Jellyfish oocyte Maturation Inducing Hormone and neuroendocrine regulation of reproduction

Noriyo Takeda[a,b], Yota Kon[c,] Gonzalo Quiroga Artigas[d], Pascal Lapébie[d], Carine Barreau[d], Osamu Koizumi[e], Takeo Kishimoto[b#], Kazunori Tachibana[b], Evelyn Houliston[d*], Ryusaku Deguchi[c*].

a. Research Center for Marine Biology, Graduate School of Life Sciences, Tohoku University, Asamushi, Aomori, Japan.

b. Laboratory of Cell and Developmental Biology, Graduate School of Bioscience, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226-8501, Japan

c. Department of Biology, Miyagi University of Education, Sendai, Japan

d. Sorbonne Universités, UPMC Univ. Paris 06, CNRS, Laboratoire de Biologie du Développement de Villefranche-sur-mer (LBDV), 06230 Villefranche-sur-mer, France.

e. Department of Environmental Science, Fukuoka Women's University, Fukuoka, Japan.

[#] Present address: Science & Education Center, Ochanomizu University, Ootsuka, Tokyo 112-8610, Japan.

* Corresponding authors: deguchi@staff.miyakyo-u.ac.jp; houliston@obs-vlfr.fr

Oocyte meiotic maturation is a critical process for all sexually reproducing animals, and its core cytoplasmic regulators are highly conserved between species. In contrast, the few known Maturation Inducing Hormones (MIHs) that act on oocytes to initiate this process have highly variable molecular natures and their evolutionary relationships are poorly understood. Using hydrozoan jellyfish species that spawn in response to opposite light cues we identified from gonad transcriptomes specific amidated tetrapeptides that directly induce maturation of isolated oocytes at nanomolar concentrations. Antibody preabsorption experiments conclusively demonstrated that these PRPamide-related neuropeptides account for endogenous MIH activity. We further showed that they are synthesized by gonad neural cells, are released following dark-light or light-dark transitions, and act on the oocyte surface. Furthermore, they are produced by male as well as female jellyfish and trigger both sperm or egg release, suggesting a role in spawning coordination. We propose an evolutionary link between hydrozoan MIH and the neuropeptide hormones that regulate reproduction upstream of MIH release in bilaterian species.

Fully-grown oocytes maintained within the female gonad are held at first prophase of meiosis until environmental and/or physiological signals initiate cell cycle resumption and oocyte maturation, culminating in release of fertilization-competent eggs. This process of oocyte maturation is a key feature of animal biology, tightly regulated to optimize reproductive success. It involves biochemical cascades activated within the oocyte that are highly conserved across animal phyla, notably involving the kinases Cdk1 (to achieve entry into first meiotic M phase) and MAP kinase (to orchestrate polar body formation and cytostatic arrest)[1-4]. These kinase regulations have been well characterized using biochemically tractable model species, notably frogs and starfish, and knowledge extended using genetic methods to other species including nematodes, drosophila and mammals. Nevertheless information is largely lacking on

certain critical steps, and in particular the initial triggering of these cascades in response to the Maturation Inducing Hormones (MIHs), which act locally in the gonad on their receptors in the ovarian oocytes; the only known examples identified at the molecular level are 1-methyladenine released in starfish[5], steroid hormones in amphibians and fish[6,7], and a sperm protein in *Caenorhabditis*[3].

Hydrozoan jellyfish provide excellent models for dissecting the molecular and cellular mechanisms regulating oocyte maturation, which in these animals is triggered by light-dark and/or dark-light transitions. Remarkably, oocyte growth, maturation and release function autonomously in gonads isolated from female jellyfish, implying that all the regulatory components connecting light sensing to spawning are contained within the gonad itself[8-10]. Furthermore, as members of the Cnidaria, a sister clade to the Bilateria, hydrozoan jellyfish can give insight into spawning regulation in early animal ancestors. In this study we identified the molecular nature and characterized the neural origin of MIH using two hydrozoan jellyfish model species *Clytia hemisphaerica* (Fig. 1A) and *Cladonema pacificum* (Fig. 1B) that are induced to spawn respectively by dark-light or dark-light transitions[11,12]. We further showed that these highly active diffusible amidated tetrapeptides have wider role in stimulating gamete release in both males and females. A complementary study of *Clytia* gonad light detection has revealed that the light-mediated MIH release reported here is dependent on an Opsin photopigment co-expressed in the same neural cell population that secretes MIH (Quiroga Artigas et al, submitted in parallel). A regulatory system for gamete maturation and release based on direct neuropeptide hormone secretion from neural cells, similar to that uncovered in these two studies in jellyfish gonads, may have promoted synchronous gamete maturation, release and fertilization in ancestral animals. The progressive involvement of additional tissues in reproductive control during the evolution of bilaterian species providing an explanation for the variable molecular nature of MIH between extant species.

Results

Active MIH can be recovered from isolated Clytia *and* Cladonema *gonads*

First we demonstrated that true MIH activity can be recovered from small drops of seawater containing isolated ovaries of either *Clytia* or *Cladonema* following the appropriate light transition, as demonstrated previously using other hydrozoan species[8,9]. Isolated oocytes incubated in endogenous MIH recovered in this way complete efficiently and with normal timing the meiotic maturation process, manifest visually by germinal vesicle breakdown (GVBD) and extrusion of two polar bodies (Fig. 1C,D). Isolated *Cladonema* gonad ectoderm, but not endoderm, tissue (see Fig. 1B) was found to produce active MIH. MIH activity from *Cladonema* gonad ectoderm resisted heat treatment at 100°C for 20 minutes (95 % GVBD, n=41), several freeze/thaw cycles (100% GVBD, n=14) and to filtration through a 3000 MW cut-off membrane (90 % GVBD, n=18), consistent with the idea that the active molecule is a small molecule, possibly peptidic [8].

MIH candidates identified from transcriptome data

Cnidarians including jellyfish, hydra and sea anemones express many low-molecular-weight neuropeptides showing various bioactivities[13-16]. These are synthesized by cleavage of precursor polypeptides and can produce multiple copies of one or more peptides, frequently subject to amidation by conversion of a C-terminal glycine[17]. We found previously that some synthetic *Hydra* amidated peptides can stimulate spawning when applied to gonads of the jellyfish *Cytaeis uchidae,* the most active being members of the GLWamide family (10^{-5} M minimum concentration)[16]. Critically, however, these did not induce meiotic maturation when applied to isolated oocytes, i.e. they did not meet the defining criterion of MIHs. These previous results were not conclusive because of the use of species-heterologous peptides, but

suggest that although jellyfish GLWamide peptides do not act as MIHs, they may be involved less directly in spawning regulation.

