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Light-induced oocyte maturation in the hydrozoan clytia hemisphaerica

Gonzalo Quiroga Artigas

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Gonzalo Quiroga Artigas. Light-induced oocyte maturation in the hydrozoan clytia hemisphaerica. Development Biology. Université Pierre et Marie Curie - Paris VI, 2017. English. NNT : 2017PA066284 . tel-01799640v2

HAL Id: tel-01799640

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Université Pierre et Marie Curie

Ecole doctorale Complexité du Vivant (ED515)

Laboratoire de Biologie du Développement de Villefranche-sur-Mer UMR8009 CNRS-UPMC

Mécanismes développementaux chez Clytia

Light-induced oocyte maturation in the hydrozoan

Clytia hemisphaerica

Par Gonzalo Quiroga Artigas

Thèse de doctorat de Biologie du Développement

Dirigée par Evelyn Houliston

La thèse sera présentée et soutenue publiquement le 23/05/2017

Devant un jury composé de :

Dr. Katja Wassmann

Présidente de Jury

Pr. Stefano Piraino

Rapporteur

Dr. Jacek Kubiak

Rapporteur

Dr. Marie-Hélène Verlhac

Examineur

Dr. Sylvie Rétaux

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Dr. Evelyn Houliston

Responsable de thèse



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If you watch animals objectively for any length of time, you are driven to the conclusion that their main aim in life is to pass on their genes to the next generation.

- David Attenborough

ACKNOWLEDGEMENTS

First of all I would like to thank my family, particularly my parents. They have always trusted in me, supported me, and helped me to find my path back when I felt lost. Thank you.

Then I would like to thank my friends, those that I still luckily keep from my childhood (Ivan, Toni, Eric, Ruth), and my friends from the UB (University of Barcelona). David, Joan, Ferran, Miquel, Martí, mes amis ;) gràcies. You have taught me how to be a better person. Thank you Marta for introducing me to Sir David Attenborough's documentaries, and for being always so shiny.

Thanks a lot Alex, cousin and friend, for being also a brother. And thanks also for formatting the 3 manuscripts of my thesis, I mean, how lucky am I!?

I thank all the people from the NEPTUNE network, students and PIs. You have made my PhD a lot more exciting and an unforgettable period of my life. What I have learned from this network... It is too good to be true. I will always be able to look back in time and think: once, I was a Neptunee, what an amazing time I had!

Thank you very much, Sir David Attenborough, for constantly reminding me why I chose to be a biologist.

I would also like to thank all the people from Dr. Evelyn Houliston's lab, who have helped me and welcomed me with open arms since the moment I started the thesis. Lucas, Pascal, Tsuyoshi, thanks a lot for all your help and for your active participation in my PhD project.

Thanks, Sophie, Loan, Laurent, Faisal, Mohamed, Philippe, Christian, Frédéric and Christelle for all your help, technical advice and for having taught me important lessons.

Stefano, Alex, thank you for always offering me your help and for mentoring me during my PhD.

Finally, I would like to thank my PhD supervisor, Dr. Evelyn Houliston. You gave me the great opportunity to do a PhD in your lab, and I will always be thankful for that. It has been a remarkable experience altogether. Thanks for your constant and thoughtful advice, which has made a better scientist. I have learned a lot from you Evelyn.

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RESUME

Un contrôle précis de la maturation ovocytaire et de la ponte sont essentiels au succès de la reproduction sexuée au sein le règne animal. Ces processus sont coordonnés précisément par des signaux endocriniens et/ou environnementaux, selon les espèces, mais beaucoup reste à apprendre sur leurs régulations. Chez les cnidaires, de nombreuses méduses du groupe des hydrozoaires sont connues pour produire des gamètes en réponse à la transition nuit/jour. Pour caractériser les machineries cellulaires et moléculaires liant la réception de la lumière à l'initiation de la maturation ovocytaire, j'ai étudié la méduse hydrozoaire *Clytia hemisphaerica*. Mon travail de thèse s'est découpé en trois parties, chacune impliquant l'identification d'un composant moléculaire clé de ce processus.

Mon étude initiale faisait partie d'une collaboration avec N. Takeda (Asamushi) et R. Deguchi (Sendai), chercheurs qui avaient, avant le début de ma thèse, identifié chez *Clytia* les Hormones d'Incitation de Maturation ovocytaire endogènes (MIH) comme étant des tétrapeptides de type WPRPamide, produit par clivage de deux précurseurs à neuropeptides. J'ai montré par hybridation *in situ* et immunofluorescence que les deux gènes précurseurs du MIH sont exprimés par un type de cellules neurosécrétrices localisées au niveau de l'ectoderme de la gonade, et que les peptides MIH sont sécrétés par ces mêmes cellules suite à une stimulation lumineuse. Cette étude a posé les bases permettant l'identification des régulateurs agissant en amont et en aval du MIH, et plus spécifiquement ceux impliqués dans la photoréception de l'ectoderme de la gonade et la réception du MIH par les ovocytes.

Pour identifier le récepteur du MIH de *Clytia* (CheMIHR) dans les ovocytes, j'ai compilé à partir de données transcriptomiques issues de tissus de gonades, une liste de 16 protéines candidates de la famille des Récepteurs Couplés aux Protéines G (GPCR). J'ai cloné les 16 cDNAs et, utilisant une méthode de « deorphelinisation » de GPCR basée sur de la culture cellulaire (collaboration avec P. Bauknecht et G. Jékély; MPI, Tübingen), j'ai pu identifier un GPCR activée par des peptides MIH synthétiques. Sa fonction *in vivo* comme récepteur essentiel du MIH a été confirmée par la méthode d'édition génétique CRISPR/CAS9. La délétion ainsi produite, entraînant un déplacement du cadre de lecture au sein du gène *CheMIHR*, a détérioré la croissance des colonies de polypes et le comportement de ponte des méduses matures. Confirmant la fonction de CheMIHR, la maturation ovocytaire chez des mutants *CheMIHR* ne pouvait pas être déclenchée par la lumière ou par addition de MIH synthétiques, mais pouvait être rétablie en utilisant des analogues au cAMP, molécule connue pour agir en aval de la réception du MIH dans les ovocytes d'hydrozoaires. Des analyses phylogénétiques ont montré que *Clytia* MIHR est affilié à un sous-ensemble de familles de neuropeptides de bilateriens impliqués dans divers processus physiologiques, notamment la régulation de la reproduction. Des hybridations *in situ* sur les méduses *Clytia*, ont en outre montré l'expression des précurseurs de CheMIH et de CheMIHR dans des cellules neurales hors de la gonade, suggérant un rôle plus large du couple CheMIH-MIHR que la seule initiation de la maturation ovocytaire.

Pour mieux comprendre la photoréception des gonades chez *Clytia*, j'ai montré que la ponte est sélectivement incitée par la lumière bleu-cyan, et mis en évidence, grâce à l'analyse de données de transcriptome de gonade, qu'un photopigment de la famille des Opsin (Opsin9) est hautement exprimé dans l'ectoderme. De façon saisissante, les hybridations *in situ* ont montré que le gène *Opsin9* est exprimé dans les mêmes cellules sécrétant le MIH. L'introduction d'une mutation de changement de cadre de lecture dans le gène *Opsin9* via la technologie CRISPR/Cas9 a empêché la maturation ovocytaire et la ponte des méduses mutantes en réponse à la lumière. Des immunofluorescences contre MIH et des expériences d'ajout de MIH synthétique sur ces mutants ont montré que la fonction essentielle de Opsin9 se situe en amont de la sécrétion du MIH. La ponte chez *Clytia* semble ainsi être réglée par une fonction double du type cellulaire photosensoriel-neurosécréteur, peut-être conservé depuis un lointain ancêtre métazoaire.

De manière générale, mon travail a contribué à la construction d'une image complète de la régulation moléculaire et cellulaire de l'initiation de la maturation ovocytaire chez *Clytia*, par l'identification et la caractérisation de 3 molécules clés. La réception de la lumière par les cellules neurosécrétrices de la gonade requière Opsin9 et cause la sortie d'un neuropeptide (MIH), qui active une GPCR spécifique (CheMIHR) sur la surface des ovocytes, ceci amorçant la maturation ovocytaire via l'élévation du cAMP cytoplasmique. La ponte de l'œuf est aussi probablement indirectement dépendante de ces trois molécules clés : Opsin9, MIH et MIHR.

ABSTRACT

Tight control of oocyte maturation and of gamete release is essential for successful sexual reproduction in the animal kingdom. These processes are precisely coordinated by endocrine and/or environmental cues, depending on the species, but much remains to be learned about their regulation. Within the Cnidaria, many hydrozoan jellyfish are known to spawn mature gametes following dark/light transitions. To characterise the cellular and molecular machinery linking light reception and oocyte maturation initiation, I have studied the hydrozoan jellyfish *Clytia hemisphaerica*. My thesis work had three parts, each involving the identification of a key molecular component of this process.

My initial study was part of a collaboration with N. Takeda (Asamushi) and R. Deguchi (Sendai), who identified the endogenous oocyte Maturation-Inducing Hormones (MIH) in *Clytia* as WPRPamide-related tetrapeptides, generated by cleavage of two neuropeptide precursors. I showed by *in situ* hybridization and immunofluorescence that *Clytia* MIH is produced by neurosecretory cells of the gonad ectoderm that co-express the two precursor genes, and that it is secreted upon light stimulation. This study paved the way for identification of regulators acting upstream and downstream of MIH release in the gonads, specifically the ones involved in photoreception in the gonad ectoderm, and in MIH reception by the oocytes.

To identify the *Clytia* MIH receptor (CheMIHR) in the oocytes, I compiled a shortlist of 16 candidate G protein-coupled receptors (GPCRs) from gonad transcriptome data. I cloned all 16 cDNAs and, using a cell culture-based "GPCR deorphanization" assay (collaboration with P. Bauknecht and G. Jékély; MPI, Tübingen), identified one GPCR that was activated by synthetic MIH peptides. Its *in vivo* function as the essential MIH receptor was confirmed by CRISPR/Cas9 gene editing. Introduction of a frame-shift mutation in the *CheMIHR* gene impaired growth of *Clytia* polyp colonies and also the spawning behaviour of mature medusae. Confirming the function of CheMIHR, oocyte maturation in *CheMIHR* mutants could not be triggered by light or by synthetic MIH, but could be restored using cell-permeable analogues of cAMP, known to act downstream of MIH reception in hydrozoan oocytes. Phylogenetic analyses showed that *Clytia* MIHR is related to a subset of bilaterian neuropeptide hormone receptor families involved in diverse physiological processes, including regulation of reproduction. Accordingly, *in situ* hybridization showed the expression of *Clytia* MIH precursors and MIHR in non-gonadal neural cells, suggesting a wider role of *Clytia* MIH-MIHR besides oocyte maturation initiation.

To address gonad photoreception, I showed that *Clytia* spawning is selectively induced by blue-cyan light, and then identified using gonad transcriptome data an opsin photopigment (Opsin9) highly expressed in the ectoderm. Strikingly, *in situ* hybridization showed that *Opsin9* is expressed in the MIH-secreting cells. Introduction of a frame-shift mutation into the *Opsin9* gene via CRISPR/Cas9 prevented oocyte maturation and spawning of mutant jellyfish in response to light. Anti-MIH immunofluorescence and rescue experiments with synthetic MIH showed that the essential function of Opsin9 is upstream of MIH release. Spawning in *Clytia* thus appears to be regulated by a dual function photosensory-neurosecretory cell type, perhaps retained from a distant metazoan ancestor.

Overall my work has helped to build a complete picture of the molecular and cellular regulation of oocyte maturation initiation in *Clytia* through the identification and characterisation of 3 key molecules. Light reception in gonad neurosecretory cells requiring Opsin9 causes release of a neuropeptidic MIH, which activates a specific GPCR (CheMIHR) on the oocyte surface to initiate oocyte maturation via cytoplasmic cAMP elevation. Egg spawning is also dependant, probably indirectly, on these three key molecules: Opsin9, MIH and MIHR.

PREFACE

This thesis manuscript is structured as follows:

The Introduction contains four sections.

- I. **Light and the rhythms of life.** In this section I will pinpoint the significance of environmental light in animals reproductive control.
- II. **Oocyte meiotic maturation.** In this section I will introduce the essential biological process of oocyte meiotic maturation, emphasizing the importance of the regulation of its initiation.
- III. **The hydrozoan *Clytia hemisphaerica* and light-induced oocyte maturation.** In this section I will introduce the model organism I have worked with during my PhD and highlight the findings that led to the establishment of the Working model for light-induced oocyte maturation in *Clytia hemisphaerica*.
- IV. **Objectives.** In this last section of the Introduction I will summarise the main objectives of my PhD work.

The Results section comprises 3 chapters, each of them containing “Context” and “Conclusions, Additional Results and Perspectives” subsections structured around manuscripts prepared for submission.

Chapter 1: Identification and characterization of *Clytia* MIH.

Chapter 2: Identification and characterization of the *Clytia* MIH receptor.

Chapter 3: Identification and characterization of the photopigment and photoreceptive cells that regulate *Clytia* spawning.

Finally, I will summarise my findings and bring them into an evolutionary context in the General Discussion.

PAPER SUBMISSION STATUS

Chapter 1 manuscript:

Jellyfish neuropeptides that act directly as oocyte maturation hormones

Noriyo Takeda, Yota Kon, **Gonzalo Quiroga Artigas**, Pascal Lapébie, Carine Barreau, Osamu Koizumi, Takeo Kishimoto, Kazunori Tachibana, Evelyn Houliston*, Ryusaku Deguchi*.

-This manuscript has been submitted to the journal *Nature Ecology & Evolution* as a sister paper to chapter 3 manuscript.

Chapter 2 manuscript:

Identification of a cnidarian oocyte maturation hormone receptor uncovers a conserved GPCR family regulating animal reproduction

Gonzalo Quiroga Artigas, Pascal Lapébie, Lucas Leclère, Philip Bauknecht, Tsuyoshi Momose, Gáspár Jékely and Evelyn Houliston*

-This manuscript is a provisional version and needs further analyses before submission. We predict to submit it to the journal *Plos Biology* during July-August 2017.

Chapter 3 manuscript:

A gonad-expressed opsin essential for light-induced spawning in the hydrozoan Clytia

Gonzalo Quiroga Artigas, Pascal Lapébie, Lucas Leclère, Noriyo Takeda, Ryusaku Deguchi, Gáspár Jékely, Tsuyoshi Momose* and Evelyn Houliston*

-This manuscript has been submitted to the journal *Nature Ecology & Evolution* as a sister paper to chapter 1 manuscript. (*Corresponding authors)

INTRODUCTION

I. Light and the rhythms of life

Environmental light is an essential factor that widely controls physiological and behavioural responses in the animal kingdom. It can provide different types of information, both visual and non-visual (Nilsson, 2009; Nilsson, 2013).

Visual light sensitivity is mediated through neuronal photoreceptor cell types which can be arranged or not in a complex organ like an ocellus or an eye, and exclusively use opsin photopigments (light-sensitive G Protein-Coupled Receptors - GPCRs) as photoreceptive proteins, which mediate direct responses to light that ultimately lead to image formation (Nilsson et al., 2009; Terakita, 2005). I will provide more detailed background information about opsins in Chapter 3.

Non-visual photoreception, which in most living organisms mediates biological responses independently of visual organs, is even more widespread than image-forming light detection, and clearly has a more ancient origin (Gehring Rosbash, 2003; Wolken and Mogus, 1979). In the next sections I will emphasize the significance of non-visual photoreception in animal life and its tight connection to sexual reproduction.

I.1. Non-visual photoreception in the animal kingdom

Non-visual photoreception is of great importance for most animals since it regulates a plethora of behavioural reactions as well as physiological responses (Martin, 2002; Peirson et al., 2009; Tessmar-Raible et al., 2011; Wolken and Mogus, 1979). Its role is based on the synchronisation of periodic biological activities to changing environmental light (Víggh et al., 2004) as well as on mediating fast behavioural and physiological reactions (Wolken and Mogus, 1979) in order to help survival of species in different ecosystems.

In contrast to visual light-sensitive cells, non-visual photoreceptors can be of dermal, muscular or neuronal nature; they can be solitary or grouped, and located in specific sites or dispersed throughout the animal. Their precise location can be lineage- or even species-specific (Martin, 2002; Peirson et al., 2009; Whitmore et al., 2000; Wolken and Mogus, 1979). Whereas in mammals non-visual photoreception is mediated entirely by ganglion cells in the retina, in non-mammalian vertebrates, specialized extra-ocular photoreceptors are located within the deep brain and at

different sites of the body (Peirson et al., 2009) (see **Figure 1**). In invertebrates, examples of extra-ocular photosensitive cells have been found in the parapodia and peristromium of marine ragworms, in dendritic arborisation neurons at the body wall of *Drosophila* larvae or in cnidocytes of the freshwater cnidarian polyp *Hydra magnipapillata* (Plachetzki et al., 2012; Wolken and Mogus, 1979; Xiang et al., 2010). These examples illustrate how animals can respond to light with more than just their eyes.

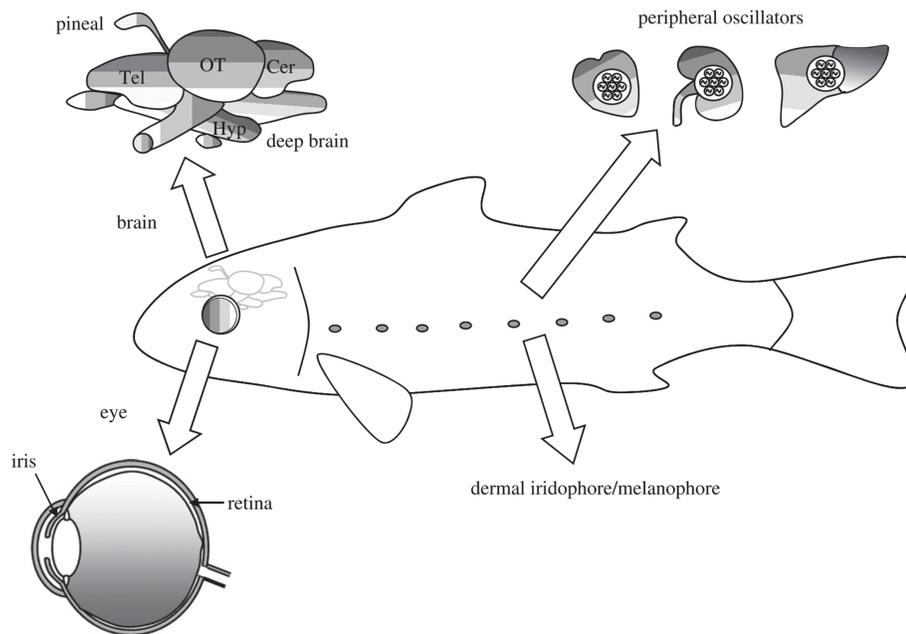


Figure 1. Different sites where non-visual photoreceptors can be found, exemplified in a fish model. Visual photoreceptors are found in the retina of animal eyes. Conversely, non-visual photoreceptors are located at different sites of the body. As an illustration, in fish, they have been identified in the iris of the lateral eye, in the pineal organ and in the deep brain, notably the hypothalamus. Dermal photoreception has also been described. Peripheral tissues such as heart, kidney or liver can entrain molecular clocks to environmental light. Taken from Peirson et al., 2009. Tel = telencephalon; OT = optic tectum; Cer = cerebellum; Hyp = hypothalamus.

Immediate behavioural non-visual photoresponses can be found across most animal lineages. The burrowing polychaete worm *Hediste diversicolor* withdraws its tail in response to light, and the photosensitive area mediating this response is located on the parapodia and peristromium. Phototropic responses, mediating orientation of the body towards the light source, were described in the ascidian *Ciona intestinalis*,

the sea anemone *Calamactis praelongus* and the alfalfa weevil *Hypera postica*. Photoavoidance and skin colour changes are also examples of direct extra-ocular responses to light (Wolken and Mogus, 1979; Xiang et al., 2010). The photopigments employed by non-visual photoreceptors mediating fast responses to light are generally rapidly responsive opsins (Nilsson, 2009). In contrast to the non-visual fast responses, entrainment of environmental light to endogenous biological clocks regulates more complex behaviours and physiological responses in a more indirect manner (**Box 1**).

Box 1 (modified from Tessmar-Raible et al., 2011)

Concepts and terminology

Environmental cycle: Periodically re-occurring natural conditions (e.g. day/night, high/low tides, moon phases, seasons).

Biological rhythm: Periodically re-occurring specific conditions within an organism (e.g. behavioural activity, metabolic state, gonadal maturity, etc.). Rhythms are typically in accordance with environmental cycles.

Zeitgeber: Environmental stimulus that serves as a synchronization cue.

Molecular clock: Molecular system that is able to maintain a given biological rhythm even under free-running conditions, i.e. in the absence of a zeitgeber. The molecularly best understood clock is the circadian clock. A unifying principle of eukaryotic circadian clocks is the existence of transcriptional/translational autoregulatory feedback loops.

Entrainment: Synchronization of a clock with the respective environmental cycle.

Periodicity: Cycles, rhythms and clocks are distinguished by their respective period length.

Most organisms sense periodic variations in environmental light conditions and synchronise them with endogenous molecular clocks. This aspect of photobiology is called photoentrainment. It allows organisms to anticipate and adapt to rhythmic environmental changes of different lengths (e.g. daily-circadian or lunar-circalunar) and to synchronise their physiology and behaviour in accordance (Roenneberg and Merrow, 2005; Tessmar-Raible et al., 2011; Zantke et al., 2013). In photobiology, a

“clock” is a molecular system that relies on negative transcriptional/translational feedback loops composed by a series of regulatory genes, the expression of which oscillates through time (i.e. molecular oscillators). A molecular clock is able intrinsically to maintain rhythmic environmental light changes even in the absence of exogenous stimuli (i.e. zeitgeber) (Roenneberg and Mellow, 2005; Tessmar-Raible et al., 2011). However, molecular clocks, in the absence of environmental light fluctuations, might not be enough to maintain the expression oscillation of regulatory genes. An example of the consequences of poor entrainment is human winter depression, taking place when the photoperiod is at its minimum, which can be efficiently treated by light (Roenneberg and Mellow, 2005). In several organisms, molecular clocks can be regulated by a class of flavoproteins called cryptochromes (Cry), which possess specialized functions in light perception and/or transcriptional regulation. In animals, two types of cryptochromes are found, type 1 Crys and type 2 Crys. Type 2 Crys are light-independent core clock components, and they interact with positive transcriptional regulators of the molecular clock, repressing their transcriptional activity. Type 1 Crys are photoreceptive, and are involved in the entrainment of environmental light signals with endogenous clocks (Oliveri et al., 2014).

Examples of opsins acting in parallel with light-entrained molecular clocks have been described in animals biology. For instance, diel vertical migrations, common in marine zooplankton, have been studied using the larvae of the ragworm *Platynereis dumerelii*. It was shown that eyespots, unable to form images but able to sense light direction through opsin photopigments, instantly changed the beating of adjacent cilia upon light stimulation, granting a phototactic swimming behaviour to the larvae (Gühhmann et al., 2015; Jékely et al., 2008). However, diel vertical migrations control is far more complex, since melatonin signalling pathways and light-entrained clocks also affect them (Gehring and Rosbash, 2003; Tosches et al., 2014). Concerning the vertebrate deep brain photoreceptor neural cells, neurohormone secretion has been hypothesized to be a direct reaction to light stimulation, affecting animals behaviour and physiology, although circadian clocks are probably coupled to these physiological responses as well, as it is described to occur in the photoperiod of birds (Fernandes et al., 2013; Halford et al., 2009; Tessmar-Raible et al., 2007). Evolution has driven a fine-tuning of biological responses with changing environmental light thanks to the appearance of sets of molecules (i.e. opsins and cryptochromes), which has granted the environmental-biological synchronization present in most animals lives.

A crucial biological process that is coordinated by non-visual photoreception of changing environmental light in a wide range of animal clades is light-dependent reproductive regulation (Halford et al., 2009; Juntti and Fernald, 2016; Lambert and Brandt, 1967; Leclère et al., 2016; Tessmar-Raible et al., 2011). In the next section I will discuss the intimate relationship between environmental light and sexual reproduction.

I.2. Light-dependent rhythmicity of sexual reproduction

An enormous variety of reproductive strategies can be found which are influenced to a certain level by ambient light (Lambert and Brandt, 1967; Leclère et al., 2016; Levy et al., 2007; Oliveri et al., 2014; Tessmar-Raible et al., 2011; Zantke et al., 2013). Many animals follow reproductive rhythms in accordance to environmental cycles of different lengths (e.g. circadian, tidal, circalunar and seasonal/circannual) to synchronise sexual reproduction between males and females when environmental conditions are favourable and ultimately increase species survival in a particular ecosystem (Juntti and Fernald, 2016; Tessmar-Raible et al., 2011; Zantke et al., 2013) (**Figure 2**). Although other factors such as temperature can be involved, changes in light intensity and spectrum bear much information about these distinct types of cycles, implying that environmental light is a key signal for reproductive regulation in many animals, even vertebrates (Halford et al., 2009; Juntti and Fernald, 2016; Lambert and Brandt, 1967; Leclère et al., 2016; Tessmar-Raible et al., 2011).

In a wide diversity of animal lineages, mostly aquatic, gamete release occurs as a result of environmental light cues such as light-dark/dark-light transitions, moonlight intensity or day length, among others (Lin et al., 2016; Martin, 2002; Tessmar-Raible et al., 2011). The fish medaka can lay eggs in a daily basis within the reproductive season, when a high light:dark ratio and warmer temperatures trigger reproductive behaviour by affecting its physiology (Juntti and Fernald, 2016). In starfish, day length increase can be an important factor triggering spawning (Lin et al., 2016). Hydrozoan jellyfish and seasonally gravid ascidians spawn in a circadian basis using light as a cue (Lambert and Brandt, 1967; Martin, 2002). Annelid worms exhibit circalunar spawning rhythms (Zantke et al., 2013). Coral mass spawning events occur in a circannual basis and are also regulated by the moon cycle and the time of the day (Brady et al., 2009; Levy et al., 2007). All these are illustrations of the importance of light in animals reproductive biology, and exemplify the wide diversification of light-dependent reproductive strategies (for more examples, see **Figure2**).

			
	biological rhythm	environmental cycle	cycle length
<i>solar influence</i>			
	circadian annual / seasonal	daily annual	24 hours 365 days
<i>lunar influence</i>			
	circalunar circasemilunar circatidal	lunar semilunar tidal	29.5 days 14.8 days 12.4 hours

<p><i>Fundulus heteroclitus</i> - Lunar and tidal spawning</p> 	<p><i>Platynereis dumerilii</i> - Lunar / semilunar spawning</p> 
<p>32 coral species of the Great Barrier Reef - Annual spawning influenced by the moon</p> 	<p><i>Mitrocoma cellularia</i> - Daily spawning</p> 

Figure 2. Common biological rhythms influenced by the sun or the moon and their tight connection to spawning in different animals. The top diagram shows different biological rhythms (left), along with their corresponding environmental cycles (middle) and their periodicity (right). Bottom pictures exemplify four species from different lineages whose spawning trigger follows a biological rhythm influenced by changing environmental light. Adapted from Tessmar-Raible et al., 2011.

Light is involved in regulating gamete release in animals as well as in unicellular and multicellular algae, which can spawn following semilunar cycles (Tessmar-Raible et al., 2011). This suggests that environmental light control of sexual reproduction arose early and/or multiple times during organismal evolution. It is conceivable to assume an early origin since rhythmic light changes were already present on Earth long before life started, and light controls fundamental biological processes of all living organisms nowadays.

The molecular intermediates involved in the light-mediated reproduction are poorly understood, with limited studies of how light-mediated reproductive rhythms are regulated at the molecular level. In the rabbitfish *Siganus guttatus* (a coral reef fish), moonlight, probably received by the pineal gland, influences circadian fluctuations of melatonin (Tessmar-Raible et al., 2011). In the coral *Acropora millipora*, expression of the cryptochrome gene *cry2* peaks at full moon (Levy et al., 2007). In the annelid *Platynereis dumerelii* core clock genes expression oscillates with circadian rhythmicity and is influenced by a circalunar clock (Zantke et al., 2013). These are some of the few examples where light-mediated reproductive control has been studied at the molecular level.

It is clear that, in order to synchronise precisely the timing of reproduction, most animals interconnect different environmental cycles to various molecular clocks, which are also affected by seasonal factors (Tessmar-Raible et al., 2011). This sophisticated scenario complicates the understanding of the molecular regulators involved in light-mediated reproductive control, and it is not unexpected to find different molecular intermediates playing a role in different animals.

Conclusion

I have briefly pointed out how non-visual photoreception is critical for most living organisms, and how fruitful sexual reproduction in animals can be widely regulated by environmental factors, notably by light. Environmental factors can be considered however as the “first layer” of regulation, acting upstream of a variety of hormonal signals that will help fine-tuning sexual reproduction, from the organismal to the cellular level. I will treat the hormonal aspects of reproductive regulation in more detail in the General Discussion.

Even though I have mainly considered light involvement in reproductive control at the level of gamete release, other levels of reproductive control exist, which may or

may not be regulated by environmental light, depending on the species. The wide variety of control points for successful sexual reproduction includes reaching sexual maturity, proper regulation of physiological steroid hormones, neurohormonal control based on nutrient availability, somatic gonadal tissue growth, germline development and gametogenesis (Herbison, 2016; Juntti and Fernald, 2016; Saberi et al., 2016; Shahjahan et al., 2014; Verlhac and Terret, 2016; von Stetina and Orr-Weaver, 2011). Fertilization, the last step of sexual reproduction, is also highly regulated. Successful fertilization requires sperm attraction and capacitation, the acrosomal reaction, gamete fusion, egg activation, and fusion of the contents of the male and female pronuclei, leading to the formation of a zygote and the onset of embryonic development (Georgadaki et al., 2016). Before fertilization can take place, the male gametes need to be activated, and the female gametes need to acquire the competency to be fertilized.

During female gametogenesis, the final step that an oocyte needs to go through in order to get fertilized is called oocyte meiotic maturation. In the next section I will introduce this fundamental biological process and highlight the diverse molecular scenarios that regulate oocyte maturation when comparing different species.

II. Oocyte meiotic maturation

The production of healthy oocytes depends on tight control of the successive steps of oogenesis, including fate specification, proliferation and migration of primordial germ cells, oocyte growth and meiotic maturation (von Stetina and Orr-Weaver, 2011). A considerable investment of energy is needed for animals to produce oocytes, making proper control of oogenesis essential for reproductive success and species survival. In a range of animals studied, belonging to different evolutionary lineages, oocytes are produced throughout adult life thanks to sustainable populations of germ cells in the gonads (Amiel et al., 2010; Verlhac and Terret, 2016; von Stetina and Orr-Weaver, 2011). In the case of eutherian mammals, only a finite number of germ cells is available, already produced and accumulated during embryogenesis (Verlhac and Terret, 2016).

During their growth in the female ovary, oocytes of all animals are arrested at the dictyate stage of Prophase I (PI) of the first meiotic division. Once they reach full size they are considered to be immature, meaning that they are still unable to be fertilised (Dupre et al., 2014; Verlhac and Terret, 2016; von Stetina and Orr-Weaver, 2011; Yamashita et al., 2000), although there are rare species where fertilization physiologically takes place at PI (Deguchi et al., 2015). The PI arrest occurs after crossing-over between parental chromosomes and it can last for only a few hours or days in some invertebrates, up to several months in some animals like rodents, or even decades in humans (Deguchi et al., 2011; Verlhac and Terret, 2016; von Stetina and Orr-Weaver, 2011). After an animal has reached sexual maturity, upon hormonal stimulation, fully grown immature post-vitellogenic oocytes complete the process of meiosis to produce cells with recombined genomes and reduced chromosome number in preparation for fertilization. In order to become haploid, oocytes generally undergo severely asymmetric divisions which lead to the formation of two tiny polar bodies and a large cell, the egg (Verlhac and Terret, 2016; von Stetina and Orr-Weaver, 2011).

Oocyte meiotic maturation is thus the biological process through which a fully grown, PI-arrested oocyte resumes meiosis, prepares to begin embryonic development and gains the ability to be fertilized. In 1988, Gary Freeman referred to it as “the transition that bridges oogenesis and embryogenesis”. Mature oocytes also contain stockpiled maternal factors that are necessary for successful fertilization and to sustain the developing embryo, until a stage of embryogenesis when development becomes entirely regulated by new transcription from the zygotic genome (von Stetina and Orr-Weaver, 2011).

During the course of maturation, oocytes experience changes associated with the progress of the meiotic cell cycle. Firstly, disassembly of the oocyte nuclear envelope, generally referred to as nuclear envelope breakdown (NEB) or “germinal vesicle” breakdown (GVBD) takes place at the prophase/metaphase transition and is considered a hallmark of maturation progression. GVBD is followed by chromosome condensation, cortical cytoskeleton reorganization, meiotic spindle assembly and formation of one or two polar bodies. Meiosis is halted again by a “cytostatic” arrest under Mos/MAPK (mitogen-activated protein kinase) control, which holds the cell cycle until arrival of the fertilizing sperm. The meiotic stage of this arrest is variable between animals, with some species having already completed meiosis before the second cytostatic arrest occurs, while others arrest at metaphase I (e.g. ascidians) or metaphase II (e.g. vertebrates) (Figure 3). Fertilization leads to the restart of the mitotic cycle in the newly formed embryo (Nagahama and Yamashita, 2008; Verlhac and Terret, 2016; von Stetina and Orr-Weaver, 2011; Yamashita et al., 2000). The mechanisms that induce gamete release, which can occur at different meiotic stages depending on the animal, are much less understood, although they are likely coupled to those inducing oocyte maturation (Deguchi et al., 2015).

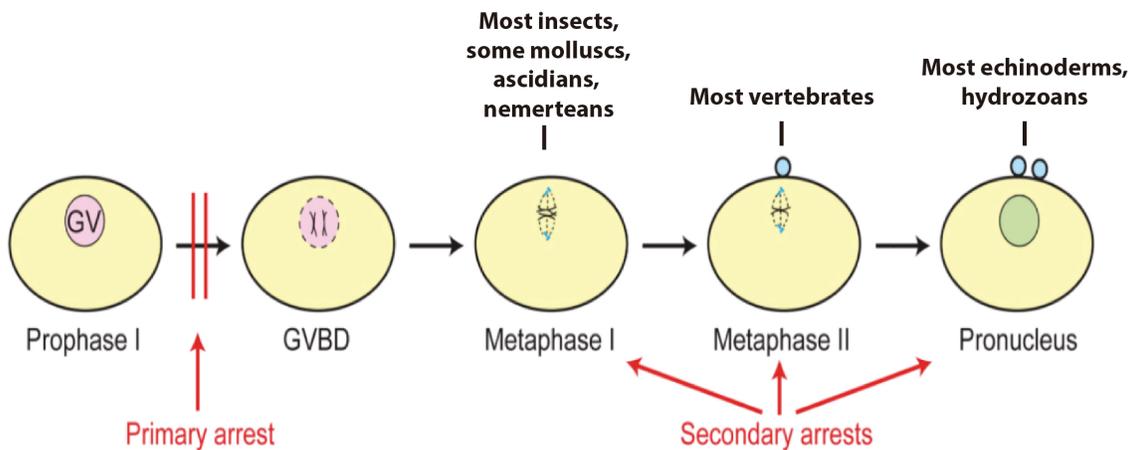


Figure 3. Primary and secondary meiotic cytostatic arrests in different animal lineages.

Fully grown ovarian oocytes from all species are arrested at Prophase I of meiosis and remain quiescent until the appropriate signal induces meiosis resumption. A second cytostatic arrest takes place at the end of oocyte maturation, coordinating meiosis completion with fertilization. The meiotic stage of the secondary cytostatic arrest is variable between animals. Blue circles represent polar bodies. Adapted from Kim et al., 2013. GV = germinal vesicle; GVBD = germinal vesicle breakdown.

Since oocyte meiotic maturation readies the female gamete for fertilization and early development, its tight regulation is of crucial importance for reproductive success and favourable embryonic development. Errors in any step can have serious consequences, including sterility, ovarian cancers and birth defects due to meiotic chromosome missegregation and resultant aneuploidies (Verlhac and Terret, 2016). Thus, research towards understanding this key physiological process should contribute significantly to the prevailing knowledge of meiosis and the hormonal regulation of reproduction, providing information with potential clinical pertinence in human fertility.

Many studies have been performed in mammalian oocytes (mainly in mice), amphibians, fish, nematodes and the fruit fly (Haccard and Jessus, 2006; Verlhac and Terret, 2016; von Stetina and Orr-Weaver, 2011). However, plenty of other “non-model” organisms, mainly marine invertebrates, have also contributed to a better understanding of this critical process (Amiel et al., 2009; Deguchi et al., 2011; Deguchi et al., 2015; Lambert, 2011; Stricker and Smythe, 2001; Takeda et al., 2006).

In the following sections I will focus on how oocyte maturation can be initiated and briefly review some of the crucial molecules and signalling pathways generally involved, since this has been the basis of my PhD work. I will do so while comparing animals from different lineages to try to offer an evolutionary perspective of this universal physiological process.

II.1. Oocyte maturation initiation

Oocyte maturation initiation, involving release of PI arrest, can be considered a physiological example of a regulated G2-M transition, one of the three major checkpoint transitions in the cell cycle (Haccard and Jessus, 2006; Hara et al., 2012; Kishimoto, 2015) (**Figure 4**). Technically the PI-arrested immature oocyte has already progressed beyond G2, since it has already entered meiosis and gone through chromosome recombination (Verlhac and Terret, 2016).

The central molecular actor in M phase entry in all cells is MPF (for M-Phase promoting factor). As “Maturation-Promoting Factor”, MPF was first identified in 1971 by Masui and Markert, who performed cytoplasm transfer assays from maturing eggs to recipient immature oocytes in frogs, demonstrating the existence of a cytoplasmic activity that upon its transfer induced oocyte maturation (**Figure 5**). Subsequent work showed that MPF can be equated with a molecular complex

composed of Cdk1 kinase (Cyclin-dependent kinase 1), also known as Cdc2 (cell division control protein 2 homolog) and its regulatory subunit, Cyclin B, and that it triggers M phase in eukaryotic cells by phosphorylating downstream targets. MPF is nowadays considered to be the universal inducer of cell division in eukaryotes, necessary and sufficient to trigger the G2-M transition in both somatic and germ cells (reviewed by Kishimoto, 2015).

