



The interrelationship of estrogen receptor and GnRH in a basal vertebrate, the sea lamprey

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The hypothalamic-pituitary system is considered to be a vertebrate innovation and seminal event that emerged prior to or during the differentiation of the ancestral agnathans. Lampreys are the earliest evolved vertebrates for which there is a demonstrated neuroendocrine system. Lampreys have three hypothalamic gonadotropin-releasing hormones (GnRHs; lGnRH-I, -II, and -III) and two and possibly three pituitary GnRH receptors involved in mediating reproductive processes. Estradiol is considered to be a major reproductive steroid in both male and female lampreys. The purpose of this study was to investigate estrogen receptor (ER) expression in the lamprey brain in adult sea lampreys. Expression of ER mRNA was confirmed in the adult lamprey brain using RT-PCR. Using digoxigenin (DIG)-labeled probes, ER expression was shown to yield moderate, but distinct reaction products in specific neuronal nuclei of the lamprey brain, including the olfactory lobe, hypothalamus, habenular area, and hindbrain. Expression of ER in the hypothalamic area of the brain provides evidence of potential interaction between estradiol and GnRH(s), and is consistent with previous evidence showing estrogen feedback on GnRH in adult lamprey brain. Earlier studies have reported that there is a close distribution of glutamic acid decarboxylase (GAD; GABA-synthesizing enzyme) and lamprey GnRH in the preoptic region in adult lampreys. The establishment of a direct estradiol–kisspeptin–GABA–GnRH interaction in lamprey has yet to be determined and will require future functional and co-localization studies. The phylogenetic position of lampreys as a basal vertebrate allows lampreys to be a basis for understanding the molecular evolution of the neuroendocrine system that arose in the vertebrates.

Keywords: estrogen receptor, lamprey, GnRH, *in situ* hybridization, lamprey hypothalamus, estradiol, basal vertebrate, agnathan

INTRODUCTION

Modern vertebrates are classified into two major groups, the gnathostomes (jawed vertebrates) and the agnathans (jawless vertebrates). The agnathans are classified into two groups, myxinioids (hagfish) and petromyzonids (lampreys), while the gnathostomes constitute all other living vertebrates including the bony and cartilaginous fishes and the tetrapods. The hypothalamic-pituitary system is considered to be a vertebrate innovation and seminal event that emerged prior to or during the differentiation of the ancestral agnathans (Sower et al., 2009). Lampreys are the earliest evolved vertebrates for which there is a demonstrated neuroendocrine system. Lampreys have three hypothalamic gonadotropin-releasing hormone (GnRHs; lGnRH-I, -II, and -III) and two and possibly three pituitary GnRH receptors involved in mediating reproductive processes (Sower et al., 2009; Hall et al., 2011).

In lampreys, there is a general pattern of GnRH distribution in the anterior-preoptic-neurohypophysial tract to the neurohypophysis of adult lampreys as determined by several immunohistochemical (IHC) studies using specific GnRH antisera to each of the lamprey GnRHs and also by *in situ* hybridization studies using specific GnRH probes (Crim et al., 1979a,b; Nozaki and

Kobayashi, 1979; King et al., 1988; Nozaki et al., 1994; Reed et al., 2002; Kavanaugh et al., 2008). Data showing GnRH immunoreactive (ir) neurons in adult lampreys using IHC showed cells restricted to a single bilateral dense arc along the third ventricle in the rostral hypothalamus and preoptic area. Of all vertebrates, only the agnathan and teleosts lack a portal vascular system (median eminence) for transferring neurohormones from the hypothalamus to the adenohypophysis (Gorbman, 1965). Studies were done to experimentally examine the functional anatomical relationship between the hypothalamus and adenohypophysis in sea lamprey (Nozaki et al., 1994). It was shown that neurosecretory peptides like GnRH diffuse from the brain (neurohypophysis) to the adenohypophysis, and thus regulate its secretory activity in lampreys. Thus, there is evidence of normal occurrence of GnRH in a part of the lamprey brain homologous with that brain region in later evolved vertebrates in which GnRH localization forms part of a neuroendocrine mechanism for gonadotropin secretion. In other words, neurosecretory peptides like GnRH are able to diffuse from the brain to the pituitary to regulate its secretory activity. In addition, Crim (1981) and King et al. (1988) showed that GnRH neurons project into the third ventricle. These authors proposed an additional route of GnRH via secretion into the third

ventricle and transported by tanyocytes to the adenohypophysis (King et al., 1988).

