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RESEARCH ARTICLE

Identification of jellyfish neuropeptides that act directly as oocyte maturation-inducing hormones

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ABSTRACT

Oocyte meiotic maturation is crucial for sexually reproducing animals, and its core cytoplasmic regulators are highly conserved between species. By contrast, the few known maturation-inducing hormones (MIHs) that act on oocytes to initiate this process are highly variable in their molecular nature. Using the hydrozoan jellyfish species *Clytia* and *Cladonema*, which undergo oocyte maturation in response to dark-light and light-dark transitions, respectively, we deduced amidated tetrapeptide sequences from gonad transcriptome data and found that synthetic peptides could induce maturation of isolated oocytes at nanomolar concentrations. Antibody preabsorption experiments conclusively demonstrated that these W/RPRPamide-related neuropeptides account for endogenous MIH activity produced by isolated gonads. We show that the MIH peptides are synthesised by neural-type cells in the gonad, are released following dark-light/light-dark transitions, and probably act on the oocyte surface. They are produced by male as well as female jellyfish and can trigger both sperm and egg release, suggesting a role in spawning coordination. We propose an evolutionary link between hydrozoan MIHs and the neuropeptide hormones that regulate reproduction upstream of MIHs in bilaterian species.

KEY WORDS: Oocyte maturation, Meiosis, Neuropeptide, Cnidaria, Hydrozoan

INTRODUCTION

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 fertilisation-competent eggs. This process of oocyte maturation is a key feature of animal biology, and is tightly regulated to optimise 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) (Amiel et al., 2009; Von Stetina and Orr-Weaver, 2011; Tachibana et al., 2000; Yamashita et al., 2000). These kinase regulations have been well characterised 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 (Kanatani et al., 1969), steroid hormones in amphibians and fish (Haccard et al., 2012; Nagahama and Yamashita, 2008), and a sperm protein in *Caenorhabditis* (Von Stetina and Orr-Weaver, 2011).

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 continue to 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 (Amiel et al., 2010; Freeman, 1987; Ikegami et al., 1978). Furthermore, as members of the Cnidaria, a sister clade to the Bilateria, hydrozoan jellyfish can provide insight into spawning regulation in early animal ancestors.

In this study we addressed the molecular nature and the cellular origin of MIH in two hydrozoan jellyfish model species, *Clytia hemisphaerica* (Fig. 1A) and *Cladonema pacificum* (Fig. 1B), which are induced to spawn by dark-light and light-dark transitions, respectively (Amiel et al., 2010; Deguchi et al., 2005; Houliston et al., 2010). Starting from the hypothesis that hydrozoan MIHs might be neuropeptides, consistent with size filtration and protease-sensitivity experiments (Ikegami et al., 1978), we screened synthetic candidate peptides predicted from gonad transcriptome data by treatment of isolated gonads (spawning assay) or isolated oocytes (MIH assay). We then raised inhibitory antibodies to confirm the presence and activity of the putative peptides in native MIH secreted from gonads in response to light/dark cues, and to characterise the MIH-producing cells and their response to light. We extended our findings by determining the activity of the identified hydrozoan MIH tetrapeptides on males as well as females, and on a selection of other diverse hydrozoan species. A parallel study of *Clytia* gonad light detection revealed that the light-mediated MIH release reported here is dependent on an opsin photopigment co-expressed in the same population of cells that secretes MIH (Quiroga Artigas et al., 2018). These specialised cells, which have neural-type morphology and characteristics, thus provide a simple and possibly ancestral mechanism to promote synchronous gamete maturation, release and fertilisation.

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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 (Freeman, 1987; Ikegami et al., 1978). Isolated oocytes recovered using this method and incubated in endogenous MIH efficiently complete the meiotic maturation process, which is manifest visually by germinal vesicle breakdown (GVBD) and extrusion of two polar bodies (Fig. 1C,D).

Further characterisation of MIH using *Cladonema* showed that isolated gonad ectoderm, but not endoderm, tissue (see Fig. 1B) could produce active MIH. MIH activity from *Cladonema* gonad ectoderm resisted heat treatment at 100°C for 20 min (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, possibly peptidic, molecule (Ikegami et al., 1978).

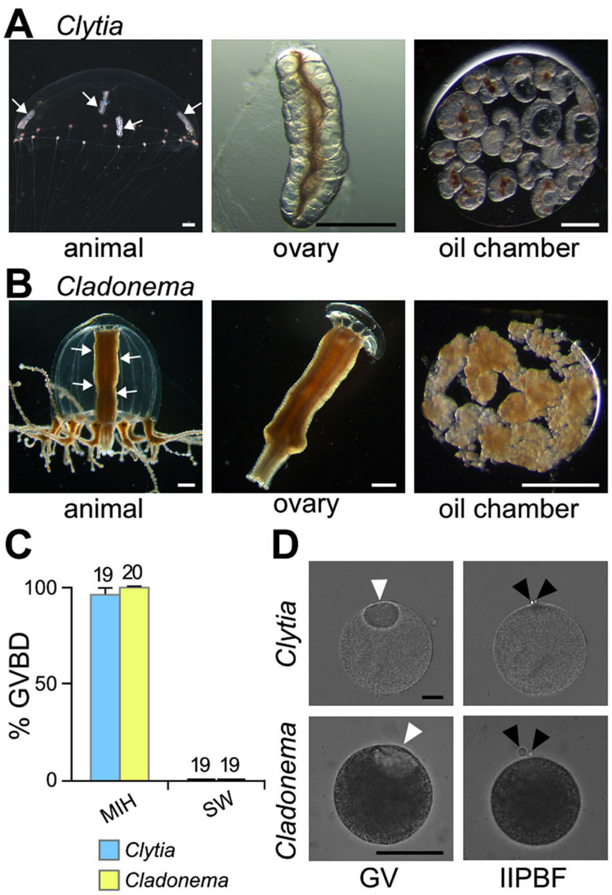

Fig. 1. Active MIH is produced by isolated jellyfish gonads. (A) *Clytia hemisphaerica* whole female jellyfish (1 cm diameter), isolated ovary and a collection of ovaries under oil used to collect MIH. (B) Equivalent samples for *Cladonema pacificum*. Arrows (A,B) point to gonads. (C) GVBD assay on isolated oocytes (number of oocytes indicated above each bar) incubated in the presence or absence of MIH from the same species. SW, seawater control. (D) Isolated oocytes from *Clytia* or *Cladonema* before [germinal vesicle (GV) stage] and 2 or 1 h, respectively, after addition of MIH at the time of second polar body formation (IIPBF stage). White arrowheads point to GVs and black arrowheads to polar bodies. Scale bars: 500 µm in A,B; 50 µm in D.

MIH candidates identified from transcriptome data

Cnidarians, including jellyfish, hydra and sea anemones, express many low molecular weight neuropeptides with various bioactivities (Anctil, 2000; Fujisawa, 2008; Takahashi et al., 2008; Takeda et al., 2013). These are synthesised by cleavage of precursor polypeptides and can produce multiple copies of one or more peptides, which are frequently subject to amidation by conversion of a C-terminal glycine (Grimmelikhuijzen et al., 1996). A previous study found 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 (active at 10^{-5} M minimum concentration) (Takeda et al., 2013). Crucially, 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 might 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 ten potential amidated peptide precursors from a mixed stage *Clytia* transcriptome (Fig. S1), and then searched for those specifically expressed in the ectoderm, the 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, only three 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 among four sequences identified in a transcriptome assembly from the *Cladonema* manubrium (which includes the gonad; Fig. 1B). Cpa-pp1 contains one 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 from the gonad 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 also of Che-pp8, a precursor not expressed in the *Clytia* gonad, was also active in this screen at 10^{-5} M. By contrast, PGLWamide, which is potentially generated from Che-pp11, did not affect the gonads at either concentration. This result placed WPRP/Aamide-related peptides as the best candidates for jellyfish MIH.

