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The Spatial Relationship of γ-Aminobutyric Acid (GABA) Neurons and Gonadotropin-Releasing Hormone (GnRH) Neurons in Larval and Adult Sea Lamprey, *Petromyzon marinus*

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Key Words
GABA · Glutamic acid decarboxylase · Gonadotropin releasing hormone · Development · Lamprey

Abstract
In this study we examined the spatial relationship of GABA-containing and GnRH-containing neurons by immunocytochemistry and in situ hybridization in larval and adult brains of sea lamprey, *Petromyzon marinus*. In immunocytochemical studies, GABA-containing neurons were detected early in lamprey development, by day 20 post-fertilization. At this time point, one population of GABA-containing neurons was visualized in the hypothalamus and preoptic area, and another population was located in the olfactory bulb of the telencephalon. By day 30 after fertilization, after the GABA neurons were detected, GnRH-containing neurons were visualized in the preoptic area/rostral hypothalamus region, adjacent to the GABA-containing neurons in the wall of the third ventricle. Similarly, in adult lamprey brains distinct populations of both GABA- and GnRH-containing neurons were located in the hypothalamus adjacent to the third ventricle. To further establish a proximate relationship between GABA and GnRH, the mRNA for glutamate decarboxylase (GAD), the enzyme catalyzing GABA synthesis from glutamate, and GnRH were examined by in situ hybridization in the brains of larval lamprey. These studies also showed that GnRH and GAD are produced in cell populations in and around the third ventricle of the hypothalamus. This close spatial relationship of GABA neurons and GnRH neurons provides a basis for a regulatory role of GABA on GnRH neurons in the sea lamprey.

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Introduction

The distribution of the amino-acid neurotransmitter γ-aminobutyric acid (GABA) in neurons in the brain and central nervous system has been examined in adult animals representing several classes of vertebrates. In the vertebrate species examined, GABA neurons were shown to be distributed throughout the brain and have been found consistently in the preoptic area of the hypothalamus and in the olfactory bulbs of the telencephalon [Domenici et al., 1988; Franzoni and Morino, 1989; Martinoli

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et al., 1990; Bennis et al., 1991; Lauder, 1993; Medina et al., 1994; Barale et al., 1996]. A few studies have examined the spatial relationship of GABA-containing neurons to regions of the brain containing gonadotropin-releasing hormone (GnRH or LHRH) neurons. In the rat, GABAergic axons synapse on LHRH neurons found in the preoptic area [Leranth et al., 1985]. In teleosts, GABA-containing cell bodies were found in the preoptic area and tuberal regions of the hypothalamus [Martinoli et al., 1990], and studies suggest that GABA is important in the early development of the teleost central nervous system [Ekström and Ohlin, 1995; Doldan et al., 1999]. Furthermore, direct innervation of the neurohypophysis and adenohypophysis by GABA neurons has been demonstrated in goldfish [Kah et al., 1987]. However, a limited number of studies examining the distribution and function of GABA as related to GnRH have been completed in fish, and none to date in lampreys. Lampreys are of particular importance in understanding endocrinological relationships since they represent the oldest lineages of extant vertebrates which evolved over 550 million years ago. Thus, these studies are the first to examine the relation of GABA and GnRH in an early-evolved vertebrate.

GABA is considered the primary inhibitory neurotransmitter in the central nervous system across all vertebrates, although its action can be excitatory if chloride concentrations are higher inside target cells than outside [Cherubini and Conti, 2001]. GABA plays an important role in the regulation of GnRH and gonadotropin (GTH) release in vertebrates [Kah et al., 1992; Sloley et al., 1992; Trudeau et al., 1993a, b, c, 2000a]. Gonadotropins (luteinizing hormone, LH, and follicle stimulating hormone, FSH) in response to GnRH are released from the pituitary gland and are the major hormones regulating steroidogenesis and gametogenesis. In teleosts, it is now generally accepted that there are two gonadotropins, GTH-I (which is FSH-like) and GTH-II (which is LH-like). The majority of studies investigating the role of GABA on GnRH and GTH function have been conducted in mammals [Flugge et al., 1986; Bergen et al., 1991; Jarry et al., 1991; Favit et al., 1993]. As an example, neurons containing GnRH expressed receptors for, and were influenced by, the action of GABA [Herbison, 1998]. In another study it was shown that GABA modulated the release of GTH from the anterior pituitary through direct inhibitory influence on GnRH secretion and regulation of adrenergic and noradrenergic systems involved in GnRH secretion [McCann et al., 1984; Adler and Crowley, 1986].

Relatively few studies have been conducted that examine the distribution and function of the neurotransmitter GABA in fish species. Some teleosts in which GABA has been studied include the adult goldfish, rainbow trout, silver eel, red seabream and deep sea armed grenadier [Martinoli et al., 1990; Trudeau et al., 1993a, b, 2000a, b; Medina et al., 1994; Mananos et al., 1999; Senthilkumaran et al., 2001]. In these studies, GABA, and agents that alter GABAergic function, significantly influenced reproductive processes by their actions on the hypothalamus and pituitary. A few studies have described GABA distribution in various parts of the agnathan nervous system, such as the retina [Rio et al., 1993, 1996; Anadón et al., 1998; Meléndez -Ferro et al., 2002], spinal cord or brainstem motor systems [Batueva et al., 1990; Brodin et al., 1990; Meléndez -Ferro et al., 2000], and olfactory system [Meléndez-Ferro et al., 2001]. No studies have been done to indicate a role of GABA in regulating reproductive processes in lampreys.

Studies have been conducted in mice, rats and humans that show a significant developmental, spatial and functional relationship between GABA and GnRH neurons [Tobet et al., 1996a; Wray et al., 1996; Fueshko et al., 1998; Bless et al., 2000]. Equivalent studies have not been reported in fish. GABA-containing neurons have been visualized along the migratory pathway of GnRH neurons originating in the olfactory placode of embryonic mice, rats and humans [Tobet et al., 1996a]. In addition, GnRH and GABA were shown in this same study to be co-expressed in neurons originating in the olfactory placode of rats and mice. In a more recent study it was shown that GnRH migration along vomeronasal fibers could be inhibited with a $GABA_A$ receptor agonist, whereas treatment of mice with a $GABA_A$ receptor antagonist led to a highly disorganized distribution of GnRH cells [Bless et al., 2000]. $GABA_A$ receptor has been shown to be the primary receptor involved in GnRH regulation [Mitsushima et al., 1994, 1996; Feleder et al., 1996].

The development of GnRH-containing neurons has been examined in prolarval and larval sea lampreys [Tobet et al., 1995, 1996b]. In these studies, GnRH neurons were visualized in the preoptic area and the hypothalamus 22 days after fertilization. In contrast to other vertebrates, GnRH neurons in the developing sea lamprey were considered not to originate from the olfactory placode, but rather within the proliferative zones of the diencephalon [Tobet et al., 1996b]. Therefore, the objective of the present study was to examine GABA-containing neurons in the brains of larval and adult sea lamprey to evaluate the potential for GABA neurons to influence GnRH neurons.

Materials and Methods

Animals

Adult sea lampreys were collected from the Cocheco River in Dover, New Hampshire, transported to the Anadromous Fish and Aquatic Invertebrate Research Laboratory (AFAIR Lab) at the University of New Hampshire, and maintained in a fresh water, flow-through system supplied with ambient local reservoir water, under natural photoperiod. The maintenance and use of lampreys adhered to guidelines established by the Animal Care and Laboratory Use Committee of the University of New Hampshire.

Embryonic and pro-larval stage sea lampreys were obtained by in vitro fertilization. Eggs were stripped from mature female sea lampreys and fertilized with sperm from mature males, in large petri dishes. Successfully fertilized eggs, as determined by their ability to adhere to the bottom of the petri dishes, were transferred to glass aquaria containing sand and well water at 18°C. The larval lampreys' development was staged following the descriptions of Piavis [1961]. Embryos hatch between days 10 and 13 at 3–5 mm in length. The pro-larval stage lasts from hatching until approximately day 40 when all traces of yolk are eliminated. Lampreys are referred to as larvae or ammocoetes from day 40 until metamorphosis, which generally occurs after 5–7 years of larval life. Larval-stage lampreys, approximately 50–150 mm in length, were collected by electrofishing streams in southern New Hampshire. The larval lampreys were maintained in the same manner as the embryonic/prolarval stage lampreys.

