GREAT LAKES FISHERY COMMISSION

Project Completion Report[1]

GnRH Neuronal Migratory Mechanisms During Lamprey Brain Development

by:

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PROGRESS (FINAL) REPORT FOR GLFC RESEARCH
SUMMARY

PROJECT TITLE: GnRH neuronal migratory mechanisms during lamprey brain development

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PROBLEM STATEMENT AND OBJECTIVES:

The proposed research program was designed to investigate gonadotropin releasing hormone (GnRH) neuron migration in developing lamprey larvae. The specific objectives included 1) determining the timing and pathway of GnRH neuron migration; 2) determining specific carbohydrates (or glycoconjugates) which may play important roles along the GnRH migratory pathway in lamprey; and 3) determining if the migration of GnRH neurons in lamprey larvae can be altered by reagents which recognize specific carbohydrates or analogs of identified carbohydrate epitopes.

RATIONALE AND RELEVANCE TO COMMISSION OBJECTIVES:

The Great Lakes Fishery Commission in its 1990 "Strategic Vision of the Great Lakes Fishery Commission for the Decade" states that one of its objectives is to "suppress sea lamprey populations to target levels by several methods including the development and use of alternate control techniques to reduce reliance on lampricides to 50% of current levels." The results from our experiments provide new information on the earliest development of GnRH neurons. Our experimental results detail the expression of selective carbohydrate structures along the olfactory system and their potential interaction with reproductive centers in the hypothalamus during lamprey development. Our developmental studies of GnRH neurons in lampreys confirm the likelihood that manipulations can be conducted within the first year of lamprey life to provide an alternative, effective management tool for the control of sea lamprey populations. The data further suggest specific carbohydrate and neurotransmitter analogs which may alter the developing GnRH neuronal system.

PROCEDURES:

Lamprey eggs were fertilized in the laboratory and larvae were collected at multiple stages and

releasing hormone-III (GnRH-III) in brains of larval lampreys (*Petromyzon marinus*). Cell and Tissure Research, 279:261-270 (1995).

Tobet, S.A., Chickering, T.W. and Sower, S.A.: Relationship of gonadotropin-releasing hormone (GnRH) neurons to the olfactory system in developing lamprey *(Petromyzon marinus).* in revision for Journal of Comparative Neurology (1996).

MacIntyre, J.K. , Sower, S.A., and Tobet, S.A: γ-aminobutyric acid neurons and their relationship to gonadotropin-releasing hormone neurons in developing lamprey, *Petromyzon marinus.* Manuscript in preparation - based on abstracts listed below.

Presentations and Abstracts:
Sower, S.A., Nozaki, M., Gorbman, A., Youson, J.H. & Tobet, S.A. Distribution of lamprey gonadotropin-releasing hormone-III in lamprey brains. 24th Meeting of the Society for Neuroscience, Miami, FL (1994). (Soc. Neurosci. Abstr. 20:1420).

Tobet, S.A., Chickering, T.W. & Sower, S.A. Relationship of gonadotropin-releasing hormone GnRH) neurons to the olfactory system in developing lamprey *(Petromyzon marinus).* 77th Meeting of the Endocrine Society, Washington, D.C. (1995). (Endocrinology suppl:554)

MacIntyre, J.K., Gamble, R., Chickering, T.W., Sower, S.A. & Tobet, S.A. Developmental relationship of neurons containing γ-aminobutyric acid to neurons containing gonadotropin-releasing hormone in the sea lamprey. American Society for Zoology, Wash., D.C. (1995). (Amer. Zool. 35:22A).

Tobet, S.A. Gonadotropin-releasing hormone containing neurons and olfactory fibers during development: from lamprey to mammals. International Conference on Hormones, Brain and Behaviour, Torino, Italy (1996). In conjunction with a manuscript to be submitted for publication.

Tobet, S.A. Development of the gonadotropin-releasing hormone neuronal system in lampreys. European Conference of Comparative Endocrinologists, Rouen, France (1996).

Reagent Identification:
The lectins **GS-1-B4** and **DBA** recognize carbohydrates in the olfactory epithelia of prolarval lamprey. Several carbohydrate analogs, including α-methyl-galactoside, alter the expression of lamprey-GnRH-III during prolarval development. **GABA** was identified as a neurotransmitter that also may influence GnRH neuron development.

SPECIFIC OBJECTIVES

The objectives of our original three year proposal were to determine and generate tools to alter the GnRH neuron system in larval sea lampreys with the goal of producing sterile adult male sea lampreys.