We then performed a 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 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

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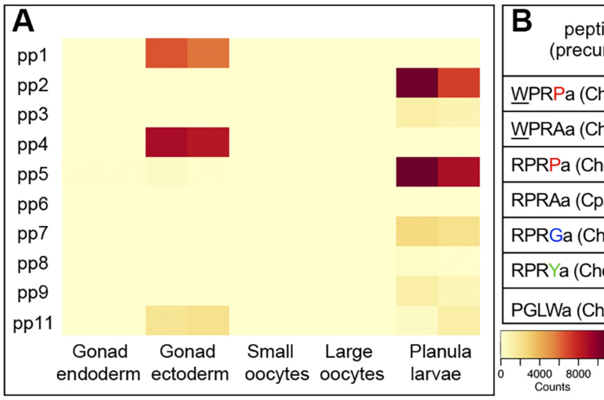

B

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)		% GVBD 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)

Fig. 2. Predicted neuropeptides from the gonad ectoderm have MIH activity. (A) Heat map representing the expression of ten candidate peptide precursor sequences from *Clytia hemisphaerica* in isolated ectoderm, endoderm, small (growing) and large (fully-grown) oocytes from mature female gonads. Illumina HiSeq 50 nt 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 synthesised 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) Synthesised amidated 3, 5 or 6 amino acid peptides, and non-amidated tetrapeptides, show poor MIH activity on isolated *Clytia* and *Cladonema* oocytes.

very potent MIH activity, and the RPRG/Yamides were also active at higher concentrations, whereas WPRP/Aamides were not active (Fig. 2C, Fig. 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, Fig. S3). The response of *Clytia* or *Cladonema* isolated oocytes to synthetic W/RPRP/A/Yamides mirrored very closely that elicited by endogenous MIH, proceeding through the events of oocyte maturation with normal timing following GVBD, and advanced by 15-20 min (*Clytia*) or 10 min (*Cladonema*) compared with light/dark-induced spawning of gonads (Fig. S4A-D). The resultant mature eggs could be fertilised and developed into normal planula larvae (Fig. S4E,F).

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 recognise 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 min 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 with 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 by neurosecretory cells in the gonad ectoderm

Single- and double-fluorescence *in situ* hybridisation 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. 5E). 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. 5A,E). Immunofluorescence with the anti-PRPamide and anti-PRAamide antibodies in both species revealed that the expressing cells have a morphology typical of cnidarian neural cells, comprising a small cell body and two or more long projections (David, 1973), and characterised by the presence of bundles of stable microtubules (Fig. 4C,F). A parallel study further revealed that in *Clytia*, these MIH-secreting cells express an opsin photoprotein with an essential function in oocyte maturation and spawning (Quiroga Artigas et al., 2018). Given their neural-type morphology, photosensory function and key role in regulating sexual

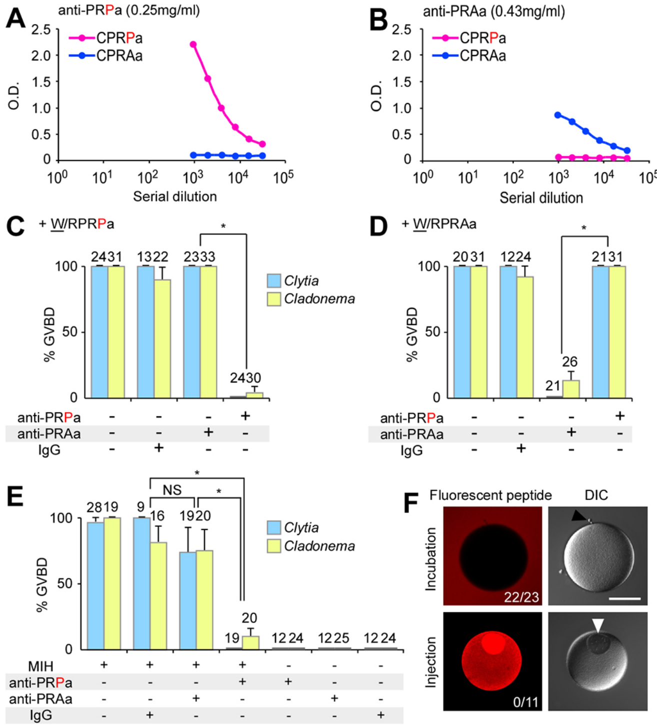

Fig. 3. Antibody inhibition shows that PRPamides are the active component of MIH. (A) ELISA assay demonstrating that the anti-PRPamide antibody binds PRPamide but not PRAamide tetrapeptides. (B) Reciprocal specificity demonstrated for the anti-PRAamide antibody. (C-E) Inhibition experiments in which 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 is indicated 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 neutralised 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*-test; **P*<0.01; NS, *P*>0.05. (F) Confocal images of *Clytia* oocytes that underwent GVBD following incubation in TAMRA-WPRPamide (top), but not following injection of TAMRA-WPRPa (bottom). Numbers indicate frequency of GVBD among oocytes tested. Black arrowhead points to polar bodies, and white arrowhead to the GV. Scale bar: 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*).

reproduction via neuropeptide hormone production, we propose that these cells have both a sensory and neurosecretory nature. Scattered endocrine cells with both sensory and neurosecretory characteristics are a feature of cnidarians (Hartenstein, 2006). Furthermore, the distribution and organisation of the gonad MIH-producing cells in *Clytia* and *Cladonema* are suggestive of the neural nets that characterise cnidarian nervous systems (Koizumi, 2016; Bosch et al., 2017; Dupre and Yuste, 2017). We could not confirm from our immunofluorescence analyses any direct connections between neighbouring cells. Future electron microscopy or calcium imaging techniques (Gründer and Assmann, 2015; Dupre and Yuste, 2017) to identify synapses or action potential transmission could resolve whether these scattered endocrine cells are integrated within a neural network. In intact *Clytia* jellyfish, both immunofluorescence and *in situ* hybridisation (Fig. 5B-D, Fig. S5A) revealed the presence of MIH peptides and their precursors at additional sites associated with neural systems: the manubrium (mouth), tentacles and the nerve ring that runs around the bell rim (Koizumi et al., 2015), as well as along the radial canals. This suggests that PRPamide family neuropeptides have other functions in the jellyfish in addition to regulating spawning. Neuropeptides can have both neural and endocrine functions in cnidarians, and are thought to have functioned in epithelial cells in primitive metazoans prior to nervous system evolution (Hartenstein, 2006; Bosch et al., 2017).

In *Clytia* gonad ectoderm, the anti-PRPamide and PRAamide antibodies decorated a single cell population, whereas in *Cladonema* the two peptides were detected in distinct cell populations (Fig. S5B, C), presumably being generated from the Cpa-pp1 and Cpa-pp2 precursors, respectively (Fig. S1). Immunofluorescence analysis of *Cladonema* gonads revealed a reduction in the anti-PRPamide signal within 20 min after darkness, whereas the anti-PRAamide signal was relatively unaffected (Fig. 4D). *Clytia* gonads showed a moderate reduction of staining with both antibodies 45 and 120 min after light stimulus (Fig. 4E). More detailed examination indicated that in each stained cell the numbers of antibody-positive dots, presumably representing peptide-filled vesicles, decreased following light exposure in both the cell body and in the microtubule-rich projections (Fig. 4F). It would be interesting to determine, for instance by live imaging techniques, whether MIH-containing vesicles are secreted from particular sites or exhibit any trafficking following the light/dark cues, or whether vesicle release occurs throughout the cell, as would be typical of a neurosecretory cell type (Hartenstein, 2006).

The similar distribution of MIH-producing cells in female and male gonads (Fig. 4A, Fig. 5E) suggests that these neuropeptides might play a general role in regulating gamete release, and not only in the initiation of oocyte maturation in female medusae. We found using male jellyfish of both *Clytia* and *Cladonema* that synthetic MIH peptides at 10^{-7} M provoked release of active sperm from the gonads (Table 1). This confirms that the oocyte maturation-stimulating effect of MIH is just one component 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 might facilitate precise synchronisation 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, WPRAamide and RPRYamide, which were 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. 6). The responsive and non-responsive species included members of two main hydrozoan groups, namely the 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 that are active

