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In situ Characterization of Gonadotropin-Releasing Hormone-I, -III, and Glutamic Acid Decarboxylase Expression in the Brain of the Sea Lamprey, *Petromyzon marinus*

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Key Words

Gonadotropin-releasing hormone · Glutamic acid decarboxylase · Gamma aminobutyric acid · Lamprey · Agnathan · In situ hybridization · LHRH

Abstract

The distribution of lamprey gonadotropin-releasing hormone (GnRH)-I and -III has been extensively characterized by immunocytochemistry in the forebrain of the sea lamprey, *Petromyzon marinus.* However, the cellular location of lamprey GnRH-III mRNA expression by in situ hybridization in the lamprey brain has not been determined. We show for the first time the location of expression of lamprey GnRH-III, as well as provide a more comprehensive in situ study of lamprey GnRH-I and glutamic acid decarboxylase (GAD; GABA-synthesizing enzyme) mRNA expression in the brain of the lamprey in different reproductive life stages. Colorimetric and dual-label fluorescent amplification methods of in situ hybridization were used on brain tissue sections of adult, juvenile, and larval sea lamprey. In each life stage of the lamprey, expression of lamprey GnRH-I was shown in the preoptic area (POA) and the hypothalamus forming the characteristic arc-like cell population extending from the preoptic nucleus (NPO) to the neurohypophysis. Lamprey GnRH-III expression was also seen in the POA of each life stage in close proximity to lamprey GnRH-I mRNA containing neurons. GAD expression was shown in distinct cell clusters in and around the POA, in the olfactory bulb, in the dorsal thalamus beneath the habenular region, and also in the ventral-medial hypothalamus stretching from the periventricular region to the anterior portion of the rhombencephalon. Using dual-label in situ hybridization, we have shown that lamprey GnRH-I and -III mRNA are colocalized in the same cells in the POA in adult lampreys. Dual-label in situ hybridization also showed close proximity of GAD mRNA containing neurons and GnRH containing neurons in the POA. These data suggest that γ-aminobutyric acid (GABA) may directly affect GnRH release in the brain of the sea lamprey.

Introduction

GnRH is the primary regulating hormone of the hypothalamo-hypophysial-gonadal axis across all vertebrates [Sower, 1998; Bless et al., 2000]. Numerous immunocytochemistry (ICC) studies [Crim et al., 1979a, b; Nozaki and Kobayashi, 1979; Nozaki et al., 1984, 2000; King et al., 1988; Wright et al., 1994; Tobet et al., 1995, 1996; Reed et

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al., 2002] have investigated the distribution of lamprey GnRH in the brain of several lamprey species. These studies have consistently demonstrated the localization of lamprey GnRH-I and -III in the preoptic area and hypothalamus extending to the neurohypophysis. Reed et al. [2002] using in situ hybridization in larval lamprey, showed that lamprey GnRH-I mRNA was expressed in the same region of the preoptic area and regions of the hypothalamus adjacent to the third ventricle. In the Reed et al. [2002] study lamprey GnRH-III had been identified, however, its cDNA had not yet been determined, preventing comparisons between lamprey GnRH-I and -III mRNA expression at different life stages of the sea lamprey.

One of the important neurotransmitters regulating GnRH neurons is γ-aminobutyric acid (GABA), the primary inhibitory neurotransmitter of the central nervous system (CNS) of vertebrates [Wild, 1994; Siegal et al., 1999]. GABA neurons are abundant throughout the brain including regions of the hypothalamus where they are believed to compose 50% of neurons [van den Pol and Obrietan, 2000]. GABA is best known for its inhibitory actions in the CNS, however, GABA has also been shown to elicit an excitatory effect on developing and immortalized hypothalamic neurons [Favit et al., 1993; Hales et al., 1994; Chen et al., 1996]. GABA is hypothesized to play an important role in the regulation of GnRH and gonadotropin (GTH) release in vertebrates such as the goldfish [Trudeau et al., 2000] by acting as either the primary neurotransmitter or as a secondary neurotransmitter in the steroidal feedback on GnRH neurons. Few studies have examined the role of GABA on GnRH in fish and to date, only one previous study has examined the possibility of GABA-GnRH interactions in the hypothalamus of the sea lamprey. Previous immunocytochemical studies by Reed et al. [2002] demonstrated the close spatial relationship between GABA-containing neurons and GnRH-containing neurons in the hypothalamus of the larval sea lamprey, hypothesizing that GABA is directly involved in controlling GnRH release. Using in situ hybridization, Reed et al. also showed the expression of the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) in close proximity to cells expressing GnRH-I in the hypothalamus of larval sea lamprey, although no comparisons between the expression of GAD and GnRH were made in the juvenile and adult sea lamprey.

The sea lamprey is a member of the oldest group of extant vertebrates, the agnathans [Furlong and Holland, 2002; Takezaki et al., 2003]. The sea lamprey is an anadramous species living the larval part of its life in freshwater streams as a filter feeder, the second phase of its life in marine environments as a parasitic animal, and finally returning to the streams as a mature adult, where it spawns and dies soon after [Hardisty and Potter, 1971]. In the present study, brains from the larval or ammocoete, the parasitic, and the mature adult stages were examined.

With the recent cloning of lamprey GnRH-III [Silver et al., 2001] and lamprey GAD [Lariviere et al., 2002] cDNAs, it is now possible to compare the relationship between mRNA expression of these genes in the sea lamprey. In the present study, two different methods of in situ hybridization were employed: (1) to fully characterize the expression patterns of the two lamprey GnRH isoforms identified; and (2) to determine GAD and GnRH spatial relationships in the hypothalamus of larval, juvenile and adult sea lamprey.