To identify endogenous species-specific neuropeptides as candidates for MIH from our model species, we first retrieved sequences for 10 potential amidated peptide precursors from a mixed-stage *Clytia* transcriptome (Fig. S1), and then searched for ones specifically expressed in the ectoderm, source of MIH, by evaluating the number of corresponding Illumina Hiseq reads obtained from manually separated ectoderm, endoderm and oocyte gonad tissues (Fig. 2A). In the ectoderm, source of MIH, only 3 putative neuropeptide precursor mRNAs were expressed above background levels, as confirmed by quantitative PCR (Fig. S2). One was a GLWamide precursor, Che-pp11, expressed at moderate levels. Much more highly expressed were Che-pp1 and Che-pp4, both predicted to generate multiple related short (3-6 amino acid) amidated peptides with the C-terminal signature (W or R)-PRP, -PRA -PRG or -PRY. Potential precursors for both GLWamide (Cpa-pp3) and PRP/Aamides (Cpa-pp1 and Cpa-pp2) were also present amongst 4 sequences identified in a transcriptome assembly from the *Cladonema* manubrium (which includes the gonad; Fig. 1B). Cpa-pp1 contains 1 copy of the RPRP motif while Cpa-pp2 contains multiple copies of RPRA motifs (Fig. S1).

Potent MIH activity of synthetic W/RPRXamide peptides

As a first screen to select neuropeptides potentially involved in regulating oocyte maturation, we incubated *Clytia* female gonads in synthetic tetrapeptides predicted from Che-pp1, Che-pp4 and Che-pp11 precursors at 10^{-5} M or 10^{-7} M (Fig. 2B). We uncovered preferential and potent activity for the WPRPamide and WPRAamide tetrapeptides, which consistently provoked oocyte maturation and release at 10^{-7} M, while the related RPRGamide and RPRYamide were also active but only at 10^{-5} M. RPRPamide a predicted product of Cpa-pp1 and of Che-pp8, a precursor not expressed in the *Clytia* gonad, was also active in this screen at

10^{-5} M. In contrast PGLWamide, potentially generated from Che-pp11, did not affect the gonads at either concentration. This result placed WPRP/Aamide-related rather than GLWamide peptides as the best candidates for jellyfish MIH, a view supported by direct MIH activity assay, i.e. treatment of isolated oocytes with the candidate peptides. For *Clytia* oocytes we detected potent MIH activity (as assessed by oocyte Germinal Vesicle breakdown; GVBD; Fig. 2C; Fig. S3) for W/RPRP/Aamide and RPRY/Gamide tetrapeptides, but not for PGLWamide. RPRAamide was more active in triggering GVBD when added to isolated oocytes than to intact gonads, perhaps because of poor permeability through the gonad ectoderm. For *Cladonema* oocytes, RPRP/Aamides showed very potent MIH activity, and the RPRG/Yamides were also active at higher concentrations, but WPRP/Aamides were not active (Fig. 2C, S3). We also tested, on oocytes of both species, pentapeptides and hexapeptides that might theoretically be generated from the Che-pp1 and Cpa-pp1 precursors, but these had much lower MIH activity than the tetrapeptides, while the tripeptide PRPamide and tetrapeptides lacking amidation were inactive (Fig. 2D, S3). The response of *Clytia* or *Cladonema* isolated oocytes elicited by synthetic W/RPRP/A/Yamides mirrored very closely that of endogenous MIH (Fig. S4A,B), and the resultant mature eggs could be fertilized and develop into normal planula larvae (Fig. S4C,D).

W/RPRPamides account for endogenous MIH activity

We demonstrated that W/RPRPamide and/or W/RPRAamide peptides are responsible for endogenous MIH activity in *Clytia* and *Cladonema* by use of inhibitory affinity purified antibodies generated to recognize the PRPamide and PRAamide motifs (as determined by ELISA assay; Fig. 3A,B). These antibodies were able to inhibit specifically the MIH activity of the targeted peptides (Fig. 3C,D). Conclusively, pre-incubation of endogenous MIH obtained from *Clytia* or *Cladonema* gonads with anti-PRPamide antibody for 30 minutes

completely blocked its ability to induce GVBD in isolated oocytes. Pre-incubation with the anti-PRAamide antibody slightly reduced MIH activity but not significantly compared to a control IgG (Fig. 3E).

Taken together these experiments demonstrate that WPRPamide and RPRPamide are the active components of endogenous MIH in *Clytia* and *Cladonema* respectively, responsible for triggering oocyte meiotic maturation. Other related peptides including RPRYamide, RPRGamide, WPRAamide (*Clytia*) and RPRAamide (*Cladonema)* also probably contribute to MIH. These peptides almost certainly act at the oocyte surface rather than intracellularly, since fluorescent (TAMRA-labelled) WPRPamide microinjected into *Clytia* oocytes, unlike externally applied TAMRA-WPRPamide, did not induce GVBD (Fig. 3F).

MIH is produced and secreted by neural cells in the gonad ectoderm

Single and double-fluorescence *in situ* hybridization showed that the *Clytia* MIH precursors Che-pp1 and Che-pp4 are co-expressed in a distinctive population of scattered cells in the gonad ectoderm in males and females (Fig. 4A,B; Fig. S5E). Similarly in *Cladonema*, the predicted RPRPamide precursor Cpa-pp1 was expressed in scattered cells in the manubrium ectoderm which covers the female or male germ cells (Fig. 4A; Fig. S5A,E). Immunofluorescence with the anti-PRPamide and PRAamide antibodies in both species revealed that the expressing cells have a neural-type morphology, with long projections running through the basal side of the ectodermal epithelia (Fig. 4C). In *Clytia* gonad ectoderm, the PRPamide and PRAamide antibodies decorated a single cell population, whereas in *Cladonema* the two peptides were detected in distinct cell populations (Fig. S6B, C). In whole *Clytia* jellyfish, both immunofluorescence and *in situ* hybridization (Fig. S5B-D, S6A) revealed the presence of MIH peptides and their precursors at other sites with conspicuous nervous systems: the manubrium (mouth), tentacles and the nerve ring that runs around the

bell rim[18], as well as the walls of the radial canals. This suggesting that related neuropeptides have other functions in the jellyfish in addition to regulating spawning.

Consistent with the model of MIH release from neural cells following light cues, both anti-PRPamide and anti-PRAamide immunofluorescence signals in the *Clytia* gonad epithelium diminished following light stimulation, becoming much weaker by the time of first meiosis (~45 min, Fig. 4D). Similarly, anti-PRPamide signals in *Cladonema* became very weak around the time of GVBD (~20 min after darkness), although the anti-PRAamide signal was not strongly reduced (Fig. 4E). A recent study has further revealed that in *Clytia* these MIH-secreting neural cells also express an Opsin photoprotein with an essential function in oocyte maturation and spawning (Quiroga Artigas et al, submitted in parallel), suggesting that in hydrozoan gonads a single neural cell population has a key role both in detecting the light signal and mediating the spawning response by releasing MIH neuropeptides.

