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In vitro and in vivo effects of GABA, muscimol, and bicuculline on lamprey GnRH concentration in the brain of the sea lamprey (*Petromyzon marinus*)

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Abstract

γ-Aminobutyric acid (GABA) is a neurotransmitter with a demonstrated neuroregulatory role in reproduction in most representative species of vertebrate classes via the hypothalamus. The role of GABA on the hypothalamus–pituitary axis in lampreys has not been fully elucidated. Recent immunocytochemical and in situ hybridization studies suggest that there may be a neuroregulatory role of GABA on the gonadotropin-releasing hormone (GnRH) system in lampreys. To assess possible GABA–GnRH interactions, the effects of GABA and its analogs on lamprey GnRH in vitro and in vivo were studied in adult female sea lampreys (*Petromyzon marinus*). In vitro perfusion of GABA and its analogs at increasing concentrations (0.1–100 μM) was performed over a 3-h time course. There was a substantial increase of GnRH-I and GnRH-III following treatment of muscimol at 100 μM. In in vivo studies, GABA or muscimol injected at 200 μg/kg significantly increased lamprey GnRH concentration in the brain 0.5 h after treatment compared to controls in female sea lampreys. No significant change in lamprey GnRH-I or GnRH-III was observed following treatment with bicuculline. These data provide novel physiological data supporting the hypothesis that GABA may influence GnRH in the brain of sea lamprey.

Keywords: Agnathan; Bicuculline; γ-Aminobutyric acid (GABA); Gonadotropin-releasing hormone (GnRH); Lamprey; LHRH; Muscimol

1. Introduction

Sexual maturation and reproduction are seasonal and synchronized processes in the sea lamprey (*Petromyzon marinus*) and regulated by the primary reproductive hormone, gonadotropin-releasing hormone (GnRH) (Youson and Sower, 1991; Sower, 2003). The central role of GnRH in the control of reproduction in the sea lamprey has been well established (Sower, 1990, 2003; Sower et al., 1993; Deragon and Sower, 1994; Gazourian et al., 1997). To date, two primary forms of GnRH, lamprey GnRH-I and lamprey GnRH-III, have been identified in the sea lamprey (Sherwood et al., 1986; Sower et al., 1993). In addition, the cDNAs of lamprey-I and lamprey-III have been characterized (Suzuki et al., 2000; Silver and Sower, 2002). Further physiological, biochemical, molecular, and immunological studies have provided substantial evidence that GnRH-I and GnRH-III are hypothalamic hormones controlling reproduction via the pituitary–gonadal axis (reviewed in Sower, 2003). Immunocytochemical studies have also shown that both GnRH-I and GnRH-III are localized in the lamprey preoptic area, extending to the neurohypophysis (Crim et al., 1979; Nozaki and Kobayashi, 1979; Nozaki et al., 1984, 2000; King et al., 1988; Wright et al., 1994; Tobet et al., 1995, 1996; Reed et al., 2002). In these same studies, GnRH was not found to be widely distributed in extrahypothalamic regions compared to GnRH systems in other vertebrates.

The neuroendocrine control of GnRH synthesis and release has been shown to be regulated both by internal and external cues such as neuropeptide Y (NPY), environmental water temperature, and estrogen feedback (Sower, 1990,

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1997, 1998, 2003; Conlon et al., 1994; Sower and Kawauchi, 2001). Recently, we hypothesized that another regulatory neurotransmitter, γ-aminobutyric acid (GABA), may also have a neuroregulatory role in lampreys (Reed et al., 2002). GABA is the major inhibitory neurotransmitter present in the central nervous system of all vertebrates studied and has been demonstrated to have many functions. One major function of GABA that has been well documented is a regulatory role in the hypothalamic–pituitary axis of representatives across vertebrates (Anglade et al., 1999). There is increasing evidence that the inhibitory neurotransmitter, GABA, is involved in regulating GnRH release in vertebrates, possibly through feedback on GnRH neurons in the hypothalamus (Trudeau et al., 2000b). Because GnRH neurons had not been shown to express steroid receptors in mammals (Herbison and Theodosis, 1992) or fish (Navas et al., 1995), it was believed that estrogen modulated GnRH activity through GABA neurons. Recently, estrogen receptor mRNAs for both α and β subunits were detected in adult mouse GnRH neurons (Skynner et al., 1999), and β subunit immunoreactivity was detected in GnRH neurons in the rat brain (Hrabovszky et al., 2000). These studies demonstrated the possibility of a direct steroid feedback system on GnRH neurons; however, due to the low expression levels detected in other studies and the overwhelming evidence of GABAergic input, it is likely that this feedback mechanism works in association with the GABAergic system. The specific effects of GABA on GnRH neurons in the sea lamprey hypothalamus are still unknown. In lampreys, it has been suggested that positive feedback by estradiol occurs at the time prior to ovulation, similar to what is seen in mammals (Sower, 1997, 2003). To date, there are no reported studies on whether lamprey GnRH neurons express estrogen receptor.

