Co-localization of three gonadotropin-releasing hormone transcripts in larval, parasitic, and adult sea lamprey brains

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ABSTRACT
RNA expression of lamprey gonadotropin-releasing hormone (lGnRH)-I, -II, and -III was demonstrated in the brains of larval, parasitic phase and adult sea lampreys, Petromyzon marinus, using a highly sensitive triple-label in situ hybridization technique. In female larval lampreys, lGnRH-I and -II were co-expressed in the same neurons throughout the olfactory bulbs, preoptic area (POA), and rhombencephalon (hindbrain); lGnRH-I, -II and -III were triple co-expressed in the hypothalamus and in the paranuclear region of neuronal somas in the rhombencephalon. In female parasitic phase lampreys, lGnRH-I and -II were co-expressed in the POA, thalamus, and preoptico-neurohypophyseal tract (PNT); lGnRH-III was minimally triple co-expressed with lGnRH-I and -II in the hypothalamus. In adult female lampreys, lGnRH-I and -II were co-expressed in the hypothalamus; lGnRH-I was also expressed in the neurohypophysis (NH). In adult male lampreys, lGnRH-I and -III were co-expressed in the primordial hippocampus, POA, thalamus, hypothalamus, NH, and PNT; lGnRH-I was also expressed in the epithalamus. In summary, we provide the first study using in situ hybridization of all three lGnRHs (lGnRH-I, -II, and -III) at three major life stages (larval, parasitic, and adult) of lampreys, which strongly supports previous immunohistological studies and suggests that lGnRH-I and -II are the predominant lGnRHs in larval and parasitic phase lampreys, and that lGnRH-I and -III are the predominant lGnRHs in adult female and male lampreys. Therefore, our results show that lGnRH-I, -II, and -III have different localization and co-expression in the development and sexual maturation of lampreys, which may suggest unique physiological roles at each life stage and sex in the developing and mature lamprey brain.

1. Introduction

Sea lampreys, Petromyzon marinus, begin life during a freshwater larval phase (5–7 years) as ammocoetes, undergo a dramatic metamorphosis and transition to a salt-water parasitic phase (1–2 years), mature into adults, and return to freshwater streams where they spawn and die (Hardisty and Potter, 1971). Along with hagfish, lampreys represent the agnathans (jawless vertebrates), whose lineage arose around 550 million years ago (Forey and Janvier, 1994). Generally, gnathostomes (jawed vertebrates) have one or two hypothalamic GnRHs; some teleosts may have up to three hypothalamic GnRHs (Decatur et al., 2013; Plachetzki et al., 2016). In comparison, lampreys have three hypothalamic GnRHs (lamprey (l)GnRH-I, -II and -III) (Kavanaugh et al., 2008; Sherwood et al., 1986; Sower et al., 1993). Each of the GnRHs exhibits differences in distribution and expression, which suggests that there are likely species-specific physiological function(s) of each peptide.

In gnathostomes, GnRH-1 neurons are primarily located in the preoptic area (POA) of the hypothalamus (Chen and Fernald, 2008; Fernald and White, 1999; Guilgur et al., 2006; Kah et al., 2007). GnRH-2 (chicken GnRH-2) neurons predominate in extrahypothalamic regions (e.g., the midbrain and numerous peripheral tissues), although in some species, GnRH-2 is found in the hypothalamic region (Guilgur et al., 2006; Kah et al., 2007). GnRH-3 (salmon GnRH-3) neurons are located in the terminal
nerve ganglion near the olfactory bulb (Guilgur et al., 2006; Kah et al., 2007). As reviewed in Sower (2015), lampreys have three distinct hypothalamic GnRhs, IgNhR-I (Type GnRH 3), -II (Type GnRH 2), and -III (Type GnRH 3) (Decatur et al., 2013). Unlike most other vertebrates, the expression of IgNhR-I, -II and -III in lamprey brains shows a general pattern of GnRH distribution in the anterior preoptic-neurohypophysial tract to the neurohypophysis of adult lampreys (Sower, 2015). Prominent changes in two of the three GnRhs (IgNhR-I and -III) have been demonstrated throughout the sea lamprey life cycle, including embryogenesis, larval development, and metamorphosis (Reed et al., 2002; Tobet et al., 1995; Youson et al., 1995; Youson and Sower, 1991), which is consistent with several studies that have shown that GnRH activity and distribution are not restricted to the final reproductive phase (Reed et al., 2002; Tobet et al., 1995; Root et al., 2005; Tobet et al., 1997; Wright et al., 1994). Similar trends have subsequently been documented in other lamprey species, as reviewed in Sower (2015).

