

Induced Spawning in the Sea Cucumber *Apostichopus japonicus* by Neuropeptide, Cubifrin

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Abstract: In hatcheries the induction of spawning in sea cucumbers has been typically carried out by regulation of rearing conditions such as temperature and light intensity. However, this method is relatively ineffective and the rate of spawning is unpredictable. In this study we established an efficient method for inducing spawning in the Japanese sea cucumber *Apostichopus japonicus* by injecting a neuropeptide, cubifrin.

We purified peptides that can induce oocyte maturation from the buccal tissues containing the nerve ring, using liquid chromatography and *in vitro* assay with ovarian fragments in combination. The effective dose of each peptide was evaluated with chemically synthesized peptides. Consequently, the most potent peptide was identified as NGIWY-amide. We also found that synthetic derivatives replaced the third amino acid, isoleucine, with a different basic amino acid could be 10-100 times more potent than the natural hormone.

When injected into the body cavity of sexually matured individuals, NGIWY-amide, or its derivative, induced spawning in both males and females. NGIWY-amide was then named cubifrin after the Japanese word “kubifuri” meaning waving head, which is a reproductive behavior of sea cucumbers. Gamete shedding started about 60 min and 80 min after the injection in males and females, respectively, and was completed almost simultaneously in the two sexes about 2 hours after the administration. The *in vitro* responsiveness of biopsied ovarian fragments was well correlated with the spawning success induced by an injection. Therefore, cubifrin can be used as an excellent detector of maturity as well as an inducer of spawning in *A. japonicus* in a hatchery setting.

Key words: reproduction, hormone, aquaculture, echinoderm

The Japanese common sea cucumber *Apostichopus japonicus* is the most commercially-important holothurian species in Japan. Until recent years the annual catch had been about 5,000-10,000 tons (wet weight) but fishing pressure has been increasing mainly because of a growing demand for export to China. Therefore, the sustainable production of the sea cucumber is crucial. For over 30 years, cultured juveniles of this species have been released into fishing grounds to supplement natural stocks. The current situation concerning sea cucumber fisheries makes this activity more important than ever.

In hatcheries, the induction of spawning in sea cucumbers is typically carried out by regulation of rearing conditions such as temperature, water exchange, and light intensity (Battaglene *et al.*, 2002; Sui, 2004; Wang and Cheng, 2004; Liu *et al.*, 2004; Shao *et al.*, 2006). In Japan, wild-caught *A. japonicus* broodstock are induced to spawn in the dark in tanks of seawater at temperatures that are ~ 5°C higher than natural seawater (Ito, 1995). However, these methods are relatively ineffective and the rate of spawning is unpredictable. Therefore, more effective method of spawning induction has been

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desired.

Starfishes have long been used as model animals for the study of reproductive biology after the finding of spawning-inducing activity in a nerve extract in the middle of the 19th century (Chaet and McConnaughy, 1959). At long last, the nervous substance was purified and identified as a relaxin-like protein (Mita *et al.*, 2009). Since both starfishes and sea cucumbers belong to the Echinodermata, their reproductive mechanism might be driven by similar endogenous factors. Thus, we started the study to discover a spawning-inducing substance in *A. japonicus*, following the strategy employed in the study in starfish.

Here, we report a native neuropeptide potent in inducing oocyte maturation and spawning in *A. japonicus*, and propose a procedure of using the peptide in a hatchery setting.

Materials and Methods

Preparation of neural extract for purification of a spawning-inducing substance: Buccal tissues containing the ring nerve were homogenized in the same volume of ice-chilled 4 N acetic acid containing 0.4 mM β -aminoethyl benzensulfonyl fluoride, 10 μ g/ml leupeptin and 4 μ M pepstatin A. The homogenates were centrifuged at 15,200 x g for 15 min. at 4°C. The resulting supernatants were then ultracentrifuged at 45,000 x g for 1 hr at 4°C. These supernatants of ultracentrifugation were kept as a crude neural extract in aliquots at -80°C. The crude extract was separated by serial ultrafiltration with AmiconUltra YM30 and YM10 (Millipore). The gonadotropic activity determined by the *in vitro* maturation assay was mainly recovered in the fraction of less than 10K daltons. After YM10 filtration, the filtrate was lyophilized and preserved for a subsequent purification process.

