Induction of Gamete Release by Gonadotropin-Releasing Hormone in a Protochordate, *Ciona intestinalis*

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Gonadotropin-releasing hormone (GnRH) of vertebrates is now believed to have multiple functions in addition to its role as a hypophysiotropic hormone, as originally defined. Recently, it has been shown that GnRH occurs also in the ascidians, which are considered ancestral chordates. Here the author shows that GnRH induces spawning of gametes from mature individuals of *Ciona intestinalis*. *Ciona* accumulates mature gametes in the gonoducts and maintains them until spawning is triggered by a photoperiodic cue(s). Injection of synthetic tunicate GnRH-I or -II into various sites of mature individuals effectively induced gamete release (spawning), although the former was more potent. Gamete release often occurred on a larger scale than in spontaneous spawning. However, moderate gamete release, similar to spontaneous spawning, was often triggered by exogenous tunicate GnRH. GnRH *in vivo* apparently is released from the GnRH-containing neurons that are distributed from the region of the cerebral ganglion to the proximal part of the ovary along the dorsal strand within the blood sinus; this indicates that both forms of tunicate GnRH may be the actual inducers of spawning. It is suggested that, in the ancestral chordate, GnRH neurons release GnRH prior to the spawning and the released GnRH acts directly on the epithelium of gonoducts or functions as a neuromodulator of other neurons innervating the gonoducts to induce spawning.

Key Words: gamete release; GnRH; injection; immunocytochemistry; protochordate; *Ciona*.

Ascidians constitute a major group of the oldest extant lineage of chordates. Therefore, it is of interest to know how their reproduction is regulated and how it relates to the evolution of the control of reproduction in vertebrates. Since Georges and Dubois (1980) reported that there are gonadotropin-releasing hormone (GnRH)-immunoreactive (-ir) neurons in *Ciona intestinalis*, similar observations have been made in the same species (Mackie, 1995; Tsutsui *et al.*, 1998) and also in *Chelyosoma productum* (Kelsall *et al.*, 1990; Powell *et al.*, 1996) and *Halocynthia roretzi* (Terakado and Ogawa, 1995). In these species, GnRH-ir neurons are distributed mainly along the dorsal strand, which is a derivative of the larval neural tube and runs parallel to the gonoducts. In *C. intestinalis*, many GnRH neurons are also present around the cerebral ganglion (Tsutsui *et al.*, 1998).

Recently, Powell *et al.* (1996) isolated two forms of GnRH, which are termed tunicate (t) GnRH-I and -II, from tissue in the regions of the neural complex, including the dorsal strand, in *C. productum* and identified the primary molecular structure of these neuropeptides. Because GnRH is the major regulator of reproduction in vertebrates, it is possible that GnRH has similar functions in ascidians. However, it has not yet been demonstrated that GnRH plays any role in the reproduction of ascidians. Here, the author examined the action of GnRH on the spawning of *C. intestinalis*, which accumulates mature eggs and sperm in the gonoducts prior to spawning.

MATERIALS AND METHODS

Adult mature individuals of *C. intestinalis*, a hermaphroditic animal, were collected at Onagawa Bay and Yokohama Bay in Japan during June and October. They were maintained in a seawater tank for 1 to 3 days at about 19 to 20° during the spring and about 22 to 23° during the summer bubbled with air under continuous fluorescent light (20W × 2, 50 cm apart) to prevent spontaneous spawning before experiments (Lambert and Brandt, 1967).

Experimental group. In each experiment, 10 mature individuals were selected and divided randomly into two groups: one for GnRH injection and the other serving as a control (saline injection only). They were gently transferred to small transparent vessels (15.0 cm wide and 8.5 cm high) and gently placed on a desk under fluorescent light for injection experiments. Each injection experiment was performed twice separately.

Forms of GnRH. Synthetic tGnRH-I (Takara Biochemicals) and tGnRH-II (Sigma Genosis, Japan) were used in the injection experiments. They were synthesized according to the formulas presented by Powell *et al.* (1996). Human (h) GnRH, salmon (s) GnRH, and chicken (c) GnRH-II were also commercially purchased and used for comparison of effectiveness and specificity in induced spawning.