The transcripts and proteins of IgNhR-I and -II were previously shown to be localized in the hypothalamus and preoptic area of larval and parasitic phase sea lampreys, as well as in neurons originating in the preoptic area, traversing the hypothalamus, and terminating in the neurohypophysis in adult sea lamprey brains (Reed et al., 2002; Tobet et al., 1995; Root et al., 2005; Wright et al., 1994; King et al., 1988; Nozaki et al., 2000). Lamprey IgNhR-II had not yet been discovered when these in situ hybridization (ISH) and immunohistochemistry (IHC) studies were completed. IgNhR-II was identified in 2008 and subsequent expression studies using ISH and IHC of IgNhR-II were performed in adult sea lamprey (Kavanaugh et al., 2008). In that study, IgNhR-II RNA transcript was widely expressed in the hypothalamus, medulla, paraventricular region, and olfactory regions; however, IgNhR-II protein was localized to the neurons coursing from the preoptic area to the neurohypophysis. The distributions of ir-IgNhR-II neurons and fibers were quite similar to the distributions of IgNhR-I and -III neurons, forming the characteristic GnRH preoptico-neurohypophysial tract in sea lampreys (Kavanaugh et al., 2008; King et al., 1988; Nozaki et al., 2000). However, until the current study, the expression of IgNhR-II had not been compared to IgNhR-I and -III.

This is the first study to examine and report the distribution of the dual and triple co-localization and co-expression of all three lamprey GnRH transcripts, IgNhR-I, -II, and -III, in the lamprey brain at the three major life stages, female larval, female parasitic phase, and adult female and male lampreys.

2. Materials and methods

2.1. Lampreys

Adult sea lampreys were collected from the fish ladder on the Cocheco River (Dover, NH, USA) from mid-May to mid-June 2015 during their upstream spawning migration from the ocean to coastal rivers. The lampreys were transported to the Anadromous Fish and Aquatic Invertebrate Research (AFAIR) Laboratory, a freshwater fish facility at the University of New Hampshire (UNH, Durham, NH, USA), and maintained in an aerated artificial spawning water fish facility at the University of New Hampshire (UNH, Durham, NH, USA), and maintained in an aerated artificial spawning water fish facility at the University of New Hampshire (UNH, Durham, NH, USA) on 9 µm sections of female ammocoete, female parasitic phase, and adult female and male gonads. The slides were visually examined on an Olympus BH2 light microscope (Olympus, Center Valley, PA) to determine the stage of gonadal maturation, as previously described (Bolduc and Sower, 1992; Fahien and Sower, 1990; Sower et al., 1985).

2.2. Tissue preparation and storage

For each sex and stage of lampreys (larval, parasitic and adult) there was one brain and pituitary sample that was dissected and prepared, as previously described (Marquis et al., 2017). Briefly, the tissues were fixed in paraformaldehyde, cryoprotected in sucrose, embedded in Tissue-Tek® OCTTM (Optimal Cutting Temperature) Compound (Sakura Finetek USA, Torrance, CA, USA), and stored at −80°C until use.