Previous studies using immunocytochemistry (ICC) for GABA in the sea lamprey have demonstrated the presence of several GABA-containing cell populations throughout the lamprey brain, specifically in regions containing GnRH (Pombal et al., 1997; Pombal and Puelles, 1999; Melendez-Ferro et al., 2001; Melendez-Ferro et al., 2002; Reed et al., 2002). A study by Reed et al. (2002) using ICC for GABA and GnRH on consecutive hypothalamic tissue sections showed the close spatial relationship of GnRH-containing neurons and GABA-containing neurons in the preoptic area of larval sea lamprey. Expression studies using in situ hybridization for lamprey GnRH and glutamic acid decarboxylase (GAD), the enzyme that synthesizes GABA, also confirmed the close localization of GABA and GnRH in the sea lamprey (Reed et al., 2002; Root et al., in press). The close proximity of both expression and protein localization in the preoptic area strongly supports the hypothesis of a neuromodulatory role for GABA on GnRH neurons.

To examine the functional relationship between GABA and GnRH, the in vivo and in vitro effects of GABA, muscimol ($GABA_A$ receptor agonist), and bicuculline ($GABA_A$ receptor antagonist) on GnRH concentration were determined in adult female sea lamprey. Since we have demonstrated that estradiol is a major steroid in female lampreys and that estradiol peaks prior to ovulation, we have focused these initial studies on females (Sower, 2003). In the present study, in vitro perfusion of adult female sea lamprey brains with increasing concentrations of GABA, muscimol, bicuculline, and lamprey GnRH-III was performed followed by radioimmunoassays (RIAs) for lamprey GnRH. In addition, in vivo studies were done in which adult female and male (for comparison) sea lampreys were injected with GABA, muscimol, and bicuculline, and then sampled over a time course and measured for GnRH concentration.

2. Materials and methods

2.1. Collection of lampreys

Adult sea lampreys (*P. marinus*) were collected from fish ladders in the Cocheco River in Dover and Exeter, NH, in the months of May and June in 2002 and 2003, during their upstream spawning migration from the ocean. The lampreys were transported to the Anadromous Fish and Aquatic Invertebrate Research (AFAIR) laboratory in Durham, NH, where they were maintained in an aerated flow-through system acting as an artificial stream as previously described (Fahien and Sower, 1990). The maintenance and use of the lampreys were performed under guidelines established by the Animal Care and Laboratory Use Committee of the University of New Hampshire.

2.2. In vitro perfusion

Five brains from adult female sea lampreys were removed following decapitation and used for in vitro perfusion using an AcuSyst-S Cell Culture multiperfusion system with GABA and analogs (Gazourian et al., 2000). The lampreys were held in water temperature of 18 °C. Briefly, each brain was placed in a perfusion chamber containing Hank's balanced salt solution (HBSS) and 25 mM HEPES (Invitrogen, Grand Island, NY, USA) 30 min after sampling. HBSS with HEPES was delivered at a rate of 67 ml/min and maintained at 18 °C. A constant volume of 400 μl of HBSS with HEPES was maintained in each chamber. HBSS with HEPES from the reservoir was in a continuous flow over each tissue for 2 h to establish the basal hormonal secretion rate. Five brains were individually perfused with one of the following: saline (control), GABA, muscimol, bicuculline, saline, or GnRH-III in concentrations of 0.1, 1.0, 10, and 100 μM, waiting 1 h after each injection. As an example, one brain was individually perfused following four successive injections with GABA at 0.1, 1.0, 10, and 100 μM. Six 400-μl fractions were col-

lected every 6 min and stored at −80 °C until extracted and assayed.