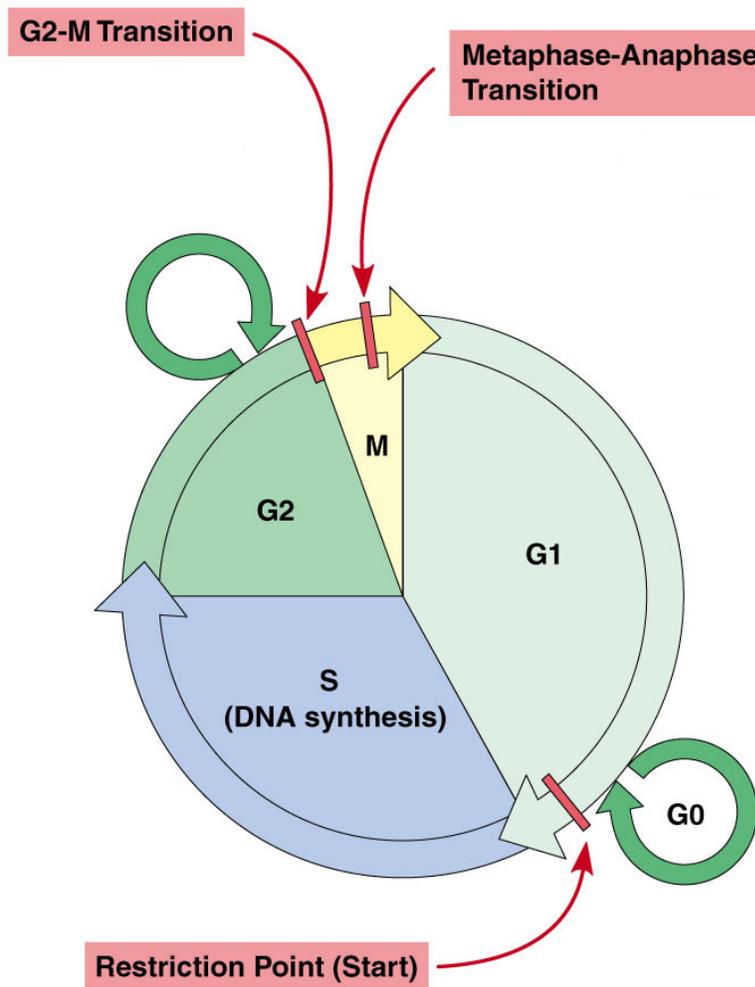


Figure 4. Eukaryotic cell cycle depicting the three major checkpoint transitions.
Modified from Pearson Education, Inc.

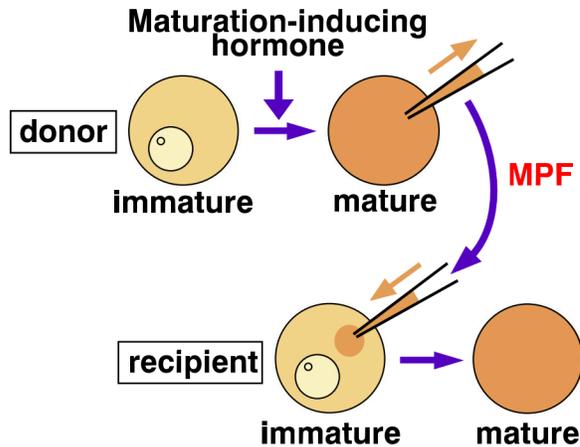


Figure 5. MPF identification by cytoplasm transfer assays. Firstly called maturation-promoting factor, MPF was originally described as a cytoplasmic activity transferable from maturing oocytes to immature (PI-arrested) oocytes, with the capacity to trigger their maturation. Taken from Kishimoto, 2015.

In frog, starfish and mammalian oocytes, MPF is produced from a pre-existing amount of inactive MPF, called Pre-MPF, already present in immature oocytes. MPF activation depends on Cdk1 dephosphorylation, which relies on the inhibition of Myt1/Wee1 kinase accompanied by the activation of the Cdc25 phosphatase, orchestrated by an “initial activator” signal inside the oocyte. Moreover, Pre-MPF can be transformed into active MPF through its own action by the so-called “MPF autoactivation loop”, in which active MPF further inactivates Myt1/Wee1 and activates Cdc25 (reviewed by Kishimoto, 2015 and Yamashita et al., 2000) (**Figure 6**).

More recent studies have revealed additional layers of complexity in the regulation of M phase entry, which is actually controlled by the coordinated activities of various protein kinases and counteracting phosphatases. In this context an important element regulating the MPF autoactivation loop is the antagonizing action of a protein phosphatase (PP2A-B55), which counteracts the phosphorylations catalysed by Cdk1/Cyclin B. This counteraction is mediated by Cdk1/Cyclin B-mediated activation of the kinase Greatwall (Gwl) (Hara et al., 2012), which in turn phosphorylates Ensa/ARPP19 (Ensa, α -endosulfine; and its close relative ARPP19, cyclic adenosine monophosphate-regulated phosphoprotein 19), activating it. Active ARPP19 leads to PP2A-B55 inhibition and reactivates the autoamplification loop. ARPP19 can also be directly phosphorylated by Cdk1/Cyclin B, and dephosphorylated by PP2A-B55. Thus, it has been suggested that Gwl along with Cdk1/Cyclin B might be considered a component of MPF, since they act as a unit, depending on one another to promote M phase entry (reviewed by Kishimoto, 2015) (**Figure 6**).

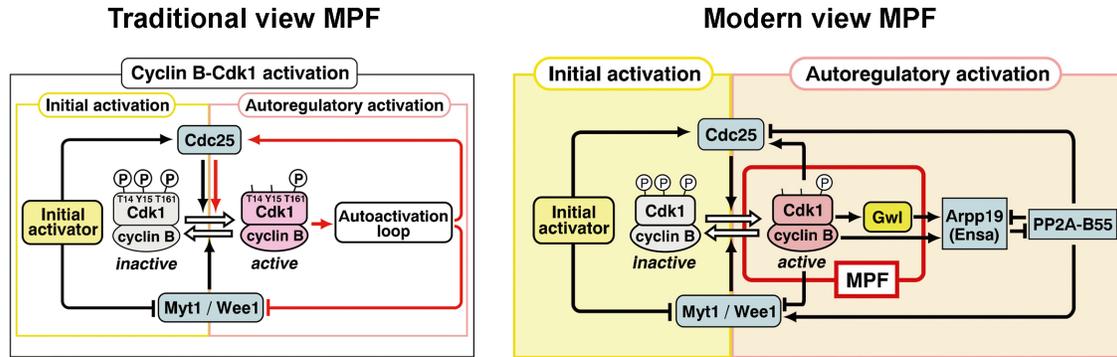


Figure 6. Traditional vs Modern view of MPF. Whereas the traditional view defined MPF as the molecular complex composed by Cdk1 kinase and Cyclin B, the modern view describes MPF as an intricate network involving Cdk1/Cyclin B and at least the kinase Greatwall (Gwl), working as a dependent entity. Modified from Kishimoto, 2015.

Other studies in the amphibian *Xenopus laevis* demonstrated the need for synthesis of Cyclin B and/or the kinase Mos in order to generate a “starter amount” of MPF to initiate the autoactivation loop of existing pre-MPF in oocytes. The involvement of Mos in initiating MPF activation is only essential if Cyclin B is inhibited, and this particular role for Mos is unique to *Xenopus* amongst different animal models studied (Haccard and Jessus, 2006). In *Xenopus* Mos/MAPK along with other kinases such as Plx1 is then also involved in the MPF autoactivation loop (Dupre et al., 2014; Haccard and Jessus, 2006). Fish immature oocytes differ from *Xenopus* oocytes in that they do not contain Pre-MPF. Cyclin B must be synthesized following reception of the maturation-inducing signal. Once the Cdk1/Cyclin B complex is assembled, it is subject to the required phosphorylations and dephosphorylations for its activation (Nagahama and Yamashita, 2008; Yamashita et al., 2000).

In parallel to MPF activation, another key kinase cascade universally activated at the onset of oocyte maturation is the Mos-MAPkinase cascade. Mos is an oocyte-specific kinase uniquely produced by translation of maternal mRNAs following maturation initiation. The Mos-MAPkinase pathway mediates oocyte-specific processes such as asymmetric spindle positioning and cytostatic arrest. Its involvement in meiosis resumption appears to be specific to *Xenopus* oocytes, as mentioned above (Dupre et al., 2014; Dupré et al., 2011; Haccard and Jessus, 2006; Verlhac et al., 2000).

II.2. Oocyte maturation initiation signals

Oocyte maturation starts when the appropriate signals are received by the quiescent oocyte, triggering downstream molecular cascades that ultimately activate MPF to resume meiosis as well as Mos mRNA translation. The environmental or hormonal signals that promote oocyte maturation act initially on somatic cells in the gonad, rather than on the oocyte itself (Deguchi et al., 2011; Haccard et al., 2012; von Stetina and Orr-Weaver, 2011; Yamashita et al., 2000). For instance, the initiation of oocyte maturation in vertebrates is controlled by a pre-ovulatory surge of luteinizing hormone (LH), received not by the oocytes but by follicle cells surrounding them. In the case of mammals, cyclic nucleotides are exchanged through gap junctions between the oocyte and follicle cells upon LH stimulation, leading to meiosis resumption (Jaffe and Norris, 2010). This shows a clear example of how cell-cell interactions between gonad somatic cells and the oocyte itself can be essential to trigger oocyte maturation.

In contrast to what happens in mammals, in other vertebrate lineages studied (e.g. fish and amphibians), as well as in many marine invertebrates, hormonal or environmental stimulation of somatic cells surrounding the oocytes induce the secretion of maturation-inducing hormones (MIHs), which act directly on oocyte receptors to activate MPF, initiating meiosis resumption.

II.3. Oocyte maturation-inducing hormones (MIHs) and MIH receptors (MIHRs)

MIHs are widely known as the molecules that are synthesized by somatic cells of the gonad, secreted upon hormonal or environmental stimulation, and able to act directly on the oocyte to trigger meiosis resumption (Nagahama and Yamashita, 2008; Yamashita et al., 2000). MIHs act as local signals on the oocyte surface, interacting with MIH receptors (MIHRs) to start the signalling cascades inside the oocyte that will lead to MPF activation and oocyte maturation, as illustrated during fish oocyte maturation initiation (**Figure 7**).

PI-arrested oocytes only acquire the ability to respond to MIHs once they have reached their fully grown stage (Yamashita et al., 2000). Whether this is due to the timing of MIHR synthesis, availability of MPF activating or other molecular components involvement is not very well known and might be a species-specific feature (Haccard and Jessus, 2006).

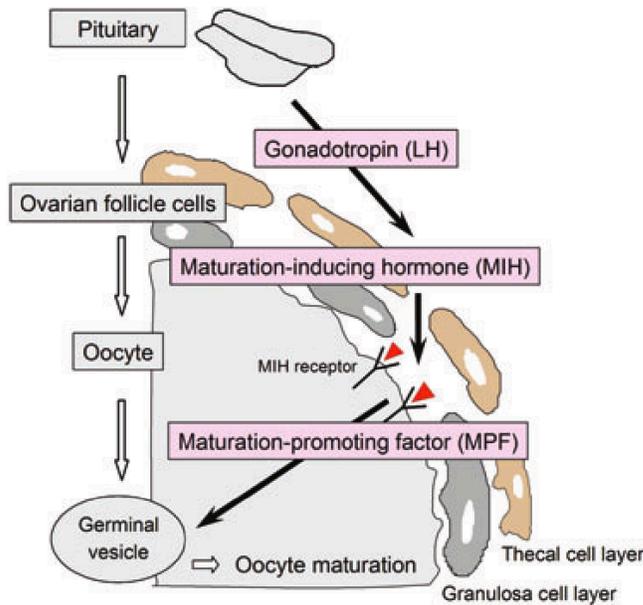


Figure 7. Simplified diagram illustrating the hormonal regulation of oocyte maturation in fish as an example of MIH secretion and reception.

Gonadotropin (luteinizing hormone, LH) released from the pituitary stimulates the production and secretion of maturation-inducing hormone (MIH) by the ovarian follicle cell layers. MIH interacts with its receptor at the oocyte surface, starting signal transduction pathways that lead to MPF activation to ultimately trigger oocyte maturation, visible as germinal vesicle breakdown. Taken from Nagahama and Yamashita, 2008.

To date very few examples of MIH have been identified at the molecular level, and their molecular nature is species specific (Haccard et al., 2012; Kanatani et al., 1969; Nagahama and Yamashita, 2008; von Stetina and Orr-Weaver, 2011) (**Figure 8**). MIH in starfish was found to be 1-methyladenine (1-MeAde) (Kanatani et al., 1969). In fish and amphibians MIHs are progesterone derivatives, enzymatically converted from cholesterol (Haccard et al., 2012; Nagahama and Yamashita, 2008). In the nematode *Caenorhabditis elegans*, where fertilising sperm initiates oocyte maturation, the major sperm protein (MSP) is believed to act as the ligand that regulates oocyte maturation (von Stetina and Orr-Weaver, 2011). Serotonin is a natural inducer of oocyte maturation in bivalve molluscs and nemertean and it affects second messenger levels in the oocytes of different animals, but it is likely not their endogenous MIH (Osada et al., 1998; Stricker and Smythe, 2001). The aforementioned example in mammals, where cyclic nucleotides pass through gap junctions between oocytes and follicle cells to induce oocyte maturation upon follicle cells LH reception does not follow the MIH principle. The MIH concept is thus not perfectly applicable to mammalian oocytes, since cyclic nucleotide transport through gap junctions may entirely regulate oocyte maturation (Shuhaibar et al., 2015).

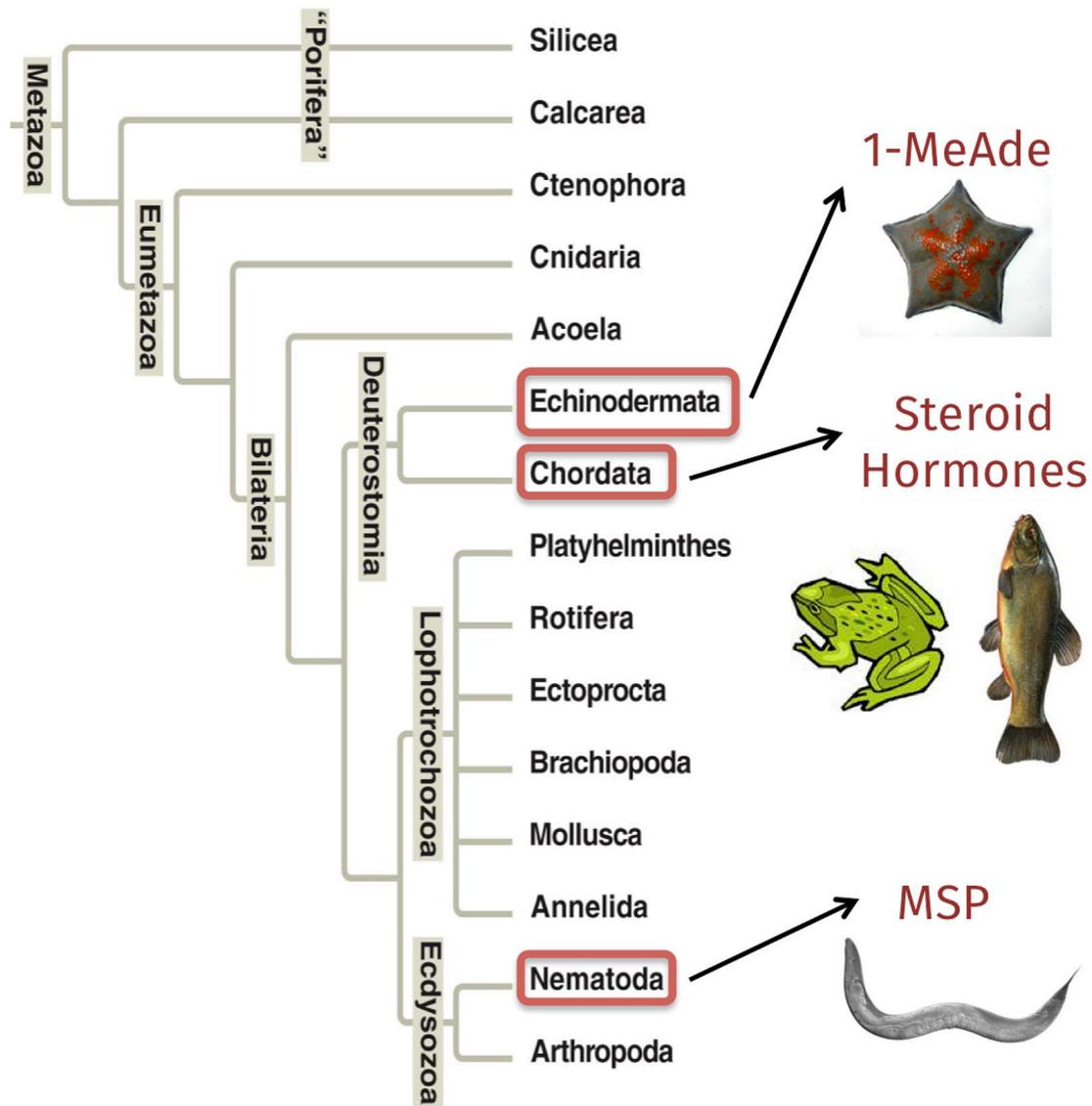


Figure 8. Phylogenetic relationships of the main animal phyla showing the few examples of MIHs found to date in the animal kingdom. Modified from Pearson Education, Inc. 1-MeAde = 1-methyladenine; MSP = major sperm protein.

The shortage of knowledge on MIHRs is even greater than for MIHs, since no MIHR has unambiguously been characterized. Membrane progesterin receptors (mPRs), belonging to the superfamily of G protein-coupled receptors (GPCRs) clearly act as intermediaries of oocyte maturation in fish and in *Xenopus* (Ben-Yehoshua et al., 2007; Zhu et al., 2003). However, the involvement of both nuclear and membrane receptors and the oocyte sensitivity to various steroid hormones raises some doubts about their universality (Haccard et al., 2012; Nagahama and Yamashita, 2008). In

Xenopus, the steroids progesterone and testosterone as well as the peptide hormone insulin are able to induce oocyte meiosis resumption (Haccard and Jessus, 2006; Haccard et al., 2012; Yamashita et al., 2000). Nuclear progesterone and testosterone receptors along with mPRs and insulin-like growth factor-1 receptors are potentially implicated in the reception of these hormones, ultimately leading to MPF activation (Haccard and Jessus, 2006; Haccard et al., 2012). In mice, a constitutively active GPCR called GPR3 and its linked $G_{\alpha s}$ G-protein are needed to maintain the PI arrest. However, no endogenous ligand for this receptor has been found, and indeed it may not have one (Mehlmann et al., 2004; Ye et al., 2014). In starfish, even though 1-MeAde receptor is predicted to be a GPCR acting through $G\beta\gamma$ and PI3 kinase, its identity has not yet been determined (Kishimoto, 2015). In any case, despite the lack of knowledge on MIHRs, it seems clear that GPCRs and G proteins are frequent candidates in the process of oocyte maturation initiation.

II.4. Signalling pathways leading to meiosis resumption: commonalities and variations between species

MPF activation is an essential feature of oocyte meiosis resumption in all animals, and the molecular components involved in its activation following MIH reception, as well as those involved in meiosis progression are largely the same. As outlined earlier, the assembly of these components into a regulatory system varies slightly between different animal lineages (Amiel et al., 2009; Deguchi et al., 2011; Deguchi et al., 2015; Haccard and Jessus, 2006; Jaffe and Norris, 2010; Nagahama and Yamashita, 2008; Stricker and Smythe, 2001; von Stetina and Orr-Weaver, 2011; Yamashita et al., 2000) (**Figure 9**). Without being exhaustive, I review below a few molecular examples of these disparate biological responses in the oocytes of various species to highlight the importance of studying a wide range of species for better understanding of the mechanisms regulating oocyte maturation and how they have evolved in the animal kingdom.

II.4.1. Cyclic AMP (cAMP) and cAMP-dependent protein kinase (PKA)

cAMP is a cyclic nucleotide that acts as a second messenger to trigger many cellular reactions by orchestrating intracellular signalling events (Cheng et al., 2008). cAMP is a key player during oocyte maturation initiation in many species, but its involvement can be widely different (**Figure 9**). The level of cAMP in the cytoplasm is maintained by balance between the activities of adenylyl cyclases (ACs) that

produce cAMP inside the cell and phosphodiesterases (PDEs), which degrade it (Omori and Kotera, 2007). When cAMP levels rise, one of the major consequences is an allosteric activation of cAMP-dependent kinase (PKA) (Cheng et al., 2008). The cAMP/PKA pathway affects many cellular events via the phosphorylation of multiple substrates. It is involved in cell cycle control and is an essential regulator of oocyte maturation (Deguchi et al., 2011; Dupre et al., 2014; Jaffe and Norris, 2010; Nagahama and Yamashita, 2008; Takeda et al., 2006; von Stetina and Orr-Weaver, 2011; Yamashita et al., 2000). cAMP also regulates cell homeostasis through other pathways, for instance interacting with a protein called Epac (exchange protein directly activated by cAMP), which regulates additional functions (Cheng et al., 2008).

PKA consists of two catalytic and two regulatory subunits, and its activity is modulated by binding of cAMP to the regulatory subunits. When cAMP levels in the cytoplasm are high, the PKA catalytic subunits are released and the enzyme becomes active. Active PKA can then affect a variety of cellular responses by phosphorylating a multitude of nuclear and cytoplasmic protein substrates, such as enzymes or transcriptional factors (Cheng et al., 2008). Inside the oocytes of many species, this kinase is crucial in regulating oocyte maturation, via phosphorylation of different substrates involved in MPF activation, mostly still unknown. (Deguchi et al., 2011; Dupre et al., 2014; Jaffe and Norris, 2010; Nagahama and Yamashita, 2008; Takeda et al., 2006; von Stetina and Orr-Weaver, 2011; Yamashita et al., 2000).

As mentioned above, plasma membrane GPCRs have been implicated in initiating oocyte maturation in mice, in fish and in *Xenopus*, and they do so by regulating cAMP levels inside the oocyte (Haccard and Jessus, 2006; Mehlmann et al., 2004; Nagahama and Yamashita, 2008). Certain classes of GPCRs are capable of tuning AC activity through the activation of the heterotrimeric G proteins to which they are coupled. G α s subunits are AC stimulators that will increase cAMP synthesis inside the cell, while G α i subunits act as AC inhibitors (Cheng et al., 2008; Nikolaev and Lohse, 2006; Shuhaibar et al., 2015). PDEs play an important role in parallel, fine-tuning the oocyte levels of cAMP through its degradation (Shuhaibar et al., 2015).

In mouse oocytes, which are surrounded by cumulus and mural granulosa cells in the ovarian follicle, high levels of cAMP sustain the P1 meiotic arrest. Constitutive synthesis of cAMP promoted by GPR3 and downstream ACs is dependent on the downregulation of PDE3A by high cGMP levels arriving via coupling to the follicle cells. This leads to permanent PKA activation, which in turn inhibits the activity of the phosphatase cdc25B and stimulates that of Wee1B and Myt1 kinases to maintain

MPF in an inactive form and hence blocks meiosis resumption (Conti et al., 2012; Jaffe and Norris, 2010; Mehlmann et al., 2004; Shuhaibar et al., 2015). cAMP degradation through PDE3A, which is activated by low levels of cGMP, is the trigger to initiate oocyte maturation (Jaffe and Norris, 2010; von Stetina and Orr-Weaver, 2011). Rather than acting through MIH, the mouse oocyte thus resumes meiosis via cGMP diffusion through gap junctions between the oocyte and mural granulosa cells upon LH reception. In this manner, cGMP in the oocyte decreases, PDE3A activity increases, cAMP decreases, and meiosis is resumed (Jaffe and Norris, 2010; Shuhaibar et al., 2015).

In resting immature fish and amphibian ovarian oocytes, cAMP is also maintained at high levels. PKA is active, phosphorylating substrates that maintain MPF in an inactive state and thus oocytes arrested at P1. As in mammals, PKA needs to be downregulated to trigger oocyte maturation, although in these animals it occurs upon MIH reception, followed by a drop in cAMP levels (Dupre et al., 2014; Haccard and Jessus, 2006; Nagahama and Yamashita, 2008). Consistent with this, inhibition of PKA activity is sufficient to induce oocyte maturation in several fish species studied (Nagahama and Yamashita, 2008). In *Xenopus* oocytes, PKA activity negatively regulates Mos and Cyclin B synthesis (Haccard and Jessus, 2006), and also has a role in M-phase progression by tuning MPF activity through phosphorylation of substrates such as Myt1, Cdc25 and ARPP19 (Dupré et al., 2014).

Together, the evidence from mammals, amphibians and fish models indicates that vertebrate oocytes depend on a decrease in cAMP levels and consequent PKA inactivation to initiate the kinase cascades that will lead to GVBD (Dupre et al., 2014).

In contrast to the situation in vertebrates, in many marine invertebrates studied so far (e.g. hydrozoans, nemertean worms and some species of bivalves, ascidians, ophiuroids and annelids), oocyte cytoplasmic cAMP elevations induce, rather than block, meiotic maturation (reviewed by Deguchi et al., 2011). In hydrozoans, exogenous applications or microinjection of cAMP analogues have a stimulatory effect on oocyte meiosis resumption, while cGMP does not seem to influence this process (Freeman and Ridgway, 1988; Takeda et al., 2006). There is also evidence that PKA mediates the early phase of meiotic maturation in hydrozoan oocytes, as is probably also the case in nemertean worms (Stricker and Smythe, 2001; Takeda et al., 2006). In contrast, cAMP elevations in ascidian oocytes from the species *Boltenia villosa* trigger GVBD through Epac activation and consequent intracellular calcium increase, rather than through PKA (Lambert, 2011). Similarly, some marine invertebrates

(mainly starfish species) have been shown to undergo GVBD in response to nuclear calcium transients (Deguchi et al., 2015), suggesting that a PKA-independent pathway might also be acting in these cases. cAMP-activated oocyte maturation is not a general rule in invertebrates. Elevated levels of cAMP inhibit GVBD in oocytes of some starfish species, as well as in two ascidian species and the annelid *Chaetopterus* (Deguchi et al., 2011; Yamashita et al., 2000). Given this variability across the animal kingdom, it is hard to decide based on current evidence whether cAMP ancestrally played a positive or inhibitory role in oocyte maturation. During evolution certain invertebrate species may have acquired the ability to resume meiosis in response to cAMP elevations inside the oocytes, or alternatively have retained this characteristic from a distant animal ancestor.

At a mechanistic level it is intriguing to compare how cAMP can elicit such divergent responses, leading to MPF activation in some animals while maintaining the PI arrest and preventing MPF activation in others. This contrasting activity of cAMP in triggering GVBD among different lineages was called the “cAMP paradox” (Deguchi et al., 2011). Follicle cells are positioned in layers surrounding the oocytes, and are intimately connected to them through gap junctions, being crucial for oocyte homeostasis and hormonal control of oocyte maturation in vertebrates and some invertebrates (Jaffe and Norris, 2010; Nagahama and Yamashita, 2008; Yamashita et al., 2000). However, in some lineages they are absent and the oocytes are only surrounded by a thin epithelial layer (Amiel et al., 2010; Stricker and Smythe, 2001). It has been suggested that the cAMP/PKA pathway inducing GVBD might be characteristic of oocytes lacking follicle cells and that, in some lineages, this pathway became segregated into the follicle cells throughout evolution, regulating substrates inside them and controlling oocyte responses through gap junctions (Deguchi et al., 2011).

Since stimulation, rather than inhibition, of meiotic maturation by intra-oocyte cAMP elevations is clearly widespread amongst animal lineages, studying non-model organisms with this characteristic could shed more light on the mechanisms initiating oocyte maturation and its evolution. In this context, hydrozoans such as our model *Clytia hemisphaerica* are well placed for an informative role (see section III of Introduction).

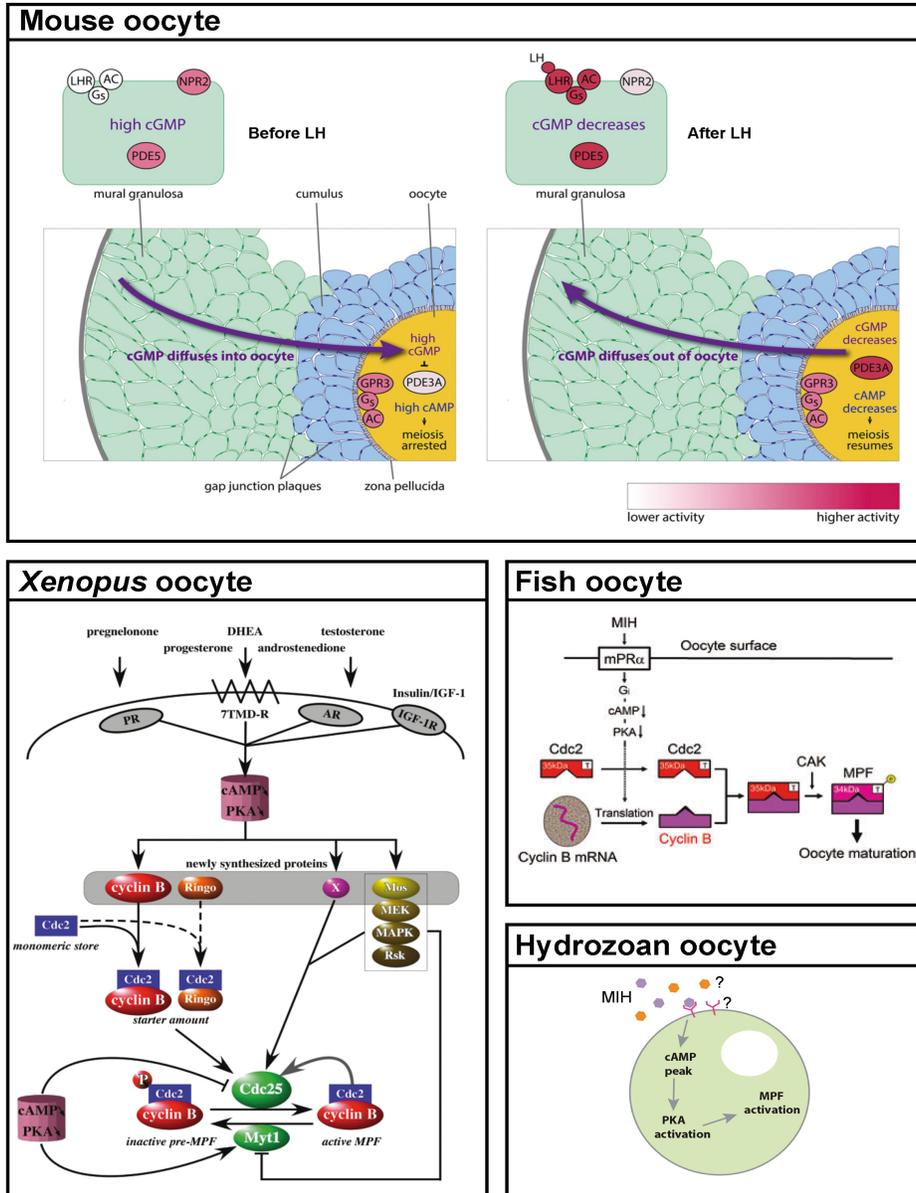


Figure 9. Signalling pathways leading to MPF activation and oocyte meiosis resumption in four well-studied models. Vertebrate oocytes require a decrease in cyclic AMP (cAMP) levels and inactivation of cAMP-dependent kinase (PKA) to trigger M phase entry, but most invertebrate oocytes studied so far (including hydrozoans) are induced to mature upon cAMP increase and PKA activation. See text for details.

Mouse oocyte scheme modified from Shuhaibar et al., 2015. LH = luteinizing hormone; LHR = luteinizing hormone receptor; AC = adenylyl cyclase; NPR2 = natriuretic peptide receptor 2; PDE5 = phosphodiesterase 5. PDE3A = phosphodiesterase 3A; cGMP = cyclic GMP. *Xenopus* oocyte scheme modified from Haccard and Jessus, 2006. DHEA = dehydroepiandrosterone; 7TMD-R = 7-transmembrane domain receptor; PR = classical progesterone receptor; AR = classical androgen receptor; IGF-1R = insulin-like growth factor-1 receptor; X = unknown synthesized protein. Fish oocyte scheme taken from Nagahama and Yamashita, 2008. MIH = maturation-inducing hormone; mPR α = membrane progestin receptor alpha. Gi = inhibitory alpha subunit of an heterotrimeric G protein; CAK = CDK-activating kinase.

II.4.2. Mos/MAPK (Mitogen-Activated Protein Kinase) pathway

Mos is a serine/threonine protein kinase essential for proper oocyte maturation that appeared early in animal evolution and whose expression is largely restricted to oocytes. The main function of Mos in the oocytes is the activation of the MAPK pathway via phosphorylation of MEK, contributing to vital meiotic processes such as asymmetric spindle positioning, polar body extrusion and second meiotic cytostatic arrest (Amiel et al., 2009; Dupré et al., 2011; Verlhac et al., 2000). Regardless of the species-specific stage at which the second meiotic arrest of unfertilized eggs occurs (**Figure 3**), this arrest has been found using several model species to be under Mos-MAPkinase regulation (Dupré et al., 2011).

In contrast to other models studied, translation of Mos protein in *Xenopus* contributes also to MPF activation, although its role is redundant with Cyclin B synthesis (Haccard and Jessus, 2006). In other amphibian species studied and in fish, Mos/MAPK is not involved in MPF activation, although may be responsible for MPF stabilisation (Yamashita et al., 2000). In mice and in marine invertebrates, Mos synthesis is not required upstream of GVBD implying that its translation is necessary for meiosis progression and/or oocyte-specific processes such as polar body formation, but not resumption (Amiel et al., 2009; Haccard and Jessus, 2006; Haccard et al., 2012; Stricker and Smythe, 2001; von Stetina and Orr-Weaver, 2011; Yamashita et al., 2000).

II.4.3. Protein synthesis during oocyte maturation initiation

Another aspect of oocyte maturation initiation that changes between animal lineages relates to whether synthesis of new protein is required. Some species are dependent on protein synthesis to initiate oocyte maturation and others are not (Yamashita et al., 2000). In general, gene transcription is silent in fully grown oocytes and only resumes after fertilization (Dupré et al., 2011). This implies that any protein synthesis occurring during maturation relies entirely on translation of maternal mRNA.

Enhanced Cyclin B synthesis upon MIH reception seems to be a common feature in oocytes between species, and in some cases like fish and some amphibians, it is essential for MPF activation (Yamashita et al., 2000). Downregulation of PKA leads to translation of both Mos and Cyclin B mRNAs, Mos activates MAPK pathway and assists to Cdc25 activation and Myt1 inhibition, supporting MPF activation.

Neosynthesized Cyclin B acts as a “starter” amount of Cdk1-Cyclin B in *Xenopus* oocytes to initiate the MPF amplification loop (Dupré et al., 2014; Haccard and Jessus, 2006). Ringo/Speedy, cell cycle regulators capable of binding and activating Cdk1, and other unknown proteins might also be synthesized and contribute to MPF amplification (Haccard and Jessus, 2006).

In some species of mammals such as pigs, oocyte protein synthesis is mandatory for GVBD, whereas in rodents it is not (Jaffe and Norris, 2010). In some marine invertebrates GVBD can take place under protein inhibition conditions (Deguchi et al., 2011; Stricker and Smythe, 2001; Yamashita et al., 2000), although in most of them it has not been determined.

Conclusion

To provide a comparative perspective on the complexity of oocyte meiosis resumption regulation, I have tried to present examples of our knowledge of the mechanisms covering various lineages of the animal tree of life, focussing on model and non-model organisms, vertebrates and invertebrates. Although a very similar set of molecules is employed, some of them are paradoxically utilized to achieve opposite outcomes. Notably, the divergent regulation of meiosis resumption by cAMP/PKA calls for further research of both upstream regulators and the target molecules of PKA in non-model organisms. In this context, future identification of MIHs and their receptors using a wider range of species may help to clarify the downstream pathways bridging MIH reception to MPF and MAPK activation.

III. The hydrozoan *Clytia hemisphaerica* and light-induced oocyte maturation

In this last part of the introduction I will introduce the experimental animal model I have worked with during my PhD, *Clytia hemisphaerica*. After briefly introducing its phylogenetic position and highlighting its advantages for scientific research, I will describe how hydrozoan sexual reproduction is regulated by environmental light changes and how isolated *Clytia* gonads can autonomously spawn mature eggs upon light stimulation. Finally, I will point out some of the findings in other hydrozoan species that led to the initial model for light-induced oocyte maturation in *Clytia*, providing the foundation of my thesis work.

III.1. Phylogenetic position and background

Clytia hemisphaerica is a hydrozoan species belonging to the phylum Cnidaria, a group of marine invertebrates comprising around 11,000 described species (Zapata et al., 2015). Cnidarians split from the bilaterian clade around 700 million years ago. They occupy a phylogenetic position as the sister group of Bilateria, close to the root from which all multicellular animals diverged (Chang et al., 2015; Dunn et al., 2014; Zapata et al., 2015) (**Figure 10**). This implies that if a range of existing bilaterian and cnidarian species share particular features, it is conceivable that these represent an ancestral state that has been conserved in both clades throughout animal evolution.

Cnidaria is composed of two main groups, Anthozoa (corals and sea anemones), containing species that are mostly sessile with either individual or colonial polyp forms, and Medusozoa (hydroids and jellyfish), which includes plenty of species exhibiting a polyp form as well as a jellyfish form in their life cycles (Brusca and Brusca, 2005). Medusozoa comprises four groups: Staurozoa, Scyphozoa, Cubozoa and Hydrozoa. More recently, phylogenetic analyses have placed a group of parasites inside the Cnidaria clade, the Myxozoa. They have been proposed to be the sister group of another clade of cnidarian parasitic species, the Polypodiozoa (Chang et al., 2015; Leclère and Röttinger, 2017; Zapata et al., 2015) (**Figure 10**).

