A Lamprey Gonadotropin-Releasing Hormone in the Bovine Brain

Thesis

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by

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ABSTRACT

Mammalian gonadotropin release from the anterior pituitary is under control of the hypothalamic luteinizing hormone-releasing hormone (LHRH) and a putative analog, follicle stimulating hormone-releasing factor (FSHRF). Analogs of mammalian LHRH and their receptors have been isolated in various animals; of these, the lamprey-gonadotropin-releasing hormone-III (lamprey-GnRH-III) analog seems the most likely candidate as a possible independent FSHRF in the mammalian system. On the basis of these studies, I hypothesized that lamprey-GnRH-III may be a FSHRF in mammals, and if so, it should be possible to demonstrate the presence of this hormone during bovine brain development. To test this hypothesis, I used immunofluorescence to determine 1) if lamprey-GnRH-III is present in the fetal bovine brain and 2) at what stage during development this hormone is expressed. Additionally, I sought 3) to localize a receptor to which lamprey-GnRH-III may bind on anterior pituitary cells, and 4) to determine the level of colocalization of GnRH receptor and the downstream effect of this ligand-receptor interaction: FSH. Three trimester ages of brain were studied in order to determine the presence of this gonadotropin-releasing hormone associated with neurons during fetal bovine development. The antibody to lamprey-GnRH-III was found bound to neuronal membranes in the diencephalon region of the fetal brain at all ages studied. Additionally, GnRH receptor antibody was found on fetal anterior pituitary cells colocalized

with FSH. These results indicate that the lamprey-GnRH-III analog or a closely related peptide is present in the bovine brain, and may be a mammalian FSHRF.

INTRODUCTION

The gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are released from anterior pituitary cells as a result of a hormonal signal from the hypothalamus. Neurons in the hypothalamus produce gonadotropin-releasing hormones (GnRHs), which travel through the hypothalamo-hypophyseal portal system to the adenohypophysis, bind to GnRH receptors, and cause gonadotropin release.

The release of LH and FSH was thought to be under the control of a single GnRH when McCann, *et al.* (1983) published a review summarizing research that suggested possible differential signaling hormones for LH and FSH at the hypothalamic level. Mammalian luteinizing hormone-releasing hormone (LHRH) was characterized based on its ability to cause LH release, but additionally had been shown to cause FSH release *in vitro* (Schally *et al.*, 1971.) LHRH, when delivered in a varying pulsatile doses or infusions, could stimulate both LH and FSH release in the rat (Schally *et al.*, 1971, Ajika *et al.*, 1972; Libertun *et al.*, 1974), and led many to conclude that LHRH was the single GnRH in the mammalian system. However, others sought to demonstrate the existence of differential gonadotropin-releasing hormones (Dhariwal, *et al.*, 1965; Iragashi and McCann, 1964). Evidence supporting the idea of separate hypothalamic agents in the control of FSH and LH release came from studies that either electrochemically stimulated different regions of the hypothalamus (Kalra, *et al.*, 1971; Chappel and Barraclough, 1976) or involved lesions in different

hypothalamic areas (Bishop, *et al.*, 1971; Lumpkin and McCann, 1979, 1982, 1984). These types of experiments suggested the anterior median eminence contained neurons associated with LHRH. Lesions or electrochemical stimulation of the posterior median eminence inhibited or initiated FSH release, respectively, and implied the existence of a separate follicle stimulating hormone-releasing factor (FSHRF). More recently, Marubayashi, *et al.* (1999) performed similar lesions experiments supporting the idea of separate gonadotropins controlling the release of LH and FSH from the anterior pituitary.

LHRH is a decapeptide cleaved from a 156-amino acid precursor; its amino terminus is primarily responsible for receptor activation, while the carboxyl terminus is involved in receptor binding (Sealfon and Millar, 1995). Variants of mammalian LHRH and their receptors have been isolated in various animals. The analogs vary primarily at the fifth, seventh, and eighth amino acid of the decapeptide, and are highly conserved at both termini (Sealfon *et al*, 1997). The receptor for GnRH has also been characterized; the cDNA sequence of the mouse GnRH receptor was the first isolated (Tsusumi, *et al.*, 1992), followed thereafter by human (Kakar *et al.*, 1992), rat (Eidne, *et al.*, 1992; Kaiser *et al.*, 1992), sheep (Brooks, et al., 1993; Illing et al., 1993), cow (Kakar et al., 1993), and pig (Weesner, et al., 1994). The vertebrate receptor is a member of the rhodopsin-like G-protein coupled receptor family, a seven-pass transmembrane protein glycosylated at the amino terminus (Sealfon, *et al*, 1997). Of the GnRH analogs and receptors studied, the non-mammalian receptors readily bind to mammalian

GnRHs, whereas mammalian receptors will only bind mammalian GnRHs (King and Millar, 1995). In current studies by Robert Millar the receptor subtypes are being investigated; of these the receptor type II is found in the mammalian pituitary and may play a role in gonadotropin release (Rahe, 2000).

Of the vertebrate GnRHs studied, the lamprey-GnRH-III analog seems the most likely candidate as an independent FSH releasing factor (FSHRF) in the mammalian system. Lamprey-GnRH-III has a potent dose-related action on FSH release (but not LH) in rat pituitary *in vitro*, and a "completely selective stimulatory effect on FSH release" in rats *in vivo* (Yu, *et al.*, 1997). Extensive studies have been performed to localize LHRH in cow, horse and sheep brain (Dees and McArthur, 1981; Dees, *et al.*, 1981; Dees *et al.*, 1981), and more recently to localize a possible FSHRF in similar regions. Antisera against the vertebrate GnRH analogs (chicken, lamprey, mLHRH) have been used to identify these proteins in rat brain; of these, the lamprey-GnRH-III analog is present in the FSH-controlling region of the hypothalamus (Dees, *et al.*, 1999).

On the basis of these studies, I hypothesized that lamprey-GnRH-III may be a FSHRF in mammals, and if so, it should be possible to demonstrate its presence during bovine brain development. Additionally, the GnRH receptor type II may be a FSHRF receptor, and may therefore have lamprey-GnRH-III as its ligand. To test these hypotheses, I endeavored to localize the lamprey-GnRH-III hormone in the fetal bovine brain using immunofluorescence microscopy. I sought to determine 1) if lamprey-GnRH-III was present in the fetal bovine brain;

2) at what stage during development this hormone was expressed; 3) the location of GnRH receptor type II in anterior pituitary; and 4) if the GnRH receptor type II colocalizes with FSH, the downstream result of the ligand-receptor interaction. Colocalization occurs when two or more labeling probes give a signal within the same volume of limited dimensions.

As positive controls, I stained for mammalian LHRH (mLHRH) in each series, and for both hormones in an adult rat brain. The migration of LHRH neurons has been established in fetal rat (Yoshida, *et al.*, 1995), and I hypothesized similar patterns for FSHRF neurons. Bovine fetal age was separated into trimester categories. All brains were cryosectioned and stained using standard techniques. Both mLHRH and l-GnRH-III were localized in the rat diencephalon; mLHRH and l-GnRH-III have been found bound to neuronal membranes in all bovine fetal ages studied thus far (days 100-260 of 280 days).

Pituitaries from both adult and fetal cows were assayed for the presence of the type II receptor, and for the colocalization of the receptor with mammalian FSH. All tissues were cryosectioned and stained using standard techniques, using the same fetal age categorizing as noted above. The receptor was found bound to anterior pituitary cells throughout the full thickness of the gland, and was colocalized with FSH in each fetal age studied. LH, like FSH, was found extracellularly in the adenohypophesis, but was not colocalized with GnRH receptor type II.

MATERIALS AND METHODS

A. Tissue acquisition and preparation

Fetuses were selected based on their size and subsequent approximated age. The presence of lamprey-GnRH-III and its receptor was sought in fetal brains of three trimester age groups, based on based on body length from crown to rump (Sakumoto, *et al.*, 2000). Three fetuses from each age group were selected to represent experimental replicates. See Table 1. In most cases, brains and pituitary glands were removed for labeling procedures.

