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Steroid and Thyroid Hormone Profiles Following a Single Injection of Partly Purified Salmon Gonadotropin or GnRH Analogues in Male and Female Sea Lamprey

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ABSTRACT The effects of exogenous administration of gonadotropin-releasing hormone (GnRH) analogues or of a partly purified salmon gonadotropin extract (GTH) on the duration of steroid and thyroid hormone levels were determined in female and male sea lampreys, *Petromyzon marinus,* tested under differing temperature and reproductive status. Plasma estradiol levels, but not androgens, were significantly elevated in response to the GnRH analogues or GTH injection compared to controls in female and male lampreys. Higher temperature and/or advance in time of maturation appeared to be inversely related to plasma estradiol levels. These data provide further evidence of hypothalamic control over reproductive function in lampreys.

Plasma thyroxine was significantly elevated after female lampreys were treated with GTH, GnRHa (10 μg/lamprey) or GnRHa (1 μg/lamprey) compared to controls. The present study is the first to demonstrate that the GnRH analogue stimulated in some way the pituitary-thyroid axis. These data suggest that a GnRH activity may activate both gonado- and thyrotropic secretion or that the endogenous hormone may itself have both functions in one of the most primitive vertebrates, the sea lamprey.

The vertebrate hypothalamus generally plays a central role in the regulation of the adenohypophysis. However, there is at this time only limited evidence that the Agnathan hypothalamus exerts any regulatory influence on the adenohypophysis. Recently, the first experimental evidence of hypothalamic control over pituitary and gonadal function in female lampreys was demonstrated, determined by stimulated steroidogenesis and induced ovulation following injections of partially purified salmon gonadotropin or gonadotropin-releasing hormone analogue (Sower et al., '83a). These studies used lampreys that were near the time of spawning, undergoing final maturation.

Further verification of the role of the hypothalamus in reproduction in lamprey is needed. Better evaluation of the hormone dose levels and timing of hormone administration, in both the male and female lampreys, under varying environmental conditions would aid in the interpretation of endocrine influences during the lampreys' final reproductive period. Therefore, in further study of these phenomena we have determined the effects of exogenous administration of GnRH analogues or salmon gonadotropin extract on the duration of steroid hormone concentrations in female lampreys tested under differing temperature and reproductive status; and second, we have determined whether these exogenously administered hormones influence steroid hormone levels in male lampreys.

In lamprey gonads steroid secretion and ovulation have been stimulated specifically by exogenous heterospecific gonadotropic preparations (Sower et al., '83a). These same preparations have been shown to induce ovulation (Hunter et al., '81) and alter steroid levels (Sower et al., '84) in coho salmon. Suspected of having thyrotropic activity since it was not highly purified, this coho salmon gonadotropin preparation was tested in adult maturing coho salmon and it did not alter thyroxine levels compared to controls (Sower,

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unpublished). Heterologous, purified gonadotropins generally stimulate the thyroid gland in teleost fishes (Fontaine, '69; Milne and Leatherland, '80; MacKenzie, '82). Thus, to gain more insight into the specificity of action of the coho salmon gonadotropin preparation and the GnRH analogues, plasma thyroid hormones were also measured in one of the experiments described here.

MATERIALS AND METHODS

Landlocked adult sea lampreys *(Petromyzon marinus)* were captured by trap in the Cheboygan River in May 1982, during their anadromous spawning migration following completion of their parasitic lake phase. They were retained in raceways at Hammond Bay Biological Station supplied with flowing lake water ranging in ambient temperature from 5.5° C to 20° C during May–August. One week prior to injection, the lampreys were separated into groups in the raceways which were divided into sections by screens. In experiments A and B, dorsal fins of all lampreys were clipped to designate the mode of treatment. In experiment C, all lampreys were identified individually with floy tags in addition to the clipped dorsal fins. The lampreys averaged 200 g in body weight and 44 cm in length.

In each of three experiments, groups of 60–80 lampreys were tested (either all males or all females) at different temperatures with single injections of various doses of GnRHa (analogue; D-ala^6, des Gly^{10} ethyl amide), GnRHant (antagonist in mammals, Ac-^{3}Pro^1, 4 FD Phe^2, DTrp^{3,6}-LRF) or partly purified coho salmon gonadotropin (Sower et al., '82). The GnRH antagonist was generously donated by J.E. Rivier and W.W. Vale of the Salk Institute, La Jolla, California. During the morning of the day when the lampreys were injected, all peptides for injections were dissolved in 0.6% NaCl water and were injected intraperitoneally into each fish. The animals first anesthetized with ethyl m-aminobenzoate methanesulfonate (MS 222) were serially bled at 8, 24, and 48 hr from the initial injection at 0 hr. Blood samples (~ 400 μl) were collected in heparinized syringes by cardiac puncture. The plasma was drawn off and stored frozen at −20°C until assayed for estradiol, androgens, and thyroid hormones. Plasma estradiol and androgens were measured by radioimmunoassay as described by Sower and Schreck ('82) and validated for lampreys (Sower et al., '83a). Plasma thyroxine and triiodothyronine were determined by an RIA validated for lamprey plasma, as previously described (Plisetskaya et al., '83).