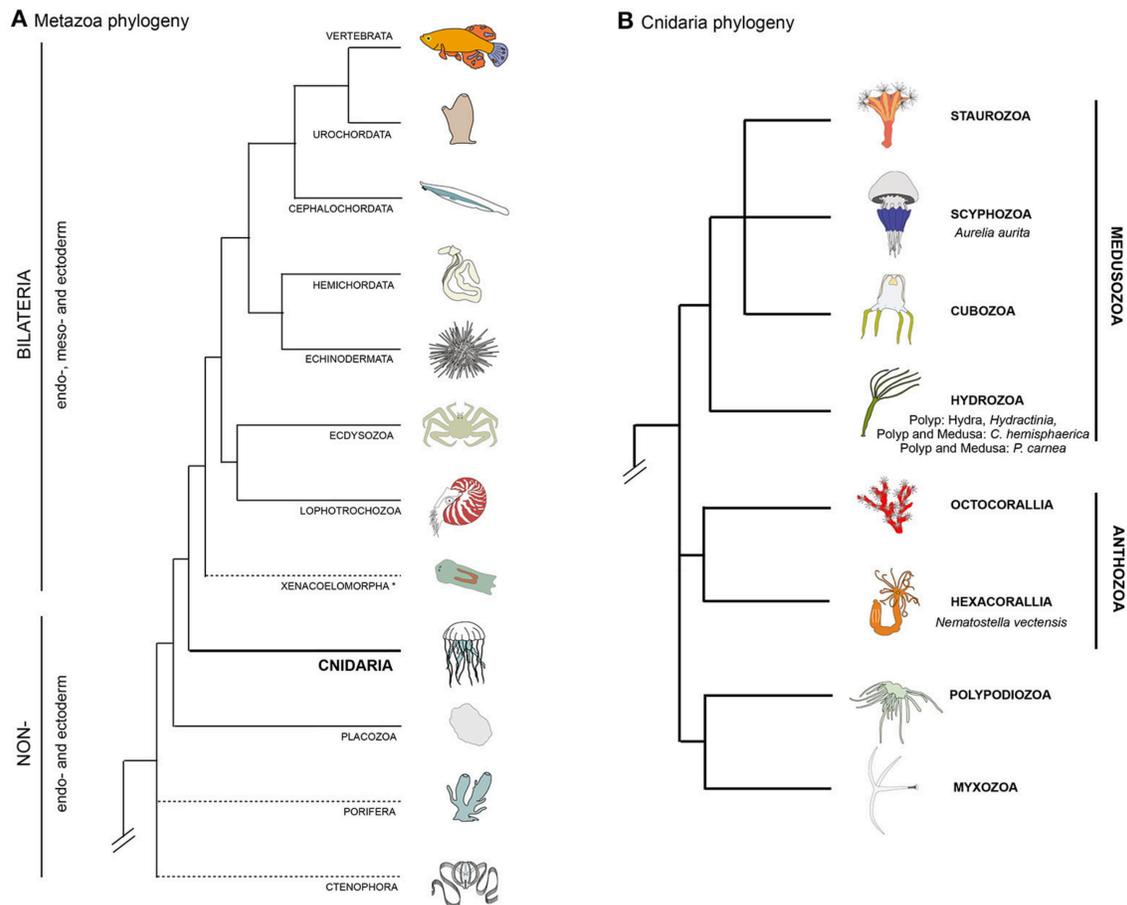


Figure 10. Phylogenetic position of Cnidaria and its different sub-groups. (A) Metazoan phylogeny, highlighting the position of Cnidaria as the sister group of Bilateria. The branches of Porifera (sponges) and Ctenophora (comb jellies) outside the bilaterians are indicated by dashed lines to point out that their exact position inside the metazoans is still debated. (B) Cnidarian phylogeny showing the relationships between the main sub-groups based on recently published data (Chang et al., 2015; Zapata et al., 2015). Taken from Leclère and Röttinger, 2017.

Cnidarians are diploblastic animals. Their body is composed only of two layers, endoderm and ectoderm, usually separated by an extracellular mesoglea. They present a single body axis, termed oral-aboral, rather than the two or three body axes found in bilaterians (dorso-ventral, antero-posterior and right-left) (Brusca and Brusca, 2005; Martindale et al., 2004). The main synapomorphy of Cnidaria (i.e. biological features only present in this clade) is the presence of a specialized cell type used for hunting and defence, the cnidocyte or nematocyte (Brusca and Brusca, 2005; Nüchter et al., 2006). Cnidarians have relatively few cell types compared to most bilaterian clades, possibly reflecting the circumstances of the common

cnidarian-bilaterian ancestor. Despite the low number of cell types, the medusa form can show a high level of complexity, including striated and smooth muscles, photosensitive reproductive organs, statocysts (balance organs), and even eyes (Brusca and Brusca, 2005; Houliston et al., 2010; Koyanagi et al., 2008; Kozmik et al., 2008; Leclère and Röttinger, 2017; Suga et al., 2008).

The cnidarian nervous system mainly consists of simple neural nets located throughout the animal body rather than having a centralized organisation (Watanabe et al., 2009). It has been argued that neural nets represent the evolutionary oldest nervous system organization, where neurons interact wherever they cross one another, and suffice to integrate environmental information with behavioural and physiological responses (Arendt et al., 2015; Hejnol and Rentzsch, 2015; Satterlie, 2011). In addition to the neural nets, the medusa form also presents complex nerve rings with high concentrations of neurons running around the jellyfish periphery. The nerve rings connect sensory organs within them and with the adjacent neural nets, and contain multiple conducting systems for swimming coordination (Satterlie, 2011).

The repertoire of developmental regulatory gene families identified in cnidarian genomes is very similar to that found in bilaterians (Chevalier et al., 2006; Houliston et al., 2010; Technau et al., 2005), although the involvement of cnidarian-specific genes in cnidarian embryonic development as well as differential deployment of conserved regulators, may contribute to the diversity of cnidarian morphologies (Lapébie et al., 2014).

The most widely used cnidarian models for scientific research are the freshwater polyp *Hydra sp.*, a hydrozoan which has lost the jellyfish form during evolution (Bosch, 2014; Galliot, 2012), and the anthozoan *Nematostella vectensis* (Layden et al., 2016). In addition, other cnidarian species have been emerging as useful experimental models during the last decades to assess different biological questions, for instance in the fields of regeneration, stem cells, alloimmunity, gametogenesis, developmental biology, evolution, neurogenesis, and photoreception (reviewed by Galliot and Schmid, 2002; Leclère et al., 2016; Rentzsch and Technau, 2016). *Clytia hemisphaerica* belongs to the largest and most diverse of all cnidarian groups, the Hydrozoa, composed of over 3,500 species. Hydrozoans have proved very useful for the scientific community thanks to their ease of culture, transparency, and convenience for experimental manipulation (reviewed by Leclère et al., 2016).

III.2. *Clytia hemisphaerica* as a laboratory model

Formerly known as *Phialidium hemisphaericum*, the hydrozoan *Clytia hemisphaerica* started to be used as a laboratory-cultured experimental model over 10 years ago (Amiel et al., 2010; Cook et al., 2016; Houliston et al., 2010) (**Figure 11**). *Clytia* species had already been a popular choice for studying gametogenesis, fertilization and developmental mechanisms (Carré and Carré; 2000; Freeman, 1987; Freeman and Ridgway 1988; Roosen-runge, 1962; Roosen-Runge and Szollosi, 1965), as well as regeneration, physiology, ecology, and characterization of Aequorin and GFP family proteins (reviewed by Leclère et al., 2016; Houliston et al., 2010).

III.2.1. Life cycle and culture

One key advantage of *Clytia hemisphaerica* as an experimental model is that its full life cycle can be conveniently reproduced under laboratory conditions, using artificial sea water (Amiel et al., 2010; Houliston et al., 2010). *Clytia* has the typical hydrozoan life cycle, comprising three life stages: planula larva, polyp colony and medusa form (**Figure 11**). Polyp and medusa stages can be routinely fed with artemia nauplii and maintained in kreisel tanks with circulating water. *Clytia* jellyfish, the pelagic, sexually reproducing adult form, have separate sexes. Males and females, once sexually mature, measure around 1cm diameter and are triggered to spawn upon light stimulation after a period of darkness (Amiel et al., 2010). Male and female gametes released in the water column undergo external fertilization and embryonic development commences (**Figure 12**). Swimming planula larvae are formed one day after fertilization, and around 2 or 3 days later, they start swimming downwards, looking for an appropriate substrate for attachment and metamorphosis into primary polyps. This step can be induced experimentally by CsCl, or by neuropeptides of the GLWamide family (Piraino et al., 2011; Takahashi and Hatta, 2011) to control planula metamorphosis onto a glass or plastic substrate. Once they start feeding, the sessile primary polyps propagate vegetatively through stolon branching and extension, forming connected colonies. The colonies are formed by two types of polyps, the gastrozooids or feeding polyps, and the gonozooids or reproductive polyps, from which baby *Clytia* medusae bud. Polyp colonies are virtually immortal, with old parts degenerating as the colony spreads. The sex of the medusae produced by some colonies is partially influenced by temperature while the colony is still young, with lower temperatures (15-18°C) favouring production of males and higher temperatures (21-24°C) females (Carré

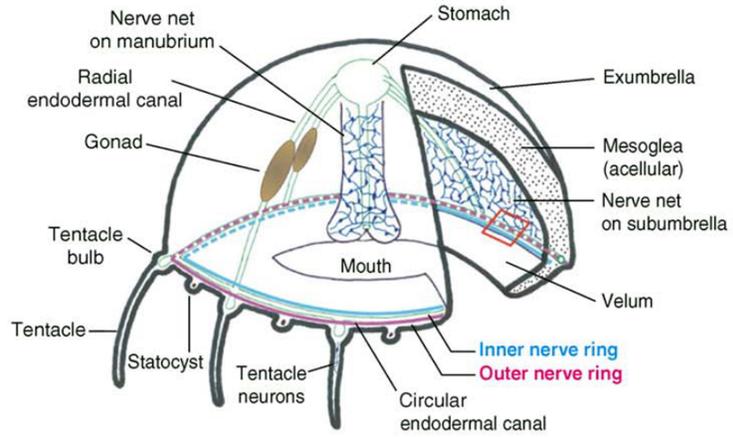
and Carré, 2000). Since a polyp colony reproduces asexually, all the medusae that bud from a particular colony can be considered genetically identical clones, a very convenient aspect for laboratory experimentation. Medusae reach sexual maturity 2 or 3 weeks after budding from the gonozooids, depending on the feeding regime and available space in the tank, and can live up to two months if conditions are favourable (reviewed by Amiel et al., 2010; Houliston et al., 2010).

III.2.2. Anatomy of the *Clytia* medusa

Clytia jellyfish do not have eyes like some other hydrozoan jellyfish do (e.g. *Cladonema radiatum* - Suga et al., 2008), but nevertheless exhibit a rather sophisticated anatomy (**Figure 11**). The aboral layer of the medusa bell is called exumbrella, and the oral surface subumbrella. They are separated by an acellular mesoglea that has a jelly consistency. A synapomorphy of the Leptothecata subclade, to which *Clytia* belongs, is the presence of a velum, an iris diaphragm located below the bell that creates a powerful water jet as the jellyfish swims (Brusca and Brusca, 2005). A mouth/manubrium, which also serves as an anus, is located in the middle of the jellyfish and connected to a stomach at its most distal part. The stomach is in turn linked to four endodermal radial canals, and the digested food is distributed through the animal along the radial canals and the connected circular canal running around the umbrella rim. One gonad lies on each of the four radial canals. Tentacle bulbs, from which tentacles arise, are positioned at regular intervals around the jellyfish border. Balance organs, the statocysts, are found on the bell rim, between tentacle bulbs. Diffuse epithelial nerve nets are present on the manubrium and subumbrella face, and two condensed nerve rings run in parallel around the periphery of the umbrella. In general terms, the outer nerve ring integrates sensory inputs and the inner nerve ring coordinates motor responses (reviewed by Houliston et al., 2010).



Clytia jellyfish anatomy



Clytia life cycle

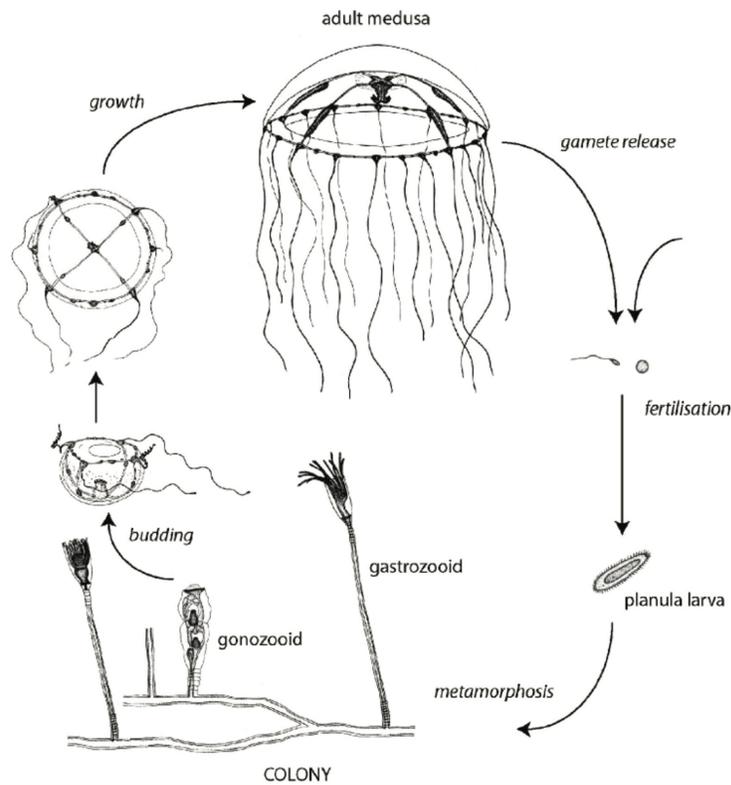


Figure 11. The hydrozoan model *Clytia hemisphaerica*, its life cycle and the anatomy of the jellyfish stage. See text for further details. Jellyfish picture and diagram of its anatomy taken from Houliston et al., 2010. *Clytia* life cycle derived from Kubota, 1978.

Clytia embryonic development

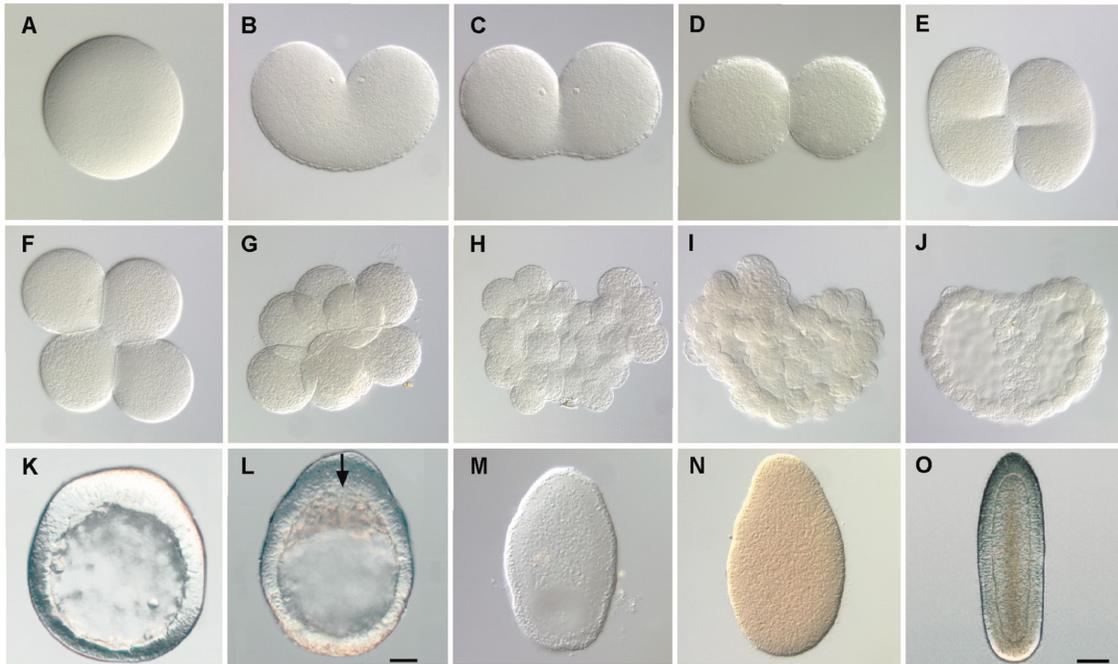


Figure 12. *Clytia hemisphaerica* embryonic development. The animal pole is always on top. (A) Unfertilised mature egg. (B-C) First mitotic division. (D) 2-cell stage embryo. (E) Second mitotic division. (F) 4-cell stage embryo. (G) 8-cell stage embryo. (H) 32-cell stage embryo. (J) Irregularly shaped, single cell-layered blastula, which produces cilia for swimming right before gastrulation starts. (K-L) Early-mid gastrula. Gastrulation occurs by unipolar cell ingression on the animal pole (black arrow). (M) Late gastrula. The blastocoel is still visible at the vegetal pole. (N) One-day old planula larva. The animal pole (gastrulation site) becomes the oral pole and the vegetal pole becomes the aboral pole of the larva. (O) Two-layered planula larva, 3-days old. Scale bars: (A-L) = 40 μ m. (M-O) = 50 μ m. Taken from Leclère 2008.

III.2.3. *Clytia* as an experimental model

The transparency, ease of culture and convenience for experimental manipulation of *Clytia* has shown the value of using *Clytia* as an experimental model. Some of the main research topics investigated using *Clytia hemisphaerica* in the last decade include regulation of oocyte maturation, embryonic polarity and patterning, and evolution of developmental mechanisms (Amiel and Houliston, 2009; Amiel et al., 2009; Chevalier et al., 2006; Denker et al., 2008; Forêt et al., 2010; Fourrage et al., 2010; Fourrage et al., 2014; Lapébie et al., 2014; Leclère et al., 2012;; Momose et al., 2012; Steinmetz et al., 2012; reviewed by Leclère et al., 2016 and Houliston et al., 2010). As an illustration, the oocyte-specific meiotic regulatory kinase Mos was

found in *Clytia*, and functionally proven via Mos antisense morpholino oligonucleotides to be crucial for meiotic spindle positioning, polar body emission and secondary cytostatic arrest prior to fertilization, reflecting its high conservation in regulating pivotal processes during oocyte meiotic progression throughout the animal kingdom (Amiel et al., 2009).

Until recently, few experimental approaches to test gene function were available in *Clytia*. Markedly, injection of morpholino antisense oligonucleotides or synthetic RNAs into eggs or single cells of the embryo have been commonly used in *Clytia*, although these techniques restricted gene function analysis to the early developmental stages (Houliston et al., 2010). The development of recent gene editing techniques in *Clytia*, such as CRISPR/Cas9, are allowing functional gene analyses at different stages of the life cycle and augur a promising venue of research for this hydrozoan model (Momose and Concordet, 2016; see chapters 2 and 3). Moreover, in addition to a collection of about 90,000 ESTs (Expressed Sequence Tags), the recent availability of an assembled genome and several transcriptomes comprising all *Clytia* life cycle stages has allowed the use of next-generation sequencing approaches (Lapébie et al., 2014; Houliston et al., 2010; see chapters 2 and 3).

In the next sections I will briefly comment on some aspects of *Clytia* sexual reproduction, how environmental light plays a crucial role in its regulation, and highlight how the simplicity and autonomy of its reproductive organs make *Clytia* a suitable model for studies of gametogenesis and reproductive control.

III.3. Light-dependent gamete release in *Clytia hemisphaerica* and other hydrozoans

Gamete release in hydrozoans is tightly synchronized; in many species it can be triggered by light-dark transitions or by dark-light transitions, or by both (Arkett, 1989; Ballard, 1942; Freeman, 1987; Martin, 2002; Miller, 1979; Mills, 1983; Takeda et al., 2006; Takeda et al., 2013). In species that show both morning and evening spawning, gamete release can occur when sexually mature adults are exposed to drastic changes in lighting intensity after acclimatization in constant conditions for several hours (Mills, 1983; Roosen-Runge, 1962). These observations indicate that hydrozoans follow circadian or semi-circadian spawning rhythms aligned with the day/night regimes, which may be influenced by endogenous 'clock' mechanisms and/or involve direct triggering of spawning by light.

In the case of hydrozoan medusae, fertilization success in wild populations is clearly improved by spawning synchronization between males and females, using changes in environmental light as direct cues for gamete release, although behavioral responses have been proposed to play a role as well (Mills, 1983). Many hydromedusae undergo light-dependent diel vertical migrations to optimize feeding or reproductive behaviors. Swimming behavior is controlled by "pacemaker" neurons in the inner nerve ring that stimulate rhythmic bell margin contractions via electrical coupling of muscles through gap junctions, and these neurons can be directly affected by light (Katsuki and Greenspan, 2013; Leclère and Röttinger, 2017; Martin, 2002; Mills, 1983). Observations in the wild as well as studies made in large tanks showed that in some hydromedusae species, notably *Aequorea Victoria*, *Gonionemus vertens* and *Bougainvillia principis*, the timing of spawning correlates with vertical migrations in the water column (Mills, 1983). Considering the massive volumes of water in any sea or ocean, synchronising spawning with vertical migrations was suggested to greatly enhance the chances of fertilization, by reducing the distances between individuals prior to spawning (Mills, 1983; Roosen-Runge, 1962). Further fertilization rate improvement during hydrozoan jellyfish spawning events may come from species-specific chemotaxis between eggs and sperm, shown for a wide range of hydromedusae (Miller, 1979). In *Clytia* species, spermatozoa and egg survive for several hours upon spawning, and successful fertilization can occur at high dilutions, which should also favour fertilization (Roosen-Runge, 1962). These studies in hydromedusae illustrate how light-mediated behavioural and physiological regulation can be coupled to optimize sexual reproduction in the animal kingdom.

Intriguingly, two different populations of *Clytia hemisphaerica*, one in the Pacific Ocean and the other in the Mediterranean Sea, show differences in their spawning regimes in response to light. *Clytia* collected in Friday Harbor (North Pacific) spawned twice a day, at dawn and at dusk, whereas in the Mediterranean they have only been observed to spawn at dawn (Roosen-Runge, 1962). In any case, the form of *Clytia hemisphaerica* we use in the laboratory, collected from the Mediterranean Sea, spawns only after dark-light transitions, and exhibits a clear vertical migration towards the surface of the tanks prior to spawning (**Figure 13**).

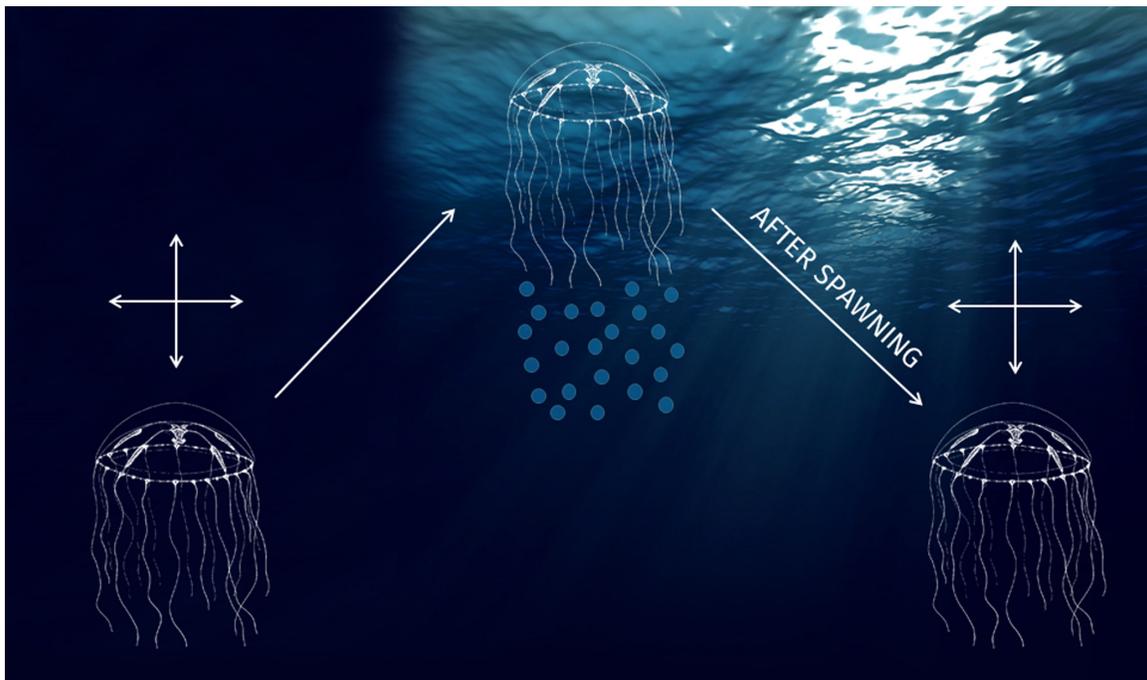


Figure 13. Scheme depicting *Clytia hemisphaerica* diel vertical migration accompanying spawning. *Clytia* jellyfish migrate towards the sea surface about 90 minutes after light stimulation, prior to spawning. Arrows indicate medusae swimming directions in the water column. Blue circles represent spawned mature eggs. This information is extrapolated from the behaviour seen under our laboratory culture conditions.

III.4. Light induces oocyte maturation and subsequent spawning in isolated *Clytia* gonads

Clytia medusae possess simple yet well-organized reproductive organs (Roosen-Runge and Szollosi, 1965). The transparency and approachability to manipulation of *Clytia* gonads offer a useful system for analysis of gametogenesis (Amiel et al., 2010). Since the main aim of my PhD work has been the characterization of the oocyte maturation initiation regulation in *Clytia*, I will describe here in detail the anatomy and physiology of the adult *Clytia* female gonads.

Each *Clytia* medusa has four gonads located halfway along the four endodermal radial canals (**Figure 14**). *Clytia* gonads consist of pools of proliferating cells, committed oocytes and vitellogenic oocytes at different growth stages, sandwiched between a layer of endodermal tissue and a thin ectodermal epithelia (Amiel et al., 2010). Proliferating cells and early differentiating oocytes are located close to the radial canals, while vitellogenic (growing) oocytes are positioned more distally, although generally lacking a clear ordered progression of growth stages inside the gonad (Amiel and Houliston, 2009) (**Figure 14**). During vitellogenesis, *Clytia* oocytes accumulate reserves of yolk, glycogen, lipids, ribosomal proteins and maternal mRNAs to prepare for embryogenesis. This accumulation of reserves is probably controlled by a combination of direct synthesis from the oocytes and uptake from surrounding endodermal cells, which are in contact with the oocytes and carry nutrients obtained from the food digestion in the gastrodermal cavity (Amiel et al., 2010). In our laboratory culture conditions, vitellogenic *Clytia* oocytes take about 16 hours to grow from stage I to reach their full size (stage III; ~180µm in diameter). At this point, the germinal vesicle (GV) has become positioned close to the animal pole and the fully grown oocytes have become competent to undergo meiotic maturation (Amiel and Houliston, 2009; Amiel et al., 2010) (**Figure 14**).

Despite the fact that *Clytia* oocytes are covered by a thin layer of ectodermal cells, these do not form an organized follicle (Amiel et al., 2010; Houliston et al., 2010), as is seen in other animals like mammals, fish, amphibians or starfish (see section II of Introduction). *Clytia* oocytes, as well as hydrozoan oocytes in general, can be separated from somatic cells of the gonad and still retain the meiotic Prophase I arrest. This implies that communication through gap junctions is not necessary for this arrest to be maintained (Amiel et al., 2010; Freeman, 1987; Freeman and Ridgway, 1988; Takeda et al., 2006).

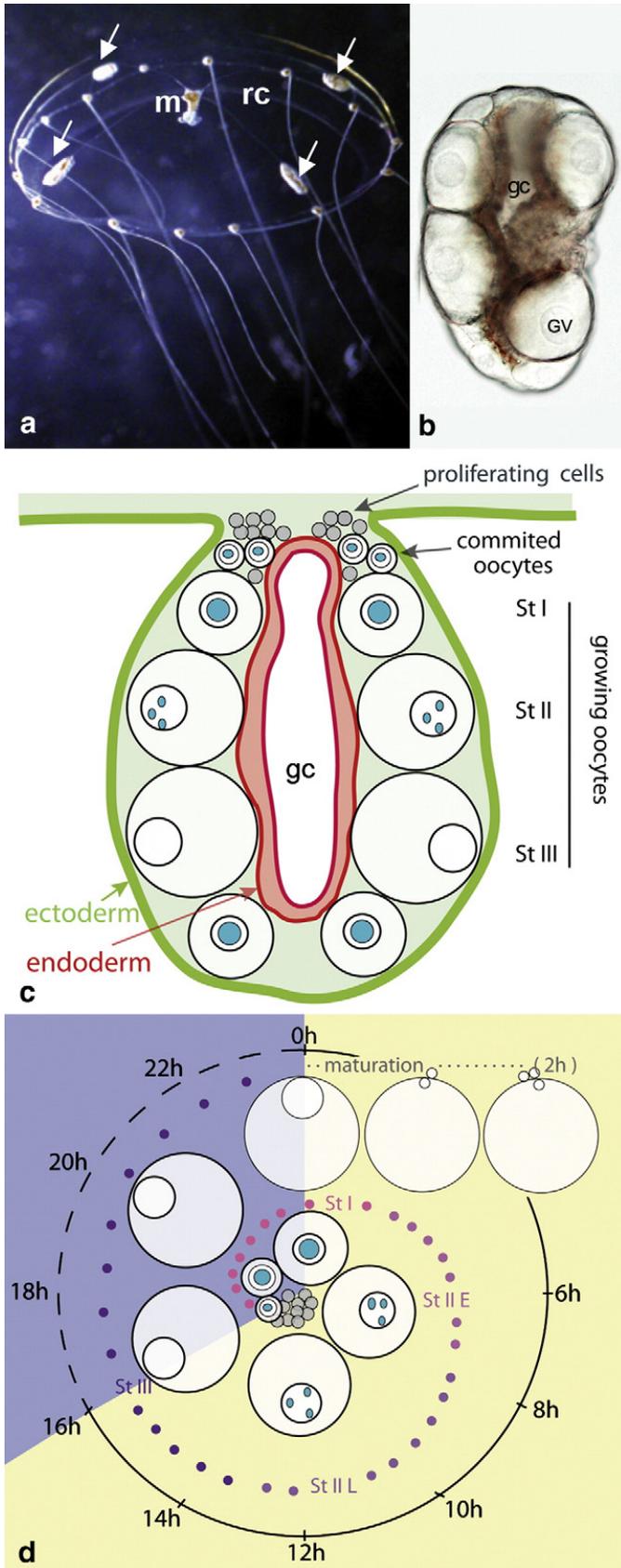


Figure 14. *Clytia* gonad structure and oogenesis.

(A) Location of the four gonads (white arrows) on the radial canals (rc) of the medusa, which distribute digestion products from the manubrium (m).

(B) Isolated gonad. gc = gastroendodermal cavity; GV = germinal vesicle.

(C) Diagram of oocyte organization within the female gonad. Oocytes are located between an endodermal pouch and a thin ectodermal epithelia, which are derived from the radial canal. Proliferating cells and small committed oocytes are situated proximally with respect to the jellyfish umbrella. Oocytes at different stages of growth are positioned more distally, although their progression is not rigorously ordered. gc = gastroendodermal cavity.

(D) Summary of oocyte growth kinetics. Time is indicated in hours after the light cue for spawning. Fully grown oocytes (St III) are competent to undergo meiotic maturation. Oocyte maturation is completed in two hours upon gonad light stimulation. Yellow and blue backgrounds represent the light and dark periods, respectively, of the standard culture conditions in the laboratory. Taken from Amiel and Houliston, 2009.

In our cultures of *Clytia hemisphaerica*, oocyte meiotic maturation is triggered by dark-light transitions, and therefore occurs in a daily basis (see above). Maturation lasts around 2 hours (**Figure 14-15**). As is typical in other hydrozoans, oocyte maturation in *Clytia* terminates meiosis; mature eggs are arrested at G1 (**Figure 3**) under Mos/MAPkinase control (Amiel et al., 2009), and only then are they competent to be fertilized. Mature eggs are released into the seawater through breaks in the gonad ectodermal epithelium when oocyte maturation is finished (Amiel et al., 2010). Remarkably, isolated gonads can survive and spawn autonomously upon light stimulation when separated from the jellyfish (**Figure 15**), and they can undergo successive cycles of oocyte growth and spawning for several days in culture (Houliston et al., 2010). This implies that all the molecular machinery required for regulating the entire process of oocyte maturation, from initial trigger (gonad light reception) to final output (spawning of mature eggs), is present in the gonads themselves.

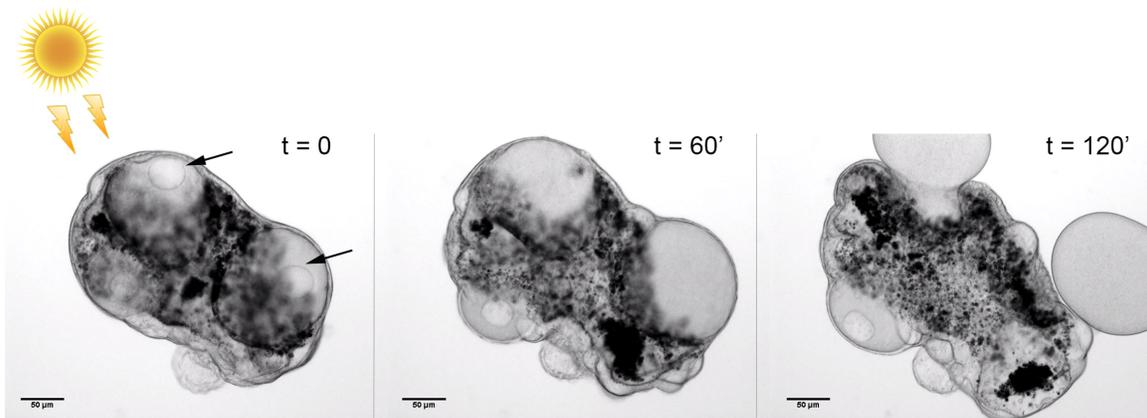


Figure 15. Light stimulation induces oocyte maturation and consequent spawning in isolated *Clytia* gonads. Bright field images of an isolated gonad showing two fully grown oocytes which were triggered to undergo meiotic maturation after light stimulation. Black arrows indicate the germinal vesicles of these two oocytes, the breakdown of which represents a hallmark of oocyte maturation (note their absence in the following time points). Times in minutes after the light cue are shown for each image. Completion of meiotic maturation and subsequent spawning takes about two hours at 18°C. Scale bars = 50µm.

My thesis work has addressed the cellular and molecular intermediates involved in gonad light reception and oocyte maturation induction in *Clytia hemisphaerica*, previously unknown.

III.5. Working model for light-induced oocyte maturation in *Clytia* gonads

Various evidence obtained from different species of hydrozoans has provided a general picture of the sequence of regulatory steps triggering oocyte maturation initiation in *Clytia* (see **Figure 17** below). As mentioned above in this section, light-dark and/or dark-light transitions provide the initial cue to trigger the release of the oocyte from its meiotic prophase I arrest in hydrozoans, followed by maturation completion and spawning.

Activation of MPF to promote GVBD is mediated by a rapid increase in the concentration of cytoplasmic cAMP, activating PKA. This was shown in the hydrozoan *Cytaeis uchidae*, which spawns mature eggs upon dark-light transitions. Physiological cytoplasmic cAMP concentrations were shown to rise inside the oocytes after stimulating the ovaries with light after a period of darkness. Drug experiments further demonstrated that this cAMP rise and PKA activation inside *Cytaeis* oocytes were required to initiate oocyte maturation (Takeda et al., 2006). Further evidence comes from experiments done by G.Freeman and E.B. Ridgway, where they showed that direct injection of cAMP into oocytes, or the addition of cell-permeable cAMP derivatives to their surrounding media, could induce maturation in a number of hydrozoan species (Freeman and Ridgway, 1988).

Downstream of the initial cAMP signal, and in parallel with active MPF downstream events, Mos mRNA translation is essential for MAPkinase activation. Inhibition of Mos translation using antisense morpholino oligonucleotides injected into *Clytia* oocytes demonstrated that the meiotic kinase Mos is essential for regulating spindle position, polar body formation, and post-meiotic arrest by activating the MAPkinase pathway, as is common in bilaterian model species (see section II of Introduction). These experiments further showed that, in *Clytia*, Mos does not participate in oocyte maturation initiation (Amiel et al., 2009).

My thesis work has focused on the initial regulatory step of the hydrozoan oocyte maturation process: how the light cue leads to a cAMP concentration rise in the oocyte. Previous studies had revealed that a Maturation Inducing Mornone (MIH - see section II of Introduction) is released by somatic cells of the gonad in response to light cues. The first experiments were performed by S. Ikegami and collaborators using the hydrozoan jellyfish *Spirocodon saltatrix*, in which oocyte maturation and gamete spawning are triggered by light-dark transitions. By transferring light-adapted isolated *Spirocodon* ovaries into a dish containing dark-stimulated male or

female gonads, they showed that a diffusible substance released into the seawater by dark-stimulated gonads could trigger oocyte maturation and consequent spawning of the non-stimulated ovaries. They further showed that this substance had a proteic nature, since its spawning-inducing activity was completely removed by treatment with trypsin or proteases. Elution of the seawater containing the maturation-inducing substance in a Sephadex column and consequent spawning assays with eluted fractions showed that the molecular weight of the spawning-inducing substance had a molecular weight lower than 1,500 Da. This was the first evidence in a cnidarian that light-controlled spawning could be mediated by diffusible molecules of low molecular weight, produced by both male and female gonads (Ikegami et al., 1978). In 1987, G. Freeman carried out similar experiments using the marine hydroid *Hydractinia echinata*, which unlike *Spirocodon* but as in *Clytia*, spawns upon dark-light transitions. Freeman performed his experiments in microdrop cultures rather than dishes. Importantly, he used not only isolated gonads but also isolated oocytes. These were non-responsive to light cues when cultured alone, but underwent maturation when transferred to microdrops containing light-induced gonads (**Figure 16**). Taken together these results strongly suggested that diffusible maturation-inducing hormones (MIHs), possibly peptidic in nature, are produced in gonadal somatic cells and secreted upon light stimulation in *H. echinata* (Freeman, 1987).