2.3. Triple-label fluorescent in situ hybridization (FISH)

Triple-label FISH was performed using an RNAscope® Multiplex Fluorescent Reagent Kit (Advanced Cell Diagnostics, Hayward, CA, USA) according to the manufacturer’s protocol and optimized, as previously described (Marquis et al., 2017). Target probes were designed using custom software (Wang et al., 2012). GenBank accession numbers and probe regions are: Pma-GnRH1, AF144480.1, region 21–650; Pma-GnRH2, DQ457017.1, region 2–356; Pma-GnRH3, AY052628.1, region 2–706. Briefly, hundreds of serial 14 µm sagittal sections of fixed frozen female larval, female parasitic phase, and adult female and male lamprey brains and pituitaries were assayed, as previously described (Marquis et al., 2017), with the following modifications: the slides were hybridized with target probes for IgNhR-I, IgNhR-II, and IgNhR-III (pre-mixed) or a three-plex negative control probe (Advanced Cell Diagnostics, Hayward, CA). Confocal images of representative expression were acquired, as previously described (Marquis et al., 2017). RNA staining signal was identified as red (IgNhR-I), green (IgNhR-II), and blue (IgNhR-III) punctate dots.

2.4. Gonadal histology staging

Routine hematoxylin and eosin staining was performed by the New Hampshire Veterinary Diagnostic Laboratory (UNH, Durham, NH, USA) on 9 µm sections of female ammocoete, female parasitic phase, and adult female and male gonads. The slides were visually analyzed on an Olympus BH2 light microscope (Olympus, Center Valley, PA) to determine the stage of gonadal maturation, as previously described (Bolduc and Sower, 1992; Fahien and Sower, 1990; Sower et al., 1985).

3. Results

3.1. Gonadal histology staging

The ovaries were in the oogonia and early vitellogenic stages for the female larval and female parasitic phase lamprey, respectively. The adult female and male lampreys were in the final stages of gonadal maturation.