2.3. In vivo studies

In 2002, 60 adult female lampreys were injected intraperitoneally with saline (control) or 200 μg/kg fish of GABA, muscimol, or bicuculline. There were 15 lampreys per treatment. Three from each treatment group were sampled for plasma, brains, and gonads every 30 min for a duration of 2.5 h. Water temperature was 15 °C. On a separate day, 48 adult male lampreys were divided into the same treatment groups and injected with saline, GABA, muscimol, or bicuculline at 200 μg/kg fish. Three from each treatment group were sampled every 30 min for 2 h. Water temperature was 13.9 °C. A 1.0-ml blood sample was collected from each animal via cardiac puncture at the time of sampling as previously described (Sower et al., 1985b). The blood was centrifuged and plasma was removed, and stored at −80°C until assayed for estradiol. Immediately following blood collection, each lamprey was decapitated and the brain was removed. Brains were snap frozen in liquid nitrogen and stored at −80 °C until assayed for GnRH. Anterior gonadal tissue was removed from each lamprey for histological examination based on methods previously described (Sower et al., 1985b). The experiment was replicated in 2003 using 100 adult females. There were 25 lampreys per treatment. Lampreys were injected with saline, GABA, muscimol, or bicuculline at 200 μg/kg fish. Five from each treatment group were sampled every 30 min for a duration of 2.5 h. Water temperature was 15 °C. Blood, brain, and gonad samples were collected from each lamprey.

2.4. Extraction and high-performance liquid chromatography (HPLC)

All brain and perfusion eluant samples were extracted for GnRH using methods previously described by Yu et al. (1987) and modified by Fahien and Sower (1990). Briefly, brains were washed, extracted, and eluted through HPLC before being assayed for GnRH.

2.5. RIA

GnRH was measured from duplicate 100-μl aliquots by RIA as previously described (Stopa et al., 1988) using antiserum 3952 at an initial dilution of 1:16,000 raised against lamprey GnRH-III. Synthetic lamprey GnRH-III (American Peptide) was used as a standard and ^{125}I lamprey GnRH-I (Peninsula Laboratories, CA, USA) was iodinated using a modification of the chloramine-T method and purified as described by Stopa et al. (1988). The lower limit of sensitivity was 9.8 pg/0.1 ml and the antibody binding ranged between 33.6% and 37.9% (2002, in vitro), 34.4% and 47.9% (2002, in vivo), and 43.2% and 46.4% (2003, in vivo). Plasma estradiol was measured from duplicate 100-μl plasma aliquots by RIA as previously described (Sower and Schreck, 1982) and validated for lampreys as described (Sower et al., 1983, 1987). The lower limit of sensitivity was 7.8 pg/0.1 ml and the antibody binding ranged between 39% and 42% (2002) and 36.2% and 43.3% (2003).

2.6. Histological analysis

Histological examination of gonadal tissues prepared for histology was performed for ovaries according to the method of Bolduc and Sower (1992); testes were examined and stages were identified based on morphology as described by Fahien and Sower (1990).

2.7. Statistical analysis

Significant changes in GnRH and estradiol concentrations in vitro and in vivo following treatment with GABA, muscimol, or bicuculline were analysed by Fisher's PLSD tests after preliminary analysis of variance. In all tests, the level of significance for different groups was <0.05.

3. Results

3.1. In vitro study

Lamprey GnRH-III and lamprey GnRH-I increased in response to lamprey GnRH-III at 1.0 and 100 μM. There was an increase of lamprey GnRH-III in response to muscimol at 10 and 100 μM, and an increase of lamprey GnRH-I in response to muscimol at 10 μM. Treatment with GABA and bicuculline had no effect on either lamprey GnRH-I or GnRH-III compared to controls (Fig. 1).

3.2. In vivo study

3.2.1. GABA effects on GnRH in vivo

In 2002, female lampreys treated with GABA showed a significant ($P<0.05$) increase of lamprey GnRH-I concentration at 0.5 and 2.5 h compared to controls (Table 1; Fig. 2). There were no significant changes in lamprey GnRH-III in female lampreys treated with GABA compared to controls at 0.5, 1.0, 1.5, 2.0, or 2.5 h (Table 1). Female lampreys treated with GABA resulted in increased lamprey GnRH-I concentrations of 35.4±10.3 ng/brain ±S.E.M. at 0.5 h compared to controls of 5.7±4.3 ng/brain. At 2.5 h, GnRH-I concentration was significantly higher (100.6±25.8 ng/brain) compared to controls (42.8±6.8 ng/brain). In 2003, there were no significant differences in lamprey GnRH between GABA-treated and control female and male lampreys (2002).