Methods and Materials

Collection and Sampling

In May and June, adult sea lampreys were collected from the fish ladder in the Cocheco River in Dover, New Hampshire, during their upstream spawning migration from the ocean. The lampreys were transported to the Anadromous Fish and Aquatic Invertebrate Research (AFAIR) Laboratory at the University of New Hampshire (UNH), where they were maintained in an artificial stream, previously described [Fahien and Sower, 1990]. Handling and care of the animals followed the guidelines established by the Animal Care and Laboratory Use Committee of UNH.

Larval lampreys (ammocoetes) were collected from the Oyster River in Durham, New Hampshire by electrofishing. The ammocoetes were transported to the AFAIR laboratory at UNH and maintained in small aquariums supplied with ambient local reservoir water or 12 °C well water, under natural photoperiod until dissection. The juvenile, or parasitic, sea lampreys were obtained from Hammond Bay Biological Station on Lake Huron in northern Michigan and sent to the University of New Hampshire and maintained at the AFAIR lab according to UNH animal care guidelines. The fish were processed for either RNA isolation or for in situ hybridization. Histological examination was performed as previously described [Fahien and Sower, 1990; Bolduc and Sower, 1992].

Digoxigenin-Labeled RNA Probe Synthesis

Digoxigenin- (DIG; Roche, Indianapolis, IN) and fluorescein- (Roche) labeled RNA probes used for in situ hybridization were generated from GAD cDNA [~ 600 bp; Lariviere et al., 2002], lamprey GnRH-I cDNA (~ 550 bp) and lamprey GnRH-III cDNA (~ 300 bp). Total RNA was isolated from ~ 100 mg lamprey brains using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH). First strand cDNA was constructed using the 1st Strand cDNA Synthesis Kit from Amersham-Pharmacia (Buckinghamshire, England, UK) with brain total RNA as template. PCR was performed using lamprey cDNA as template with gene-specific primers:

RG1 5′-TGCTGTCGAGAGAAGAAACCCCT-3′ (GnRH-I)

RG4 5′-GCCACGCATAGCACAGTGTAT-3′ (GnRH-I)

pmarinus-lGnRH3-5′ end 5′-GACCGTCTGGAATCATCACAGAAGC-3′ (GnRH-III)

pmarinus-lGnRH3-3′end-RC 5′-CGCCTTGTTGTTACGCGTGGCC-3′ (GnRH-III).

GADprobe1 5′-TATGAGGGTTCCAGGTCACAGAGTTCGC-3′

GADprobe2a 5′-CCCCCTCTGGTCGGCTTTGATGAGA-3′

GADprobe2b 5′-TATCATCAAAGCCGACCAGAGGG-3′

GADprobe3 5′-GGTGAAGATGAAGGGGATGGCGGC-3′

GAD-RT-3′ 5′-TCTCGACCCCGTTGAGCTTGTGAC-3′

GAD-RT-5′ 5′-TGGCCGCTCGCTACAAGTTCTTCC-3′

The oligonucleotide primers were obtained from Integrated DNA technology (Coralville, IA). PCR reactions were prepared [10× Advantage 2 PCR Buffer, 1 m*M* dNTPs, 1.25 units AdvanTaq DNA polymerase (Clonetech Laboratories Inc, Palo Alto, CA), 2 μ*M* each primer, 1 m*M* $MgCl_2$, 1 μl template cDNA]. PCR reactions were performed using a Perkin Elmer Thermal Cycler under the following conditions: activation step of 94°C for 5 min; 94°C for 30 s/74°C for 2 min × 5 cycles; 94°C for 30 s/72°C for 2 min × 10 cycles; 94°C for 30 s/70°C for 2 min × 10 cycles; 94°C for 30 s/68°C for 2 min × 15 cycles; 4°C hold. Reaction product was analyzed on 1% agarose gel electrophoresed at 80 V for 1 h and stained with ethidium bromide for purposes of visualization.

PCR products were gel purified as described in the QIAEX II Gel Purification Kit© (QIAGEN, Valencia, CA). GnRH-I, -III, and GAD purified PCR product were then inserted into the pGEM-T Easy vector (Promega, Madison, WI) and subsequently transformed into *E. coli* JM1090 cells (Promega). Ligations and transformations were performed following the protocol described in the pGEM T-easy Vector System (Promega). Overnight cultures were used for plasmid preparation with the Wizard Plus Miniprep system (Promega), following the manufacturer's protocol. Purified plasmid (5 μl) containing insert was then digested overnight at 37°C with either Sal I or Nco I [2 μg BSA and 1× Buffer D (Promega)] restriction enzymes producing singly digested linearized plasmid. Digestions were analyzed by 1% agarose gel electrophoresis to confirm presence of expected clones and for quantification of the DNA fragments for sequencing preparation. DNA sequencing was performed by The Huntsman Cancer Institute DNA Sequencing Facility at the University of Utah.

Digoxigenin-labeled riboprobes were synthesized using the SP6 Riboprobe Synthesis (Promega), and either digoxigenin-11-UTP or fluorescein-12-UTP as previously described [Rubin et al., 1997]. This protocol was slightly modified in that the transcription reactions were allowed to continue overnight instead of 2 h.