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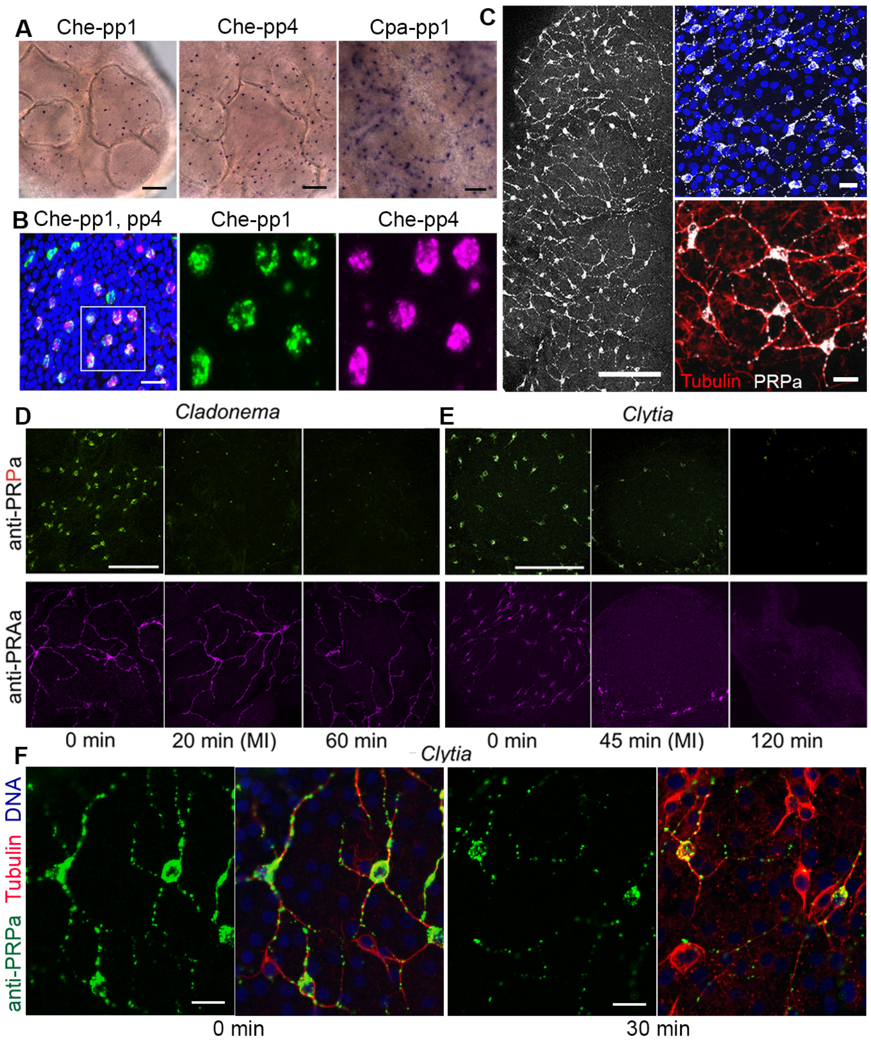

Fig. 4. MIH is generated by gonad ectoderm cells with neural characteristics. (A) *In situ* hybridisation detection of Che-pp1 and Che-pp4 mRNAs in *Clytia* (left, centre) and of Cpa-pp1 in *Cladonema* (right) in scattered ectoderm cells of female gonads. (B) Double fluorescence *in situ* hybridisation reveals co-expression of Che-pp1 (green) and Che-pp4 (magenta); nuclei in blue (Hoechst). Single channels are shown for the boxed region. (C) Immunofluorescence of *Clytia* female gonads showing the neural-type morphology of MIH-producing cells, which are characterised by two or more long projections containing microtubule bundles. Staining with anti-PRPamide (white), anti-tubulin (red) and Hoechst (blue). (D) Loss of anti-PRPamide staining from *Cladonema* gonads during dark-induced meiotic maturation (MI, first meiotic M phase). The distinct anti-PRAamide-stained cells were not obviously affected. (E) Equivalent experiment in *Clytia*, in which the two antibodies decorate the same cell population (see Fig. S5). (F) High-magnification images of PRPa-stained cells in the ectoderm of isolated *Clytia* gonads before (0 min) or 30 min after light exposure. The dots of anti-PRPa staining (green) presumably represent peptide-filled vesicles, which become less abundant in both cell bodies and in the microtubule-rich projections (anti-tubulin staining in red, Hoechst in blue). All fluorescence panels are confocal images. Scale bars: 50 µm in A; 20 µm in B; 100 µm in C (left), D,E; 10 µm in C (right), F.

between related species might reduce stimulation of spawning between species in mixed wild populations.

DISCUSSION

We have demonstrated that in the hydrozoan jellyfish *Clytia* and *Cladonema*, short amidated peptides with the prototype sequence W/RPRPamide are responsible for inducing oocyte maturation, resulting ultimately in release from the gonad of active mature gametes (Fig. 7A). These peptides act as bona fide MIHs, i.e. they interact directly with the surface of resting ovarian oocytes to initiate maturation. Related W/RPRXamide peptides act as MIHs also in other hydrozoan jellyfish species. Regarding the later events of the spawning process, we hypothesise that egg release through the gonad ectoderm in female medusae is not triggered directly by MIH but via a secondary signal emitted by the oocyte towards the end of the meiotic maturation process. More specifically, it could depend on Mos-MAP kinase activation in the oocyte, since maturation in the absence of spawning can occur when Mos translation is inhibited in ovarian oocytes (Amiel et al., 2009). In male gonads, MIH peptides presumably act on inactive, postmeiotic spermatozoids to initiate the spawning response, but the mechanisms involved are not yet known.

Some GLWamide family peptides are also able to provoke oocyte maturation and spawning, albeit at higher concentrations than the PRPamides, but do not induce maturation of isolated oocytes (this study; Takeda et al., 2013), suggesting that the role of these peptides in regulating spawning is indirect. We can imagine that inhibitory or sensitising factors, possibly including GLWamides, could act either in the gonad MIH-secreting cells or in other ectodermal cells to 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 the well-known mass annual spawning events seen in tropical reef corals (Harrison et al., 1984).

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 trigger oocyte maturation is known in surprisingly few animal species, notably 1-methyladenine in starfish and steroid hormones in teleost fish and amphibians (Kanatani et al., 1969; Nagahama and Yamashita, 2008; Yamashita et al., 2000; Haccard et al., 2012). The very different molecular natures of these known MIH examples from across the (bilaterian plus cnidarian) clade could be explained by an evolutionary scenario in which secretion of neuropeptide MIHs from cells positioned near 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. 7B). Such interpolation of

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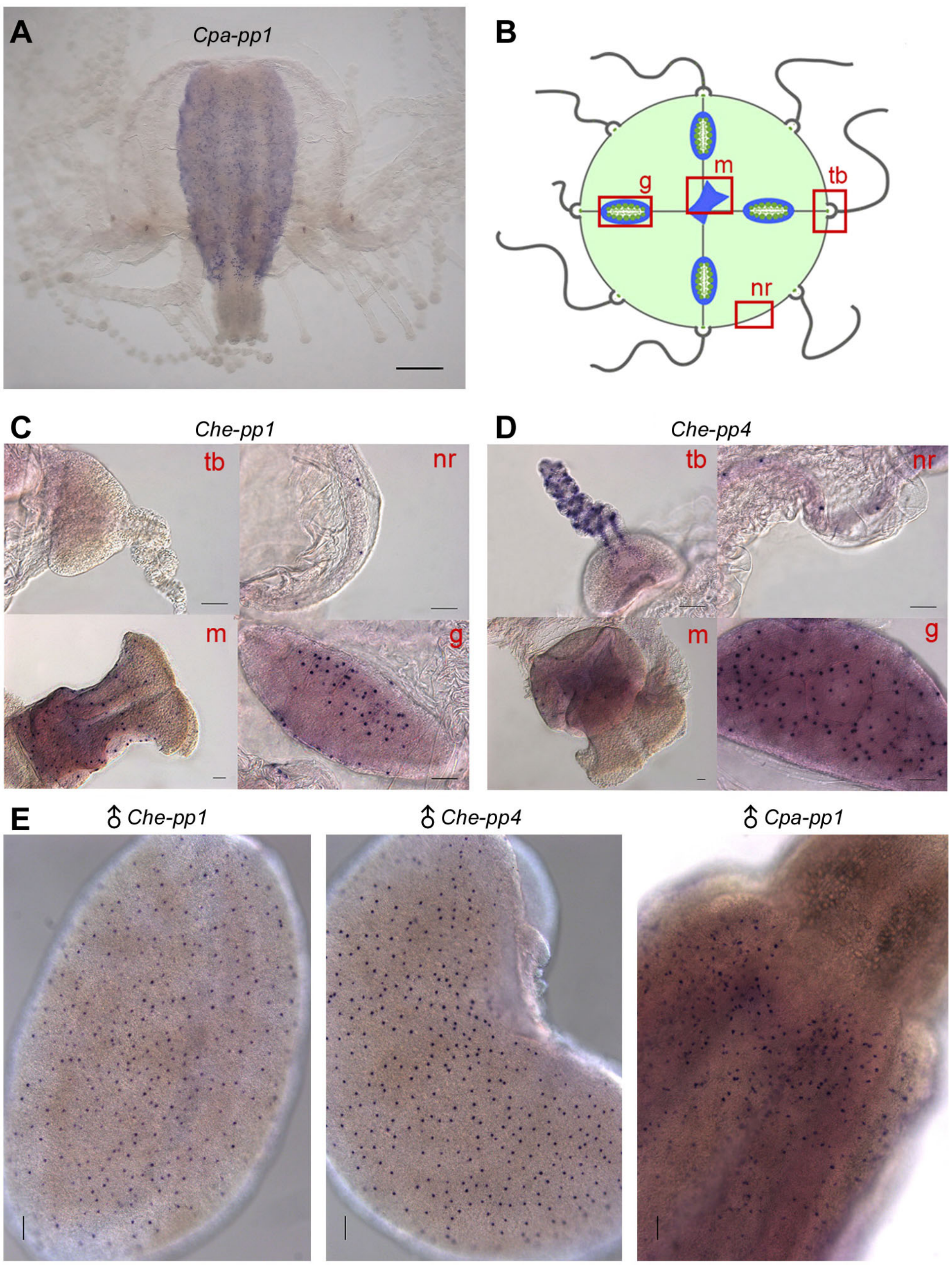