Liquid chromatography: The lyophilized preparation was dissolved in MilliQ water. After centrifugation, the supernatant was applied to a Develosil C8-UG-5 column (20 x 50 mm) and eluted with 80 % acetonitrile containing 0.1 % trifluoro acetic acid (TFA) for desalting. The desalted effluent was lyophilized. The lyophilized extract was fractionated by reversed-phase high performance

liquid chromatography (RP-HPLC) using a Develosil RPAQUEOUS-AR-5 column (10 x 250 mm) with a linear gradient of acetonitrile from 10 to 40 % with 0.1 % TFA. Each fraction was lyophilized, then dissolved in 0.5 ml MilliQ water to detect the gonadotropic activity by the *in vitro* maturation assay. The fractions containing the gonadotropic activity were separated by a Develosil RPAQUEOUS-AR-5 column (10 x 250 mm) with a linear gradient of acetonitrile from 10 to 30 % containing 20 mM ammonium acetate (pH 6.0). The fractions were lyophilized and assayed as mentioned above. The active fractions were separated by a Develosil RPAQUEOUS-AR3 column (2 x 250 mm) with a linear gradient of acetonitrile from 15 to 22 % with 0.1 % TFA. The gonadotropic activity was detected in fractions 30 and 33 of the last chromatography step.

Determination of chemical structure of gonadotropic substances: The fractions 30 and 33 were analyzed for accurate mass values and amino acid sequences by liquid chromatograph-tandem mass spectrometers (Quattro Premier, Waters; nanoFrontier LD, Hitachi High-Technologies) and a protein sequencer (491 cLC, Applied Biosystems).

Preparation of peptides: NGIWY-amide (cubifrin) and its derivatives were chemically synthesized. A stock solution of 1 mM dissolved in MilliQ water was stored at -80°C. Prior to use, it was diluted with filtered seawater to the desired concentrations.

***In vitro* maturation assay:** A portion of ovary was excised through a short incision in the body wall. The excised ovarian tissue was cut into small fragments about 3 mm long. For the purification of a maturation-inducing substance, ovarian fragments were incubated with each fraction for 1.5 hr at 20°C. The gonadotropic activity was determined by germinal vesicle breakdown (GVBD) and also by the incidence of ovulation of oocytes from ovarian fragments.

For the detection of the responsiveness to peptides, ovarian fragments were incubated either with the peptide solution at 100, 10, 1 nM, or 100 pM in filtered seawater or with filtered seawater alone. The test was duplicated for each individual. The incidence of GVBD were scored on a scale of 0-3 in which 3 indicates GVBD in over two-thirds

of oocytes in the fragment; 2, in over one-third of oocytes; 1, in up to one-third of oocytes; 0.5, in a few percentage of oocytes; 0, no response of oocytes. The sum of scores of all treated ovarian fragments from one individual (8 fragments for each individual) was regarded as the oocyte maturation score of that individual. After the assay, oocytes in the control ovarian fragments were mechanically separated by gentle pipetting and the diameters of 20 well-developed oocytes were measured.

Injection of cubifrin and observation of reproductive behavior: Cubifrin solution (10 μ M) or filtered seawater was injected into the body cavity of thesea cucumbers; the injection volume was 0.1% of body weight (v/w). Each sea cucumber was then separately placed on the bottom of a 21-l tank, and its behavior was continuously monitored until the completion of spawning, or for 2 hr if no spawning occurred. We recorded the times at which the underside of the water surface was reached, when head waving commenced, and when spawning was started and completed. After the completion of spawning, the number of spawned eggs in 10 ml of tank water was counted to estimate the total number of spawned eggs for each female.

