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# Characterization and Localization of Gonadotropin-Releasing Hormone Receptors in the Adult Female Sea Lamprey, *Petromyzon marinus**

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**ABSTRACT**

Quantitative *in vitro* autoradiography was used to characterize and localize putative GnRH receptors in the anterior pituitary of the adult female sea lamprey, *Petromyzon marinus*. Pituitaries were sectioned at 20 μm and incubated for 3 h at 4 C with DAla$^6$,Pro$^9$ NEt mammalian GnRH as both the labeled and unlabeled ligand. Scatchard analysis revealed two classes of high affinity binding sites with $K_d$s of $1.5 \times 10^{-12}$ M and $5 \times 10^{-9}$ M. Binding to the GnRH receptors was saturable, reversible, tissue specific, and time and temperature dependent. Displacement studies showed that labeled peptide could be displaced by chicken GnRH-I, chicken GnRH-II, synthetic mammal, salmon, lamprey GnRH-I, lamprey GnRH-III, DAla$^6$,Pro$^9$ NEt mammalian GnRH and DPhe$^{2,6}$,Pro$^3$ lamprey GnRH. The proximal pars distalis region of the anterior pituitary contained most of the GnRH binding sites with slight binding in the rostral pars distalis. These data provide direct evidence of GnRH activity on the Agnathan pituitary and are the first to demonstrate that a vertebrate pituitary contains two high affinity binding sites for GnRH. (*Endocrinology* **134:** 492–498, 1994)

GnRH is a major hypothalamic regulatory peptide whose action in various reproductive processes has been well defined in mammals and birds and, to a lesser extent, in some amphibians, reptiles, teleosts, and agnathans. There is considerable diversity in the structure of GnRH and related molecular forms. The primary structures of eight GnRHs in vertebrates have been determined (Table 1) (1). Two of these eight GnRH molecules have been identified in the sea lamprey (*Petromyzon marinus*), GnRH-I (2) and lamprey GnRH-III (1). Certain regions of the GnRH molecule have been highly conserved throughout evolution including the $NH_2$-terminal, pGlu$^1$-His$^2$ and Ser$^4$ and the COOH-terminal α-aminated dipeptide. These regions and the length of the molecule have remained unchanged during 500 million years of evolution. The conservation of the $NH_2$- and COOH-termini suggests these regions are of functional significance for conformation, receptor binding, resistance to enzymatic degradation and in receptor-mediated events required for gonadotropin release (3).

Agnathans are of particular importance in understanding the evolution of GnRH since they represent the oldest lineage of vertebrates that diverged over 550 million years ago. Lampreys are one of the only two living representatives groups of this group. Since the elucidation of lamprey GnRH-I, immunocytochemical and physiological studies have demonstrated that GnRH-I regulates reproduction by stimulating the pituitary-gonadal axis (4). In these studies, plasma estradiol and progesterone were measured in the lamprey as potential indicators of pituitary responsiveness to GnRH. Although there is strong evidence for the presence of gonadotropin in the lamprey pituitary, as well as gonadotropic functions (5, 6), gonadotropin(s) (GTH) have not yet been isolated from the lamprey pituitary glands. Preliminary studies have indicated that lamprey GnRH-III is also a neurohormone involved in reproduction (1). In addition, there are seasonal correlations between changes in brain GnRH and gametogenic and steroidogenic activity of the gonads in male and female adult sea lampreys (7, 8). This structural information combined with later immunocytochemical (9–13) and physiological studies (14–17), provide evidence for the regulatory influence of the hypothalamus on the pituitary-gonadal axis implying that certain aspects of the GnRH molecule have been conserved throughout vertebrate evolution. To date, no direct action of GnRH on the lamprey pituitary has been demonstrated despite the above described studies. Thus, the characterization of the GnRH binding site in the sea lamprey would provide the first evidence of GnRH exerting its regulatory actions on the pituitary.

In addition, the development of an assay for GnRH pituitary receptors could be used for a variety of physiological experiments on pituitary responsiveness, including factors that may be modulating the receptors to further our understanding of the interrelationship of the structure and function of the GnRH peptides in the hypothalamic-pituitary-gonadal axis. The identification of GnRH receptors in the oldest lineage of vertebrates is important especially in understanding the evolution of these peptides in vertebrates. Therefore, the objective of this study was to localize and characterize GnRH binding sites within the pituitary of the adult female sea lamprey, using *in vitro* autoradiography techniques.

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Mammalian [DAla$^6$,Pro$^9$ NEt]GnRH was selected as the ligand because this analog has been shown to be a potent stimulator of gametogenesis and steroidogenesis in the lamprey (4).

## Materials and Methods

### *Lampreys and preparation of pituitaries*

Adult female sea lampreys were captured during their final spawning migration from the ocean at the New Hampshire Fish and Game fish ladder on the Cocheco river in Dover, NH, during the months of May and June 1991 and 1992. Lampreys were transferred and maintained in an artificial stream at the University of New Hampshire Anadromous Fish and Aquatic Invertebrate Research laboratory. Lampreys were killed by decapitation, and the pituitary was removed and frozen on dry ice until transferred to a −80 C freezer or used immediately for sectioning.