Another set of experiments showed that *C. uchidae* oocytes isolated from the ovaries right after light stimulation did not undergo meiosis resumption (Takeda et al., 2006). A similar situation was also reported in *H. echinata* (Freeman, 1987). This raised the possibility that presumptive diffusible MIHs are produced after light stimulation and their effect on the oocytes is delayed. Alternatively, the presumptive MIHs might need some time upon light stimulation to be secreted and/or perform their biological activity (Takeda et al., 2006).

Based on these various findings it was proposed that small peptides, and more specifically neuropeptides, were strong candidates for hydrozoan MIH (Takeda et al., 2013). This was hypothesized because cnidarians nervous systems are strongly peptidergic (discussed in more detail in chapter 1). The involvement of neuropeptides in oocyte maturation induction was tested in *C. uchidae* using a series of neuropeptides identified in *Hydra magnipapillata* (Fujisawa, 2008). Gonads from sexually mature *C. uchidae* jellyfish could be induced to spawn by treatments with a GLWamide family peptide (NPYPGLWamide). However, fully grown oocytes isolated from the gonads did not resume meiosis in the presence of these neuropeptides.

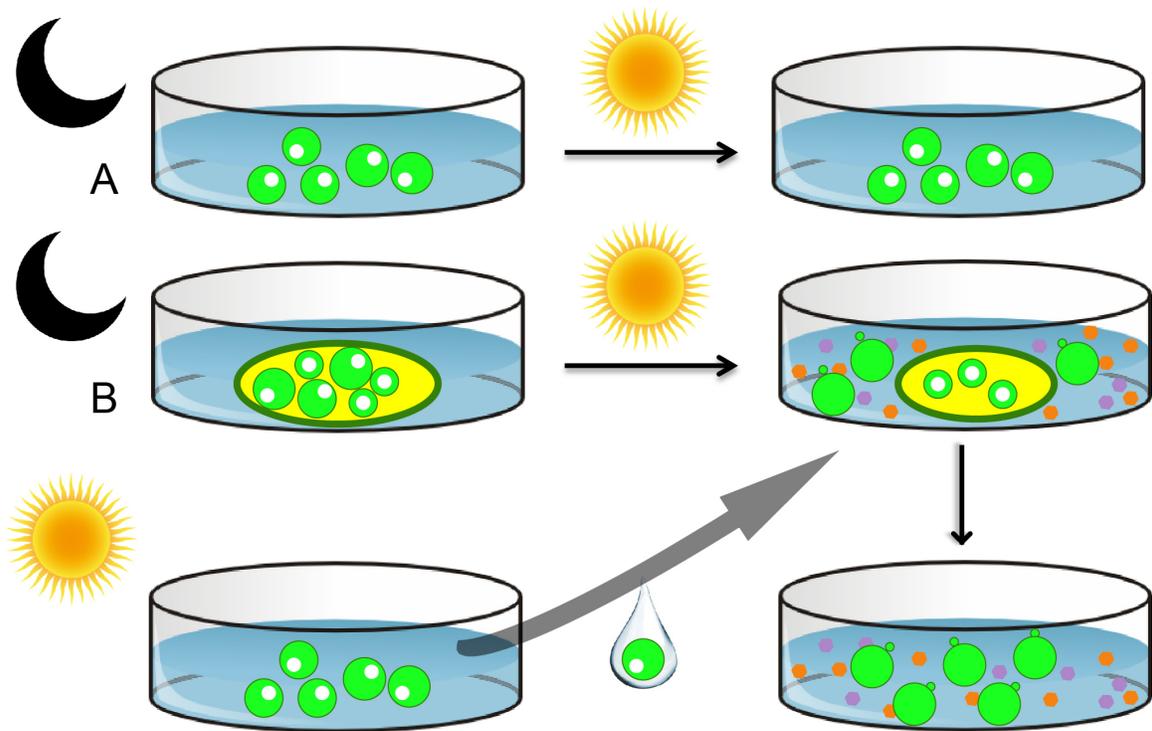


Figure 16. Microdrop experiments with hydrozoan gonads and oocytes.

Schematic representation of the experiments done by G.Freeman in 1987 with isolated ovaries and oocytes from *Hydractinia echinata* in seawater microdrops. (A) Isolated fully grown oocytes (green) subject to a dark-light transition fail to undergo meiotic maturation. (B) Dark-light transitions trigger the maturation and ensuing spawning of fully grown oocytes when they are cultured inside isolated ovaries. When light-adapted isolated oocytes are transferred to the microdrop containing spawning gonads they are induced to mature, indicating that diffusible MIHs (purple and orange symbols) are released from somatic gonadal tissues upon light stimulation. Similar experiments were performed by N. Takeda and by myself with *Clytia* isolated gonads and oocytes, obtaining the same results.

Furthermore the concentrations of NPYPGLWamide needed to trigger gonads to spawn ranged high micromolar levels. Although the poor activity could partially reflect the species differences, these results indicated that, even though neuropeptide signalling may be involved in oocyte maturation and spawning in *C.uchidae*, GLWa family peptides probably act on the somatic cells of the gonads, rather than being directly responsible for MIH activity. Immunostaining with anti-GLWamide antibodies decorated neural cells and arrays of axon-like structures containing dense-core synaptic vesicles in *C.uchidae* gonad ectodermal epithelia overlying the oocytes (Takeda et al., 2013). These results suggested that ectodermal neural cells and GLWamide family neuropeptides could play a role in regulating oocyte maturation and spawning in hydrozoans, although probably not as MIHs.

Regarding the ability of hydrozoan gonads to detect light cues, the expression of opsin photopigment mRNAs in the gonad ectodermal epithelia of the hydromedusae *Cladonema radiatum* was shown (Suga et al., 2008). This raised the possibility that opsin photopigments could be involved in the control of spawning in hydrozoans.

Put together, these findings in various hydrozoans suggested the following complete Working model for light-induced oocyte maturation in *Clytia* gonads, illustrated in **Figure 17**. Gonad ectoderm photosensitive cells of unknown nature are responsible for detecting light cues. In response to changes in light intensity, the photosensitive cells promote the secretion of maturation-inducing hormones (MIHs), possibly neuropeptides, either from the same cells or from interacting cells. MIHs bind to unknown receptors, most probably located in the oocyte plasma membrane. Upon MIH reception, a cytoplasmic downstream signaling cascade elevates cAMP levels, which in turn activate PKA. This leads to MPF activation through PKA-mediated phosphorylation of unknown substrates, and thus meiotic progression. In parallel, Mos translation and subsequent MAPK activation orchestrate polar body formation and post-meiotic arrest.

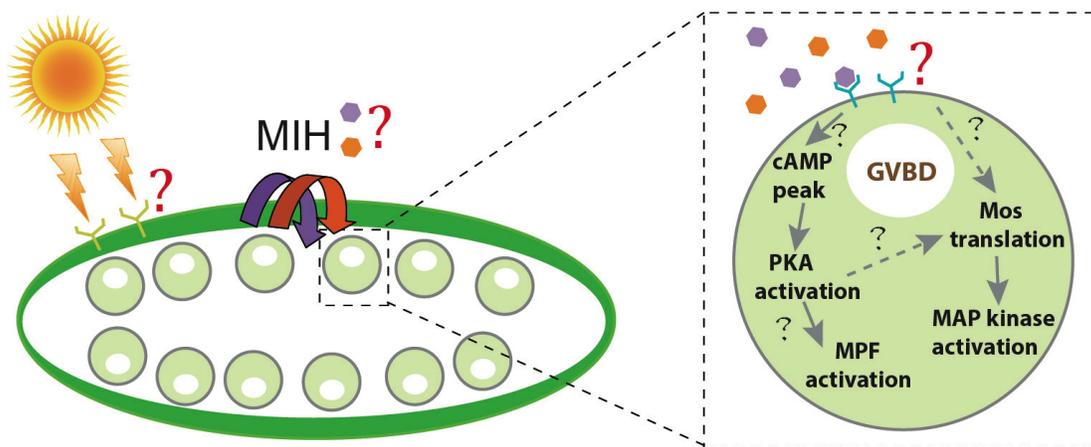


Figure 17. Working model for light-induced oocyte maturation in *Clytia hemisphaerica* isolated gonads. See text for explanations.

IV. Objectives

In the introduction of my thesis I have highlighted some aspects of animal biology that are fundamental for the regulation of sexual reproduction but remain poorly understood. I have illustrated how changes in environmental light can be of crucial importance for reproductive control in the animal kingdom, although little is known about the cellular and molecular regulation of these processes. I have also pointed out the complexity and variable regulation of the pivotal process that is oocyte meiotic maturation in different animal lineages. Finally, I have introduced the animal experimental model I have used during my PhD, the hydrozoan *Clytia hemisphaerica*. I have tried to emphasize the interest of its phylogenetic position, as well as the accessibility to experimentation of its gonads, making *Clytia* an excellent model for studying the mechanisms regulating oocyte meiotic maturation.

Based on previous studies that allowed the establishment of a working model for light-induced oocyte maturation in *Clytia hemisphaerica* (**Figure 17**), I commenced my PhD with three main questions to answer (the 3 big red question marks in **Figure 17**):

- 1) To determine the molecular identity of *Clytia* MIH, produced by gonad somatic cells and released following a light stimulus, and characterize the cells which produced it. This work, based on screening of candidate neuropeptides, was already advanced when I started my PhD. See chapter 1.
- 2) To identify and characterize the *Clytia* MIH receptor in the oocyte and test its function in triggering oocyte maturation initiation by gene editing techniques. Prime candidates were oocyte-expressed GPCRs, given the probable neuropeptide nature of MIH and the immediate cAMP response to its activation. See chapter 2.
- 3) To determine the molecular identity of the photopigments used for gonad light reception in *Clytia* and characterize the gonad cells that express them, as well as their relationship with MIH-producing cells and their role in MIH release upon light stimulation. Opsins were chosen as prime candidate photopigments, since opsin mRNAs had been detected in the gonad ectodermal epithelia in *Cladonema radiatum* (Suga et al., 2008). See chapter 3.

The next three chapters of my thesis manuscript, present in paper manuscript format, show the data I obtained through a variety of approaches, and report how each of these initial objectives was largely attained. As explained in detail in the context of each chapter, each study involved collaborations with colleagues in the group as well as in Japan and Germany. However, I was responsible for the majority of the work reported in chapters 2 and 3.

RESULTS

Preface

As an important common initial step towards achieving the three main aims of my PhD, and more specifically to identify candidates for *Clytia* MIH, its receptor, and upstream light-responsive photopigments from different *Clytia* gonad tissues, I generated RNA-Seq data (i.e. short sequences generated quantitatively from the pool of mRNA in each tissue) to allow assessment of the expression levels of candidate genes. In the light of the working model presented in **Figure 17** we predicted that transcripts for MIH and photosensitive proteins would be present, probably preferentially, in gonad ectoderm, whereas those for the MIH receptor would be abundant in the oocyte.

I pooled tissues obtained by manually dissecting around 150 *Clytia* gonads under identical conditions; all gonads were isolated from jellyfish that had been under constant illumination for 20 hours after the last spawning event. I separated the gonad into endoderm, ectoderm and two different oocyte growth stages (**Figure 18**), and extracted their mRNA. I collected enough samples to have two biological replicates of each gonad tissue for Illumina Hi-seq sequencing.

In order to check for cross-contamination between mRNA samples, I synthesized cDNA by reverse transcription and performed qPCR analyses using primers specific for three endogenous *Clytia* GFP (Green Fluorescent Protein) genes that are differentially expressed in *Clytia* medusa tissues (Fourrage et al., 2014). Oocyte samples were enriched in GFP2 mRNA, whereas the gonad ectoderm ones were enriched in GFP4 mRNA, matching perfectly previously obtained results (Fourrage et al., 2014). GFP expression in the gonad endoderm had not been assessed before, but since small committed oocytes remain attached to it and are almost impossible to get rid of, it is possible that the GFP2 enrichment I obtained in both gonad endoderm biological replicates was due to the presence in the samples of GFP2 from small *Clytia* oocytes. Another possibility would be that GFP2 is also expressed and predominant in the gonad endoderm (**Figure 18**).

Illumina Hi-seq reads from each sample were mapped against a comprehensive *Clytia* reference transcriptome recently assembled in the group, to which the new gonad transcriptome sequences also contributed (analysis performed by Pascal Lapébie). To allow comparisons between samples and genes, read counts for each biological replicate were normalized per total amount of reads and per transcript

sequence length. The normalized number of mapped reads was taken as a measure of transcript level.

These RNA-Seq data were used for the identification of candidate molecular regulators for each step of maturation initiation, as explained in detail in each chapter.

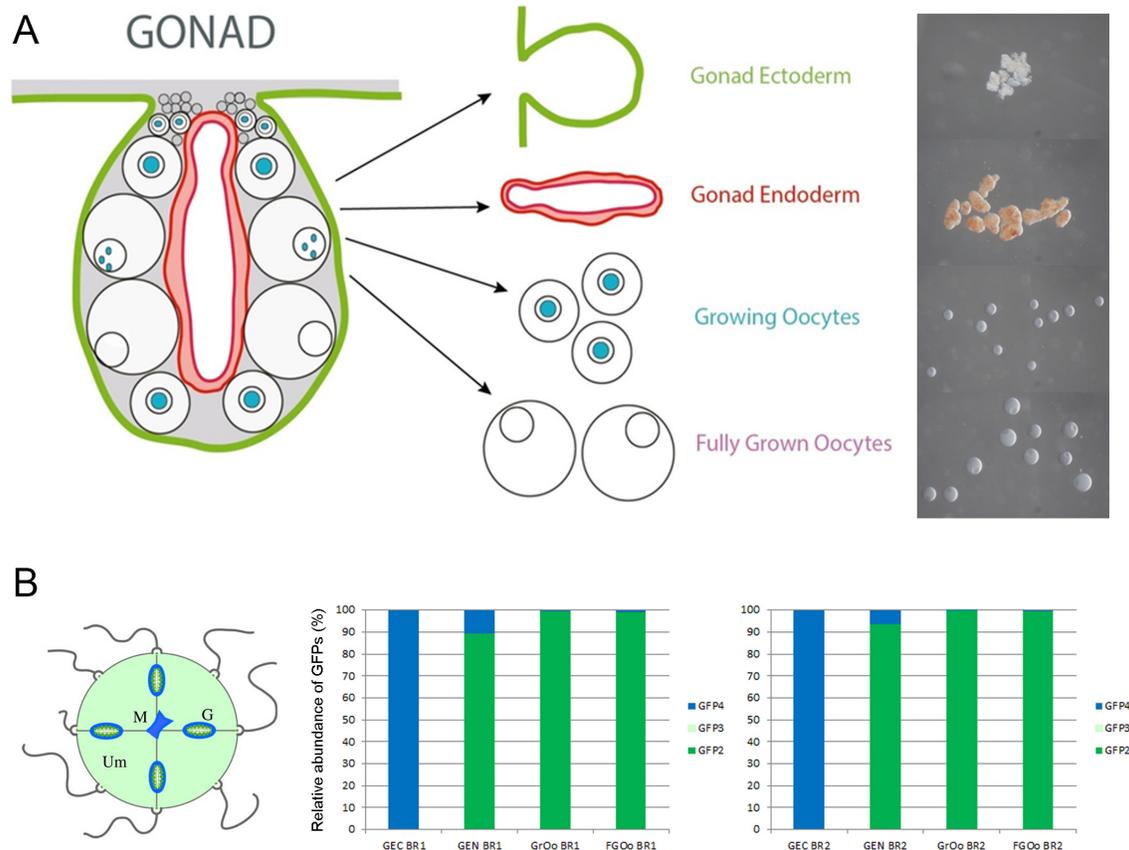


Figure 18. Sample collection for mRNA sequencing.

(A) Scheme of the *Clytia* gonad showing the four tissues collected (modified from Amiel and Houlston, 2009). Images of each tissue under the dissecting scope are shown on the right, all at the same magnification (Fully grown Oocytes are about 180µm in diameter).

(B) Schematic drawing of a medusa colour-coded to show sites of endogenous GFP gene expression (modified from Fourrage et al., 2014); Oocytes predominantly express *GFP-2* and the gonad ectoderm *GFP-4*, while *GFP-3* is expressed in bell tissue. Graphs show the relative expression of three endogenous GFP genes for each sample, analysed by qPCR, confirming lack of contamination between the oocyte and ectoderm samples. M = manubrium; Um = umbrella; G = gonad; BR1/2 = biological replicate 1/2; GEC = gonad ectoderm; GEN = gonad endoderm; GrOo = growing oocytes; FGOo = fully grown oocytes.

CHAPTER 1

Identification and characterization of *Clytia* MH

Context

The molecular identification of *Clytia* MIH was initiated by Dr. Noriyo Takeda during a collaborative visit to Villefranche. Her starting hypothesis was that hydrozoan MIHs were likely to be neuropeptides, since earlier experimental studies had indicated that small, protease-sensitive molecules released from gonadal somatic tissues act as MIHs in various hydrozoans (see section III of Introduction; Freeman, 1987; Ikegami et al., 1978). Dr. Takeda and her collaborators had previously demonstrated that certain neuropeptides identified in *Hydra* could induce oocyte maturation and spawning when applied to *Cytaeis* gonads (Takeda et al., 2013), although the relatively high concentrations required and their inability to cause maturation of isolated oocytes left the identity of MIH open.

A brief introduction to Neuropeptides

Neuropeptides constitute the most diverse and largest class of neuron-secreted signalling molecules. They can function as peptide neurotransmitters or neuromodulators, mediating chemical communications among neurons, or as peptide neurohormones, regulating endocrine systems. Neuropeptides can be released at considerable distances from their target sites, allowing the neuron-mediated control of peripheral endocrine functions (Baraban and Tallent, 2004; Hook et al., 2008; Jékely, 2013) (**Figure 19**).

Neuropeptides are generally small molecules, ranging from 3 to 40 amino acid residues. They are synthesized from inactive peptide precursors (proneuropeptides or neuropeptide precursors), which contain a single or multiple copies of active peptides and require proteolytic processing in order for the neuropeptides to reach their final biologically active form (Grimmelikhuijzen et al., 1996; Hook et al., 2008). The N-terminus of each neuropeptide precursor amino acid sequence features a signal peptide that is cleaved by signal peptidases at the rough endoplasmic reticulum during translation. The resulting proneuropeptide is translocated to the Golgi apparatus and parcelled into large, dense-core secretory vesicles, also called regulated secretory vesicles, together with proteolytic enzymes. Proteolytic processing of the precursors occurs at canonical dibasic or monobasic sites flanking the NH₂ and COOH ends of the neuropeptide sequences, and is executed by various enzymes (including prohormone convertases, aminopeptidases, endoproteinases and carboxypeptidases) (Grimmelikhuijzen et al., 1996; Hook et al., 2008; Southey et al., 2006) (**Figure 19**). Many neuropeptides require C-terminus α -amidation for their biological activity. A glycine in the COOH terminus of the neuropeptide is

converted into peptidyl-amide by a peptidylglycine alpha-amidating monooxygenase (PAM), the enzymatic activities of which can be separated by endoproteolysis (Burget et al., 2003; Grimmelikhuijzen et al., 1996). Biologically active neuropeptides then await cellular stimuli for regulated secretion (Hook et al., 2008).

Neuropeptides are widespread in the animal kingdom, being present in placozoans, ctenophores, cnidarians and bilaterians (Conzelmann et al., 2013a; Grimmelikhuijzen and Hauser, 2012; Hewes and Taghert, 2001; Janssen et al., 2010; Jékely, 2013; Mirabeau and Joly, 2013; Moroz et al., 2014; Nikitin, 2015; Osugi et al., 2015; Semmens et al., 2016; Takahashi and Takeda, 2015), and have been proposed to represent an ancient mode of neuronal communication and neuroendocrine control (Grimmelikhuijzen and Hauser, 2012; Jékely, 2013). Neuropeptides can act as important modulators of neuronal circuits, and widely regulate developmental, physiological and behavioural responses, such as planktonic larvae swimming behaviour (Conzelmann et al., 2011), life cycle transitions (Conzelmann et al., 2013b; Schoofs and Beets, 2013; Takahashi and Hatta, 2011), feeding and reproduction (Mita, 2013; Shahjahan et al., 2014; Wójcik-Gładysz and Polkowska, 2006).

Cnidarian nervous systems are strongly neuropeptidergic, while other neurotransmitters have been less commonly identified (Grimmelikhuijzen and Hauser, 2012; Kass-Simon and Pierobon, 2007). Cnidarian neuropeptides are processed from precursors as in bilaterians, but their cleavage sites are rather unconventional since acidic residues are commonly used to guide proneuropeptide cleavage on the N terminal side of each peptide, in contrast to bilaterian cleavage residues, which are always basic (Gajewski et al., 1996; Grimmelikhuijzen et al., 1996; Schmutzler et al., 1994) (**Figure 19**). The use of a broad range of enzymes for proneuropeptide cleavage is also characteristic of cnidarian neuropeptide systems (Grimmelikhuijzen et al., 1996). Cnidarian neuropeptides have been shown to be involved in the regulation of important biological processes. These include the swimming of planula larvae (Katsukura et al., 2004), metamorphosis from larva to polyp (Takahashi and Hatta, 2011), gamete release (Takeda et al., 2013; Tremblay et al., 2004), feeding, growth, muscle contraction and relaxation, neuron differentiation and morphogenesis (reviewed by Grimmelikhuijzen et al., 1996; Takahashi and Hatta, 2011; Takahashi and Takeda, 2015). Moreover, the nerve rings of various hydrozoan species can be decorated using antibodies against RFamide, GLWamide and PRGamide family neuropeptides (Koizumi et al., 2014), suggesting a wide role of these particular families in cnidarian nervous systems.

The wide neuropeptide involvement in cnidarians biology makes them very attractive candidates to be the endogenous MIH in *Clytia hemisphaerica*, and possibly in other cnidarians.

Overview of the findings of the study

Briefly, the findings presented in this manuscript are as follows:

- A list of putative neuropeptide precursor sequences was compiled from *Clytia hemisphaerica* combined transcriptome data (10 sequences) and a related hydrozoan species (*Cladonema pacificum*) gonad (+manubrium) transcriptome data (4 sequences). RNA-Seq analysis identified which ones were expressed preferentially in each gonad tissue.
- Synthetic versions of neuropeptides predicted from the candidate precursors were tested first on isolated gonads for spawning induction, and then on isolated fully grown oocytes for meiosis resumption (MIH assay). By far the most active in both assays were a subset of amidated tetrapeptides with the signature W/RPRP (and closely related sequences), able to induce these processes at nanomolar concentrations in both species when added to the media.
- The activity of endogenous MIH collected from gonads of both species could be neutralised with antibodies specific to the PRPamide epitope.
- Artificially synthesized WPRPamide (MIH) injected in *Clytia* oocytes could not trigger oocyte maturation, in contrast to its potent activity when applied extracellularly.
- *in situ* hybridization showed that the proneuropeptides precursors of *Clytia* MIH are co-expressed in a scattered population of gonad ectoderm cells. *Clytia* MIH precursor mRNAs were also detected in other parts of the jellyfish besides the gonads (e.g. manubrium, tentacles and nerve ring). One of the predicted *Cladonema* MIH precursors was also found to be expressed in a sparse population of cells in the gonads ectoderm.

- Immunofluorescence analyses with antibodies against MIH demonstrated the existence of a neural population in the gonad ectoderm in both *Clytia* and *Cladonema*, and showed that MIHs were secreted upon the corresponding environmental light transition in each species.
- Synthetic MIH neuropeptides were able to induce male spawning in both species.

PhD student contribution: When I started my PhD, Noriyo Takeda together with Dr Ryusaku Deguchi and their colleagues, had made considerable progress in the identification of *Clytia* endogenous MIH. They had found that short amidated peptides predicted from *Clytia* putative neuropeptide transcripts had extremely potent MIH activity on isolated oocytes, and then identified closely related peptides in another hydrozoan, *Cladonema pacificum*, with equivalent activities. Furthermore, antibodies specifically recognizing these peptides could neutralize the activity of endogenous MIH produced in the gonads by dark-light transitions (or light-dark transitions in *Cladonema*). To complete this study I performed all the *in situ* hybridizations (single and double) on both species to identify the cells that produce MIH. I then performed some of the immunofluorescence analyses in *Clytia*. I also participated actively in compiling figures and preparing a manuscript for submission to the journal *Nature Ecology & Evolution*, as a sister paper to the one presented in chapter 3. This manuscript is reproduced in the following pages.

Jellyfish neuropeptides that act directly as oocyte maturation hormones

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Short title: Gonad neuropeptides as oocyte maturation hormones

ABSTRACT

Oocyte maturation is the pivotal process that transforms ovarian oocytes into mature eggs via meiotic divisions. Here, we identify small specific neuropeptides that function as maturation-inducing hormones (MIHs) in two jellyfish model species. Among candidate peptides identified from gonad transcriptomes, we found that amidated tetrapeptides with the amino-acid signature W/RPRP at nanomolar concentrations can induce oocyte maturation and subsequent egg release. Antibody preabsorption experiments demonstrated that these PRPamide family neuropeptides account for the endogenous MIH activity released from the gonad following physiological light-dark

transitions. Localization studies further showed that PRPamides are synthesized by neural cells close to the oocytes and are released following light-dark transitions to act on the oocyte surface. Moreover, the same neuropeptides are produced by male jellyfish gonads, and trigger sperm release. A direct regulatory system using neuropeptide hormones, similar to that in jellyfish gonads, may have allowed synchronous gamete maturation, release and fertilization in ancestral animals.

Keywords: oocyte, meiosis, neuropeptide, spawning, reproduction, Cnidaria, *Clytia*, *Cladonema*.

INTRODUCTION

Fully-grown oocytes maintained within the female gonad are held at first prophase of meiosis until environmental and/or hormonal 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 studying the initiation and regulation of oocyte maturation, which in these animals is triggered by light cues. 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 cellular 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).

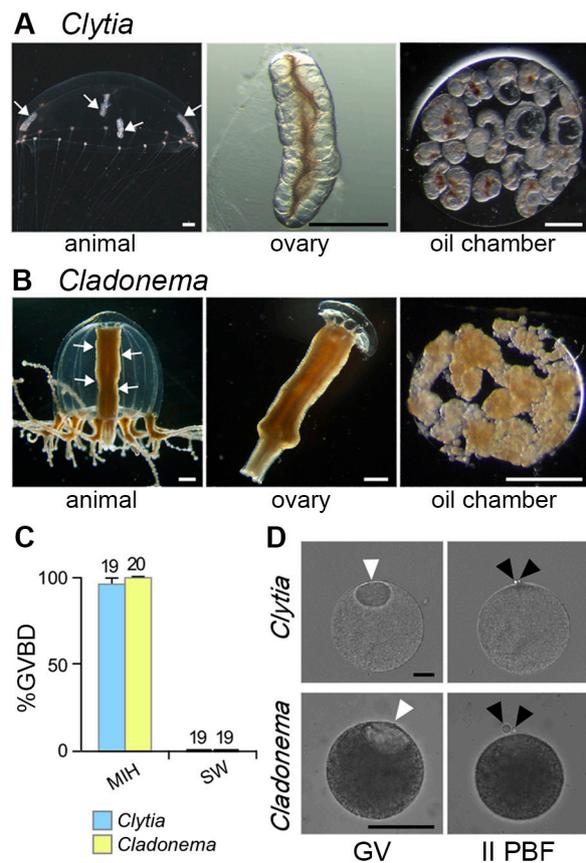


Figure 1. Active MIH is produced by isolated jellyfish gonads

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) MIH assay on isolated oocytes (% undergoing GVBD; 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 GV and black arrowheads to polar bodies. Scale bars: 500 μ m in A, B and 50 μ m in D.

RESULTS

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

We first demonstrated that 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 in other hydrozoan species (8, 9). Oocytes incubated in MIH obtained 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 results are 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

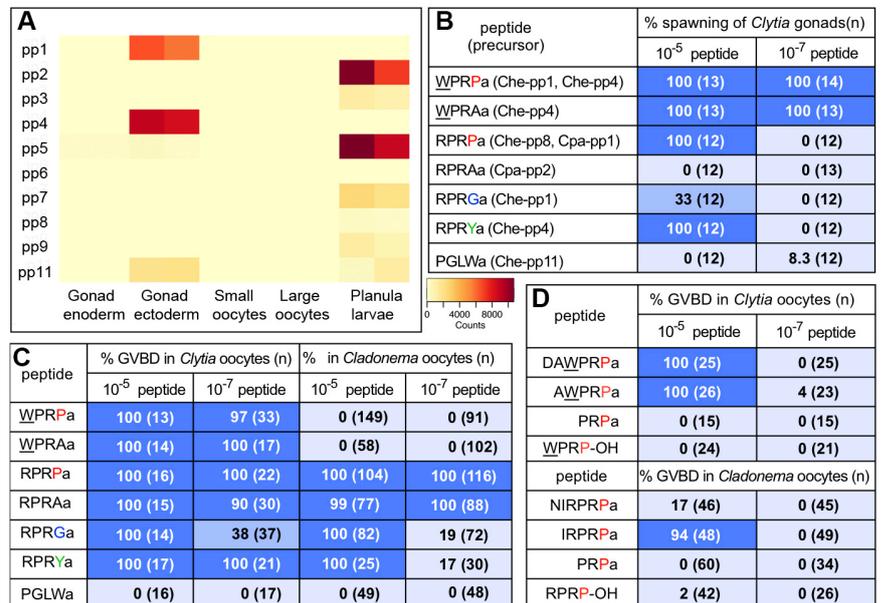


Figure 2. Predicted neuropeptides from the gonad ectoderm have MIH activity

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. Illumina 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.

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 PRPamides (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 provide endogenous MIH activity

We demonstrated that W/RPRPamide and/or W/RPRAamide peptides are responsible for

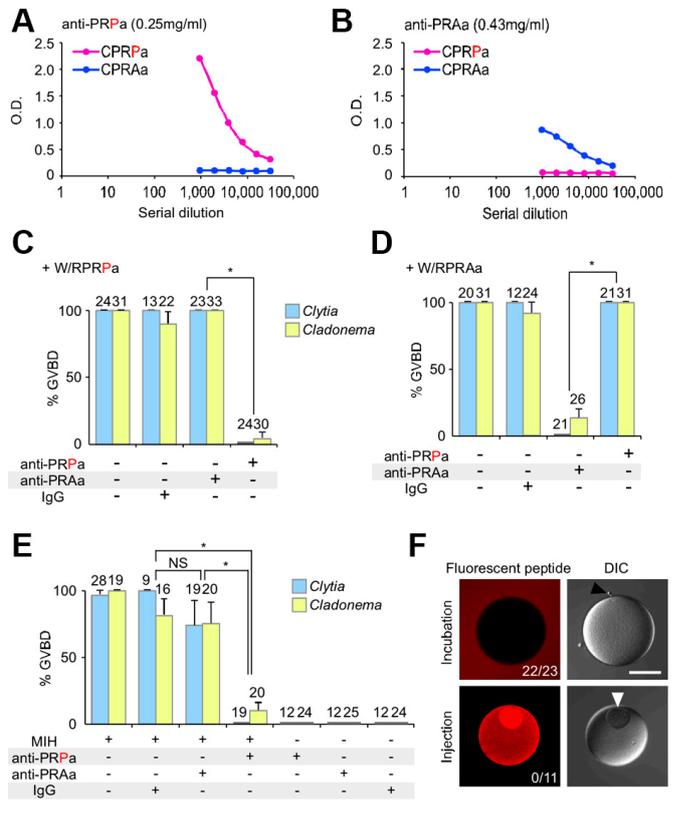


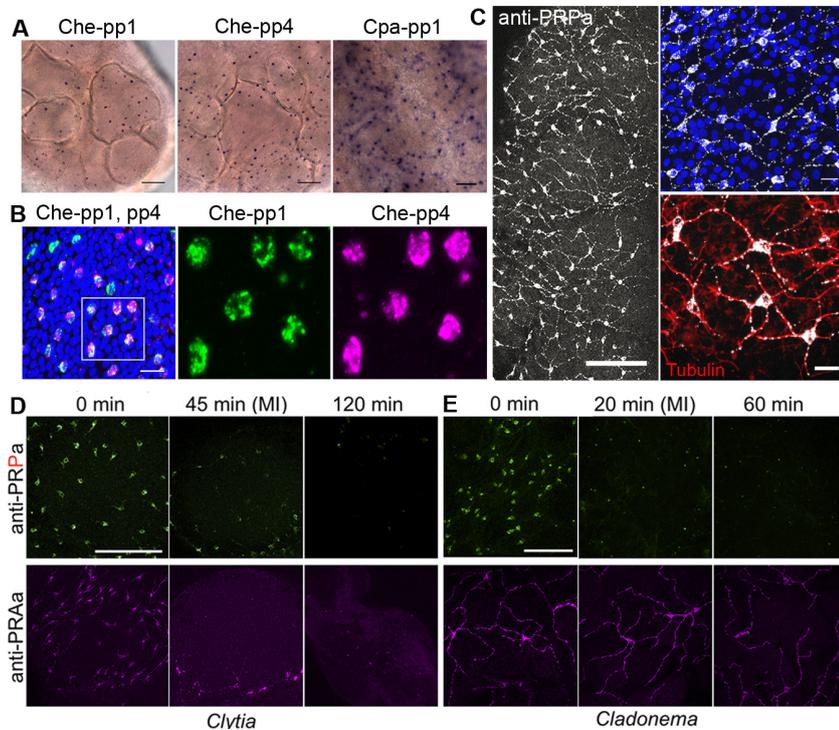
Figure 3. Antibody inhibition shows that PRPamides are the active component of MIH

A) ELISA assay demonstrating that the anti-PRPamide antibody binds PRPamide but not PRAamide tetrapeptides.
B) Reciprocal specificity 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 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 μ m. 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*).

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

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, E) Release of peptides detected with anti-PRPamide and anti-PRAamide antibodies from *Clytia* (D) and *Cladonema* (E) gonads at successive times during meiosis completion following light exposure as indicated (MI= first meiosis). Scale bars: 50 μ m in A; 20 μ m in B; 100 μ m in C (left), D, E; 10 μ m in C (right).

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

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
<i>Clytia</i>	WPRPamide	100 (16)	100 (15)
<i>Clytia</i>	WPRP-OH	0 (11)	0 (11)
<i>Cladonema</i>	RPRPamide	94 (16)	86 (22)
<i>Cladonema</i>	RPRP-OH	0 (10)	0 (11)

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 suggests 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).

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.

A

Peptide	conc. at M	% GVBD in Leptomedusa oocytes (n)			% GVBD in Anthomedusa oocytes (n)				
		<i>Obelia sp.</i>	<i>Aequorea coerulescens</i>	<i>Eutonina indicans</i>	<i>Bouillonactinia misakiensis</i>	<i>Sarsia tubulosa</i>	<i>Nemopsis doffeini</i>	<i>Rathkea octopunctata</i>	<i>Cytaeis uchidae</i>
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

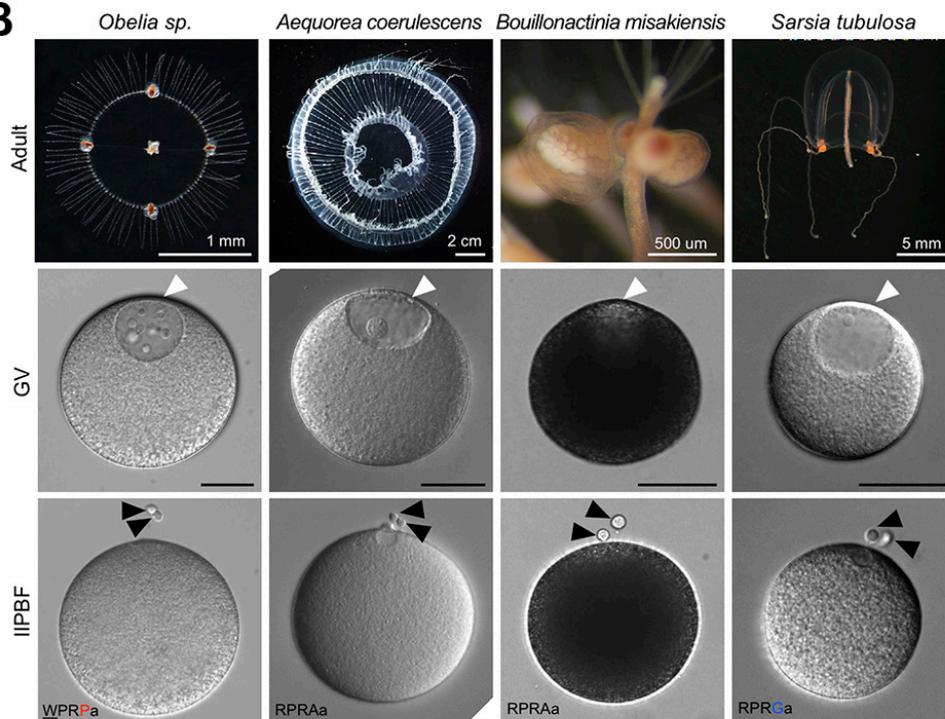


Figure 5. Synthetic peptides show MIH activity in a subset of hydrozoan jellyfish species

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 GV and polar bodies, respectively.

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 this 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, 5-7). 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

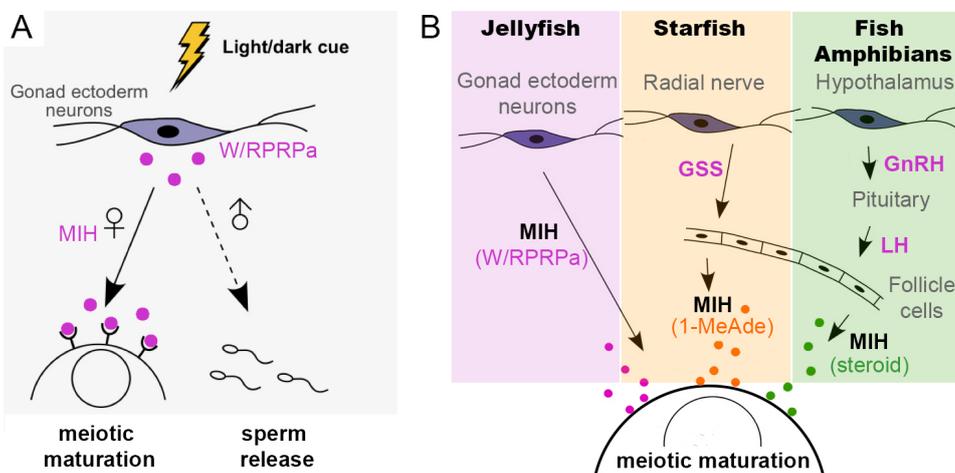


Figure 6. MIH action in jellyfish compared with other animals

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/dark 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.