Results and Discussion

Purification of an oocyte maturation-inducing substance: The neural extract was separated by three serial HPLCs (Fig. 1). On the third chromatography, the gonadotropic activity was separated into two independent fractions, Fractions 30 and 33 (arrowheads in Fig. 1c). Both fractions were analyzed by mass spectrometry and protein sequencing. The amino acid sequence of Fraction 30 was NGIWY, and Fraction 33's sequence was QGLFSGV. While the calculated monoisotopic mass values of these sequences were 651.320 and 706.365, analyses by mass spectrometry indicated mass signals of 650.35 and 705.42, respectively. Further *de novo* sequencing analyses of these mass signals suggested that these had the same sequences as those given through protein sequencing except for anamidation at each C-terminal end of the peptides. With these analyses we determined that the sequences of the components in Fractions

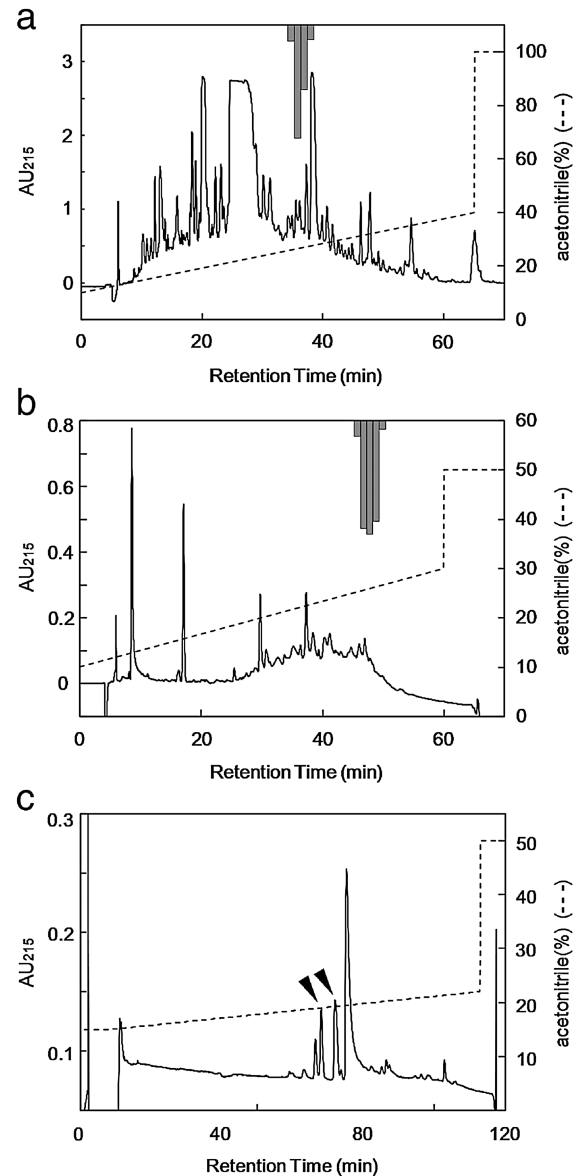


Fig. 1. Separation of the gonadotropic activity by RP-HPLC. Dotted lines in the chromatograms indicate changes of programmed concentrations of acetonitrile in the eluents. Hormonal activity in each fraction is shown with the help of bars hanging from the top side of the chromatograms (a, b). Arrowheads in (c) indicate fractions 30 and 33 containing the bioactivity. Reproduced from Kato *et al.* (2009) with permission.

30 and 33 were NGIWY-amide and QGLFSGV-amide, respectively. Unexpectedly, both of these peptides completely differ from a relaxin, which is an oocyte maturation-inducing substance in starfish. NGIWY-amide has been reported as a peptide associated with contractions of muscle, intestine and tentacles in *A. japonicus* (Inoue *et al.*, 1999),

whereas QGLFSGV-amide has not been reported ever.