Fig. 5. Distribution of MIH-expressing cells detected by *in situ* hybridisation. (A) Cpa-pp1 in scattered ectodermal cells in the gonad of a *Cladonema* female jellyfish. (B) Schematic representation of a *Clytia* jellyfish indicating the position of the tentacle bulbs (tb), nerve ring (nr), gonads (g) and manubrium (m). (C,D) *In situ* hybridisation detection of Che-pp1 and Che-pp4 in different structures of young *Clytia* jellyfish. Both these precursors are expressed in the gonad ectoderm and nerve ring, whereas in the manubrium mainly Che-pp1 is detected, and in the tentacle mainly Che-pp4. (E) *In situ* hybridisation detection in male gonads from *Clytia* and *Cladonema* showing Che-pp1, Che-pp4 and Cpa-pp1 expression in scattered ectoderm cells. Scale bars: 500 µm in A; 50 µm in C-E.

additional layers of regulation is a common feature of endocrine system evolution (Hartenstein, 2006). During the evolution of reproductive regulation, various neuropeptides, including vertebrate gonadotropin-releasing hormones (GnRHs) (Roch et al., 2011) as well as modulatory RFamide peptides such as Kisspeptins and gonadotropin-inhibitory hormone (GnIH) (Parhar et al., 2012), 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 (LH), from the pituitary. Similarly, starfish gonad-stimulating substance (GSS/Relaxin) (Mita et al., 2009) 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 (Shuhaibar et al., 2015). Regulation of reproduction by GnRHs probably predated the divergence of deuterostomes and protostomes (Roch et al., 2011; Tsai, 2006), the best evidence coming from mollusc species in which peptides structurally related to GnRH, and synthesised at various neuroendocrine sites, regulate various reproductive processes (Osada and Treen, 2013).

Cnidarians use neuropeptides to regulate multiple processes including muscle contraction, neural differentiation and

Table 1. MIH peptides induce male spawning

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

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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)	-	-	-

B

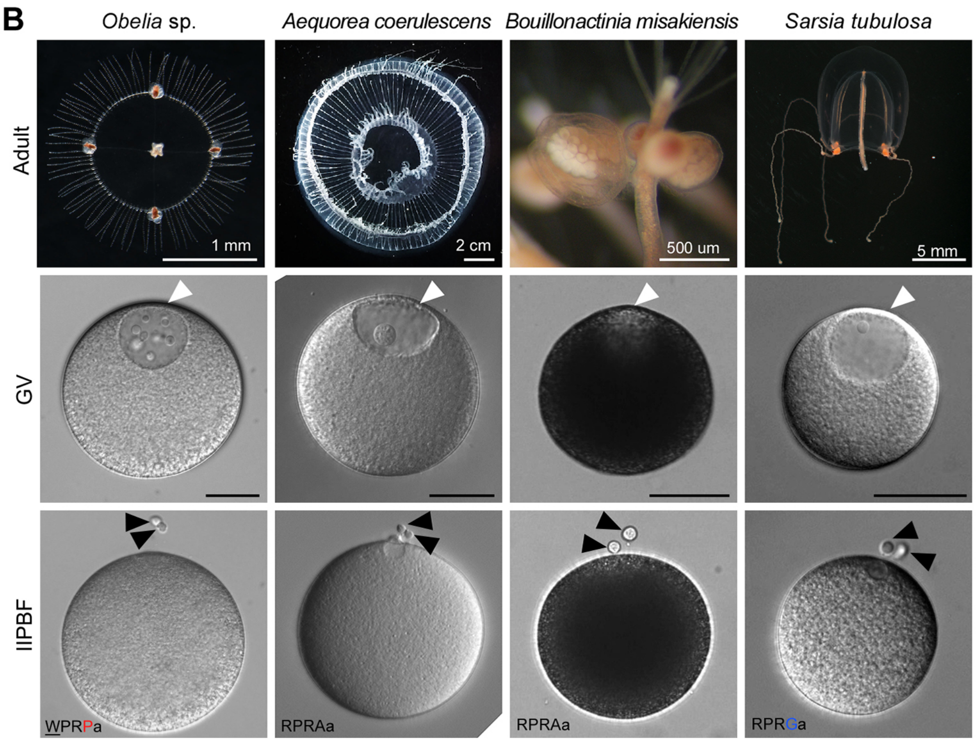

Fig. 6. Synthetic peptides show MIH activity in a subset of hydrozoan jellyfish species. (A) Synthetic W/RPRP/A/Gamides were tested for their ability at 10^{-5} M and 10^{-7} M to induce GVBD of oocytes of the eight species indicated. Highest success of GVBD is emphasised by the darker blue shading. (B) Examples of four of the species tested, showing 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: 50 µm. White and black arrowheads indicate GVs and polar bodies, respectively.

metamorphosis from larva to polyp (Anctil, 2009; Fujisawa, 2008; Takahashi and Hatta, 2011). Transcript sequences predicted to produce many copies of short neuropeptides have also been found in ctenophore and placozoan genomes (Moroz et al., 2014; Nikitin, 2015), and neuropeptides are thought to have been the predominant neurotransmitters in the ancient common ancestor of these groups (Grimmelikhuijzen and Hauser, 2012). 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 (Takeda et al., 2013; Tremblay et al., 2004) as well as bilaterians (see above), suggests that neuropeptide signalling played a central role in coordinating sexual reproduction in the bilaterian-cnidarian ancestor, and might have been involved in coordinating spawning events in the marine environment. In *Clytia* medusae we found cells producing PRPamide family peptides not only in the gonad but also in the manubrium, tentacles and bell margin (Fig. 5C, D), so these presumably have wider functions beyond 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 neuroendocrine regulation of reproduction.

MATERIALS AND METHODS

Animal cultures

Laboratory strains of *Clytia hemisphaerica* (‘Z colonies’), *Cladonema pacificum* (6W, NON5, UN2) and *Cytaeis uchidae* (17) were maintained throughout the year (Deguchi et al., 2005; Houliston et al., 2010; Takeda et al., 2006). Wild specimens of *Cladonema* as well as *Eutonina indicans*, *Nemopsis dofleini*, *Obelia* sp., *Rathkea octopunctata* and *Sarsia tubulosa* were collected from Sendai Bay, Miyagi Prefecture, and *Aequorea coerulescens* and *Bouillonactinia misakiensis* from Mutsu Bay, Aomori Prefecture. The brand of artificial seawater (ASW) used for culture and for

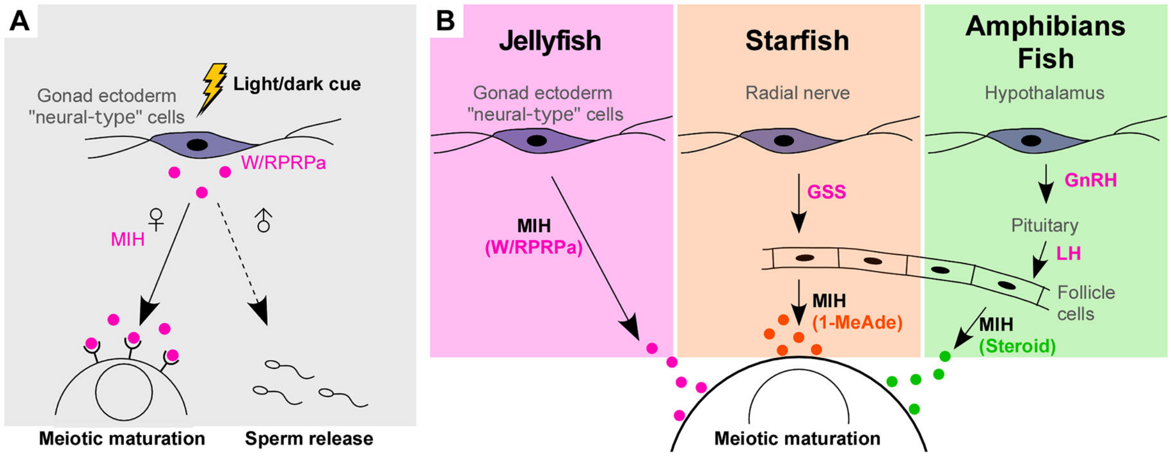

Fig. 7. MIH action in jellyfish compared with other animals. (A) Summary of the findings of this study. Jellyfish MIH, consisting of PRPamide family peptides in *Clytia* and *Cladonema*, is secreted by neural-type cells in the gonad directly in response to light cues and causes oocyte maturation as well as spawning in males and females. (B) Comparison of the regulation of oocyte maturation by peptide hormones (pink) in jellyfish, starfish and fish/amphibians. GSS, gonad-stimulating substance; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone.