Riboprobe Cross-Reactivity Screening

Cross-reactivity between anti-sense probes was measured using a modified Digoxigenin Northern blot protocol [Allen et al., 2000]. In place of total RNA, full-length probe template cDNA used for each riboprobe synthesis was cross-linked to a nylon membrane and hybridized with the different anti-sense and sense riboprobes. Cross-reactivity was measured colorimetrically. Briefly, template cDNA was denatured for 2 min at 100°C, spotted onto nylon membrane and UV cross-linked. Hybridization solution (50% dextran sulfate, 4× SSC, 1× Denhardt's, 1 mg/ml yeast tRNA, 10 m*M* DTT) was prepared and mixed with 0.5 mg/ml DIG-11-UTP or fluorescein-12-UTP-labeled RNA diluted in DEPC water. Probes were applied to each template cDNA and allowed to hybridize overnight at 55°C. Following hybridization, membranes were washed twice in 2× SSC for 5 min each at RT, 1× SSC for 15 min at 55°C, and maleate buffer (0.1 *M* maleaic acid, 0.3% Tween, 0.15 *M* NaCl; pH 7.5) for 2 min at RT. Membranes were blocked in blocking buffer for 30 min at RT and then incubated in anti-DIG or anti fluorescein-alkaline phosphatase (AP; Roche) antibody (1:5000) for 30 min at RT. Membranes were washed twice in maleate buffer twice for 15 min each at RT, and then incubated in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate [NBT/BCIP (Roche) 1:50 diluted in 100 m*M* Tris-HCl, 100 m*M* NaCl, 50 m*M* Mg_2Cl_2; pH 9.5] in darkness for 1–3 h at RT. Cross-reactivity was determined by the presence of dark purple precipitate. No cross-reactivity was seen between any probes used in this study.

In situ Hybridization

Individual ammocoetes, juvenile parasitic and adult lampreys were sampled for brains and pituitaries. The fish were killed by decapitation, and the brains were removed and immediately placed on dry ice. Brains were either used immediately or stored at –80°C for future use. Sagittal, horizontal or coronal tissue sections of 14–18 μm were cut on a cryostat (Reichert-Jung, Leica Instruments, Heidelberg, Germany) at –12°C, mounted onto Vectabond (Vector Laboratories, Burlingame, California) coated slides and immediately moved into tissue preparation steps of day 1 of the in situ hybridization protocol as previously described by Rubin et al. [1997]. Modifications to this protocol included a shorter stringency wash in 0.1× SSC in 10 m*M* DTT (7.5 min) as well as the application of counter-stains for some tissues using either hematoxylin or Fast Red© (Vector Laboratories). Sections were viewed under light microscopy using an Olympus BH2 microscope and digital photographs were captured with a Micropublisher 3.3 digital camera (Qimaging, Richmond, VA) and saved in TIFF format. Photographs were processed with Adobe Photoshop 6.0 for size, brightness and contrast modification only.

Double in situ Hybridization

Dual label in situ hybridization was performed on adult sea lamprey brains using the TSA-plus Cyanine 3/Fluorescein System (Perkin Elmer, Boston, MA) in conjuction with the previously described colorimetric procedure. Riboprobes labeled with either digoxigenin-11-UTP (lamprey GAD) or fluorescein-12-UTP (lamprey GnRH-III) were prepared as described above. Tissues were sampled, dehydrated and delipidated as described by Rubin et al. [1997]. Signal detection used a modified protocol from Xi et al. [2003]. Modifications included the addition of a 30-min 0.3% hydrogen peroxide in methanol wash before the blocking step and between signal amplification steps to eliminate endogenous peroxidase and remaining horseradish peroxidase (HRP) activity respectively, as well as the use of counter-stains previously described. Visualization was performed using an Olympus BH2 microscope with fluorescence filters and photographs were processed as before.

Results

In situ Hybridization

Anti-sense probes showed strong, distinct reaction product in specific hypothalamic nuclei, whereas tissue sections treated with respective sense probes showed no positive results and little or no background. Positive expression patterns were consistent in all brains tested, with respect to each anti-sense probe. In parasitic and