3.2. Fluorescent in situ hybridization (FISH)

In female larval lampreys, IgNhR-I was highly expressed in the olfactory bulb (OB) (Fig. 1: A, B1), preoptic area (POA) (Fig. 1: A, C1), hypothalamus (Fig. 1: A, D1; Table 1), and rhombencephalon (Fig. 1: A, E1). IgNhR-II was highly expressed in the POA (Fig. 1: A, C2), and moderately expressed in the OB (Fig. 1: A, B2), hypothalamus (Fig. 1: A, D2), and rhombencephalon (Fig. 1: A, E2) in larval lampreys. IgNhR-III was minimally expressed only in the hypotha-
Fig. 1. Triple-label FISH of lGnRH-I, -II, and -III in female larval lamprey. lGnRH-I (red) was highly expressed in the OB (A, B1), POA (A, C1), Hyp (A, D1), and Rho (A, E1). lGnRH-II (green) was highly expressed in the POA (A, C2), and moderately expressed in the OB (A, B2), Hyp (A, D2), and Rho (A, E2). lGnRH-III (blue) was minimally expressed in the Hyp (A, D3) and Rho (A, E3). lGnRH-I and -II were highly co-expressed (yellow) in the OB (A, B4), POA (A, C4), Hyp (A, D4), and Rho (A, E4). lGnRH-III was also triple co-expressed with lGnRH-I and -II (light teal) in the perinuclear region of a neuronal soma in the Rho (A, E1-E8). Neurons from the OB (B7), POA (C7), Hyp (D7), and Rho (E7) are outlined with white dotted lines. Probe specificity was confirmed by a negligible background signal in negative controls in the OB (B9), POA (C9), Hyp (D9), and Rho (E9). Arrowheads in merged and individual images indicate the regions of co-expression of the lGnRHs, lGnRH-I, -II, and -III. Scale bars (A) 100 µm, (b-d) 20 µm. GnRH gonadotropin-releasing hormone, OB olfactory bulb, Pr.Hip primordial hippocampus, POA preoptic area, Epi epithalamus, Thal thalamus, Hyp hypothalamus, NH neurohypophysis (posterior pituitary), PNT preoptico-neurohypophyseal tract, Rho rhombencephalon, P pineal, PON preoptic nucleus, OC optic chiasm, RPD rostral pars distalis of adenohypophysis (AH, anterior pituitary), PPD proximal pars distalis of AH, PI pars intermedia of AH, III third ventricle.
Fig. 2. Triple-label FISH of lGnRH-I, -II, and -III in female parasitic phase lamprey. lGnRH-I (red) was highly expressed in the Thal (A, E1) and Hyp (A, D1), and moderately expressed in the POA (A, B1) and PNT (A, C1). lGnRH-II (green) was moderately expressed in the POA (A, B2) and Hyp (A, D2), and minimally expressed in the Thal (A, E2) and PNT (A, C2). lGnRH-III (blue) was minimally expressed only in the Hyp (A, D3). lGnRH-I and -II were moderately co-expressed (yellow) in the POA (A, B4), Thal (A, E4), Hyp (A, D4), and PNT (A, C4). lGnRH-III was minimally triple co-expressed (light teal) with lGnRH-I and -II in the Hyp (A, D1-D8). Neurons from the POA (B7), PNT (C7), Hyp (D7), and Thal (E7) are outlined with white dotted lines. Probe specificity was confirmed by a negligible background signal in negative controls in the POA (B9), PNT (C9), Hyp (D9), and Thal (E9). Scale bars (A) 100 µm, (b-d) 20 µm. For abbreviations, see Fig. 1.
In larval parasitic phase lampreys, lGnRH-I was highly expressed in the thalamus (Fig. 2: A, E1) and hypothalamus (Fig. 2: A, D1), and moderately expressed in the POA (Fig. 2: A, B1) and preoptic-neurohypophyseal tract (PNT) (Fig. 2: A, C1). lGnRH-II was moderately expressed in the POA (Fig. 2: A, B2) and hypothalamus (Fig. 2: A, D2), and minimally expressed in the thalamus (Fig. 2: A, E2) and PNT (Fig. 2: A, C2) in parasitic phase lampreys. lGnRH-III was minimally expressed only in the hypothalamus (Fig. 2: A, D2) in parasitic phase lampreys. lGnRH-I and -II were moderately co-expressed in the POA (Fig. 2: A, B4), thalamus (Fig. 2: A, E4), hypothalamus (Fig. 2: A, D4), and PNT (Fig. 2: A, C4) in parasitic phase lampreys. lGnRH-III was minimally triple co-expressed with lGnRH-I and -II in the hypothalamus (Fig. 2: A, D1-D8) in parasitic phase lampreys. Regions of co-expression (Fig. 2: B8, C8, D8, E8) are magnified in (Fig. 2: B7, C7, D7, E7), respectively.

In female parasitic phase lampreys, lGnRH-I was highly expressed in the OB (Fig. 1: A, B4), POA (Fig. 1: A, C4), hypothalamus (Fig. 1: A, D4), and rhombencephalon (Fig. 1: A, E4) in larval lampreys. lGnRH-I and -II were highly co-expressed in the OB (Fig. 1: A, B4), POA (Fig. 1: A, C4), hypothalamus (Fig. 1: A, D4), and rhombencephalon (Fig. 1: A, E4) in larval lampreys. lGnRH-III was also triple co-expressed with lGnRH-I and -II in the perinuclear region of a large neuronal soma in the rhomencephalon (Fig. 1: A, E1-E8) in larval lampreys. Regions of co-expression (Fig. 1: B8, C8, D8, E8) are magnified in (Fig. 1: B7, C7, D7, E7), respectively.