The similar distribution of MIH-producing cells in female and male gonads (Fig. 4A, S5E) suggests that these neuropeptides may play a general role in regulating gamete release. Consistently, synthetic MIH peptides at 10^{-7} M induced sperm release from male gonads from both *Clytia* and *Cladonema* (Table 1). This indicates that the oocyte maturation stimulating effect of MIH is part of a wider role in reproductive regulation. It also raises the intriguing possibility that MIH neuropeptides released into the seawater from males and females gathered together at the ocean surface during spawning may facilitate precise synchronization of gamete release during the periods of dawn and dusk.

Selective action of MIH peptides between hydrozoan jellyfish species

Our experiments revealed some selectivity in the MIH activity of different peptides between *Clytia* and *Cladonema*. The most potent MIH peptides for *Clytia* oocytes were the main Che-pp1/Che-pp4 derived tetrapeptides WPRPamide (the main component of endogenous MIH),

WPRAamide and RPRYamide, clearly active even at 10^{-8} M (Fig. S3). The best candidate for *Cladonema* MIH is RPRPamide (from Cpa-pp1), while RPRAamide (from Cpa-pp2) was slightly less active (Fig. S3). Correspondingly, the RPRP sequence is not found in precursors expressed in the *Clytia* gonad, while WPRP/Aamides are not predicted from any *Cladonema* precursors (see Fig. 2A; Fig. S1).

Further testing on oocytes from eight other hydrozoan jellyfish species revealed responsiveness with different sensitivities to W/RPRP/A/G/Yamide type tetrapeptides in *Obelia, Aequorea, Bouillonactinia* and *Sarsia,* but not *Eutonina, Nemopsis, Rathkea* or *Cytaeis* (Fig. 5). The responsive and non-responsive species included members of two main hydrozoan groups, leptomedusae and anthomedusae. These comparisons suggest that W/RPRXamide type peptides functioned as MIHs in ancestral hydrozoan jellyfish. We can speculate that variation in the peptide sequences active between related species might reduce inter-species stimulation of spawning in mixed wild populations.

Discussion

We have demonstrated that short neuropeptides with sequence W/RPRPamide are responsible for inducing oocyte maturation and also for provoking gamete spawning in the hydrozoan jellyfish *Clytia* and *Cladonema* (Fig. 6A). These act as *bona fide* MIHs, i.e. they interact directly with the surface of resting ovarian oocytes to initiate maturation. Related neuropeptides (W/RPRXamide type peptides) act as MIHs also in other hydrozoan jellyfish species. Some GLWamide family peptides can also induce spawning, albeit at relatively elevated concentrations, but require the presence of somatic gonad tissue to induce oocyte maturation (this and previous[16] studies) and so may participate indirectly in regulating these processes. Inhibitory or sensitizing factors that act either in the MIH-secreting neural cells or in other ectodermal cells could modulate the light response, and account for species-specific

dawn or dusk spawning. It remains to be seen whether regulation of spawning by MIH neuropeptides related to those in *Clytia* and *Cladonema* extends beyond hydrozoan jellyfish to other cnidarians. If so, further layers of regulation could allow the integration of seasonal cues and lunar cycles to account for well known mass annual spawning events seen in tropical reef corals[19].

The identification of MIH in *Clytia* and *Cladonema* is a significant step forward in the oocyte maturation field because the molecular nature of the hormones that in this way trigger oocyte maturation is known in surprisingly few animal species, notably 1-methyladenine in starfish and steroid hormones in teleost fish and amphibians[1,5]. The very different molecular natures of these known MIH examples from across the (bilaterian+cnidarian) clade could be explained by an evolutionary scenario in which secretion of neuropeptide MIHs from neural cells close to the oocyte was the ancestral condition, with intermediate regulatory tissues, such as endocrine organs and ovarian follicle cells, evolving in the deuterostome lineage to separate neuropeptide-based regulation from the final response of the oocyte (Fig. 6B). Thus various neuropeptides including vertebrate GnRHs (gonadotropin-releasing hormones)[20], as well as modulatory RFamide peptides such as Kisspeptins and GnIH (gonadotropin-inhibitory hormone)[21], regulate various aspects of reproduction including gamete release in both males and females. Chordate GnRHs are PGamide decapeptides, which stimulate the release of peptidic gonadotropic hormones (GTHs) such as vertebrate luteinizing hormone from the pituitary. Similarly, starfish gonad-stimulating substance (GSS/Relaxin)[22] is a GTH produced at a distant "neuroendocrine" site, the radial nerve. In both cases, these peptidic GTHs in turn cause oocyte maturation by inducing MIH release from the surrounding follicle cells, or in the case of mammals GAP junction-mediated exchange of cyclic nucleotides between these cells[23]. Regulation of reproduction by GnRHs probably predated the divergence of deuterostomes and protostomes[20,24], the best evidence coming from mollusc species in which peptides structurally

related to GnRH, synthesized at various neuroendocrine sites, regulate various reproductive processes[25].

Cnidarians use neuropeptides to regulate multiple processes including muscle contraction, neural differentiation and metamorphosis from larva to polyp[15,26,27]. Transcript sequences predicted to produce many copies of short neuropeptides have also been found in ctenophore and placozoan genomes[28,29], and neuropeptides are thought to have been the predominant neurotransmitters in the ancient common ancestor of these groups[30]. Although independent evolution of neuropeptide regulation or reproduction between animal clades cannot be ruled out, the identification of the MIH neuropeptides in *Clytia and Cladonema* along with other evidence from cnidarians[16,31] as well as bilaterians (see above), suggests that neuropeptide signaling played a central role in coordinating sexual reproduction in the bilaterian-cnidarian ancestor, and may have been involved in coordinating spawning events in the marine environment. MIH-producing neural cells in hydrozoan jellyfish are found not only in the gonad but also in the manubrium, tentacles and bell margin (Fig. S5C,D), so presumably have wider function than orchestrating gamete release. It will be of great interest to investigate the activities of related peptides across a wide range of species in order to track the evolutionary history of the neurendocrine regulation of reproduction.

Methods

Animal cultures

Laboratory strains of *Clytia hemisphaerica* ("Z colonies"), *Cladonema pacificum* (6W, NON5, UN2), and *Cytaeis uchidae* (17) were maintained throughout the year [11,12,32]. *Cladonema* were also collected from Sendai Bay, Miyagi Prefecture. The brand of artificial seawater (ASW) used for culture and for functional assays in Japan was SEA LIFE (Marine Tech, Tokyo), and for *Clytia hemisphaerica* culture, transcriptomics and microscopy in France was Red Sea Salt.