There are several important brain neurohormones/factors that have been shown to stimulate/modulate GnRH and/or gonadotropin synthesis and/or release in vertebrates. In some teleosts, those neurohormones/factors include dopamine, neuropeptide Y, gamma-aminobutyric acid (GABA), and more recently gonadotropin-inhibitory hormone (GnIH) and kisspeptin (KiSS; Kah and Dufour, 2010). In lampreys, GABA and neuropeptide Y have been shown to be involved with brain GnRH and reproduction (Conlon et al., 1994; Root et al., 2004, 2005). Glutamic acid decarboxylase (GAD; GABA-synthesizing enzyme) mRNA was 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 in adult lamprey (Root et al., 2005). The close distribution of GAD and lamprey GnRH in the preoptic region supports the hypothesis that GABA might act on the reproductive axis through the feedback on GnRH neurons (Reed et al., 2002; Root et al., 2005).

Data supporting estrogen feedback on GnRH has been shown in lamprey. Sower (1997) observed that adult female lamprey injected with microencapsulated E2 experienced an elevation in GnRH concentrations, and activity of E2 in the hypothalamic region is supported *in vitro* autoradiography studies demonstrating binding of E2 in that area (Kim et al., 1980, 1981). However, it is unclear if the E2–GnRH interaction in lamprey occurs through a direct or an indirect mechanism. Within the preoptic anterior hypothalamus, co-localization studies of GABA and GnRH in lamprey have indicated GABA and GnRH-containing cells are closely associated (Reed et al., 2002; Root et al., 2005), supporting the possibility of a GABA-mediated (indirect) mechanism of E2–GnRH interaction in lamprey.

With the cloning of an estrogen receptor (ER), it is now possible to examine the expression of the receptor in the adult lamprey brain (Thornton, 2001). In contrast to teleost fish that have three types of ER (Hawkins et al., 2000; Menuet et al., 2002), lampreys apparently only have one ER (Thornton, 2001). Earlier studies in mammals suggested that the mechanism of E2 action on GnRH is largely indirect, and neurons of the anteroventral periventricular brain region that express the neurotransmitter, gamma amino butyric acid (GABA), are believed to be responsible for facilitating interaction between E2 and GnRH (Petersen et al., 2003). The discovery of kisspeptin and subsequent studies now suggest that steroid feedback may also be occurring on GnRH neurons via kisspeptin and its receptor (Roa et al., 2009).

The proximity of GnRH to GABAergic neurons and the effects of GABA/muscimol on GnRH support a role for GABA in the regulation of lamprey GnRH. However, such studies do not necessarily imply E2 involvement in GABA–GnRH interaction. Until recently, it was not clear that a classical ER existed in lamprey. Thornton's (2001) discovery and cloning of a lamprey provides an opportunity to enhance our understanding of the relationships between GABA, GnRH, and E2 in lamprey. Therefore, in the present study, we examined the expression patterns of ER in the adult sea lamprey, *Petromyzon marinus*, brain in relation to GnRH and GABA.

MATERIALS AND METHODS

COLLECTION AND SAMPLING

Adult female sea lampreys were collected from the fish ladder on the Cocheco River in Dover, New Hampshire. Collection of lamprey occurred in May and June during two successive seasons during the lamprey's upstream spawning migration from the ocean to coastal rivers. The lampreys were transported to the freshwater fish hatchery at the University of New Hampshire and maintained in an artificial spawning channel supplied with flow-through water from a nearby stream-fed reservoir at an ambient temperature range of 13–20°C, under natural photoperiod following the University of New Hampshire Institutional Animal Care and Use guidelines.

RT-PCR

Expression of ER was examined in the lamprey brain using RT-PCR, both for the purpose of verifying expression and to generate cDNA template for the synthesis of *in situ* hybridization probes. Expression was also assessed in ovary, testis, and muscle tissue for the purpose of comparison with expression in the brain. One adult, female lamprey was killed by decapitation. Tissues for were snap frozen in liquid nitrogen and stored at –80°C. Total RNA was isolated from ~100 mg of each tissue using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Gene specific primers were selected against the partial cDNA sequence of ER cloned from the sea lamprey by Thornton (2001), Accession #AY028456, using Primer3¹ and were obtained from Integrated DNA technology (Coralville, IA, USA).