Pituitaries were embedded in Tissue Tek OCT and the entire pituitary was sectioned (20 μm) on a cryostat at −16 C. Sections were placed on alternate subbed slides in order to determine total and nonspecific binding on adjacent sections. Sections were then thaw mounted and dried for 30 min in a vacuum desiccator at −20 C. Pairs of slides were randomly assigned to treatment groups to control for differences among pituitaries.

### *Binding procedures*

In most experiments, sections were incubated with $1 \times 10^{-7}$ M $I^{125}$-DAla$^6$,Pro$^9$ NEt mammalian GnRH in 300 μl 8.0 mM TRIS-HCl buffer (pH 7.4) with 0.2 g/ml BSA. DAla$^6$,Pro$^9$NEt mammalian GnRH (Sigma, St. Louis, MO) was iodinated using a modification of the chloramine-T method and purified as described in Stopa *et al.* (18). Specific activity (80 Ci/mmol) was determined by a self-displacement assay. Nonspecific binding sections were incubated in the same medium with the addition of $1 \times 10^{-4}$ M unlabeled DAla$^6$,Pro$^9$ NEt mammalian GnRH. Slides were incubated for 3 h at 4 C unless otherwise indicated. After incubation, slides were washed in two 1 ½ min rinses of ice-cold buffer (8.0 mM TRIS-HCl) and dipped in ice cold water. Slides were dried under a gentle stream of cool air. Sections were removed using a moist Whatman GF/C filter and counted on a LKB γ-counter, or used for film autoradiography as described below.

Saturation studies used DAla$^6$,Pro$^9$ NEt mammalian GnRH at 10 concentrations ranging from $1 \times 10^{-8}$ M to $7 \times 10^{-12}$ M for determination of nonspecific binding with the addition of unlabeled DAla$^6$,Pro$^9$ NEt mammalian GnRH ($1 \times 10^{-4}$ M). Competition studies used mammalian GnRH, lamprey GnRH I and salmon GnRH (Peninsula labs), chicken I and II GnRH (gifts from Dr. Robert P. Millar and Dr. Judy King), lamprey III (1), DPhe$^{2,6}$,Pro$^3$ lamprey GnRH and DAla$^6$,Pro$^9$-OH lamprey GnRH (2). Results from the binding studies were analyzed using EBDA and Ligand (19) computer programs from Biosoft Inc. Dissociation studies consisted of incubating slides for 3 h at 4 C then were immersed in TRIS-HCl buffer at 4 C for increasing time periods of 0, 10, 20, 30, and 60 min. Tissue specificity studies consisted of tissues (pituitary, skin, muscle, and intestine) being removed from a single lamprey and frozen on dry ice. All tissues were sectioned at 20 μm and incubated and similar procedures were followed as stated above.

**TABLE 1.** Primary structures of the eight known vertebrate GnRH molecules

| Species | Amino acid position | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Lamprey | pGLU | HIS | TYR | SER | LEU | GLU | TRP | LYS | PRO | GLY-NH2 |
| Lamprey III | pGLU | HIS | TRP | SER | HIS | ASP | TRP | LYS | PRO | GLY-NH2 |
| Catfish | pGLU | HIS | TRP | SER | HIS | GLY | LEU | ASN | PRO | GLY-NH2 |
| Salmon | pGLU | HIS | TRP | SER | TYR | GLY | TRP | LEU | PRO | GLY-NH2 |
| Dogfish | pGLU | HIS | TRP | SER | HIS | GLY | TRP | LEU | PRO | GLY-NH2 |
| Chicken II | pGLU | HIS | TRP | SER | HIS | GLY | TRP | TYR | PRO | GLY-NH2 |
| Chicken I | pGLU | HIS | TRP | SER | TYR | GLY | LEU | GLN | PRO | GLY-NH2 |
| Mammal | pGLU | HIS | TRP | SER | TYR | GLY | LEU | ARG | PRO | GLY-NH2 |

### *Autoradiography*

Slides to be used for localization were dried in a vacuum desiccator for 30 min at room temperature. They were removed and apposed to Amersham $^3$H Hyperfilm, along with one Amersham $I^{125}$ Microscale. The film was exposed for 1 week at room temperature and developed in Kodak D-19 developer for 4 min at room temperature.

Localization of the GnRH binding sites was determined using a Drexel Image Analysis system consisting of a Macintosh IIci computer with the Brain 1.0 software developed at Drexel University (20), and a Sony CCD video camera mounted on a Chromapro 45 light box. Standard curves were based on Amersham $I^{125}$ Microscales. Films were analyzed for differences in optical densities which were converted to NanoCuries per mg polymer by the Brain 1.0 software.

## Results

Measurements of optical density on the autoradiograms revealed high concentrations of $I^{125}$-DAla$^6$,Pro$^9$ NEt mammalian GnRH binding sites in the proximal pars distalis region of the anterior pituitary. There was slight binding within the rostral pars distalis whereas binding was not present within the pars intermedia (Plate 1). Liver, skin, intestine and muscle were tissues examined for tissue specificity. Determination of possible binding sites in the liver, skin, intestine, and muscle revealed no specific binding (data not shown).