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 anaesthetised 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 (Freeman, 1987). A chamber formed between a plastic dish and a coverslip separated by two pieces of 400 or 500 µm-thick double-sided sticky 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 two *Clytia* jellyfish or several ovarian epithelium fragments stripped from three to five *Cladonema* jellyfish was inserted into the oil space (Fig. 1A,B). The oil chambers were subjected to light-dark changes (light after dark for *Clytia*, and dark after light for *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 were discarded. MIH assays were performed at 14-16°C for *Eutonina*, *Nemopsis*, *Obelia*, *Sarsia* and *Rathkea* or at 18-21°C for *Clytia*, *Cladonema*, *Cytaeis* and *Bouillonactinia*.

Identification of peptide precursors

Potential amidated peptide precursor sequences were recovered from a *Clytia* reference transcriptome derived from mixed larva, polyp and jellyfish samples. Open reading frames (ORFs) and protein sequences were predicted using an R script (Lapébie et al., 2014). Potential secreted proteins were identified by the presence of signal peptide using SignalP 4.0 (Petersen et al., 2011). Then, sequences rich in the amidated pro-peptide cleavage motifs GR/K and lacking domains recognised by InterProScan-5.14-53.0 (EMBL-EBI) were selected. Finally, sequences containing repetitive motifs of fewer than 20 amino acids were identified using TRUST (Szklarczyk and Heringa, 2004). Among this final set of putative peptide precursors, some known secreted proteins with repetitive structures were identified by BLAST (NCBI) and removed.

To prepare a *Cladonema* transcriptome, more than 10 µg total RNA was isolated from the manubrium of female jellyfish (6W strain) using the NucleoSpin RNA purification kit (MACHEREY-NAGEL). RNA-seq library preparation and sequencing (Illumina 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 the pGEM-T Easy vector (Promega), or retrieved from our *Clytia* EST collection cDNA library prior to probe synthesis. Sequences and accession numbers are given in Fig S1.

For *Clytia* gonad tissue transcriptome comparisons, Illumina HiSeq 50 nt reads were generated from mRNA isolated using the RNAqueous Micro Kit (Ambion Life Technologies) from ectoderm, endoderm and oocytes manually dissected from ∼150 *Clytia* female gonads. Quantitative PCR was performed to check for contamination between samples using endogenous GFP genes expressed in oocyte, ectoderm and bell tissue (Fourrage et al., 2014), and to quantify the expression of selected peptide precursors (see Fig. S2B for primers). The reads were mapped against a *Clytia* reference transcriptome using Bowtie 2 (Langmead and Salzberg, 2012). The counts for each contig were normalised per total reads of each sample and per sequence length and visualised 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, DAWPRP-NH_2, AWPRP-NH_2, FNIRPRP-NH_2, NIRPRP-NH_2, IRPRP-NH_2, PRP-NH_2, WPRP-OH and RPRP-OH were synthesised by GenScript or Life Technologies. These peptides were dissolved in deionised water at 10^{-2} M or 2×10^{-3} M, stored at −20°C, and diluted in ASW at 10^{-5}-10^{-10} M prior to use. TAMRA-WPRPamide (TAMRA-LEKRNWPRP-NH_2) was synthesised by Sigma and a 5×10^{-4} M solution in H_2O was injected at 2-17% of the oocyte volume, to give an estimated final oocyte concentration of 1-9×10^{-5} M (Deguchi et al., 2005).

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-Aldrich). For MIH inhibition experiments, antibodies or control normal rabbit IgG (MBL, MP035) were concentrated using a 30,000 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* hybridisation

For single or double anti-PRPamide/anti-PRAamide staining, specimens were anesthetised using excess Mg^{2+} ASW and fixed overnight at 4°C in 10% formalin-containing ASW, then rinsed three times for 10 min each in phosphate buffered saline (PBS) containing 0.25% Triton X-100 (PBS-

Triton). They were incubated in anti-PRPamide or anti-PRAamide antibody diluted 1/1000-1/10,000 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, both 1/1000; Invitrogen, A-11034, A-11036) for 2 h at room temperature and nuclei stained using 50 µM Hoechst 33258 or 33342 (Invitrogen) for 5-20 min. Zenon antibody labelling kits (Molecular Probes, Z-25313, Z-25302, Z-25306) were used for double peptide staining. In control experiments, PBS-Triton alone or normal rabbit IgG (3 mg/ml; Zymed, 100500C) diluted 1/1000 in PBS-Triton 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 18°C 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% Tween 20 (PBS-T). Treatment on ice with 50% methanol in PBS-T, then 100% methanol, plus storage in methanol at −20°C improved visualisation of microtubules in the MIH-producing cells. Samples were rehydrated, washed in PBS containing 0.02% Triton X-100, blocked in PBS with 3% BSA overnight at 4°C, then incubated in anti-PRPamide antibody and anti-alpha tubulin (YL1/2, Sigma-Aldrich, 92092402; 1/500) in PBS with 3% 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, both 1/100; Jackson ImmunoResearch, 111-025-003, 712-175-153) overnight in PBS at 4°C and nuclei stained using Hoechst 33258 for 20 min.

For *in situ* hybridisation, isolated gonads or whole jellyfish were processed as described previously (Fourrage et al., 2014) except that 4 M urea was used instead of 50% formamide in the hybridisation buffer (Sinigaglia et al., 2017). For double fluorescent *in situ* hybridisation, female *Clytia* gonads were fixed overnight at 18°C in HEM buffer containing 3.7% formaldehyde, washed five times in PBS-T, then dehydrated on ice using 50% methanol/PBS-T then 100% methanol. *In situ* hybridisation (Lapébie et al., 2014; Sinigaglia et al., 2017) was performed using a DIG-labelled probe for Che-pp1 and a fluorescein-labelled probe for Che-pp4. A 3 h incubation with a peroxidase-labelled 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 Plus Fluorescence Amplification Kit (PerkinElmer) and Cy3 fluorophore [1/400 in TSA buffer (PBS with 0.0015% H_2O_2)] at room temperature for 30 min. After three 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-labelled anti-fluorescein antibody was followed by washes in MABT. The anti-Che-pp4 fluorescence signal was developed using the TSA kit with Cy5 fluorophore. Nuclei were stained using Hoechst 33258. Images were acquired using a Leica SP5 confocal microscope and maximum intensity projections of *z*-stacks prepared using ImageJ software (NIH).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Methodology: O.K.; Formal analysis: N.T.; Investigation: N.T., Y.K., G.Q.A., P.L., C.B., R.D.; Writing - original draft: R.D.; Writing - review & editing: E.H.; Supervision: T.K., K.T.; Project administration: E.H., R.D.; Funding acquisition: E.H., R.D.

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Data availability
Putative precursor amino acid sequences have been deposited in GenBank under accession numbers KX496947- KX496961 as detailed in Fig. S1. *Clytia* gonad transcriptome data are available at the NCBI Gene Expression Omnibus repository under accession number GSE101072.

Supplementary information
Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.156786.supplemental