Doses. Knowing that a dose of about 0.8 to 1.6 × 10^{-7} M (10–20 ng/g body weight) of tGnRH-I effectively induces gamete release in preliminary experiments (Fig. 1), each form of GnRH was injected mainly at a dose of 1.6 × 10^{-7} M into each individual. For tGnRH-II, the dose is calculated as about 0.8 × 10^{-7} M. The GnRH was dissolved in a 3% saline solution. As controls, 3% saline solution was injected. For injection, disposable Tuberculin syringes and G25 needles (Terumo) were used. Injections into the posterior body cavity, ovary, lumen of the stomach, or gonoducts (about 2 cm below the gonoductal openings) were selected. Injected animals were carefully watched for 60 min or more to discern whether and to what extent they release sperm and/or eggs.

Immunocytochemistry. Tissues, including the neural complex, dorsal strand, gonoducts, and gonads, were fixed in a mixture of an equal part of 8% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4) and saturated aqueous picric acid (1:1 mixture) for 4 h at 4° and stored in 70% ethanol until use. Some pieces were dehydrated through a graded series of ethanol, infiltrated with xylene, and embedded in paraplast for sections. Others were used for whole-mount preparations. Sections or pieces were treated with 0.3% hydrogen peroxide in ethanol to reduce endogenous peroxidase activity for 30 min. The immunostaining was performed by using the Vectastain avidin–biotin peroxidase complex (ABC) Elite kit (Vector Laboratories, Burlingame, CA). As the primary antibody, anti-synthetic hGnRH antibody (Sanbio) or anti-synthetic sGnRH antibody (Biogenesis) diluted 1:1000 was used. They were incubated for 3 h at room temperature or overnight at 4°. The immunoreaction product was visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB) reaction. Control sections were incubated in the antiserum preabsorbed with synthetic tGnRH-I or -II at 10 μg/ml overnight at 4°. For whole-mount preparations, procedures were nearly the same, but each step was somewhat prolonged, during which small pieces were sucked and squeezed several times with a wide-bore pipette.

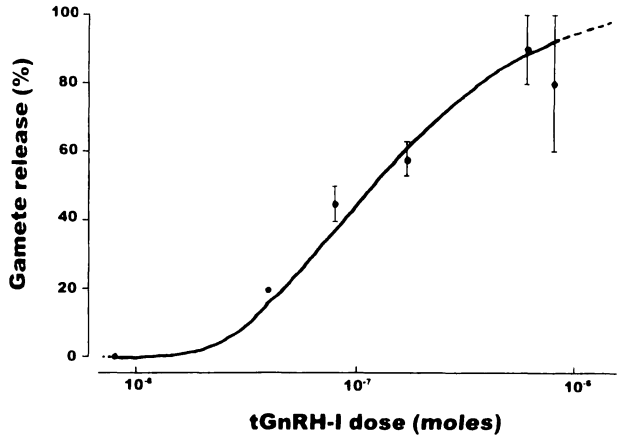
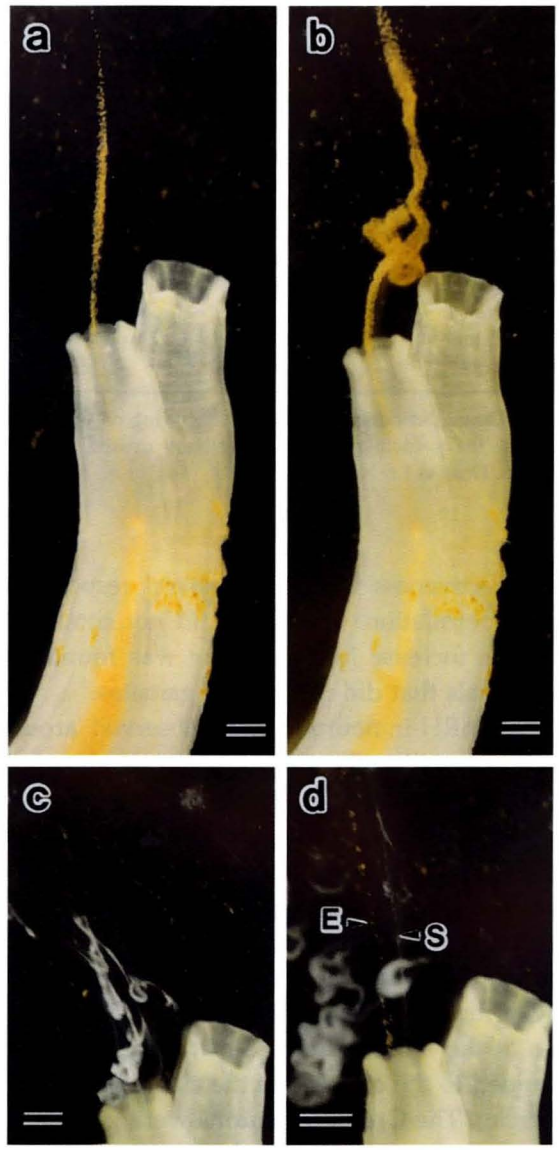