The fetal size was measured with a tape measure gauging standard crown-to-rump length in inches. The fetus was beheaded, and the top of the cranium was removed with a Stryker bone saw. Fetal brains were removed and placed in a dish containing sterile phosphate buffered saline (PBS) solution (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4). See Appendix 1. In most cases (group two and three fetuses) the lateral cerebrum was trimmed, leaving preoptic area, diencephalon, and midbrain intact. The remaining brain was partitioned into approximately 5 mm pieces with a sterile steel blade, using the optic chiasma and mammillary bodies as landmarks. See Figures 1&2. Pituitary bodies were cut away from the sella turcica with a sterile blade. The brain sections and pituitaries were fast-frozen in hexane in an ethanol/dry ice bath, sealed in individually labeled Ziploc bags, and placed in a cooler with dry ice for transport to the lab. All specimens were stored at -80°C until cryosectioning and immunostaining (see part B).

Table 1. Fetal sizes and ages

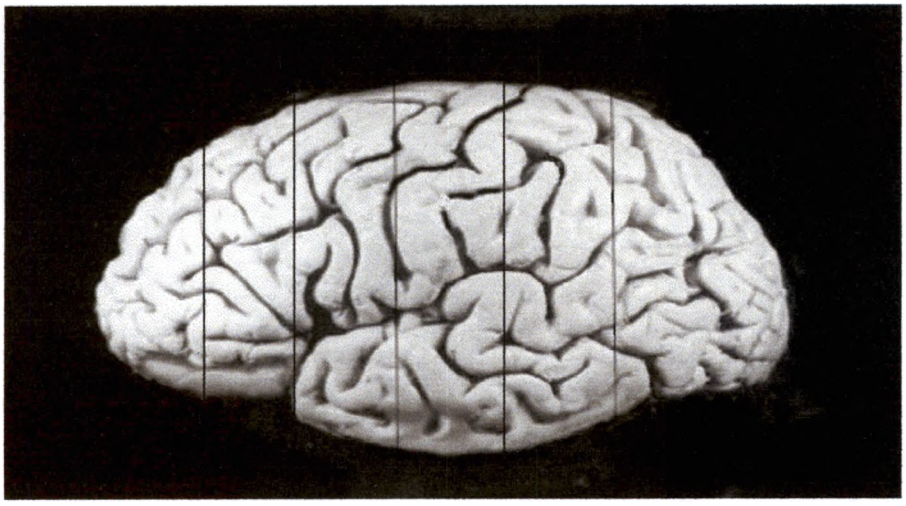
Cow #	sex	length (in)	age (days)	# sections	group #	labeled	pit labeled
adult #1	f			5+P	n/a		✓
adult A				P	n/a		✓
adult B				P	n/a		✓
adult C				P	n/a		✓
5		35	260	4+P	3	✓	
6		21	180	3+P	2	✓	
7	f	14.5	150		2	✓	
8	m	14	150		2		✓
9	f	16	160		2		✓
10	f	18	170		2		
11	f	16.5	160		2		
12	f	16.5	160		2		✓
13		30	>210	5+P	3		
14		8	100	3	1	✓	
15		12	120	3+P	1	✓	
16		30	>210	5+P	3	✓	
17		24	200	5+P	3		✓
18		24	200	4+P	3	✓	
19		12	120	4+P	1		✓
20		11	110	4+P	1	✓	
21		13	150	4+P	2	✓	
22		12	120	4+P	1		✓
23		21	180	5+P	3		✓
24		17	165	P	2		✓

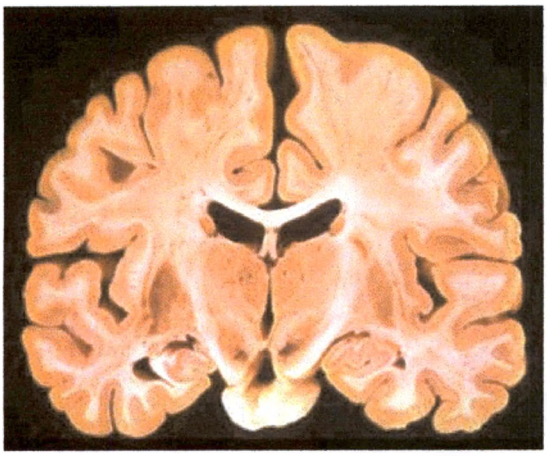
Group 1 = <120 days Group 2 = 120-180 days Group 3 = 180+ days

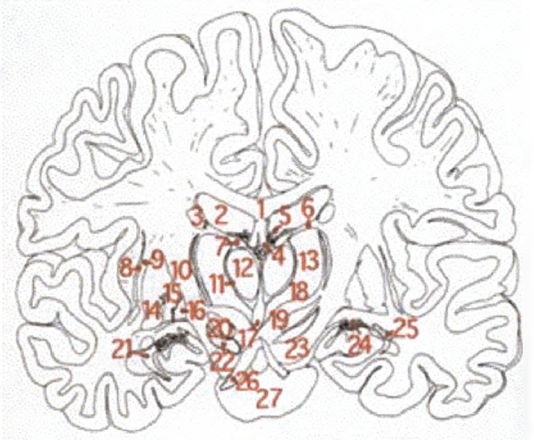
P = pituitary gland

Figure 1. Lateral view of the brain. The lines represent approximate sections.

Figure 2. Coronal section through the thalamus. This represents roughly the fourth section from the anterior surface in Figure 1. Image obtained through the University of Iowa's Health Center Virtual Hospital web site www.vh.org.







1.Body of corpus callosum 2. Central part of lateral ventricle 3. Tail of caudate nucleus 4. Body of fornix 5. Choroid plexus of lateral ventricle 6. Stria terminalis 7. Anterior thalamic nuclear group 8. Claustrum 9. External capsule 10. Internal capsule 11. Internal medullary lamina of thalamus 12. Medial thalamic nucleus 13. Dorsal lateral thalamic nucleus 14. Putamen 15. Lateral part of globus pallidus 16. Medial part of globus pallidus 17. 3rd ventricle 18. Ventral anterolateral thalamic nucleus 19. H1 field of Forel 20. Subthalamic nucleus 21. Alveus of hippocampus 22. Substantia nigra 23. Basis pedunculi 24. Choroid plexus of temporal horn of lateral ventricle 25. Tail of caudate nucleus 26. Oculomotor nerve 27. Pons

B. Lamprey-GnRH-III in the fetal bovine hypothalamus

Specimens were transferred from -80°C to -20°C twenty-four hours before cryosectioning on the Micron Cryotome (Thornwood, New York). Tissues were sectioned at -25°C in 20-50 μm increments. Typically, two sections each for controls and experimentals were collected, twenty sections were discarded, and the above repeated until the full thickness of the brain segment was represented. All slices were collected on collagen-coated cover slips and dried overnight at 4°C (see Appendix 1).

The samples were treated with a protein blocking solution of 10% non-fat powdered milk in PBST (PBS containing 0.2% Tween-20) for twenty minutes, then labeled using a combination of immuno- and other cytochemical probes on the subbed cover slips. See Table 2. The primary antibody used was highly specific for lamprey-GnRH-III (Dees, *et al.*, 2001). All samples were stained with a fluorescent secondary antibody; a few samples were treated additionally with TO-PRO 3 iodide to label nuclei in order to determine cell density and number in the hypothalamus. Staining was performed using standard reagents and procedures (see Appendix 2). All samples were mounted in 90% glycerol in PBS containing 1mg/ml p-phenylenediamine. An Olympus IX-70/Bio-Rad MRC 1024 Laser Scanning Confocal Microscope (LSCM, Hercules, CA) was used to collect images of the sections stained with fluorescent probes. Image acquisition and primary processing was done using Bio-Rad's Lasersharp software. Images were written to CD-ROM for storage. From the CD-ROM, image finishing and

printing of was done using Adobe Photoshop (Adobe Systems Inc; www.adobe.com/) software on a Macintosh G4 computer (Apple Computer, Cupertino, CA).

C. Controls for brain section staining

Negative controls for all immuno-preparations (cow and rat) were prepared by use of the appropriate normal rabbit serum or PBST in place of the primary antibody. All other procedures were performed using standard reagents and protocols (see Appendix 2).

Positive controls were performed using the lamprey-GnRH-III antisera in domestic rat brain; localization of this hormone previously had been shown in the rat (Dees, *et al.*, 1999). The control brains (two) were removed from rats euthanized by carbon dioxide asphyxiation (SWT IACUC #00-075). The brains were fast-frozen using the same technique as described above (see Part A), and similarly cryosectioned and stained (see Appendix 2).