Oocyte isolation and MIH assays

Fully-grown oocytes were obtained from ovaries of intact jellyfish or pre-isolated ovaries placed under constant illumination for 20–24 h following the previous spawning. Ovarian oocytes were aspirated using a mouth pipette or detached using fine tungsten needles. During oocyte isolation, jellyfish were in some cases anesthetized in excess Mg^{2+} ASW (a 1:1 mix of 0.53 M $MgCl_2$ and ASW). Pre-isolated ovaries of *Clytia, Aequorea*, and *Eutonina* were bathed in ASW containing 1 mM sodium citrate to facilitate the detachment of oocytes from ovarian tissues.

Active MIH was recovered from cultured ovaries of *Clytia* and *Cladonema* by a similar approach to that used previously [9]. A chamber formed between a plastic dish and a coverslip separated by two pieces of 400 or 500 μm-thick double-stick tape was filled with silicon oil (10 cSt; TSF451-10, Momentive Performance Materials), and a drop of ASW (0.5-1 μL) containing several ovaries separated from 2 *Clytia* jellyfish or several ovarian epithelium fragments stripped from 3-5 *Cladonema* jellyfish were inserted into the oil space (Fig. 1A,B). The oil chambers were subjected to light-dark changes (light after dark in *Clytia* and dark after light in *Cladonema*) and the ASW with MIH activity was collected 60 min later. Prior to

MIH assays, isolated oocytes were cultured in seawater for at least 30 min and any oocytes showing damage or GVBD discarded. All MIH assays were performed at 18-21°C.

Identification of peptide precursors

Potential amidated peptide precursor sequences were recovered from a *Clytia* reference transcriptome derived from mixed larva, polyp and jellyfish samples. ORFs and protein sequences were predicted using an R script [33]. Potential secreted proteins were identified by the presence of signal peptide, using SignalP 4.0 [34]. Then sequences rich in the amidated pro-peptide cleavage motifs GR/K and lacking domains recognized by Interproscan-5.14-53.0 were selected. Finally, sequences containing repetitive motifs of less than 20 amino-acids were identified using TRUST [35]. Among this final set of putative peptide precursors, some known secreted proteins with repetitive structures were identified by BLAST and removed.

To prepare a *Cladonema* transcriptome, more than 10 μg of total RNA was isolated from the manubrium of female jellyfish (6W strain) using NucleoSpin RNA purification kit (MACHEREY-NAGEL, KG). RNA-seq library preparation and sequencing Ilumina HiSeq 2000) were carried out by BGI (Hong-Kong, China). Using an assembled dataset containing 74,711 contigs and 35,957 unigenes, local BLAST searches were performed to find peptide precursors using published cnidarian neuropeptide sequences or the *Clytia* pp1 and pp4 sequences as bait.

The ORFs of putative candidate *Clytia* and *Cladonema* peptide precursors were cloned by PCR into pGEM-T easy vector, or retrieved from our *Clytia* EST collection cDNA library prior to probe synthesis. Sequences and accession numbers are given in Table S1.

For *Clytia* gonad tissue transcriptome comparisons, Illlumina Hi-seq 50nt reads were generated from mRNA isolated using RNAqueous micro kit (Ambion Life technologies, CA) from ectoderm, endoderm and oocytes manually dissected from about 150 *Clytia* female

gonads. Q-PCR was performed to check for contamination between samples using endogenous GFP genes expressed in oocyte, ectoderm and bell tissue [36], and to quantify expression of selected peptide precursors (primer list in Fig. S2B). The reads were mapped against a *Clytia* reference transcriptome using Bowtie2 [37]. The counts for each contig were normalized per total of reads of each sample and per sequence length and visualized using the heatmap.2 function in the "gplots" R package.

Peptides and antibodies

WPRP-NH_2, WPRA-NH_2, RPRP-NH_2, RPRA-NH_2, RPRG-NH_2, RPRY-NH_2, PGLW-NH_2, DAWPRR-NH_2, AWPRP-NH_2, NIRPRP-NH_2, IRPRP-NH_2, PRP-NH_2, WPRP-OH and RPRP-OH were synthesized by GenScript or Life Technologies. These peptides were dissolved in deionized water at 10^{-2} M or $2x10^{-3}$ M, stored at -20°C, and diluted in ASW at 10^{-5}-10^{-10} prior to use. TAMRA-WPRPamide (TAMRA-LEKRNWPRP-NH_2); was synthesized by Sigma and a 10^{-2} solution in H_2O was injected at 2-17% of the oocyte volume, to give an estimated final oocyte concentration of 1 to $9x10^{-5}$ M [12].

Polyclonal antibodies against XPRPamide and XPRAamide were raised in rabbits using keyhole limpet hemocyanin (KLH)-conjugated CPRA-NH_2 and CPRP-NH_2 as antigens, and antigen-specific affinity purified (Sigma-Ardrich Japan). For MIH inhibition experiments, antibodies or control normal rabbit IgG (MBL) were concentrated using a 30000 MW cut-off membrane (Millipore), giving a final protein concentration of 10^{-6} M, and the buffers were replaced with seawater through repeated centrifugations.

Immunofluorescence and in situ hybridization

For single or double anti-PRPamide /anti-PRAamide staining, specimens were pre-anesthetized using excess Mg^{2+} ASW and fixed overnight at 4°C in 10% formalin-containing

ASW and rinsed 3x 10 min in Phosphate Buffered Saline (PBS) containing 0.25% Triton X-100. They were incubated in anti-PRPamide or anti-PRAamide antibody diluted in PBS-Triton overnight at 4˚C. After washes in PBS-Triton, the specimens were incubated with secondary antibody (Alexa Fluor 488 or 568 goat anti-rabbit IgG; Invitrogen, Carlsbad, CA) for 2 h at room temperature and nuclei stained using 50 μM Hoechst 33258 or 33342 (Invitrogen) for 5-20 min. Zenon antibody labeling kits (Molecular Probes, Eugene, OR) were used for double peptide staining. In control experiments, PBS-Triton alone or normal rabbit IgG (3 mg/ml; Zymed, San Francisco, CA) in PBS-Triton (1/1000 dilution) replaced the anti-PRPamide or anti-PRAamide antibodies. Images were acquired using a laser scanning confocal system (C1, Nikon).