FIG. 1. Dose–response curve for tGnRH-I on gamete release in *C. intestinalis*. Vertical bars indicate means ± SEM (n = 10).

Electron microscopy. Neural complexes were excised and fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 4 h at 4°. After washing in the same buffer containing 7% sucrose overnight at 4°, they were postfixed with 2% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at 4°, dehydrated through an ethanol series, and embedded in epon–araldite. For immunoelectron microscopy, tissues were fixed with a mixture of 4% paraformaldehyde and 0.5% glutaral-



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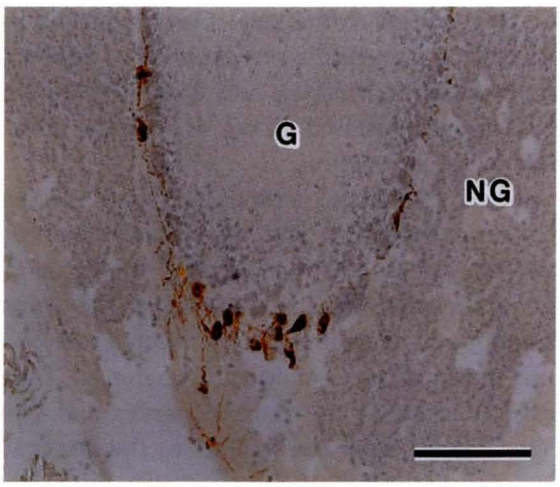

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dehyde in 0.1 M cacodylate buffer (pH 7.3) for 4 h at 4°. They were postfixed with 0.5% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at 4°. The tissues were dehydrated through a graded ethanol series, infiltrated with LR White resin (London Resin, Basingtoke, UK), and then placed in the pure resin. They were polymerized in gelatin capsules at 55° for 48 h. Ultrathin sections were mounted on Formvar-coated nickel grids and preincubated with 1% bovine serum albumin (BSA) in 0.01 M phosphate-buffered saline (PBS, pH 7.5) for 1 h. After washing in PBS, the sections were incubated with the primary antibody raised against synthetic sGnRH (UCB–Bioproducts, 1:100) in 1% BSA/PBS overnight at 4°. The sections were washed well with PBS, reacted with gold-labeled (10-nm) secondary antibody (British BioCell International, Cardiff, UK, 1:20) for 2 h, and then washed with PBS and distilled water. After air-drying, the sections were double-stained with aqueous uranyl acetate and lead citrate.

RESULTS

Injections of tGnRH-I at any of the four selected sites (posterior body cavity, ovary, stomach, gonoducts) all induced gamete release. The latent period between the administration of GnRH and the initiation of gamete release and its extent varied according to the injection sites and between individuals. Administration of the neuropeptide also increased the water flow by an activation of ciliary movement of stigmata and a bodily contraction. When injected into the posterior body cavity, the latent period was about 40 min or more. This was the longest lag time among the injection sites. The extent of gamete release was moderate and very similar to spontaneous spawning. The

FIG. 2. Gamete release induced by injection of tGnRH-I into the lumen of the stomach. (a) Beginning of ovulation a few minutes after injection. (b) One minute later [from (a)]. Eggs are further extruded in a form of thick thread. (c) Massive release of sperm. (d), Normal release of eggs (E) and sperm (S) following massive release of sperm. Bar, 1 cm.