In adult female lampreys, lGnRH-I was highly expressed in the hypothalamus (Fig. 3: A, B1) and NH (Fig. 3: A, C1) only. lGnRH-II was not expressed anywhere in the brain of adult female lampreys. lGnRH-III was moderately expressed only in the hypothalamus (Fig. 3: A, B3) in adult female lampreys. lGnRH-I and -III were moderately co-expressed only in the hypothalamus (Fig. 3: A, B3) of adult female lampreys. There was no extra-hypothalamic expression of any of the lGnRHs in adult female lampreys. Regions of co-expression (Fig. 3: B8, C8) are magnified in (Fig. 3: B7, C7), respectively.

In adult male lampreys, lGnRH-I was highly expressed in the primordial hippocampus (Pr.Hip) (Fig. 5: A, F1), POA (Fig. 4: A, B1), epithalamus (Fig. 5: A, C1), thalamus (Fig. 5: A, D1), hypothalamus (Fig. 4: A, D1), NH (Fig. 4: A, E1), and PNT (Fig. 4: A, C1). lGnRH-II was not expressed anywhere in the brain of adult male lampreys. lGnRH-III was highly expressed in the Pr.Hip (Fig. 5: A, F3), POA (Fig. 4: A, B3), thalamus (Fig. 5: A, H3), hypothalamus (Fig. 4: A, D3), and NH (Fig. 4: A, E3), and moderately expressed in the PNT (Fig. 4: A, C3) in adult male lampreys. lGnRH-I and -III were highly co-expressed in the Pr.Hip (Fig. 5: A, F1, F3, F5), POA (Fig. 4: A, B1, B3, B5), thalamus (Fig. 5: A, H1, H3, H5), hypothalamus (Fig. 4: A, D1, D3, D5), and NH (Fig. 4: A, E1, E3, E5), and moderately co-expressed in the PNT (Fig. 4: A, C1, C3, C5) of adult male lampreys. Regions of co-expression (Fig. 4: B8, C8, D8, E8; Fig. 5: B8, C8, D8, E8) are magnified in (Fig. 4: B7, C7, D7, E7), respectively.

![Image](image_url)

**Fig. 3.** Triple-label FISH of lGnRH-I, -II, and -III in adult female lamprey. lGnRH-I (red) was highly expressed in the Hyp (A, B1) and NH (A, C1) only. lGnRH-II was not expressed (no green) anywhere in the brain. lGnRH-III (blue) was moderately expressed only in the Hyp (A, B3). lGnRH-I and -II were moderately co-expressed (purple) only in the Hyp (A, B5). Neurons from the Hyp (B7) and NH (C7) are outlined with white dotted lines. Probe specificity was confirmed by a negligible background signal in negative controls in the Hyp (B9) and NH (C9). Scale bars (A) 100 µm, (b-d) 20 µm. For abbreviations, see Fig. 1.

Fig. 4. Triple-label FISH of lGnRH-I, -II, and -III in adult male lamprey. lGnRH-I (red) was highly expressed in the POA (A, B1), Hyp (A, D1), NH (A, E1), and PNT (A, C1). lGnRH-II was not expressed (no green) anywhere in the brain. lGnRH-III (blue) was highly expressed in the POA (A, B3), Hyp (A, D3), and NH (A, E3), and moderately expressed in the PNT (A, C3). lGnRH-I and -III were highly co-expressed (purple) in the POA (A, B1, B3, B5), Hyp (A, D1, D3, D5), and NH (A, E1, E3, E5), and moderately co-expressed in the PNT (A, C1, C3, C5). Neurons from the POA (B7), PNT (C7), Hyp (D7), and NH (E7) are outlined with white dotted lines. Probe specificity was confirmed by a negligible background signal in negative controls in the POA (B9), PNT (C9), Hyp (D9), and NH (E9). Scale bars (A) 100 μm, (b-d) 20 μm. For abbreviations, see Fig. 1.
Triple-label FISH of lGnRH-I, -II, and -III in adult male lamprey. lGnRH-I (red) was highly expressed in the Pr.Hip (A, F1), Epi (A, G1), Thal (A, H1). lGnRH-II was not expressed (no green) anywhere in the brain. lGnRH-III (blue) was highly expressed in the Pr.Hip (A, F3) and Thal (A, H3). lGnRH-I and -III were highly co-expressed (purple) in the Pr.Hip (A, F1, F3, F5) and Thal (A, H1, H3, H5). Neurons from the Pr.Hip (F7), Epi (G7) and Thal (H7) are outlined with white dotted lines. Probe specificity was confirmed by a negligible background signal in negative controls in the Pr.Hip (F9), Epi (G9) and Thal (H9). Scale bars (A) 100 μm, (b-d) 20 μm. For abbreviations, see Fig. 1.
4. Discussion