Experiment A from May 25–27, 1982, included only female lampreys. The water temperature ranged between 5° and 6° C. The treatments of groups of 12 lampreys were: *control a)* no injection, plasma samples only at 0 and 48 hr; *control b)* saline; GnRHa, 10 μg/lamprey; GnRHa, 1 μg/lamprey; GnRHant, 10 μg/lamprey; GnRHant, 1 μg/lamprey; or salmon GTH, 20 μg/lamprey.

Experiment B from June 2–4, 1982, included only male lampreys . The water temperature ranged between 8.3° and 8.8° C. The treatments in groups of 12 male lampreys each were: *control a)* no injection, plasma samples only at 0 and 48 hr; *control b)* saline; GnRHa, 10 μg/lamprey; GnRHant, 10 μg/lamprey; or salmon GTH, 20 μg/lamprey.

Experiment C from July 15–17, 1982, included only female lampreys. The temperature was 11° C. The treatment schedules of individuals in groups of 12 lampreys each were identical to the treatments in Experiment A.

RESULTS

Experiment A

Plasma estradiol in female lampreys was significantly elevated at 8, 24, and 48 hr after treatment with GnRHa (10 μg/lamprey) (5.16 ± 0.51, 5.51 ± 0.61, and 6.16 ± 0.82 ng/ml), GnRHa (1) (4.66 ± 0.48, 5.00 ± 0.49, and 3.80 ± 0.56 ng/ml), or GTH (2.84 ± 0.48, 3.45 ± 0.66, and 3.01 ± 0.44 ng/ml), compared to controls (1.59 ± 0.31, 1.36 ± 0.23, and 1.15 ± 0.10 ng/ml) (Fig. 1). Plasma estradiol was not significantly different from controls after injections of GnRHant at 10 or 1 μg/lamprey. However, plasma estradiol significantly decreased within the group of lampreys treated with GnRH at 10 μg/lamprey from 8–48 hr following injection. Estradiol levels did not vary significantly at the different sampling times in all treatment groups except for GnRHant.

Plasma thyroxine in females was significantly elevated at 8 hr after lampreys were treated with GnRHa (10 μg/lamprey) (1.8 ± 0.03 ng/ml) or GnRHa (1) (1.8 ± 0.1 ng/ml), compared to controls (1.4 ± 0.1 ng/ml) (Fig. 1). Plasma thyroxine was significantly elevated at 24 hr after treatment with 1 μg/lamprey of GnRHant (2.1 ± 0.2 ng/ml) or GTH (2.8 ± 0.3 ng/ml), compared to controls

(1.4 ± 0.1 ng/ml). In the GnRHant (1) treated lampreys, plasma thyroxine did not vary between 24 and 48 hr, but was significantly lower (2.0 ± 0.1 ng/ml) compared to saline control (2.6 ± 0.2 ng/ml) at 48 hr. Except for the GnRHant (1) treated lampreys, plasma thyroxine did not vary significantly between the treatment groups and controls at 48 hr.

Plasma triiodothyronine (1.2 ± 0.1, 0.7 ± 0.04, and 2.1 ± 0.1 ng/ml) varied significantly at 8, 24, and 48 hr in saline controls (Fig. 1). Plasma triiodothyronine was significantly lower at 8 hr after lampreys were treated with GnRHant (1 μg/lamprey) (0.8 ± 0.04 ng/ml) or GTH (0.8 ± 0.1 ng/ml), compared to controls (1.2 ± 0.1 ng/ml). Plasma triiodothyronine was significantly higher at 24 hr after lampreys were treated with GnRHant (1) (1.3 ± 0.1 ng/ml) or GTH (1.8 ± 0.1 ng/ml), compared to controls (0.7 ± 0.04 ng/ml).

Plasma androgens were not measured at all sampling hours due to the lack of adequate quantities of plasma. However, of the samples assayed, plasma androgens did not vary significantly between the controls and treatment troups (Table 1).