FIG. 3. GnRH-ir neurons around the cerebral ganglion (G). They send fibers posteriorly (under side). NG, neural gland. Bar, 100 μm.

increase in water flow always preceded spawning. This was noticed by an opening of siphons, swaying of water surface, and active movement of minute particles along excurrent water following administration of GnRH. Bodily contraction also became frequent. When directly injected into the ovary, gamete release began within a few minutes and continued for about 10 min. Massive extrusion of eggs and sperm sometimes occurred. It was motivated by a bodily contraction when the extruded gametes from the gonoducts were accumulated in the atrium. Injection into the lumen of the stomach unexpectedly also caused gamete release. Most individuals that released gametes initiated spawning within 10 min that continued for about 30 min, with interruptions. Large-scale extrusion was very common; massive release and often continuous extrusion of eggs or sperm in the form of thick thread usually occurred during active spawning (Fig. 2). Injection directly into the lumen of the gonoducts caused an instantaneous release of gametes. Large-scale extrusion (i.e., the massive extrusion or release of thread-like aggregate more than 10 cm long) often occurred as soon as the injected individuals were returned to water vessels. In many cases, it continued for about 10 min and then ceased abruptly. Control saline injections in each experiment caused no gamete release. As a double control, tGnRH-I was injected into the control animals at the experimental dose just after the end of the injection experiment. This caused a gamete release that was very similar to that of the actual injection experiments. The time course and effectiveness of gamete release after injection of tGnRH-I into the experimental sites is summarized in Table 1.

The tGnRH-II is a homodimer of the decapeptide, unique to ascidians. Its function is currently unknown, but the author investigated whether and how this second form of GnRH may be concerned with reproduction in ascidians. Injections into the same sites and doses at equivalent of the tGnRH-I (doses are nearly halves of the tGnRH-I, as molar concentration) caused gamete release, but it was generally less effective than that evoked by tGnRH-I. Usually, only a weak spermiatin occurred. However, direct injections into the lumen of the oviduct sometimes also caused ovulation, and it continued for a few minutes.

Nontunicate forms of GnRH (e.g., hGnRH, sGnRH, cGnRG-II) also induced gamete release, but the relative effectiveness was low. General response was a weak spermiation. Ovulation did not commonly occur. The increase in water flow was found even in individuals that did not release gametes.

TABLE 1

Time Course of Occurrence and Duration of Gamete Release after Injection of tGnRH-I in the Four Different Sites

Site of injection	Range of latent period	Mean	Spawning caused (%)	*n*
Posterior body cavity	40–80 min	45 min	67	10
Within the ovary	2–25 min	8 min	60	10
Lumen of the stomach	1–40 min	10 min	80	10
Lumen of the gonoducts	Several seconds–10 min	2 min	87	10

Note. Latent period greatly varies depending on the site of injection, but the gamete release is effectively caused in all sites of injection. Dose is 1.6×10^{-7} M.

The GnRH-ir neurons were observed around the cerebral ganglion (Fig. 3) and along the dorsal strand, which is a thin tubular structure connecting the posterior part of the neural gland and the gonads. The GnRH-ir neurons are numerous on the posteroventral surface of the cerebral ganglion facing the neural gland. They send fibers posteriorly along the dorsal strand, partly associated with visceral nerves. Anteriorly, a few GnRH-ir fibers that originated from neurons at the anterior surface of the cerebral ganglion extended to the opening of the ciliated funnel (dorsal tubercle). The GnRH-ir neurons were generally bipolar and connected to each other via fibers, suggesting that they act synchronously. These neurons around the cerebral ganglion were counted at about 130 in fully matured individuals and face circulating blood. GnRH-ir neurons and fibers are scarce within the adult cerebral ganglion.