Estrogen receptor left primer (first set): 5'-CCTCGTGCACAGAG TTCT-3'

Estrogen receptor right primer (first set): 5'-GTAGCGATCCGGA GCTGA-3'

Estrogen receptor left primer (second set): 5'-GACATGTTTCGACA TGCTGCT-3'

Estrogen receptor right primer (second set): 5'-AGCGGGATCAC ATTCTTACG-3'

RT-PCR reactions were prepared (16.75 µl H₂O, 2.5 µl buffer, 1.5 µl MgCl₂, 0.5 µl dNTPs, 0.25 µl Taq Polymerase, 500 nM primer concentration, and 1 µl template). RT-PCR reactions were performed using an Eppendorf Thermal Cycler under the following conditions: 48°C/1 min; (95°C/15 s; 60.1°C/1 min; 72°C/1 min) × 35 cycles; 72°C/5 min; 10°C/hold. The reaction products were analyzed on 1% agarose gels electrophoresed at 90 V for 1 h and stained with ethidium bromide for purposes of visualization.

For the purpose of verifying the sequence identity of candidate ER-bands, RT-PCR products were first gel purified as described in the QIAEX II Gel Purification Kit[®] (QUIGEN, Valencia, CA, USA). Purified products were then inserted into the pGEM-T-Easy vector (Promega, Madison, WI, USA) and subsequently transformed into *E. coli* JM109 cells (Promega). Ligations and transformations were performed following the protocol described in the pGEM-T-Easy Vector System (Promega). Overnight cultures were used

¹http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

for plasmid preparation with the Wizard Plus Miniprep system (Promega), following the manufacturer's protocol. Purified plasmid was then sent for sequencing at the University of Utah's Health Science Center².

RNA PROBE SYNTHESIS

Templates for probe synthesis were prepared in plasmids as described above. Purified plasmid containing the ER insert was then digested overnight at 37°C with either *Sall* or *NcoI* [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. Riboprobes were synthesized using the SP6 Riboprobe Synthesis Kit (Promega), and either ³⁵S- or Digoxigenin (DIG)-labeled 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.

EVALUATION OF RNA PROBE YIELD

The yield of DIG-labeled probes was evaluated by spectrometric analysis of total RNA and a modified DIG Northern blot protocol (Allen et al., 2000). For the Northern blot, in the 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. 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 mM DTT) was prepared and mixed with 0.5 mg/ml DIG-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 maleic 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-alkaline phosphatase (AP; Roche) antibody (1:5000) for 30 min at RT. Membranes were washed twice in Maleate Buffer 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 mM Tris-HCl, 100 mM NaCl, 50 mM Mg₂Cl₂; pH 9.5] in darkness for 3 h at RT.

For ³⁵S-labeled probes, radioactivity in the waste products from the LiCl precipitation and washing of probes was measured and compared against the activity of the purified probes. Based on this relationship, the % incorporation of ³⁵S-UTP was calculated. And based on the % of label incorporated, product yield from the synthesis reactions was calculated.

IN SITU HYBRIDIZATION

Forty-seven adult, female lamprey were killed by decapitation. Brains were dissected, immersed in O.C.T. Compound (Miles Inc. Elkhart, IN), and then frozen on dry ice. Sagittal tissue sections of 16–18 µm were cut the day of dissection on

a cryostat (Reichert-Jung, Leica Instruments, Heidelberg, Germany) at –12°C. Sections were then mounted onto Vectabond (Vector Laboratories, Burlingame, CA, USA) coated slides and immediately moved into tissue preparation steps of day 1 of the *in situ* hybridization protocol. Tissue sections were incubated with either anti-sense (odd numbered slides) or sense (even numbered slides) riboprobes for lamprey ER. The application and signal development of DIG-labeled probes followed the protocol previously described by Rubin et al. (1997), and characterized for use in lamprey by Root et al. (2005).

The application and signal development of ³⁵S-labeled probes followed the protocol characterized by Root et al. (2005), with the exception of signal development, which followed the protocol described by Hrabovszky et al. (2004). The concentration of probe in the hybridization solution, the concentration of antibody applied, and the stringency of the washes were all varied to optimize the results. Following signal development, both ³⁵S and DIG-labeled tissue sections were viewed under light microscopy using an Olympus BH2 microscope, and digital photographs were captured with a Nikon Coolpix 5000 camera. A detailed visual assessment was made of signal deposition for each slide. In addition, images were also collected at Tufts Medical School using a Zeiss Axioscope and Spot RT color camera (Diagnostic Instruments, Inc.), and submitted for analysis by Optimus™ analysis software, which quantitatively compared the signal intensity between sense and anti-sense slides.