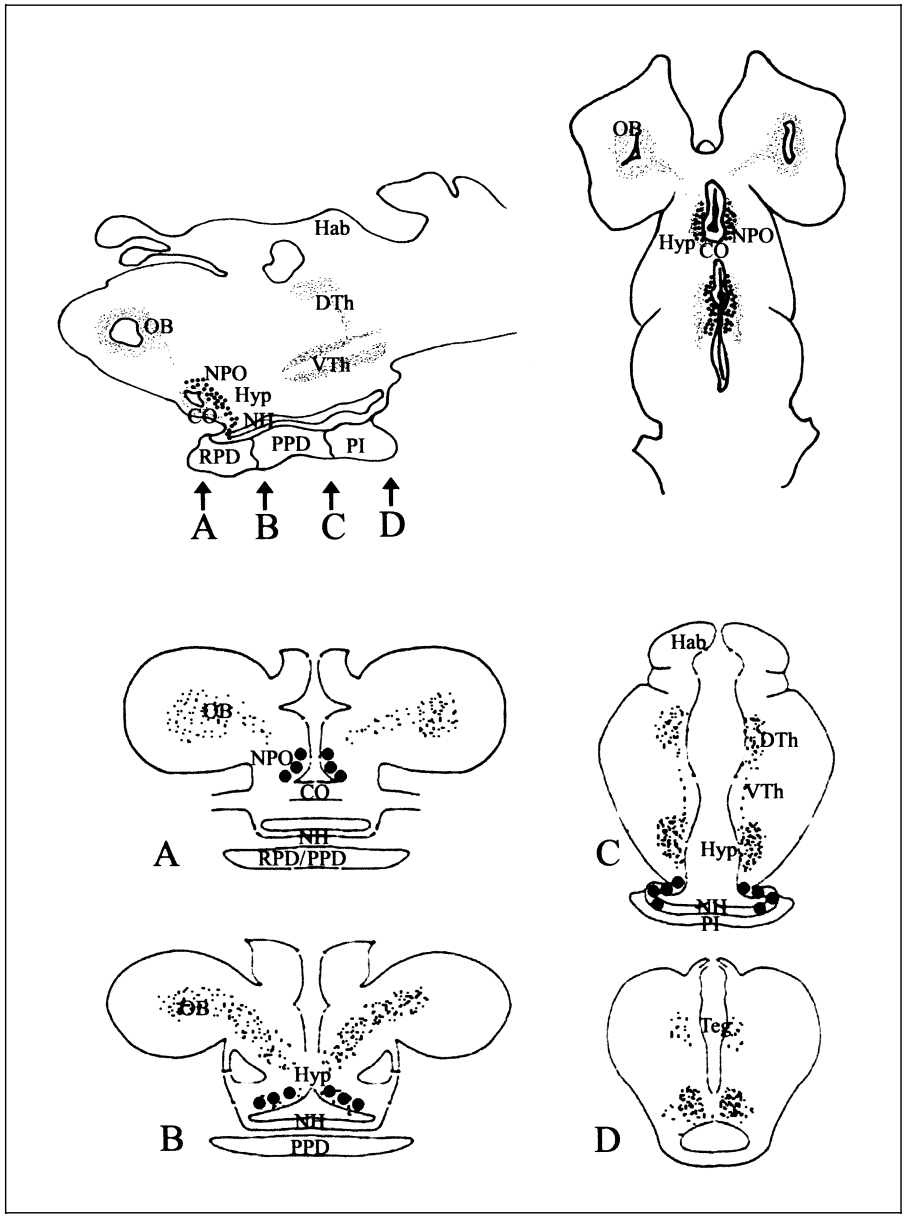


Fig. 1. Schematic representation of mRNA expression in adult lamprey. Topographical distribution of lamprey GnRH I and III (circles) and GAD mRNA (dots) in sagittal, horizontal, and coronal tissue sections. Coronal sections correspond to the arrows in the sagittal plane. OB = Olfactory bulb; NPO = preoptic nucleus; Hyp = hypothalamus; CO = optic chiasm; NH = neurohypophysis; Hab = habenula; DTh = dorsal thalamus; VTh = ventral thalamus; RPD = rostral pars distalis; PPD = proximal pars distalis; PI = pars intermedia; Teg = tegmentum. Terminology is based on that used by Nieuwenhuys [1977]. Diagrams are not drawn to scale.

adult sea lamprey, no noticeable differences were observed between males and females, however quantitative analyses were not conducted.

Lamprey GnRH-I. In adults, lamprey GnRH-I mRNA expression was seen clearly in cell clusters in the ventral hypothalamic area, extending from the preoptic area (POA) and the postoptic commisural nucleus (PCN), running dorsally to the optic chiasm (CO) to the ventral anterior hypothalamus and eventually to the neurohypophysis (fig. 1, 2; table 1). Dense expression signal was found in the cell bodies of the neurons of the POA. Intense expression signal was observed near the sagittal midline in regions adjacent to the third ventricle. Expression was detected in the same regions of the brain of juvenile and larval life stages of lamprey with similar expression patterns. In horizontal tissue sections of the ventral hypothalamus, lamprey GnRH-I expression was seen in two bilateral-neuron populations bordering the third ventricle with the majority of the signal in the POA. Coronal sections showed signal bordering the third ventricle in the POA and further into the brain lamprey GnRH-I-expressing cells were seen in the ventral hypothalamus and