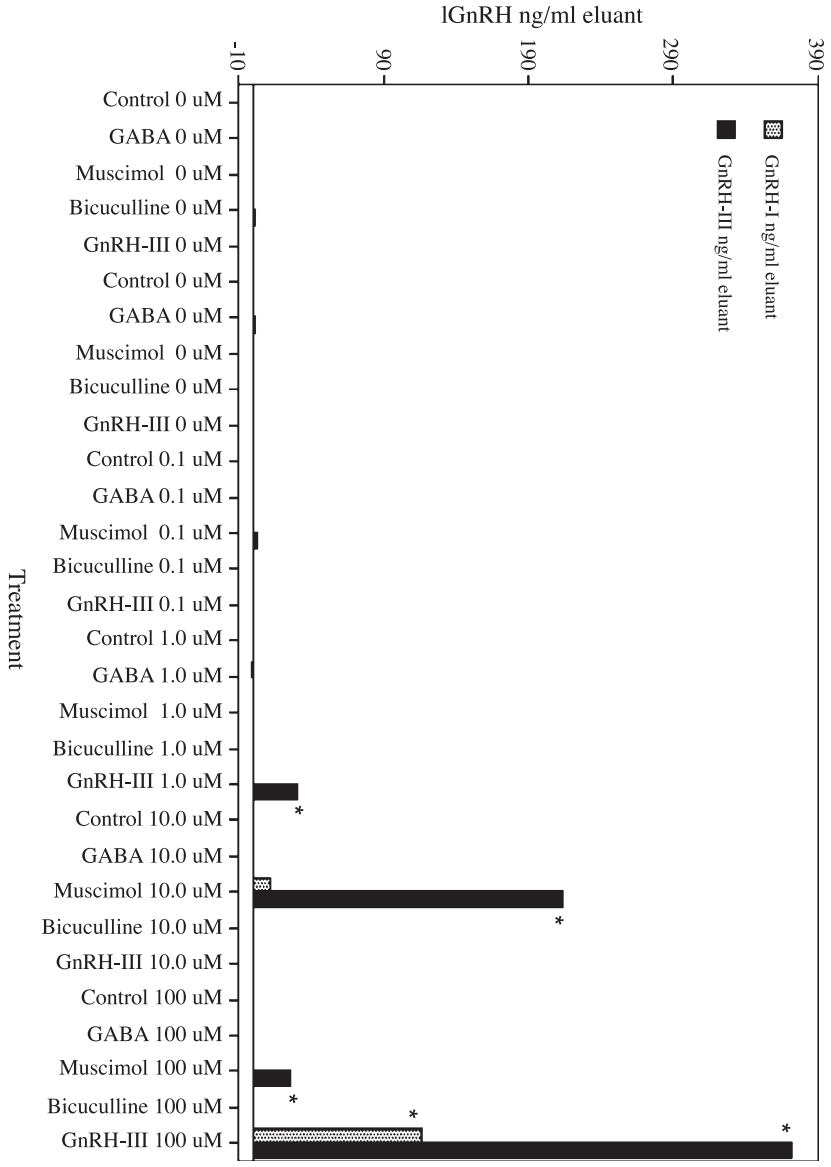

Fig. 1. Effects of in vitro perfusion. Perfusion effects of increasing concentrations (0.1–100 μM) of GABA, muscimol, and bicuculline on in vitro release of lamprey GnRH-I (lGnRH; ng/ml) and lamprey GnRH-III (ng/ml) in adult female sea lamprey (*P. marinus*) brains. Five brains were individually perfused with one of the following: saline (control), GABA, muscimol, bicuculline, or lamprey GnRH-III in concentrations of 0.1, 1.0, 10, and 100 μM, waiting 1 h after each injection. As an example, one brain was individually perfused following four successive injections with GABA at 0.1, 1.0, 10, and 100 μM. *Denotes increase in lamprey GnRH concentration.