Table 2. Probes and antibodies used.

Probe/ Antibody to (Catalog #)	mAB/pAB	Conjugated to	Dilution	Supplier
Lamprey-GnRH-III (Code No. 3952)	pAB (rabbit)	-	1:200 in PBST	Stacia Sower, University of New Hampshire, Biological Science Center
Rabbit IgG (#T2767)	pAB (goat)	TRITC	1:200 in PBST	Sigma Chem. Co, St. Louis, MO
TO-PRO 3 iodide (T-3605)	-	-	1:1000 in PBST	Molecular Probes, Eugene, OR
GnRH Receptor Type II	pAB (rabbit)	-	1:200 in PBST	Bob Millar, MRC Human Reproductive Biology Unit, Edinburgh, Scotland
Bovine LH (#518B7)	mAB (mouse)	-	1:200 in PBST	Janet Roser, Dept. of Animal Science, UC Davis
Mouse IgG (#40885)	pAB (goat)	Cy-5	1:200 in PBST	Jackson ImmunoResearch Laboratories, Westgrove, PA
Human FSH (# A57) FluoReporter (#F-6153)	pAB (rabbit)	FluoReporter Oregon Green 488	1:100 in PBST	Biomeda Corp., Foster City, CA Molecular Probes, Eugene, OR

D. Staining for GnRH receptor on bovine pituitary

Pituitaries were removed from adult cows, fast-frozen, stored, and cryosectioned as reported above. In addition to pituitary from one adult cow, two pituitaries from each fetal age group were selected (see Part A). Sections on cover slips were triply stained by sequential staining with three primary antibodies: antisera against receptor type II, LH, and FSH. Secondary antibodies specific to the primary antibody host animal were used to label the receptor primary antibody and the LH antibody; the FSH primary antibody was labeled directly using a FluoReporter tagging kit (see Table 2). These three antibodies were used to determine the presence of the receptor on the anterior pituitary (adenohypophesis) cells, and to try to colocalize FSH, the downstream product of lamprey-GnRH-III on its receptor. All staining procedures were performed using standard reagents and protocols (see Appendix 2); slides were prepared and images collected by confocal microscopy as described above (see Part B).

E. Controls for pituitary staining

Negative controls for all immuno-preparations in the pituitary were prepared by use of PBST in place of the primary antibodies. All other procedures were performed using standard reagents and protocols (see Appendix 2).

RESULTS

A. Demonstration of lamprey-GnRH-III in the bovine brain

Using the techniques described above, I found lamprey GnRH-III antibody to bind an antigen in multiple regions of fetal brain (see figures 3 and 4). Bovine fetuses were separated into three trimesters of <120 days, 120-180 days, and 180+days, based on length (Sakumato, R., *et al.*, 2000). Comparison to positive and negative controls supported identification of the antigen as a lamprey-GnRH-III-like peptide.

The labeled antibody was found bound to membranes of neuronal processes in the paraventricular region of the hypothalamus (see Figure 4). Texas Red TRITC was conjugated to the secondary antibody, and may be seen bound to multiple neurons in this region of the brain.

The labeled antibody was also found bound to membranes of neuronal processes in the dorsomedial region of the hypothalamus (see Figure 5). Texas Red TRITC was conjugated to the secondary antibody, and may be seen bound to multiple neurons. The cell bodies of these neurons also may be visualized.

Additionally, the labeled antibody was also found bound to membranes of neuronal processes in the ventromedial region of the hypothalamus (see Figure 6), again visualized with Texas Red TRITC. Previous studies have identified lamprey-GnRH-III in the dorsomedial and ventromedial regions of the preoptic area in the adult rat brain (Dees, *et al.*, 1999). These regions are consistent with those reported to control FSH release.

Figure 3. Regions of the bovine brain showing labeled antibody to lamprey-GnRH-III. Paraventricular (A), dorsomedial (B), ventromedial (C), and arcuate (D) regions surrounding the 3rd ventricle of the fetal bovine brain containing labeled antibody to lamprey-GnRH-III.

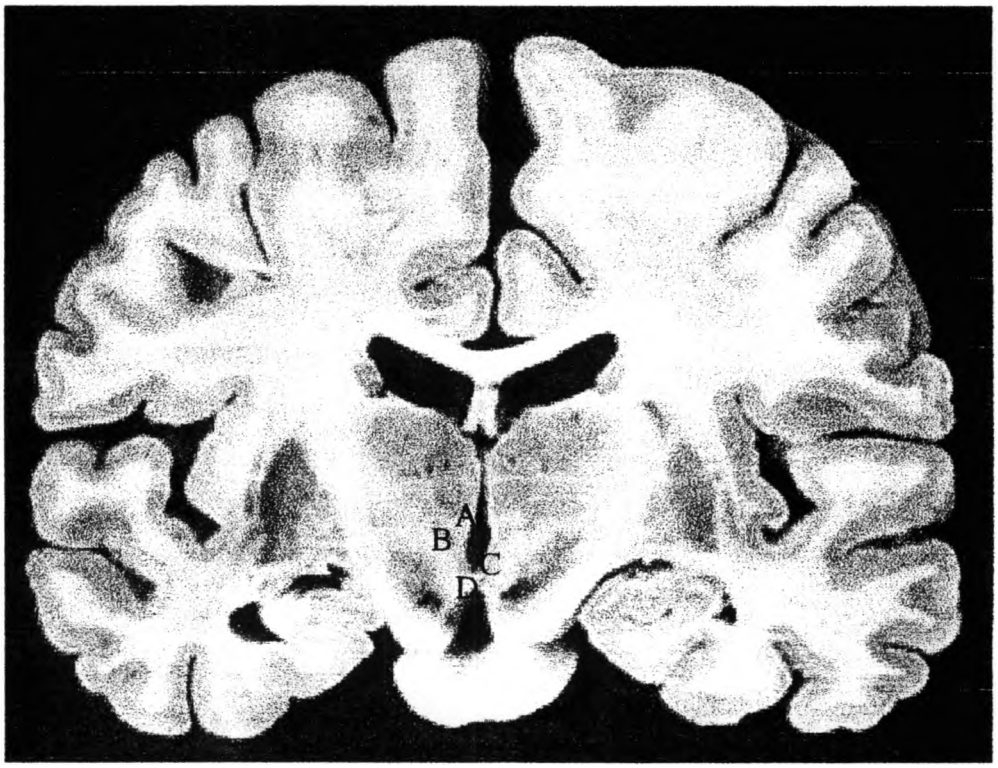
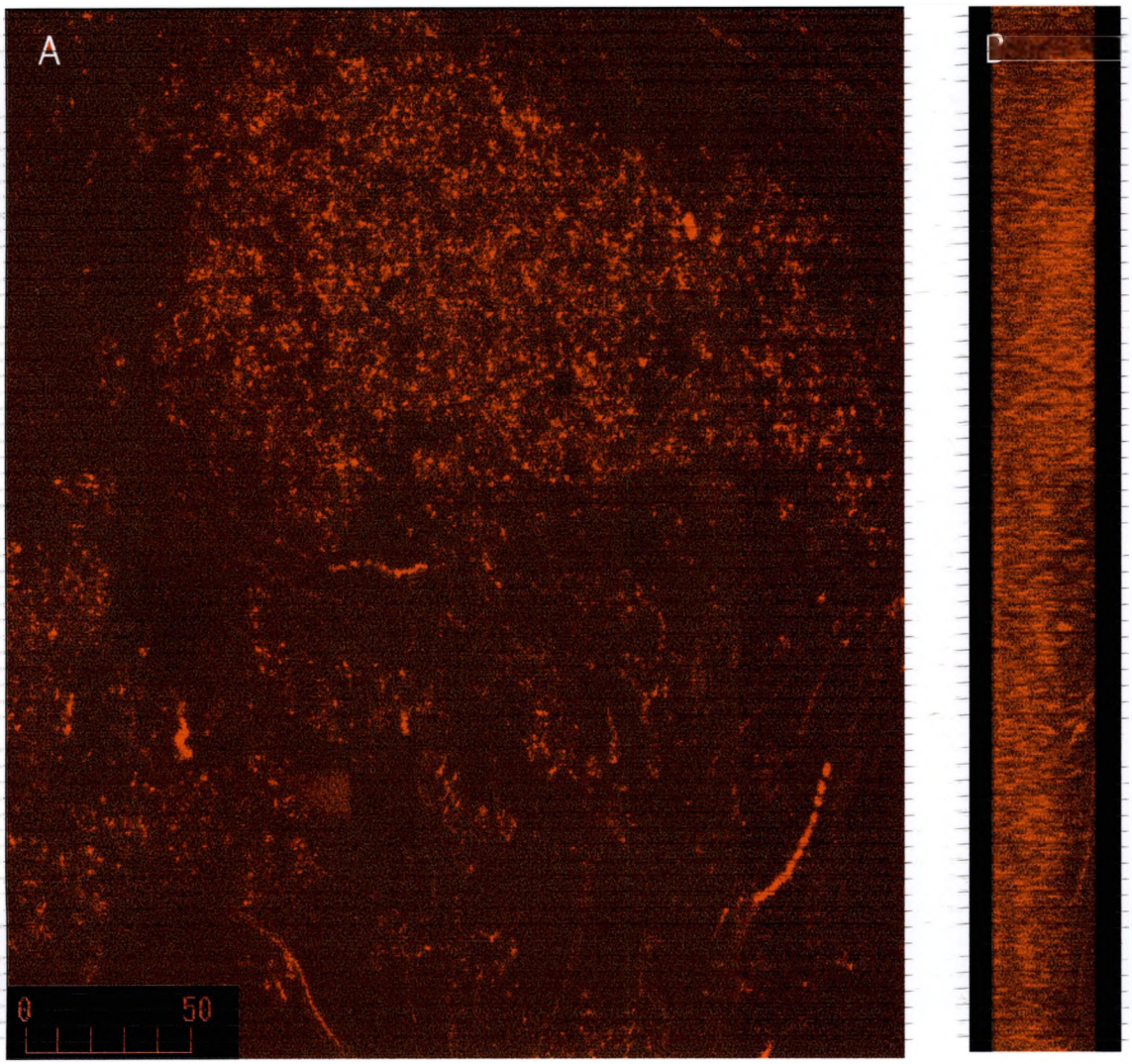