RESULTS

RT-PCR

The first set of primers for lamprey ER yielded specific transcripts in ovarian tissue, but not in brain, testis, or muscle tissue (results not shown). In contrast, the second set primers for lamprey ER yielded specific transcripts in all tissues examined, with strongest expression in ovarian and muscle tissues (Figure 1).

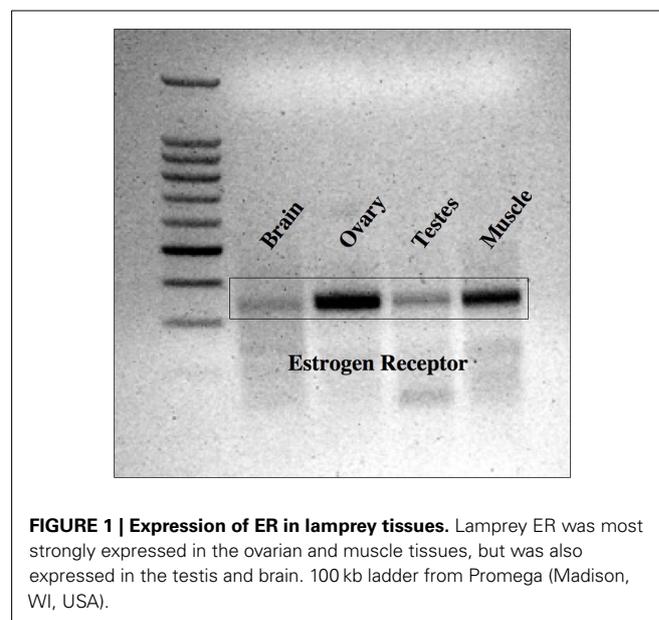


FIGURE 1 | Expression of ER in lamprey tissues. Lamprey ER was most strongly expressed in the ovarian and muscle tissues, but was also expressed in the testis and brain. 100 kb ladder from Promega (Madison, WI, USA).

²<http://hci-w1.hci.utah.edu/OrderTracking/index.html>

Notably, the expression of ER in the target tissue (brain) was exceptionally weak. Megalign™ analysis software was used to compare sequenced PCR products with the published sequence for lamprey ER. Specifically, the transcript cloned from the first set of primers (ER1 = 407 bp total length) had 100% identity with the published sequence for lamprey ER (Thornton, 2001). In contrast, the transcript cloned from second set of primers (ER2 = 341 bp total length) contained a single deletion at position 285, relative to the published sequence. The sequence confirms the analyzed RT-PCR product is from the ER gene; as to the single base-pair difference noted – while presumably changing the reading frame – it is most likely an artifact. A pseudogene would be expected to be much more different over the 341-bps sequenced, thus was not pursued further.

EVALUATION OF RNA PROBE YIELD

The fidelity of DIG-labeled probes was supported through Dot Blot analysis that revealed binding of ER1, ER2, and LIII (GnRH-III) to respective cDNAs (Figure 2). In addition, probes yield was determined to high, as determined by absorption at 260 nm. For ³⁵S-labeled probes, a high amount of label was determined to have been incorporated into all probes, generating a high yield of product that was also consistent between probes (data not shown).

IN SITU HYBRIDIZATION

A total of 44 lamprey brains were used for DIG-labeled *in situ* hybridization experiments. DIG-labeled, anti-sense ER probes demonstrated moderate, yet distinct reaction product in specific neuronal nuclei across many regions of the lamprey brain, including the olfactory lobe, hypothalamus, habenular area, and hindbrain. Major brain regions were scored for signal intensity on every slide. Patterns of staining were consistent between all brains examined. Slides were subjected to image analysis performed at Tufts University, and in most cases, indicated that staining on sense slides was significantly less robust than that on anti-sense slides. In addition, similar results were observed in both sagittal (Figure 3) and transverse tissue sections (Figure 4).