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Name	Access number	Amino acid sequence [a]	Predicted peptides
Che-pp1	KX496947	MERKILACLFLLIVLLNLNDGKNIAILIEPDDNLASELEWLGSDMTDSHSLNAGAWPRPGDARSSHD AWPRPGKREFYGNEMFEKRPFPGQQMQFSWPRPGKKETKEDTWPRPGKRESYSEGDMDSRSGALRRS EEKETNEDEKLENAWPRPGKREFYASRKMDVRPRGGRDSKSHKISKRNSEAISNDEIDMMIREEAWP RPGKRDYHMLSATRPRGGKDARPRGGKDSSRPRGGKNAKPRGGKDSVRPRGGKKDSWPRPGKDAFVK EINGSRPRGGKDASKWPRPGKKDLK	7 x WPRPa 6 x RPRGa 1 x KPRGa
Che-pp2	KX496948	MKIYFGCLFVILSVNQIGCYPSSNQNSERELVRRIYKTVHPNPHYQVNEIQRVKEALKRRVLENVHR VDSLKASLKRVLGQDAGNGFHMSSSKIFQKKRSKARLPHSYMFRKRQNSPGALGLWGREVEAPGDIG PPGIWGDVVPDETRKDKPGAVQGLWGKDERVIRALLKTLKR	QNSPGALGLWa KPGAVQGLWa
Che-pp3	KX496949	MNCVLIFLVLFLANNVYSASLTREEDALVTKLLDTIEKRDAVPRLGKREVPRLGREIEDVPRLGREI ELVPRLGREAEEVPRLGREAEEVPRLGREVELVPRLGREVELVPRLGREVEVPRLGREVPRLGRGID EVPRLGREVPRLGRELEVPRLGREVPRLGKREIPRLGKREVPRLGRREVPRLGREASTYDLKQLYNQ LKREVSNDMIEAEIKEEKRVLKAFDLGTRGLILRRIGDKLNKDGFSDQKRDMNEKESSRPFLHVKRT NLLSLIEKLTSEE	15 x VPRLa 1 x IPRLa
Che-pp4	KX496950	MNLLVSIPVICAIVLKLTESAPISNVRKIGSNELLLKLTVSDLAKLLSRLQNVHEDGHKEDLNKVSV EGMIADYLDEKQYKDRPRYGKDLKEASRPRYGKEMSEGGNHNVIEQLIEKLVNQSSESDTKDDGNIK SDGKVDNLVSLLHGLDEEKEWPRPGKDWPRAGKDWPRAGKDWPRAGKDWPRAGKDWPRAGKDWPRPG KDQPNGIARGGKRSLGEILSDLMSKRPRYGKDESSTNGPSYQYTLEDMMTEIISGDRPRYGRKSEAS RPRYGKESADKTLEEALSNLGTKSTKKDTSSRSSLARGGKKRGVEDLILEYIENHLDDKKDYESARK DTPK	5 x RPRYa 5 x WPRAa 2 x WPRPa IKSDa IARGa LARGa
Che-pp5	KX496951	MELKYFLASFIFVIIAIQLASCSSKAEEYKQMKKEVDGLLKEIVSQENAKQHTSEKKSSQWLNGRFG KRQLVSGRFGRELKQWLNGRFGREATEQWLNGRFGKREDDQWLNGRFGREVEQWLNGRFGRDAAEQW LNGRFGKRSANQWLNGRFGKRSADQWLNGRFGKRSADQWLNGRFGREASADQWLNGRFGREAADQWL NGRFGREAKEQWLNGRFGREVEQWLNGRFGREAGQWLNGRFGREMGQWLNGRFGREADQWLNGRFGR EADQWLNGRFGRDAAPLAARYGDEPAHVESQTIAAPEEAKPKIVAAVKVVKKPVAVSE	17 x QWLNGRFa
Che-pp6	KX496952	MARESLLFFLLALHCCEAFYNGDVPRRRSEASRLLMAKDASQKHASSDTSRLLFGKDAPQRHVSSDS RLLFGKDAPQRHVLSDTSRLLFGKDAPQRHVSSDSRLLFGKDAPQRHISSDSRLLFGKDAPQRHVSS DSRLLFGKDAPQRHVSSDTSRLLFGKDAPQRHVSSDTSRLLFGKDVPQRPAGGSDSRTSYTAHLMPT LGKDEYVNALKERLLNDYRMKLLQQQRQQQHQQQEDDDELYFDHSFRRGNNPSASAFRRRYSDQTGQ PLSGSRDQKVNQDENARDTLEKKQQASLDQTKEELKRSLLKDFYKKMITEKREKQEFAKKSDAPVSN DFDDEILRHLVEFKLKKEDPMKRMRR	8 x SRLLFa
Che-pp7	KX496953	MRFCSWTNLFLLGITCLCLTNGMPNKQHVRNKKNLIDNTVKMADHGKTLVKKSAHPMKIKDVSKKST GGGSDIANSDDTFDRAADGTDNSLYGRQEKEQGTENSGVGKFEGPPCRWGCGKREAGIDGPPGRWGG RKRGMRRVGPPGRWGGRKRGELPGRWGGKKRGELPGRWGGKKRSELPGHWGGKKRSELPGHWGGKKR SEIPGRWGGKKRSEIPGRWGGKNRSELPLGWSQKEGNQRPPSKET	2x LPGRWGa 2x GPPGRWGa 2x IPGRWGa 2x LPGHWGa
Che-pp8	KX496954	MLSSETTIRIFCFFIAVGFAVGSSSPEEEGQLLHVKRETWLNPGFDSMLHRRESQELLNRPRPGRRE LFDLMNQDSILKKRALLHRPRPGRRELFRNQGLDSMLHKRAMLNRPRPGRRELFRNPGLDSMLHKRG QEFLSGPRPGREIRPRPGRRERHPDSMLHRRSSDTDLDYQHLLRNPRPGRRELFRPRPGHRELQHLD SLLHRRSEEMWGRPRPGKREVYYEDDGRSEDEKLLRVLDELKRDIIDELWDRFQN	[b] 6x --RPRPa 1x --GPRPa 1x --NPRPa
Che-pp9	KX496955	MYLSVGFLLFLCHQFQDTHGLSIRGPNDAQQLINSHGENDLPSGGMWETAKSQAMETYRKDSRRGIP KRGRSSFLAIGKKDDSLSLGSKMKKSLESPSLSVWRRGDSLHSILRVNPSLGIWKRGDSLMRRFGRK NFGKDNSRRSLVREIENDAFGMNRKDEFPSPGMWEKAKSQYFGKREFRNSILGKHGKDEFPSPAMWE KAKNQYFGKRDIRNSILGKHGKDEFPSPAMWEKAKNQYFGKGR	3x --QYFa 2x LGKHa SLMRFa
Che-pp11	KX496956	MDQSLSSILLLLCCWVALTTCMSVQRKEAGDALSALDKENAKKSANSITEELARNLMEHLYDEIRKR SNSNEETISNFRRASSDTHRQQQAPKGLWGRELQPGNPPGLWGREASEAENTDSNDGPIPGMWGRRE ADDKNAHEKFQ	QQQAPKGLWa QPGNPPGLWa GPIPGMWa
Cpa-pp1	KX496957 KX496958	MISKAIIVYSLVLLILPVYSKFPKSRFSIRGNQLDNGYPRGEIPKIKPDMKDRERSNLLMKFAEALR ILRTNQNYHDGKPYSKDIEEDFRKISSNRFNIRPRPGKRFLKNKFAACDEHFANE	RPRPa
Cpa-pp2	KX496959	MAMNITFISFICFAISIILLNVQSAPLDDIRERSIEHEMAQNQRESLVHLLTLGYSLGARDDSNTLS KLITYVDNLKDDVWNTDSEGCRNIETTLYSDLLSQPNYDLTDLESRELDTYENERPTAGKRFSSMNI IDNDSFRPRAGRRDIRPRAGRREFRPRAGKRYLAESESFDAYRPRAGKRELRPRAGKRDEDNEKMRD TANRQSQREVLKRNSIYKELMTANESNERPRAGKRETETDEGKHDMSTFLEINSDKREERPRAGKKE LRPRAGKKELRPRAGKKE	9x RPRAa
Cpa-pp3	KX496960	MKTTMLYHTVLCICVINIYGNGKFVYTENAIDEKELTDLKDLQLDAESNQIQSRITKDLSRILADKI YNQLKESTNTEFKKNLDLFQFQPEIGSFNGKRPPLPKPPGLWGKDVHSRFQIDSHDISIKSSGDISK REAEKRKLKNNLKSDDASKYADYINMDNKRSEILNGPPGLWGREAEKLNGPPGLWGRDEKVNDALNG KLGKVHEPRDLLKRENGKVNGPPGLWGREIGKRNGPPGLWGRDAEMNNGPPGLWGRDAEKVNGPPGL WGRDIGKINAPSSLQDHTFEKNRGPPGLWGREIEKVNGPPGLWGREIEKNNGPPGLWGRELGMNNGP PGLWGRDFTTIKENGDNDATRKEKPTDTKMENSDKKGGFGQKDDSKYNAKWKRNERNLLKDRNQIET QKRESSRKESSADKQIKNRNSKDSKTAKKDSRNDVTIDNIFPWHKSKHDDNTASNLRDIIENEF	10x NGGPPGLWa
Cpa-pp4	KX496961	MNLILPIFASILLCLADSASITTKKDSENDVERQIETLLNELLEGADNNLYDKKEVDQWLKGRFGRE SSDQWLKGRFGRDADQLLKGRFGREAEQWLKGRFGREAGDQWLKGRFGREAEQWLKGRFGREAGQWL KGRFGREAEQWLKGRFGREVEQWLKGRFGRETEEAEQWLKGRFGRDAITKDSNPLLRGRYGRDSESE VTENAPKQLDRGRYGREQWLKGRFGREAQSENETKKEIDNANKKVDVNAKKEVSKTAM*	9x QWLKGRFa 2X RGRYa