The animals were sacrificed by decapitation. The brains of the adult lampreys and whole heads from the developing lampreys were fixed in either 2% acrolein for immunocytochemistry [King et al., 1988], or 4% paraformaldehyde (PFA)/0.1 *M* phosphate buffer (PB) for in situ hybridization, pH 7.4 at room temperature (RT). The tissues were fixed overnight before transfer to 25% sucrose (w/v), 0.1 *M* PB for storage at 4°C. Eight to ten lampreys were utilized for each stage (prolarval days 10, 20, 30 and 40, larval and adult) for immunocytochemistry and 8 animals were used for the in situ hybridization experiments.

Antibodies for Immunocytochemistry

Previously characterized antisera directed against lamprey GnRH-III, antiserum 3952 [Sower et al., 1993; Tobet et al., 1995], and lamprey GnRH-I, 1467 [King et al., 1988], were used at dilutions of 1/10,000 and 1/2,000, respectively. The lamprey GnRH-III antisera, 3952 was used in the pro-larval lamprey studies, whereas 3952 and antisera to lamprey GnRH-I, 1467, were used in the adult lamprey studies. Three different antisera directed against GABA were used to provide an additional control for non-specific immunoreactions. One was a rabbit polyclonal antiserum obtained from DiaSorin Corp. (Stillwater, MN), and is the antisera used to generate the results presented herein. The other two were guinea pig polyclonal antisera obtained from Chemicon International, Inc. (Temecula, CA) and Eugene Tech International (Ridgefield Park, NJ). The rabbit polyclonal antiserum was optimized at 1/40,000 and the guinea pig antisera at 1/1,000 [Lauder et al., 1986; Tobet et al., 1996b]. Comparison of results using each antiserum under its optimal conditions yielded similar results supporting the accuracy of the localization of GABA. Antisera were diluted in 1.0% BSA/0.05 *M* phosphate buffered saline (PBS)/0.1% Triton X-100 (pH 7.4). Negative controls included omission of primary antisera and preabsorption of the antisera with 20 μ*M* lamprey GnRH-III (generously supplied by R. Doolittle) or lamprey GnRH-I peptide (Peninsula Laboratories, Belmont, Calif., USA).

Immunocytochemistry

The fixed whole heads of individual pro-larval lampreys, 10–40 days old, or brains from adult lampreys were embedded in 5% agarose gel. Forty-micrometer coronal, horizontal or sagittal sections were sliced into cold 0.05 *M* PBS using a Vibratome. Sections from each lamprey were placed into alternating plastic containers to allow us to compare matched sections for GABA and GnRH; the containers had nitex mesh bottoms to facilitate the changing of solutions. Histochemical procedures for GnRH and GABA experiments were adapted from procedures previously described [Lauder et al., 1986; Tobet et al., 1996a, b]. Acrolein-fixed sections were washed in 0.05 *M* PBS followed by a 1/2 h treatment in 0.1 *M* glycine and another wash with 0.05 *M* PBS. Sections were then treated with sodium borohydride, followed by a wash in 0.05 *M* PBS. Next the sections were blocked in 5.0% normal goat serum (NGS) with 1.0% hydrogen peroxide followed by the Vector avidin/biotin blocking reagents. Tissues were incubated in the primary antisera for GnRH or GABA overnight at 4°C. The following day, the tissues were washed with PBS containing 1% NGS and 0.02% Triton X-100 followed by a 2 h incubation with the secondary antisera (goat anti-rabbit biotinylated secondary antibodies from Vector Laboratories, Burlingame, CA, or donkey anti-guinea pig biotinylated secondary antibodies from Jackson Immunoresearch, West Grove, PA) in NGS/PBS, as appropriate. After another wash in PBS containing 0.02% Triton X-100, the sections were incubated with Vectastain ABC reagent (Vector Laboratories) for 1 h. Additional washes used in TRIS-buffered-saline (TBS; 0.05 *M*, pH7.4, @RT). A dark gray reaction product was produced using 0.025% 3,3-diaminobenzidine with 0.2% nickel ammonium sulfate in TBS as substrate with 0.02% hydrogen peroxide for 5 min.

In situ Hybridization

Tissues fixed in 4% PFA were embedded in OCT compound and 14-μm sections were cut on a cryostat (Reichert-Jung, Leica Instruments, Heidelberg, Germany) at –22°C and mounted onto vectabond coated slides (Vector Laboratories). Sections were probed with either digoxigenin (Dig)-labeled ribo- or DNA oligonucleotide probes. Dig-labeled riboprobes were prepared by in vitro transcription of a 300 base pair, linearized cDNA template of lamprey GnRH-III [Suzuki et al., 2000] in the presence of Dig-labeled uridine triphosphate (UTP) and unlabeled nucleotides. Oligonucleotides (25-mers) to lamprey GAD (Antisense: CGAGTGTAGTCACTA; Sense: TAGTGACTACACTCG; Accession AF432157) were labeled by tailing with Dig-dUTP and cold dATP by terminal transferase [Schmitz et al., 1991]. A simplified in situ hybridization protocol was used to detect GnRH and GAD mRNAs in tissue sections [Braissant and Wahli, 1998]. Briefly, following pretreatment, the sections were prehybridized for 2 h at 58°C with a solution containing 50% formamide, 5× SSC (0.75 *M* NaCl, 0.75 *M* Na-Citrate) and 100 μg/ml yeast tRNA. Sections were then hybridized in the same solution containing probes that had been denatured for 5 min at 80°C (either 0.5 μg/ml riboprobe or 0.1 μg/ml oligoprobe), and incubated overnight at 58°C in a box saturated with a 5× SSC-50% formamide solution to avoid evaporation. The next day sections were vigorously washed and incubated for 2 h with AP-coupled Dig antibody (Roche Molecular Biochemicals, Mannheim, Germany) diluted 1:5,000 in Dig Buffer 1 (100 m*M* Tris, pH 7.5; 150 m*M* NaCl) at RT. To visual-