Experiment B

Plasma estradiol levels in males were significantly elevated at 8, 24, and 48 hr after lampreys were treated with GnRHa (10) (1.71 ± 0.26, 1.35 ± 0.18, and 1.11 ± 0.18 ng/ml), compared to controls (0.75 ± 0.10, 0.70 ± 0.07, and 0.65 ± 0.10 ng/ml) (Fig. 2). Estradiol was not significantly different from controls after a single injection of GnRHant at 10 μg/lamprey. Plasma estradiol did not vary significantly between the different sampling times in all treatment groups. Plasma androgens were only significantly higher at 24 hr after lampreys were treated with GnRHa (10 μg/lamprey), compared to controls. Following injection of GnRHa (10 μg/lamprey), plasma androgens decreased significantly from 8 to 48 hr. In the saline controls, plasma androgens (0.145 ± 0.018 ng/ml) at 8 hr were significantly different compared to uninjected controls (0.101 ± 0.005 ng/ml) at 0 hr and saline controls (0.099 ± 0.014 ng/ml and 0.093 ± 0.005 ng/ml) at 24 and 48 hr. Plasma estradiol or androgens were not significantly different from controls after injections of GnRHant.

Experiment C

Plasma estradiol in females was significantly elevated at 8, 24, and 48 hr after lampreys were treated with GnRHa (10), GnRH (1), or GTH (20), compared to saline controls and at 8 and 48 hr after lampreys were treated with GnRHa (10 μg/lamprey) and GTH (20 μg/lamprey), compared to uninjected controls (Fig. 3). Following injection of GnRHa at 1 μg/kg, plasma estradiol decreased significantly from 8 hr to 24 hr from 2.47 ± 0.32 to 1.25 ± 0.19 ng/ml and then increased at 48 hr to 1.94 ± 0.18 ng/ml. Saline injected lampreys showed a similar trend from 8, 24, to 48 hr of 1.18 ± 0.15 ng/ml, 0.74 ± 0.09 ng/ml. Plasma estradiol was not significantly different from controls after injec-

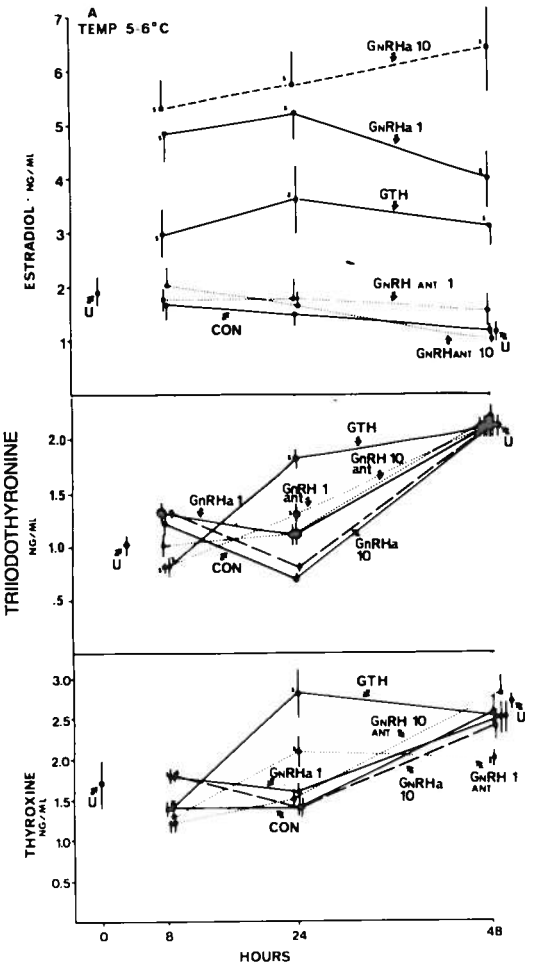


Fig. 1. Plasma estradiol, thyroxine and triiodothyronine (ng/ml) at 0, 8, 24, and 48 hr of female lampreys uninjected (U) or injected at 0 hr with saline (CON), GnRHa (10 μg/lamprey), GnRHa (1 μg/kg), GnRHant (10 μg/kg), GnRHant (1 μg/kg), or GTH (20 μg/lamprey). S is significant at $P < 0.05$.