Binding of $I^{125}$-DAla$^6$,Pro$^9$ NEt mammalian GnRH was temperature dependent with binding reaching equilibrium at 22 C and 4 C (Fig. 1). Binding was also time dependent with equilibrium being reached between 2 and 3 h (Figs. 1 and 2) and remaining stable up to 24 h at 4 C (data not shown). Association rate was also determined in a separate experiment at 4 C (Fig. 2) and at 22 C (data not shown). The binding of GnRH to the receptor at 22 C reached a maximum at 30 min, then decreased rapidly thereafter. The observed association rate constant for 4°C ($K_{obs}$) was determined as 0.03 from the slope of the line (see *inset*, Fig. 2).

Specifically bound $I^{125}$-DAla$^6$,Pro$^9$ NEt mammalian GnRH was released in a time-dependent manner (Fig. 3). The best fit line was determined to be linear. The rate constant for dissociation ($K_{-1}$) was calculated from the slope of the line as −0.02 (see *inset*, Fig. 3). After 60 min, only 10% of specifically bound radiolabeled mammalian GnRH remained.

The binding was also determined to be saturable and of high affinity. Binding sites saturated around $3.90 \times 10^{-10}$ M of labeled ligand was the saturation point of the receptor (Fig. 4). A Scatchard replot best fit a two site model and yielded two dissociation constants ($K_d$) of $1.5 \times 10^{-12}$ M (site I) and $5 \times 10^{-9}$ M (site II). The respective $B_{max}$'s were determined to be $8.4 \times 10^{-14}$ M and $5 \times 10^{-11}$ M and Hill coefficients of 0.936 and 0.966 (Fig. 5).

Displacement experiments showed a dose-dependent inhibition of binding to lamprey pituitary sections by several unlabeled GnRH peptides (Fig. 6, Table 2). All GnRH peptides examined were able to displace the radiolabeled ligand except one, a lamprey analog, DAla$^6$,Pro$^9$-OH (free carboxylic acid) lamprey GnRH. The neuropeptide TRH also was unable to displace the radiolabeled GnRH.