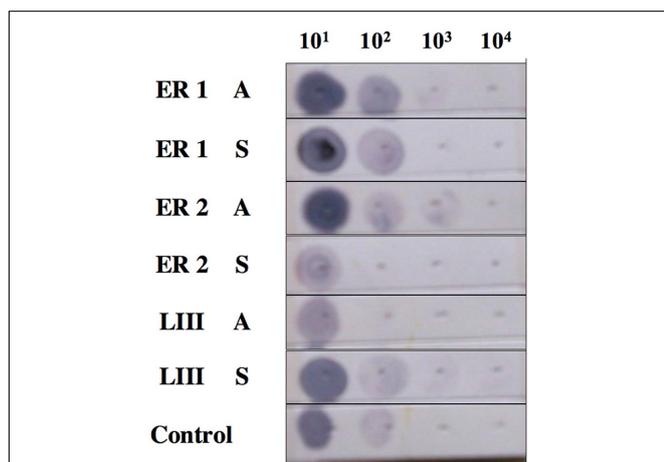


FIGURE 2 | Fidelity of estrogen receptor and GnRH-III (DIG-labeled) probes. Probe fidelity was supported through Dot Blot analysis that revealed binding of ER1, ER2, and LIII (GnRH-III) to respective cDNAs.

For ³⁵S-labeled tissue sections, a total of three lamprey brains were used, and for samples in which the high stringency wash was omitted, results were similar to those seen using DIG-labeled probes. However, while anti-sense slides tended to be more heavily stained than sense slides, a high degree of background staining prevented a detailed analysis (Figure 5). In contrast, for ³⁵S-labeled tissue sections exposed to 10 min of a high stringency wash, no signal was observed (results not shown). The limited range of stringencies attempted (0 and 10 min) using ³⁵S-labeled probes limits the derivative power of these results.

DISCUSSION

In this study, ER was shown to be expressed in the lamprey brain by RT-PCR and DIG-labeled *in situ* hybridization. Specifically, *in situ* hybridization indicated that ER is expressed in the olfactory bulb, hypothalamus, habenular area, midbrain, and hindbrain of the lamprey. As stated earlier, in lampreys, there is a general pattern of GnRH distribution in the anterior-preoptic-neurohypophysial tract to the neurohypophysis of adult lampreys (Crim et al.,