Around the dorsal strand, the GnRH-ir neurons were most numerous and were distributed continuously from the areas surrounding the cerebral ganglion to the anterior part of the ovary. They run along the gonoducts and some fibers projected to them in the under parts of the gonoductal openings. They also partly attached to the visceral nerves and GnRH-ir fibers derived from the regions of the cerebral ganglion. Preabsorption of the antiserum with synthetic

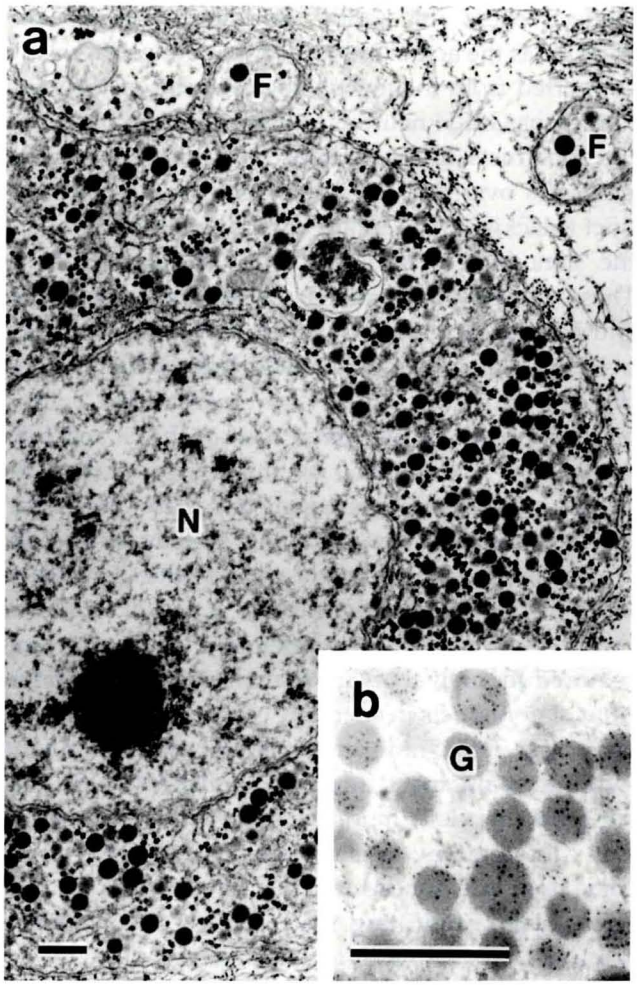

FIG. 4. Electron micrographs of GnRH neurons. (a) Numerous dense granules measuring 100 to 250 nm in diameter distributed throughout the cytoplasm. Some fibers (F) from other neurons lie nearby. (b) Immunogold particles localized on the granules (G). N, nucleus. Bar, 0.5 μm.

tGnRH-I or -II (10 ng/ml) greatly diminished immunoreactivity.

GnRH neurons are large in general, measuring about 10 to 20 μm in diameter. They contain numerous dense granules about 100 to 250 nm in diameter throughout the cell body and fibers (Fig. 4a). Rough endoplasmic reticulum and a well-developed Golgi apparatus, which are characteristic of endocrine cells, are present. Distribution of immunogold-labeled neurons completely coincided with that of light microscopic GnRH-ir neurons. Most immunogold particles were localized on the granules (Fig. 4b). The GnRH neurons were always covered with thin connective tissue filaments. The GnRH neurons surrounding the cerebral ganglion often formed groups of several cells and were separated by a layer of connective tissue filaments from the cerebral ganglion itself. The granule-rich (GnRH-ir) fibers often ran toward other granule-rich neurons but did not form synapses.

DISCUSSION

It is commonly accepted that spawning of *C. intestinalis* occurs with reference to a light cue, which we often experience when animals are transferred to a bright light from the dark. Mature individuals of *C. intestinalis* from North America normally spawn shortly after dawn (Conklin, 1905; Berrill, 1947; Lambert and Brandt, 1967), like those of other solitary ascidians (Conklin, 1905; Huus, 1939, 1941; Costello *et al.*, 1957), while those from the Mediterranean spawn at darkness following light (Georges, 1968). This shows that the photoperiod cue(s) naturally triggers spawning, which may be advantageous for animals of the same species because it makes possible a coordinated simultaneous gamete release.