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 neuroendocrine 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 Mg²⁺ ASW (a 1:1 mix of 0.53 M MgCl₂ 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 (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 pGEM-T easy vector, or retrieved from our *Clytia* EST collection cDNA library prior to probe

synthesis. Sequences and accession numbers are given in Figure S1.

For *Clytia* gonad tissue transcriptome comparisons, Illumina 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-NH₂, WPRA-NH₂, RPRP-NH₂, RPRA-NH₂, RPRG-NH₂, RPRY-NH₂, PGLW-NH₂, DAWPRR-NH₂, AWPRP-NH₂, NIRPRP-NH₂, IRPRP-NH₂, PRP-NH₂, 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-NH₂); was synthesized by Sigma and a 10⁻² solution in H₂O 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-NH₂ and CPRP-NH₂ 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 Mg²⁺ 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 18°C in HEM buffer (0.1 M HEPES pH 6.9, 50 mM EGTA, 10 mM MgSO₄) 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 -20°C 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 4°C, 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 4°C 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. Details of this protocol optimization will be published elsewhere. For double fluorescent *in situ* hybridization, female *Clytia* gonads were fixed overnight at 18°C 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) was performed using a DIG-labeled probe for Che-pp1 and a fluorescein-labeled probe for Che-pp4. A 3 h 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/H₂O₂ 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.

ACKNOWLEDGMENTS

We thank P. Dru, S. Chevalier and L. Leclère for generating and assembling *Clytia* reference transcriptome, A. Ruggiero and C. Sinigaglia for sharing information on *in situ* hybridization optimization, Syunsuke Yaguchi for useful advice on immunofluorescence. We also thank our group members, “Neptune” network colleagues, Clare

Hudson and Hitoyoshi Yasuo for useful discussions. Work was supported by JSPS KAKENHI Grant Numbers 26440177 & 26840073, the French ANR (“OOCAMP”), the Marie Curie ITN “Neptune” and the Tokyo Institute of Technology GCOE program from JSPS (NT’s visit to Villefranche).

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SUPPLEMENTARY FIGURES

Che-pp1 (KX496947)

MERKILACFLLLVLLNLNDGKNIAILIEPDDNLASELEWLGSDMTDSHSLNAGAWPR
 PGDARSSHDAWPRPGKREFYGNEMFEKRPFPQGQMQFSWPRPGKKEETKEDTWP
 RPKRESYSEGDMDSRSGALRRSEEKETNEDEKLENAWPRPGKREFYASRKMDV
 RPRGGRDSSKSHKISKRNSEAINDEIDMMIREAWPRPGKRDYHMLSATRPRGGK
 DARPRGGKSSRPRGGKNAKPRGGKDSVRPRGGKDSWPRPGKDAFVKEINGS
 RPRGGKASKWPRPGKDLK

Predicted peptides: 7 x WPRPamide, 6 x RPRGamide, KPRGamide

Che-pp2 (KX496948)

MKIYFGCLFVILSVNQIGCYPSSNQNSERELVRRRIYKTVHPNPHYQVNEIQRVKEAL
 KRRVLENVHRVDSLKASLKRVLGQDAGNGFHMSSSKIFQKKRSKARLPHSYMFRK
 RQNSPGALGLWGREVEAPGDIGPPGIWGDVVPDET RKDKPGAVQGLWGKDERVI
 RALLKTLKR

Predicted peptides: QNSPGALGLWamide, KPGAVQGLWamide

Che-pp3 (KX496949)

MNCVLIFLVFLANNVYSASLTREEDALVTKLLDTIEKRD AVPRLGKREVPRLGREIE
 DVPRLGREIELVPRLGREAEVVPRLGREAEVVPRLGREVELVPRLGREVELVPRLG
 REVEVPRLGREVPRLGRGIDEVPRLGREVPRLGRELEVPRLGREVPRLGKREIPRL
 GKREVPRLGRREVPRLGREASTYDLKQLYNQLKREVSNDMIEAEIKEEKRVLKAFD
 LGTRGLILRRIGDKLNKDGFSQKRDMEKESSRPFLHVKRTNLLSLIEKLTSEE

Predicted peptides: 15 x VPRL, IPRL

Che-pp4 (KX496950)

MNLLVSIPVICAIVLKLTESAPISNVRKIGSNELLKLTVSDLAKLLSRLQNVHEDGHKE
 DLNKVSVEGMIADYLDEKQYKDRPRYGKDLKEASRPRYGKEMSEGGNHNVIEQLIE
 KLVNQSSSESDTKDDGNIKSDGKVDNLVSLHGLDEEKEWPRPGKDWPRAGKDWPR
 RAGKDWPRAGKDWPRAGKDWPRAGKDWPRPGKDWPNGIARGGKRSLGEILSDL
 MSKRPRYGKDESSTNGPSYQYTLEDMMTEIISGDRPRYGRKSEASRPRYGKESAD
 KTLEEALSNLGTKSTKKDTSSRSSLARGGKKRGVEDLILEYIENHLDDKKDYESARK
 DTPK

Predicted peptides: 5 x RPRYamide, 5 x WPRamide, 2 x WPRPamide, IKSDamide,
 IARGamide, LARGamide

Che-pp5 (KX496951)

MELKYFLASFIFVIAIQLASCSSKAE EYKQMKKEVDGLLKEIVSQENAKQHTSEKKS
SQWLNGRFGKRQLVSGRFRGRELKQWLNGRFGREATEQWLNGRFGKREDDQWLN
GRFGREVEQWLNGRFRDAAEQWLNGRFGKRSANQWLNGRFGKRSADQWLNG
RFGKRSADQWLNGRFRGASADQWLNGRFRGAADQWLNGRFRGAKEQWLN
GRFGREVEQWLNGRFRGAGQWLNGRFRGEMGQWLNGRFRGREADQWLNGRF
GREADQWLNGRFRDAAPLAARYGDEPAHVESQTIAAPEEAKPKIVA AVKVVKKPV
AVSE

Predicted peptides: 17 x QWLNGRFamide

Che-pp6 (KX496952)

MARESLFFLLALHCCEAFYNGDVPRRRSEASRLLMAKDASQKHASSDTSRLLFGK
DAPQRHVSSDSRLLFGKDAPQRHVLS DTSRLLFGKDAPQRHVSSDSRLLFGKDAP
QRHSSDSRLLFGKDAPQRHVSSDSRLLFGKDAPQRHVSSDTSRLLFGKDAPQRH
VSSDTSRLLFGKDVQRPAGGSDSRTSYTAHLMPTLGKDEYVNALKERLLNDYRM
KLLQQQRQQHQQQEDDDELYFDHSFRRGNPSSASAFRRRYS DQTGQPLSGSR
DQKVNQDENARDTLEKKQQASLDQTKEELKRSLLKDFYKKMITEKREKQEF AKKSD
APVSNDFDDEILRHLVEFKLKKEDPMKRMRR

Predicted peptides: 8 x SRLFamide

Che-pp7 (KX496953)

MRFCSWTNLFLLGITCLCLTN GMPNKQHVRNKKNLIDNTVKMADHGKTLVKKSAHP
MKIKDVSKKSTGGGSDIANSDDTFDRAADGTDNSLYGRQEKEQGTENSGVGKFEF
PPCRWGCGKREAGIDGPPGRWGGRKRGMRVGPGRWGGRKRGELPGRWGG
KKRGE LPGRWGGKKRSELPGHWGGKKRSELPGHWGGKKRSEIPGRWGGKKRS
EIPGRWGGKNRSELPLGWSQKEGNQRPPSKET

Predicted peptides: 2 x LPGRWGamide, 2 x GPPGRWGamide, 2 x IPGRWGamide,
2 x LPGHWGamide

Che-pp8 (KX496954)

MLSSETTIRIFCFFIAGVAVGSSSPEEEGQLLHVKRETWLNPGFDSMLHRRSESEL
LNRPRPGRRELFDLMNQDSILKKRALLHRPRPGRRELFNRNQLDSMLHKRAMLNR
PRPGRRELFNRNPLDSMLHKGQEF LSGPRPGRREIRPRPGRREIRHPDSMLHRRS
SDTDLDYQHLLRNPRPGRRELFRRPGRHRELQHLD SLLHRRSEEMWGRPRPGKR
E VYYEDDGRSEDEKLLRVLDELKRDIIDELWDRFQN

Predicted peptides: Predicted to produce 6 amidated peptides with the C-terminal
motif RPRP and two slight variants

Che-pp9 (KX496955)

MYLSVGFLFLCHQFQDTHGLSIRGPNDAQQLINSHGENDLPSGGMWETAKSQAM
ETYRKDSRRGIPKRGRSSFLAIGKKDDSLSLGSKMKKSLESPSLSVWRRGDSLHSI
LRVNP SLGIWKRGDSL MRRFGRKNFGKDNSRRSLVREIENDAFGMNRKDEFPSPG
MWEKAKSQYFGKREFRNSILGKHGKDEFSPAMWEKAKNQYFGKRDIRNSILGKH
GKDEFSPAMWEKAKNQYFGKGR

Predicted peptides: Predicted to produce 3 peptides with the C-terminal motif QYFamide and 2 ending LGKHamide. Possibly also an MRRFamide

Che-pp11 (KX496956)

MDQSLSSILLLLCCVALLTTCMSVQRKEAGDALSALDKENAKKSANSITEELARNLM
EHL YDEIRKRSNSNEETISNFRRASSDTHRQQQAPKGLWGRELQPGNPPGLWGRE
ASEAENTDSNDGPIPGMWGRREADDKNAHEKFQ

Possible peptides: PGLWamide, QQQAPKGLWamide, QPGNPPGLWamide, GPIPGMWamide

Cpa-pp1 (two variants identified in transcriptome assemblies: accession numbers KX496958 and KX496958)

MISKAIIVYSLVLLILPVYSKFPKSRFSIRGNQLDNGYPRGEIPKIKPDMKDRERSNLL
MKFAEALRILRTNQNYHDGKPYSKDIEEDFRKISSNRFNIRPRPGKRFLKNKFAACD
EHFANE

Predicted peptides: 1x RPRPamide

MISKAIIVYSLVLLILPVYSKFPKSRFSIRGNQLDNGYPRGEIPKIKPDMKDRERSNLL
MKFAEALSILRTNQNYHDGKPYSKDIEEDFRKISSNRFNIRPRPGKRFLT NK FVGCD
EHFEN

Predicted peptides: 1x RPRPamide

Cpa-pp2 (KX496959)

MAMNITFISICFAISIILLNVQSAPLDDIRERSIEHEMAQNRQRESLVHLLTLGYS LGAR
DDSNTLSKLITYVDNLKDDVWNTDSEGCRNIETTL YSDLLSQPNYDLTDLESRELD T
YENERPTAGKRFSSMNIIDNDSFRPRAGRRDIRPRAGRREFRPRAGKRYLAESSEF
DAYRPRAGKRELRPRAGKREDEDNEKMRDTANRQSQREVLKRNSIYKELMTANES
NERPRAGKRETTETDEGKHDMSTFLEINSDKREERPRAGKKELRPRAGKKELRPRA
GKKE

Predicted peptides: 9x RPRAamide

Cpa-pp3, GLWamide precursor (KX496960)

MKTTMLYHTVLCICVINIYNGK FVY TENAIDEKELTDLKDLQLDAESNQIQSRITKDL
SRILADKIYNQLKESTNTEFKKNLDFQFQPEIGSFNGKRPLPKPPGLW **GKD** VHSR
FQIDSHDISIKSSGDISKREAEKRKLKNNLKSDDASKYADYINMDNKRSEILNGPPGL
W **GRE** AEKLN GPPGLW **GRDE** KVNDALNGKLGKVHEPRDLLKRENGKVNGPPGLW
GRE IGKRN GPPGLW **GRDA** EMNNGPPGLW **GRDAE** KVNGPPGLW **GRDIGK** INAPSS
LQDHTFEKNR GPPGLW **GREI** EKVNGPPGLW **GREI** EKNN GPPGLW **GRE** LGMNNGP
PGLW **GRD** FTTIKENGDNDATRKEKPTDTKMENS DKKGGFGQKDDSKYNAKWKRN
ERNLLKDRNQIETQKRESSRKESSADKQIKNRNSKDSKTAKKDSRNDVTIDNIFPW
HKSKHDDNTASNLRDIIENEF

Predicted peptides: 11x PPGLWamide

Cpa-pp4, RFamide precursor (KX496961)

MNLILPIFASILLCLADSASITTKK DSENDVERQIETLLNELLEGADNNLYDKKEVDQW
LKGRF **GRE** SSDQWLKGRF **GRD** ADQLLKGRF **GRE** AEQWLKGRF **GRE** AGDQWLK
RF **GRE** AEQWLKGRF **GRE** AGQWLKGRF **GRE** AEQWLKGRF **GRE** VEQWLKGRF **GR**
E TEEAEQWLKGRF **GRDA** ITKDSNPLLRGRY **GRD** SESEVTENAPKQLDRGRY **GRE**
QWLKGRF **GRE** AQSENETKKEIDNANKKVDVNAKKEVSKTAM*

Predicted peptides: 9x QWLKGRFamide, 2X RGRYamide

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.

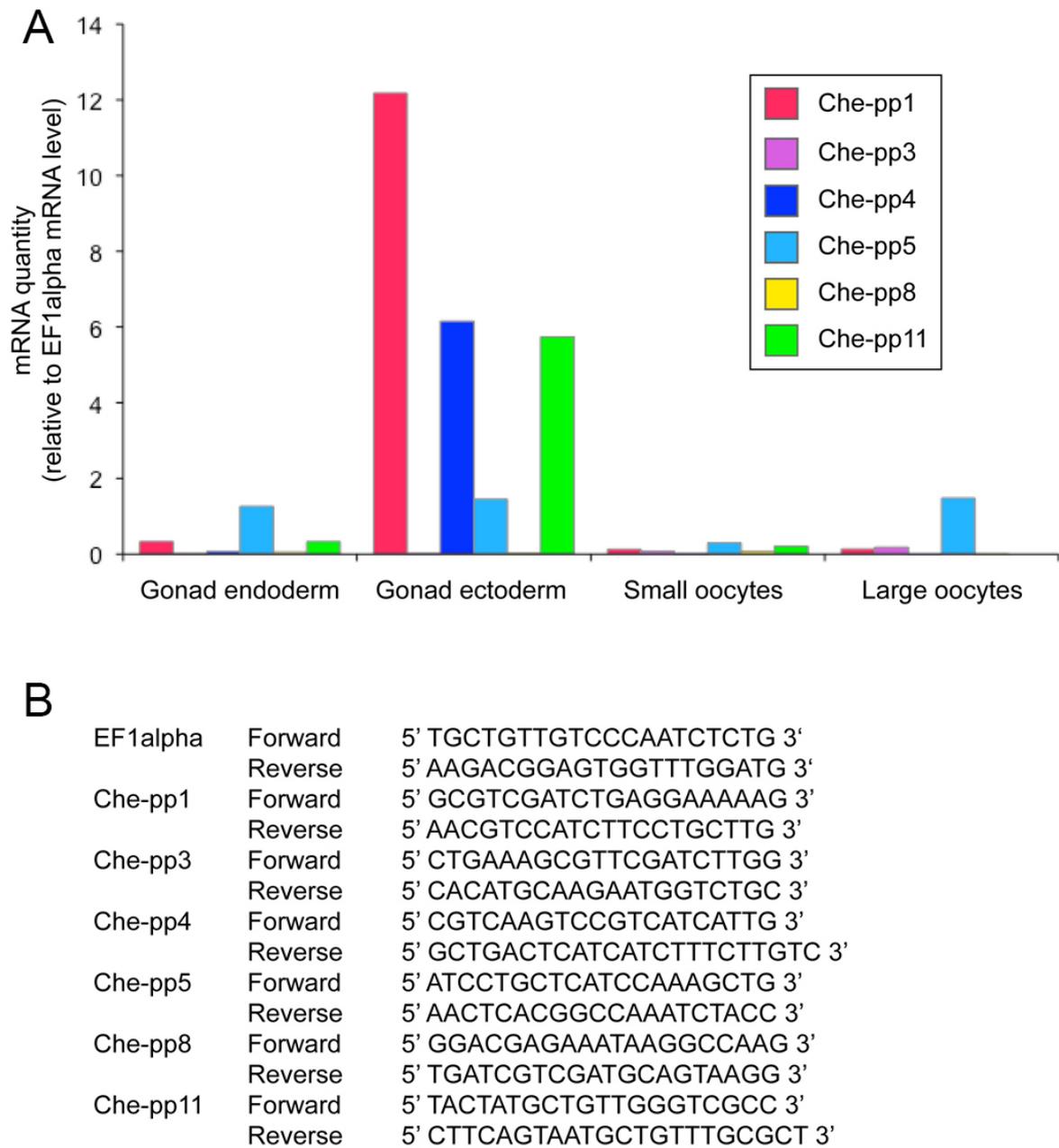


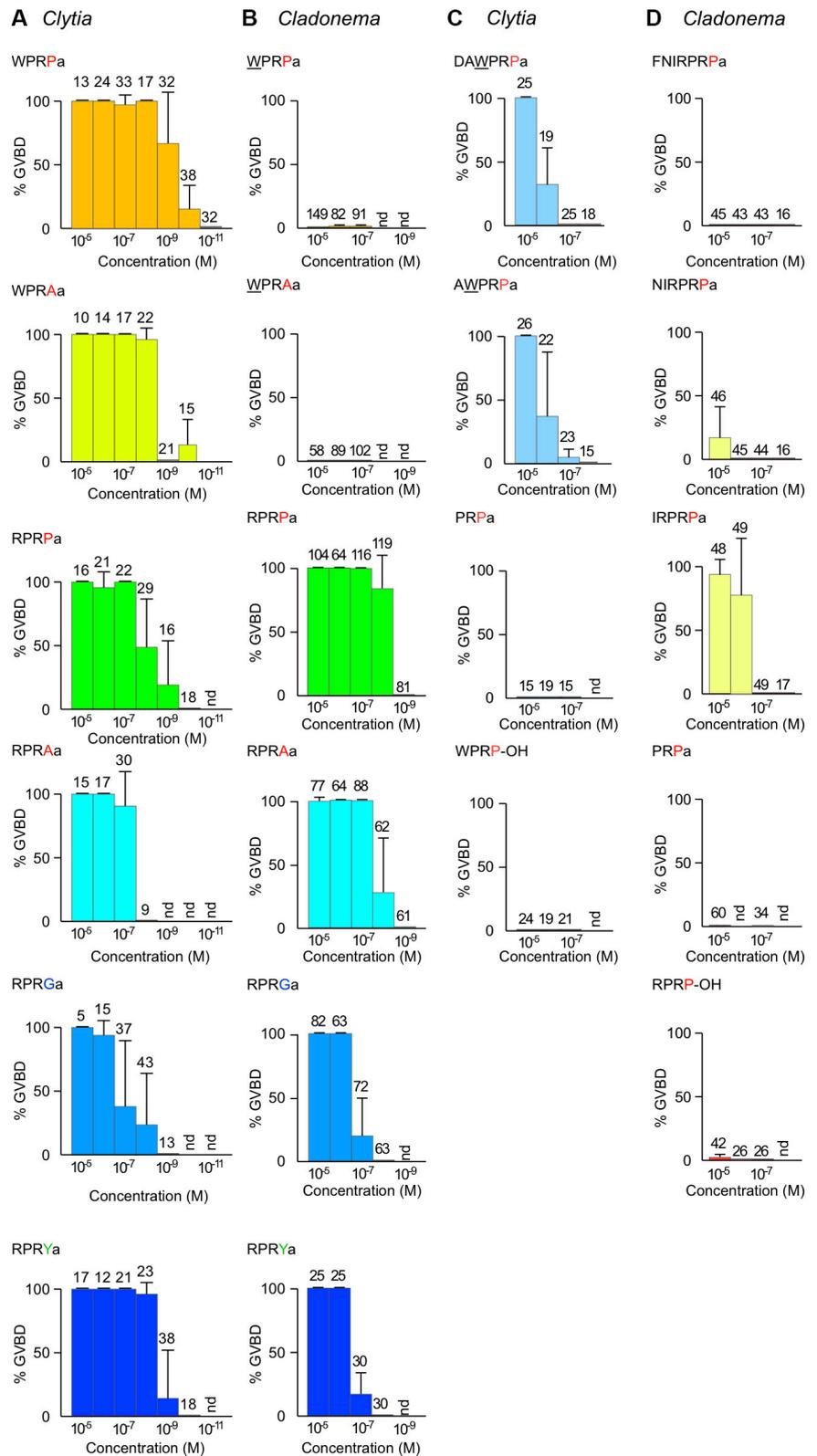
Figure S2. Quantification of neuropeptide transcript levels between gonad tissues.

A) Quantitative RT-PCR analysis of neuropeptide precursor expression in manually separated tissues from *C. 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.

Figure S3. MIH activity of synthetic amidated peptides.

Clytia (columns A and C) or *Cladonema* (columns B and D) oocytes were incubated in ASW 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, D.



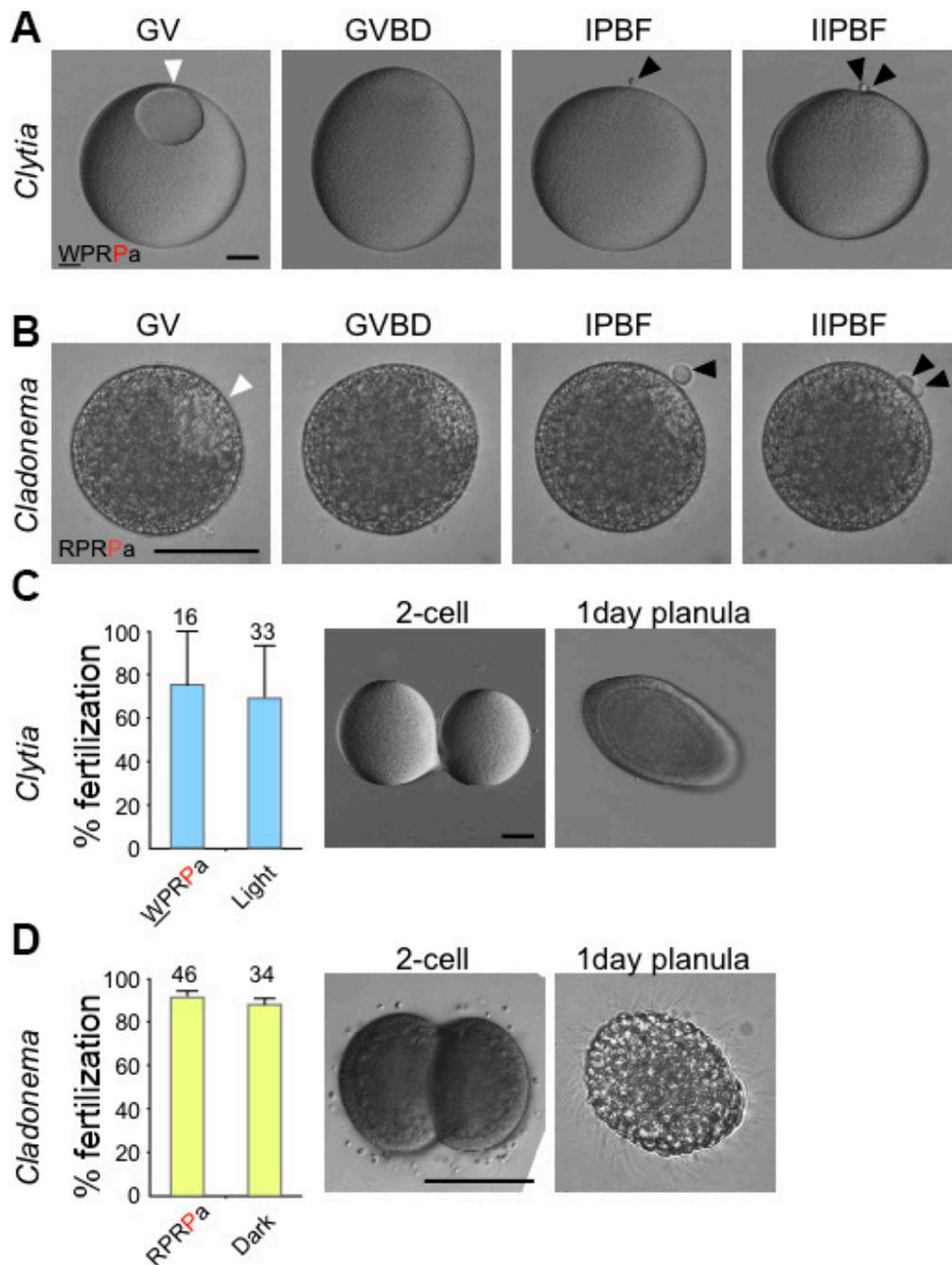


Figure S4. Synthetic tetrapeptides induce normal oocyte maturation.

A, B) Images of *Cladonema* and *Clytia* oocytes respectively, incubated in $10^{-7}\mu\text{M}$ W/RPRPamide as indicated, undergoing successive steps of meiosis: GVBD; First polar body formation (IPBF); Second polar body formation (IIPBF).

C) Fertilization 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).

D) Equivalent fertilization success documented for *Cladonema* eggs incubated in 10^{-7} M RPRPamide. Scale bars: 50 μm . White and black arrowheads indicate GV and polar bodies, respectively.

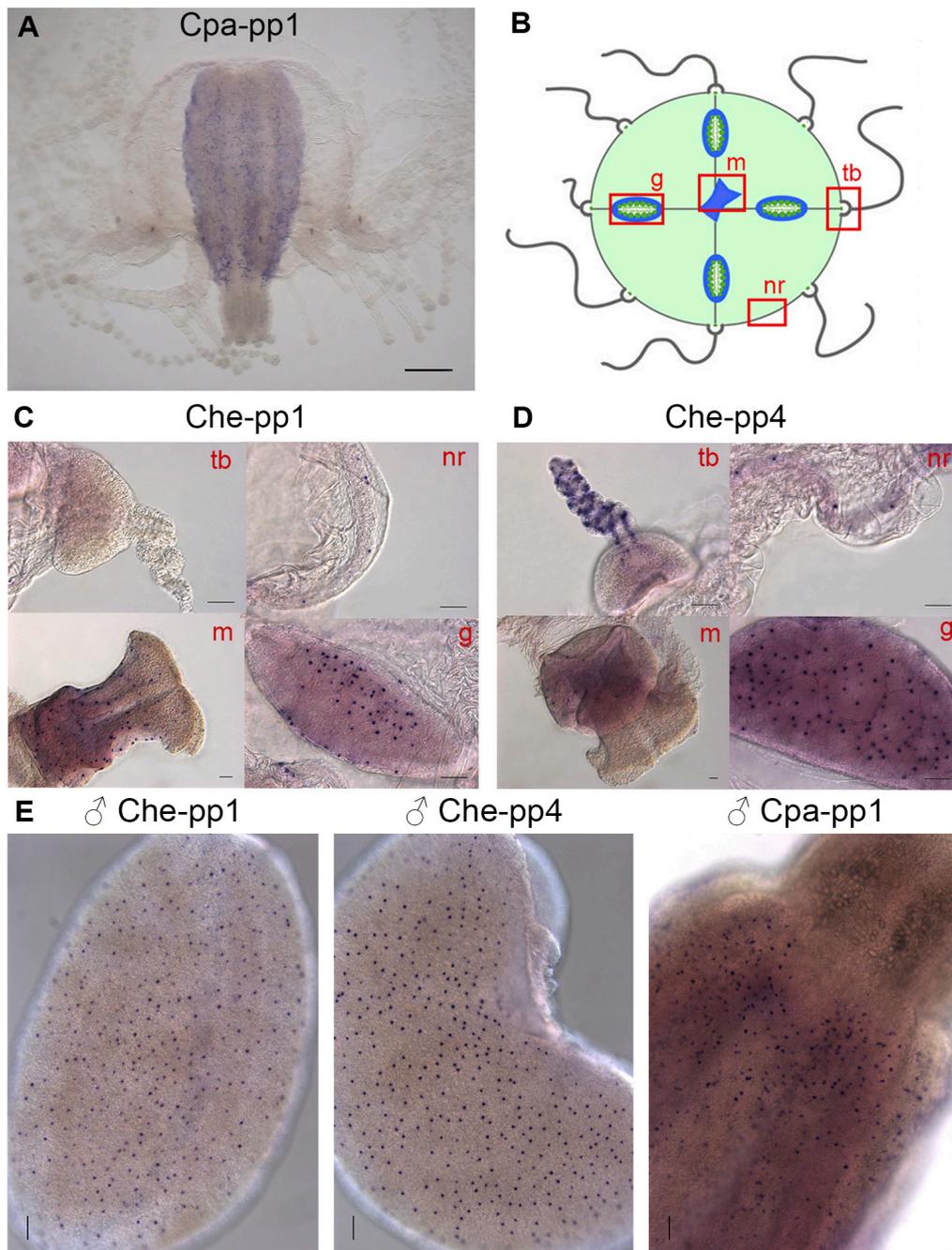


Figure S5. Distribution of MIH-expressing cells detected by *in situ* hybridization.

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* hybridization detection of Che-pp1 and Che-pp4 in different structures of young *Clytia* jellyfish as indicated in red. Both these precursors are expressed in the gonad ectoderm and nerve ring, but in the manubrium mainly Che-pp1 is detected, and in the tentacle Che-pp4.

E) *In situ* hybridization detection in male gonads from *Clytia* and *Cladonema* showing Che-pp1, Che-pp4 and Cpa-pp1 expression in scattered ectoderm cells. Scale bars: 500 μ m in A; 50 μ m in C-E.

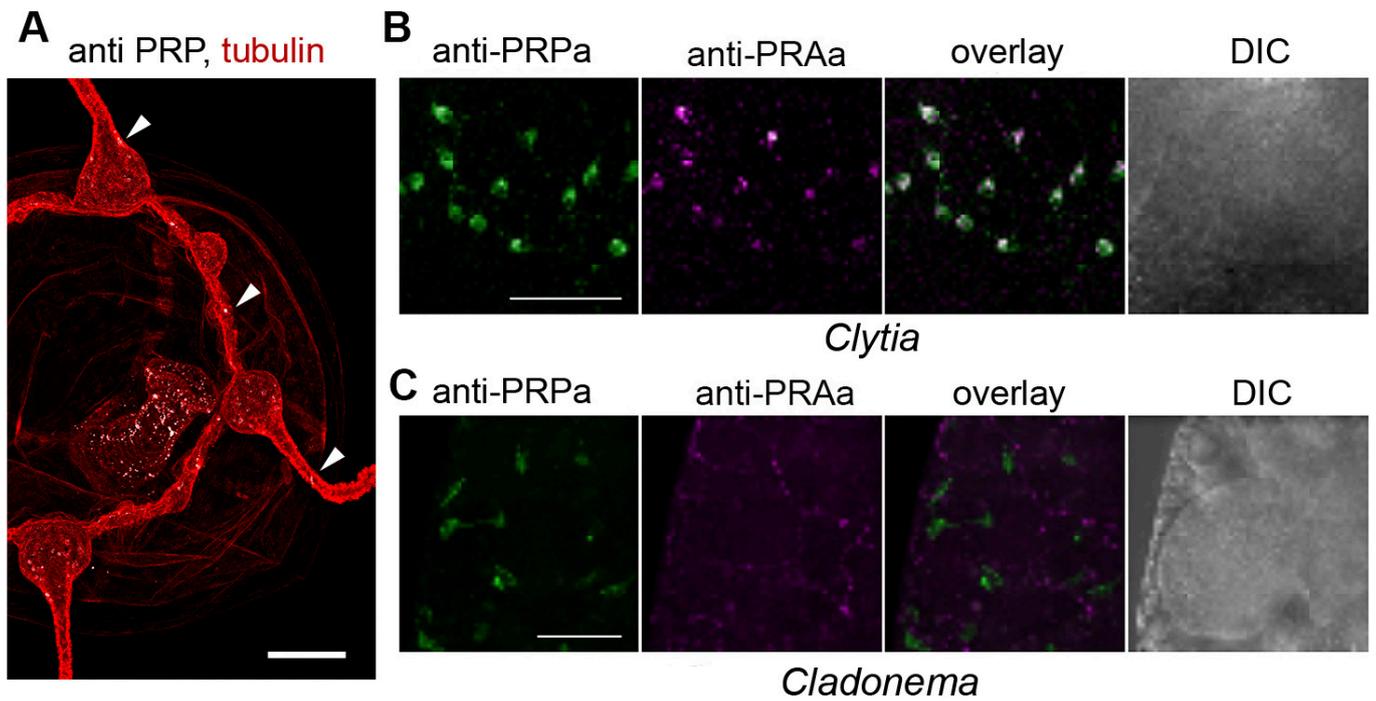


Figure S6. MIH distribution in *Clytia* jellyfish and MIH-immunopositive cell populations in *Clytia* and *Cladonema* gonad ectoderm.

A) Confocal image (summed Z stack) of a *Clytia* baby jellyfish following immunofluorescence performed with anti-PRPamide and anti-Tyr 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 μ m.

Conclusions, Additional Results and Perspectives

The study documented in this manuscript showed that MIH in *Clytia hemisphaerica*, and also in *Cladonema radiatum*, are neuropeptides with the amino acid signature W/RPRPamide (or closely related sequences). We demonstrated that these neuropeptides are synthesized in scattered cells in the gonad ectoderm and secreted upon dark-light or light-dark transitions, depending on the species. We also showed the ability to trigger male spawning, implying that the same neuropeptides account for both female and male gamete release. The reactivity of other hydrozoan species oocytes to *Clytia* and *Cladonema* MIH suggests that amidated neuropeptides might be the endogenous MIH in Hydrozoa. Our findings represent the first identification of MIH in a cnidarian.

These findings opened a path for further studies on *Clytia* MIH reception and the downstream cascades that lead to oocyte maturation, as well as on the upstream pathways coordinating gonad light reception to MIH release, as described in chapters 2 and 3. They also could serve as a basis for further analysis of the organization and function of *Clytia* nervous system. For instance, the distribution and function of GLWamide family neurons and their relation with PRPamide neurons is of particular interest.

Neuropeptides of the GLWamide (GLWa) family have been broadly documented in cnidarians, and evidence shows that cnidarians employ them to regulate different biological processes. To illustrate some examples, in *Hydra*, GLWa peptides can induce elongation of the body column as well as bud detachment from the founder polyp. In the anthozoan *Anthopleura elegantissima*, peptides from the GLWa family trigger contraction reactions of the retractor muscle (reviewed by Takahashi and Takeda, 2015). Another described function of GLWa peptides in cnidarians is the induction of planula larval metamorphosis into polyp. This life cycle transition induction by GLWa peptides has been described in *Hydractinia echinata* and in coral larvae from the genus *Acropora* (reviewed by Takahashi and Hatta, 2011; Takahashi and Takeda, 2015). Accordingly, a dense population of GLWamide-immunoreactive neurons was detected in the planula larva of the hydroid *Clava multicornis*, with the highest number localized at its most anterior part (Piraino et al., 2011). The precise mechanisms of GLWa family peptides involvement in cnidarian metamorphosis induction are not clear. In *Hydractinia*, larvae metamorphosis can be triggered in response to chemical stimuli secreted by bacteria in the surrounding environment

(Takahashi and Takeda, 2015). This raises the intriguing question of whether GLWa neurons are chemosensory and have a dual sensory-neurosecretory function, directly regulating larval settlement and metamorphosis induction when environmental conditions are appropriate (e.g. nutrient-rich), or whether this process is mediated by a more complex system involving more types of neurons besides the GLWa ones. Neuropeptides showing sequence similarities to cnidarian GLWamides were found in the annelid *Platynereis dumerelii* to regulate larval settlement, suggesting a conserved role for this neuropeptide family in mediating life-cycle transitions (Conzelmann et al., 2013b; Schoofs and Beets, 2013).

In the context of spawning regulation, it was shown that GLWa neuropeptides could trigger spawning in the hydrozoan *Cytaeis uchidae*, and GLWa-immunopositive neurons were found in its gonad ectodermal epithelia (Takeda et al., 2013). This raises the intriguing possibility of a relationship between MIH-secreting cells in *Clytia* gonads and GLWa neurons in the regulation of oocyte maturation and spawning.

GLWa family neuropeptides and their relation to spawning regulation

As mentioned above, previous studies showed that some GLWa family peptides can induce spawning in the hydrozoan *Cytaeis uchidae*, albeit at high concentrations relative to the MIH PRPamide family peptides. Importantly, GLWamides were shown to require the presence of somatic gonadal tissues in order to induce oocyte maturation (Takeda et al., 2013). These findings suggest that GLWa-secreting neurons participate indirectly in regulating oocyte maturation. For instance, they could allow coupling of the regulation of MIH secretion with other environmental factors such as food availability.

In *Clytia*, our RNA-Seq analyses revealed the expression in the gonad ectoderm of a neuropeptide precursor coding for neuropeptides of the GLWa family (i.e. pp11 – see figure S1 in this chapter manuscript). An artificially synthesized tetrapeptide predicted from pp11 (PGLWamide) did not induce oocyte maturation and spawning in isolated *Clytia* gonads at any concentration (see Figure 2 in this chapter manuscript). However, it is possible that the biologically active neuropeptides cleaved from the pp11 precursor have a longer amino acid sequence that we have not assessed. In order to locate the cells expressing pp11 in *Clytia* medusae and to understand their relationship with the MIH neuropeptide precursors (pp1 and pp4), I performed *in situ* hybridizations with pp11 antisense probes in young *Clytia* jellyfish and in isolated adult gonads, as well as double fluorescent *in situ*

hybridization of pp11 and pp4 in isolated gonads (**Figure 20**). pp11 mRNA was detected in scattered cells in the young jellyfish gonads, manubrium, and most strikingly associated with the circular and radial endodermal canals. In the adult gonads, pp11 expression was mainly detected in a few sparse cells positioned at the junction between the gonad and the radial canal. The double fluorescent *in situ* hybridization showed that it was not co-expressed with the pp4/pp1 (i.e. MIH) expressing neural population.