EXPERIMENTAL SERIES I:

In various vertebrate species, multiple forms of GnRH have been found to coexist in the same brains (Calvin et al., 1993; Sherwood et al., 1993), usually in different cells (see Lepretre et al., 1993). A new form of lamprey GnRH (referred to as lamprey GnRH-III) was sequenced after isolation from extracts of adult lamprey brains (Sower et al., 1993). Radioimmunoassay and HPLC data from extracts of brains of lampreys during their metamorphosis from larvae (ammocoetes) to adults suggest that the levels of lamprey GnRH-III (identified previously as a second GnRH-like molecule) may actually be higher than lamprey GnRH-I at early stages of development (Youson and Sower, 1991). The first series of experiments were conducted to characterize the anatomical distribution of lamprey GnRH-III in larval lamprey brains.

We used antisera preferentially directed against either lamprey GnRH-I or -III in immunocytochemical procedures (Tobet et al., 1995). Dense reaction product was seen in cell bodies in the rostral hypothalamus and preoptic area. Reaction product was also dense in fibers to and within the neurohypophysis, in addition to numerous fibers which projected caudally, beyond the neurohypophysis through the mesencephalon. The majority of immunoreactive GnRH was lamprey GnRH-III, and when lamprey GnRH-I was seen, it was in cells that appeared to contain both forms of GnRH. A small number of cells found in the caudal hypothalamus contained only immunoreactive lamprey GnRH-III, and these may constitute a functional subgroup within the population of GnRH neurons. In animals undergoing metamorphosis there was a large increase in reaction product in all GnRH containing cells and fibers. A striking change within the distribution of GnRH cells was localized to a distinct group of GnRH immunoreactive cells (GnRH-I and -III) in the ventral anterior hypothalamic area. These cells were minimally detectable in larvae, but during metamorphosis became densely filled with immunoreactive product in perikarya and distal processes.

The results are consistent with the hypothesis that lamprey GnRH-III is an important form of GnRH during the maturation of GnRH cells and fibers, and further indicates that these cells have attained their normal positions in the preoptic area and hypothalamus before metamorphosis.

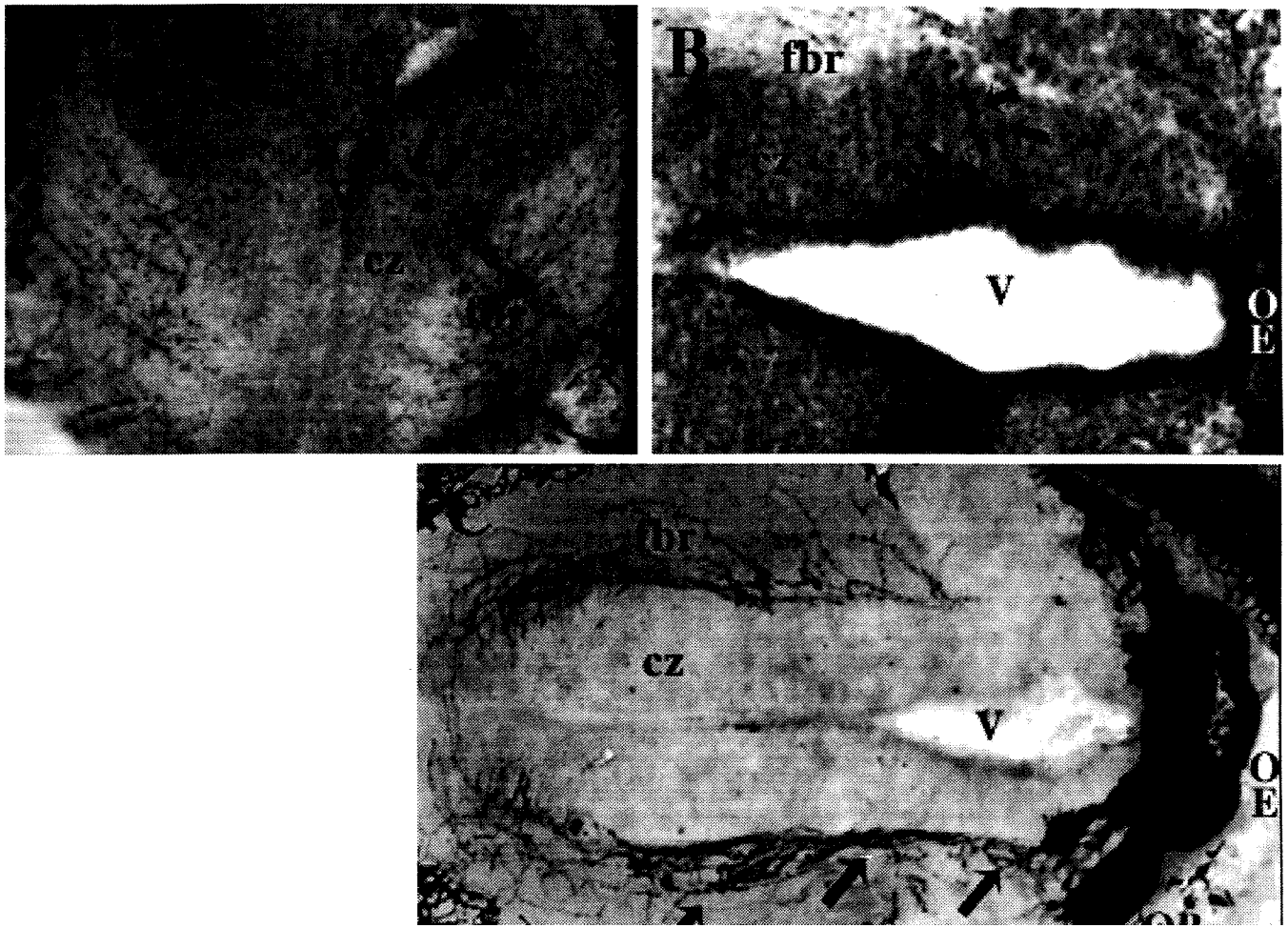
EXPERIMENTAL SERIES II:

The regulation of GnRH is intimately related to information from the olfactory system. Additionally, in the majority of vertebrates GnRH neurons are thought to be derived from progenitor cells in medial olfactory placodes (Muske, 1993). Our second series of experiments were conducted to characterize the earliest development of GnRH neurons in lamprey and determine their relationship to cells and fibers derived from the olfactory system.

Eggs from fertile adult sea lamprey were fertilized in the laboratory and larvae were maintained for up to 180 days. GnRH neurons were visualized within the lamprey preoptic area and hypothalamus as soon as GnRH was detectable (within 4 weeks after fertilization - see Figure 1A,B). The number of GnRH neurons increased slowly over the 100 days for which cells were counted. GnRH neurons were never seen within the olfactory system. The cells and fibers of the olfactory system were identified

olfactory fibers and GnRH containing fibers from prolarval stages to metamorphosis, olfactory stimuli may play a major role in the regulation of GnRH neuron development and secretion in lamprey.

Figure 1: Photomicrographs of coronal (A) and horizontal (B,C) sections from larval lamprey 33-42 days after fertilization. Arrows in A and B indicate the positions of cells containing immunoreactive lamprey GnRH-III. In coronal sections (A) immunoreactive cells were only noted within the dense medial cell zone (cz). In horizontal sections (B) immunoreactive cells were seen caudal to the olfactory bulb (OB) and medial to the lateral fiber rich region (fbr). At the same time, strong bundles of GS-1-B4 reactive fibers (panel C; black arrows) stretched caudally from the olfactory epithelia (OE) toward the preoptic area and hypothalamus by traveling outside of the dense medial cell zone. The surface of the olfactory bulb (OB) was also densely reactive. The magnification in B is twice as high as in C. Hb = habenula, V = ventricle.



placed lamprey into water containing α-methyl-galactoside, melibiose, fucose, the lectin GS-1-B4, or nothing extra. Lamprey were raised in this environment for 16-22 days and then immersed in BHS fixative. The following numbers are counts of neurons per brain containing immunoreactive lamprey GnRH-III in 36 and 42-day old lamprey; the number of animals is given in parentheses.

Control:	17.8 ± 3.2 (n=8)
α-methyl-galactoside (0.02M):	30.7 ± 3.4 (n=9)
melibiose (0.02M):	20.9 ± 4.3 (n=9)

The initial counts were striking; exposure to α-methyl-galactoside caused a 50% increase in neurons with immunoreactive GnRH. The processing of additional animals, and including data from lamprey treated with fucose or the lectin GS-1-B4, provided information that is still highly suggestive of treatment effects, but less encouraging for specificity. All carbohydrate treatments caused 3-fold increases in GnRH neuron numbers.