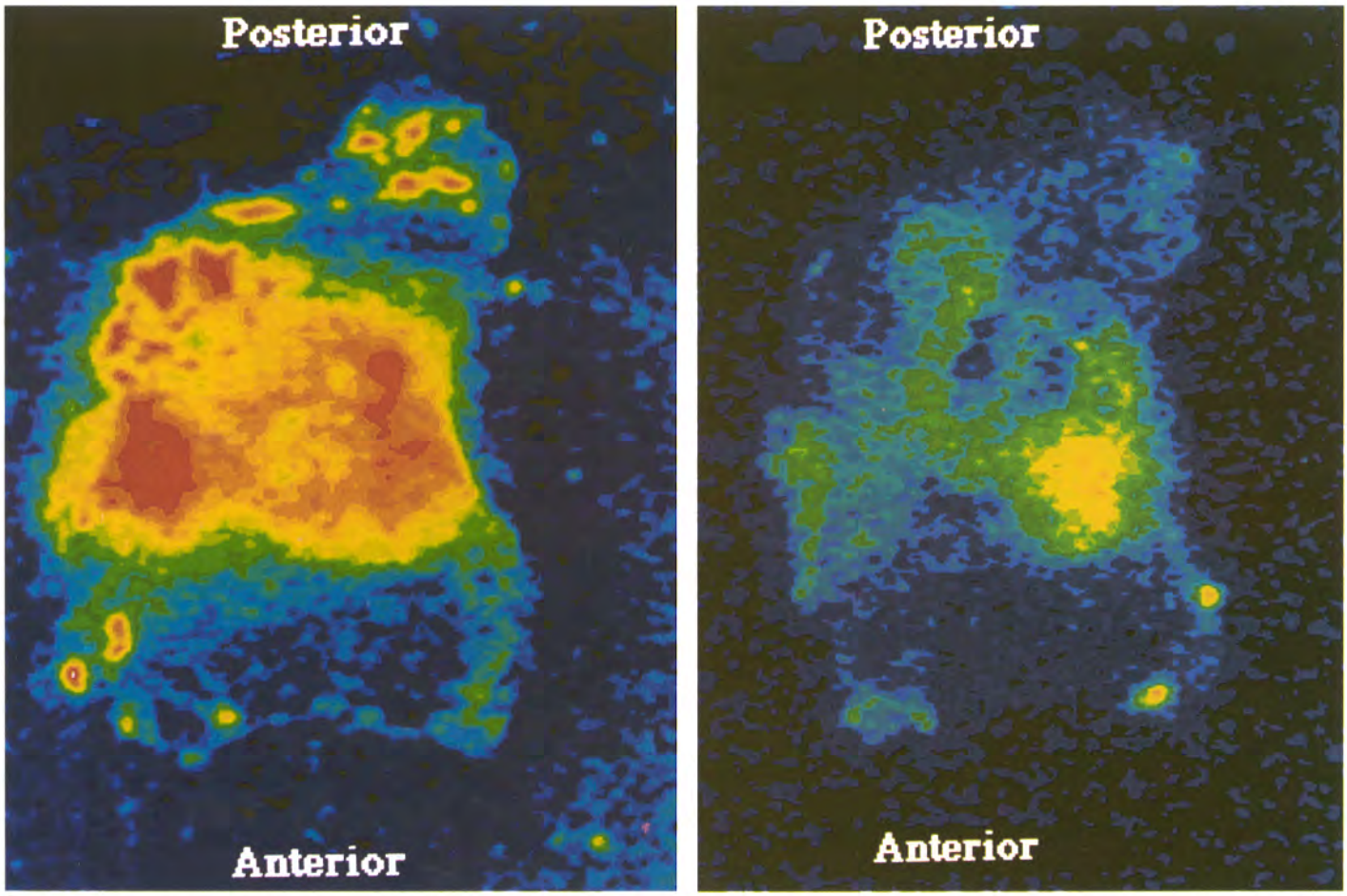


PLATE 1. Localization of the GnRH binding sites in the anterior pituitary sections from the female sea lamprey. A, Autoradiogram of total binding section incubated with [$^{125}$I] DAla$^6$,Pro$^9$ NEt mGnRH for 3 h at 4 C. Red and orange indicate areas of high binding, whereas blue and green represent areas of little or no binding. B, Autoradiogram of adjacent nonspecific binding section incubated with [$^{125}$I] DAla$^6$,Pro$^9$ NEt mGnRH, and unlabeled DAla$^6$,Pro$^9$ NEt mGnRH.

## Discussion

These studies demonstrate specific GnRH binding in an agnathan pituitary. Two specific classes of high affinity binding sites were characterized and localized in the sea lamprey using *in vitro* autoradiography techniques. The binding was saturable, reversible, time, and temperature dependent and tissue specific. GnRH binding sites were located primarily in the proximal pars distalis area of the anterior pituitary. These studies provide further evidence that GnRH exerts its regulatory effects on the hypothalamic-pituitary-gonadal axis by interacting with specific receptors located in the pituitary.

The present study provides evidence of two high affinity, specific classes of receptors in a single vertebrate pituitary. To date, every vertebrate examined has shown the presence of two or more forms of GnRH (1). In teleosts, in particular, at least two GnRH forms have been identified in each of the species examined (1). However, with the exception of goldfish (21), only a single class of GnRH binding site has been demonstrated in these same teleosts: stickleback, $K_a$ = 0.71 $10^9$ $M^{-1}$ (22), African catfish, $K_a$ = 0.66 $10^9$ $M^{-1}$ (23), the seabream, $K_a$ = 7.08 $10^9$ $M^{-1}$ (24), and winter flounder, $K_a$ = 2.1 $10^9$ $M^{-1}$ (25). In the goldfish pituitary there are only high affinity and low affinity sites with $K_d$'s of 17.6 × $10^{-9}$ M and 0.02 × $10^{-9}$ M, respectively (26). In lamprey, on the other hand, analysis of the Scatchard plot revealed two classes of high affinity binding sites, $K_d$ = 1.5 × $10^{-12}$ M and $K_d$ = 5 × $10^{-9}$ M. The displacement experiments also suggest the presence of two binding sites within the lamprey pituitary.

The primary structures of lamprey GnRH I and III have been determined (1, 2). As stated earlier, lamprey GnRH I has been demonstrated to induce both steroidogenesis and gametogenesis in female lampreys (14). Lamprey GnRH III has been shown to induce steroidogenesis in female lampreys (1). These two forms may therefore bind to different classes of receptors and have different biological actions. In lampreys, all of the GnRH peptides and analogs tested displaced the radiolabeled ligand from the receptor except the lamprey analog and TRH. In *in vivo* studies, variant analogs of the GnRH peptide have been shown to induce biological actions (14–17). In the present study, two of these analogs, DAla$^6$, Pro$^9$ NEt mammalian GnRH and DPhe$^{2,6}$Pro$^3$ lamprey GnRH, were tested and shown to displace I$^{125}$-DAla$^6$,Pro$^9$ NEt mammalian GnRH. In goldfish, displacement analysis

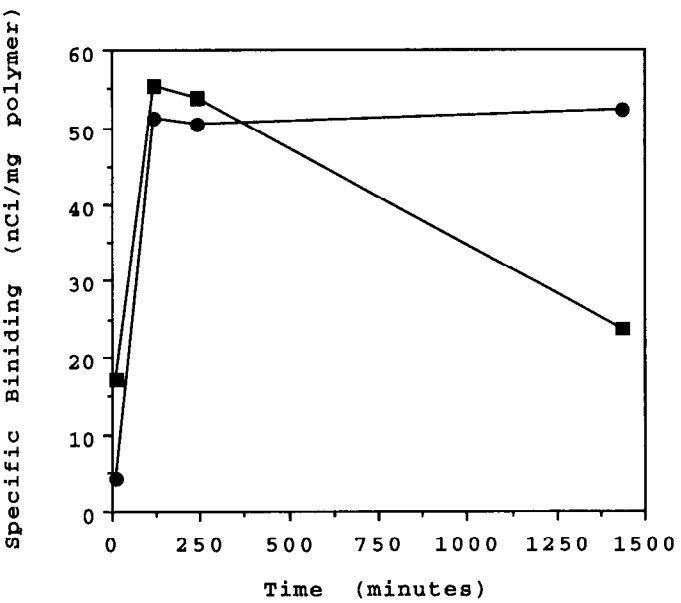


FIG. 1. Association plot to determine optimum temperature for incubation. Three temperatures were examined: 4 C (●), 13 C, and 22 C (■) (data from 13 C not shown). Binding was determined from films using image analysis and Brain 1.0.

demonstrated that all native and synthetic GnRH forms bind to the one high affinity class of receptor site (26–28) and bioactivity occurs with the binding of the GnRH to these high affinity receptors only. Further experiments will be needed to determine the actual binding site for these analogs in lampreys.