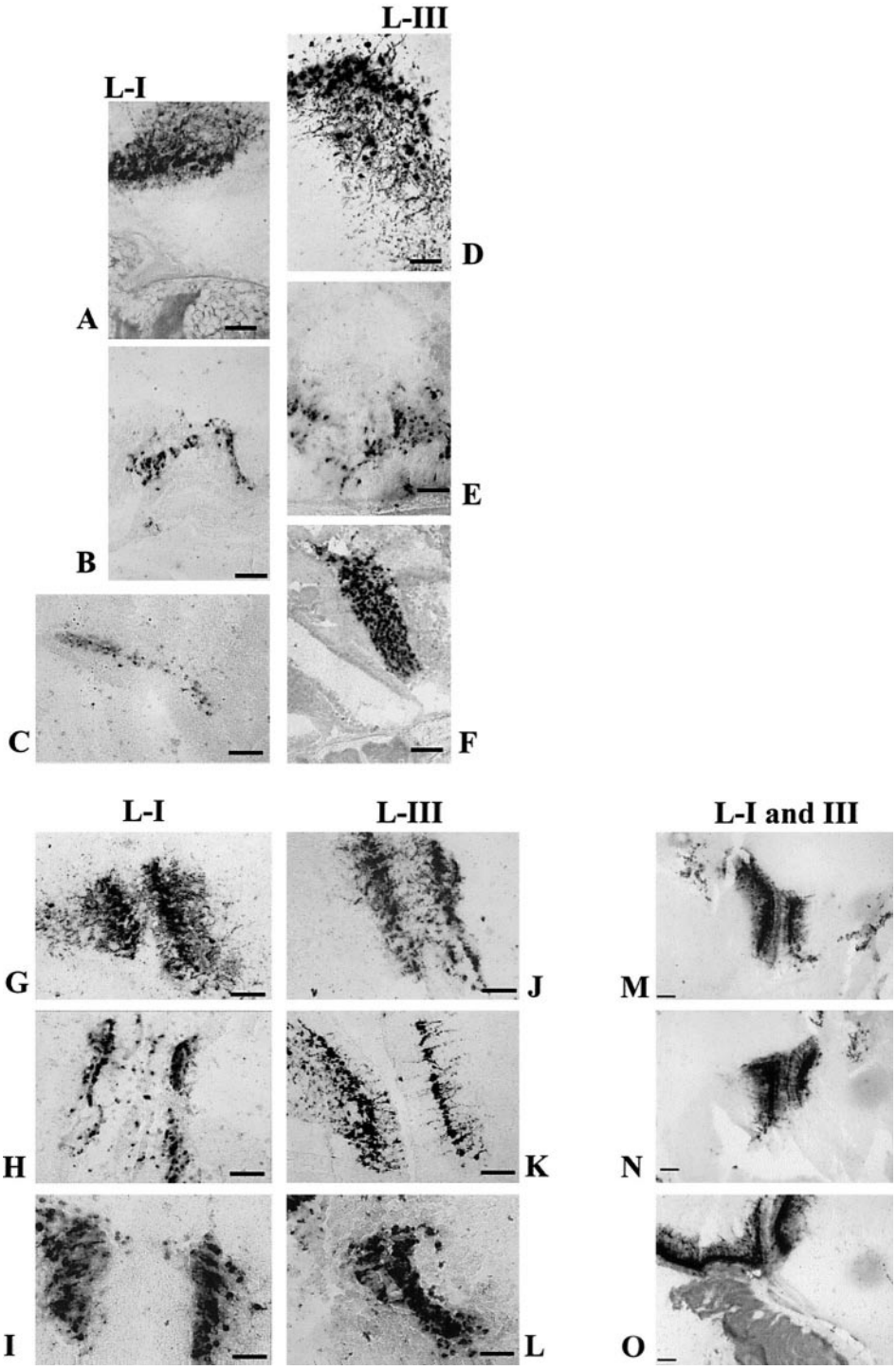


Fig. 2. DIG-labeled in situ hybridization for lamprey GnRH-I and -III. Digital photographs of sagittal and horizontal tissue sections from adult (**A**, **D**, **G**, **J**), juvenile (**B**, **E**, **H**, **K**), and larval (**C**, **F**, **I**, **L**), sea lamprey and coronal (**M–O**) tissue sections showing distinct cell populations expressing lamprey GnRH-I or -III mRNA. The GnRH expressing cells are located in the preoptic area and extend to the neurohypophysis. Coronal sections correspond to figure 1 as follows: **M** = **A**, **N** = **B**, **O** = **C**. Scale bars = 25 μm.

Table 1. Location of expression

Structures	GnRH-I, -III-expressing neurons	GAD-expressing neurons
Diencephalon		
Anterior preoptic area		+
Habenula	– – –	+
Thalamus, dorsal	– – –	+
Thalamus, ventral	– – –	+ +
Periventricular arcuate nucleus		
Dorsal hypothalamus	+ + +	+ + +
Ventral hypothalamus	+ + +	+ + +
Pretectal area	– – –	+ +
Neurohypophysis	+ +	+
Postoptic commissural nucleus	+ + +	+
Telencephalon		
Olfactory bulb		
Glomerular layer	– – –	+ +
Granular layer	– – –	+ +
Mitral layer	– – –	+ +

Location of expression of lamprey GnRH-I, -III and GAD in the adult sea lamprey brain. + = light staining; + + = moderate staining; + + + = intense staining; – – – = no staining.

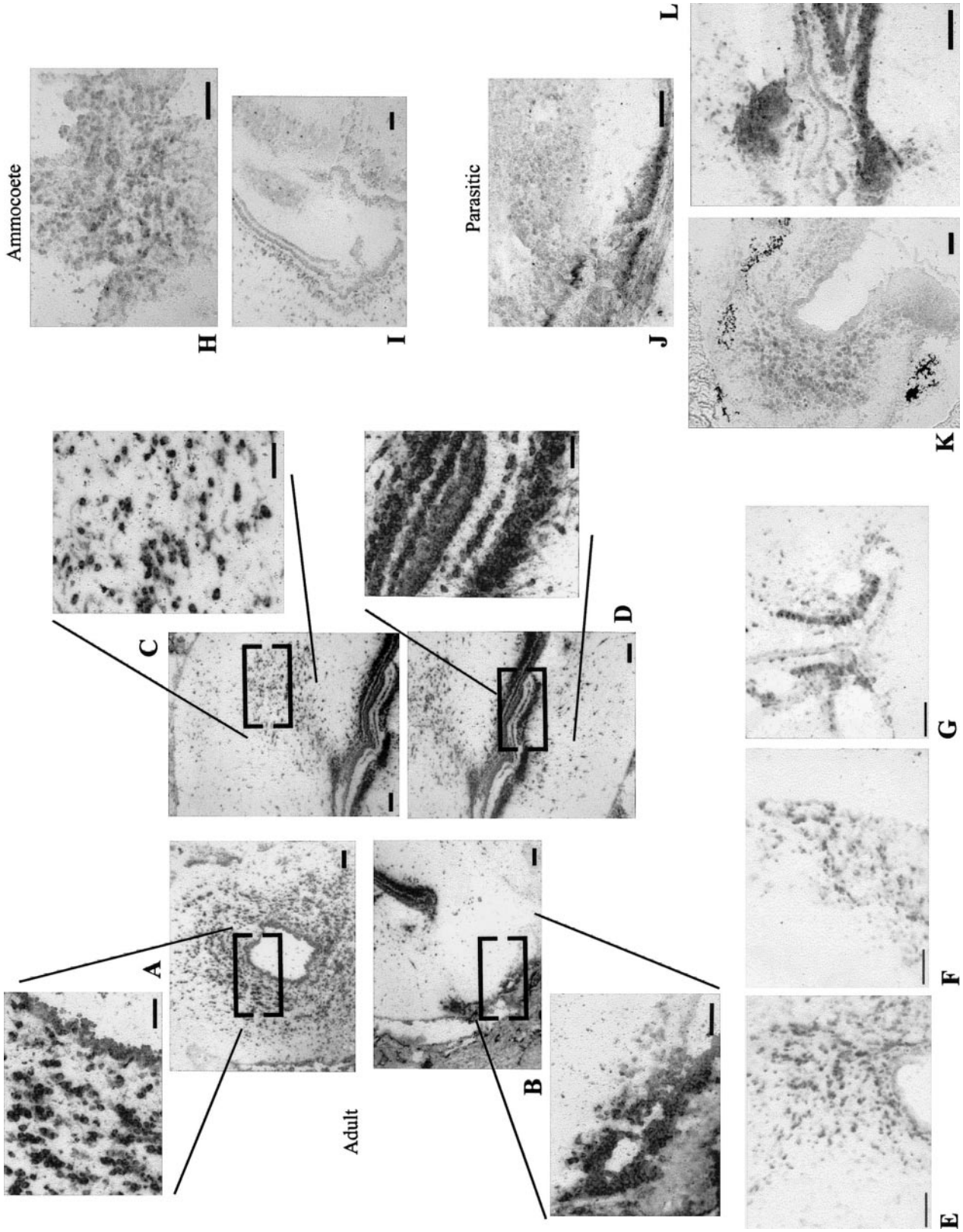
neurohypophysis. Expression in male and female adult sea lamprey showed no differences in terms of localization, distribution or density. No expression of lamprey GnRH-I was seen in other parts of the hypothalamus or in areas of the forebrain, midbrain or hindbrain.