A
B
C
D

Figure 4. Confocal micrograph of fetal bovine brain stained with the lamprey-GnRH-III antibody. Texas Red TRITC was conjugated to the secondary antibody labeling the primary antibody to lamprey-GnRH-III. Here, the labeled antibody was found bound to paraventricular hypothalamic neurons. Image (A) shows a projection of a 35μm-thick cryosection of the hypothalamus adjacent to the third ventricle. The concentrated area of labeling at the top of the image represents neurons projecting into the tissue (perpendicular to the plane of the visible neurons). Image (B) shows the same section of tissue rotated 88°. The neurons at the top of the image may be seen traversing the entire 35μm section. The scale bar represents 50μm.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0
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Figure 5. Confocal micrograph of fetal bovine brain stained with the lamprey-GnRH-III antibody. Here, the labeled antibody was found bound to a dorsomedial hypothalamic neuron. The fluor labeling the neuron in this projection is Texas Red TRITC, conjugated to the secondary antibody. A second fluor, TO-PRO 3 iodide (violet) is a DNA-specific dye and was used to label the neuronal nuclei to determine cell number and density in this region of the hypothalamus. Original magnification 400X.

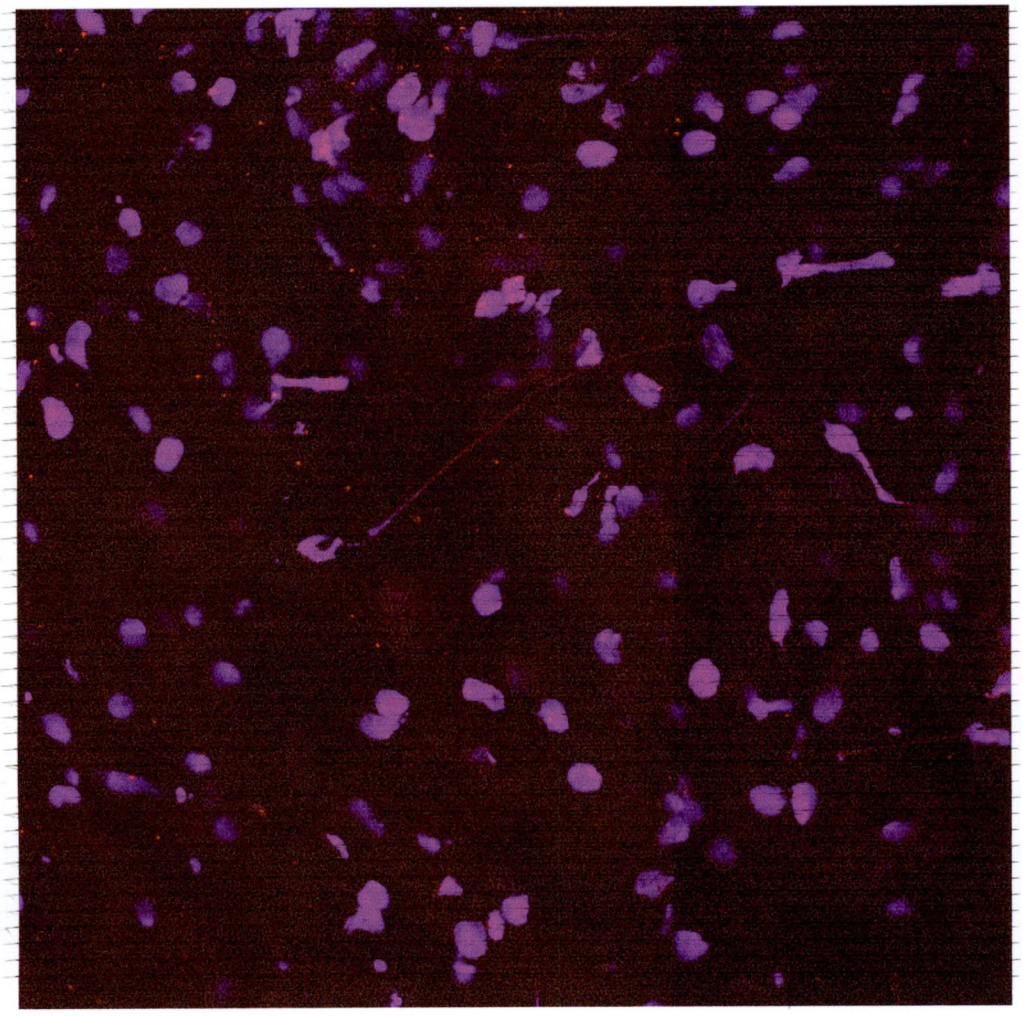


Figure 6. Confocal micrograph of fetal bovine brain stained with the lamprey-GnRH-III antibody. Here, the labeled antibody was found bound to ventromedial hypothalamic neurons. The fluor labeling the neuron in this projection is Texas Red TRITC, conjugated to the secondary antibody. A DNA-specific dye, TO-PRO 3 iodide (violet), was used to label the neuronal nuclei to determine cell number and density in this region of the hypothalamus. Image (A) shows a 30μm-thick cryosection lateral and ventral to the paraventricular nuclei (see Fig. 4). Multiple neurons bound by the antibody to lamprey-GnRH-III may be seen in this projection. The yellow and blue arrows indicate neurons that extend perpendicularly into the tissue, as seen by Image (B), an 88° rotation of the same section. The scale bar represents 50μm.