For co-staining of neuropeptides and microtubules (Fig. 4C,D), dissected *Clytia* gonads were fixed overnight at 18ºC in HEM buffer (0.1 M HEPES pH 6.9, 50 mM EGTA, 10 mM $MgSO_4$) containing 3.7% formaldehyde, then washed five times in PBS containing 0.1% Tween20 (PBS-T). Treatment on ice with 50% methanol/PBS-T then 100% methanol plus storage in methanol at -20ºC improved visualization of microtubules in the MIH-producing cells. Samples were rehydrated, washed in PBS-0.02% Triton X-100, blocked in PBS with 3% BSA overnight at 4ºC, then incubated in anti-PRPamide antibody and anti-alpha tubulin (YL1/2) in PBS/BSA at room temperature for 2 h. After washes, the specimens were incubated with secondary antibodies (Rhodamine goat anti-rabbit and Cy5 donkey anti-rat-IgG; Jackson ImmunoResearch, West Grove, PA) overnight in PBS at 4ºC and nuclei stained using Hoechst dye 33258 for 20 min.

For *in situ* hybridization, isolated gonads or whole jellyfish were processed as previously [36] except that 4 M Urea was used instead of 50% formamide in the hybridization buffer [38]. For double fluorescent *in situ* hybridization, female *Clytia* gonads were fixed overnight at 18ºC in HEM buffer containing 3.7% formaldehyde, washed five times in PBS containing 0.1%

Tween20 (PBS-T), then dehydrated on ice using 50% methanol/PBS-T then 100% methanol. *In situ* hybridization[33,38]was performed using a DIG-labeled probe for Che-pp1 and a fluorescein-labeled probe for Che-pp4. A 3 hour incubation with a peroxidase labeled anti-DIG antibody was followed by washes in MABT (100 mM maleic acid pH 7.5, 150 mM NaCl, 0.1% Triton X-100). For Che-pp1 the fluorescence signal was developed using the TSA (Tyramide Signal Amplification) kit (TSA Plus Fluorescence Amplification kit, PerkinElmer, Waltham, MA) and Cy3 fluorophore (diluted 1/400 in TSA buffer: PBS/H_2O_2 0.0015%) at room temperature for 30 min. After 3 washes in PBS-T fluorescence was quenched with 0.01 M HCl for 10 min at room temperature and washed again several times in PBS-T. Overnight incubation with a peroxidase labeled anti-fluorescein antibody was followed by washes in MABT. The anti Che-pp4 fluorescence signal was developed using TSA kit with Cy5 fluorophore. Nuclei were stained using Hoechst dye 33258. Images were acquired using a Leica SP5 confocal microscope and maximum intensity projections of z-stacks prepared using ImageJ software.

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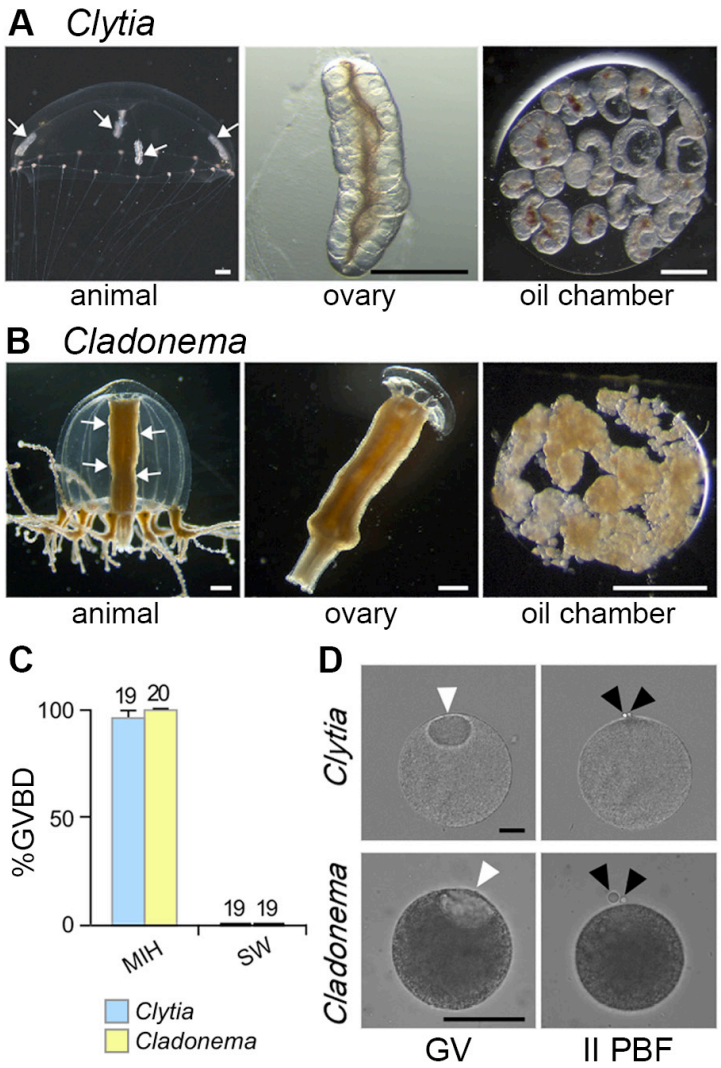
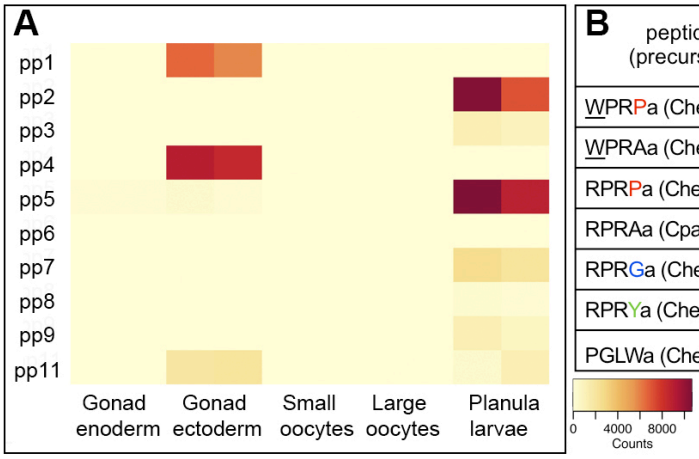
Figure legends

Figure 1. Active MIH is produced by isolated jellyfish gonads.



A) *Clytia hemisphaerica* whole female jellyfish (1cm diameter), isolated ovary and a collection of ovaries under oil used to collect MIH. B) Equivalent samples for *Cladonema pacificum*. Arrows point to gonads in A and B. C) GVBD assay on isolated oocytes (number of oocytes marked above each bar) incubated in presence or absence of MIH from the same species. D) Isolated oocytes from each species before ('GV' stage) and 2 or 1 h respectively after addition of MIH at the time of 2nd polar body formation ("II PBF" stage). White arrowheads point to GVs and black arrowheads to polar bodies. Scale bars: 500 µm in A, B and 50 µm in D.