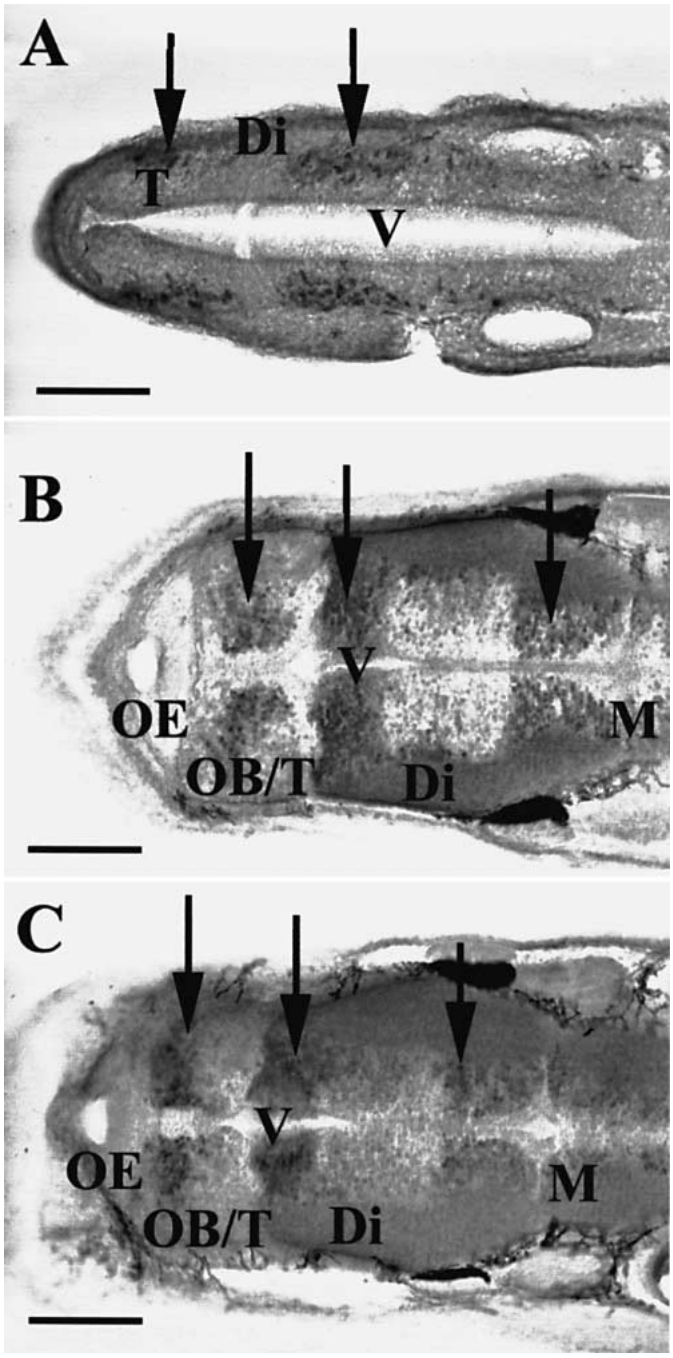

Fig. 1. Photomicrographs of horizontal sections from larval lamprey 10 (A), 20 (B), and 30 (C) days post-fertilization. Populations of GABA immunoreactive cells, using rabbit polyclonal antiserum, were present in the telencephalon/olfactory bulb region, the preoptic area/rostral hypothalamus and the midbrain. Black arrows indicate the location of immunoreactive GABA cell populations. Di = diencephalon, M = midbrain, OB = olfactory bulb, T = telencephalon, OE = olfactory epithelium, V = ventricle. Scale bars = 100 μm.

ize hybridization products, the sections were incubated overnight in boxes, as previously described, with Dig Buffer 2 (100 m*M* Tris, pH 9.5, 100 m*M* NaCl, 50 m*M* $MgCl_2$) containing 20 μl/ml NBT/BCIP (Roche Molecular Biochemicals, Mannheim, Germany). On the third day sections were rinsed, dehydrated through successive baths of EtOH (70, 95 and 100%) and mounted to glass slides with Permount (Fisher Scientific, Pittsburgh, Pa.).

Results

Immunocytochemistry

Cells and fibers containing immunoreactive (ir) GnRH and GABA were detected in the brains of all pro-larval and adult lampreys. GABA-immunoreactive cells were apparent in several brain regions by day 20 after fertilization, prior to the appearance of GnRH-ir neurons. One population of GABA-containing neurons was visible within the olfactory bulb region of the telencephalon, and another population was evident in the rostral preoptic area of the diencephalon (fig. 1A). On days 30 and 40 after fertilization these two populations of GABA-ir cells were still present and a third population of GABA-ir cells appeared in the midbrain (fig. 1B, C). All the antisera (rabbit polyclonal and guinea pig polyclonal) directed against GABA utilized in this study revealed identical patterns of immunoreactivity, thus confirming our results. However, only the results obtained using the rabbit polyclonal antisera are presented.

In agreement with previous results [Tobet et al., 1996b], the GnRH-ir neurons (GnRH-III) were apparent by day 30 after fertilization in the rostral preoptic area, adjacent to the third ventricle, with GnRH-ir fibers extending into a lateral fiber-rich region (fig. 2). Sections from lampreys 30 and 40 days after fertilization were processed for GABA immunochemistry and matched in the coronal plane with those processed separately for GnRH immunocytochemistry. Although the GnRH-ir neurons were found in the same regions as the GABA-ir neurons, they still appeared to comprise distinct populations. GABA-ir neurons appeared to surround GnRH-ir neurons (fig. 2, 3). The morphology of cells containing immunoreactive GABA was rounder than for those containing immunoreactive GnRH further suggesting that they were different cells. Adult lamprey brains were also processed in the coronal plane for immunoreactivity to GnRH-I, GnRH-III and GABA (fig. 4). Both GABA-ir and GnRH-ir cells were located in or near the walls of rostral regions of the third ventricle. GnRH-ir fibers extended caudally from the third ventricle to the midbrain and neurohypophysis. The GnRH-I-ir and GnRH-III-ir cells comprised

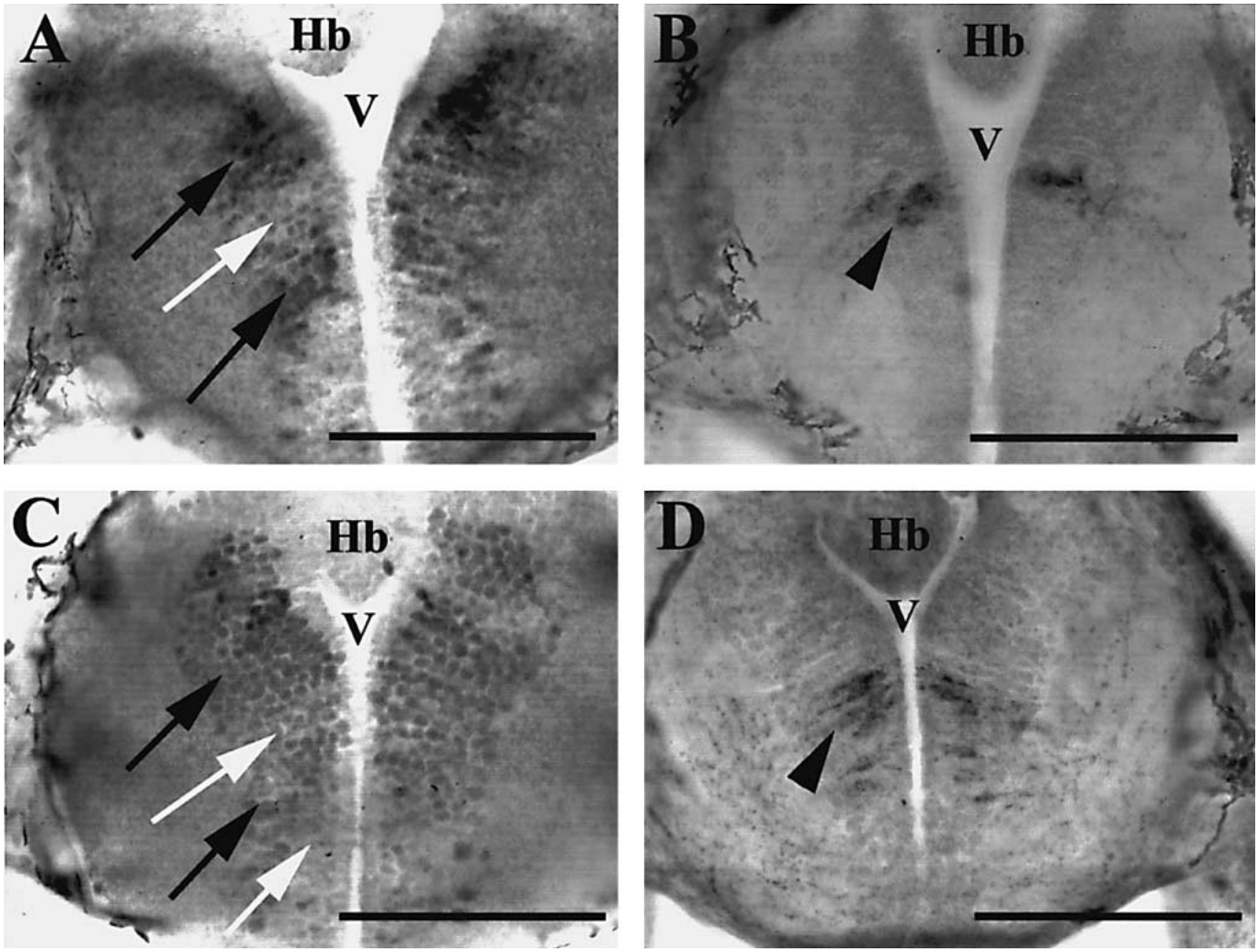

Fig. 2. Photomicrographs of coronal sections from larval lamprey 30 (A, B) and 40 (C, D) days post-fertilization, matched for GABA and GnRH immunoreactivity. Plates A (day 30) and C (day 40) were processed for GABA immunoreactivity (rabbit polyclonal antiserum) and matched with plates B (day 30) and D (day 40) which were processed for lamprey GnRH-III (Ab 3952) immunoreactivity. Black arrows indicate cells containing immunoreactive GABA, arrowheads indicate GnRH immunoreactive cells in the preoptic area/rostral hypothalamus, and white arrows indicate the approximate positions of lamprey GnRH cells. Hb = habenula, V = ventricle. Scale bars = 100 μm.

overlapping populations and will be referred to as GnRH-ir cells. In coronal sections, the majority of GABA-ir cells were noted in regions dorsal and ventral to GnRH-ir neurons. Additionally, several GABA-ir cells appeared interspersed in the region of GnRH neurons. In horizontal sections of adult lamprey brains processed for immunoreactivity to GnRH-I and GABA (fig. 5), and to GnRH-III (data not shown), GABA-ir cells were noted in regions rostral to GnRH-ir neurons as well as interspersed in the region of GnRH neurons. Similar to the larval lamprey, the morphologies of GnRH-ir and GABA-ir were mostly different. Occasional cells displaying the same shape might indicate that in adults there may be a very small subpopulation of cells reactive for both GABA and GnRH. Immunoreactive GnRH-I fibers coursed away from the third ventricle towards the midbrain and hypothalamus. It was impossible to trace the trajectories of GABA-ir fibers as they merged into the surrounding neuropil.