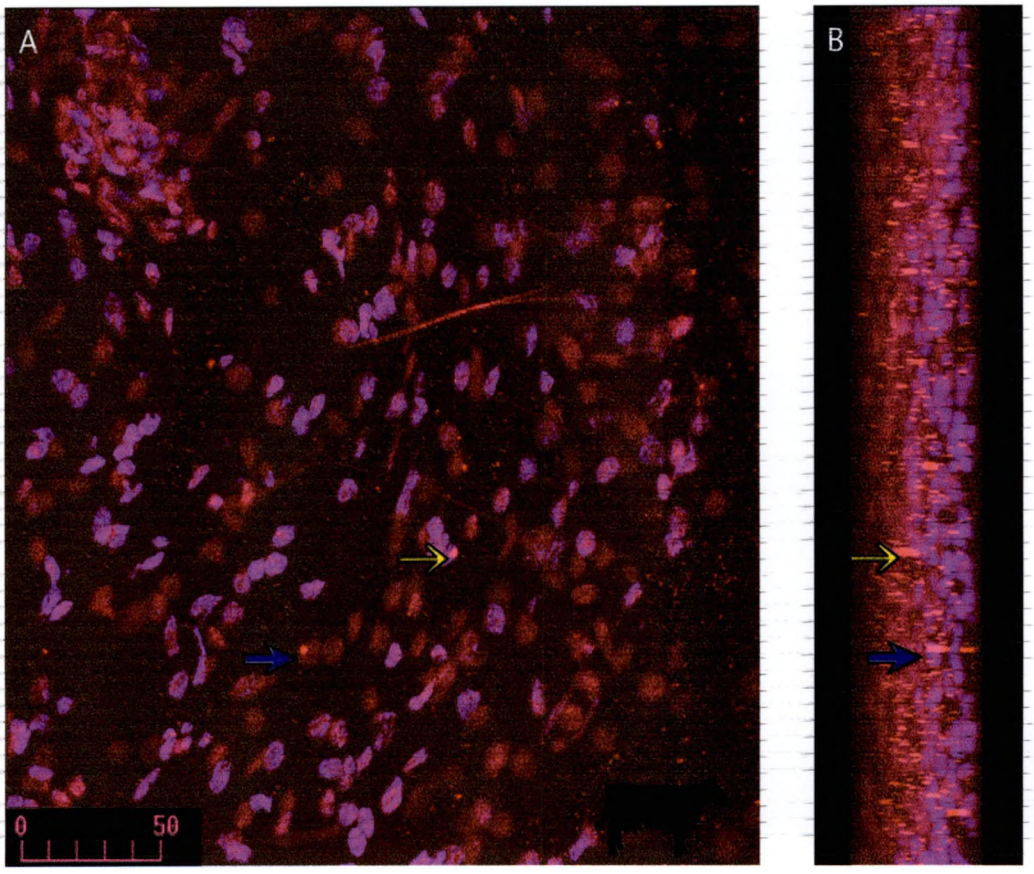
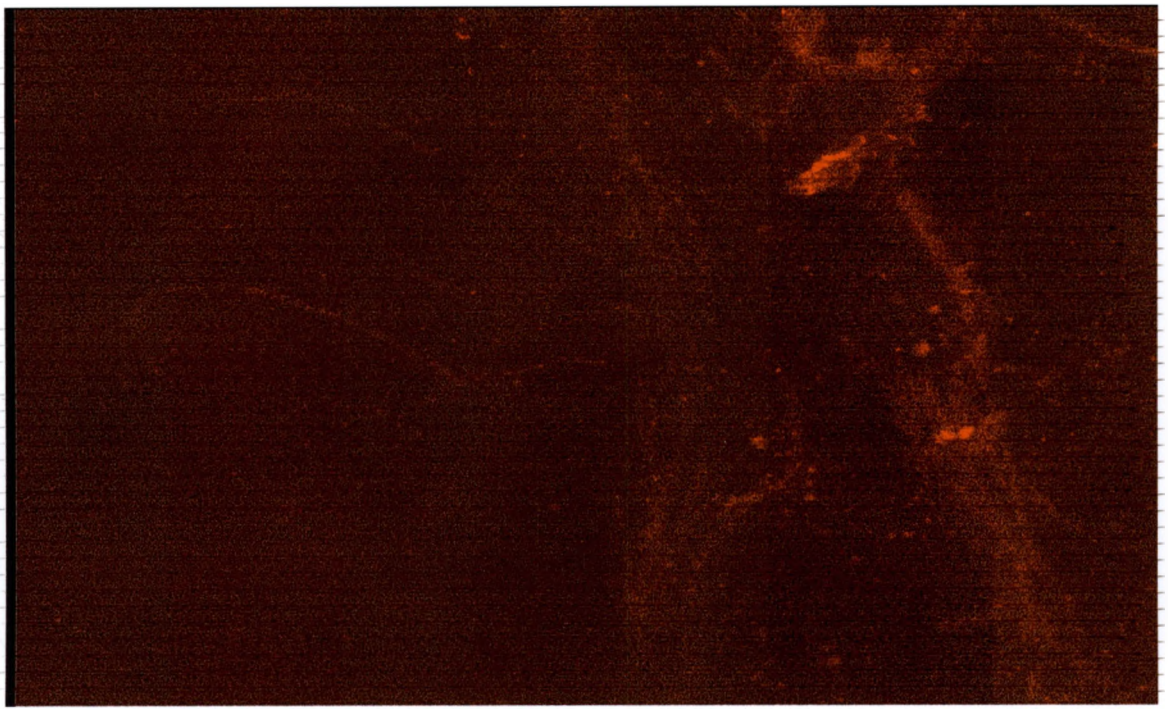
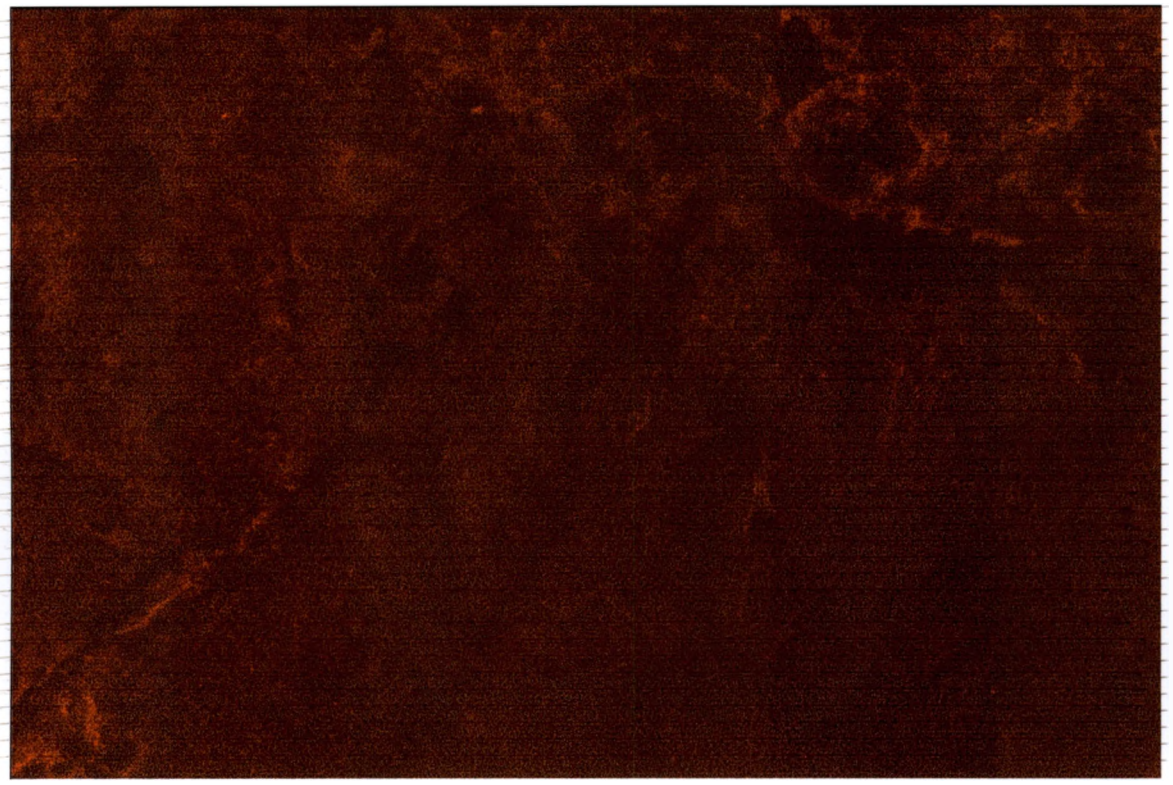

A
B
0
50

Figure 7. Confocal micrographs of adult rat brain stained with lamprey-GnRH-III antibody. Texas Red TRITC was conjugated to the secondary antibody. Lamprey-GnRH-III antibody may be seen bound to neuronal processes in the paraventricular and dorsomedial regions of the rat brain. Images such as these served as positive control for the lamprey staining in the bovine. Original magnification 400X for both images.