Figure 2. Predicted neuropeptides from the gonad ectoderm have MIH activity



B

peptide (precursor)	% spawning of *Clytia* gonads(n)	
	10^{-5} peptide	10^{-7} peptide
WPRPa (Che-pp1, Che-pp4)	100 (13)	100 (14)
WPRAa (Che-pp4)	100 (13)	100 (13)
RPRPa (Che-pp8, Cpa-pp1)	100 (12)	0 (12)
RPRAa (Cpa-pp2)	0 (12)	0 (13)
RPRGa (Che-pp1)	33 (12)	0 (12)
RPRYa (Che-pp4)	100 (12)	0 (12)
PGLWa (Che-pp11)	0 (12)	8.3 (12)

C

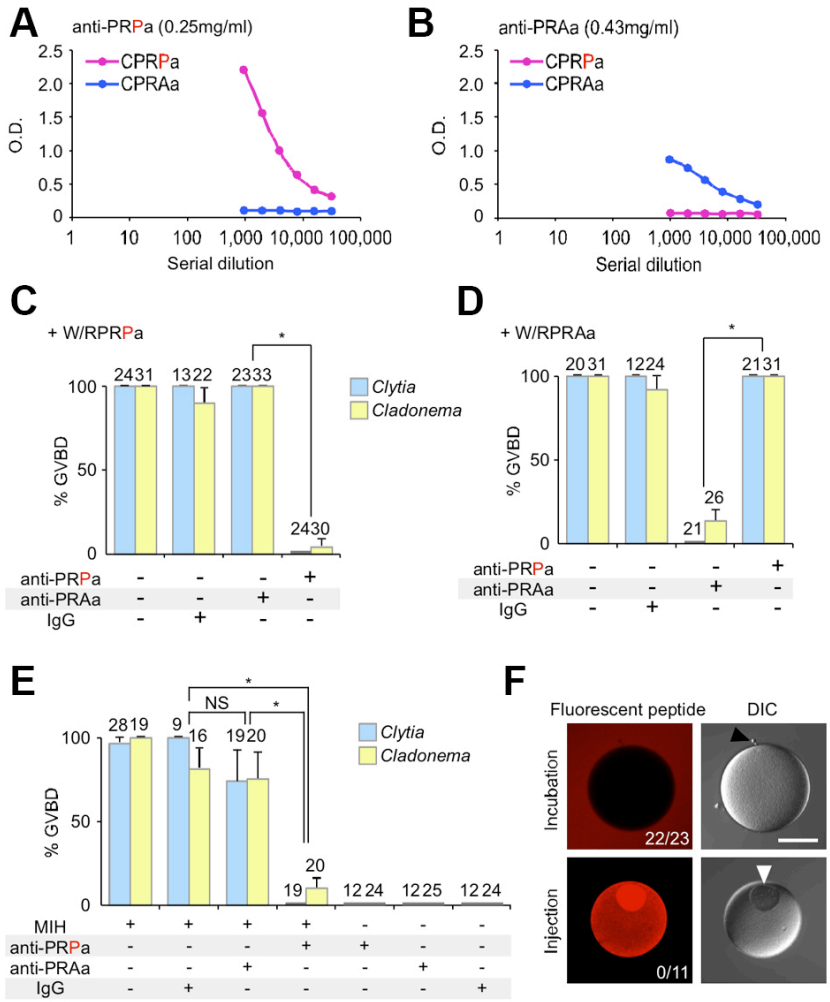
peptide	% GVBD in *Clytia* oocytes (n)		% in *Cladonema* oocytes (n)	
	10^{-5} peptide	10^{-7} peptide	10^{-5} peptide	10^{-7} peptide
WPRPa	100 (13)	97 (33)	0 (149)	0 (91)
WPRAa	100 (14)	100 (17)	0 (58)	0 (102)
RPRPa	100 (16)	100 (22)	100 (104)	100 (116)
RPRAa	100 (15)	90 (30)	99 (77)	100 (88)
RPRGa	100 (14)	38 (37)	100 (82)	19 (72)
RPRYa	100 (17)	100 (21)	100 (25)	17 (30)
PGLWa	0 (16)	0 (17)	0 (49)	0 (48)

D

peptide	% GVBD in *Clytia* oocytes (n)	
	10^{-5} peptide	10^{-7} peptide
DAWPRPa	100 (25)	0 (25)
AWPRPa	100 (26)	4 (23)
PRPa	0 (15)	0 (15)
WPRP-OH	0 (24)	0 (21)
peptide	% GVBD in *Cladonema* oocytes (n)	
NIRPRPa	17 (46)	0 (45)
IRPRPa	94 (48)	0 (49)
PRPa	0 (60)	0 (34)
RPRP-OH	2 (42)	0 (26)

A) Heat map representing the expression of 10 candidate peptide precursor sequences from *Clytia hemisphaerica* in isolated ectoderm, endoderm, small (growing) and large (fully-grown) oocytes from mature female gonads. Illlumina High-seq 50nt reads generated from ectoderm, endoderm and oocyte mRNA were mapped against a *Clytia* reference transcriptome. Data from a sample of 2 day old planula larvae are included for comparison. B) Results of spawning assay on isolated *Clytia* gonads using synthesized amidated tetrapeptides; WPRPamide and WPRAamide, generated from Che-pp1 and Che-pp4 precursors, induced 100% spawning even at 10^{-7} M. C) MIH assay using isolated *Clytia* or *Cladonema* oocytes showing strong MIH activity of related amidated tetrapeptides. D) Synthesized amidated 3, 5 or 6 amino acid peptides, and non-amidated tetrapeptides, show poor MIH activity on isolated *Clytia* and *Cladonema* oocytes.