In this study, we are the first to report the dual and triple co-localization and co-expression of the three lamprey GnRHs, lGnRH-I, -II, and -III, at the three major life stages, female larval, female parasitic phase, and adult female and male sea lampreys. Former studies have focused extensively on the expression of one or two of the lGnRHs, predominantly in a single life stage. Using a new triple-label fluorescent in situ hybridization (FISH) assay system, RNAscope, we have shown that lGnRH-I and -II are the predominant lGnRHs in larval and parasitic phase lampreys and that they are co-localized and co-expressed, and that lGnRH-I and -III are the predominant lGnRHs in adult female and male lampreys and that they are co-localized and co-expressed. Thus, we provide new supporting evidence for stage-specific expression and distribution of each of the three lGnRHs likely having specific physiological roles in males and females.

In female larval sea lampreys, lGnRH-I and -II were localized to the olfactory bulb (OB), hypothalamus and rhombencephalon (hindbrain), and were highly co-expressed in the same neurons. Prior to the current study, lGnRH-II expression had not previously been studied in larval lampreys. lGnRH-III exhibited minimal expression in the hypothalamus, and thus the co-expression with lGnRH-I and -II in this region was limited. lGnRH-III was clearly localized in the perinuclear region of a large neuronal soma in the rhombencephalon and was highly co-expressed with lGnRH-I and -II in the soma. This is consistent with earlier studies that showed lGnRH-I transcript and protein expression in the hypothalamus and neurohypophysis, and lGnRH-I protein expression in the preoptic area (POA)/rostral hypothalamus (Reed et al., 2002; Root et al., 2005; Wright et al., 1994). In the larval phase of the pouched lamprey, Geotria australis, a member of the Southern Hemisphere lamprey, ir-lGnRH-I was not as widely distributed in the brain as compared to larval sea lamprey; in this species, lGnRH-I was restricted to the dorsal POA, with simple tracts projecting only to the rostral and caudal NH (Sower et al., 2000). These distinctions may reflect differences in species, water temperature of the lampreys, and/or particular stage of development.

In female parasitic phase lampreys, lGnRH-I and -II transcripts were expressed in the POA, thalamus, hypothalamus, and preoptico-neurohypophyseal tract, and exhibited co-expression in the same neurons in these regions. lGnRH-III was also minimally expressed in the hypothalamus, and was co-localized with lGnRH-I and -II. These results are similar to the distribution of lGnRH-I protein noted in parasitic phase sea lampreys (Wright et al., 1994). Our results are also similar to Youson and Sower (1991), who reported that sea lampreys in the late stages of metamorphosis and transitioning into the parasitic phase exhibited a significant elevation of lGnRH-I, and to a lesser extent, lGnRH-III (Sherwood et al., 1986; Sower et al., 1993). Prior to the current study, there had not been any previous reports on lGnRH-II and -III expression in parasitic phase lamprey brains. The earlier reported studies all used lampreys in the early parasitic phase (approximately the first six months of parasitic phase). There have not been any studies examining expression of GnRHs in lampreys in the later parasitic phase (after six months of this phase). Further studies will be needed to examine not only the expression of the GnRHs and respective receptors, but also the functions of each GnRH during the duration of the parasitic phase, which can span up to two or three years (Sower, 2003).