Positive control assays in adult rat brain yielded positive labeling of antibodies to both lamprey-GnRH-III in the ventromedial and paraventricular hypothalamus (see Figure 7) and mLHRH (results not shown). Negative controls for all immuno-preparations (cow and rat) were prepared by use of the appropriate normal rabbit serum or PBST in place of the primary antibody, and no labeling was detected (results not shown).

B. Determination of timing of expression of lamprey-GnRH-III during development

The labeled antibody to lamprey-GnRH-III was found bound to neuronal membranes of paraventricular, dorsomedial, arcuate (images not shown) and ventromedial regions of the hypothalamus in all age groups studied. Due to the methodology of tissue acquisition, it is impossible to determine whether the processes of these neurons extend rostrally or caudally. Nor is it possible to determine at what stage during fetal development these neurons migrate to and from the preoptic area; although based on these observations the neurons most likely migrate very early in the first trimester.

C. Lamprey-GnRH-III receptor on pituitary cells

Tissue sections from the anterior pituitary were stained with antibodies that recognize GnRH receptor type II, FSH, and LH. Tissue sections were stained

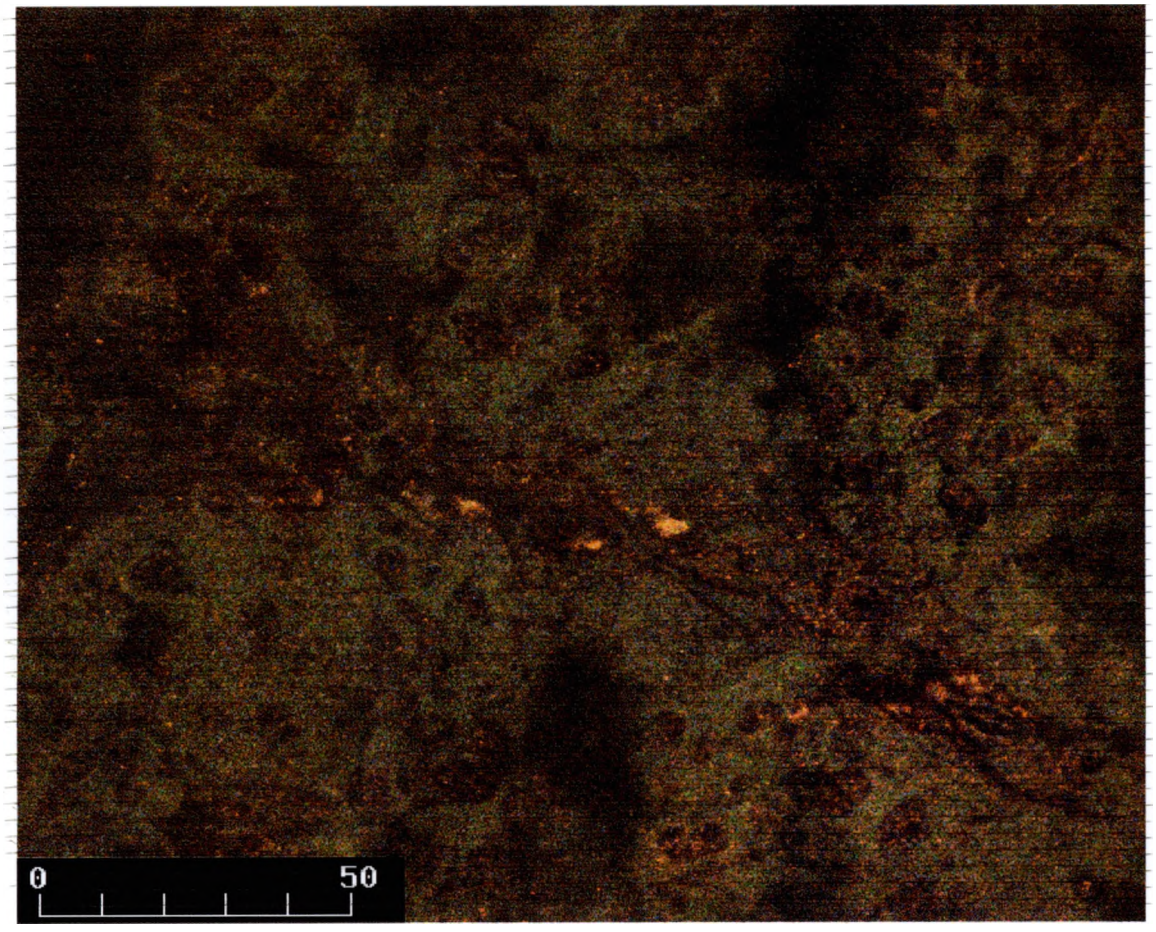
for receptor, FSH, and LH in order to determine (1) the presence of these proteins and (2) if receptor would be found in close proximity to the downstream effect of that receptor binding its ligand. Colocalization is a technique employed in confocal microscopy to determine whether two antigens occupy the same area of a limited dimension within the tissue or cell being viewed. Here, the presence of FSH was sought colocalized with receptor type II, exclusive of LH.

The labeled antibody to GnRH receptor type II was found on anterior pituitary cells throughout the full thickness of the gland (see Figure 8). The immunostaining techniques reveal the location of the receptor on cell surfaces, while the gonadotropin hormones appeared extracellularly. FSH appeared to be colocalized with cells bearing the GnRH receptor type II antibody, whereas LH was not colocalized with the receptor. Colocalization analysis using BioRad software shows the extent to which these fluorescently tagged antibodies appeared together in some areas of the pituitary (see Figure 9).

Figure 10A demonstrates another pituitary wherein labeling of the receptor, FSH, and LH antigens occurred. The labeled antibody for all three proteins were present in anterior pituitary samples in adult bovine brain and for all fetal ages studied. Again, colocalization of GnRH receptor type II and FSH, but not LH, is apparent (see Figure 11). No labeling was detected in samples not treated with the primary antibodies (see Figure 10B).

Figure 8. Confocal micrograph of anterior pituitary cells stained with the labeled antibodies to receptor type II, FSH, and LH. The receptor is labeled with Texas Red TRITC, FSH with Oregon Green, and LH with Cy-5 (blue). FSH and LH may be seen intercellularly, while the receptor type II is associated with the cell surface. The yellow spots (see arrows) indicate colocalization of the red and green channels (see Figure 9), thereby signifying colocalization of lamprey-GnRH-III receptor and the downstream product of this receptor-ligand interaction: FSH. No colocalization of LH and receptor is apparent (as would be indicated by white areas of colocalized blue and red channels). The scale bar represents 50 μm.

Figure 9. Colocalization charts of GnRh receptor type II and FSH. These charts were obtained using BioRad software. Figure A measures the degree of colocalization of signals from the red and green channels (receptor and FSH); figure B measures the colocalization (or lack thereof) from of the red and blue channels (receptor and LH). The greater the distance along an axis a color travels, the stronger the signal. The more the two signals are combined, the more the mixed color shows on the graph, and the farther toward the middle of the graph the colors extend.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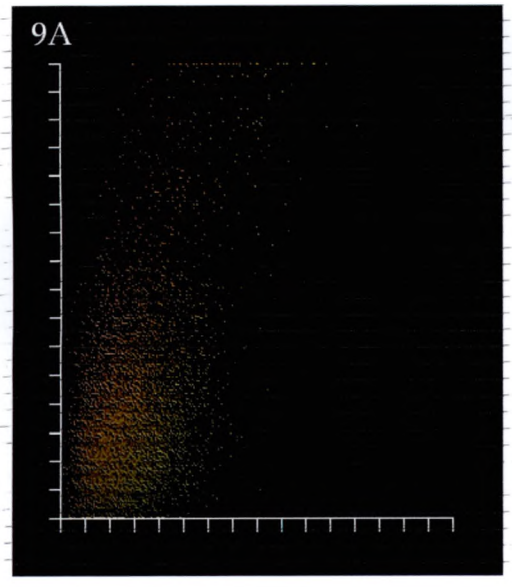