Another analog ([DAla$^6$,Pro$^9$-OH free carboxylic acid] lamprey GnRH), was unable to displace the radioactive ligand in the present study. In *in vivo* studies, this same lamprey analog stimulated plasma progesterone levels but inhibited spermiation in male lampreys (17) and inhibited ovulation in female lampreys (16). The apparent lack of binding or competitive inhibition of GnRH in the pituitary and little or no biological activity of this analog implies that the sixth position and α-aminated COOH terminal are significant for receptor binding and in receptor-mediated events for biological activity. Previous studies have suggested that GnRH acts directly on the pituitary and does not directly influence steroidogenesis in lampreys. In *in vitro* studies, lamprey GnRH-I (range of dose: 1–1000 ng peptide/ml media) had little or no direct effect on estradiol or progesterone as determined from media of testes culture or ovary cultures compared to controls (4). In addition, GnRH has not been detected in circulating plasma (3, 7). These data along with the lack of binding in the liver, skin, intestine, and muscle in the present study suggest that GnRH's action occurs at the pituitary. Both lamprey GnRH-I and -III are the only vertebrate GnRH molecules to have substitutions in the sixth position, Glu$^6$ and Asp$^6$, respectively (1). These data and the data from the present study would suggest that the receptor requirements for GnRH are different in the lamprey from those in other vertebrates, *i.e.* the presence of the high $K_d$ value for site I ($1.5 \times 10^{-12}$ M).

The highest concentration of GnRH binding sites occurred in the proximal pars distalis of the pituitary with little specific binding in the rostral pars distalis. In review of light and electron microscopy studies, both the rostral and proximal pars distalis have been shown to have PAS positive staining basophilic cells that may be the gonadotropes (5, 6, 29, 30). The lampreys, as well as hagfish, are unique and unlike other

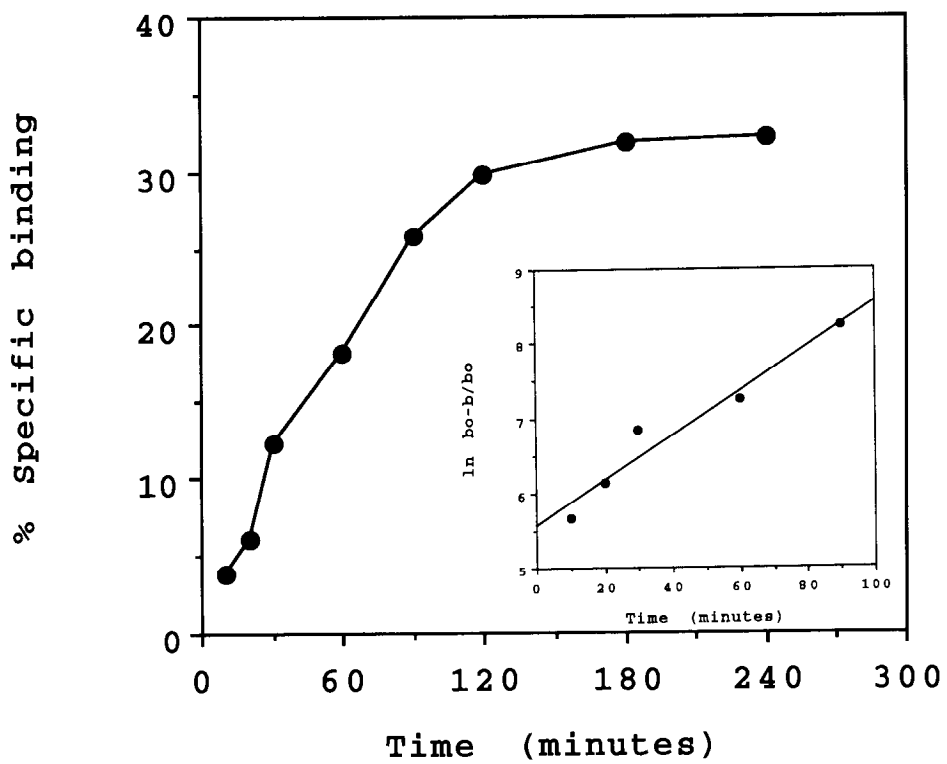


FIG. 2. Rate of association of [$^{125}$I] DAla$^6$,Pro$^9$ NEt mGnRH at 4 C. A log relationship best fit the line (Y = −1686.2+ 1808.7 LOG (X); $r^2$ = 0.96). All data in the remaining figures are in bound counts per min per section. *Inset*, Pseudo-first-order association plot. The observed rate constant ($K_{obs}$) was determined from the slope of the line which was linear (slope = 0.03; y-intercept = 5.530; $r^2$ = 0.95). b = binding of [$^{125}$I] DAla$^6$,Pro$^9$ NEt mGnRH at indicated time; $b_0$ = specific binding of [$^{125}$I] DAla$^6$,Pro$^9$ NEt mGnRH at equilibrium (mean values for 120, 180, and 240 min).