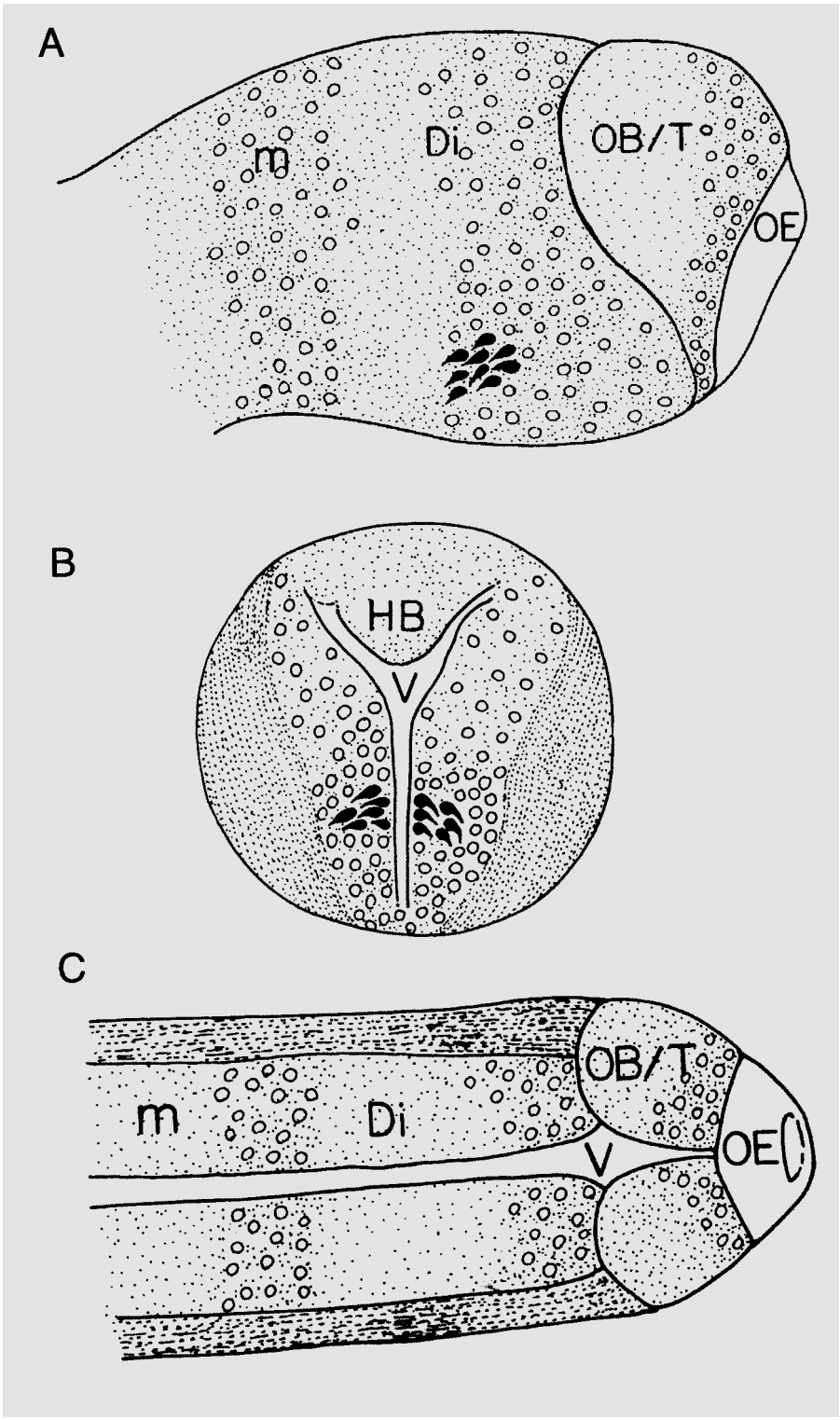

Fig. 3. Schematic diagrams of sagittal (A), coronal (B), and horizontal (C) sections illustrate the positions of GABA and lamprey GnRH immunoreactive cells in the heads and brains of larval sea lamprey (days 30 or 40; fig. 1 and 2). Round circles represent GABA immunoreactive cells and filled teardrop shapes represent lamprey GnRH immunoreactive cells within the preoptic area/rostral hypothalamus. The horizontal plane chosen (C) shows the different rostral-caudal populations for cells containing immunoreactive GABA and is located dorsal to the region containing GnRH neurons. HB = habenula, Di = diencephalon, m = midbrain, OB/T = olfactory bulb/telencephalon, OE = olfactory epithelium, V = ventricle.