TABLE 1. Plasma androgens (ng/ml) ($\bar{x}$ ± SE (n)) at 8, 24, and 48 hr of female lampreys uninjected (U) or injected at 0 hr with saline (SAL), GnRHa (10 μg/lamprey), GnRHa (1), GnRHant (10), GnRHant (1), or GTH (20)

	Total androgens		
	Hours		
	8	24	48
U Control	No data	No data	0.116 ± 0.024 (8) ND (1)
SAL Control	0.086 (1)	ND* (3)	0.094 ± 0.016 (8) ND (1)
GnRHa (10)	No data	0.108 ± 0.020 (3) ND (1)	0.297 ± 0.105 (6)
GnRHa (1)	0.063 (1)	0.104 ± 0.041 (2) ND (3)	0.164 ± 0.050 (8)
GnRHant (10)	No data	0.068 ± 0.18 (2) ND (2)	0.142 ± 0.019 (7)
GnRHant (1)	No data	0.079 ± 0.030 (2)	0.186 ± 0.049 (10)
GTH (20)	0.128 (1)	0.081 ± 0.018 (2) ND (1)	0.217 ± 0.088 (7)

*ND = nondetectable

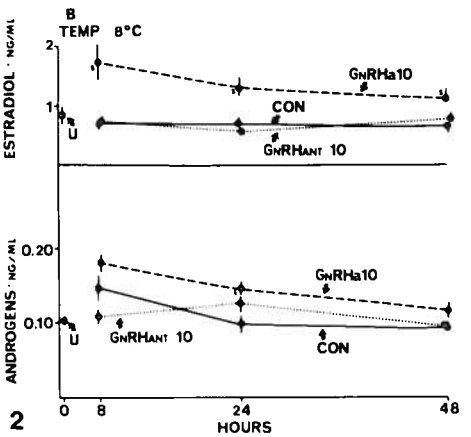


Fig. 2. Plasma estradiol (ng/ml) and total androgens (ng/ml) at 0, 8, 24, and 48 hr of male lampreys uninjected (U) or injected at 0 hr with saline (CON), GnRHa (10 μg/lamprey) or GnRHant (10). S is significant at $P < 0.05$.

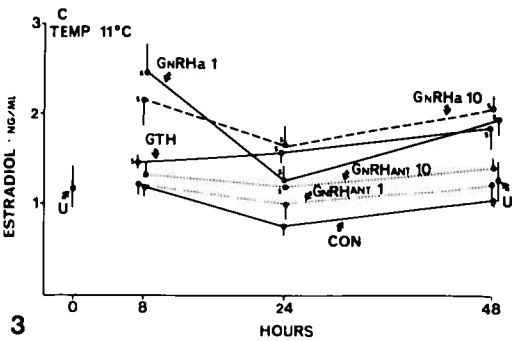


Fig. 3. Plasma estradiol (ng/ml) at 0, 8, 24, and 48 hr in female lampreys uninjected (U) or injected at 0 hr with saline (CON), GnRHa (10 μg/lamprey), GnRHa (1), GnRHant (10), GnRHant (1), or GTH (20). S is significant at $P < 0.05$.

tions of GnRHant at 10 or 1 μg/lamprey, except at 24 hr where estradiol was significantly higher in lampreys treated with GnRHant at 10 μg/lamprey (1.19 ± 0.08 ng/ml), compared to saline control (0.74 ± 0.09 ng/ml).

DISCUSSION

In lampreys, the role of the hypothalamus in reproductive processes has yet to be established. Circumstantial evidence such as synchronized seasonality of gonadal maturation and breeding (Hardisty,'79) and immunohistochemical evidence have strongly indicated that there is neuroendocrine control over lamprey reproduction. Crim et al. ('79) have demonstrated the presence of immunoreactive gonadotropin-releasing hormone (GnRH) in the preoptico-neurohypophysial system of the Pacific lamprey *(Lampetra tridentata)*. More recently, brains from spawning sea lampreys *(Petromyzon marinus)* were shown to contain a peptide at a concentration of 1.27 ng/brain that was chromatographically and immunologically similar to the mammalian form of GnRH (Sower et al., '83b; Sherwood and Sower, '85). The first experimental evidence of hypothalamic control over pituitary and gonadal function in lampreys was demonstrated by injections of salmon gonadotro-

pin or gonadotropin-releasing hormone analogue and observation of stimulated steroidogenesis and induced ovulation (Sower et al., '83a). In this study, we have found that the gonadotrophic response is not sexually differentiated and that the stage of maturation and temperature modify the response, and that GnRH may function as a thyrotrophic as well as a gonadotropic releasing hormone in this primitive Agnathan species.