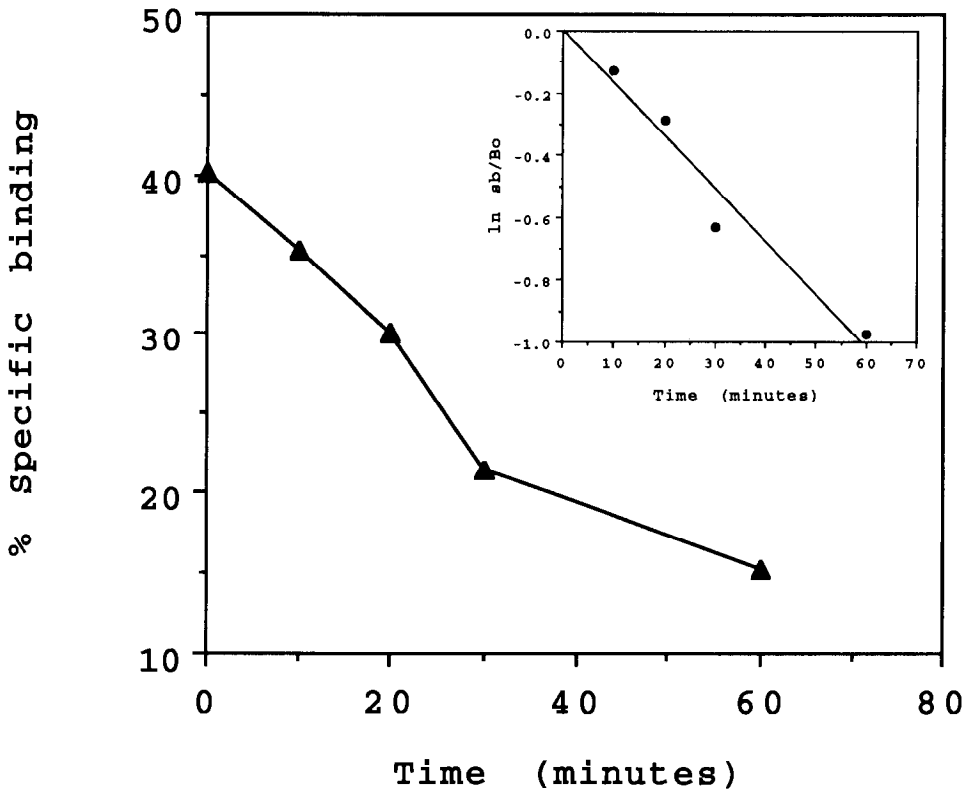


FIG. 3. Reversibility of [$^{125}$I] DAla$^6$,Pro$^9$ NEt mGnRH binding. Curve fitting analysis demonstrated the line was linear (slope = −91.523; y-intercept = 8287.9; $r^2$ = 0.934). *Inset*, First order dissociation rate plot. The rate constant for dissociation ($K_{-1}$) was determined by the slope of the line which was linear (slope = 0.02; y-intercept = 0.000179; $r^2$ = 0.968) where sb = specific binding; $B_0$ = specifically bound [$^{125}$I] DAla$^6$,Pro$^9$ NEt mGnRH at 60 min.

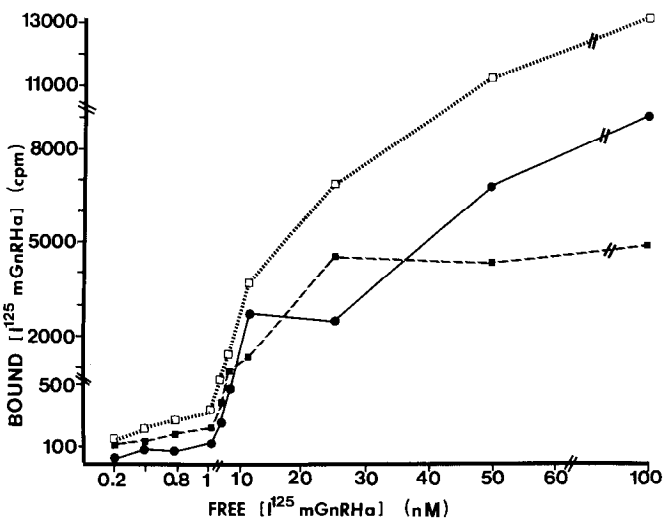


FIG. 4. Saturability of the GnRH binding sites. Specific binding (●) was determined by the subtraction of nonspecific binding (■) values from total binding values (□).