3.2.2. Muscimol effects on GnRH in vivo

In 2002, female lampreys treated with muscimol at 0.5 h showed a significant (P<0.05) increase of lamprey GnRH-I and GnRH-III compared to controls (Table 1; Figs. 2 and 3). In female lampreys treated with muscimol at 0.5 h, lamprey GnRH-I (49.3±8.3 ng/brain) and lamprey GnRH-III (115.8±15.4 ng/brain) were increased compared to controls of 5.7±4.3 and 16.8±8.9 ng/brain, respectively (Figs. 2 and 3). No significant differences were noted in lamprey GnRH between treated and control male lampreys in 2002. In 2003, female lampreys treated with muscimol at 2 h had a significant (P<0.05) increase of lamprey GnRH compared to controls (Table 2). At 2 h after muscimol treatment, there was an increase of lamprey GnRH of 122.9±7.8 ng/brain compared to controls of 88.5±9.4 ng/brain.

3.2.3. Bicuculline effects on GnRH in vivo

In 2002, female lampreys treated with bicuculline at 0.5 h showed no significant change at P<0.05 in lamprey GnRH-I or GnRH-III compared to controls. Female lampreys treated with bicuculline at 1 h showed a significant (P<0.05) increase of lamprey GnRH-III of 136.6±47.7 ng/brain compared to controls of 45.3±5.4 ng/brain. Treated and control groups in male lampreys in 2002 did not differ significantly. In 2003, there were no significant differences

Table 1
2002 In vivo effects of GABA, muscimol, and bicuculline

	Male			Female		
	lGnRH-I (ng/brain)	lGnRH-III (ng/brain)	Estradiol (ng/ml)	lGnRH-I (ng/brain)	lGnRH-III (ng/brain)	Estradiol (ng/ml)
Control						
0.5	37.7±19.7	143.4±36.4	1.66±0.53	5.7±4.3	16.8±8.9	1.35±0.30
1	39.3±9.3	98.0±8.6	0.86±0.05	22.0±3.4	45.3±5.4	1.43±0.24
1.5	62.1±37.6	116.1±27.7	1.47±0.25	49.1±5.0	121.4±31.7	0.96±0.20
2	42.5±16.8	89.0±14.0	0.82±0.24	43.1±4.7	108.0±7.3	0.84±0.24
2.5				42.8±6.8	107.5±15.9	1.18±0.09
GABA						
0.5	48.6±12.6	113.6±13.0	2.14±0.18	35.4±10.3*	10.9±19.7	1.24±0.45
1	32.2±8.2	97.3±10.6	1.27±0.62	58.3±13.5	105.8±4.3	1.12±0.09
1.5	52.5±5.7	107.8±8.4	1.03±0.07	24.8±7.6	60.9±20.1	1.10±0.16
2	32.1±6.6	87.5±18.1	0.62±0.04	66.7±48.8	109.4±54.4	1.03±0.48
2.5				100.6±25.8*	166.5±22.8	0.92±0.23
Muscimol						
0.5	40.7±8.2	104.3±14.1	1.55±0.09	49.3±8.3*	115.8±15.4*	1.10±0.10
1	67.0±39.7	134.3±45.7	1.21±0.16	41.8±5.7	94.7±3.5	1.45±0.34
1.5	28.6±4.3	90.0±14.3	1.30±0.17	54.6±15.3	95.8±18.6	1.02±0.10
2	69.2±49.2	122.2±82.5	1.14±0.24	53.4±12.9	98.6±10.4	1.23±0.37
2.5				35.1±18.1	77.8±30.2	0.73±0.10
Bicuculline						
0.5	36.0±10.4	92.0±24.5	0.86±0.20	17.4±5.6	54.6±21.6	1.40±0.27
1	46.7±12.5	130.5±17.9	1.18±0.11	68.3±42.7	136.6±47.7*	0.89±0.21
1.5	72.4±18.0	89.1±29.5	1.16±0.28	31.9±5.1	95.0±18.2	1.19±0.06
2	31.4±9.4	98.2±15.4	1.59±0.25*	34.1±2.7	105.5±8.1	1.33±0.15
2.5				34.6±10.9	83.7±18.3	0.95±0.24

Concentrations of lamprey GnRH-I (ng/brain), lamprey GnRH-III (ng/brain), and plasma estradiol (ng/ml) in adult male and female sea lampreys (*P. marinus*) following injections of GABA, muscimol, and bicuculline (200 μg/kg fish) at time points 0.5–2.5 h. Data are represented as mean±S.E.M. of three fish per treatment.