Electrophysiological studies in *H. roretzi* shows that high-frequency spontaneous discharges, which are characteristic of GnRH neurons, can be recorded in the dark and are completely inhibited by light (Ohkuma *et al.*, 2000). This inhibition of electrophysiological activity by light of GnRH neurons seems very important with respect to the neurophysiological response to light. This might mean that a light cue is associated with a control of the activity of GnRH neurons and presumably also GnRH release. In *C. intestinalis*, GnRH neurons run parallel to the gonoducts and some fibers projected to the wall of the sperm duct and the oviduct posterior to the openings of the gonoducts. The walls of both the oviduct and sperm duct are comprised of a single epithelium. The oviduct is richly ciliated, but the sperm duct is nonciliated. These cells of sperm duct are especially characterized by the development of a microfilament system in the distal region of each cell, which may contribute to the contraction of the sperm duct preceding spawning (Woollacott, 1974). The neurons that project to the wall of the gonoducts may be involved in the control of spawning. It is also possible that the numerous GnRH neu-

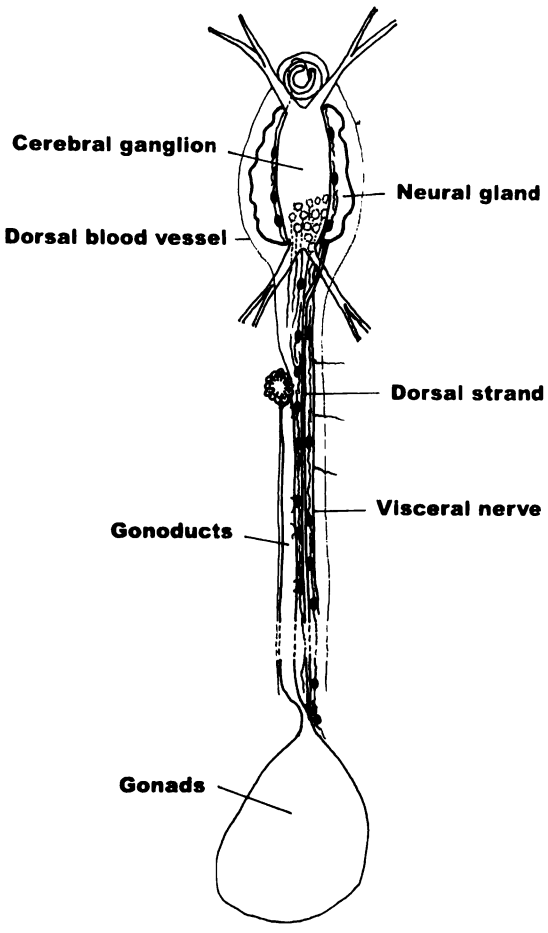

FIG. 5. Schematic illustration showing the distribution of GnRH-ir neurons (viewed from dorsal side). GnRH-ir neurons are distributed from the surrounding regions of the cerebral ganglion to the gonads along the dorsal strand. They are wholly surrounded by the blood circulation.

rons in the blood sinus along the gonoducts release GnRH into the blood circulation in response to light following a dark adaptation and that the released GnRH stimulates ciliary beating in the oviduct and also induces contraction of the microfilament-rich epithelium of the sperm duct, by which eggs and sperm are extruded. The distribution of GnRH-ir neurons is schematically shown in Fig. 5.

Because the sperm duct of *Ciona* is nonciliated, the development of microfilament systems in the epithelial cells of the sperm duct may be responsible for the effective extrusion of accumulated spermatozoa by contraction (Woollacott, 1974).