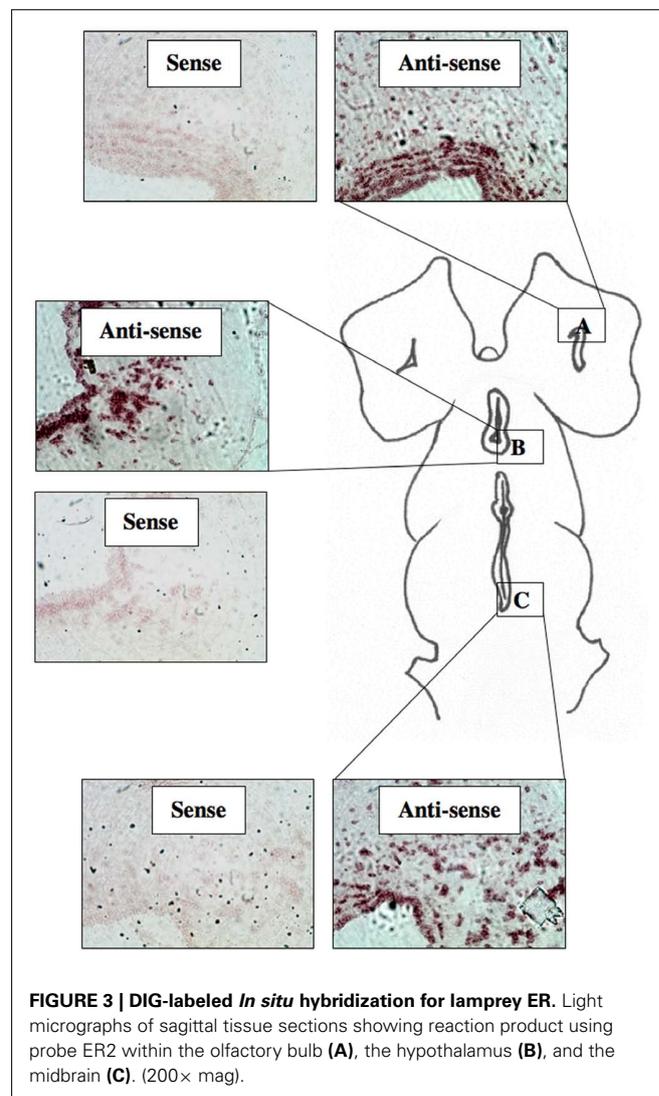
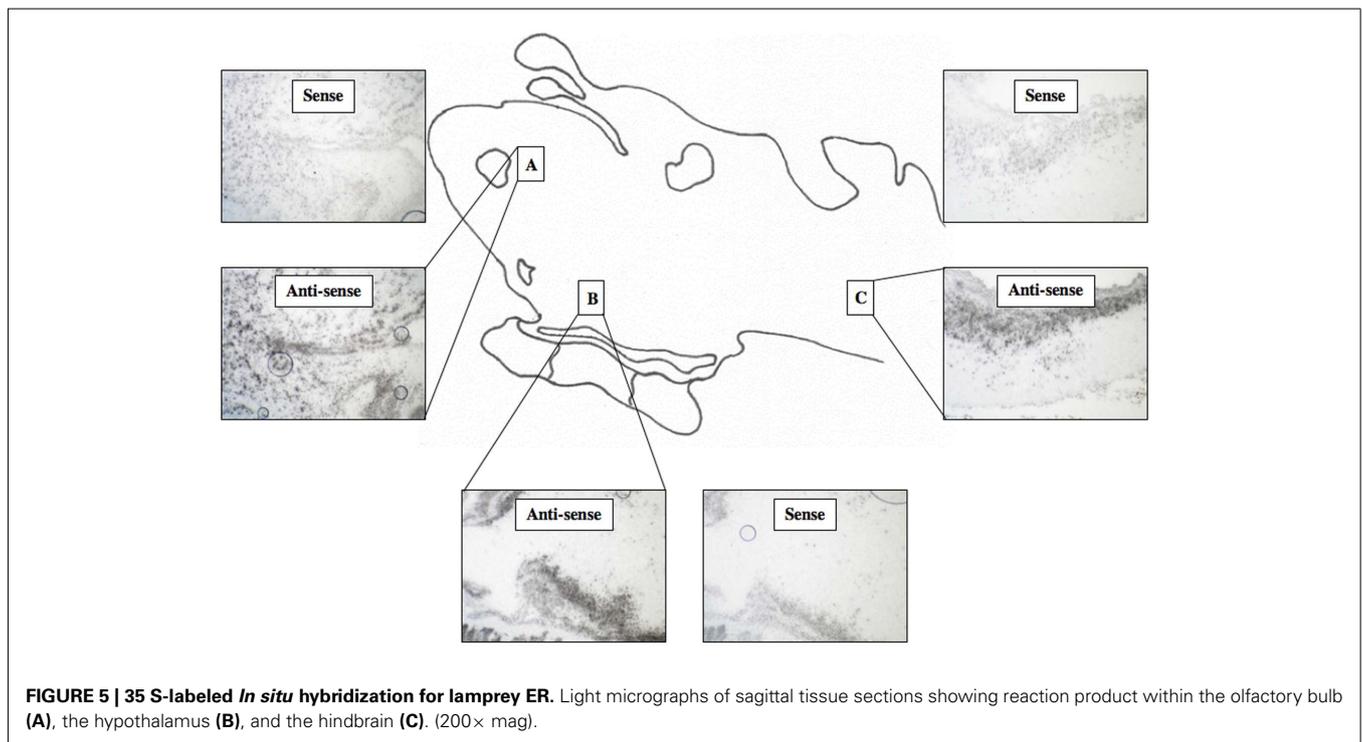
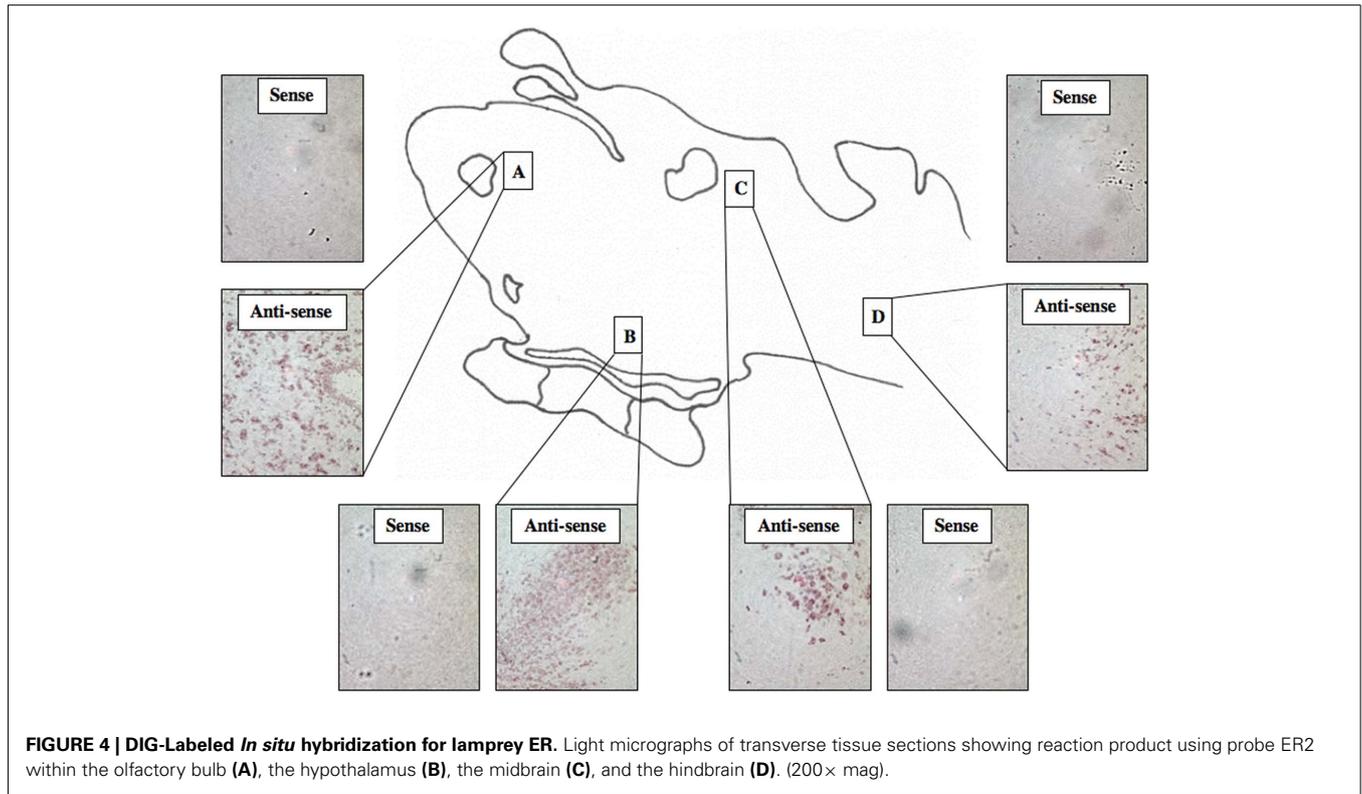