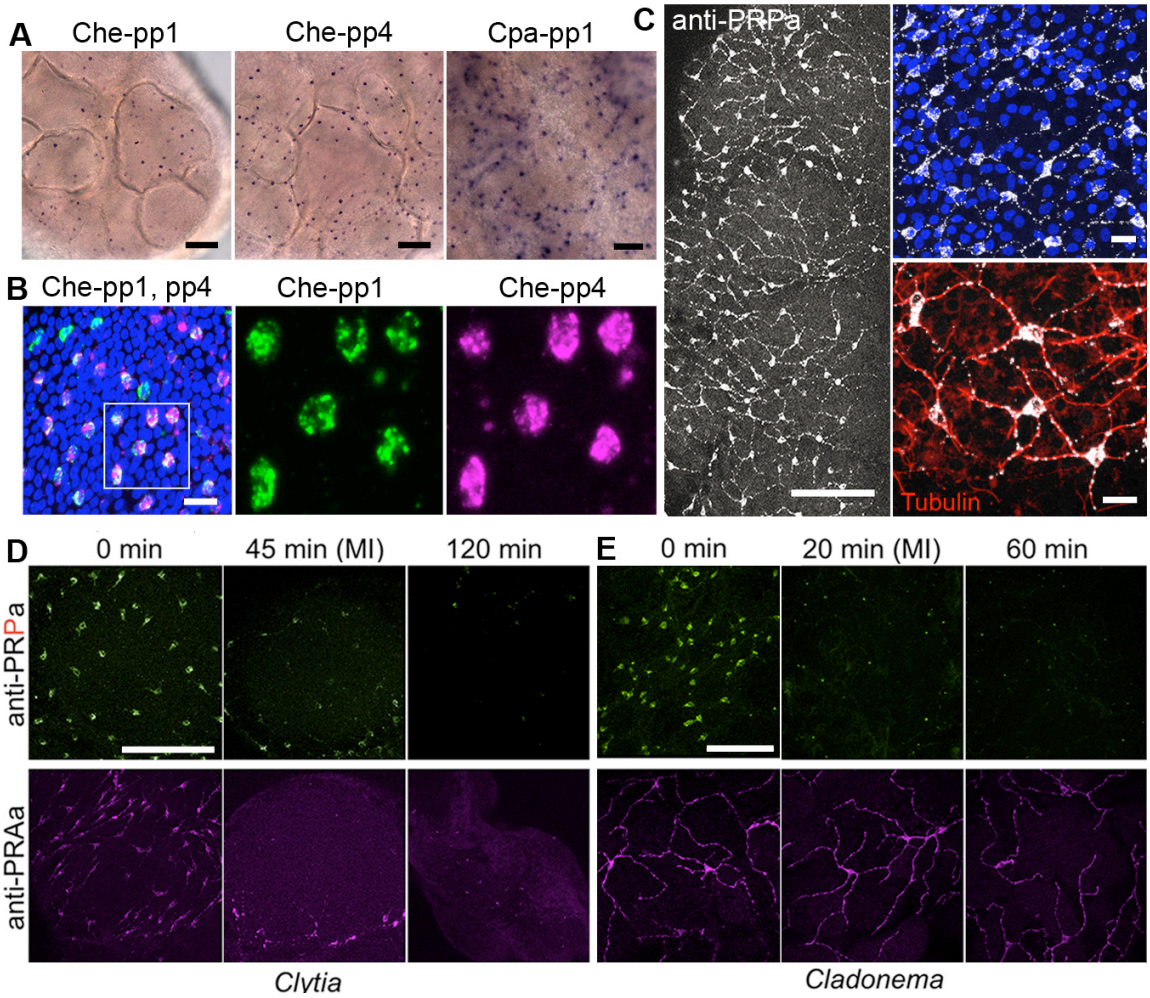
Figure 3. Antibody inhibition shows that PRPamides are the active component of MIH



A) ELISA assay demonstrating that the anti-PRPamide antibody binds PRPamide but not PRAamide tetrapeptides. B) Reciprocal specificity for the anti-PRAamide antibody. C-E) Inhibition experiments in which either anti-PRPamide or anti-PRAamide antibody was pre-incubated with W/RPRPamide, W/RPRAamide or natural MIH prior to the MIH assay (number of oocytes tested above each bar). Oocyte maturation induced by WPRPamide (*Clytia*) or RPRPamide (*Cladonema*) was inhibited by anti-PRPamide but not anti-PRAamide antibodies, while PRAamide activity was specifically neutralized by anti-PRAamide antibodies. The activity of endogenous MIH produced by either *Clytia* or *Cladonema* gonads was inhibited by anti-PRPamide antibody. Inhibition by the anti-PRAamide antibody was not statistically significant (Student's *t*-tests ; asterisk: $P < 0.01$; NS: $P > 0.05$). F) Confocal images of *Clytia* oocytes which underwent GVBD following incubation in TAMRA-WPRPamide (top), but not following injection of TAMRA-WPRPa (bottom). Numbers indicate GVBD/oocytes tested. Scale bars: 100 µm. In C-F, oocytes that did not mature underwent normal GVBD induced by subsequent

addition of excess neuropeptides (10^{-5}-10^{-7} M WPRPamide for *Clytia*; 10^{-7} M RPRP/Aamide for *Cladonema*).

Figure 4. MIH is generated by neural cells in the gonad ectoderm



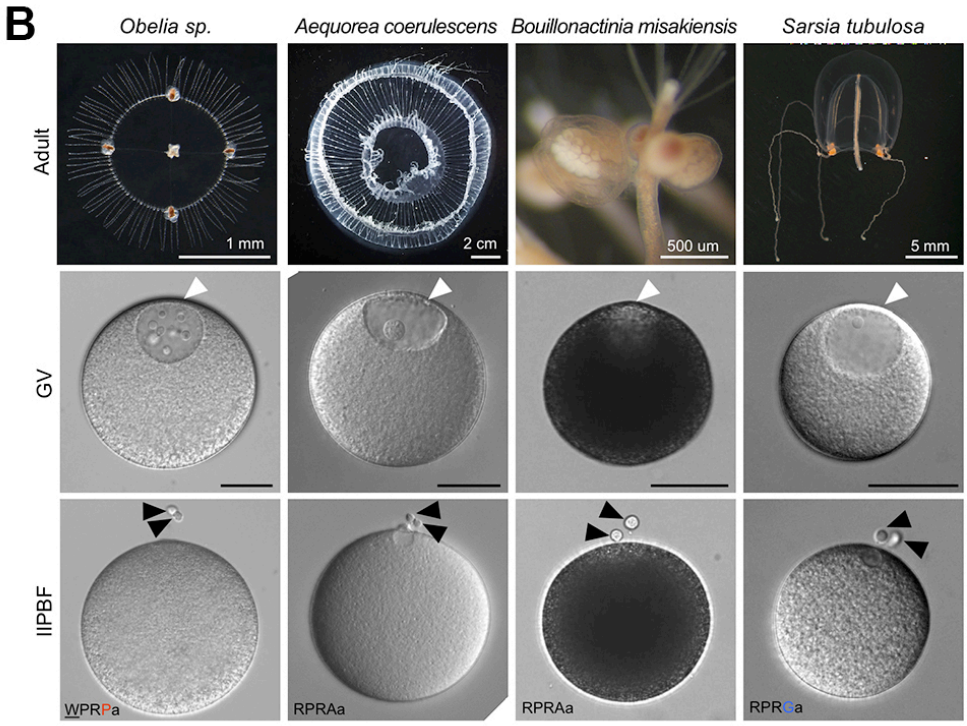
A) *In situ* hybridization detection of Che-pp1 and Che-pp4 mRNAs in *Clytia* (left, center) and Cpa-pp1 in *Cladonema* (right) in scattered ectoderm cells of female gonads. B) Double fluorescent *in situ* hybridization reveals co-expression of Che-pp1 (green) and Che-pp4 (magenta); nuclei (Hoechst) in blue. Single channels are shown for the outlined zone in the left image. C) Immunofluorescence of *Clytia* female gonads showing the neural morphology of MIH-producing cells. Staining with anti-PRPamide (white), anti-tubulin (red) and Hoechst (blue). D) Equivalent Immunofluorescence of a whole young jellyfish showing PRPamide-