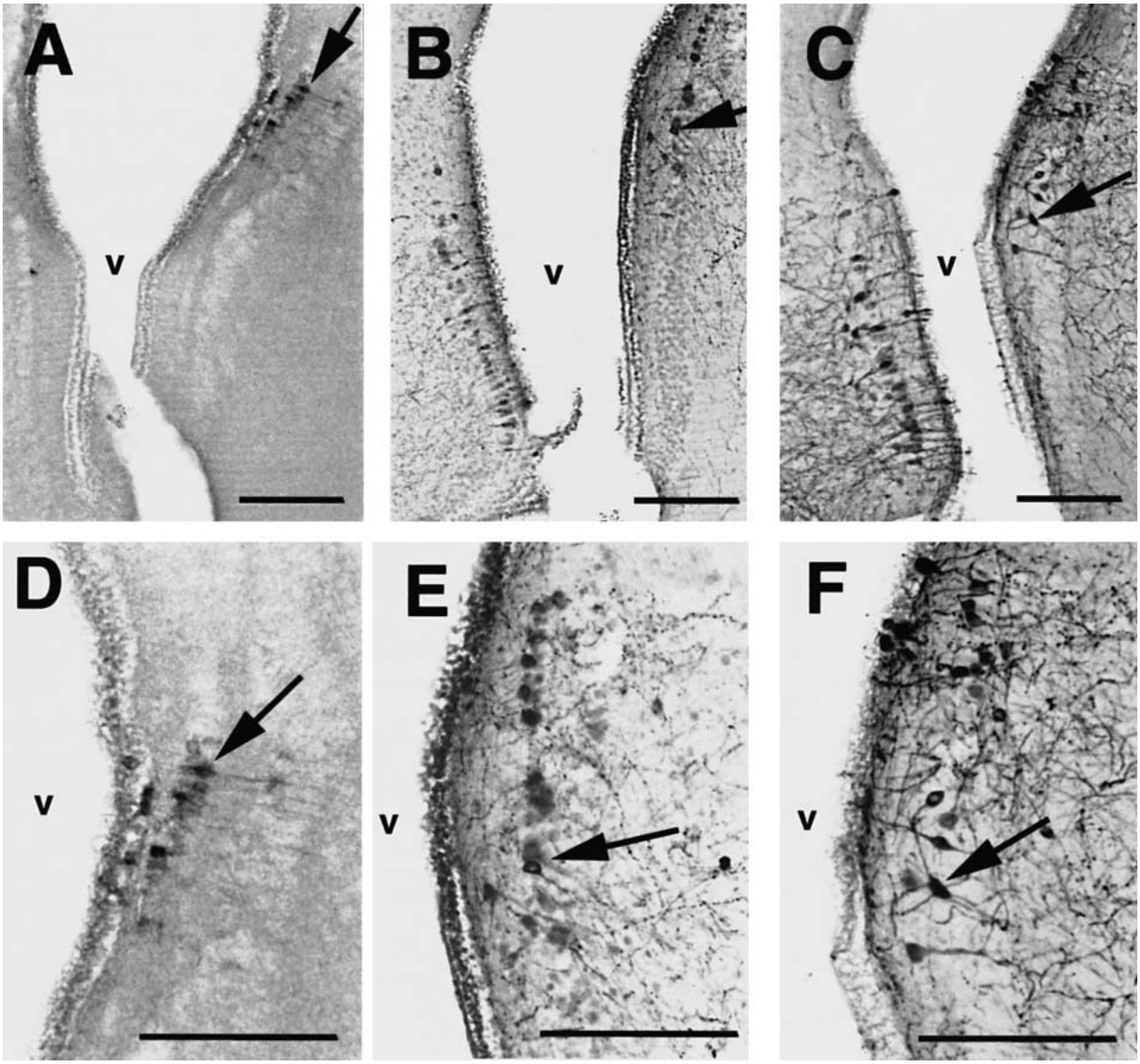

Fig. 4. Photomicrographs of consecutive coronal sections from adult lamprey showing distinct populations of cells immunoreactive for GABA, using rabbit polyclonal antiserum (A, D), GnRH-III, Ab 3952, preabsorbed with GnRH-I peptide (B, E) and GnRH-I, Ab 1467 (C, F). The immunoreactive cells are located in the hypothalamus adjacent to the third ventricle, and the GABA cell populations are positioned immediately anterior to both GnRH-I and GnRH-III cell populations. The immunoreactive cells shown in (D, E, F) are taken from sections shown at lower magnification (A, B, C). The solid arrows indicate the locations from which the higher magnification photomicrographs were taken. V = ventricle. Scale bars = 100 μm.

In situ Hybridization

To further corroborate a relationship between GABA and GnRH, GAD and GnRH mRNA expression was examined by in situ hybridization in the brains of larval lamprey. GnRH-III and GAD transcripts were localized in sagittal sections of ammocoete brains (fig. 6) by in situ hybridization with either antisense GnRH-III riboprobe or GAD oligoprobe. Cells containing GnRH-III or GAD mRNA were detected based on a purple reaction product. Similar to the results obtained by immunocytochemistry,

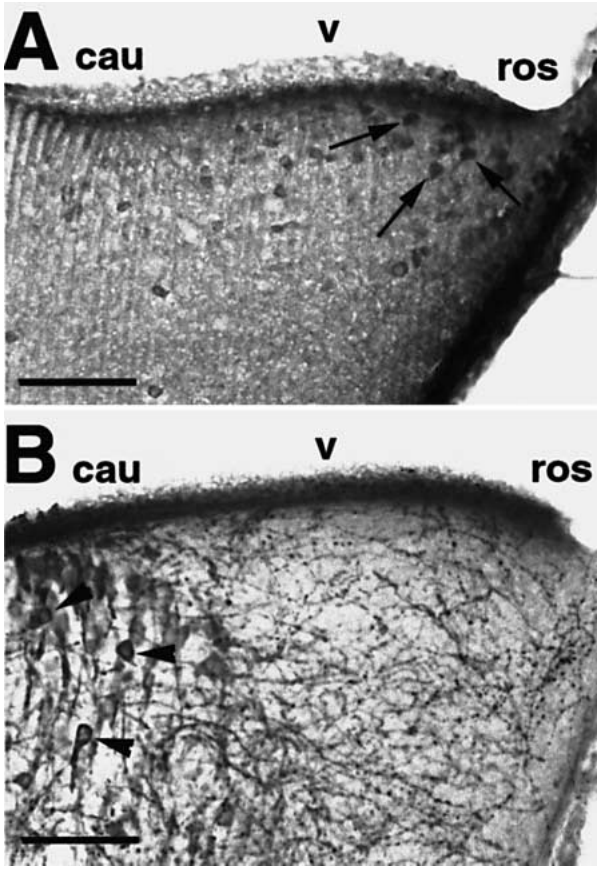

Fig. 5. Photomicrographs of consecutive horizontal sections from adult lamprey showing distinct populations of cells immunoreactive for GABA, using rabbit polyclonal antiserum (A) and lamprey GnRH-I, Ab 1467 (B). The GABA immunoreactive cells, located in the hypothalamus adjacent to the third ventricle, are rostral to the GnRH-I immunoreactive cell populations. cau = caudal, ros = rostral, V = ventricle. Scale bars = 100 μm.

GnRH and GAD mRNA was present in cell populations in and around the third ventricle of the hypothalamus. Cells in adjacent sections that were hybridized with either sense GnRH-III riboprobe or excess unlabeled GAD oligoprobe, as negative controls, were not stained purple, indicating the specificity of the probes.

Discussion

In this study we examined the topographic distribution of GABA- and GnRH-containing cells in the brains of developing and adult sea lamprey. In the prolarval sea lamprey, distinct populations of GABA-containing cells were visible in the forebrain by 20 days after fertilization. These GABA-containing cells occurred throughout the olfactory bulb region of the telencephalon and in the diencephalon, particularly in the periventricular region of the rostral preoptic area. The GABAergic cells remained distributed in these separate populations throughout the lamprey prolarval developmental stages. In the adult sea lamprey, GABAergic elements appeared ubiquitous throughout the brain making cell body of origins difficult to discern. Nonetheless, cell bodies were discernible in the rostral hypothalamus. The distribution of cells containing GABA was then compared to that of GnRH cells using brain sections matched for coronal or horizontal planes within the diencephalon from both larval and adult lamprey. The GnRH-containing cells were found in the same distribution as described previously [Tobet et al., 1996b], with GnRH-containing cells arising in the rostral diencephalon after 20 days of development. This study demonstrated that in the lamprey, GABA-containing cells are discernable earlier in development than GnRH-containing cells. The GABA-containing cells were first visualized in the hypothalamus at 10–20 days after fertilization, whereas GnRH appeared in the same region as GABA between 20 and 30 days after fertilization. The matched section analysis in the current study suggests that GABA and GnRH cell populations in the rostral hypothalamus and preoptic area are closely apposed, yet likely distinct.

To further establish a proximate relationship between GABA and GnRH, GAD and GnRH mRNA expression were examined by in situ hybridization in the brains of larval lamprey, thus providing the first GAD expression data for agnatha. Similar to the results obtained by immunocytochemistry, GnRH and GAD mRNA were present in cell populations in and around the third ventricle of the hypothalamus. If the close proximity of these elements in the developing and adult hypothalamus provides for specific neural communication, then there is the potential for a regulatory role for GABA on GnRH neuronal development and reproductive function in the lamprey.

GABA is one of the earliest neurotransmitters to appear in the brain during development [Lauder et al., 1986; Roberts et al., 1987; Barale et al., 1996; Anadón et al., 1998], thus it is not surprising that GABA might affect GnRH neuronal development in the lamprey. Studies in the early zebra fish *(Danio rerio)* demonstrated that GABA was distributed in discrete brain regions during development [Doldan et al., 1999]. Another study also described the developmentally dependent appearance of GABA-ir neurons in the early brain of the teleost, *Gasterosteus aculeatus* L.; GABA appeared to be expressed in