Control:	15.5 ± 7.5 (n=2)
α-methyl-galactoside (0.02M):	44.0 ± 13.5 (n=4)
α-methyl-galactoside (0.2M):	53.0 ± 10.0 (n=2)
Melibiose (0.02M):	50.5 ± 20.2 (n=4)
Fucose (0.02M):	54.0 ± 10.3 (n=4)
GS-1-B4 (80μg/100ml):	51.5 ± 4.1 (n=4)

An additional experiment was conducted to assess the potential of α-galactosidase enzyme treatments to influence olfactory carbohydrates. Using fixed tissue from 30-day-old prolarvae, sections were treated overnight with 250mU of green coffee bean α-galactosidase (Sigma Chemical Co.) or 500μl of the over the counter α-galactosidase derived from aspergillus niger, and sold under the trade name BEANO (AkPharma, Inc.). Reactions were run in 0.05M sodium citrate buffer, pH 5.0 at 37°C. Such treatments either eliminated or significantly reduced (respectively) the ability of GS-1-B4 to recognize elements of the lamprey olfactory system, obvious in the controls. This experiment suggests that it may be possible to design a live treatment paradigm with these enzymes, which could then potentially influence functions of the lamprey olfactory system.

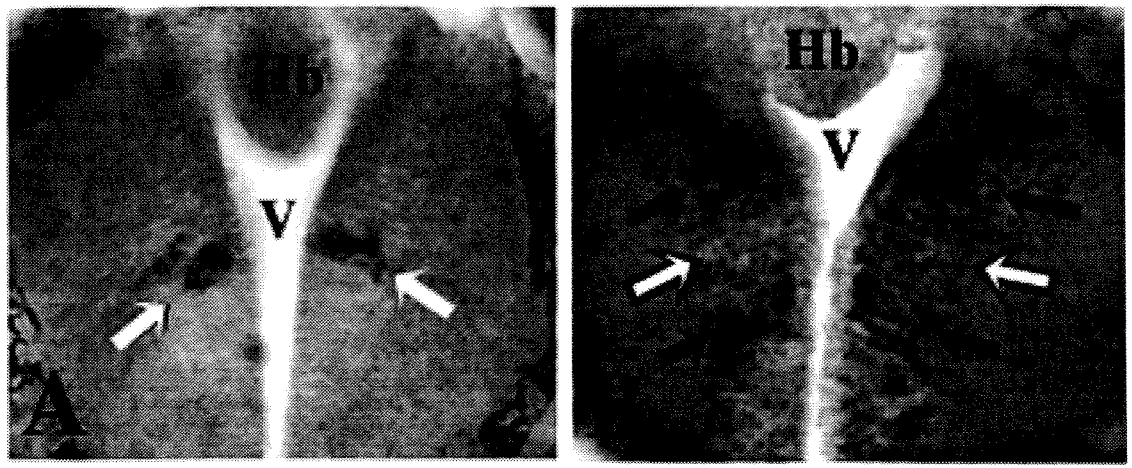
Our data are consistent with the hypothesis that environmental exposure to specific carbohydrates influences the development of the GnRH system in lamprey. Exogenous carbohydrate exposure may influence the olfactory system either by binding to receptors on the surface of olfactory neuroepithelia or by interfering (e.g., competitive inhibition) with endogenous interactions between α-galactose-linked glycoconjugates and the carbohydrate binding proteins with which they normally interact. Carbohydrate and binding protein interactions have been postulated to mediate axon development in the rodent olfactory system (Mahanthappa et al., 1994). Interestingly, in the rodent studies, the β-galactose analog thiodigalactoside was able to interfere with olfactory neuron adhesion in their studies carried out in vitro. Future experiments need to determine the full range of selectivity and the longevity of such effects in lamprey.

EXPERIMENTAL SERIES IV:

receptor stimulation on cell migration have been indicated in cerebral cortical and spinal cord neurons (Behar et al, 1994; Ma & Barker, 1995). In rats, in vivo perinatal administration of the GABA agonist muscimol (Bach et al, 1992) or the benzodiazepine receptor agonist diazepam (Perez-Laso et al, 1994) resulted in morphological changes in the preoptic area and accessory olfactory bulb, respectively. We examined the developmental relationship of GABA and GnRH neurons in prolarval lamprey to determine the potential for GABAergic influences on the developing lamprey GnRH system.