In vitro maturation-inducing activity of peptides: Two peptides, NGIWY-amide and QGLFSGV-amide, were chemically synthesized and GVBD-inducing activity was estimated with *in vitro* maturation assay. NGIWY-amide was extremely potent even at 1 nM or less (Fig. 2). QGLFSGV-amide was less potent in inducing GVBD, with effective concentrations being 1 μ M or more. Thus, NGIWY-amide is strongly suspected to be involved in the regulation of oocyte maturation. In contrast, QGLFSGV-amide does not appear to be the primary endocrine regulator of oocyte maturation.

The activities of the two derivatives, NGLWY-amide and NGIWY-COOH, were compared with that of the natural NGIWY-amide (Fig. 3). NGLWY-amide was effective at 10 pM or less, a hundred times more potent than the natural peptide, NGIWY-amide. However, NGIWY-COOH barely induced GVBD at 100 nM. Accordingly, C-terminal amidation of the

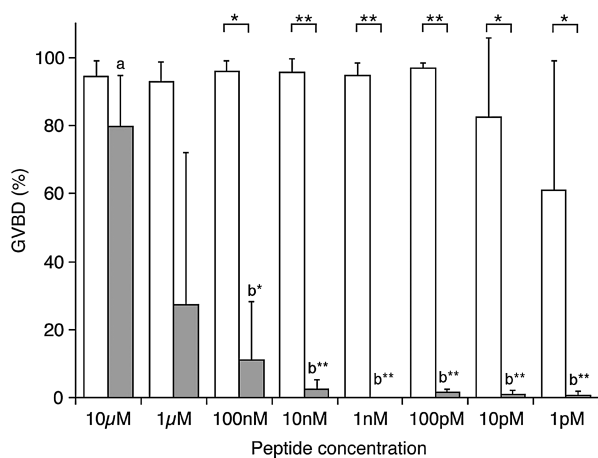


Fig. 2. Concentration dependence of NGIWY-amide and QGLFSGV-amide to induce GVBD in ovarian fragments. Ovarian fragments were incubated with NGIWY-amide (white bars) and QGLFSGV-amide (gray bars) at various concentrations. Bars represent percentages of GVBD (means \pm SD of duplicate determinations of four separate experiments). Bars with different labels differ significantly ($P < 0.05^*$, $P < 0.01^{**}$). The grouping symbol at the top side of the graph indicates a significant difference between NGIWY-amide and QGLFSGV-amide at each concentration. Significant differences were determined using Wilcoxon's rank sum test. Reproduced from Kato *et al.* (2009) with permission.

peptide is indispensable for the bioactivity.

Effect of NGIWY-amide and NGLWY-amide in inducing spawning: NGIWY-amide and NGLWY-amide were injected into sexually matured males and females to examine the effect on inducing spawning behavior. Injections of NGIWY-amide induced spawning in males and females at 10 nM. NGLWY-amide more effectively induced spawning in males at 1 nM and in females at 100 pM. NGLWY-amide was therefore at least ten times more potent than NGIWY-amide, as in the case of *in vitro* experiments. Sea cucumbers injected with NGIWY-amide or NGLWY-amide exhibited reproductive behaviors that were independent of sex and typically included: (1) climbing up the side wall of the tank to the underside of the water surface; (2) throwing back and swaying of the head; (3) shedding gametes from the gonopore in the head region. Gamete release occurred 45-60 min after