Effective induction of gamete release by administration of exogenous tGnRH-I into *C. intestinalis* and the ineffectiveness of saline injection indicate that tGnRH-I is the actual inducer of spawning. This also is supported from the morphological point of view that numerous GnRH neurons and fibers run in parallel to the gonoducts. These neurons send numerous fibrous processes over the walls of the sperm duct and oviduct (Mackie, 1995). Innervation by motor neurons of the gonoducts was not found (Woollacott, 1974). These careful observations reveal that some of the GnRH neurons project to the wall of sperm duct and oviduct. This makes it possible that GnRH neurons directly control the ciliary movement of the oviduct and contraction of the sperm duct or that it acts indirectly (neuromodulately) on the neurons within the gonoducts. Instantaneous gamete release after direct injection into the lumen of the gonoducts and the requirement of a long latent period when injected in more distant sites imply a direct action of GnRH on the gonoducts. The latent period may be the time required for arrival of GnRH molecules at the epithelium of gonoducts in adequate concentrations to cause spawning. The 27-min latent period required for light-induced spawning (Lambert and Brandt, 1967) also supports this view. In *Molgula manhattensis,* spawning was caused at any time by injection of tGnRH at the same dose into gonads within a few minutes (unpublished data), indicating that tGnRH is the common inducer of spawning in ascidian species.

The second form of GnRH (tGnRH-II) also induced a weak but appreciable gamete release. This indicates that tGnRH-II is the real co-inducer of spawning but that its role might be secondary. Differing from the result that the nontunicate forms of GnRH had the tendency to induce only a weak spermiation, tGnRH-II induced both the spermiation and ovulation and to a larger extent than the former, even the molar concentration is half of other forms of GnRH. A dimeric motif may play an important role in receptor activation (Craig *et al.,* 1997), as both the N- and C-terminus of the molecule that are known to play in receptor binding are the same.

The nontunicate forms of GnRH also caused a weak spawning response (i.e., mostly a weak spermiation). This is apparently due to the similarity of amino acid sequence in the GnRH peptides at both ends and the same structures at the carboxy- and amino-terminals of the GnRH molecules. However, for effective activation of spawning in *C. intestinalis,* the characteristic

four-amino-acid sequence in the central region of tGnRH may be important for receptor binding and activation.

The acceleration of the water flow following the administration of GnRH, which gives the impression of an "awakening" of ascidians, may also be due to the activation of ciliary movement by GnRH in stigmata of the branchial basket as well as increased bodily contraction at irregular intervals. This phenomenon is normally initiated preceding or in concert with spawning. Fine GnRH-ir fibers also run within the branchial basket (Mackie, 1995), but they do not extend side branches ending in synaptic boutons to the ciliated cells of the branchial stigmata like those of the motor nerve fibers visualized by cholinesterase histochemistry in *Corella* (Arkett *et al.*, 1989). Naturally, increased water flow may occur in concert with or slightly before the spawning, possibly due to activation of ciliary movement in both stigmata and epithelial cells of oviduct by GnRH secreted into the bloodstream and/or modulation of cholinergic neurons projected to the ciliated cells of the branchial stigmata by GnRH neurons.

Maturation of oocytes and spermatocytes is an event that is prerequisite to spawning. It has been claimed that injection of tGnRH into the visceral blood sinus of adult ascidians results in an increase in estradiol content of the gonads (Sherwood *et al.*, 1997) and that treatment of *Ciona* gonads with mGnRH or cGnRH-I stimulates the release of sex steroids (DiFiore *et al.*, 2000). However, GnRH, including tGnRH-I and -II, did not induce the maturation of oocytes *in vitro* in *H. roretzi* (unpublished data). It is then strongly suggested that tGnRH -I or -II causes the release of already matured gametes from the gonoducts but might not directly induce the maturation of the eggs.

Abundant peripheral localization of GnRH neurons in the blood sinus along the gonoducts is peculier to ascidians. The new function of GnRH (i.e., the induction of spawning) may be added for control of reproduction in ascidians, in addition to its originally established hypothalamo–hypophysiotropic function (Matsuo *et al.*, 1971; Burgus *et al.*, 1972), and as a neuromodulator in the synpathetic nervous systems (Jones, 1987; Lescheid *et al.*, 1996), as well as a paracrine regulator in the gonads (Kelly *et al.*, 1991; Habibi *et al.*, 1994), in vertebrates.

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