Female lampreys that were tested in May at relatively low temperatures and at times when normal circulating plasma estradiol was about 1 ng/ml were relatively more responsive to salmon gonadotropin or GnRH in terms of elevated plasma estradiol (4–6 ng/ml), compared to lampreys tested later in the season. At this earlier time, the oocytes contained granular yolk, and almost every ovarian histological sample contained at least one or two small eggs which had a thickened follicular epithelium (Sower et al., '85). In July, the temperature averaged 11° C and normal circulatory levels of estrogen in the females were comparable (mean, 1 ng/ml) and yet their gonadotropic steroidogenic response was less (2–3 ng/ml) than by females treated in May. In most ovaries yolk was largely liquified rather than granular with some possible polar development (Sower et al., '85). In both experiments, plasma estradiol remained elevated for 48 hr. Plasma estradiol in males treated with salmon gonadotropin or GnRHa responded in a pattern similar to that in the second group of females at 11° C, however, plasma estradiol had started to decline by 48 hr in the treated male animals. Higher temperature and/or time of maturation appear to be related to lower plasma estradiol profiles. Therefore, these data suggest that higher temperature and/or advance in time of maturation may be inversely related to plasma estradiol levels.

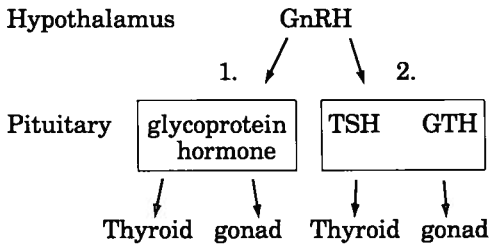
In the present study, surprisingly, plasma estradiol but not androgens were elevated in response to the GnRHa injection in female or male lampreys. The physiological role of the sex steroids in lampreys remains an enigma. For instance, plasma testosterone and 5 α-dihydrotestosterene are very low or absent in adult sea lampreys (Katz et al., '82; Sower et al., '85), so it is difficult to recognize their physiological role. Plasma estradiol levels have been found equivalent in both male and female adult lampreys undergoing gonadal maturation (Katz et al., '82; Sower et al., '85). Furthermore, Belvedere and Colombo ('83) have shown that testicular and ovarian steroidogenesis are similar with respect to the quantity and type of steroids produced in vitro in the Brook lamprey, *Lampetra zanandrea*. Our present findings provide further evidence that the role of estradiol may not be sex-specific during the final maturational processes.

The coho salmon pituitary gonadotropin preparation which had no apparent thyrotropic activity in adult coho salmon (Sower, unpublished) did elevate both plasma thyroxine and triiodothyronine in the lamprey. Thus, this study possibly demonstrates heterothyrotropic activity in an Agnathan, supporting the assertion that the evolutionary divergence in the structure and function of thyrotropin molecules and their receptors (Dodd and Dodd, '69; Fontaine, '80; MacKenzie and Licht, '84) may have occurred at the cyclostome level. However, these interpretations require confirmation, using a purified salmon gonadotropin since the preparation we have used may contain some thyrotropic activity. It is possible that there may not normally be differentiated glycoproteins in the lamprey, but rather a single, ancestral glycoprotein.

The hypothalamic hormone(s) involved in the regulation of secretion of adenohypophysial glycoprotein(s) may have evolved in parallel with the differentiation of thyrotropic and gonadotropic function. Even though the lamprey GnRH molecule appears to be chromatographically and immunologically similar to mammalian GnRH, this molecule could play very different physiological roles in different primitive species. The GnRH analogue to the mammalian GnRH used in the previous (Sower et al., '83a) and present studies clearly stimulated the pituitary-gonadal axis. However, the mammalian GnRH molecules in our studies further stimulated in some way the pituitary-thyroid axis. Direct effects of GnRH or its analogues on any other vertebrate pituitary-thyroid axis have not been reported. The present study is the first to demonstrate that the GnRH analogues either directly or indirectly increase plasma thyroxine and triiodothyronine levels.

The GnRH antagonist which did not alter steroid levels in the males or females significantly increased both thyroxine and triiodothyronine levels at 24 hr after the injection. GnRHa at 8 hr only induced elevated plasma thyroxine levels in the lampreys. These data further suggest that a

GnRH activity may activate both gonado- and thyrotropic secretion, or that the endogenous hormone may itself have both functions. There certainly may be other possible explanations, but the two possibilities are diagrammed below.



In the lower vertebrates, though the data are few, TRH has not been demonstrated to stimulate thyroid hormone levels (reviewed by Crim et al., '78). It is possible to speculate that independent control of the pituitary-thyroid and pituitary gonadal axes developed in vertebrates that evolved after the cyclostomes, and that in the most ancient class of vertebrates, these two axes may be under the control of one form of glycoprotein hormone. This releasing hormone might at first have been a more generally occurring brain peptide (Barker, '77; Knigge et al., '78).

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