Lamprey GnRH-III. Lamprey GnRH-III mRNA expression was found in the same region of the anterior preoptic region as lamprey GnRH-I in each lamprey life stage studied (fig. 1, 2; table 1). Expression signal for lamprey GnRH-III in the adult lamprey brain showed the same expression patterns observed in lamprey GnRH-I, where the primary signal was seen in the cell bodies. Signal in larval and parasitic lamprey appeared more abundant for lamprey GnRH-III than lamprey GnRH-I however, quantitation was not performed. This was most evident in the larval lamprey where only light signal was seen for lamprey GnRH-I whereas lamprey GnRH-III showed abundant expression. Horizontal tissue sections showed expression in neurons bordering the third ventricle, with the primary expression seen in the POA. Coronal sections showed signal for lamprey GnRH-III adjacent to the third ventricle in the hypothalamus. GnRH-III expression was also detected further into the brain in the neurohypophysis. No noticeable differences were seen in localization, distribution or density of expression between adult male and female lamprey. No expression was seen in any other region of the brain. The closeness of lamprey GnRH-I and -III in the ventral anterior hypothalamus in each life stage suggests they are co-expressed and that both play active roles in lamprey development and reproduction.

Lamprey GAD. Lamprey GAD mRNA expression in all life stages of lamprey was observed in several well-defined regions throughout the brain (fig. 3; table 1). Expression was seen abundantly in the olfactory bulbs in the glomerular, mitral and granular cell layers. This population also appeared to project ventro-caudally to the POA. A second GAD-expressing cell population was seen in the POA and the ventral hypothalamus dorsal to the neurohypophysis in decreased signal as compared to GAD expression in the olfactory bulb. GAD-expressing cells were clearly abundant in a third population in the medial hypothalamus extending along the dorsal and ventral periventricular arcuate nuclei to the anterior regions of the rhombencephalon. This population showed the strongest signal of all GAD cell populations indicating the potential of these cells to synthesize larger quantities of GABA. GAD-expressing neurons were also present in the dorsal thalamus bordering the third ventricle scattered between the ventral habenular region and the optic tectum in an arc-like pattern. This grouping of GAD-expressing cells appeared to then project caudo-ventrally to the medial region of the thalamus and joined the more densely populated GAD-expressing cell population extending to the rhombencephalon.

Lamprey GnRH-I and -III Co-expression. Lamprey GnRH-I mRNA labeled with a cyanine-3 tyramide fluorophore was detected at 570 nm emitting a bright red signal. Lamprey GnRH-III mRNA labeled with a fluorescein tyramide fluorophore was detected at 517 nm emitting a bright green signal. Lamprey GnRH-I mRNA was detected in the same regions of POA as seen using the colorimetric in situ hybridization method (fig. 4). This was also seen in lamprey GnRH-III mRNA (fig. 4). Fluorescent images for both GnRH forms taken on the same tissue section were overlayed. The yellow cells indicated co-expression of lamprey GnRH-I and -III (fig. 4), which was observed.

Lamprey GnRH and GAD Expression. Adult tissue sections treated with digoxigenin-labeled GAD and fluorescein-labeled GnRH-III showed GAD-expressing cell populations in close proximity to GnRH-expressing cell populations in the preoptic area and in the ventral hypothalamus, however the mRNAs did not show co-expression (fig. 4).