9A

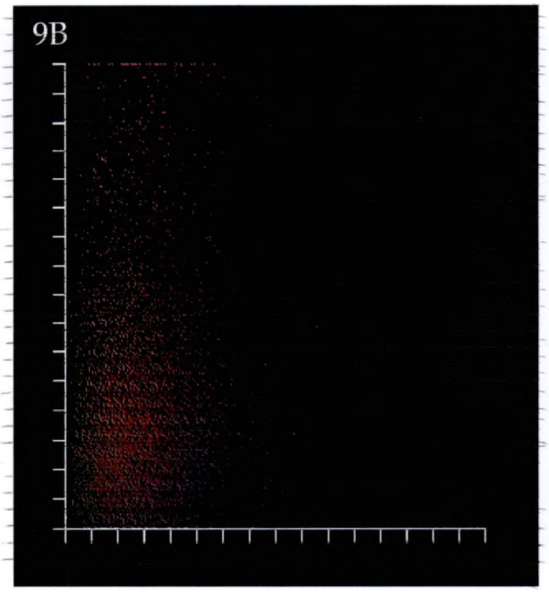
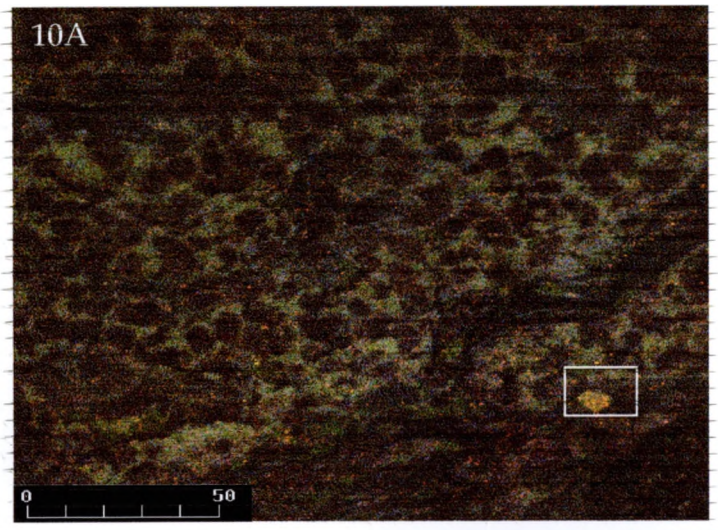

9B

Figure 10A. Confocal micrograph of anterior pituitary cells stained with the labeled antibodies to receptor type II, FSH, and LH. The receptor was labeled with Texas Red TRITC, FSH with Oregon Green, and LH with Cy-5 (blue). FSH and LH may be seen intercellularly, while the receptor type II is associated with the cell surface. The bright yellow spot (see box) indicates colocalization of the red and green channels on that cell (see Fig. 11), thereby signifying colocalization of GnRH receptor type II and the downstream product of a receptor-ligand interaction: FSH. Figure 10B shows controls for the three proteins in fetal bovine tissue. No labeling was seen when PBST was used in place of the primary antibodies. Scale bars represent 50 μm.

Figure 11. BioRad software colocalization chart of GnRH receptor type II and FSH (see figure 10A). Figure A measures the degree of colocalization of signals from the red and green channels (receptor and FSH); Figure B measures the colocalization (or lack thereof) from of the red and blue channels (receptor and LH). The degree of colocalization of the green and red channels on this one cell in the adenohypophesis demonstrates the colocalization of the GnRH receptor type II and the downstream effect of the hormone-ligand bond: FSH.


10A
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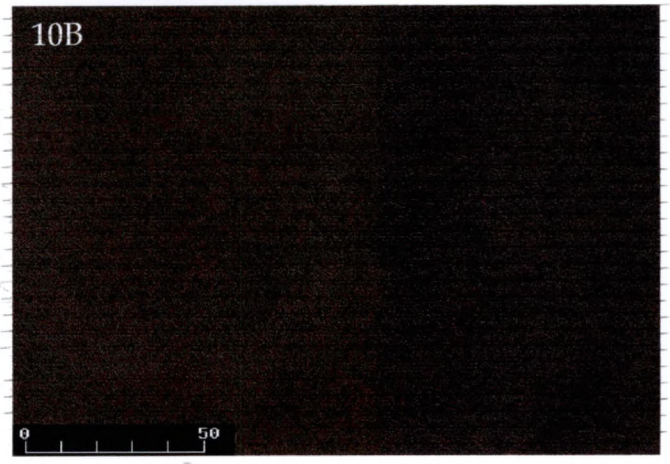
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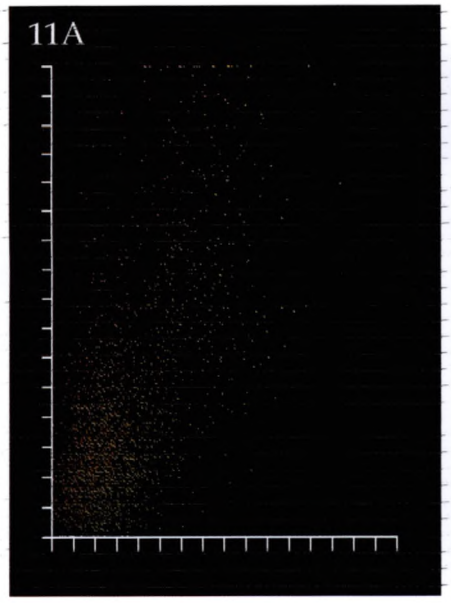
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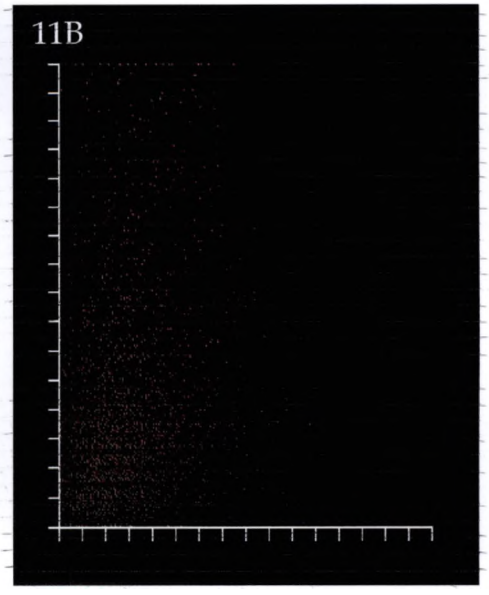
11B

DISCUSSION

This study involved the localization of a lamprey-GnRH-III-like peptide and the GnRH type II receptor in the bovine brain. Lamprey-GnRH-III has been proposed as the mammalian follicle stimulating hormone-releasing factor, and based on previous research I hypothesized that (1) lamprey-GnRH-III would be localized in fetal bovine brain, (2) the hormone would be expressed during the second trimester of development, (3) a possible receptor for this class of gonadotropin-releasing hormone (FSHRF receptor) would be present in the adenohypophesis, and (4) the GnRH receptor type II would be colocalized in the pituitary with FSH.

A. Demonstration of a lamprey-GnRH-III-like peptide in the bovine brain

Lamprey-GnRH-III was sought in the fetal bovine brain using immunofluorescence microscopy. Microscopic and macroscopic tissue morphology was highly conserved using the techniques listed in Material and Methods Part A. In beginning trials, the brain samples were fixed in a 4% paraformaldehyde (PF) solution (freshly made from paraformaldehyde [Electron Microscopy Sciences, Ft. Washington, PA]) and buffered with PBS for 24 hours, washed in PBS, then cryo-protected in 30% sucrose in distilled water for 48 hours at room temperature. The tissues were then stored at -20°C in PBS until cryosectioning on the Micron Cryotome (Zeiss, Thornwood, NY). These

techniques have been used countless times in immunohistochemical assays with no deleterious effects, but in this case initial tissue samples proved to be of poor quality, having microscopic morphological distortions (a shotgun hole appearance). Fixation time was increased to 48 hours, with similar results. During this change in procedure, we also changed staining protocols, which facilitated maintaining the integrity of the gross morphology of the brain sections. Initial trials were performed using a protocol wherein sections from PF-fixed brains were obtained using a vibratome and collected into tissue culture wells containing PBS. All staining was performed in these wells, and tissue sections were mounted on a glass slide with a paintbrush for microscopic examination. Although this is a standard method for immunohistochemical staining, this procedure often resulted in poor quality sections in our initial trials. The sections often floated in PBS (containing peroxidase staining reagents) for up to 36 hours, and thereby resulted in compromised gross morphological tissue integrity. The same results occurred in brains fixed using the fast-freeze method as described in Materials and Methods Part A. Additionally, I wanted to ultimately be able to identify multiple proteins in the brain and pituitary, and therefore changed protocols to the fluorescence immunohistochemical protocol listed in Appendix II (Bolanos, *et al.*, 1998). The resulting fixation and staining protocols yielded conserved tissue morphology, and allowed for visualization of multiple labeled proteins.