FIGURE 3 | DIG-labeled *In situ* hybridization for lamprey ER. Light micrographs of sagittal tissue sections showing reaction product using probe ER2 within the olfactory bulb (A), the hypothalamus (B), and the midbrain (C). (200× mag).



1979a,b; Nozaki and Kobayashi, 1979; Nozaki and Gorbman, 1984; King et al., 1988; Reed et al., 2002; Kavanaugh et al., 2008). Expression of ER in the hypothalamic area of the brain supports the

possibility of interaction between E2 and GnRH, and is consistent with previous evidence that E2 feedback is occurring indirectly or directly via GnRH neurons (Sower, 1987).

The current study revealed much broader expression of ER than that of previous studies in lamprey. Autoradiographic studies using ^3H -labeled E2 previously documented binding only within the hypothalamic region and regions immediately adjacent the hypothalamus (Kim et al., 1980, 1981). In the current study, staining was observed in and around the hypothalamus, but also within the olfactory lobe, habenular area, and hindbrain. In vertebrates other than lamprey, expression of ER occurs widely in the brain (Shima et al., 2003), and it may be that the distribution of ER in the lamprey brain more closely parallels that of other vertebrates than was previously thought. The discrepancy between autoradiographic and *in situ* hybridization studies may have occurred due to the presence of another as yet unknown ER isoform in lamprey or due to non-specific binding. There is increasing evidence suggesting that ERs are capable of activation by means other than E2 (Blaustein, 2004), therefore *in situ* hybridization may reveal regions of ER expression that function independently of E2, and are otherwise undetectable by autoradiographic methods using ^3H -labeled E2.