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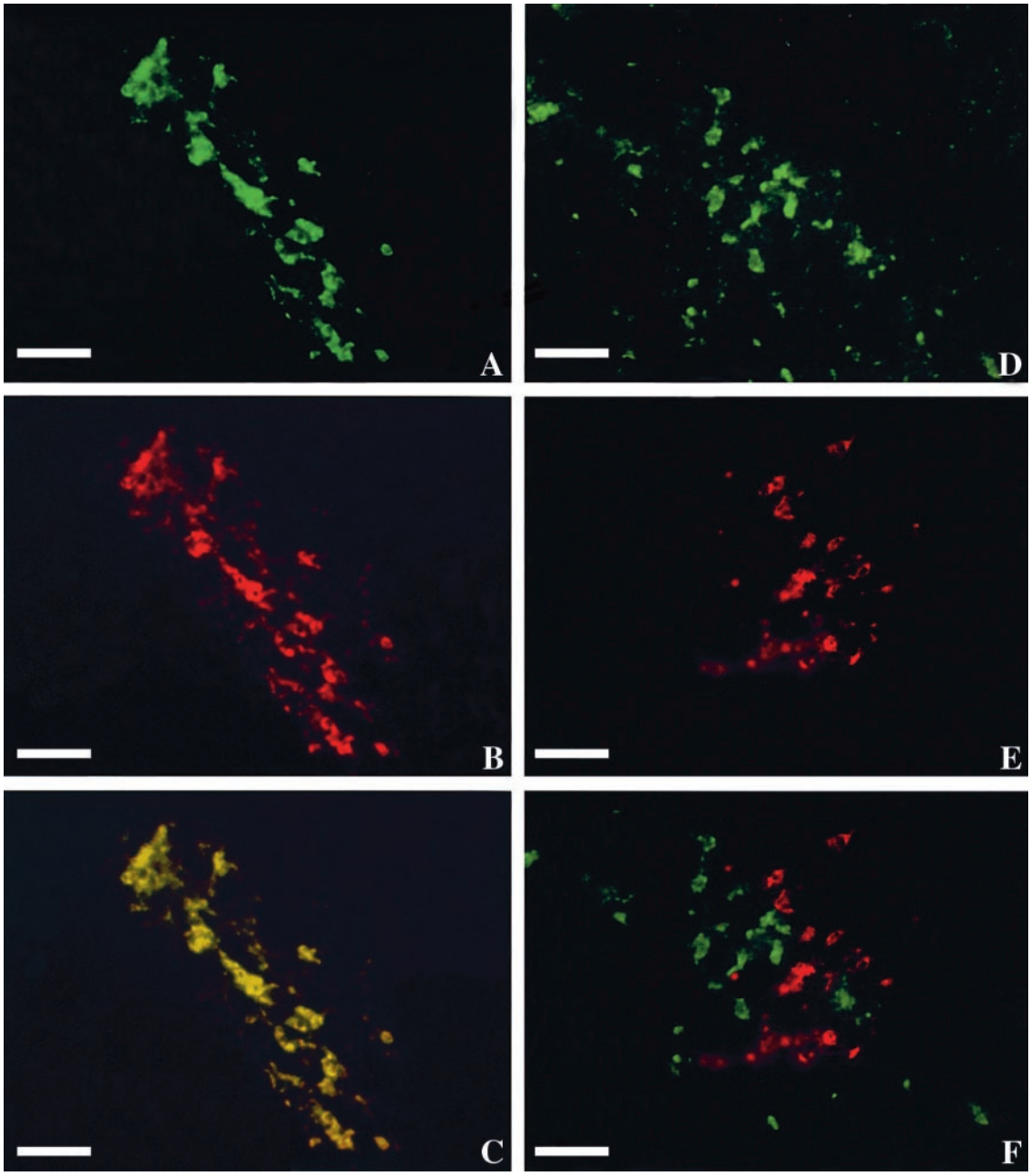

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Fig. 3. DIG-labeled in situ hybridization for lamprey GAD in adult, parasitic and juvenile lamprey. Digital photographs of sagittal tissue sections from adult (**A–D**), parasitic (**J–L**) and juvenile (**H, I**) sea lamprey brains, and coronal tissue sections from adult (**E–G**) sea lamprey, showing distinct cell populations expressing lamprey GAD mRNA. Reaction product was detected in the olfactory bulb (**A**), preoptic nucleus and ventral hypothalamus (**B**), dorsal thalamus extending to the base of the habenular region (**C**), and ventral and dorsal periventricular arcuate nuclei (**D**). Coronal sections correspond to figure 1 as follows: **E = A, F = B, G = C.** Scale bars = 25 μm.

Fig. 4. Dual-label in situ hybridization for lamprey GnRH-I and -III (**A–C**), and lamprey-III and GAD (**D–F**). Digital photographs of sagittal tissue sections from adult sea lamprey brains, showing distinct cell populations expressing lamprey GnRH and GAD. Tissue sections were treated with fluorescein-labeled lamprey GnRH-III (**A**, **D**) and digoxigenin-labeled lamprey GnRH-I (**B**) or GAD (**E**). Co-expression was detected (**C**) in approximately 70–80% of lamprey GnRH-expressing cells, however no co-expression was detected between lamprey GnRH-III and GAD (**F**). Scale bars = 25 μm.

Discussion

Data obtained in this study and previous immunocytochemical studies for lamprey GnRH-I and -III in adult and larval lamprey have consistently shown localization in the same regions of the POA. This has led to the hypothesis that the two forms of GnRH in the lamprey are co-expressed in the same cell. This study shows that lamprey GnRH-I and -III are expressed in the same cells of the preoptic region of adult sea lamprey, therefore implying that the mature proteins are co-localized. In the adult sea lamprey brain, cells expressing lamprey GnRH-I and lamprey GnRH-III mRNA were found in the arc-shaped preoptic-anterior region and the ventral hypothalamic area. Lamprey GnRH-I and -III mRNA expression was also seen in the same regions of the hypothalamus in larval lamprey and juvenile parasitic lamprey. Dual-label in situ hybridization also showed close proximity of GAD mRNA-containing neurons and GnRH-containing neurons in the POA.

The present study establishes that both forms of lamprey GnRH are expressed during the larval, parasitic, and adult phases of the lamprey life cycle. The level of expression was indistinguishable between the two GnRH mRNAs in all adult male and female lampreys tested. These data along with previous ICC and physiological studies support the hypothesis that both forms are functional and play active roles in the hypophysiotropic axis in the lamprey [Sower et al., 1993; Sower, 1997, 2003; Silver et al., 2001]. In larval lamprey, lamprey GnRH-III expression was more abundant than lamprey GnRH-I as was demonstrated in a study by Tobet et al. [1995], which showed that the majority of immunoreactive GnRH in the brain was lamprey GnRH-III, and when lamprey GnRH-I was seen, it was in cells that appeared to contain both forms of GnRH. This suggested that lamprey GnRH-III is the primary functional form of GnRH during the larval stage of the lamprey life cycle. The study by Tobet et al. [1995] was the first to use multiple antisera in larval sea lamprey directed at both lamprey GnRHs, and based on the similarities in staining between the two forms, the authors proposed that lamprey GnRH-I and -III were co-localized in the same cells. An ICC study in adult sea lamprey [Nozaki et al., 2000] showed similar patterns of GnRH localization to the larval studies of Tobet et al. [1995]. However, based on cell structure data Nozaki et al. [2000] suggested that the two forms were not co-localized, but rather were the product of different hypothalamic neurons. Using dual-label fluorescence in situ hybridization, the present study shows that lamprey GnRH-I and -III are co-expressed and therefore co-localized within the same cell.