Labeled antibody to lamprey-GnRH-III was found bound to neuronal

membranes of paraventricular, dorsomedial, arcuate, and ventromedial regions of the fetal bovine hypothalamus. This probe is highly specific for lamprey-GnRH-III (Dees, *et al.*, 2001), and comparison to positive and negative controls supported the identification of the antigen as lamprey-GnRH-III or a closely related molecule. The lamprey-GnRH-III analog has been shown present in the FSH-controlling region of the hypothalamus (Dees, *et al.*, 1999), and the results of this study support previous evidence of a non-mammalian gonadotropin-releasing hormone in the mammalian brain.

B. Determination of timing of expression of lamprey-GnRH-III during development

Brain samples were categorized into three trimester age groups (Sakumoto, *et al.*, 2000), and three replicates were performed for each age group. Yoshida, et al. (1995) reported the mLHRH neurons in the developing rat migrate from the olfactory placode to the brain from embryonic day 14 of 21. Based on this study, I proposed the putative FSHRF neurons would migrate in a similar pattern, and would be localized in the developing bovine brain around embryonic day 180 of 280 (late second trimester). However, labeled antibodies to lamprey-GnRH-III and mLHRH were found bound to the membranes of neurons in multiple regions of the hypothalamus in all fetal ages studied thus far. The neurons controlling FSH release may migrate earlier in the bovine than in the rat, or lamprey-GnRH-III may not be the peptide associated with FSH release.

C. GnRH receptor type II and gonadotropins on pituitary cells

Receptor type II was sought in the anterior bovine pituitary using immunofluorescence microscopy. Receptors for the gonadotropin-releasing hormones have been characterized (Sealfon, 1995), and the receptor subtypes have been isolated; of these, the receptor type II is present on the mammalian pituitary. Labeled antibody to GnRH receptor type II was found bound to adult and fetal bovine anterior pituitary cells. The labeled antibody was bound to antigens present throughout the full thickness of the gland, was located in both adult brain and in all fetal ages studies this far. Additionally, GnRH receptor type II has been colocalized with the downstream product of FSHRF and its receptor binding: FSH. Colocalization analysis is a powerful tool used in confocal microscopy to determine the extent to which two or more labeling probes give a signal within the same volume of limited dimensions. LH was present in the anterior pituitary, but was not colocalized with the type II receptor.

D. Conclusions and further studies

I have thus far been able to give preliminary evidence of the presence of an antigen in the bovine brain that binds the highly specific antibody to lamprey-GnRH-III. Further immunohistochemical trials must be run with primary antibody preabsorbed with its antigen. Assays must be performed on younger

fetuses (less than 100 of 280 days) to determine the initial presence of this hormone in the diencephalon and pre-optic regions. Further staining must also be performed to localize this hormone as the neurons to which it associates migrate through fetal tissue; the migration most likely occurs early during the first trimester.

I have also demonstrated the presence of an antigen in the anterior bovine pituitary binding the antibody to GnRH receptor type II. I have colocalized FSH with this receptor (and the lack of colocalization of LH and this receptor) on anterior adult and fetal pituitary cells. Further colocalization assays must be performed to show the lamprey-GnRH-III hormone bound to GnRH receptor type II on anterior pituitary cells in order to suggest these compounds to be specific receptor and ligand.

Lamprey-GnRH-III also must be shown to selectively release FSH from the bovine pituitary, as occurs in rat (Yu, *et al.*, 1997), and *in vitro* and *in vivo* stimulation of FSH release in mammals by the lamprey-GnRH-III peptide will strongly support the concept of independent hypothalamic control of FSH release (Yu, *et al.*, 2000). Experiments are currently underway to determine this hormone's action *in vivo* in the bovine (Dees, *et al.*, 2001). Positive results will more conclusively suggest lamprey-GnRH-III (or a closely related compound) to be a mammalian FSHRF, and entail extensive biomedical implications. Once characterized, the mammalian FSHRF will have widespread uses in agricultural

and human reproductive sciences in both the facilitation and inhibition of FSH release.

Appendix 1. Recipes for Reagents

A. 0.5% Collagen-coated cover slips

Heat 200 mL of distilled water to 55°C. Turn heat to low, add 0.1 g Chromium Potassium Sulfate. SLOWLY add 1.0 g gelatin until dissolved. Dip cover slips in solution and allow to dry (a test tube rack works well) overnight. Store up to two weeks at room temperature.

B. PBS

To make 10X PBS, combine the following with 800 mL distilled water: 80.0 g NaCl, 2.0 g KCl, 11.5 g $Na_2HPO_4 \cdot 7H_2O$ (dibasic), and 2.0 g KH_2PO_4 (monobasic). Bring volume to 1 L with distilled water, pH 8.0-8.5. Store at room temperature.

To make 1X PBS (PBS), combine 100 mL of 10X PBS with 900 mL distilled water. Store at room temperature.

C. 4% Paraformaldehyde (PF)

Place 20.0 g paraformaldehyde in a 500 mL beaker. Bring volume to 250 mL with distilled water. Heat and stir under a fume hood to 60°C. Add three to six drops of 1.0 M NaOH or KOH until the cloudy solution turns clear. Turn off heat. Filter solution under fume hood, and bring the total volume to 250 mL with distilled water. Add 50 mL of 10X PBS (see above) to the paraformaldehyde solution, and bring the final volume to 500 mL with distilled water.

Appendix 2. Protocol for Immunofluorescence Staining

1. (Sections on cover slips are dried overnight at 4°C in labeled sterile petri dishes.)
2. Sections are rinsed 3 times with 200 μL PBST. **After each treatment of each reagent used, excess reagent is removed with a micropipette and discarded.**
3. Sections are treated with 200 μL of a protein blocking solution of 10%-20% powdered milk in PBST for 20-30 minutes.
4. Sections are rinsed 3 times with 200 μL PBST.
5. Sections are treated with 100-200 μL of first primary antibody solution, diluted with PBST according to manufacturer's recommendations. ***All antibodies are stored at -80°C until immediately before use.** *Negative control sections are treated with 100-200 μL of PBST or appropriate serum instead of primary antibody solution.* **Covered** sections may sit in primary antibody for 2 hours at room temperature or 24 hours at 4°C. (Should the cover slips be left for 24 hours, tape filter paper to the petri dish lid, and moistened with distilled water to prevent the sections from drying out.)
6. Sections are rinsed 3 times with 200 μL PBST.
7. Sections are treated with 200 μL of appropriate secondary antibody (including any negative control sections). **Covered** sections may sit in

secondary antibody for 2 hours at room temperature or 24 hours at 4°C. (Should the cover slips be left for 24 hours, tape filter paper to the petri dish lid, and moistened with distilled water to prevent the sections from drying out.) ***Secondary antibodies conjugated to a fluor must be diluted, used, and stored in dark conditions (low to no white light). Sections treated with a secondary antibody or with a fluorescent tag must be stored in the dark.**

8. (If desired, TO-PRO 3 may be added to cover slips in last 30 minutes of step 7.)
9. Sections are rinsed 3 times with 200 μL PBS.
10. (*Steps 5-8 may be repeated sequentially for multiple antibodies used*).
11. After the final rinse with PBS, sections are rinsed once with 200 μL distilled water. Excess water is removed with a micropipette.
12. Clean slides are labeled and one drop of 90% glycerol in PBS containing 1mg/ml p-phenylenediamine is applied to the slide for each cover slip.
13. Cover slips are removed from petri dish, inverted, and place on the drop of glycerol.
14. Slides are inverted onto a clean absorbent cloth and pressure is applied to remove excess glycerol.
15. Slides are placed in a slide container, **and stored at -20°C in the dark.**

Adapted from Bolanos, et al., *1998.*

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