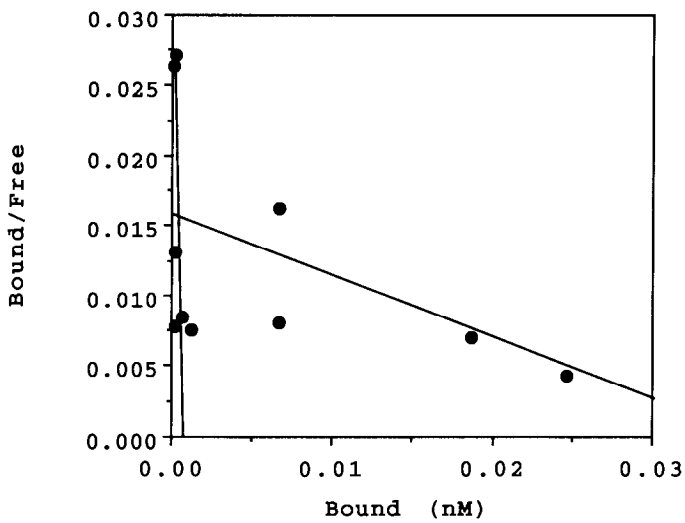


FIG. 5. Scatchard replot of saturation data showing two classes of binding sites within the lamprey pituitary. Site I $K_a$ = 1.5 × $10^{12}$ M; $B_{max}$ = 8.4 × $10^{-14}$ M; Hill coefficient = 0.936. Site II $K_a$ = 5 × $10^9$ M; $B_{max}$ = 5 × $10^{-11}$ M; Hill coefficient = 0.966.

vertebrates, in that they lack a hypothalamo-hypophysial portal vascular or innervation system. In the lamprey, the neurohypophysis and adenohypophysis are separated by avascular connective tissue (31). However, there is anatomical evidence to support the concept of hypothalamic control of adenohypophysial function by diffusion of the neurohormones from the neurohypophysis to the pars distalis of the adenohypophysis (13, 31).

In summary, these data provide direct evidence of GnRH activity on the pituitary in an Agnathan and are the first to demonstrate that a vertebrate pituitary contains two high affinity binding sites. The binding was located primarily in the proximal pars distalis of the pituitary. The characterization of GnRH binding in the pituitary of an agnathan implies that evolution of GnRH most likely antedated the origin of all known vertebrates.

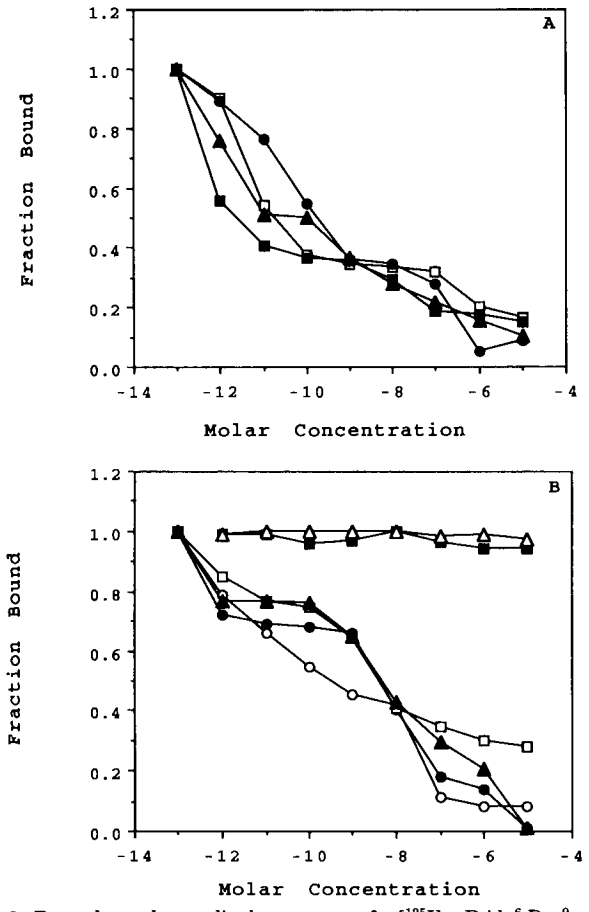


FIG. 6. Dose-dependent displacement of [$^{125}$I] DAla$^6$,Pro$^9$ NEt mGnRH from GnRH binding sites within the lamprey pituitary. Fraction bound is equal to the natural log of specific binding/the specific binding minus the nonspecific binding. A, Displacement of [$^{125}$I] DAla$^6$,Pro$^9$ NEt mGnRH by synthetic GnRH peptides. Mammalian GnRH (□), chicken GnRH II (▲), salmon (●), and chicken GnRH I (■). B, Displacement of [$^{125}$I] DAla$^6$,Pro$^9$ NEt mGnRH by native lamprey GnRH I (○) and III (●), lamprey GnRH analogs DPhe$^{2,6}$,Pro$^3$ lamprey GnRH (□), DALa $^6$,Pro$^9$-OH lamprey GnRH (■), mammalian GnRH analog DAla$^6$,Pro$^9$ NEt mGnRH (▲) and TRH (△).

**TABLE 2.** Inhibition constants ($K_I$) and biological activities for GnRH peptides and analogs

| Ligand | $K_I$ | Biological activity in lamprey |
|---|---|---|
| DAla$^6$, Pro$^9$ NEt mGnRH | $1.025 \times 10^{-10}$ | Yes |
| Mammalian GnRH | $1.79 \times 10^{-11}$ | NT |
| Chicken GnRH I | $2.59 \times 10^{-14}$ | NT |
| Chicken GnRH II | $2.84 \times 10^{-13}$ | NT |
| Salmon GnRH | $1.97 \times 10^{-11}$ | Yes |
| Lamprey GnRH I | $1.07 \times 10^{-10}$ | Yes |
| Lamprey GnRH III | $3.99 \times 10^{-9}$ | Yes |
| DPhe$^{2,6}$, Pro$^3$ Lamprey GnRH | $1.73 \times 10^{-8}$ | Yes |
| DAla$^6$, Pro$^9$-OH Lamprey GnRH | No displacement | Yes/No |
| TRH | No displacement | NT |

NT, Not tested in lamprey.