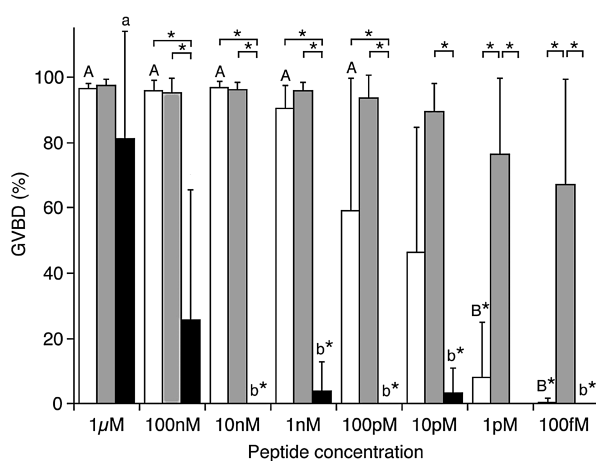


Fig. 3. Concentration dependence of NGIWY-amide and its derivatives to induce GVBD in ovarian fragments. Ovarian fragments were incubated with synthetic analogues at various concentrations. NGIWY-amide (white bars), NGLWY-amide (gray bars) and NGIWY-COOH (black bars) were examined. Bars represent percentages of GVBD (means \pm SD of triplicate determinations of six different experiments), and bars with different labels differ significantly in each peptide ($P < 0.05^*$, $P < 0.01^{**}$). The grouping symbol at top side of the graph indicates a significant difference among two peptides at each concentration. Significant differences between means were determined using Steel-Dwass's pair-wise comparisons. Reproduced from Kato *et al.* (2009) with permission.

the peptide injections in males and 70–90 min in females, and was completed almost simultaneously in the two sexes about 2 hr after the administration (Fig. 4). This series of actions from climbing to spawning observed in the experiment seemed to be a typical behavior of spawning by sea cucumbers (McEuen, 1988; Battaglione, 2002). NGLWY-amide and NGLWY-amide were named cubifrin and cubifrin-L, respectively, after the Japanese word “kubifuri” meaning waving head, which is a reproductive behavior of sea cucumbers.

Association between oocyte size and responsiveness to cubifrin: The competence of ovarian fragments to undergo *in vitro* oocyte maturation and the induction of spawning by cubifrin-L stimulation were examined in relation to oocyte size. All ovarian fragments with oocytes >155 μ m in diameter (animal nos. 17–25 in Fig. 5) responded to cubifrin-L *in vitro*, as indicated by their oocyte maturation score, which was a measure of their ability for ovulation and GVBD. In contrast, all fragments but one (animal no. 11 in Fig. 5)

with oocytes <155 μ m failed to respond to the treatment. The injection of cubifrin-L into the body cavity induced spawning in all females with ovaries that responded to cubifrin-L *in vitro*. Females with unresponsive ovaries *in vitro* did not spawn following the injection, with the exception of two females (nos. 12 and 13). The number of spawned eggs per 100 g body weight varied from 765 to 1,826,000, and there was a significant correlation between this value and the oocyte maturation score ($r = 0.84$). The number of eggs spawned by the two non-responsive females in *in vitro* experiment was especially low (765 eggs per 100 g body weight in female no. 12 and 23,700 in female no. 13).

These results indicate that potential spawners in broodstock can be selected by *in vitro* examination of the cubifrin or cubifrin-L responsiveness of ovarian fragments or by measuring oocyte size. Furthermore, the number of spawned eggs can be roughly estimated by *in vitro* examination.

Procedure of spawning induction in a hatchery: In conclusion, spawning induction by cubifrin is an