* Denotes significant difference between treatment mean and control mean ($P<0.05$; one-way ANOVA followed by post-hoc Fisher's PLSD test).

in lamprey GnRH between bicuculline-treated and control female lamprey.

3.2.4. Effects of in vivo injections on estradiol levels

In 2002, male lampreys treated with bicuculline at 2 h had a significant ($P<0.05$) increase of estradiol at 1.5±0.25 ng/ml compared to controls at 0.82±0.24 ng/ml (Table 1). In 2003, female lampreys treated with GABA, muscimol, and bicuculline at 0.5 h showed a significant ($P<0.05$) decrease in plasma estradiol at 0.37±0.09, 0.30±0.07, and 0.34±0.07 ng/ml compared to controls at 0.71±0.17 ng/ml, respectively (Table 2). In lampreys treated with bicuculline at 2.5 h, we noticed a significant ($P<0.05$) increase of estradiol (0.37±0.03 ng/ml) compared to controls (0.20±0.02 ng/ml) (Table 2).

3.3. Histology

The reproductive stage of the gonad was determined based on the methods previously described for males (Fahien and Sower, 1990) and females (Bolduc and Sower, 1992). In adult females, the ovary was in the final maturational stages (preovulatory). In adult males, testes were in stages IV–VII (stage IV=spermatids and immature sperm; stage V=immature sperm; stage VI=immature and mature sperm; and stage VII=mature sperm).

4. Discussion

Many factors, which are able to modulate reproductive events via the hypothalamic–pituitary–gonadal axis, have been identified in vertebrates. One such factor is GABA, which has an important neuroregulatory role in the regulation of GnRH and gonadotropin (GTH) release in vertebrates such as the goldfish (Kah et al., 1992; Sloley et al., 1992; Trudeau et al., 1993b,c, 2000a,b) and the rainbow trout (Mananos et al., 1999) by acting as either the primary neurotransmitter or as a secondary neurotransmitter in the steroidal feedback on GnRH neurons. In the present study, GABA and muscimol, a GABA agonist, stimulated GnRH concentration and release in adult female lamprey during final reproduction. Bicuculline, a known GABA antagonist, had no effect on GnRH concentration or release in female lamprey. Similar to other vertebrates, data from the present study suggest that GABA may have a neuroregulatory influence on GnRH.

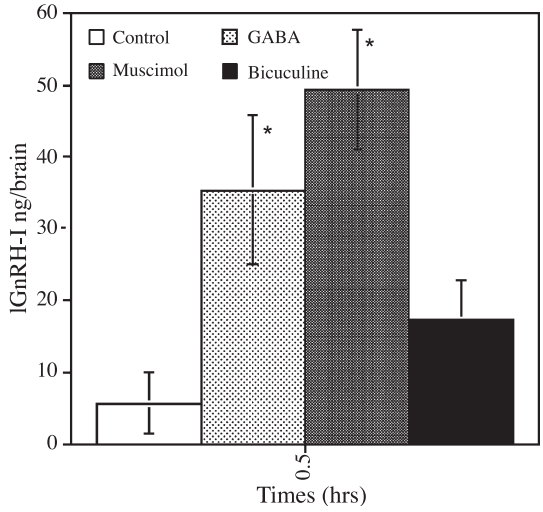

Fig. 2. 2002 In vivo effects on lamprey GnRH-I. In vivo effects of GABA, muscimol, and bicuculline (200 μg/kg fish) on lamprey GnRH-I concentration (lGnRH; ng/brain) in adult female sea lampreys (*P. marinus*) at 0.5 h following injections. Bars represent mean±S.E.M. of three fish. *Denotes significant difference between treatment mean and control mean ($P<0.05$; one-way ANOVA followed by post hoc Fisher's PLSD test).

In vitro administration of muscimol at 10.0 and 100.0 μM concentrations stimulated lamprey GnRH, most notably lamprey GnRH-III, from adult female sea lamprey brains. In vivo injection of muscimol also showed an apparent response in lamprey GnRH concentration in adult female sea lamprey. Treatment with muscimol and, to a lesser extent, GABA showed elevated levels of lamprey GnRH in the brain 0.5 h after injection. This increase of lamprey GnRH