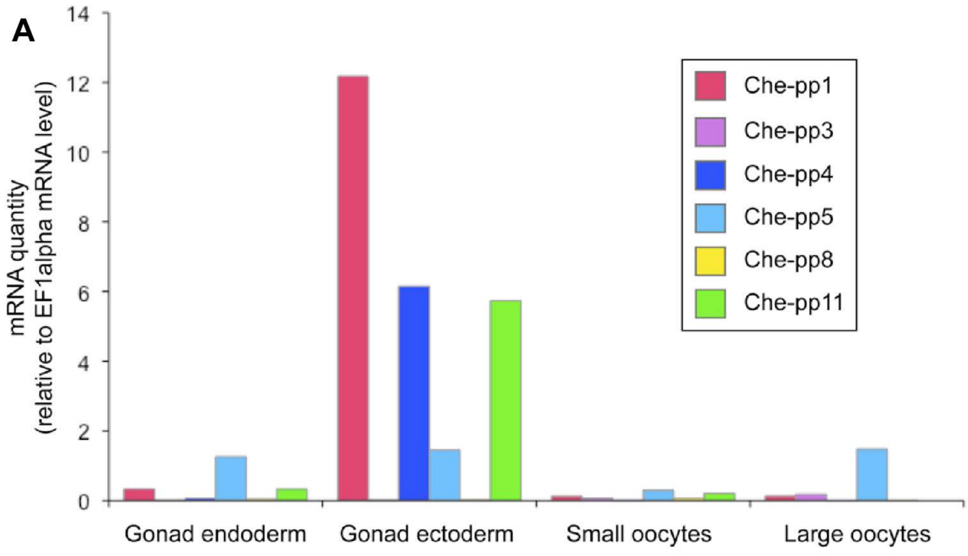
[a] N terminal secretion signal sequences are highlighted in pink. Probable mono- or di-basic cleavage sites, highlighted in blue, are preceded by Glycine residues (yellow) that would be converted to C terminal amides. N terminal protolysis limits of each liberated peptide (best estimates in grey) during precursor cleavage in cnidarians are difficult to predict, but are often associated with additional basic or acidic residues (red).

[b] N terminal limits of possible peptides generated for pp8 are particularly hard to predict, but all have the C-terminal sequence PRPa

Figure S1. Amino acid sequences of putative precursors of amidated peptides identified from *Clytia hemisphaerica* (Che) and *Cladonema pacificum* (Cpa) transcriptome data.

N terminal secretion signal sequences are highlighted in pink. Probable mono- or di-basic cleavage sites highlighted in blue are preceded by Glycine residues (yellow) that would be converted to C terminal amides in the final peptides. The limits of proteolysis on the N terminal side of each liberated peptide during precursor cleavage in cnidarians are variable and difficult to predict but are often associated with additional basic or acidic (red highlighted here) residues. Genbank Accession numbers for each peptide precursor sequence are indicated in parentheses.

A



B

EF1alpha	Forward	5' TGCTGTTGTCCCAATCTCTG 3'
	Reverse	5' AAGACGGAGTGGTTTGGATG 3'
Che-pp1	Forward	5' GCGTCGATCTGAGGAAAAAG 3'
	Reverse	5' AACGTCCATCTTCCTGCTTG 3'
Che-pp3	Forward	5' CTGAAAGCGTTCGATCTTGG 3'
	Reverse	5' CACATGCAAGAATGGTCTGC 3'
Che-pp4	Forward	5' CGTCAAGTCCGTCATCATTG 3'
	Reverse	5' GCTGACTCATCATCTTTCTTGTC 3'
Che-pp5	Forward	5' ATCCTGCTCATCCAAAGCTG 3'
	Reverse	5' AACTCACGGCCAAATCTACC 3'
Che-pp8	Forward	5' GGACGAGAAATAAGGCCAAG 3'
	Reverse	5' TGATCGTCGATGCAGTAAGG 3'
Che-pp11	Forward	5' TACTATGCTGTTGGGTCGCC 3'
	Reverse	5' CTTCAGTAATGCTGTTTGCGCT 3'

Figure S2. Quantification of neuropeptide transcript levels between gonad tissues.

A) Quantitative RT-PCR analysis of neuropeptide precursor expression in manually separated tissues from *Clytia hemisphaerica* gonads confirmed that Che-pp1, Che-pp4 and Che-pp11 are the 3 main peptide precursors expressed in the ectoderm. Q-PCR was run in triplicate and EF1alpha used as the reference control gene. B) Sequences of forward and reverse primers used for each gene.

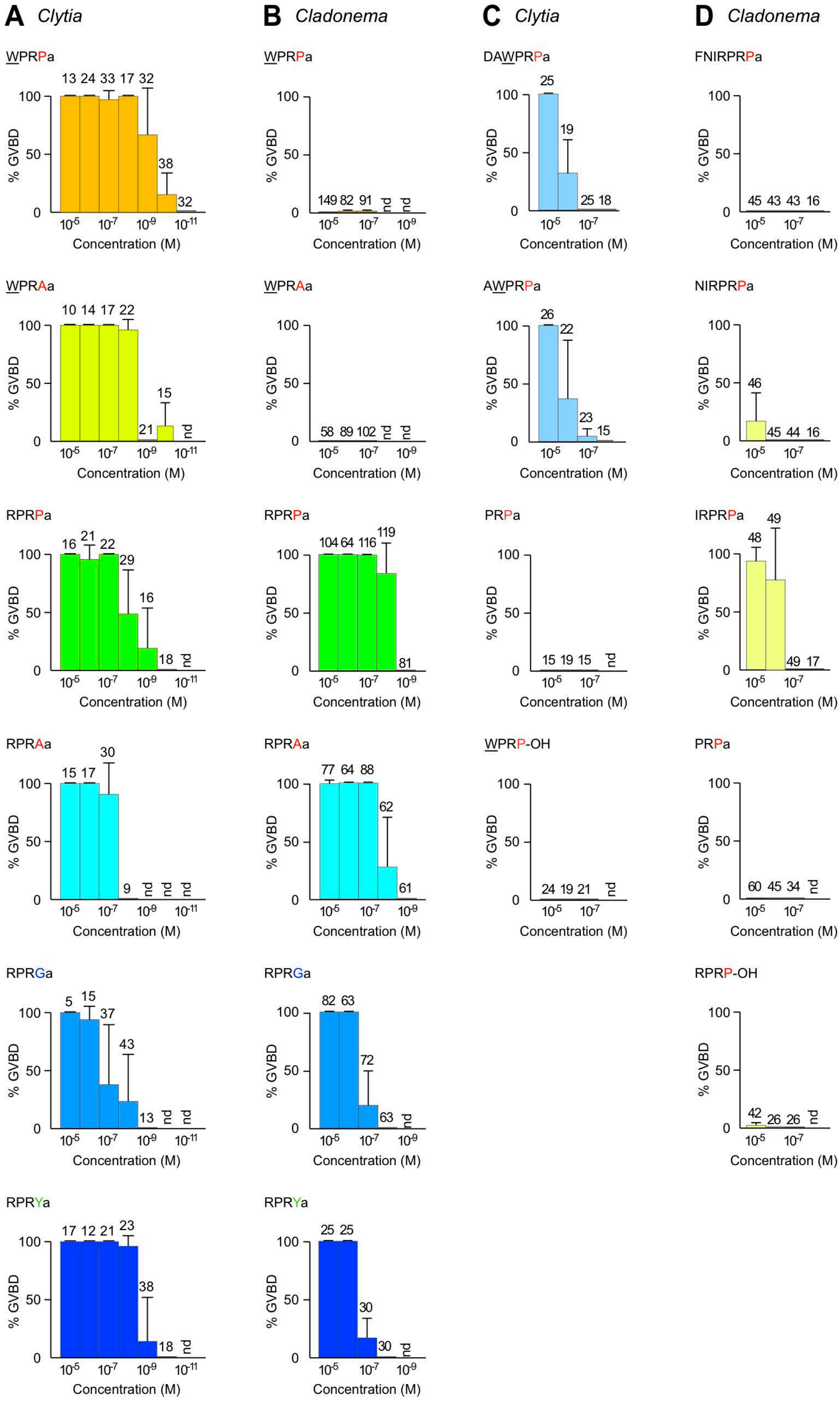
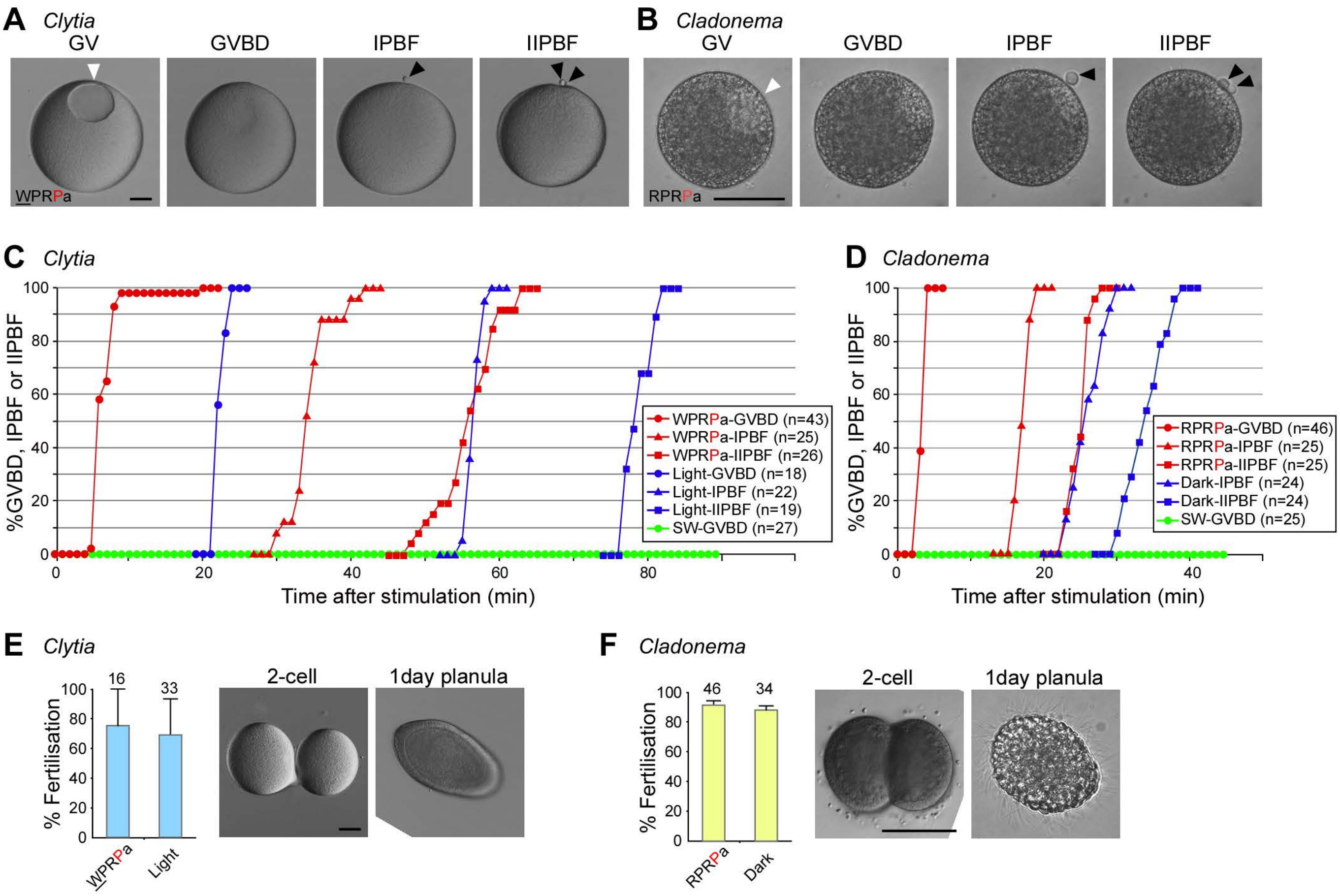