To visualize in more detail the morphologies of the cells and their relationship to one another I performed immunofluorescence on *Clytia* gonads with antibodies recognising PRPa and LWa antigens (kindly provided by Dr. Takeda and Dr. Koizumi, respectively). This confirmed the presence of two different neural populations in the gonad, the abundant ectodermal MIH-secreting cells, and a few LWa-immunopositive neurons positioned near the junction of the gonad with the radial canal (**Figure 20**). The LWa-immunopositive neurons showed long projections (generally longer than those exhibited by the PRPa-immunopositive neurons) mainly along the junction of the gonad with the radial canal, with a few projections directed towards the main body of the gonad. From this analysis it was not clear whether both PRPa and LWa neural populations were interconnected. It would be of interest to repeat the same immunofluorescence with whole medusae, since the structure of the gonad junction with the radial canal would be intact and it should allow a better clarification of the relationship between these two neural populations at the gonad level.

These additional results show that the cells expressing pp11 can be found in different sites of the jellyfish, possibly relating to the utilization of GLWa family neuropeptides for various functions in *Clytia* medusae. They also show that at least two different populations of neural cells co-exist in *Clytia* gonads and that each of them synthesizes a different family of neuropeptides.

On the basis of these preliminary observations I would like to propose some scenarios about the possible relationship between GLWa, MIH and oocyte maturation in *Clytia*. A possible scenario would be that GLWa-positive neural cells in the gonad might be light sensitive and secrete GLWa neuropeptides upon light stimulation. These neuropeptides would then stimulate the MIH-secreting neural cells to induce MIH release. Another possibility would be that GLWa-positive neural cells release GLWa neuropeptides in response to other environmental or physiological signals of different nature, and input into the MIH-secreting cells to favour MIH secretion in response to light (i.e. provide a facultative signal). Since

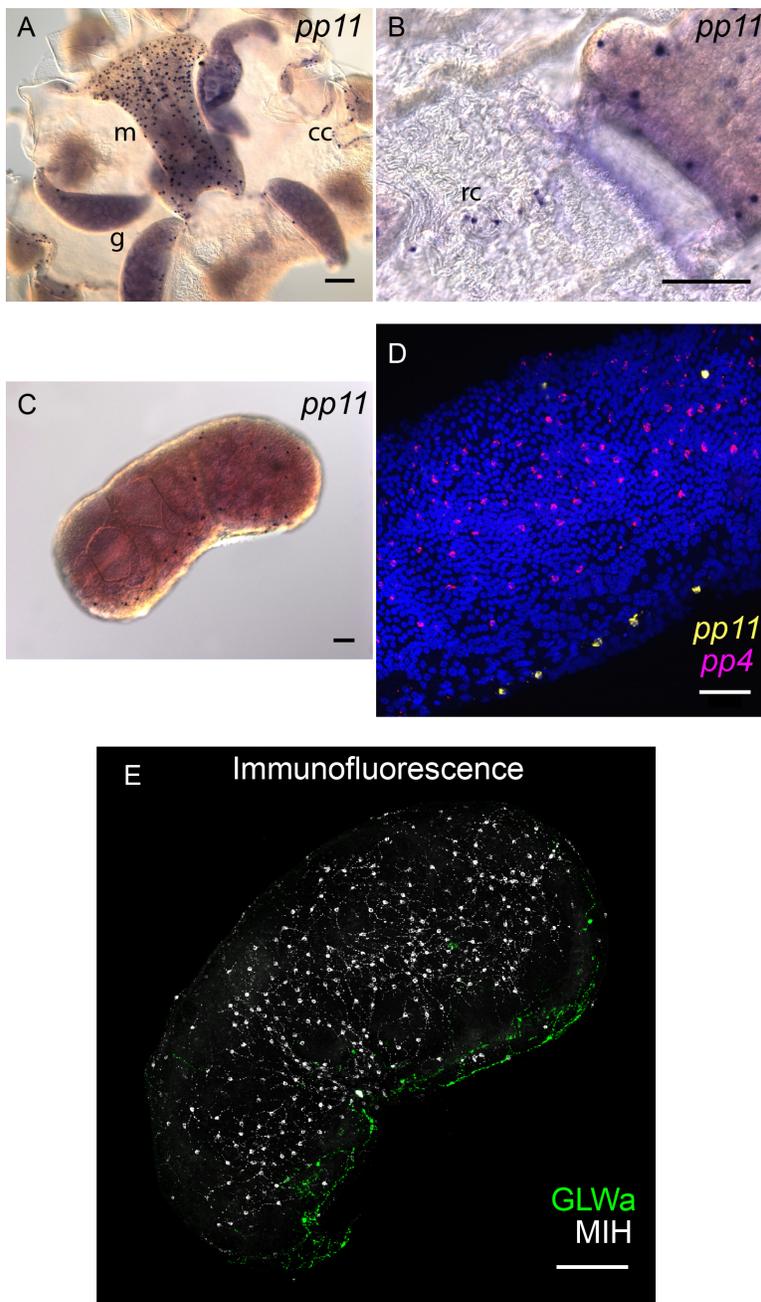


Figure 20. Expression of GLWamide neuropeptides in *Clytia* jellyfish .

(A-B) *In situ* hybridization detection of mRNAs for the GLWamide precursor pp11 in different structures of young *Clytia* jellyfish. pp11 expression is detected in scattered cells at the manubrium (m), circular canal (cc), radial canals (rc) and gonads (g).

(C) *In situ* hybridization detection of pp11 expression in adult *Clytia* gonad showing a sparse distribution mainly close to the radial canals.

(D) Double fluorescent *in situ* hybridization detection of pp11 and pp4 in *Clytia* adult gonads. The expression pattern of each proneuropeptide gene shows two independent cell populations.

(E) Immunofluorescence of *Clytia* female gonads showing two independent populations of neurons, the MIH-immunopositive neurons and the GLWa-immunopositive neurons. Staining with anti-PRPamide (white) and anti-GLWamide (green).

Scale bars: (A, E) = 100 μ m; (B-D) = 50 μ m.

they are mainly located in the junction of the gonads with the endodermal radial canals, the GLWa cells might be sensitive for instance to chemical stimuli informative of the nutrition level and availability, and secrete the GLWa neuropeptides when these factors reach an optimal state, similarly to how vertebrates integrate metabolic signals to control the reproductive system through neuropeptides (Shahjahan et al., 2014; Wójcik-Gładysz and Polkowska, 2006). This could help tuning MIH secretion in addition to environmental light changes and favour oocyte maturation and spawning events when the radial canals of the jellyfish are nutrient rich. This idea would be consistent with the hypothesis that GLWa neural cells in cnidarian larvae might be chemosensory to nutrition levels.

As possible experiments to explore the mentioned scenarios in *Clytia*, the fluorescence of LWa-immunopositive neural cells could be compared between light-adapted gonads and gonads that have undergone a dark-light transition (similarly to the experiments done for MIH quantification in chapters 2 and 3). This would be informative to assess if GLWamide family neuropeptides, like the MIH peptides, are released upon light stimulation after a period of dark, (see Figure 4 in this chapter manuscript). Another possible experiment would be to check whether overfed female *Clytia* jellyfish are able to spawn more than once a day under normal day-night cycles when compared to jellyfish maintained under the standard feeding regime in our culture system, which only spawn after dark-light transitions. If this were the case, checking the GLWamide neuropeptide secretion at the gonad level by immunofluorescence quantification (see above) in parallel with MIH secretion, after a second spawning event during the same day, would be very informative to assess a possible relationship between nutrition levels, GLWa neuropeptides and MIH in *Clytia*.

CHAPTER 2

Identification and characterization of the *Clytia* MH Receptor

Context

The molecular identification of *Clytia* MIH opened the way to study other steps of oocyte maturation initiation. Specifically, it provided a route to identify the MIH receptor in *Clytia* oocytes.

We had several reasons to hypothesize that *Clytia* MIH receptor could belong to the superfamily of G protein-coupled receptors (GPCRs), seven transmembrane domain proteins located at the plasma membrane that transduce extracellular signals into the intracellular milieu (Audet and Bouvier, 2012; Kristiansen, 2004). 1)

Experiments reported in Chapter 1 demonstrated that MIH (PRPamides) injected in the oocytes did not induce oocyte maturation whereas external application could indeed trigger meiosis resumption, implying that a presumptive MIH receptor should be located on the oocyte plasma membrane.

2) In mice, amphibians and fish, GPCRs have been shown to be involved in the regulation of meiosis resumption (Haccard et al., 2012; Mehlmann et al., 2004; Nagahama and Yamashita, 2008) (see section II of introduction).

3) An endogenous rise in cAMP concentrations in the oocyte cytoplasm has been documented at the onset of maturation in *Cytaies uchidae* (Takeda et al., 2006). For several classes of GPCR, cAMP commonly acts as a second messenger, with cytosolic cAMP levels rising through adenylyl cyclases activity stimulated downstream of GPCR activation. This is illustrated by the case of the human β 2 adrenergic receptor (Pierce et al., 2002).

4) We now know that *Clytia* MIHs are short neuropeptides, and these generally signal through (a large subgroup of class-A) GPCRs (Bauknecht and Jékely, 2016; Bauknecht and Jékely, 2015; Jékely, 2013; Mirabeau and Joly, 2013).

Background on GPCRs

GPCRs represent the largest and most ubiquitous family of transmembrane proteins in the animal kingdom, present across phyla including sponges (Jékely, 2013; Kobilka, 2007; Krishnan et al., 2014; Mirabeau and Joly, 2013). They mediate cellular responses to a wide variety of environmental and physiological stimuli, such as light, neurotransmitters, lipids or hormones (Audet and Bouvier, 2012; Kobilka, 2007; Kristiansen, 2004; Zhang et al., 2015). Ligand-GPCR interactions regulate a vast number of biological processes, from neuromodulation to cell differentiation, and are thus a rich source of targets in pharmacology (Civelli et al., 2012; Gloriam et al., 2009).

GPCRs (or more correctly 7TM proteins as not all are associated with G proteins) share a universal structural architecture, exhibiting a seven transmembrane helical array connected by three intracellular loops and three extracellular loops, an extracellular NH₂-terminus and an intracellular COOH-terminus (**Figure 21**). Members of the GPCR family share greatest sequence homology within the transmembrane segments, the most variable structures being the NH₂- and COOH-termini as well as the intracellular and extracellular loops. The NH₂-terminus is the region with highest variability among different GPCRs; its length and structure can help define different GPCR subfamilies (reviewed by Kobilka, 2007; Kristiansen, 2004; Zhang et al., 2015).

Members of the 7TM/GPCR superfamily have been grouped in five or six classes using different criteria such as phylogenetic analyses, the use of their native ligands or gene clustering in the human genome (Audet and Bouvier, 2012; Kristiansen, 2004; Schiöth and Fredriksson, 2005). Most GPCRs belong to class-A (rhodopsin-like), B (secretin-like) and C (metabotropic glutamate-like), defined by the conservation of singular structural features (Audet and Bouvier, 2012; Kristiansen, 2004). Each main class is further subdivided into several subfamilies, class-A being the most diverse and numerous, comprising opsins, biogenic amine receptors, odorant receptors, protease-activated receptors, small peptide receptors and glycoprotein receptors. The last of these two together are referred to as neuropeptide class-A GPCRs (Jékely, 2013; Kristiansen, 2004), although some neuropeptide hormones like glucagon, secretin and vasoactive intestinal peptide interact with class-B GPCRs (Kristiansen, 2004). Class-A neuropeptide GPCRs form the largest subgroup, mediating a large range of physiological functions in neuromodulation and neuroendocrine regulation.

GPCRs commonly signal through heterotrimeric guanine nucleotide-binding proteins (heterotrimeric G proteins), composed of α -, β - and γ -subunits (**Figure 21**). Alpha subunits are very diverse, and are classified in various families and subtypes. They are regarded as the core of the G protein, and the name given to the G proteins is usually based on the $G\alpha$ -subunit family. When active, each subtype interacts with different effectors, or with the same effector in an antagonistic manner, leading to varied cell activities. Beta and gamma subunits are intimately associated and can be considered as one functional unit, also mediating cellular responses when activated (reviewed by Cabrera-Vera et al., 2003). G proteins are bound to cytoplasmic domains of a GPCR when it is in an inactive state, and the $G\alpha$ -subunit of the G protein is bound to guanosine diphosphate (GDP). Ligands of diverse molecular nature and size can interact with various binding sites on the GPCR to regulate its

activity. In the canonical model of GPCR signalling, a ligand-bound receptor activates the cytoplasmic G proteins via nucleotide exchange reaction from GDP to guanosine triphosphate (GTP) on the $G\alpha$ -subunit. This causes the release of the $G\beta\gamma$ subunit from the GTP-bound $G\alpha$ -subunit and of both of them from the receptor. Consequently, active G proteins transmit the signal to effectors, such as enzymes or ion channels, which promote the intracellular reactions that lead to cell responses (Audet and Bouvier, 2012; Cabrera-Vera et al., 2003; Kristiansen, 2004; Pierce et al., 2002) (**Figure 21**). Signalling termination occurs when GTP is hydrolyzed back to GDP by the intrinsic GTPase activity of the $G\alpha$ -subunit, a process that can be aided by regulatory proteins. The $G\alpha$ -subunit thus associates again with the $G\beta\gamma$ -subunit and the inactive heterotrimer with the receptor (Cabrera-Vera et al., 2003; Kristiansen, 2004).

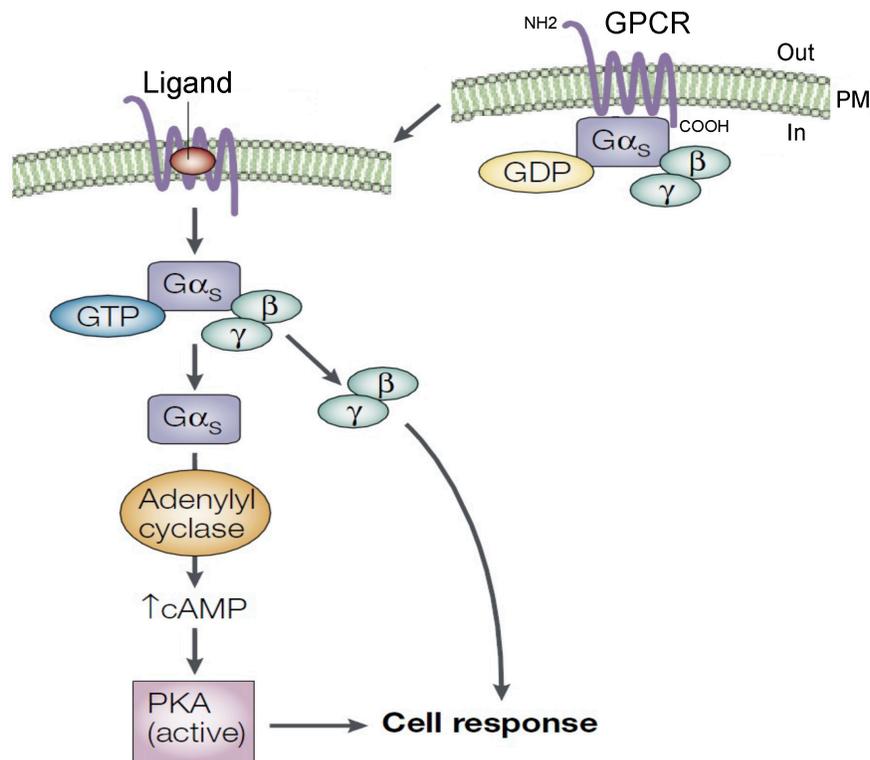


Figure 21. Schematic representation of a GPCR signalling event. In the canonical model, the GPCR remains in an inactive state in the absence of a ligand. After ligand binding, a transient complex of ligand-bound GPCR and G protein is formed. GDP is released from the $G\alpha$ -subunit and is replaced by GTP, activating the G protein. This leads to the $G\alpha$ -subunit and $\beta\gamma$ dimer dissociation, both activating different effectors, leading to cellular responses. In this example, the $G\alpha$ -subunit activates adenylyl cyclase, which increases the intracellular cAMP and in turn activates PKA, leading to phosphorylation of many different substrates. PM = plasma membrane; Out = extracellular milieu; In = intracellular milieu. Modified from Pierce et al., 2002.

Regulation of GPCR signalling is generally more complex than the canonical model. GPCRs ligands can act as agonists, antagonists or partial agonists, regulating GPCR activity in different ways (Kobilka, 2007). Individual receptors can engage multiple G proteins with varying kinetics and efficacy (Cabrera-Vera et al., 2003; Masuho et al., 2015) and it has been suggested that the diverse effects of a particular GPCR on cellular physiology might be determined by its varied engagement to multiple G proteins (Masuho et al., 2015). Some GPCRs can signal independently of G proteins, through direct interactions with other effectors, like β -arrestin (Audet and Bouvier, 2012; Rajagopal, 2005). In some cases, GPCRs can even dimerize, although the functional basis is still not clear (Audet and Bouvier, 2012).

Cnidarians have large and diverse GPCR repertoires, including phylum-specific subgroups (Anctil et al., 2007; Forêt et al., 2010). Almost nothing is known, however, about the function of any cnidarian GPCR, and no ligand-GPCR couple has yet been validated. Based on sequence similarity and phylogenetic analyses, some likely cnidarian orthologs of glycoprotein receptors (Roch and Sherwood, 2014) and odorant receptors (Churcher and Taylor, 2011) have been identified in anthozoans. A group of cnidarian GPCRs was found to have sequence similarities with chordate insulin-like peptide relaxin receptors (Jékely, 2013). Opsin family GPCRs are widespread in cnidarians (Feuda et al., 2012), as discussed in detail in chapter 3. Four GPCRs were found in the coral *Acropora millepora*, distinct from any member of bilaterian rhodopsin class GPCRs, suggesting the formation of new GPCR groups in cnidarians (Anctil et al., 2007). Even though most neuropeptides bind to GPCRs, ligand-gated ion channels can be activated by neuropeptides in cnidarians, giving fast responses (Grimmelikhuijzen and Hauser, 2012). No neuropeptide GPCR has been yet characterized in cnidarians.

The results described in the following manuscript document the identification and characterization of the MIH receptor in *Clytia* oocytes from a list of candidate GPCRs.

Overview of the findings of the study

Briefly, the findings presented in this manuscript are as follows:

- A list of putative GPCR candidates was compiled from *Clytia* transcriptome data on the basis of expression in oocytes combined with neuropeptide class-A GPCR sequence similarities.
- ORFs from the top 16 shortlisted candidates were cloned in a specific vector, and their ability to bind *Clytia* MIH neuropeptides was assessed by a cell culture-based GPCR deorphanization assay. Only one GPCR was activated by *Clytia* MIHs, showing similar concentration-dependent reactivity to MIH as *in vivo* oocyte maturation assays with artificial MIH. This result confirmed the identification of *Clytia* MIH receptor (MIHR).
- CRISPR/Cas9 gene editing technique was used to invalidate *Clytia* MIHR in order to test its *in vivo* function. One highly mutant strain (*CheMIHR* KO) displayed hampered polyp growth although could generate mutant jellyfish.
- *CheMIHR* KO jellyfish were unable to spawn upon dark-light transitions and displayed grossly inflated gonads full of immature oocytes due to spawning incapability.
- *CheMIHR* KO isolated gonads and oocytes were unable to react to artificial MIH and induce oocyte maturation and consequent spawning; a phenotype that could be rescued using cAMP analogues, acting downstream of MIH reception.
- Phylogenetic analyses showed that *Clytia* MIHR is related to a subset of bilaterian neuropeptide hormone receptor families involved in various physiological processes, including neurohormonal regulation of reproduction.
- *In situ* hybridization preliminary analyses showed the expression of *CheMIHR* in non-gonadal sites, notably in tentacle cells.

PhD student contribution: Pascal Lapébie performed the bioinformatics analyses shown in Figure S2, as well as the RNA-Seq mapping (see preface). Lucas Leclère carried out the clustering and molecular phylogeny analyses shown in Figure 4. I performed the rest of the experimental work, prepared the rest of the figures and drafted the following provisional manuscript, which will be completed following some additional experiments to characterize G α involvement downstream of the receptor and to describe better MIHR expression at other sites in the medusa (described in the Conclusion and Perspectives section of this chapter). The GPCR deorphanization assays were performed during a visit to G. Jékély's laboratory (MPI, Tübingen), with the help, advice and surveillance of Philipp Bauknecht, and that CRISPR-Cas9 gene KO was performed with the help and advice from Tsuyoshi Momose.

Identification of a cnidarian oocyte maturation hormone receptor uncovers a conserved GPCR family regulating animal reproduction

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Key words: GPCR, oocyte, reproduction, neuropeptide, *Clytia*

Short title: A cnidarian oocyte maturation hormone receptor

ABSTRACT

Maturation-inducing hormones (MIHs) act directly on ovarian oocytes to trigger meiosis completion and oocyte maturation, a process critical for successful fertilization and thus for sexual reproduction. Bona fide MIHs are known in only very few animal species and no MIH receptor has yet been unambiguously characterized. Here, using the molecularly accessible hydrozoan jellyfish *Clytia hemisphaerica*, we identified an oocyte MIH receptor (MIHR) amongst selected G protein-coupled receptor (GPCR) candidates using a calcium-aequorin-based “GPCR deorphanisation” assay, with the amidated tetrapeptides that comprise *Clytia* MIH as ligands. The essential *in vivo* function of *Clytia* MIHR was confirmed by CRISPR/Cas9 gene editing to introduce a frame-shift mutation into the gene. Resultant *MIHR* mutant

Clytia generated sexually mature jellyfish in which immature oocytes accumulated abnormally and failed to spawn following the natural dark-light cue. They also showed defects in growth and swimming, relating to MIH neuropeptide function in non-gonad neural cells. Confirming the essential oocyte function of MIHR, maturation of *MIHR* mutant oocytes could not be triggered by synthetic MIH, but could be restored using analogues of cAMP, an essential downstream mediator acting downstream of MIH reception. Amongst the large GPCR repertoire in cnidarians, *Clytia* MIHR is the first to be “deorphanised”. Its sequence clustered in phylogenetic analyses with a subset of bilaterian neuropeptidic hormone receptor families, supporting the existence of a conserved GPCR family regulating animal reproduction.

INTRODUCTION

Oocyte meiotic maturation is the key process that transforms the quiescent, fully grown, meiotic prophase I-arrested oocyte into an ovulated egg ready for fertilization (Verlhac and Terret, 2016; von Stetina and Orr-Weaver, 2011). It involves activation of the Cdk1-CyclinB complex (known as M-phase promoting factor or MPF) to resume meiosis and promote entry into M phase, accompanied by germinal vesicle breakdown (GVBD), chromosome condensation, cortical cytoskeleton reorganization, meiotic spindle assembly, and later as meiosis progresses generation of polar bodies and cytostatic arrest (Amiel et al., 2009; Verlhac and Terret, 2016; von Stetina and Orr-Weaver, 2011).

The tight regulation of oocyte maturation by environmental and/or hormonal stimuli is of vital importance for successful sexual reproduction. In all known examples across the animal kingdom, these signals act not directly on the oocyte, but via various somatic cells of the gonad that generally secrete maturation-inducing hormones (MIH) which in turn trigger the initiation of oocyte maturation (Deguchi et al., 2015; Yamashita et al., 2000). Despite extensive research using a range of model systems, much remains to be understood about the molecular relays acting in the somatic cells and oocytes during this process. In particular, the identity of the molecules that initiate oocyte maturation, i.e. the MIH molecules and their receptors in the oocyte, has remained largely elusive.

To date only a few examples of MIH have been identified at the molecular level: steroid hormones in fish and amphibians (Haccard et al., 2012; Nagahama and Yamashita, 2008), 1-methyladenine in starfish (Kanatani et al., 1969), a sperm protein in *C.elegans* (von Stetina and Orr-Weaver, 2011), and PRPamide family tetrapeptides in several hydrozoan jellyfish (Takeda et al., submitted). Furthermore, no oocyte MIH receptor has been unambiguously identified. In fish and amphibians, membrane progesterin receptors (mPR) have been shown to be intermediaries in oocyte maturation (Josefsberg Ben-Yehoshua et al., 2007; Zhu et al., 2003), although the involvement of more than one receptor and steroid hormone in the triggering of oocyte maturation leaves some uncertainty about their universality as MIH receptors (Haccard et al., 2012; Nagahama and Yamashita, 2008). In starfish no receptor has been yet found for 1-methyladenine, but is predicted to be a GPCR acting through $G\beta\gamma$ and PI3 kinase (Kishimoto, 2015). In mice, a

constitutively active GPCR coupled to $G\alpha_s$ (GPR3) was identified at the oocyte surface and shown to be crucial to maintain meiotic arrest by keeping cAMP levels high inside the oocyte (Mehlmann et al., 2004). Although some potential agonists have been described, this receptor remains an orphan (Ye et al., 2014).

The hydrozoan *Clytia hemisphaerica* is an attractive model for identification and characterisation of a MIH receptor, and is a convenient model for studies on oocyte maturation regulatory mechanisms using autonomously functioning gonads isolated from the medusae (Amiel et al., 2010). *Clytia* MIH has recently been characterised as a group of short WPRPamide related neuropeptides, released from gonad ectoderm neural cells upon light stimulation, which trigger oocyte maturation as well as gamete release in males and females (Takeda et al., submitted).

We hypothesized that *Clytia* MIH receptor (MIHR) was likely to be a G-protein-coupled receptor (GPCR), since neuropeptides generally signal through GPCRs (Bauknecht and Jékely, 2015), the largest superfamily of integral transmembrane receptors (Kristiansen, 2004). GPCRs have been categorized into five or six classes, depending on the criteria used for classification (Audet and Bouvier, 2012; Kristiansen, 2004). Most of them belong to 3 main classes (A: rhodopsin-like; B: secretin-like; C: metabotropic glutamate-like), widely defined by the conservation of particular structural features. Class-A GPCRs are the most abundant and diverse throughout the animal kingdom and can be split into various subfamilies, neuropeptide receptors being the largest subgroup amongst class-A GPCRs (Kristiansen, 2004). The ligands of class-B GPCRs can also be peptides, but the amino acid sequence of these is generally much longer than the endogenous *Clytia* MIH neuropeptide hormones (Jékely, 2013; Takeda et al. submitted), implying that class-B GPCRs are less likely to be *Clytia* MIHR. GPCRs commonly signal via a variety of $G\alpha$ subunits (although not exclusively – see Rajagopal, 2005), which act through different cytoplasmic signalling intermediates, including cAMP. Their involvement would thus be consistent with the known role of cAMP as an essential mediator of oocyte maturation initiation in hydrozoan oocytes, an immediate rise in the concentration of cytoplasmic cAMP being required for MPF activation (Freeman and Ridgway, 1988; Takeda et al., 2006). Moreover, addition of MIH neuropeptides to the sea water triggers *Clytia*

oocyte maturation, but their injection into the oocyte does not (Takeda et al., submitted), implying that MIH receptors are located on the oocyte plasma membrane rather than acting in the cytoplasm.

In this study we show that *Clytia* MIH neuropeptides act as ligands for one of the few *Clytia* GPCRs to show sequence conservation with characterised

neuropeptide GPCRs from bilaterian species. Gene knockout conclusively demonstrated the essential *in vivo* function of the *Clytia* MIHR in initiating oocyte maturation, as well as non-essential roles in growth and swimming, probably related to its expression in non-gonadal cells. This finding sheds new light into the ancestral role of neuropeptide GPCRs and their involvement in animal reproductive biology.

RESULTS

Selection of candidate MIH GPCRs

As a first step to search for candidate MIH receptors in the oocyte we compiled a comprehensive catalogue of *Clytia* GPCRs from a *Clytia* reference transcriptome covering all life cycle stages. We conceived a bioinformatics pipeline to retrieve all predicted protein sequences coding for 7 transmembrane domain proteins (7TM domain) and bearing GPCR-related Pfam tags (see methods). This generated an initial dataset of 761 putative *Clytia* GPCR sequences assigned by Pfam to the 3 main GPCR classes. Sequences belonging to none of the 3 aforementioned classes (e.g. sweet-taste receptors, cAMP-like receptors, etc.) were classified as “other”. Redundant *Clytia* GPCR sequences were removed from the collection, obtaining a final dataset of 536 class-sorted putative *Clytia* GPCRs. The real number of *Clytia* GPCRs in the transcriptome might be slightly lower due to the presence of some incorrectly identified incomplete sequences in this dataset. The 377 class-A GPCRs were considered preferential candidates to be a *Clytia* MIH receptor given that most neuropeptide GPCRs belong to class-A (Jékely, 2013). Clustering analyses of all *Clytia* putative GPCRs together with known and predicted metazoan neuropeptide GPCRs, as well as GPCRs from other classes, supported Pfam class categorization and defined a relatively small subset of *Clytia* sequences closely related to known bilaterian neuropeptide GPCRs within class-A (Fig. S1).

MIH receptor candidates were also selected from the class-A GPCR list on the basis of enrichment in oocytes. We mapped Illumina HiSeq reads previously obtained from different *Clytia* gonad tissue mRNAs (Takeda et al., submitted) and life cycle stage mRNAs (Fig.1A) against all putative GPCR sequences. Tissue/life cycle stage expression enrichment was visualised as a heat map, and profile clustering revealed three groups of sequences with oocyte-enriched expression (Fig.S2). Candidate selection based on combined sequence

clustering, Pfam analysis and expression profile groups allowed us to narrow down the number of potential MIHRs to 96 sequences. From this we compiled a short-list of 16 candidates for functional testing, using high expression level in oocytes as the final selection criterion (Fig.1A).

Identification of the *Clytia* MIH receptor by GPCR deorphanisation assay

We used a “GPCR deorphanisation” assay to identify the MIH receptor from our candidate shortlist. cDNAs for each candidate were transfected into CHO-K1 cells along with an aequorin-GFP luminescence reporter that measures Ca²⁺ mobilization downstream of a promiscuous G α protein (Bauknecht and Jékely, 2016; Bauknecht and Jékely, 2015). We first screened the 16 candidate *Clytia* GPCRs against a mixture of a total of 33 synthetic amidated peptides (including MIHs) predicted to be generated by cleavage from the 10 characterised *Clytia* neuropeptide precursors (Takeda et al., submitted; list in file S4) as well as 5 additional putative neuropeptide precursor sequences identified from transcriptome data. Pro-peptide cleavage sites were selected based on previous knowledge of their processing in cnidarians (Grimmelikhuijzen et al., 1996). Only one GPCR was functionally activated by this peptide mixture (not shown).

Detailed analyses demonstrated that the one GPCR active in the mixed-peptide assay responded selectively to known MIH tetrapeptides (Fig. 1B). Cells expressing this GPCR were challenged with two different peptide submixtures, and individually with active MIH amidated tetrapeptides previously deduced from the MIH precursors identified in *C. hemisphaerica* and in another hydrozoan species, *Cladonema radiatum*, as well as penta- and tripeptides similar to *Clytia* MIH, previously shown to be almost inactive triggering oocyte maturation *in vivo* (Takeda et al., submitted). All peptides were used at 1 μ M, a concentration at which the synthetic

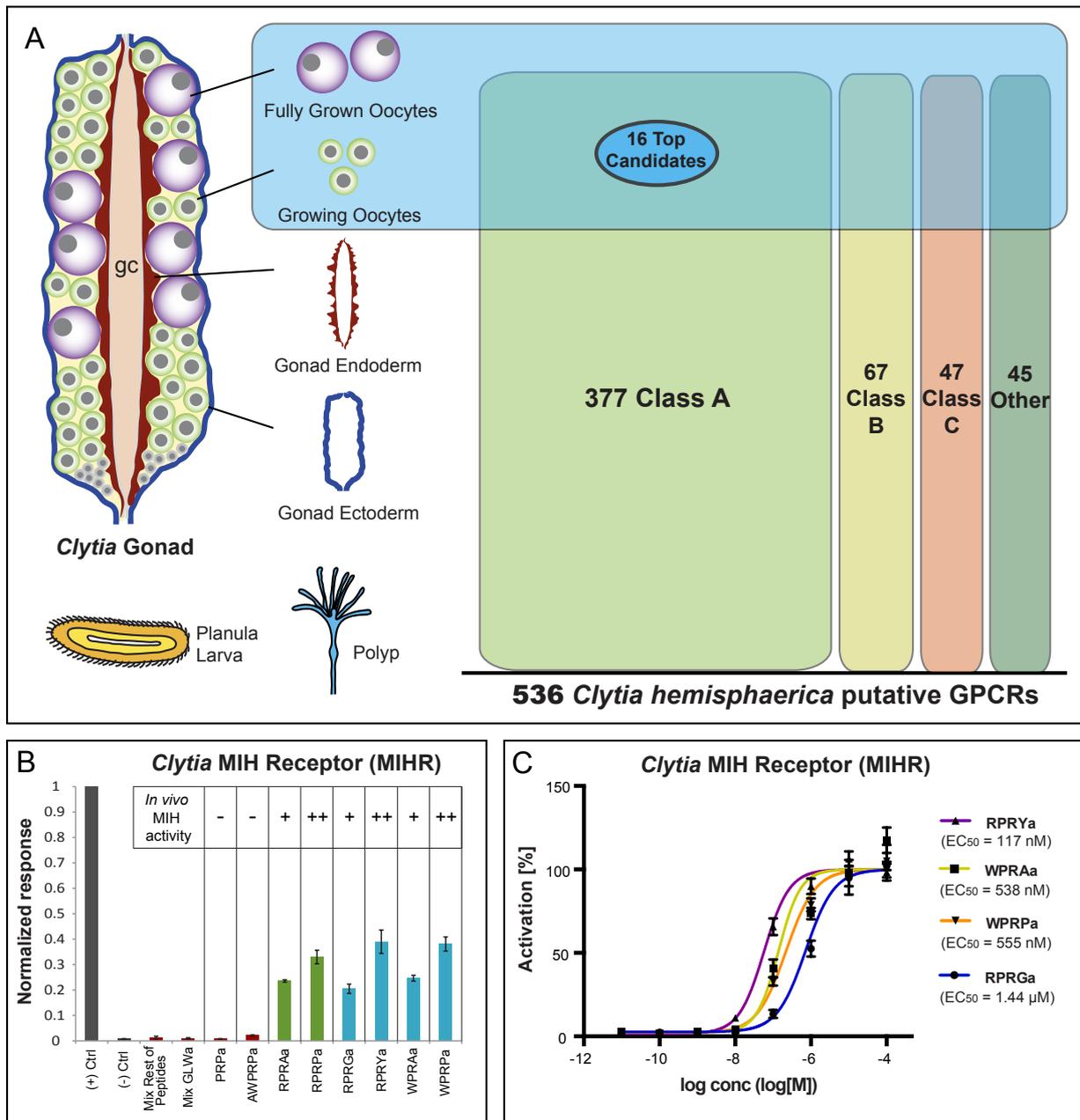


Figure 1. Identification of *Clytia* MIH receptor (MIHR)

A) Schematic representation of a *Clytia* gonad and of the different tissues and life cycle stages used for RNA-seq and GPCR expression comparisons. 536 putative GPCRs identified in *Clytia* mixed-stages transcriptome were assigned to the three main GPCR classes (A, B, C) or “other” based on Pfam signatures. 16 top candidate GPCRs to be MIHR were selected based on oocyte enrichment, relatedness to known bilaterian class-A neuropeptide GPCRs and Pfam information. gc = gastroendodermal cavity.

B) Activation response of CHO-K1 cells expressing the putative *Clytia* MIHR or control receptors, treated with different combinations of neuropeptides, MIHs or control peptides. All peptides were added at 1 μM final concentration. Similarly to oocyte maturation assays, MIHs from *Cladonema* (green bars) and *Clytia* (blue bars) were able to activate *Clytia* MIHR at this concentration, whereas other peptides failed to activate it (red bars). The responses closely matched the *in vivo* MIH activity of these peptides in inducing oocyte maturation, indicated on top of the graph (Takeda et al., submitted). Empty pcDNA3.1 vector was used as negative control, while *Platynereis* FLamide receptor and peptide were used as positive control (dark grey bars). Absolute units of luminescence were normalized based on the (+) Ctrl response and data are shown as mean ± standard error of the mean (n= 3).

C) Dose–response curves of *Clytia* MIHR treated with varying concentrations of ligand (*Clytia* MIHs). A representative of 3 independent experiments is shown. Luminescence values were normalised relative to the maximum of the fitted dose–response curves, and are shown as mean ± standard error of the mean (n= 3). Half maximal effective concentration (EC₅₀) values for each MIH were calculated as means of 3 independent experiments and are shown next to the curves.

MIH tetrapeptides selectively trigger *in vivo* spawning. All the tested *Clytia* and *Cladonema* MIH neuropeptides were able to activate our candidate receptor, whereas other *Clytia* peptide submixtures and penta-/tripeptides were not (Fig.1B). The responses closely matched the *in vivo* MIH activity of these peptides in inducing oocyte maturation (Fig.1B). This GPCR is capable of binding all 4 synthetic *Clytia* MIH tetrapeptides tested as well as the related *Cladonema* MIH peptides, which can also activate oocyte maturation in *Clytia*. All these peptides are structurally similar with the generic amino acid signature W/R-P-R-P/A/Yamide, and likely bind the same site with different affinities. Dose-response curves for each of the 4 *Clytia* MIH neuropeptides, obtained in three independent experiments showed half-maximal effective concentrations (EC50) values in the high-nanomolar or low-micromolar range for all 4 MIHs, RPRYa showing the highest activity (Fig. 1C). *Clytia* MIH and its receptor (*Clytia* MIHR or CheMIHR) are the first neuropeptide ligand-GPCR pair demonstrated in a cnidarian.

***Clytia* MIHR invalidation impairs polyp growth and jellyfish spawning**

The cell culture-based assay identified *Clytia* MIHR as specifically able to bind MIH peptides, but did not assess its *in vivo* function in the jellyfish. Multiple receptors and possibly parallel hormone systems may be involved in triggering oocyte maturation. Conversely, the MIHR might have functions outside the gonad; especially since MIH neuropeptides are synthesized in neural cells associated with the jellyfish tentacles, nerve ring and manubrium as well as the gonad (Takeda et al., submitted) and the polyp (Fig S4).