Table 1
Summary table of expression results from current (highlighted in yellow) and past studies. FISH fluorescent in situ hybridization, ISH in situ hybridization, IHC immunohistochemistry, lGnRH lamprey gonadotropin-releasing hormone, lGnRH-I, -II, -III, OB olfactory bulb, Pr.Hip primordial hippocampus, POA preoptic area, Epi epithalamus, Thal thalamus, Hyp hypothalamus, NH neurohypophysis (posterior pituitary), PNT preoptico-neurohypophyseal tract, Rho rhombencephalon. (Kavanaugh et al., 2008; Reed et al., 2002; Tobet et al., 1995; Root et al., 2005; Wright et al., 1994; King et al., 1988; Nozaki et al., 2000; Youson et al., 2006).

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In adult female lampreys, lGnRH-I and -III were co-expressed in the hypothalamus. In adult male lampreys, lGnRH-I and -III had much more widespread distributions; they were individually and co-expressed in the same neurons of the primordial hippocampus, POA, thalamus, hypothalamus, neurohypophysis (NH), and preoptico-neurohypophyseal tract; lGnRH-I was also localized separately in the epithalamus of adult male lampreys.

Our results are similar to previous studies that have shown localization and co-expression of lGnRH-I and -III transcripts and protein in the POA, hypothalamus, NH, and preoptico-neurohypophyseal tract in sea lamprey and American brook lamprey, Lampetra appendix (Kavanaugh et al., 2008; Reed et al., 2002; Tobet et al., 1995; Root et al., 2005; Wright et al., 1994; King et al., 1988; Nozaki et al., 2000; Youson et al., 2006). However, in contrast to these studies, there is one reported study in which lGnRH-III cells appeared to be distinct from lGnRH-I cells in adult sea lampreys (Nozaki et al., 2000). We suggest from the current study, that it is likely that there are cells that express either both forms of GnRH or individually express one GnRH. lGnRH-II transcript was not present in adult female or male lamprey brains in the current study. This is contrast to our previous study in which lGnRH-II transcript was expressed in the OB, POA, and rhombencephalon with lGnRH-II protein being expressed in the POA, hypothalamus, and NH (Kavanaugh et al., 2008). The timing of the sampling and/or water temperature of these adults for determination of lGnRH-II could have produced different results.

Sower et al. (2011) showed that brain GnRH concentrations, measured by high performance liquid chromatograph (HPLC) and radioimmunoassay (RIA), fluctuated throughout the summer, from May to July, in correlation with temperature in male and female adult sea lampreys. In this same study, lGnRH-II protein concentrations displayed a sharp decrease from the middle of May to the end June in females; a similar trend was seen in males, but with an additional spike and subsequent decrease in the middle of June. Furthermore, when lampreys were sampled at 15°C, the average lGnRH-II concentrations in male and female brains were below two nanograms (Sower et al., 2011). In the current study, adult lampreys were sampled at 15°C, which may be the reason that lGnRH-II expression was not detected in the adult lampreys. Whether the differences or lack of expression are due to the timing of sampling, water temperature, and/or use of different expression techniques is not known.

Unlike most other vertebrates, the expression of lGnRH-I, -II and -III in lamprey brains frequently shows a distinctive pattern of GnRH distribution in the preoptico-neurohypophyseal tract (Sower, 2015). Although extra-hypothalamic lGnRH mRNA transcript expression has previously been reported in sea lampreys, lGnRH protein expression has been consistently shown to be more highly localized to the preoptico-neurohypophyseal tract (Kavanaugh et al., 2008; Sower, 2015). Therefore, in lampreys, translation of the lGnRH transcripts into protein may occur only in hypothalamic regions and not in extra-hypothalamic regions. Additional triple-label IHC and functional studies are needed in order to determine if the lGnRH proteins are co-expressed in larval, parasitic phase, and adult sea lamprey brains, and to fully understand the role of each lGnRH in sea lamprey physiology and reproduction.