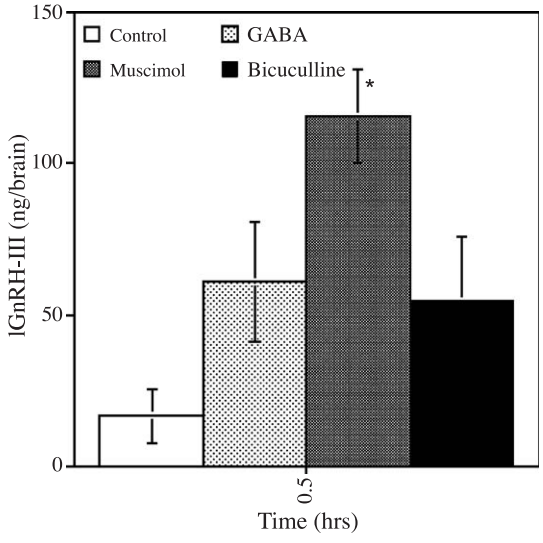

Fig. 3. 2002 In vivo effects on lamprey GnRH-III. In vivo effects of GABA, muscimol, and bicuculline (200 μg/kg fish) on lamprey GnRH-III concentration (lGnRH; ng/brain) in adult female sea lampreys (*P. marinus*) at 0.5 h following injections. Bars represent mean±S.E.M. of three fish. *Denotes significant difference between treatment mean and control mean ($P<0.05$; one-way ANOVA followed by post hoc Fisher's PLSD test).

Table 2
2003 In vivo effects of GABA, muscimol, and bicuculline

	Female lGnRH (ng/brain)	Estradiol (ng/ml)
Control		
0.5	81.4±10.7	0.71±0.17
1	97.6±7.3	0.31±0.04
1.5	85.5±3.2	0.29±0.03
2	88.5±9.4	0.29±0.05
2.5	113.7±7.7	0.20±0.02
GABA		
0.5	75.0±8.6	0.37±0.09*
1	77.2±10.8	0.41±0.08
1.5	68.8±13.4	0.40±0.14
2	95.5±4.8	0.31±0.03
2.5	95.5±7.2	0.25±0.07
Muscimol		
0.5	95.6±4.8	0.30±0.07*
1	93.1±3.2	0.39±0.13
1.5	93.2±4.7	0.22±0.04
2	122.9±7.8*	0.31±0.09
2.5	117.6±11.5	0.21±0.03
Bicuculline		
0.5	95.0±7.6	0.34±0.07*
1	79.5±9.9	0.37±0.10
1.5	106.3±18.4	0.27±0.03
2	96.1±7.7	0.33±0.04
2.5	103.5±23.0	0.37±0.03*

Concentrations of total lamprey GnRH (ng/brain) and plasma estradiol (ng/ml) in adult female sea lampreys (*P. marinus*) following injections of GABA, muscimol, and bicuculline (200 μg/kg fish) at time points 0.5–2.5 h. Data are represented as mean±S.E.M. of five fish per treatment.

* Denotes significant difference between treatment mean and control mean ($P<0.05$; one-way ANOVA followed by post hoc Fisher's PLSD test).

compared to the control group suggested that muscimol, and therefore GABA, is stimulating the increase in GnRH concentration. However, future studies will be required to verify these results.

The GnRH neuronal system in the sea lamprey shares many characteristics with that of other vertebrates including the neuroregulation of hypothalamic GnRH neurons. Several neuropeptides and neurotransmitters are believed to play a role in GnRH regulation; however, to date, only NPY (Conlon et al., 1994) and estrogen (Sower, 1997, 2003) have been examined in the lamprey. The present study is the first to examine the functional role GABA plays in GnRH content and release in the sea lamprey. Based on previous studies investigating GABA's role in mammalian and other vertebrate reproduction, it was hypothesized that GABA acts similarly in the brain of the sea lamprey (Reed et al., 2002). In fish, GABA has been implicated in the control of GTH release (Kah et al., 1992; Trudeau et al., 1993a,b, 2000c), although it appears that GABA does not primarily regulate GTH at the pituitary level, but rather indirectly through stimulating GnRH secretion (Kah et al., 1992; Trudeau et al., 2000c).

Senthilkumaran et al. (2001) showed with in vitro static cultures that GABA had a stimulatory effect on GnRH release in the red seabream (*Pargus major*). This hypothesis of a GABA neuroregulatory effect on GnRH was first supported in the sea lamprey by Reed et al. (2002) and later supported by Root et al. (in press). The experiments in these studies showed collectively that GABA is ideally localized to interact with the GnRH system in lampreys. Together with the previous studies performed in the lamprey, the present data have provided further evidence of a relationship between GABA and GnRH; however, further studies are needed to determine the interrelationship and interactions between GABA and GnRH in the reproductive axis.