To address both these issues, we generated several *Clytia* MIHR knockout (KO) polyp colonies by means of CRISPR/Cas9 (see methods). This gene editing technique is very effective in *Clytia*, allowing extensive bi-allelic mutation of target genes already in F0 polyp colonies (Momose and Concordet, 2016). We targeted the region of *MIHR* encoding the 3rd transmembrane domain (Fig. 2A). Injected embryos developed into planula larvae, most of which could metamorphose into polyps. Genotyping revealed that 5 out of 7 polyp colonies generated showed a high frequency of a 4-bp frame-shift deletion, which introduced a premature STOP codon (Fig.2A), and displayed low levels of mosaicism. We found that growth of mutant colonies was poor compared to wild type colonies, characterised by much more frequent degeneration of feeding polyps (Fig.2B-D).

This suggests that MIHR has a role in regulating growth and/or feeding of the polyps.

We collected jellyfish from mutant colonies to assess the function of MIHR in adult medusae. Only one colony (which we will refer to as *CheMIHR* KO) produced female jellyfish that reached sexual maturity; the others either produced no jellyfish or jellyfish that grew poorly. Jellyfish derived from the *CheMIHR* KO colony swam less vigorously than wild type medusae and, importantly, failed to undergo oocyte maturation or spawning after dark-light transitions. Their gonads were grossly inflated in size due to accumulation of fully-grown immature oocytes (Fig.2E-F).

The phenotypes observed upon MIHR knockout confirm that this receptor is essential for oocyte maturation, but also suggest that it may have non-essential functions in polyp growth, colony growth and medusa swimming. Regulation of feeding and bell muscle contractions functions could be mediated by the MIH-positive neural cells of the mouth and tentacles (polyp and jellyfish) as well as by the nerve ring (Fig.S4). *In situ* hybridization detection of *CheMIHR* mRNA correspondingly revealed a wide expression in the jellyfish, notably in some tentacle cells (Fig.S5).

***Clytia* MIHR is the essential oocyte receptor of MIH**

To detail the *in vivo* function of MIHR in *Clytia* oocytes, we performed a series of oocyte maturation and spawning assays, comparing the responses of wild type and *CheMIHR* KO isolated gonads and oocytes to different treatments (Fig.3A-D). Light stimulation and 100nM synthetic WPRPamide (MIH) application induced oocyte maturation and spawning in dissected wild type *Clytia* gonads, as previously shown (Takeda et al., submitted), but had no effect on *CheMIHR* KO gonads (Fig.3A-B). In contrast, treatment with 4mM 5Br-cAMP, a cell-permeable analogue of cAMP which induces hydrozoan oocyte maturation by mimicking the natural cytoplasmic cAMP rise downstream of MIH reception (Amiel et al., 2010; Freeman and Ridgway, 1988), rescued the phenotype of *CheMIHR* KO gonads, triggering oocyte maturation and spawning (Fig.3C). 5-Br-cAMP treatments also promoted maturation of isolated fully grown *CheMIHR* KO oocytes (Fig.3D). Br-cAMP-matured *CheMIHR* KO oocytes extruded two polar bodies and could fertilize and develop to form planula larvae, although development and metamorphosis rates were lower than for wild type oocytes (not shown).

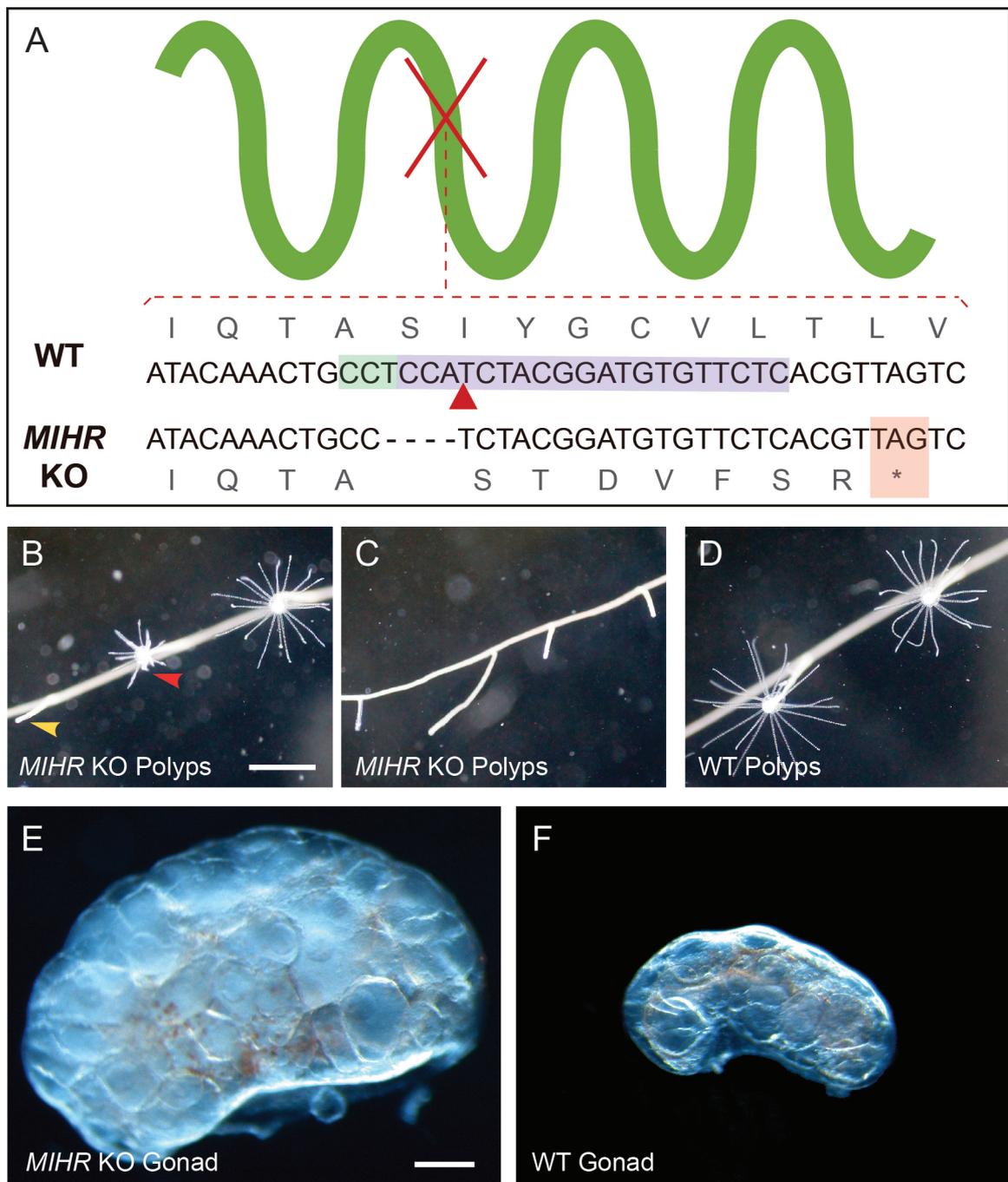


Figure 2. Invalidation of *Clytia* MIHR by CRISPR/Cas9 gene editing impairs polyp growth and jellyfish spawning

A) Scheme of *Clytia* MIHR (a GPCR) and sequence of the genomic region within the 3rd transmembrane domain targeted by *CheMIHR* CRISPR sgRNA. Corresponding amino acids are shown. Purple box indicates the target site of sgRNA. Green box is the PAM sequence (NGG). The expected cleavage site of Cas9 is indicated by a red triangle. The predominant 4-bp deletion of MIHR KO is shown. This mutation leads to a frame-shift and an early STOP codon in MIHR (red box).

B-C) Polyps from different areas of the *CheMIHR* KO mutant polyp colony. Feeding polyps tend to degenerate (red arrowhead) and often disappear leaving their stalk behind (yellow arrowhead in B).

D) Representative wild type *Clytia* polyps for comparison.

E) Representative inflated gonad of a *CheMIHR* KO mutant jellyfish containing an abnormal accumulation of immature oocytes due to spawning failure upon dark-light transitions.

F) Representative wild type *Clytia* gonad for comparison. Gonads in E and F were dissected from 3-week old medusae. Pictures were taken 10 hours after light stimulation, which induces oocyte maturation and subsequent spawning in wild type *Clytia*. Scale bars= 1 mm in B; 200 μm in E.

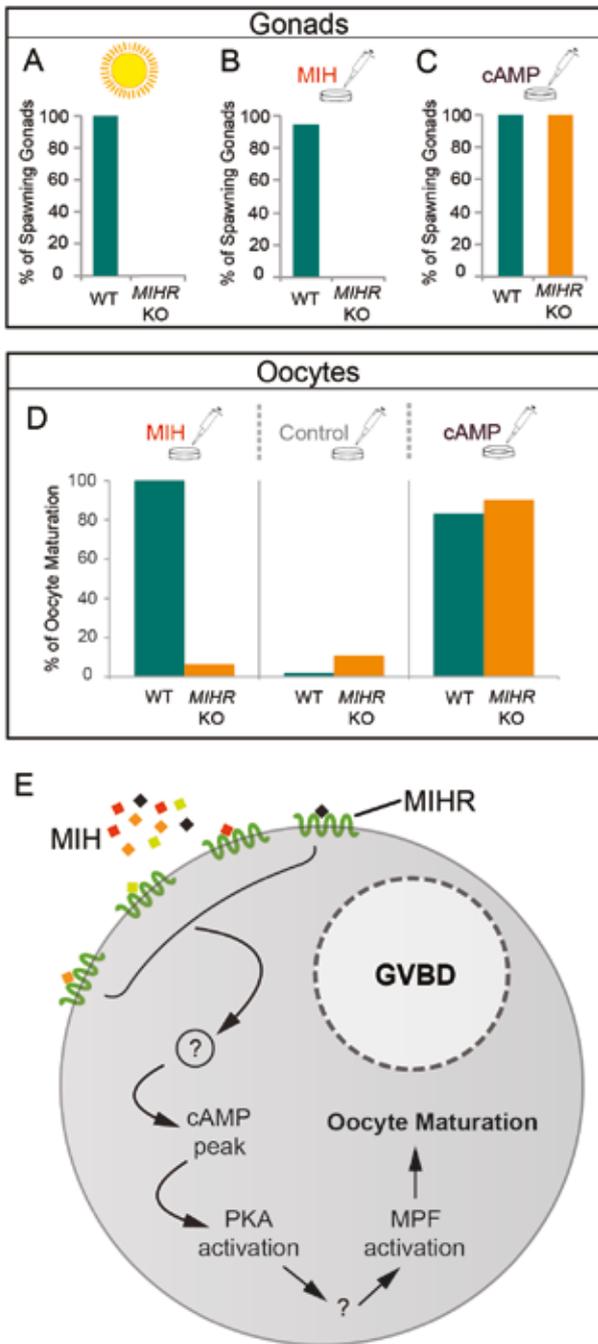


Figure 3. Confirmation of *Clytia* MIHR function in oocytes

A-C) Oocyte maturation and spawning capacity of wild type (WT) and *CheMIHR* KO gonads following different treatments (A: light stimulation; B: 100nM synthetic MIH treatment; C: 4mM 5-Br-cAMP treatment). Percentage of spawning gonads combined from 3 independent experiments is shown in all cases. Whereas oocyte maturation and spawning were impaired in *CheMIHR* KO gonads upon light stimulation or MIH treatment, 5-Br-cAMP, acting downstream of MIH reception, completely rescued this phenotype. In A, n= 75 gonads for WT and n= 82 gonads for *CheMIHR* KO; in B, n= 64 gonads for WT and n= 82 gonads for *CheMIHR* KO; in C, n= 32 gonads for WT and n= 29 gonads for *CheMIHR* KO. The Fisher exact test showed significant differences (F= 0) between wild types and mutants in A and B, and did not show significant differences (F= 1) in C.

D) Equivalent analysis performed with fully grown oocytes manually isolated from WT and *CheMIHR* KO gonads. Percentage of maturation induction combined from 3 independent experiments is shown in all cases. Oocyte maturation, seen as GVBD, was triggered by MIH and 5-Br-cAMP treatment in WT oocytes, but only the latter induced maturation of *CheMIHR* KO oocytes. Control experiments with the 5-Br-cAMP solvent (distilled water) showed a low level of spontaneous maturation in both cases. In the control assay, n= 50 WT oocytes and n= 46 *CheMIHR* KO oocytes. Fisher exact test did not show significant differences (F= 0.101). In the MIH assay, n= 47 WT oocytes and n= 46 *CheMIHR* KO oocytes. Fisher exact test showed significant differences (F= 0). In the 5-Br-cAMP assay, n= 59 WT oocytes and n= 92 *CheMIHR* KO oocytes. Fisher exact test did not show significant differences (F= 0.216). E) Working model of *Clytia* oocyte maturation initiation through MIH-MIHR interaction. An as yet uncharacterised signalling cascade downstream of MIH reception raises cAMP levels inside the oocyte, which in turn activates PKA to ultimately trigger oocyte maturation, as a result of PKA-dependent MPF activation.

Anti-MIH immunofluorescence analysis revealed no obvious differences in the distribution of MIH-secreting cells in the gonad ectoderm and showed that MIH was released upon light stimulation, as in wild types (Fig.S3). Quantification of overall MIH immunofluorescence levels showed lower values in the mutants compared to wild types (p= 0, Mann-Whitney test), possibly due to progressive loss of MIH over time in the particular physiological conditions of the non-spawning gonads (Fig.S3).

Taken together these analyses of mutant jellyfish

confirmed that the essential *in vivo* function of the *Clytia* MIHR GPCR is in the oocyte as the receptor of MIH.

***Clytia* MIHR is related to a subset of bilaterian peptide hormone receptors**

We performed sequence-similarity based clustering to explore the relationship of the *Clytia* MIHR sequence with known GPCR families (Bauknecht and Jékely, 2015; Jékely, 2013). We found that MIHR is one of the few *Clytia* class-A GPCRs that cluster

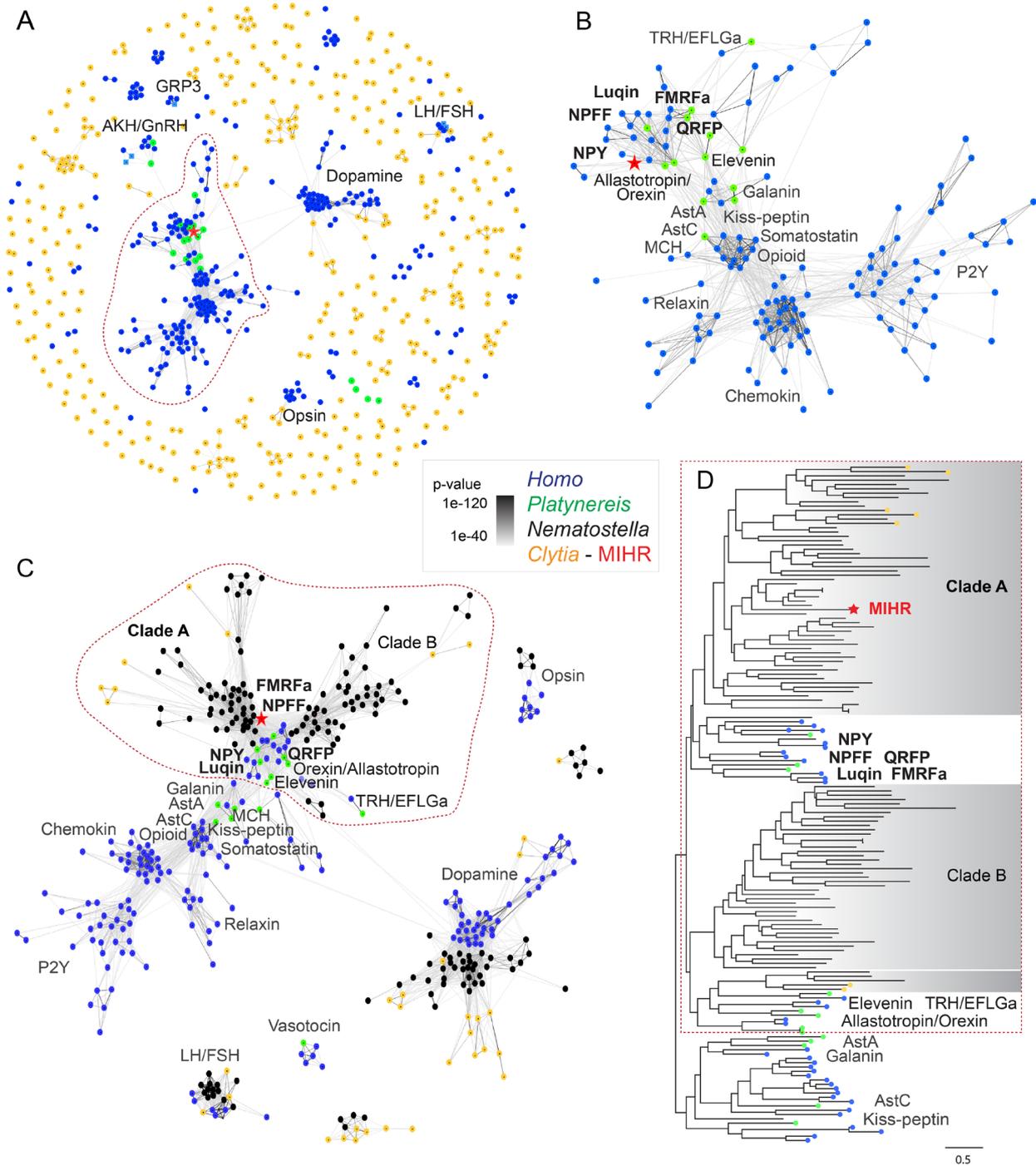


Figure 4. Phylogenetic analyses of the *Clytia* MIHR

A) Sequence-similarity-based clustering using Clans2 of class-A GPCRs from *Clytia*, human (olfactory receptors excluded) and *Platynereis* (deorphanized GPCRs only – Bauknecht and Jékely, 2015). BLASTP p-value < 1e-40.

B) Cluster map of the largest cluster (circled in red in A) keeping only sequences that show at least 2 connections with the central cluster. BLASTP p-value < 1e-40.

C) More stringent cluster map (p-value < 1e-50) of the same sequences as in (A) plus all *Nematostella* GPCR-A sequences. Only clusters containing at least 5 sequences from at least 2 species were kept. All connections with p-value < 1e-40 are shown.

D) Maximum likelihood analysis using RAXML (PROTGAMMAGTR) of the receptor sequences contained inside the dashed area shown in (C). AstA, Galanin, AstC and Kiss-peptin receptors were included as outgroup. AKH, adipokinetic hormone; AstC, allatostatin-C; AstA, allatostatin-A; GnRH, gonadotropin releasing hormone; LH/FSH, Luteinizing/Follicle-stimulating hormone receptor; GPR3, G protein-coupled receptor 3; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; NPFF, neuropeptide FF; PRLH, prolactin releasing hormone; P2Y, purinoreceptor; QRFP, pyroglutamylated RFamide peptide; TRH, thyrotropin releasing hormone.

with any bilaterian GPCR-A families (Fig.4A). Its sequence is most related to a subset of human peptide hormone receptors including neuropeptide Y, neuropeptide FF, Orexin/allastropin, Elevenin, Luqin and EFLGa/Thyrotropin releasing hormone (Fig.4B). It should be noted that the GPCRs most closely involved in controlling directly or indirectly oocyte maturation in vertebrates, namely GPR3, gonadotropin releasing hormone (GnRH), or Luteinizing/Follicle-stimulating hormone (LH/FSH) receptors are not closely related to *Clytia* MIHR or the aforementioned bilaterian groups. By including *Nematostella* GPCR-A sequences in our analysis we could further show that *Clytia* MIHR belongs to one of two large clusters of undescribed cnidarian GPCRs that cluster with the bilaterian peptide hormone receptor families listed above (Fig.4C). Maximum Likelihood phylogenetic analyses allowed us to tentatively associate these two clusters of cnidarian sequences into two distinct sets of bilaterian GPCRs (Fig.4D). The group containing *Clytia* MIHR (labelled Clade A in Fig.4D)

is monophyletic, and most closely related to a small set of bilaterian peptide hormone receptor families: the neuropeptide Y/neuropeptide F, neuropeptide FF/GnIH), Luqin/RYa and pyroglutamylated RFamide peptide (QRFP) receptor families (Fig. 4D). Neuropeptides associated with the NPY, GnIH and QRFP receptor families have been shown to regulate the release of GnRH and/or LH in vertebrates (reviewed by Kriegsfeld, 2006; Shahjahan et al., 2014). A second group of GPCRs (clade B in Figure 4), slightly less closely related to CheMIHR, included receptors of a more structurally diverse group of ligands including Elevenin, EFLGa/Thyrotropin releasing hormone and Orexin/allastropin (Fig.4D). This phylogenetic pattern strongly suggests independent diversifications of peptide hormone receptors in Cnidaria and Bilateria, with both *Clytia* MIHR and a very large group of *Nematostella* GPCRs having a common ancestor with bilaterian receptors for certain peptide hormone families involved in neurohormonal control of sexual reproduction.

DISCUSSION

We have identified and functionally characterised *Clytia* MIHR as a neuropeptide GPCR highly expressed in the oocytes and essential for the initiation of oocyte meiotic maturation. Our findings provide the first example of a neuropeptide GPCR in Cnidaria, and the first “deorphanised” cnidarian GPCR. The *Clytia* MIHR is also the first oocyte receptor identified in any animal to function as the sole receptor mediator of maturation initiation. We can propose a model in which this receptor responds to MIH, secreted from gonad ectoderm neural cells in response to light, to promote an immediate rise in cytoplasmic cAMP (Fig.3E). Good candidates as molecular intermediates of these events would be $G\alpha_s$ and adenylate cyclase activation. Elevated cytoplasmic cAMP in turn would activate cAMP-dependent Protein Kinase (PKA) as documented in other hydrozoans (Takeda et al., 2006), which phosphorylates unknown substrates essential for MPF activation and thus promotes entry into first meiotic M phase.

The evolutionary relationship of *Clytia* MIHR to a subset of hormone receptors in bilaterians provides an evolutionary framework to understand the bewildering diversity of MIH molecules and oocyte second messenger involvement in oocyte maturation. Specifically we propose that neuropeptide GPCRs acting via cAMP were involved more generally in regulating the sexual reproduction and associated behaviours in the

common eumetazoan (cnidarian + bilaterian) ancestor. To decide whether the direct role of *Clytia* MIHR in initiating oocyte maturation has been selectively retained from a common eumetazoan ancestor, or has arisen specifically in the hydrozoan lineage, will require future functional studies of CheMIHR orthologues in other cnidarian species.

Early appearance of neuropeptide GPCRs

Cnidarian nervous systems are strongly peptidergic and neuropeptides regulate vital functions in cnidarian biology (reviewed by Takahashi and Takeda, 2015). Some cnidarian neuropeptides activate ligand-gated ion channels, leading to fast responses (Grimmelikhuijzen and Hauser, 2012), but until now no cnidarian neuropeptide GPCR had been identified. Our identification of *Clytia* MIHR implies that neuropeptide GPCRs were already present in the common ancestor of cnidarians and bilaterians, at least 700 million years ago, prior to the divergence of these animal clades. This strengthens the belief that ancestral nervous systems mainly used neuropeptides as transmitters, regulating fundamental physiological functions (Grimmelikhuijzen and Hauser, 2012).

The *Clytia* MIHR is one of only very few *Clytia* GPCRs, even amongst class-A, to show a clear relationship with any bilaterian GPCR family (Jékely, 2013). On the other hand, we identified

many anthozoan (*Nematostella*) sequences related to neuropeptide class-A GPCRs. Given the large number of neuropeptides in Hydrozoans (Grimmelikhuijzen et al., 1996; Takahashi and Takeda, 2015; Takeda et al., submitted), we suspect that hydrozoans such as *Clytia* also have a large family of neuropeptide GPCRs, but that rapid sequence evolution led to their exclusion from recognised families by any methods based on sequence similarity. In this context, we can wonder what functional constraints underlie the stability of the *Clytia* MIHR sequence relative to other *Clytia* neuropeptide GPCRs during evolution. Presumably, particular structural interactions with pathway regulators, such as $G\alpha$ subunits or other specific cytoplasmic regulator proteins, have restrained the rate of *Clytia* MIHR sequence evolution.

In bilaterians it is common that neuropeptide hormones have broad functions, depending on the site where or time when they are secreted. For example, neuropeptides such as neuropeptide Y (NPY) are strong candidates to modulate the link between nutrition and reproduction at the level of the central nervous system in vertebrates, since they can control gonadotropic hormones secretion as well as enhancing appetite arousal (Shahjahan et al., 2014; Wójcik-Gładysz and Polkowska, 2006). Similarly, *Clytia* MIH and MIHR are expressed both in the gonad and at other sites in both polyp and jellyfish stages (Fig.S4, S5), and swimming and polyp growth were correspondingly impaired in *Clytia* MIHR mutants. Indeed, most mutant colonies failed to generate sexually mature jellyfish through poor growth of the colony and/or the medusae. Taken together, these observations suggest an involvement of MIH neuropeptides and MIHR in different physiological and behavioural regulations during the *Clytia* life cycle. Specific studies of their roles in non-gonad tissues are required for a better understanding of this matter.

Neuropeptides and neuropeptide GPCRs in reproductive control

A wide variety of neuropeptides and neuropeptide GPCRs can be found in bilaterian animals, and coevolution of respective pairs has been proposed (Jékely, 2013; Mirabeau and Joly, 2013). Plenty of neuropeptides act as neuroendocrine hormones and are involved in regulating the reproductive physiology (Garrison et al., 2013; Kriegsfeld, 2006; Pasquier et al., 2014; Saberi et al., 2016; Shahjahan et al., 2014). For instance, NPY ligands can play a stimulatory role on the hypothalamic-pituitary-gonadal (HPG) axis in vertebrates,

inducing the release of GnRH from hypothalamic GnRH neurons and LH from the pituitary in fish and mammals (Shahjahan et al., 2014; Wójcik-Gładysz and Polkowska, 2006). NPY signalling, acting at the central nervous system (CNS) level, is also associated with the systemic regulation of reproductive development in planarians (Saberi et al., 2016). GnIH regulates the HPG axis at different levels in birds and mammals, inhibiting GnRH release from the hypothalamus and gonadotropins synthesis and release at the pituitary level (Tsutsui et al., 2010). Systemic administration of QRFP in female rats, as well as its *in vivo* intracerebral injection, were shown to significantly increase LH levels in the serum (Navarro et al., 2006). Our sequence analyses indicate that the GPCRs for these neuropeptide hormones, which have been found to be expressed in bilaterian CNS neurons and/or pituitary endocrine cells (Klenke et al., 2010; Navarro et al., 2006; Saberi et al., 2016; Tsutsui et al., 2010), derived from a common ancestral receptor with a large group of cnidarian receptors, including *Clytia* MIHR (Fig.4). The role of *Clytia* MIH-MIHR in oocyte maturation initiation and the extensive involvement of neuropeptides and neuropeptide GPCRs in the nervous system-mediated reproductive control of bilaterians may thus have a common evolutionary origin. More specifically we hypothesize that a common ancestral neuropeptide GPCR gave rise to a large cnidarian family of putative neuropeptide receptors, including *Clytia* MIHR, and to the bilaterian NPY-NPFF-Luqin-QRFP group of bilaterian GPCR families, and that it functioned in the physiological regulation of reproduction as well possibly as in more general physiological functions through an unknown neuropeptide ligand. In these animals, the ancestral receptor may have been involved in directly triggering oocyte meiosis resumption, like in *Clytia* today, or alternatively this specific role may have arisen more recently within the hydrozoan clade. More studies from other cnidarian and bilaterian groups would help to resolve this issue.

An evolutionary perspective on the signalling pathways initiating oocyte maturation

In oocytes from different animal species, similar cytoplasmic signalling pathway components, notably cytoplasmic cyclic nucleotides, have been implicated in initiating the maturation response. However, their integration into regulatory systems differs markedly between species (Deguchi et al., 2011). In particular, the second messenger cAMP has opposite roles in oocyte maturation initiation in different animals (Deguchi et al., 2011; Takeda

et al., 2006). In starfish and vertebrate oocytes cytoplasmic cAMP is held at elevated levels until the meiotic arrest at prophase I is released, in vertebrates critically inhibiting meiosis initiation. In contrast, in diverse groups of marine invertebrates, including nemerteans, hydrozoans, ascidians, bivalves and ophiuroids, elevation in cytoplasmic cAMP concentration is an early and essential step in meiosis initiation (Deguchi et al., 2011). Precisely how cAMP elicits such contrasting responses remains unclear. It has been proposed that cAMP-induced oocyte maturation might have appeared several times in evolution as an adaptation to different aspects of sexual reproduction shared by distantly related animals, and might be linked to differing involvement of follicle cells as signalling intermediates (Deguchi et al., 2011). Under this scenario, the evolutionary appearance of cAMP stimulatory role in oocyte maturation in hydrozoans would be linked to the expression of *Clytia* MIHR-related GPCRs in the oocyte, rather than acting in 'relay' somatic tissues. In this light it will be of great interest to search for GPCRs related to *Clytia* MIHR in the oocytes of nemertean, ascidian, bivalves or ophiuroid species that depend on cAMP signalling for maturation, and determine their ligands and function.

METHODS

Animals

Sexually mature jellyfish generated from laboratory maintained *Clytia hemisphaerica* polyp colonies ("Z strains") (Amiel et al., 2010) were fed regularly with *Artemia nauplii* and cultured under light-dark cycles to allow daily spawning. Red Sea Salt brand artificial seawater (ASW) was used for all culture and experiments.

Selection of candidate *Clytia* MIH Receptors

Using a comprehensive *Clytia* reference transcriptome (86,606 contigs) derived from mixed larva, polyp and jellyfish samples as input; we ran a homemade script in R (<https://www.r-project.org/>) to predict and translate all ORFs (Lapébie et al., 2014), obtaining a catalogue of predicted protein sequences from complete and incomplete ORFs. This dataset was screened using TMHMM 2.0c to produce a list of *Clytia* complete protein sequences coding for 7 transmembrane domain proteins (7TMD) including putatively incomplete sequences containing 2 to 7 transmembrane domains. This file was used as a query to locally scan for Pfam

The different molecular nature of the MIHs described so far (Haccard et al., 2012; Kanatani et al., 1969; Nagahama and Yamashita, 2008; von Stetina and Orr-Weaver, 2011; Takeda et al., submitted) whose involvement correlates with the involvement in some animal lineages of follicle cells and/or endocrine organs involved in reproductive regulation that produce these hormones, adds layers of complexity to understanding the evolution of meiosis resumption regulation. In mice, cGMP diffusion through gap junctions from oocytes to somatic cells upon luteinizing hormone reception by mural granulosa cells regulates meiosis resumption (Shuhaibar et al., 2015), indicating that the MIH concept has been significantly modified during evolution of the mammalian ovary.

To conclude, *Clytia* MIHR is not closely related to any GPCR characterised so far in the oocytes of any animal. Rather it forms part of a conserved GPCR family that has diversified independently in cnidarians and bilaterians, and whose members have frequently been implicated in the neurohormonal control of sexual reproduction.

annotations via Interproscan 5.22, and only those sequences with Pfam tags related to 7TMD receptors were retained, compiling a dataset of 761 potential *Clytia* GPCRs arranged by class, defined by Pfam. Cd-hit (Fu et al., 2012) was run with 95% identity to eliminate sequence duplicates, obtaining a final dataset of 536 *Clytia* GPCRs.

Illumina HiSeq 50nt reads were generated from mRNA isolated using RNAqueous micro kit (Ambion Life technologies, CA) from ectoderm, endoderm, growing oocytes and fully grown oocytes manually dissected from around 150 *Clytia* ovaries (Takeda et al., submitted), and from other *Clytia* life cycle stages (e.g. polyp and planula larva). The reads were mapped against all candidate GPCR sequences using Bowtie2 (Langmead & Salzberg, 2012). The counts for each contig were normalized per total of reads of each sample and contig length to allow expression comparisons between genes and samples.

Presumptive GPCR sequences were separated in groups using a hierarchical method based on the correlation of their expressions (gplots package in

R). Z-scores were obtained after standardization of the counts in the different sequenced samples for each GPCR candidate and plotted in a heat map using R (Fig.S2). For clustering, a previous collection of GPCRs (Jékely, 2013) was complemented with several bilaterian class C GPCRs retrieved from UniProtKB and all putative *Clytia* GPCRs. Clustering analysis was performed using CLANS2 (Frickey and Lupas, 2004) with a BLOSUM62 matrix and a p-value cutoff of $1.e-30$ (Fig.S1). Finally, based on this information, Pfam signatures, reciprocal BLASTs and high expression level in oocytes, a subset of 16 top candidates was manually selected. FASTA sequences are available in Files S1 and S2.

Receptor cloning

The selected *Clytia* GPCRs were cloned from cDNA into pcDNA3.1(+) (Thermo Fisher Scientific, Waltham, MA, USA) using the Gibson Assembly® Cloning Kit (New England Biolabs) (Gibson et al., 2009). pcDNA3.1(+) vector was linearized with BamHI and NotI restriction enzymes. Primers were designed using Geneious v8.1 and the Gibson Cloning option. Forward primers consisted of the overhang left after BamHI vector linearization followed by the Kozak consensus sequence (CGCCACC), a start codon (ATG), and a sequence corresponding to the target sequence. Reverse primers consisted of the overhang left after NotI vector linearization followed by a STOP codon, and a reverse complementary sequence to the target sequence. The list of primers for all cloned GPCRs is available in FileS3. Polymerase chain reaction was performed using Phusion polymerase (New England Biolabs). Cloned GPCRs were sequenced using primers for T7: TAATACGACTCACTATAGGG and BGHrev: TAGAAGGCACAGTCGAGG.

Cell culture and receptor deorphanisation

Cell culture assays were performed as described (Bauknecht and Jékely, 2016; Bauknecht and Jékely, 2015). Briefly, CHO-K1 cells were cultured in Ham's F12 Nut Mix medium (Thermo Fisher Scientific) with 10% fetal bovine serum. Cells were seeded in 96-well plates at approximately 10.000 cells/well and transfected the following day with pcDNA3.1(+) plasmids encoding a GPCR, the promiscuous G α -16 protein (Offermanns and Simon, 1995), and a reporter construct GFP-apoaequorin (Baubet et al., 2000) (60 ng each) using the transfection reagent TurboFect (Thermo Fisher Scientific). After 2 days of expression, the medium was removed and replaced with Hank's Balanced Salt Solution (HBSS) supplemented with 1.8 mM Ca $^{2+}$, 10 mM glucose,

and 1 mM coelenterazine h (Promega, Madison, WI, USA). After incubation at 37 °C for 2 hours, cells were tested by adding synthetic peptides (GenScript) in HBSS supplemented with 1.8 mM Ca $^{2+}$ and 10 mM glucose. A list of all synthetic peptides used is given in FileS4. Luminescence was recorded for 45 s in a plate reader (BioTek Synergy Mx or Synergy H4; BioTek, Winooski, VT, USA). Data were integrated over the 45-s measurement period and recorded as technical triplicates in each case. Data were normalized using the response of *Platynereis* FLamide receptor to 1 μ M AKYFL-NH2 (Bauknecht and Jékely, 2015). Dose-response curves were obtained using concentrations between 0.01 nM and 100 μ M for each peptide. Data for dose-response curves were recorded in triplicate for each concentration and the experiment was repeated independently 3 times. Dose-response curves were fitted with a four-parameter curve using Prism 6 (GraphPad, La Jolla, CA, USA) and were normalized to the calculated upper plateau values (100% activation).

Generation of CRISPR-Cas9 mutant *Clytia* colonies

MIHR small guide RNA (sgRNA) was assembled by hybridising crRNA and tracrRNA synthesized at IDT (Integrated DNA Technologies), obtaining a final concentration of 50 μ M. sgRNA was kept at -80°C until use. The crRNA MIHR sequence is shown in FileS5. Purified Cas9 protein dissolved in Cas9 buffer (10 mM HEPES, 150 mM KCl) was kindly provided by J-P Concordet (MNHN Paris) and diluted to 5 μ M. sgRNA was added to Cas9 protein in excess (~2:1) prior to injection and incubated for 10 minutes at room temperature. The final Cas9 concentration was adjusted to 4 μ M and for sgRNA to 10 μ M. The mixture was centrifuged at 14,000 rpm for 10 minutes at room temperature. 2-3% of egg volume was injected into unfertilized eggs within 1 hour after spawning, prior to fertilization.

Injected embryos were cultured for 3 days in Millipore-filtered sea water (MFSW) at 18°C. Metamorphosis of planula larvae into polyps was induced about 72 hours after fertilization by placing larvae (20-80/slide) on double-width glass slides (75 x 50mm) in drops of 3-4ml MFSW containing 1 μ g/ml synthetic metamorphosis peptide (GNPPGLW-amide), followed by overnight incubation. Slides with fixed primary polyps were transferred to small aquariums kept at 24°C, a temperature which favours the establishment of female colonies (Carré and Carré, 2000). Primary polyps and young polyp colonies were fed twice a day with smashed

Artemia nauplii until they were grown enough to be fed with swimming nauplii. Following colony vegetative expansion, a single well-growing colony on each slide was maintained as a founder. After several weeks of growth, polyp colonies were genotyped to assess mutation efficiency and mosaicism, and medusae were collected from the most strongly mutant colony (MIHR KO) for further experimentation.

Mutant genotyping

Genomic DNA from *Clytia* polyps was purified using DNeasy blood/tissue extraction kit (Qiagen). The MIHR target site was amplified by PCR using Phusion DNA polymerase. Primers used for genotyping are listed in FileS5. PCR products were sequenced and mutation efficiency was assessed using TIDE analyses, which estimates the mutation composition from a heterogeneous PCR product in comparison to a wild type sequence (Brinkman et al., 2014). The MIHR KO polyp colony used for jellyfish phenotyping experiments displayed a dominant 4 base pair frame-shift mutation, expected by microhomology-mediated end joining (MMEJ – Momose et al., in preparation) in >75% of the PCR products, with no other significant mutation or wild type products.

We scanned a *Clytia* genome for possible off-targets of MIHR crRNA at <http://crispor.tefor.net>. The 1 possible off-target site was not adjacent to a PAM sequence and corresponded to a non-coding sequence.