The pattern of ER expression observed in this study is similar to that of ER expression previously observed in the brains of teleost fish (Menuet et al., 2003; Pellegrini et al., 2005). Interaction between E2 and GnRH has been demonstrated in teleosts (Trudeau et al., 1993), and the absence of ER expression in the GnRH neurons of teleosts suggests that E2–GnRH interaction in this vertebrate class occurs indirectly (Navas et al., 1995). While lamprey have a single identified ER (Thornton, 2001), teleost fish have three types of ER (Hawkins et al., 2000; Menuet et al., 2002). These ERs are differentially expressed in the brain (Hawkins et al., 2000; Pellegrini et al., 2005), and also appear to have distinct functions in the neuroendocrine control of reproduction and behavior (Hawkins et al., 2005). In comparison to teleosts, the possession of only a single ER by lamprey may suggest alternative signaling pathways for E2 in the lamprey brain.

The current study identified ER expression within the same hypothalamic region where GnRH expression occurs, but did not offer any insight into how E2 may interact with GnRH in lamprey. A key question remains as to whether E2 acts directly on GnRH-expressing neurons, or indirectly through a mechanism utilizing GABA or kisspeptin. Root et al. (2005) identified GABA-expressing neurons in close proximity to neurons expressing lamprey GnRH, supporting the possibility of an indirect action of E2 on GnRH. However, the critical evidence needed in order to establish the direct and/or indirect nature of E2–GnRH interaction in lamprey lies in the determination of cellular co-localization between GABA, GnRH, kisspeptin, and ER. Kisspeptin and its receptor is another system that may also be involved in feedback studies. The Kiss1/GpR54 system was discovered and shown to be the central gatekeeper in the regulation of GnRH and puberty in mammals (Seminara et al., 2003; Seminara and Crowley, 2008). In mammals, the kisspeptin system acts in regulating many aspects of reproduction functions including the mediation of steroid feedback (Roa et al., 2009). The identification and function of kisspeptin(s) and respective receptors in lampreys has not yet been elucidated. Future studies will be required to examine the inter-relationship between the kisspeptin, GnRH, GABA, and steroid

feedback systems and to determine whether there is another ER in lampreys.

Estradiol is considered to be a major reproductive hormone in both male and female lampreys (Sower and Kawauchi, 2010). The role of estradiol in reproduction is supported by the cloning of an estrogen-like receptor in sea lamprey (Thornton, 2001). In sea and Japanese river (*Lethenteron camtschaticum*) lampreys, estradiol concentrations increased during spermiation (Fukayama and Takahashi, 1985; Sower et al., 1985; Fahien and Sower, 1990) and decreased during ovulation (Sower et al., 1985; Bolduc and Sower, 1992). In the first reported study examining sex steroid profiles in the Pacific lamprey (*Entosphenus tridentatus*) during overwintering and sexual maturation, estradiol levels were usually higher in males than in females and increases coincided with the development of secondary sex characteristics (Mesa et al., 2010). In another study, there were higher plasma concentrations of estradiol in females compared to males and in both sexes, plasma estradiol significantly increased as the season progressed correlating with a temperature increase that is in general agreement with these earlier studies (Sower and Kawauchi, 2010). In males, higher estradiol concentrations corresponded to males that have mature sperm as shown in maturing lampreys (Fukayama and Takahashi, 1985; Sower et al., 1985; Linville et al., 1987) and are consistent with the presence of an ER in the male testes (Ho et al., 1987). While estradiol is considered to be a major steroid involved in reproductive processes, the precise function(s) of estradiol and its corresponding receptor(s) in both male and female lampreys need to be elucidated. There are still many questions remaining as to the type of steroids that are synthesized and their respective functions (reviewed in Bryan et al., 2008). For example, there is growing evidence that all lampreys produce gonadal steroids that are different from those of other vertebrates by possessing an additional hydroxyl group at the C15 position (Bryan et al., 2006, 2008).

In conclusion, the current study identifies for the first time expression of ER in the lamprey brain using both RT-PCR and *in situ* hybridization. A broad expression of ER was observed using *in situ* hybridization, including expression in the olfactory lobe, hypothalamus, habenular area, and hindbrain. Expression of ER in the hypothalamic region supports the possibility of an E2 feedback mechanism the GnRH system, similar to that observed in other vertebrate groups. By demonstrating ER expression in the lamprey brain using *in situ* hybridization, the current study provides a means for further investigation of possible mechanisms of interaction between E2 and GnRH in lamprey and other critical neurohormone systems.

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