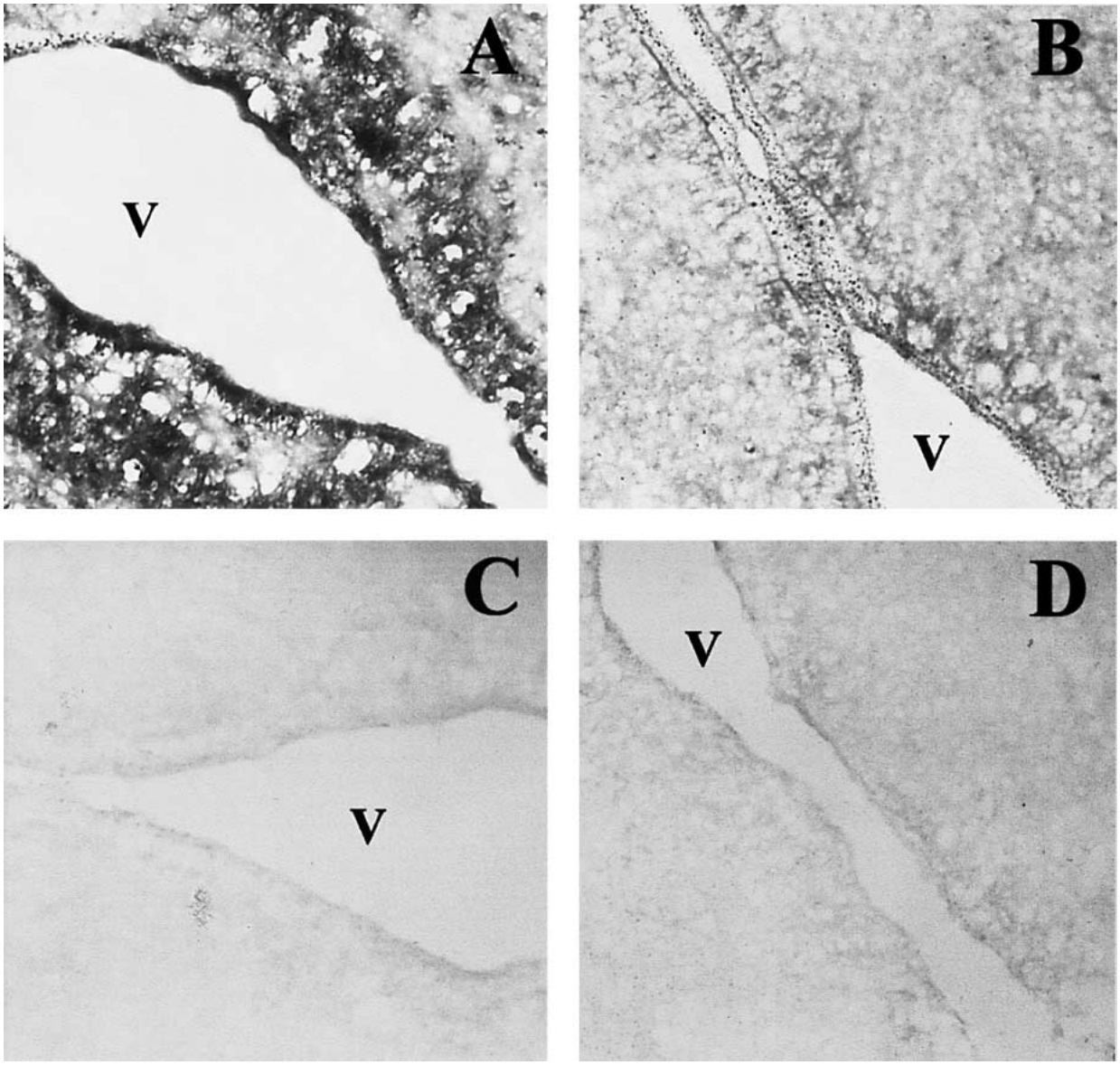

Fig. 6. Localization of GnRH and GAD transcripts in ammocoete brains by in situ hybridization. Coronal sections were hybridized with either Dig-labeled GnRH-I transcribed riboprobe (A, C) or GAD oligoprobe (B, D). The sections in A and B were hybridized with antisense probes, while C and D were hybridized with sense and excess unlabeled oligo-probe, respectively. The GnRH-I and GAD mRNA-containing cells are both located in the hypothalamus near the third ventricle (V). Magnification = 100×.

the first differentiated neuronal populations of the teleost brain [Ekström and Ohlin, 1995]. In the *Xenopus laevis* tadpole, GABA was found in the prosencephalon along the prosencephalic vesicle, and in the ventral thalamus and the hypothalamus early in embryonic development [Barale et al., 1996]. In rats, a population of GABAergic neurons was found in the diencephalon, including the hypothalamus, early in development [Lauder et al., 1986; Tobet et al., 1997]. In teleosts, GABA neurons emerge early in development within the rostral prosencephalon [Ekström and Ohlin, 1995; Doldan et al., 1999]. In Amphioxus, GABA-ir cells were localized caudal to the infun-

dibular organ, which is thought to correspond to the GABA-ir fibers observed in the ventral hindbrain of *Xenopus* embryos [Roberts et al., 1987; Anadón et al., 1998]. Taken together with the results obtained in the present study, it is suggested that the early establishment and development of GABAergic systems appears to be a phylogenetically old developmental pattern. The early appearance of GABA could be due to its suggested dual role as a trophic factor as well as a neurotransmitter [Lauder, 1993].

Glutamate decarboxylase or GAD catalyzes the α-decarboxylation of l-glutamic acid to produce GABA. GAD has recently been cloned from the brains of several species including the zebra finch *(Taeniopygia guttata)*, turtle *(Trachemys scripta)*, goldfish *(Carassius auratus)*, zebrafish *(Danio rerio)* and armored grenadier *(Coryphaenoides armatus)* [Bosma et al., 1999]. GADs have recently been cloned from representatives of all major vertebrate classes [Trudeau et al., 2000a; Lariviere et al., 2002]. The presence of both GAD65 and GAD67 in rays, teleosts and tetrapods suggests that an early gene duplication event gave rise to a least two GADs before the emergence of the Chondrichthyes more than 400 million years ago. A single divergent GAD from both *P. marinus* and one hagfish species has also been identified. Although most similar to GAD67, these agnathan GADs do not consistently group with either GAD65 or GAD67 in our molecular phylogenetic analysis [Lariviere et al., 2002]. The existence of other GAD forms in agnathans is not known. In situ hybridization studies for GAD allow for the site of actual GABA production to be determined. In one study, the cellular distribution of GAD was examined in mid-gestation mouse embryos by in situ hybridization, and it was determined that the embryonic location of GAD in the brain developed into regions with strong GABAergic contribution [Katarova et al., 2000]. The authors concluded that GAD mRNA are translated efficiently into enzymatically active GAD which produces GABA, and that the pattern of GAD expression is consistent with the notion that GAD and its product, GABA, play a signaling role during development. In another study, utilizing the polymerase chain reaction, it was shown that the hypothalamic expression of GAD 65 was significantly greater in the male deep sea armed grenadier *(Coryphaenoides (Nematonurus) armatus)* than in the female indicating that the synthesis of GABA is sexually dimorphic in this species [Trudeau et al., 2000b]. In the present study we demonstrated, by in situ hybridization, that in larval lamprey, both GABA and GAD were present in the same population of cells. These results are similar to those obtained in the African lungfish *(Protopterus annectans)* by analysis of GAD using specific antibodies [Trabucchi et al., 2000]. In this species, as in the lamprey, populations of immunoreactive cells were particularly abundant in the periventricular preoptic nucleus and the hypothalamic region of the diencephalon.

In most vertebrates, a population of GnRH neurons originates in the olfactory placode [Muske, 1993]. In rats, mice and humans, some cells containing GABA were found in the nasal compartment along the migration route of GnRH neurons and likely also originated in the olfactory placode [Tobet et al., 1996a]. In the sea lamprey, in contrast to other vertebrates, GnRH neurons might not originate in the olfactory placode, but instead in the cell dense proliferative zone of the diencephalon [Tobet et al., 1996b, 1997]. Similarly, in the present study neither GABA-ir nor GnRH-ir was detected in the olfactory placode, which is consistent with a non-placodal origin for both cell types. Nonetheless, it remains possible that the GABA-ir and GnRH-ir cells originate in the lamprey olfactory placode, migrate from the site of origin, and do not express these molecules until they are distant from their site of origin.

If the GABA-containing cells defined in the current study communicate with the GnRH-containing cells that we found in close proximity then this could provide a mechanism for GABA in influencing the development and establishment of GnRH cell populations in the sea lamprey. The physiological role of GABA, in relation to GnRH, at any stage during the life cycle of the sea lamprey is presently undetermined. In mice, it has been shown that GABA influences the development of the GnRH system [Fueshko et al., 1998; Bless et al., 2000]. GABA is transiently expressed during development [von Bartheld and Rubel, 1989; Barale et al., 1996; Tobet et al., 1996a], and in explants from embryonic mice, synaptic input from GABAergic cells caused spontaneous activity in GnRH neurons [Kusano et al., 1995]. The published results in other species suggest that GnRH neurons possess GABA receptors and are responsive to GABA early in development.