Treatment with bicuculline in the adult sea lamprey showed no immediate effect on GnRH concentration in vivo or in vitro. Bicuculline is known to bind strongly to and block the $GABA_A$ receptor; however, in this study, it appeared to have little or no effect on GnRH concentration. Previous studies examining activation of GABA receptors and their effects on GnRH release in vertebrates have shown that GnRH neurons are regulated by $GABA_A$ receptors and not $GABA_B$ receptors (Bolduc and Sower, 1992; Martinez de la Escalera et al., 1994; Mitsushima et al., 1994; Kang et al., 1995; Leonhardt et al., 1995; Fueshko et al., 1998). In vitro administration of muscimol in immature seabream demonstrated an increase of seabream GnRH release (Senthilkumaran et al., 2001). The present study showing stimulation of GnRH release following treatment with muscimol further supports this hypothesis, whereas treatment with bicuculline had no effect. This suggests that the receptor and/or ligand–receptor interactions and/or signaling pathway may be different in lampreys. Further studies will be needed to identify the GABA receptor and its mechanism of action in the sea lamprey.

In the sea lamprey, estradiol is considered to be one of the major sex steroids in the regulation of reproduction in both male and female sea lampreys (Sower et al., 1985b; Fahien and Sower, 1990; Bolduc and Sower, 1992; Sower, 1997). It has been hypothesized that estradiol in adult female sea lampreys is involved in a positive feedback system on GnRH neurons prior to ovulation, similar to that of mammals (Sower, 1997). Previous studies have demonstrated significant changes of plasma estradiol during spawning of female Japanese river lamprey (*Lampetra japonica*; Fukayama and Takahashi, 1985), and sea lamprey (Sower et al., 1985a; Bolduc and Sower, 1992; Sower, 1997), with estradiol increasing significantly prior to final gonadal development and decreasing significantly at ovulation. Previous autoradiography studies (Kim et al., 1980, 1981) have demonstrated the presence of estrogen receptive cells in the forebrain of river lampreys and larval sea lampreys indicating possible steroid binding sites, further supporting the hypothesis of a hypothalamic feedback system of estradiol. In the present study, increased estradiol concentrations in female lampreys occurred after treatment with GABA, muscimol, and bicuculline, which may be due to direct or indirect effects. Increased estradiol concentrations only occurred in male lampreys after treatment with bicuculline. It can be hypothesized that many GABA neurons act as intermediary neurons in steroid feedback, similar to mammals. It is important to note that where significant responses of lamprey GnRH to GABA and muscimol in females were seen in the 2002 study, no significant differences were seen in plasma estradiol concentrations. However, in 2003, no significant responses of lamprey GnRH to GABA or muscimol were seen at the same time point, but plasma estradiol concentrations were significantly decreased. Temperature is considered an important and critical environmental factor for reproduction in lampreys (Sower, 1990, 2003). Upstream-spawning sea lampreys kept at temperatures below 15.5 °C will not ovulate or spermiate unless the temperature is elevated close to their optimal spawning temperature of 21 °C. Furthermore, in previous studies, injections of salmon gonadotropin or GnRH analog sufficient to elevate plasma estradiol will not evoke ovulation in female lampreys held at a lower water temperature of 13 °C (Sower, 1990). It is suggested from the current studies that the actions and differences between the in vivo and in vitro studies and between male and female lampreys are dependent on temperature and/or stage of reproduction, likely reflecting differences in metabolic turnover or degradation rates of GnRH, GTH, and/or their receptors. These data may suggest that changes in endogenous estradiol, different reproductive stages representing a different hormone milieu, different environmental factors such as temperature, or a combination of these factors can influence or alter the action of GABA and its analogs on GnRH.

In summary, this study has provided novel physiological evidence of possible interactions of GABA on GnRH in the brain of the sea lamprey. In vitro perfusion of muscimol noticeably stimulated the release of lamprey GnRH, and in vivo treatment with both muscimol and GABA significantly affected the concentration of lamprey GnRH in the sea lamprey brain. Further research is still needed to fully understand the interactions between GABA and GnRH, and to determine if GABA is, in fact, involved in steroid feedback on GnRH, either directly or indirectly.

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