohypophyseal region of the brain of adult lampreys (King et al., '88). Therefore, we can only assume that it is in this region of the brain of metamorphosing animals that GnRH is increasing in concentration. However, it is now imperative that an immunohistochemical study be performed on the brains of metamorphosing lampreys to try to locate the site of synthesis and/or storage of GnRH. In light of recent observations on very early detection of GnRH-containing neurons in the mammalian fetus (Ronnekliev and Resko, '90), it would also be of interest to examine lamprey embryos for the presence of this hormone.

The identification of a second GnRH or GnRH-like hormone was surprising and we have no immediate explanation for why it should appear at this interval of the life cycle. However, the GnRH-like molecule cannot be overlooked, for it appears more consistently at an earlier stage of metamorphosis than lamprey GnRH. An earlier study on brains of upstream migrant *P. marinus* (Sherwood et al., '86) indicated that a second form of GnRH in lampreys (lamprey II GnRH) represented 10% of the total GnRH-immunoreactive material of the brain. Although the amino acid sequence is not know, lamprey II GnRH differs from the more abundant lamprey GnRH by three residues (Ile, Phe, and His instead of Glu, Lys, and Tyr) which make it more hydrophobic. However, on the basis of the fact that it is detected with mammalian GnRH antiserum 1245 and with two lamprey GnRH antibodies, it is believed that the second GnRH has the same terminal residues as lamprey and mammalian GnRH. Because the GnRH-like molecule was detected in 14 of the 16 samples of stages 4–7 inclusive, and it seems to represent a higher proportion of total brain GnRH than is seen in adult brain, implies that it is an important molecule that warrants further investigation.

The underlying importance of this present study is that it provides the first conclusive evidence, at least in *P. marinus,* that metamorphosis is probably essential before final phases of maturation of gonads can occur. Although it has long been recognized through morphological examination of gonads that gametogenesis is accelerated to varying degrees during and following metamorphosis (Hardisty, '71; Youson, '80; Hardisty et al., '86), the present study may provide an explanation for this phenomenon. The accelerated oocyte growth during the 6 months of metamorphosis of the Southern Hemisphere lamprey, *Geotria australis,* led Hardisty et al. ('86) to suggest that pituitary gonadotropins or other endocrine factors are involved. Previously, partial hypophysectomy of ammocoetes of *G. australis* had implied that the caudal pars distalis and its basophils (gonadotrophs?) become active at stage 3 of metamorphosis and are important in the initiation of metamorphosis (Joss, '85). Recently, it has been established that in adult, upstream migrant *P. marinus* there is a relationship between brain GnRH and gametogenesis (Fahien and Sower, '90). In *Xenopus laevis* tadpoles undergoing metamorphosis, increasing immunoreactive GnRH was demonstrated and the authors suggested that this increase may indicate a function in reproductive maturation (King and Millar, '81). As significant changes occur in the adenohypophysis during metamorphosis of *P. marinus* (Wright, '89), it will be of interest to try to correlate these changes with the timing of increased concentration of brain GnRH.

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