In conclusion, this study has shown that GABA is present early in the development of the prolarval sea lamprey as well as in the larval and adult stage lamprey brains. The GABA cells clustered in several distinct populations within the forebrain. One of the populations of GABA cells, in the rostral hypothalamus/preoptic area, was closely apposed to GnRH cells in the same region. The results from this study are consistent with the hypothesis that GABA influences the development and function

of GnRH neurons in the sea lamprey. In addition, it is suggested that the early establishment and development of GABAergic systems within the lamprey brain, particularly the forebrain, appears to be a phylogenetically ancient pattern.

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References

Adler, B.A., and W.R. Crowley (1986) Evidence for gamma-aminobutyric acid modulation of ovarian hormonal effects on luteinizing hormone secretion and hypothalamic catecholamine activity in the female rat. Endocrinology, *118:* 91–97.

Anadón, R., F. Adrio, and I. Rodriguez-Moldes (1998) Distribution of GABA immunoreactivity in the central and peripheral nervous system of amphioxus *(Branchiostoma lanceolatum Pallas)*. J. Comp. Neurol., *401:* 293–307.

Anadón, R., M. Meléndez-Ferro, E. Pérez-Costas, M. A. Pombal, and M. C. Rodicio (1998) Centrifugal fibers are the only GABAergic structures of the retina of the larval sea lamprey: an immunocytochemical study. Brain Res., *728:* 297–302.

Barale, E., A. Fasolo, E. Girardi, C. Artero, and M.F. Franzoni (1996) Immunohistochemical investigation of gamma-aminobutyric acid ontogeny and transient expression in the central nervous system of *Xenopus laevis* tadpoles. J. Comp. Neurol., *368:* 285–294.

Batueva, V., E.I. Suderevskya, N.P. Vesselkin, J. Pierre, and J. Repérant (1990) Localisation of GABA-immunopositive cells in the river lamprey spinal cord. J. Hirnforsch., *31:* 739–745.

Bennis, M., A. Calas, M. Geffard, and H. Gamrani (1991) Distribution of GABA immunoreactive systems in the forebrain and midbrain of the chameleon. Brain Res. Bull., *26:* 891–898.

Bergen, H.T., J.F. Hejtmancik, and D. W. Pfaff (1991) Effects of gamma-aminobutyric acid receptor agonists and antagonist on LHRH-synthesizing neurons as detected by immunocytochemistry and in situ hybridization. Exp. Brain Res., *87:* 46–56.

Bless, E.P., W.A. Westaway, G.A. Schwarting, and S. A. Tobet (2000) Effects of gamma-aminobutyric acid(A) receptor manipulation on migrating gonadotropin-releasing hormone neurons through the entire migratory route in vivo and in vitro. Endocrinology, *141:* 1254–1262.

Bosma, P.T., M. Blazquez, M.A. Collins, J. D. Bishop, G. Drouin, I. G. Priede, K. Docherty, and V.L. Trudeau (1999) Multiplicity of glutamic acid decarboxylases (GAD) in vertebrates: molecular phylogeny and evidence for a new GAD paralog. Mol. Biol. Evol., *16:* 397–404.

Braissant, O., and W. Wahli (1998) Differential expression of peroxisome proliferator-activated receptor-alpha, -beta, and -gamma during rat embryonic development. Endocrinology, *139:* 2748–2754.

Brodin, L., N. Dale, J. Christenson, J. Storm-Mathisen, T. Hökfelt, and S. Grillner (1990) Three types of GABA-immunoreactive cells in the lamprey spinal cord. Brain Res., *508:* 172–175.

Cherubini, E., and F. Conti (2001) Generating diversity at GABAergic synapses. Trends Neurosci., *24:* 155–162.

Doldan, M.J., B. Prego, B.I. Holmqvist, and E. de Miguel (1999) Distribution of GABA-immunolabeling in the early zebrafish *(Danio rerio)* brain. Eur. J. Morphol., *37:* 126–129.

Domenici, L., H.J. Waldvogel, C. Matute, and P. Streit (1988) Distribution of GABA-like immunoreactivity in the pigeon brain. Neuroscience, *25:* 931–950.

Ekström, P., and L.M. Ohlin (1995) Ontogeny of GABA-immunoreactive neurons in the central nervous system in a teleost, *Gasterosteus aculeatus* L. J. Chem. Neuroanat., *9:* 271–288.

Favit, A., W.C. Wetsel, and A. Negro-Vilar (1993) Differential expression of gamma-aminobutyric acid receptors in immortalized luteinizing hormone-releasing hormone neurons. Endocrinology, *133:* 1983–1989.

Feleder, C., H. Jarry, S. Leonhardt, W. Wuttke, and J.A. Moguilevsky (1996) The GABAergic control of gonadotropin-releasing hormone secretion in male rats during sexual maturation involves effects on hypothalamic excitatory and inhibitory amino acid systems. Neuroendocrinology, *64:* 305–312.

Flugge, G., W.H. Oertel, and W. Wuttke (1986) Evidence for estrogen-receptive GABAergic neurons in the preoptic/anterior hypothalamic area of the rat brain. Neuroendocrinology, *43:* 1–5.

Franzoni, M.F., and P. Morino (1989) The distribution of GABA-like-immunoreactive neurons in the brain of the newt, *Triturus cristatus carnifex*, and the green frog, *Rana esculenta*. Cell Tissue Res., *255:* 155–166.

Fueshko, S.M., S. Key, and S. Wray (1998) GABA inhibits migration of luteinizing hormone-releasing hormone neurons in embryonic olfactory explants. J. Neurosci., *18:* 2560–2569.

Herbison, A.E. (1998) Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. Endocr. Rev., *19:* 302–330.

Jarry, H., S. Leonhardt, and W. Wuttke (1991) Gamma-aminobutyric acid neurons in the preoptic/anterior hypothalamic area synchronize the phasic activity of the gonadotropin-releasing hormone pulse generator in ovariectomized rats. Neuroendocrinology, *53:* 261–267.

Kah, O., P. Dubourg, M. G. Martinoli, M. Rabhi, F. Gonnet, M. Geffard, and A. Calas (1987) Central GABAergic innervation of the pituitary in goldfish: a radioautographic and immunocytochemical study at the electron microscope level. Gen. Comp. Endocrinol., *67:* 324–332.

Kah, O., V.L. Trudeau, B.D. Sloley, J.P. Chang, P. Dubourg, K.L. Yu, and R.E. Peter (1992) Influence of GABA on gonadotrophin release in the goldfish. Neuroendocrinology, *55:* 396–404.

Katarova, Z., G. Sekerkova, S. Prodan, E. Mugnaini, and G. Szabo (2000) Domain-restricted expression of two glutamic acid decarboxylase genes in midgestation mouse embryos. J. Comp. Neurol., *424:* 607–627.

King, J.C., S.A. Sower, and E.L. Anthony (1988) Neuronal systems immunoreactive with antiserum to lamprey gonadotropin-releasing hormone in the brain of *Petromyzon marinus*. Cell Tissue Res., *253:* 1–8.

Kusano, K., S. Fueshko, H. Gainer, and S. Wray (1995) Electrical and synaptic properties of embryonic luteinizing hormone-releasing hormone neurons in explant cultures. Proc. Natl. Acad. Sci. USA, *92:* 3918–3922.

Lariviere, K., L. MacEachern, G. Majchrzak, V. Greco, S. Chiu, G. Drouin, and V. L. Trudeau (2002) Multiplicity of glutamic acid decarboxylase: early gene duplication and the existence of novel vertebrate forms. Mol. Biol. Evol., *submitted*.

Lauder, J.M. (1993) Neurotransmitters as growth regulatory signals: role of receptors and second messengers. Trends Neurosci., *16:* 233–240.

Lauder, J.M., V.K. Han, P. Henderson, T. Verdoorn, and A. C. Towle (1986) Prenatal ontogeny of the GABAergic system in the rat brain: an immunocytochemical study. Neuroscience, *19:* 465–493.

Leranth, C., N.J. MacLusky, H. Sakamoto, M. Shanabrough, and F. Naftolin (1985) Glutamic acid decarboxylase-containing axons synapse on LHRH neurons in the rat medial preoptic area. Neuroendocrinology, *40:* 536–539.

Mananos, E.L., I. Anglade, J. Chyb, C. Saligaut, B. Breton, and O. Kah (1999) Involvement of gamma-aminobutyric acid in the control of GTH-1 and GTH-2 secretion in male and female rainbow trout. Neuroendocrinology, *69:* 269–280.