Cells containing immunoreactive GABA were found in the lamprey CNS at the earliest age examined, 10 days after fertilization (MacIntyre et al., 1995abstr; 1996abstr). Our initial impression was that the GABA cells were in the same region in which the GnRH neurons would appear 10-20 days later. After GnRH neurons began appearing, we compared the distribution of GABA cells to GnRH cells based on matching sections from the same aged animals. Unfortunately, double-label analyses were thwarted by technical difficulties of antisera and fixation requirements. Nonetheless, the matched section analysis suggests that GnRH neurons (Figure 2A) and GABA neurons (Figure 2B) are closely opposed, but segregated, from the earliest points in development for GnRH neurons. This close anatomical arrangement is similar to the rodent model in which they are also closely opposed early in development in the nasal cavity. Our ongoing rodent experiments suggest direct effects of GABA on GnRH neuron development. Future experiments are needed to test directly whether GABAergic stimulation or inhibition influences GnRH neuronal development in lamprey.

Figure 5: Photomicrographs of coronal (A,B) sections from prolarval lamprey (30 days after fertilization). White arrows in A indicate the positions of cells containing immunoreactive lamprey GnRH-III and black arrows in B and C indicate the position of cells containing immunoreactive GABA. The white/black arrows in B indicate positions where GnRH neurons would appear if the sections were double-labeled. The matched sections suggest a close relationship between GABA- and GnRH-containing neurons in the developing lamprey. Hb = habenula, V= ventricle.



FUTURE DIRECTIONS

For the GLFC, one long-term benefit of extending our basic knowledge of carbohydrate influences on GnRH development would be related to learning more about the contribution of indigenous plants to the carbohydrate content of various tributaries to the Great Lakes. It may very well be that particular streams are detrimental for lamprey reproduction due to the presence of specific plant (or other) carbohydrate contributions to local ecosystems. An alternative by-product of the carbohydrate findings in the olfactory system is the possibility that such carbohydrates are important for olfactory function. In this respect, specific carbohydrate signals may act as attractants or repellents within particular streams. The GLFC would again benefit again by determining if such mechanisms were already at work within particular tributaries of the Great Lakes.

Since no one has reported on the effects of treatment with GABAergic drugs on the developing reproductive axis of live animals, studies are needed in treating prolarvae with GABA itself, or the selective GABA agonists muscimol ($GABA_A$) and baclofen ($GABA_B$) beginning on day 10 after fertilization and examining the position of neurons containing immunoreactive GABA and GnRH in prolarvae after several weeks of treatment. The effects of agonists need to be compared with those of the selective antagonists such as bicuculline and picrotoxin. In the context of the Great Lakes one striking aspect of GABA function is the effect of organochlorine pesticides on $GABA_A$ receptors (Pomes et al., 1994). This class of environmental toxins may play a key role relative to the reproductive axis by influencing the GnRH neuronal system through GABA receptor-dependent events. Streams that contain significant levels of particular pesticides may be particularly advantageous or disadvantageous for lamprey reproduction. It would be a particular benefit to the GLFC if heavy pesticide presence was good for lamprey reproduction; then an effective "solution" would be in line with the goals of several agencies to remove such environmental hazards. As indicated above, more information about the plants of the Great Lakes region could be very useful, since they may contain significant GABAergic agents. For example, the GABA antagonist picrotoxin is derived from the seeds of *Anamirta cocculus* L., and is already known to be highly toxic to fish. Such factors, in addition to carbohydrates, may provide a scientific basis for existing differences in stream quality for lamprey development.

METHODS DEVELOPED DURING THE PROJECT

We localized GnRH containing cells in brains of lamprey ammocoetes and embryos using immunocytochemical procedures which we have optimized first for ammocoetes (Tobet et al., 1995) and subsequently for prolarval lamprey (Tobet et al., submitted). Briefly, lamprey heads are immersion-fixed in Bouin's-Hollande Sublimate, and the brains stored with or without 25% sucrose in 0.1M phosphate buffer. Vibratome sections are cut 30-40 microns thick, and GnRH containing neurons are visualized using Vectastain ABC procedures and Nickel-DAB reaction product.

We localized GABA (MacIntyre et al., 1995; 1996) and BrdU (Tobet et al., submitted) containing cells in brains of developing lamprey using immunocytochemical procedures which we have optimized for prolarval lamprey. In this case, lamprey are immersion-fixed in 2% acrolein, and the brains stored in 0.1M phosphate buffer. Again, Vibratome sections are cut 30-40 microns thick, and GABA or BrdU containing neurons are visualized using Vectastain ABC procedures and Nickel-

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