The presence of at least two co-localized GnRHs at each of three major life stages of lampreys is relatively unique among vertebrates. In general, two or three GnRHs within any one species typically have distinct distributions throughout the brain (Guilgur et al., 2006; Kah et al., 2007). However, the sea lamprey is unique from most vertebrates because it only spawns once in its lifetime and then dies. There are few studies that have documented and suggested functional roles for the co-expression of multiple GnRHs. Co-expression of two GnRHs has been reported in non-mammalian vertebrates, including African catfish (Bogerd et al., 1992; Zandbergen et al., 1995) and frog (Rana ridibunda) (Collin et al., 1995). GnRH-1 (catfish GnRH-1) and GnRH-2 (chicken GnRH-2) were co-localized in the anterior hypothalamic neurosecretory cells of African catfish, and were co-expressed in the same fibers and neurosecretory granules of the pars distalis of the anterior pituitary in close proximity to gonadotropic cells (Bogerd et al., 1992; Zandbergen et al., 1995). Collin et al. (1995) reported the co-localization of GnRH-1 (mammalian GnRH-1) and GnRH-2 (chicken GnRH-2) in the septal-anterior preoptic area in R. ridibunda. These authors suggested that co-expression of GnRHs may activate distinct secondary messenger pathways and/or distinct GnRH receptors at different anterior pituitary cell types (Zandbergen et al., 1995; Chang and Jobin, 1991).

The actions of lamprey GnRHs at different life stages depend in part upon the ligand-receptor interactions. In lampreys, there are three identified lGnRH receptors (lGnRH-R-1, -2, and -3) (Joseph et al., 2012; Silver et al., 2005; Silver and Sower, 2006). All three receptor transcripts are expressed in parasitic phase brain and adult brain and pituitary tissues (Hall et al., 2013). In these reported studies, only lGnRH-R-1 was expressed in the larval brain and pituitary. It is not known at this time whether lGnRH-II or -III can activate lGnRH-R-1 in parasitic phase lampreys, but it has been shown that lamprey GnRH-I, -II and -III can all activate IP3 production at lGnRH-R-1 (Joseph et al., 2012; Silver et al., 2005; Silver and Sower, 2006). In adult lampreys, quantitative in vitro autoradiography was used to characterize and localize GnRH receptors in the anterior pituitary of the adult female sea lamprey (Knox et al., 1994). Scatchard analysis revealed two classes of high-affinity binding sites with Kds of 1.5 × 10^{-12} M and 5 × 10^{-9} M for GnRH in the proximal pars distalis in adult sea lampreys (Knox et al., 1994). In addition to these binding studies, a novel cell type, called a proto-glycotrope, was discovered in the pituitary of the sea lamprey and shown to be the dominant cell type in the pars distalis and pars intermedia at all three major life stages in the sea lamprey; additionally, it is responsible for producing the subunits of the two lamprey glycoprotein hormones (lamprey glycoprotein hormone (lGPH) and thyrostimulin) (Marquis et al., 2017). Further studies will be needed to examine and understand the role of each of the lamprey GnRHs in regulating the pituitary and the synthesis and/or release of the glycoprotein hormones at each of the three major life stages of the lamprey.

In summary, we show that lGnRH-I, -II, and -III have different localization and co-expression in the development and sexual maturation of lampreys, which may suggest unique physiological roles at each life stage and sex in the developing and mature lamprey brain.

The collective evidence from extensive anatomical, expression, and functional studies suggests that each lGnRH may have unique functional role(s) throughout the complex and highly synchronized sea lamprey life cycle, reviewed in Sower (2015). The delineation of the hypothalamic and pituitary hormones and respective receptors in the control of reproduction and metabolism/development in lampreys will further allow us to understand the evolution of the neuroendocrine system from the perspective of a basal vertebrate.

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