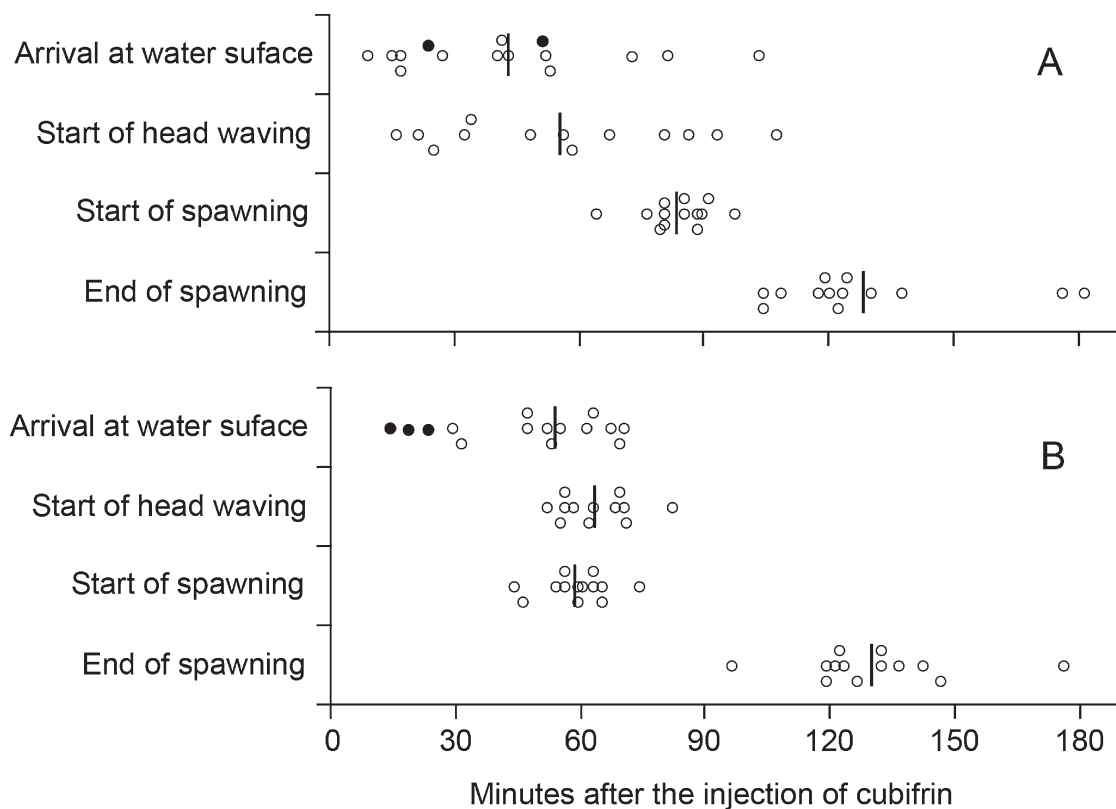


Fig. 4. Progress of spawning behavior in females (A) and males (B). Open and closed circles indicate the data point of each NGLWY-amide-injected male and female ($n=14$ each) and control sea cucumber ($n=4$ each). Vertical bars show the average of the NGLWY-amide-injected group.