Martinoli, M.G., P. Dubourg, M. Geffard, A. Calas, and O. Kah (1990) Distribution of GABA-immunoreactive neurons in the forebrain of the goldfish, *Carassius auratus*. Cell Tissue Res., *260:* 77–84.

McCann, S.M., E. Vijayan, A. Negro-Vilar, H. Mizunuma, and H. Mangat (1984) Gamma aminobutyric acid (GABA), a modulator of anterior pituitary hormone secretion by hypothalamic and pituitary action. Psychoneuroendocrinology, *9:* 97–106.

Medina, M., J. Repérant, S. Dufour, R. Ward, N. Le Belle, and D. Miceli (1994) The distribution of GABA-immunoreactive neurons in the brain of the silver eel (*Anguilla anguilla* L.). Anat. Embryol. (Berl.), *189:* 25–39.

Meléndez-Ferro, M., E. Pérez-Costas, M.J. González, M.A. Pombal, R. Anadón, and M.C. Rodicio (2000) GABA-immunoreactive internuclear neurons in the oculomotor system of lampreys. Brain Res., *855:* 150–157.

Meléndez-Ferro, M., E. Pérez-Costas, R. Rodríguez-Muñoz, M.P. Gómez-López, R. Anadón, and M.C. Rodicio (2001) GABA immunoreactivity in the olfactory bulbs of the adult sea lamprey. Brain Res., *893:* 253–260.

Meléndez-Ferro, M., B. Villar-Cheda, X.M. Abalo, E. Pérez-Costas, R. Rodríguez-Muñoz, W.J. Degrip, J. Yáñez, M. C. Rodicio, and R. Anadón (2002) Early development of the retina and pineal complex in the sea lamprey: Comparative immunocytochemical study. J. Comp. Neurol., *442:* 250–265.

Mitsushima, D., D.L. Hei, and E. Terasawa (1994) gamma-Aminobutyric acid is an inhibitory neurotransmitter restricting the release of luteinizing hormone-releasing hormone before the onset of puberty. Proc. Natl. Acad. Sci. USA, *91:* 395–399.

Mitsushima, D., F. Marzban, L.L. Luchansky, A.J. Burich, K.L. Keen, M. Durning, T.G. Golos, and E. Terasawa (1996) Role of glutamic acid decarboxylase in the prepubertal inhibition of the luteinizing hormone releasing hormone release in female rhesus monkeys. J. Neurosci., *16:* 2563–2573.

Muske, L.E. (1993) Evolution of gonadotropin-releasing hormone (GnRH) neuronal systems. Brain Behav. Evol., *42:* 215–230.

Piavis, G.W. (1961) Embryological stages in the sea lamprey and effects of temperature on development. US Fish Wildlife Serv. Fish. Bull., *182:* 111–143.

Rio, J.P., N.P. Vesselkin, E. Kirpitchnikova, N. B. Kenigfest, C. Versaux-Botteri, and J. Anadón Repérant (1993) Presumptive GABAergic centrifugal input to the lamprey retina: a double-labeling study with axonal tracing and GABA immunocytochemistry. Brain Res., *600:* 9–19.

Rio, J.P., N.P. Vesselkin, J. Repérant, N.B. Kenigfest, D. Miceli, and V. Adanina (1996) Retinal and nonretinal inputs upon retinopedal RMN neurons in the lamprey: a light and electron microscopic study combining HRP axonal tracing and GABA immunocytochemistry. J. Chem. Anat., *12:* 51–70.

Roberts, A., N. Dale, O.P. Ottersen, and J. Storm-Mathisen (1987) The early development of neurons with GABA immunoreactivity in the CNS of *Xenopus laevis* embryos. J. Comp. Neurol., *261:* 435–449.

Schmitz, G.G., T. Walter, R. Seibl, and C. Kessler (1991) Nonradioactive labeling of oligonucleotides in vitro with the hapten digoxigenin by tailing with terminal transferase. Anal. Biochem., *192:* 222–231.

Senthilkumaran, B., K. Okuzawa, K. Gen, and H. Kagawa (2001) Effects of serotonin, GABA and neuropeptide Y on seabream gonadotropin releasing hormone release in vitro from preoptic-anterior hypothalamus and pituitary of red seabream, *Pagrus major*. J. Neuroendocrinol., *13:* 395–400.

Sloley, B.D., O. Kah, V.L. Trudeau, J.G. Dulka, and R.E. Peter (1992) Amino acid neurotransmitters and dopamine in brain and pituitary of the goldfish: involvement in the regulation of gonadotropin secretion. J. Neurochem., *58:* 2254–2262.

Sower, S.A., Y.C. Chiang, S. Lovas, and J.M. Conlon (1993) Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. Endocrinology, *132:* 1125–1131.

Suzuki, K., R.L. Gamble, and S.A. Sower (2000) Multiple transcripts encoding lamprey gonadotropin-releasing hormone-I precursors. J. Mol. Endocrinol., *24:* 365–376.

Tobet, S.A., T.W. Chickering, J.C. King, E.G. Stopa, K. Kim, V. Kuo-Leblank, and G.A. Schwarting (1996a) Expression of gamma-aminobutyric acid and gonadotropin-releasing hormone during neuronal migration through the olfactory system. Endocrinology, *137:* 5415–5420.

Tobet, S.A., T.W. Chickering, and S.A. Sower (1996b) Relationship of gonadotropin-releasing hormone (GnRH) neurons to the olfactory system in developing lamprey *(Petromyzon marinus)*. J. Comp. Neurol., *376:* 97–111.

Tobet, S.A., M. Nozaki, J.H. Youson, and S.A. Sower (1995) Distribution of lamprey gonadotropin-releasing hormone-III (GnRH-III) in brains of larval lamprey *(Petromyzon marinus)*. Cell Tissue Res., *279:* 261–270.

Tobet, S.A., S.A. Sower, and G.A. Schwarting (1997) Gonadotropin-releasing hormone containing neurons and olfactory fibers during development: from lamprey to mammals. Brain Res. Bull., *44:* 479–486.

Trabucchi, M., N. Chartrel, G. Pelletier, M. Vallarino, and H. Vaudry (2000) Distribution of GAD-immunoreactive neurons in the diencephalon of the african lungfish, *Protopterus annectens*: colocalization of GAD and NPY in the preoptic area. J. Comp. Neurol., *419:* 223–232.

Trudeau, V.L., B.D. Sloley, and R.E. Peter (1993a) GABA stimulation of gonadotropin-II release in goldfish: involvement of $GABA_A$ receptors, dopamine, and sex steroids. Am. J. Physiol., *265:* R348–355.

Trudeau, V.L., B.D. Sloley, and R.E. Peter (1993b) Norepinephrine turnover in the goldfish brain is modulated by sex steroids and GABA. Brain Res., *624:* 29–34.

Trudeau, V.L., B.D. Sloley, and R.E. Peter (1993c) Testosterone enhances GABA and taurine but not N-methyl-D,L-aspartate stimulation of gonadotropin secretion in the goldfish: possible sex steroid feedback mechanisms. J. Neuroendocrinol., *5:* 129–136.

Trudeau, V.L., D. Spanswick, E.J. Fraser, K. Lariviere, D. Crump, S. Chiu, M. MacMillan, and R.W. Schulz (2000a) The role of amino acid neurotransmitters in the regulation of pituitary gonadotropin release in fish. Biochem. Cell. Biol., *78:* 241–259.

Trudeau, V.L., P.T. Bosma, M. Collins, I.G. Priede, and K. Docherty (2000b) Sexually dimorphic expression of glutamate decarboxylase mRNA in the hypothalamus of the deep sea armed grenadier, *Coryphaenoides (Nematonurus) armatus*. Brain Behav. Evol., *56:* 269–275.

von Bartheld, C.S., and E.W. Rubel (1989) Transient GABA immunoreactivity in cranial nerves of the chick embryo. J. Comp. Neurol., *286:* 456–471.

Wray, S., S.M. Fueshko, K. Kusano, and H. Gainer (1996) GABAergic neurons in the embryonic olfactory pit/vomeronasal organ: maintenance of functional GABAergic synapses in olfactory explants. Dev. Biol., *180:* 631–645.

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