Many factors have been identified in vertebrates that are able to modulate reproductive events through their influence on the hypothalamic-pituitary-gonadal axis. One major factor is GABA, which has been found to have an inhibitory action on GnRH neurons in vertebrates [Jarry et al., 1991; Scott and Clark, 1993; Mitsushima et al., 1994]. A fundamental question that has arisen however, regards the steroid effects on GnRH neurons and whether these effects are exerted directly or indirectly [Petersen et al., 2003]. There is new evidence that in addition to steroids acting indirectly on GnRH neurons through other systems such as GABA, steroids can act directly on GnRH neurons [Petersen et al., 2003]. In lampreys, there is estradiol feedback on hypothalamic GnRH [Sower, 1997], estrogen binding has been identified in the hypothalamic region [Kim et al., 1980, 1981] and an estrogen receptor has been cloned [Thornton, 2001], but there have been no estrogen expression studies performed in lamprey brains. The expression data from the current study suggest an interrelationship between GABA and GnRH neurons. However, whether the steroid feedback occurs directly or indirectly via GABA or some other neurotransmitter system will require further study. It is conceivable that feedback effects of GABA are a regulatory mechanism that occurs throughout all vertebrates.

In the present study, GAD-expressing cells were distributed in several populations throughout the adult sea lamprey brain. A population of GAD-expressing cells was localized in the olfactory bulb of the telencephalon, a second smaller cell population was seen in the ventral anterior hypothalamic region, and a third larger cell population was identified, stretching from the medial ventral hypothalamus and neurohypophysis along the dorsal and ventral divisions of the periventricular arcuate nucleus to the anterior region of the rhombencephalon. GAD-expressing cells were also detected in the dorsal thalamus, widely scattered between the habenular region and the optic tectum. A similar distribution of GAD populations was observed in the larval and metamorphosing parasitic lampreys although the reaction product did not appear to be as concentrated as in adults.

These data indicate a possible relationship of the GnRH- and GABA-expressing neurons in the ventral hypothalamus. Dark reaction product was detected in the hypothalamus near the preoptic region stretching along the ventral hypothalamus and neurohypophysis in the adult sea lamprey. In the parasitic lamprey brain, GAD-expressing neurons were detected in this same region of

the lamprey hypothalamus along the neurohypophysis. In the ammocoete, GAD mRNA was seen in the developing medial and ventral hypothalamic regions. What is significant is that these GAD-expressing cells were observed in similar regions of the hypothalamus as GnRH-expressing cell populations in all three lamprey life stages. Under the fluorescent microscope, GAD-expressing cells were seen remarkably close to those populations expressing GnRH in the preoptic area suggesting direct interaction between these neurons. These data are in agreement with previous immunocytochemistry data from Reed et al. [2002] where GABA-immunoreactive neurons were detected near GnRH-immunoreactive neurons. This possible interaction between GABAergic neurons and GnRH neurons is further supported by recent in vitro and in vivo studies. In vitro administration of muscimol (GABA receptor A agonist) in adult female sea lampreys showed an increase in GnRH-III release compared to controls, whereas in vivo administration of GABA and muscimol showed an increase of both forms of GnRH compared to controls in adult female sea lamprey brains [Root et al., 2003]. These data suggest that GABA has a direct action on GnRH neurons as a neurotransmitter.

The occurrence of GAD mRNA expression in the forebrain of the sea lamprey is supported by previous immunocytochemical studies for GABA in larval [Melendez-Ferro et al., 2001, 2002; Reed et al., 2002] and adult [Pombal et al., 1997; Pombal and Puelles, 1999] lamprey, by a GAD microassay study [Wald et al., 1981], and by GAD in situ hybridization studies in other fish [Anglade et al., 1999]. These studies have shown collectively that both GABA and GAD are present and that GAD is functionally active in the sea lamprey. Melendez-Ferro et al. [2002] and Reed et al. [2002] separately demonstrated that GABA is present in the forebrain of the embryonic and larval lamprey, appearing in the telencephalon and diencephalon 20 days after fertilization. In the olfactory bulb, Melendez-Ferro et al. [2001] reported that at least 5 types of cells containing GABA were present in all of the olfactory layers, with the majority present in the glomerular layer and the regions surrounding the olfactory nerve. In the present study, GAD-expressing cells were detected in the glomerular layer and mitral and granular cell layers agreeing well with data presented by Melendez-Ferro et al. [2001] with regard to distribution and intensity. These data suggested that GABA is involved in processing in the olfactory bulb of lampreys. GAD-expressing cells located in the dorsal thalamus near the optic tectum suggest a possible role for GABA as a neurotransmitter affecting the optic nerve in the lamprey. Neurons projecting to the medial hypothalamus and eventually to the rhombencephalon support a role in the oculomotor system as suggested by Melendez-Ferro et al. [2000]. Although these data offer support to the above hypotheses, they are not definitive evidence and as such, further research is needed.

In conclusion, the present study has shown that lamprey GnRH-I and -III are expressed in the POA and hypothalamus of larval, juvenile parasitic, and adult sea lamprey, and that lamprey GnRH-I and -III are co-expressed in the adult sea lamprey. This study has also shown that GAD mRNA is expressed in four distinct cell populations in the lamprey brain, ranging from the telencephalon and diencephalon of the forebrain to the mesencephalon and rhombencephalon of the midbrain and hindbrain. The close distribution of GAD and lamprey GnRH in the preoptic region also reported here further supports the hypothesis that GABA might act on the reproductive axis through the feedback on GnRH neurons.

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