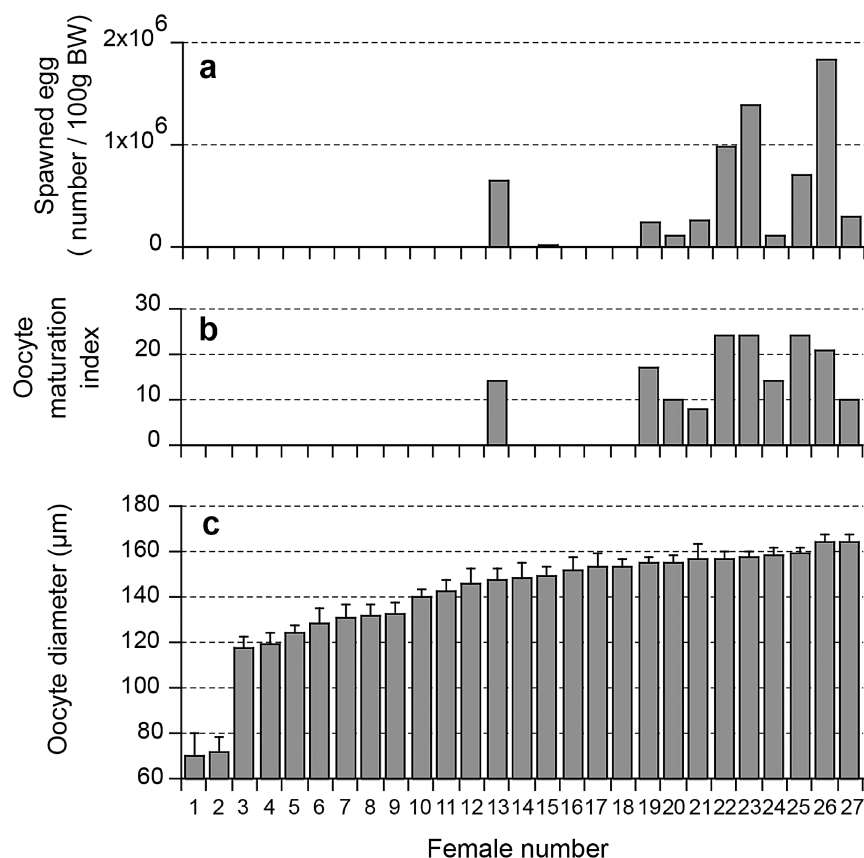


Fig. 5. Association between oocyte size and responsiveness to cubifrin. Responsiveness to NGLWY-amide was determined by spawning success, as indicated by the number of spawned eggs per 100 g body weight (a) and by *in vitro* maturation assay, evaluated from the oocyte maturation score (b). See the Materials and methods for a description of the oocyte maturation score. Oocyte sizes (mean diameter \pm SD, $n = 20$) of each female are shown in (c).

effective and simple method for practical application during the artificial culture of *A. japonicus*. We propose the procedure of spawning induction as follows:

- (1) preparation of sea cucumbers during the spawning season;
- (2) sex determination and gross evaluation of maturity from the appearance of biopsied gonad;
- (3) evaluation of definite maturity with biopsied ovary by *in vitro* test;
- (4) Rearing mature individuals below 15°C to suppress unanticipated natural spawning until spawning induction;
- (5) spawning induction by the injection of 10 μ M cubifrin solution (0.1% of body weight (v/w));
- (6) standard fertilization and cultivation of fertilized eggs.

Acknowledgements

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Kato S., Tsurumaru S., Taga M., Yamane T., Shibata Y., Ohno K., Fujiwara A., Yamano K., and Yoshikuni M., 2009: Neuronal peptides induce oocyte maturation and gamete spawning of sea cucumber, *Apostichopus japonicus*. *Dev. Biol.*, **326**, 169-176.

Extracts prepared from tissues containing buccal ring nerve or longitudinal radial nerve of sea cucumber induce oocyte maturation and ovulation from ovarian tissues. We purified two small peptides, a pentapeptide and a heptapeptide, from the buccal tissues of Japanese common sea cucumber, *Apostichopus japonicus*. Both peptides induced oocyte maturation and gamete spawning. The pentapeptide was identified as NGIWY-amide. This peptide induced *in vitro* germinal vesicle breakdown and ovulation of fully-grown oocytes at less than 1 pM and *in vivo* spawning at 10 nM. A synthetic derivative of the pentapeptide, NGLWY-amide, was 10-100 times more potent compared to the natural NGIWY-amide. The heptapeptide was less potent, inducing ovulation at 1 μM. NGIWY-amide and NGLWY-amide induced a characteristic spawning behavior when injected into sexually matured individuals. Mature eggs artificially spawned were fertilized, and developed normally and metamorphosed into young sea cucumbers. The details of the production and the mechanism of action of NGIWY-amide are still unclear, but the high biopotency of the peptide will aid understanding of the neuronal and hormonal control of reproduction of sea cucumber.

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The neuropeptide cubifrin-I and its derivative cubifrin-L have recently been demonstrated as potent substances that induce oocyte maturation *in vitro* and spawn in the Japanese common sea cucumber *Apostichopus japonicus*. Here, the reproductive behavior provoked by injection of cubifrin-L into the body cavity of *A. japonicus* was examined with a view to the practical application

of the peptide for induction of spawning in the hatchery. Ovarian fragments with oocytes larger than 155 μm in diameter responded to cubifrin-L *in vitro*. The *in vitro* responsiveness of ovarian fragments was well correlated with the spawning success induced *in vivo* by a cubifrin-L injection. Mature sea cucumbers injected with cubifrin-L displayed sequential reproductive behaviors, which comprised climbing the side wall of the tank toward the water surface, waving of the head, and shedding of gametes. Gamete shedding started about 60 min and 80 min after the injection in males and females, respectively, and was completed almost simultaneously in the two sexes about 2 hours after the administration. Repeated injections of cubifrin-L at intervals of about 10 days successfully induced multiple spawnings in males and females. This study demonstrated that cubifrin-L is an effective inducer of spawning in Japanese sea cucumber cultivation.