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## Erratum

In the article, "Regulation of gonadotropin-releasing hormone (GnRH) and galanin gene expression in GnRH neurons during lactation in the rat," by Daniel L. Marks, M. Susan Smith, Donald K. Clifton, and Robert A. Steiner (*Endocrinology* **133:** 1450–1458, 1993), on page 1454 Fig. 2 was incorrectly positioned and should be as shown below. The printer regrets the error.

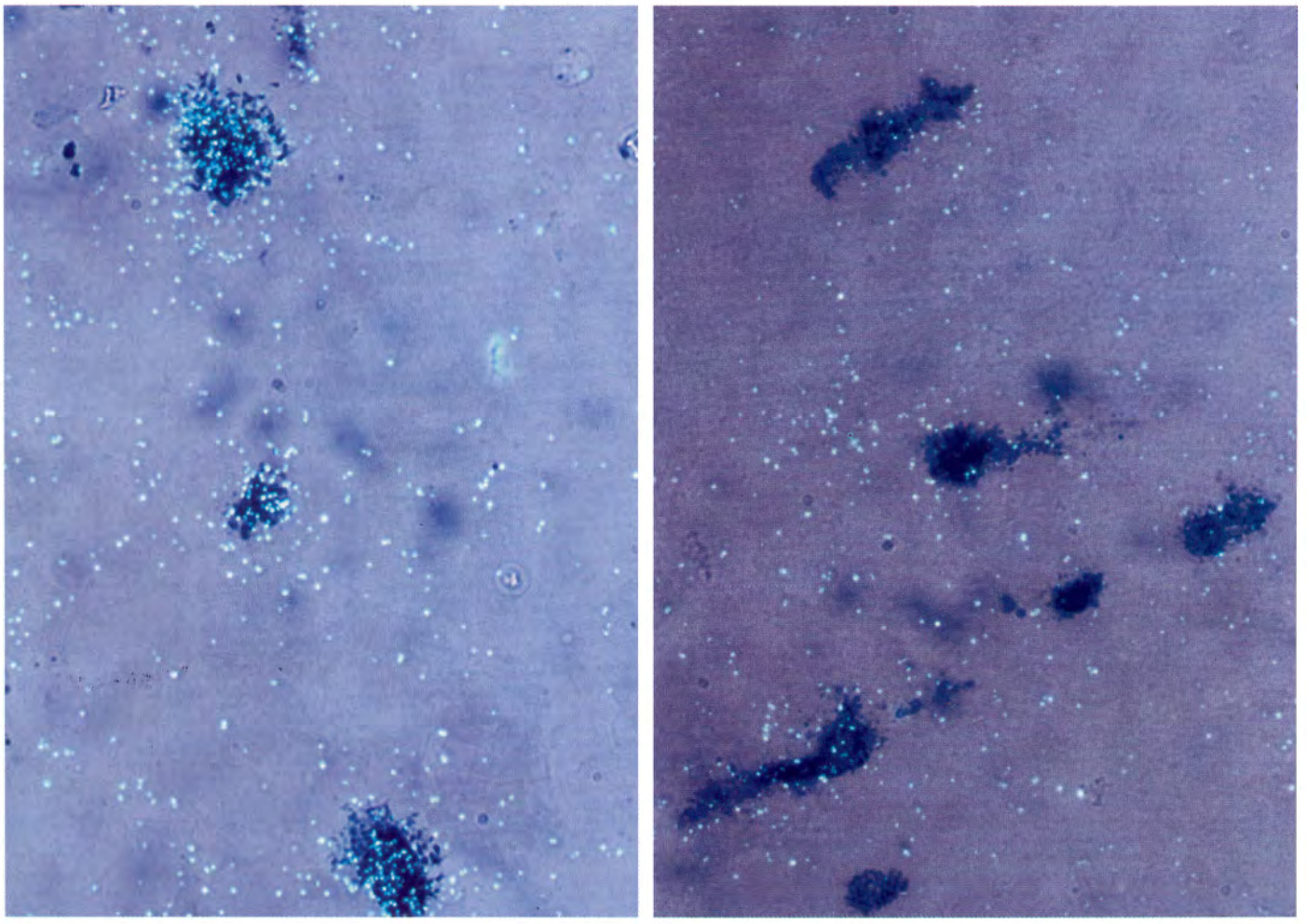

FIG. 2. Simultaneous bright- and darkfield photomicrographs (×40 objective) of the medial POA of adult female rats, showing cells labeled with a digoxigenin-conjugated cRNA probe for GnRH mRNA and a cRNA probe for galanin mRNA labeled with $^{35}S$ in sections obtained from animals killed at 1000 h on diestrus-1 (*left panel*) and on day 10 of lactation with eight pups (*right panel*).