expressing cells in the nerve ring, manubrium and tentacles (white arrowheads). E) Release of peptides detected with anti-PRPamide and anti-PRAamide antibodies from *Clytia* (top) and *Cladonema* (bottom) gonads at successive times during meiosis completion following light exposure as indicated (MI= first meiosis). F) Epifluorescence images of gonad ectoderm following double immunofluorescence performed with anti-PRPamide and anti-PRAamide antibodies. Overlaid images shown in the third panel of each row. In *Clytia* gonads (left) these decorated a single cell population, whereas in *Cladonema* (right) the two peptides were detected in distinct cell populations. Scale bars: 50 μm in A; 20 μm in B; 100 μm in C (left), D, E; 10 μm in C (right).

Figure 5. Synthetic peptides show MIH activity in a subset of hydrozoan jellyfish species

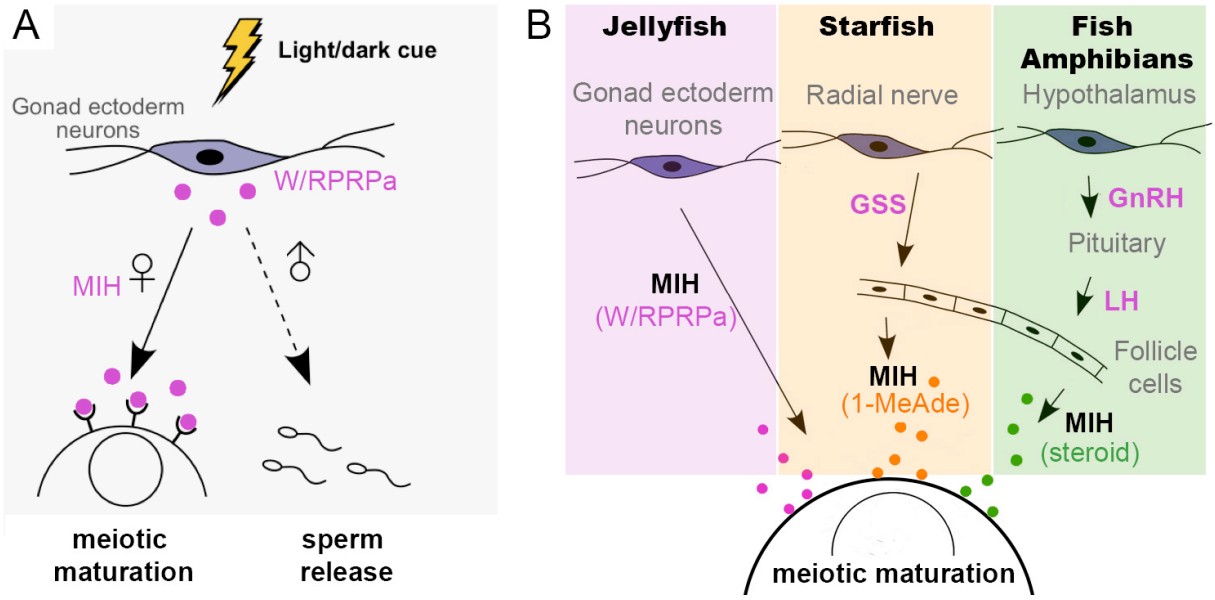
A

Peptide	conc. at M	% GVBD in Leptomedusa oocytes (n)			% GVBD in Anthomedusa oocytes (n)				
		Obelia sp.	*Aequorea coerulescens*	*Eutonina indicans*	*Bouillonactinia misakiensis*	*Sarsia tubulosa*	*Nemopsis dofleini*	*Rathkea octopunctata*	*Cytaeis uchidae*
WPRPa	10^{-5}	100 (6)	98(40)	6 (17)	100 (14)	5 (20)	0 (12)	0 (12)	0 (18)
	10^{-7}	100 (13)	67 (30)	-	84 (19)	0 (19)	-	-	-
WPRAa	10^{-5}	100 (6)	91 (32)	0 (13)	100 (13)	60 (20)	0 (13)	0 (18)	0 (20)
	10^{-7}	100 (19)	28 (32)	-	88 (25)	0 (23)	-	-	-
RPRPa	10^{-5}	100 (6)	95 (21)	0 (10)	87 (39)	92 (25)	0 (17)	0 (14)	0 (20)
	10^{-7}	100 (7)	88 (26)	-	100 (24)	21 (33)	-	-	-
RPRAa	10^{-5}	100 (8)	47 (34)	0 (14)	75 (28)	94 (36)	0 (14)	0 (11)	0 (19)
	10^{-7}	46 (11)	71 (24)	-	96 (54)	83 (60)	-	-	-
RPRGa	10^{-5}	100 (7)	96 (25)	0 (10)	85 (33)	81 (36)	0 (12)	0 (11)	0 (20)
	10^{-7}	0 (9)	61 (23)	-	69 (26)	89 (45)	-	-	-



A) Synthetic W/RPRP/A/Gamides were tested for their ability at concentrations of 10^{-5} M and 10^{-7} M to induce GVBD of oocytes of the 8 species indicated. Highest success of GVBD is emphasized by the darker blue colors. B) Examples of four of the species tested, showing the adult females (top row), isolated oocytes (middle row) and mature eggs with two polar bodies (bottom row) generated by incubation in the peptides indicated. Scale bars for oocytes: 50µm. White and black arrowheads indicate GVs and polar bodies, respectively.

Figure 6. MIH action in jellyfish compared with other animals



A) Summary of the findings of this study: jellyfish MIH, consisting of PRPamide family peptides in *Clytia* and *Cladonema*, is secreted by neural cells in the gonad directly in response to light cues and causes oocyte maturation as well as spawning in males and females.

B) Comparison of the regulation of oocyte maturation by peptide hormones (pink) in jellyfish, starfish and fish/amphibians.

Table 1. MIH peptides induce male spawning.

Species	Test peptide	% testes that released sperm (number of testes)	
		Peptide at 10^{-5} M	Peptide at 10^{-7} M
Clytia	WPRPamide	100 (16)	100 (15)
Clytia	WPRP-OH	0 (11)	0 (11)
Cladonema	RPRPamide	94 (16)	86 (22)
Cladonema	RPRP-OH	0 (10)	0 (11)