A Clytia
B Cladonema
C Clytia
D Cladonema
WPRPa
WPRAa
RPRPa
RPRAa
RPRGa
RPRYa
DAWPRPa
AWPRPa
PRPa
WPRP-OH
FNIRPRPa
NIRPRPa
IRPRPa
PRPa
RPRP-OH
% GVBD
Concentration (M)

Figure S3. MIH activity of synthetic amidated peptides.

Clytia (columns A and C) or *Cladonema* (columns B and D) oocytes were incubated in SW drops containing synthetic amidated peptides as indicated, and GVBD scored after 2 hours. The numbers of oocytes tested is shown above each bar. Black lines indicate standard deviation between experiments. Simplified representations of these data are shown in Fig. 2C.



A Clytia
GV
GVBD
IPBF
IIPBF
WPRPa
B Cladonema
GV
GVBD
IPBF
IIPBF
RPRPa
C Clytia
%GVBD, IPBF or IIPBF
Time after stimulation (min)
WPRPa-GVBD (n=43)
WPRPa-IPBF (n=25)
WPRPa-IIPBF (n=26)
Light-GVBD (n=18)
Light-IPBF (n=22)
Light-IIPBF (n=19)
SW-GVBD (n=27)
D Cladonema
%GVBD, IPBF or IIPBF
Time after stimulation (min)
RPRPa-GVBD (n=46)
RPRPa-IPBF (n=25)
RPRPa-IIPBF (n=25)
Dark-IPBF (n=24)
Dark-IIPBF (n=24)
SW-GVBD (n=25)
E Clytia
% Fertilisation
16
33
WPRPa
Light
2-cell
1day planula
F Cladonema
% Fertilisation
46
34
RPRPa
Dark
2-cell
1day planula

Figure S4. Synthetic tetrapeptides induce normal oocyte maturation.

A, B) Images of *Clytia* and *Cladonema* oocytes respectively, incubated in 10^{-7} M W/RPRPamide as indicated, undergoing successive steps of meiosis: GVBD; First polar body formation (IPBF); Second polar body formation (IIPBF). C) Time course of *Clytia* oocyte maturation. The percentages of oocytes undergoing GVBD, IPBF, and IIPBF after application of 10^{-7} M WPRPamide or SW alone (control), or light stimulation are indicated as a function of time. The time of IPBF and IIPBF after light stimulation was determined in oocytes isolated from light-stimulated ovaries following GVBD. WPRPamide- and light-induced meiotic maturation progressed at a similar speed, although there was a lag time of 15-20 minutes. D) Time course of *Cladonema* oocyte maturation. Meiotic maturation in isolated oocytes treated with 10^{-7} M RPRPamide was advanced by about 10 minutes compared to that induced by dark treatment of gonads. At 11~12 minutes after dark initiation, oocytes just undergoing GVBD were isolated from the gonads for subsequent observation of IPBF and IIPBF. E) Fertilisation rates for *Clytia* oocytes matured by incubation in 10^{-7} M WPRPamide were equivalent to those released from gonads stimulated by light. Numbers of gonads tested and standard deviation between experiments are shown for each bar. They went on to undergo normal cleavage divisions (center panel) and form swimming planula larvae (right panel). F) Equivalent fertilisation success documented for *Cladonema* eggs incubated in 10^{-7} M RPRPamide. Scale bars: 50 µm. White and black arrowheads indicate GVs and polar bodies, respectively.

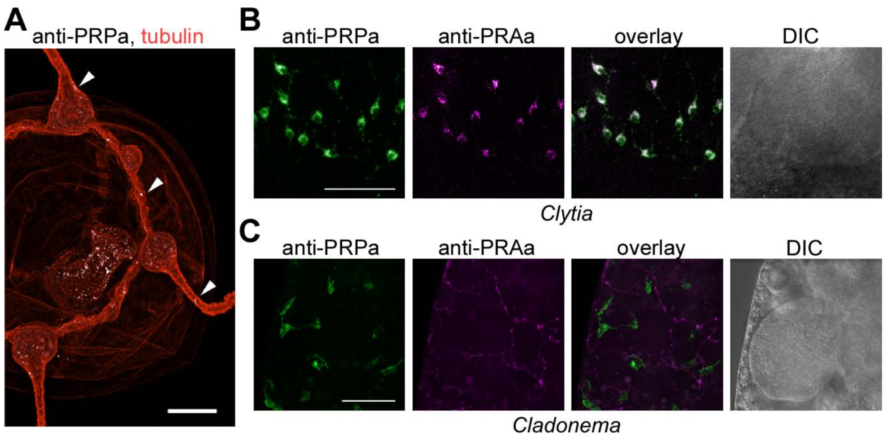


Figure S5. Immunodetection of MIH in *Clytia* jellyfish and in *Clytia* and *Cladonema* gonad ectoderm.

A) Confocal image (summed Z stack) of a *Clytia* baby jellyfish following immunofluorescence performed with anti-PRPamide and anti-tubulin. Arrowheads point to MIH-immunopositive cells in the nerve ring and tentacles. B, C) Epifluorescence images of gonad ectoderm following double immunofluorescence performed with anti-PRPamide and anti-PRAamide antibodies as indicated. Overlaid images are shown in the third panel of each row. In *Clytia* gonads (B) these decorated a single cell population, whereas in *Cladonema* (C) the two peptides were detected in distinct cell populations. Scale bars: 50 µm.