Gonad spawning assays

Sexually mature wild type and MIHR KO mutant medusae of the same age and adapted to the same day-night cycle were collected for gonad dissection. Individual gonads were transferred to 100µl MFSW in 96-well plastic plates. Plates were covered with aluminium foil overnight and brought back to white light the following day. In parallel, wild type and MIHR mutant gonads adapted to light conditions were dissected, transferred to 96-well plastic plates and acclimatised for two hours. For some wells, 100µl of a stock of 2x100nM synthetic WPRPamide (MIH) in MFSW were added to give a final concentration of 100nM. For other wells, 40µl of a stock of 20mM bromo adenosine 3'5'cyclic MonoPhosphate (5-Br-cAMP – Fluka; Sigma Aldrich) in distilled water were added to give a final concentration of 4mM. Gonads were washed after 5 minutes incubation in 5-Br-cAMP. In each experimental setup oocyte maturation

was scored as GVBD after 30 minutes. Spawning followed in all cases where oocyte maturation was triggered. Gonads that spawned prematurely due to manipulation stress were excluded from analysis.

Oocyte maturation assays

Fully grown oocytes were isolated manually from dissected gonads of wild type and MIHR KO mutant jellyfish and transferred into small wells of 96-well plastic plates. Five to eleven oocytes were added per well. For control conditions, 20µl of 5-Br-cAMP solvent (distilled water) were added to wells containing fully grown oocytes in 80µl MFSW. Spontaneous maturation occurs at a low frequency in control oocytes. For experimental conditions, 10µl of a stock of 1µM synthetic WPRPamide (MIH) in MFSW were added to give a final concentration of 100nM or, 20µl of a stock of 20mM 5-Br-cAMP in distilled water were added to give a final concentration of 4mM. Oocytes were washed after 5 minutes incubation in 5-Br-cAMP. In each experimental setup GVBD was scored after 30 minutes.

GPCR molecular phylogeny

Clustering was performed as for the selection of the *Clytia* MIHR receptor candidates (see above). Multiple alignments were generated using Muscle (Edgar, 2004). Positions containing more than 80% gaps were excluded. Phylogenetic analyses were performed using RAXML and the model PROTGAMMAGTR. The resulting trees were visualized with FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>).

In situ hybridization

For *in situ* hybridization, isolated gonads were processed as previously (Fourrage et al., 2014) except that 4M Urea was used instead of 50% formamide in the hybridization buffer. Details of this protocol optimization will be published elsewhere. Images were taken with an Olympus BX51 light microscope.

Immunofluorescence

For co-staining of neuropeptides and tyrosinated tubulin, dissected *Clytia* gonads, whole medusae and polyp heads were fixed overnight at 18°C in HEM buffer (0.1 M HEPES pH 6.9, 50 mM EGTA, 10 mM MgSO₄) 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 -20°C improved visualization of microtubules in neural cells. Samples were rehydrated, washed several times in PBS-0.02% Triton X-100, then one time in PBS-0.2% Triton X-100 for 20 minutes, and again several times in PBS-0.02% Triton X-100. After overnight incubation at 4°C in PBS with 3% BSA they were incubated in a rabbit anti-PRPa antibody (Takeda et al., submitted) and a rat monoclonal anti-Tyr tubulin (YL1/2, Thermo Fisher Scientific) 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 4°C, and nuclei stained using Hoechst dye 33258. Images were acquired using a Leica SP8 confocal microscope and maximum intensity projections of z-stacks prepared using ImageJ software.

For MIH fluorescence quantification, 5-6 independent gonads for each of the two conditions (light-adapted and dark-adapted after light stimulation) and *Clytia* strains (WT and CheMIHR KO) were fixed as mentioned above and co-stained

for MIH and Tyr-tubulin. All the fixations were done in parallel. Confocal images were acquired using the same scanning parameters (i.e. magnification, laser intensity and gain). In all cases, 10 confocal Z planes were summed over 3 µm depth at the gonad surface using ImageJ software. With ImageJ, we separated the two channels (MIH and Tyr-tubulin) and selected the contour of MIH-positive cells using the Tyr-tubulin staining as guidance. Using the “Integrated Density” option, we recovered the “RawIntDen” values of the MIH-stained channel, which refer to the sum of the pixel intensity values in the selected region of interest. These values divided by 1000 correspond to the RFU (Relative Fluorescence Units) in Figure S3.

Statistics

Fisher exact tests and Mann-Whitney U tests were performed at <http://www.socscistatistics.com>. Mann-Whitney U tests were chosen for MIH fluorescence quantification comparisons since the results did not follow a normal distribution as determined by the Shapiro-Wilk test.

ACKNOWLEDGEMENTS

We thank C. Sinigaglia for sharing her optimised *in situ* hybridisation method, S. Chevalier for contributing to transcriptome sample preparation, M. Khamla for producing the 3D jellyfish model shown in Figure S4, J-P Concordet (MNHN Paris)

for generously providing Cas9 protein, and all our research colleagues for stimulating and critical discussions. Funding was provided by the Marie Curie ITN NEPTUNE and French ANR grant OOCAMP-ANR-13-BSV2-0008.

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SUPPLEMENTARY FIGURES

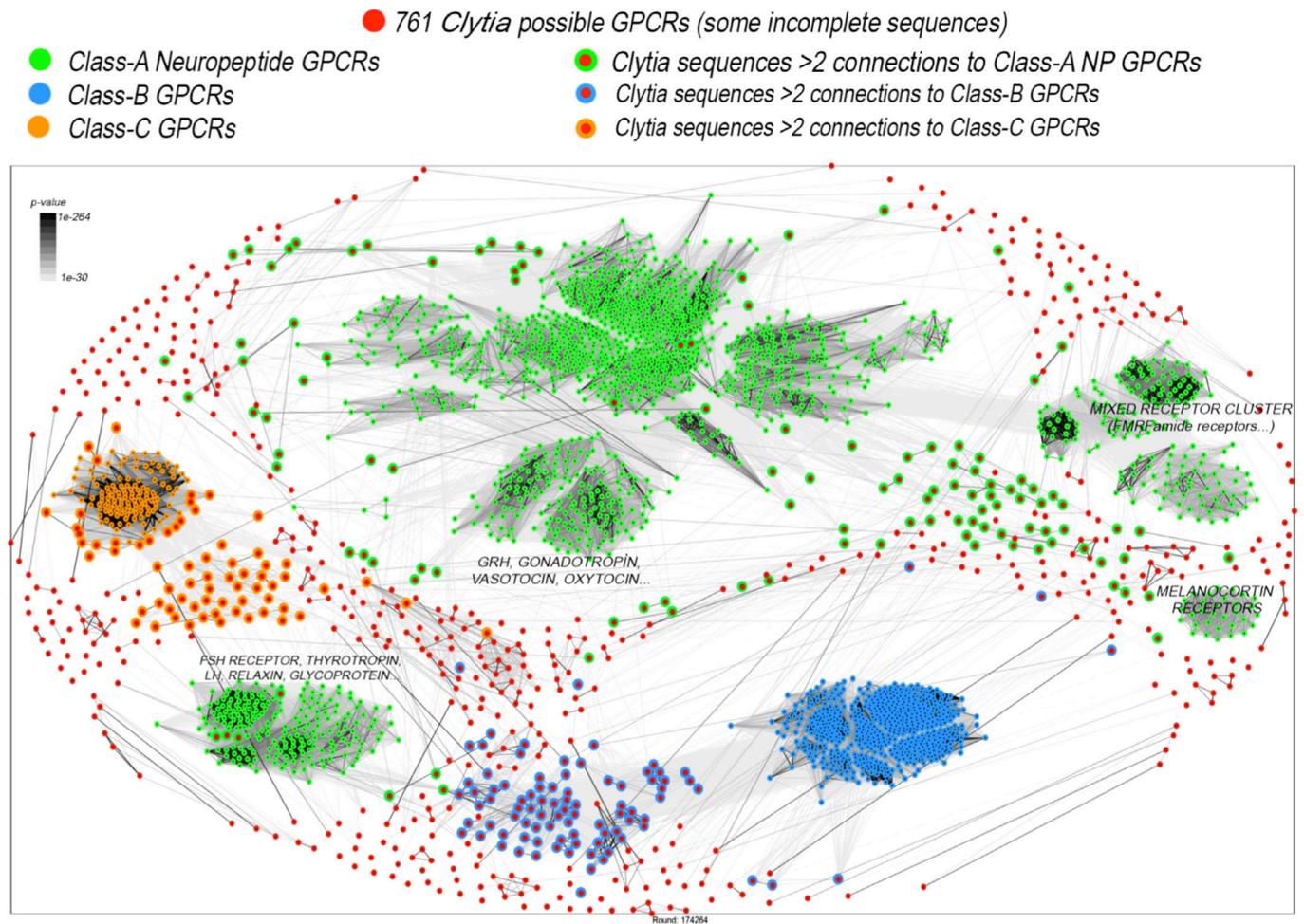


Figure S1. Relatedness of putative *Clytia* GPCRs to different metazoan GPCR classes

BLOSUM62 cluster map of 761 potential *Clytia* GPCRs combined with over 2000 known and predicted metazoan class-B, class-C and neuropeptide class-A GPCRs. Nodes correspond to GPCR protein sequences and are coloured based on GPCR class as depicted in the legend. Red nodes represent *Clytia* sequences. Edges are coloured according to BLASTP p-values. Those *Clytia* sequences most closely related to known families of Class-A neuropeptide GPCRs are indicated by green-red circles. *Clytia* sequences with significant relatedness to Class-B and Class-C GPCRs are also highlighted, supporting Pfam information and enabling rapid narrowing down of the number of *Clytia* candidate MIH receptor GPCRs.

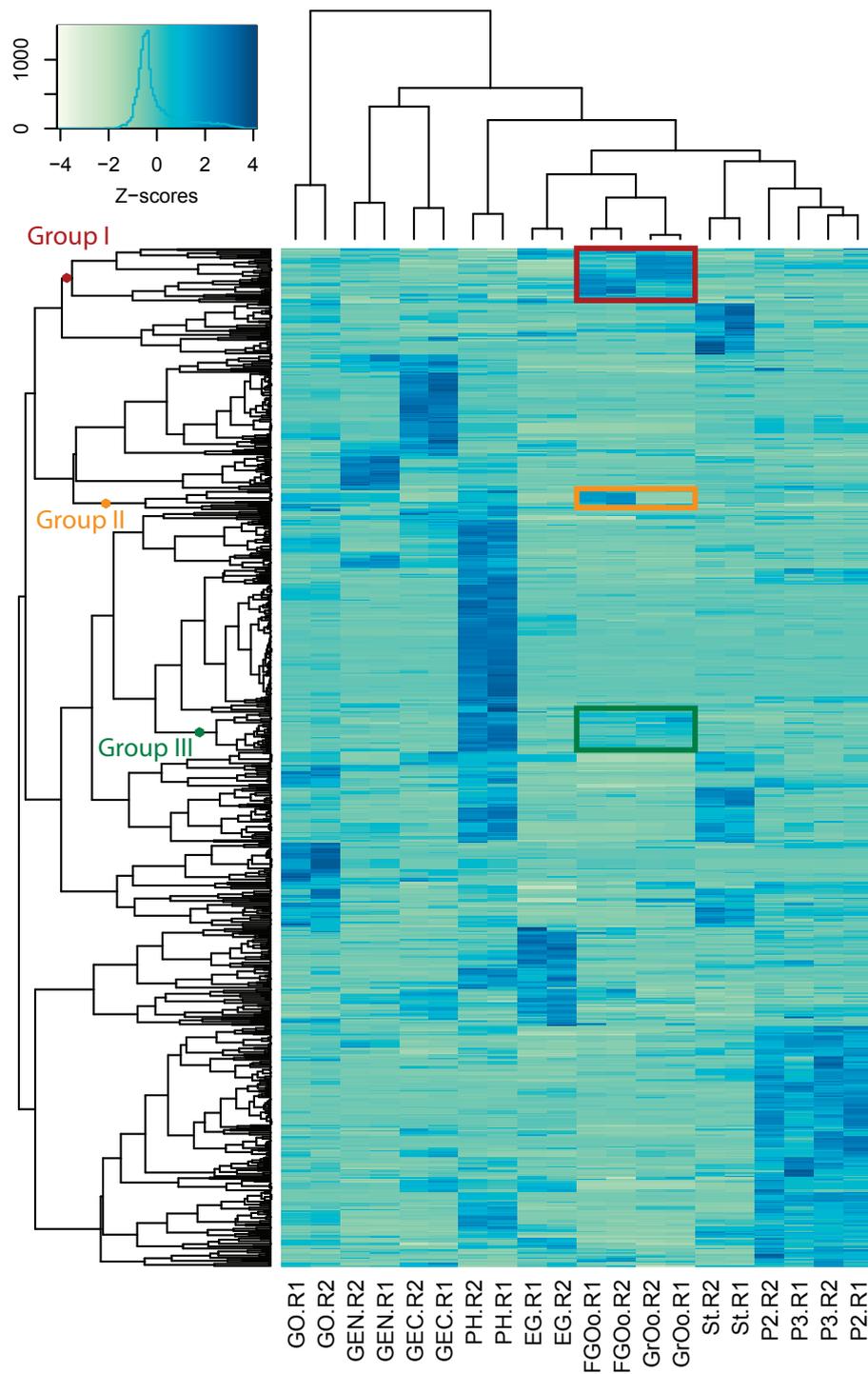


Figure S2. *Clysia* GPCRs expression across tissues and life cycle stages

Heat map representing the expression of putative *Clysia* GPCRs in the different samples studied derived from RNA-seq data (see methods), in which sequences are clustered according to similarity of their profiles across tissues and stages. Z-score values are colour-coded to reflect significantly higher (dark blue) or lower (pale green) than average values -see z value distribution in inset. Three main profile groups showed expression enriched in the oocytes (coloured boxes). Abbreviations: R1/R2= Biological Replicate1/2 for Illumina sequencing; GO= Gonozoid; GEN= Gonad Endoderm; GEC= Gonad Ectoderm; PH= Polyp Head; EG= Early Gastrula; FGOo= Fully Grown Oocytes; GrOo= Growing Oocytes; St= Stolon; P2/3= 2/3-day old Planula larvae.

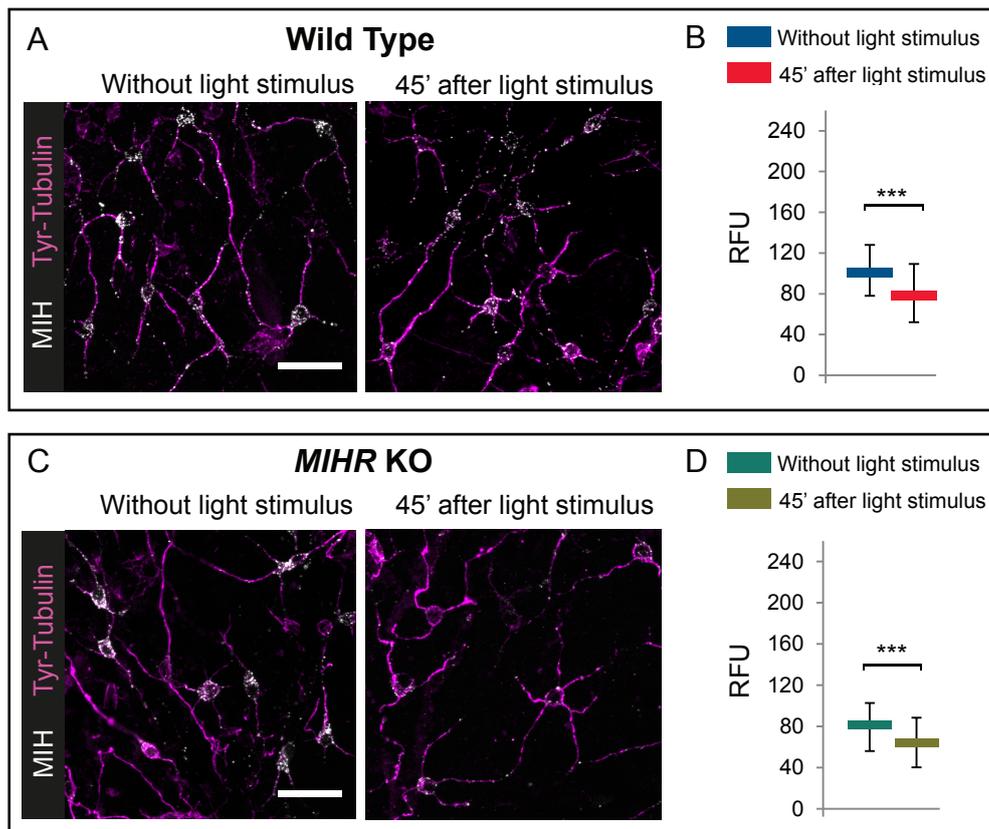


Figure S3. MIH-secreting neural cells in *CheMIHR* KO jellyfish

In all cases, the confocal images were taken during the same day, under the same scanning parameters (i.e. magnification, laser intensity and gain), and 10 confocal Z planes were summed over 3 μm depth at the gonad surface.

A) Representative immunofluorescence images of wild type *Clytia* gonad MIH-secreting cells before and after light stimulation. Anti-PRPa (MIH - white) and anti-tyrosinated tubulin (magenta) antibodies were used.

B) Graph showing the medians of RFU (Relative Fluorescence Units) values after fluorescence quantification in wild type MIH-secreting cells for the two conditions (number of cells quantified= 203 and n= 215, respectively). Limits correspond to 1st and 3rd quartiles. The Mann-Whitney U test showed a significant reduction in signal per cell following light stimulation ($p < 0.001$).

C) Representative immunofluorescence images of *MIHR* KO *Clytia* gonad MIH-secreting cells before and after light stimulation. The lack of MIH staining in some neural cells in the absence of light stimulation suggests that MIH might be lost from these cells under the abnormal physiological conditions of *MIHR* KO mutant gonads.

D) Graph showing the medians of RFU values after fluorescence quantification in *MIHR* KO MIH-secreting cells for the two conditions (number of cells quantified= 258 and n= 232, respectively). Limits correspond to 1st and 3rd quartiles. The Mann-Whitney U test showed a significant reduction in signal per cell following light stimulation ($p < 0.001$). Scale bars = 20 μm .

This experiment has only been done once and needs repetition.

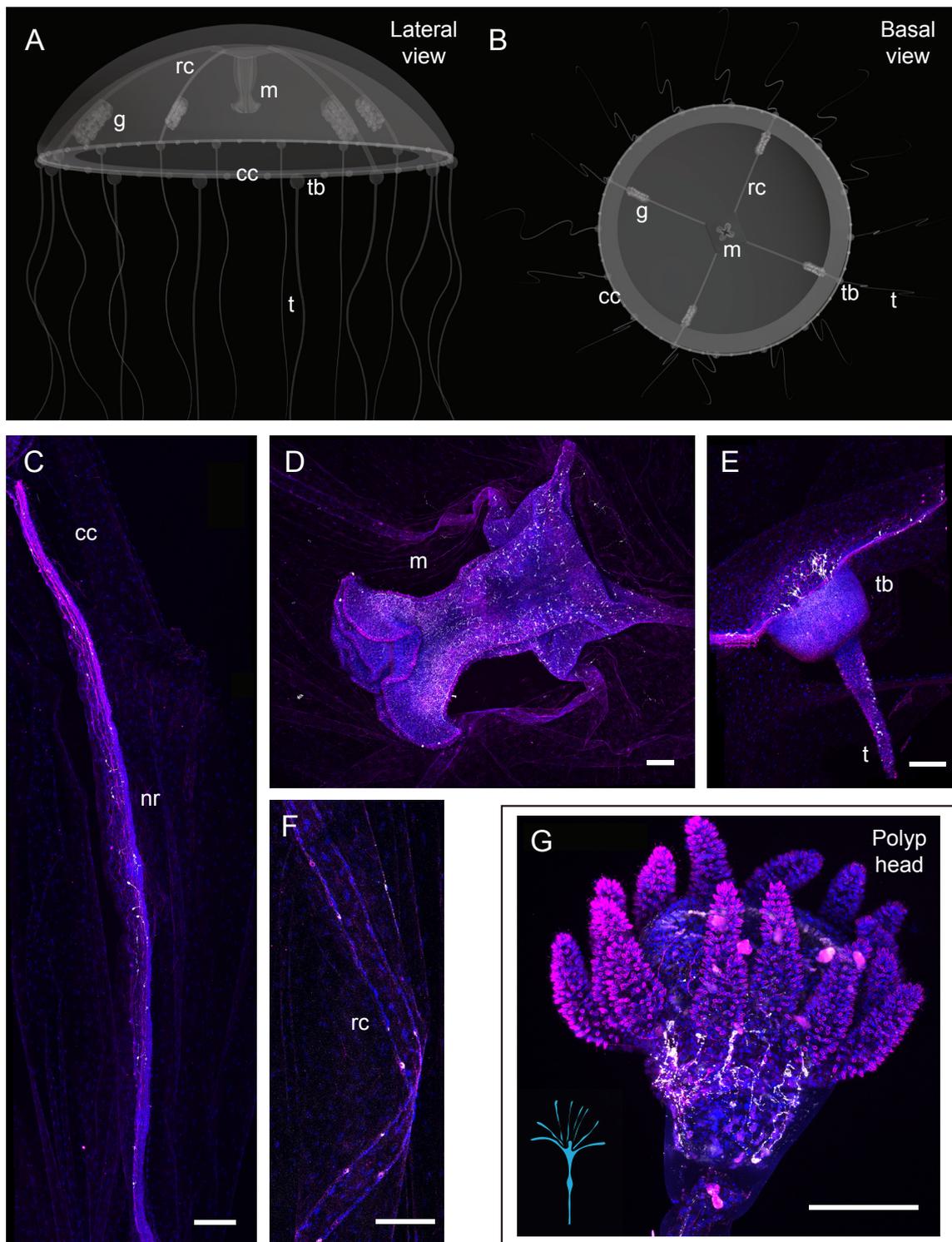


Figure S4. MIH in neural cells in *Clytia* adult jellyfish and polyps

A-B) Lateral and basal views of an adult *Clytia* jellyfish 3D schematic model indicating the different structures imaged in C-G: g= gonad; rc= radial canal; m= mouth/manubrium; cc= circular endodermal canal; tb= tentacle bulb; t= tentacle.
C-G) Confocal images (summed Z stacks) of the *Clytia* jellyfish and feeding polyp, stained with Hoechst dye (for nuclei - blue) and antibodies to PRPa (MIH - white), and tyrosinated tubulin (magenta). MIH staining decorates neural populations in the bell periphery (C), manubrium (D), tentacle (E), radial canals (F) and polyp heads (G). At the bell periphery the cells are associated with two condensed nerve rings (nr). All scale bars= 100 μ m.

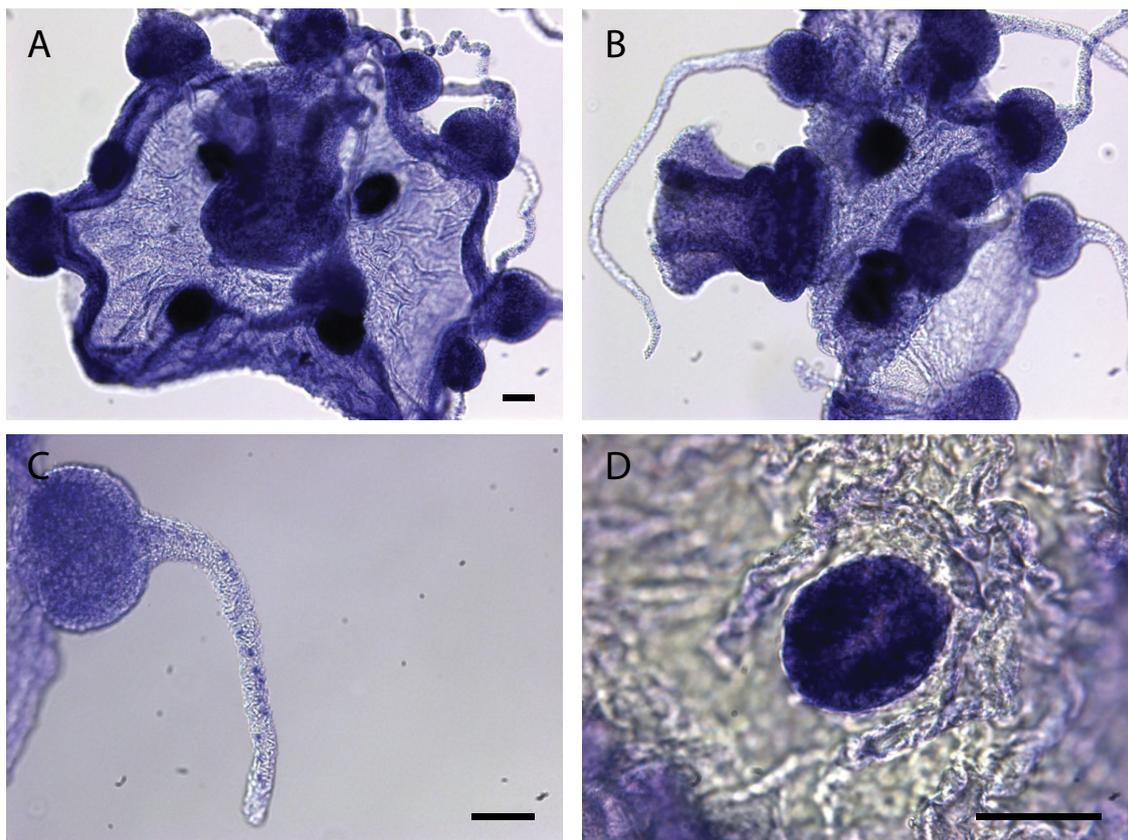


Figure S5. MIHR is expressed at different sites of *Clytia* jellyfish

In situ hybridization (ISH) detection of MIHR mRNA in *Clytia* jellyfish.

A-B) Basal and lateral views of young medusae showing heavy MIHR signal in gonads, tentacles and possibly manubrium and nerve ring.

C) Detail of a tentacle bulb and its tentacle showing expression of MIHR in some tentacle cells.

D) Detail of a young jellyfish gonad, the small oocytes of which are strongly positive for MIHR mRNA. All scale bars= 50 μ m. ISH needs to be repeated and Immunofluorescence tests with an MIHR mouse antiserum are underway.

Conclusion and Perspectives

The work presented in this chapter shows that *Clytia* MIHR is the essential receptor of MIH in the oocyte. It was identified by testing oocyte-expressed GPCR candidates for MIH responsiveness using a GPCR deorphanization assay. Conclusively, the oocytes of *Clytia* MIHR jellyfish mutants could not be induced to mature with artificial MIH, but matured in response to cAMP analogues, which act downstream of MIH reception.

The localization of *Clytia* MIH and MIHR in non-gonadal sites, combined with the phylogenetic position of CheMIHR, close to a subset of bilaterian GPCRs involved in the regulation of various physiological functions, strongly suggest a wider role of CheMIHR besides oocyte maturation.

To complete the studies of CheMIHR role in oocyte maturation initiation, I am planning to perform a set of experiments to try to elucidate the signalling cascade between MIH reception and cAMP rise inside *Clytia* oocytes. This would provide a complete description of the chain of events from MIH - receptor binding to cAMP signalling. Specifically, I will use inhibitory and stimulatory drugs affecting G α s subunits (e.g. NF449 and cholera toxin, respectively) as well as adenylyl cyclases (e.g. Forskolin). We hypothesize that this could be the most likely pathway involved since G α s and adenylyl cyclases are common molecular intermediates of GPCR pathways, and the activation of G α s leads to adenylyl cyclases activation, which in turn rises the cAMP levels in the cells cytoplasm (Stricker and Smythe, 2001).

Clytia MIH-MIHR represents the first cnidarian neuropeptide-GPCR couple characterized. *Clytia* MIHR does not have a direct bilaterian orthologue, but is part of an independently amplified cnidarian gene clade closely related to groups of bilaterian GPCRs involved in various physiological functions, including the neurohormonal control of sexual reproduction (see the manuscript in this chapter and General Discussion). This observation suggests that neuropeptidergic regulation of animal reproduction through neuropeptide-GPCR interactions may have been already a feature of the eumetazoan ancestor and then evolved in parallel in Cnidarians and Bilaterians. Based on the non-gonad expression and synthesis of MIH described in this chapter and in Chapter 1 (Figure S5 and S6 in Chapter 1 manuscript and Figure S4 in this chapter manuscript), it is likely that the MIH-MIHR neuropeptidergic system in *Clytia* is involved in the regulation of other physiological processes as well as the direct induction of meiosis resumption. To address this issue and obtain a wider view of CheMIH-MIHR physiological regulation in *Clytia* I will need to fully characterize the distribution of cells expressing CheMIHR by *in situ*

hybridization and immunofluorescence. Performing behavioural analyses with both wild types and *MIHR* KO *Clytia* jellyfish after MIH addition should also provide some insights in the involvement of this neuropeptide-GPCR couple on other physiological aspects of *Clytia* biology.

Comparisons between the complexity of the neurohormonal regulation of endocrine systems controlling oocyte maturation in bilaterians, and the relatively simple scenario found in *Clytia* gonads can allow us to propose evolutionary scenarios concerning the regulation of meiosis resumption in the animal kingdom. I will discuss this matter in more depth in the General Discussion.

With the finding of *Clytia* MIH and MIHR, we have identified two essential players in the regulation of *Clytia* oocyte maturation initiation. The work presented in the third chapter of my thesis concerns the upstream events: how light signals are detected by somatic cells of the gonad to lead to MIH secretion.

CHAPTER 3

**Identification and characterization
of the photopigment and
photoreceptive cells that regulate
Clytia spawning**

Context

The results presented in Chapter 1 showed that *Clytia* MIH is released from gonad ectoderm neural cells upon dark-light transitions (Figure 4 in Chapter 1 manuscript). More precisely, we have shown that MIH fluorescence levels diminish significantly 45 minutes upon reception of the light cue by *Clytia* gonads (see Figure 4 in chapter 1 and Figure S3 in chapter 2 manuscripts), implying its light-mediated release. Importantly, this response likely occurs within 20 minutes following light stimulation after a period of darkness, since GVBD, the hallmark of oocyte maturation initiation (see section II of introduction), is already visible at this point (Amiel et al., 2010). These observations suggest the involvement of fast-responsive photopigments in the *Clytia* gonad.

As the starting point for the work presented in the following manuscript, we hypothesized that light-induced oocyte maturation and subsequent spawning in *Clytia* might be regulated by opsin photopigments. We anticipated that these should be present in cells located in the gonad ectoderm because our collaborator R. Deguchi demonstrated in *Cladonema pacificum* that isolated gonad ectoderm, but not endoderm, tissue was found to produce active MIH (observations mentioned in Chapter 1 manuscript). Moreover, eighteen opsin genes were previously identified in *Cladonema radiatum*, and five of them were shown to be expressed in the manubrium, where the gonads of hydrozoan anthomedusae like *C. radiatum* are located, while the rest were found expressed in other body parts such as eyes or tentacles. In particular, one opsin (CropC) was detected by *in situ* hybridization in some cells of the gonad ectodermal epithelia covering the oocytes (Suga et al., 2008). All this information strengthens our hypothesis that opsin photopigments expressed in *Clytia* gonad ectoderm cells might be the responsible molecular actors of light-induced MIH release.

Background on opsins

Opsins are photosensitive proteins that belong to the GPCR superfamily of membrane receptors (see context chapter 2). The first opsin characterized was bovine rhodopsin (**Figure 22**) in 1983. Today several thousands of opsins have been identified throughout the animal kingdom (Terakita, 2005; Terakita et al., 2012). For convenience, the nomenclature used for amino acid position comparisons of important residues in newly found opsins are generally based on the positions held by the bovine rhodopsin (Terakita, 2005). The spectrum of light

absorption in the opsin family ranges from near ultraviolet to infrared (340nm to >700nm) (Martin, 2002).

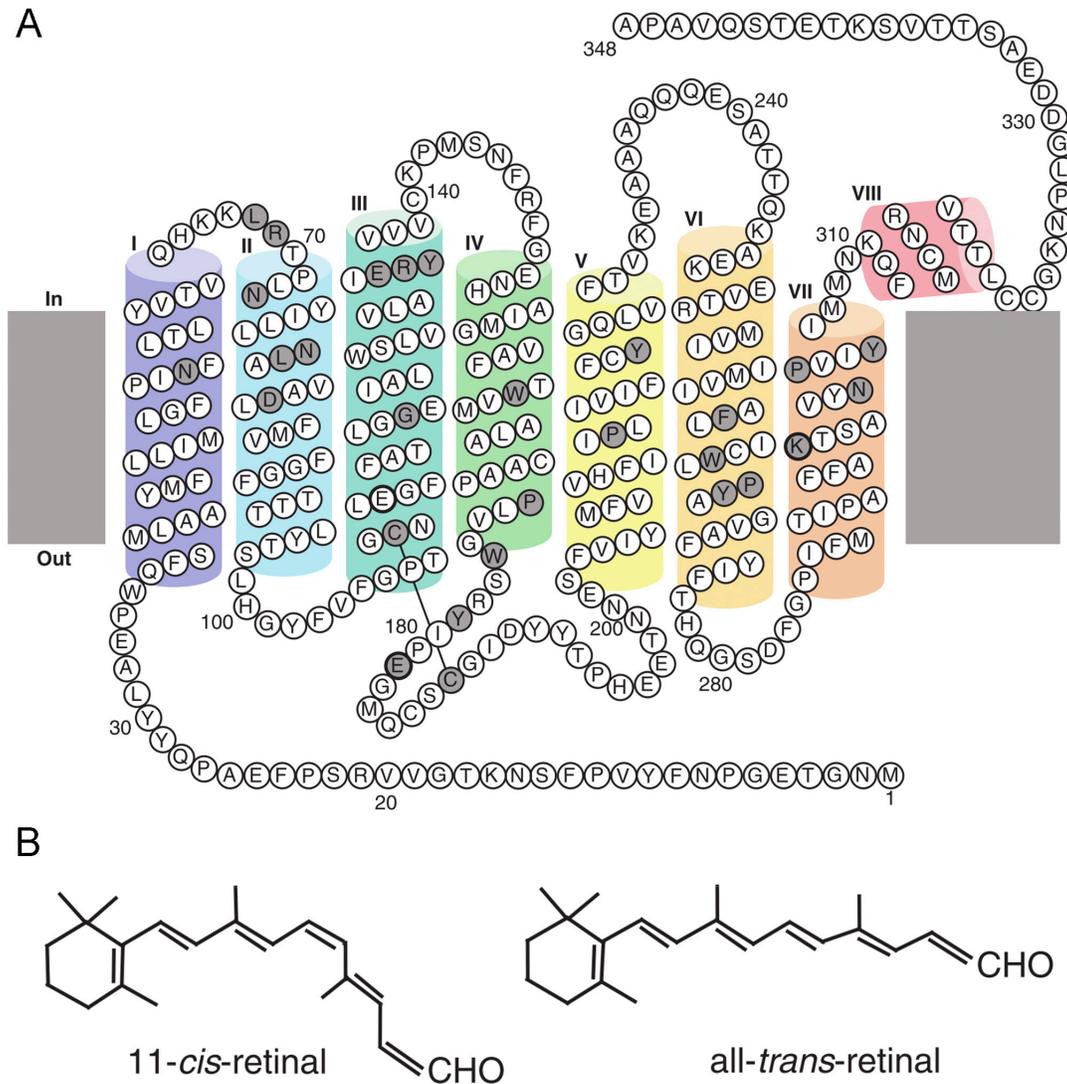


Figure 22. Structure of bovine rhodopsin and the chromophore retinal.

(A) Model of the secondary structure of bovine rhodopsin. Amino acid residues that are highly conserved in the opsin family are shown with a grey background. The retinal-binding site (K296) is marked with a bold circle. Residues E113 and E181 are also highlighted, and represent the two possible counterion positions, which help to stabilize the protonated Schiff base linkage. C110 and C187 form a disulphide bond important for opsin folding.

(B) The chemical structures of the 11-*cis* and all-*trans* forms of the chromophore retinal.

Taken from Terakita, 2005.

In order for the opsins to be photosensitive, a light-sensitive chromophore binds to a conserved lysine residue buried in the seventh transmembrane domain via a protonated Schiff base linkage (Terakita, 2005). This key lysine is necessary for light absorption and its presence can serve to judge whether a novel GPCR is an opsin or not. The chromophore is a vitamin-A derivative and is generally referred to as chromophore retinal. The chromophore retinal reacts to light stimulation by isomerizing from an 11-cis to an all-trans conformation (**Figure 22**), causing a structural change of the opsin GPCR and resulting in the activation of the G protein to which the opsin is coupled to. In consequence, a phototransduction cascade starts inside the photoreceptor cell, leading to the activation of effector enzymes which in turn activate ion channels that mediate electrophysiological cell responses (reviewed by Fain et al., 2010; Nilsson et al., 2009; Terakita, 2005; Terakita et al., 2012).

It is noteworthy that the chromophore retinal can be classified in two types, bleaching or bistable, and its characteristics depend on the type of opsin it is bound to (Nilsson et al., 2009; Terakita et al., 2012). Bistable pigments, when isomerized to the all-trans isoform, stay bound to the opsin and can be converted back to the 11-cis form by subsequent light absorption. Bleaching pigments are released from the opsin once converted to the all-trans form and need a separate enzymatic system to regenerate them (Nilsson et al., 2009; Terakita et al., 1989; Terakita et al., 2012). This reversion is generally mediated by a particular type of opsins, called photoisomerases, which instead of using light stimulation for cell signalling, use light to convert all-trans chromophore forms to the 11-cis forms. The regenerated 11-cis chromophore retinal is then released and can be used by conventional opsins (Chen et al., 2001; Nilsson et al., 2009; Terakita et al., 2012).

As mentioned in section I of the introduction, opsins are involved in both visual and non-visual photoreception, leading to rapid responses inside the photoreceptor cells (Nilsson et al., 2009). In visual photoreception, all photoreceptor cells are of neural nature and enlarge the surface of their membranes to increase the storage area of photopigments. The way they do so leads to the categorization of the two main types of photoreceptors, the ciliary and the rhabdomeric photoreceptor cells. Ciliary photoreceptors fold the membrane of their cilia, while rhabdomeric photoreceptors fold their apical cell surface (Arendt, 2003). Non-visual photoreception can be mediated by neural or non-neural cell types and can accommodate changes in environmental light without any membrane morphological specializations (Martin, 2002; Nilsson et al., 2009).

Opsins have been found in cnidarians (Liegertová et al., 2015; Mason et al., 2012; Suga et al., 2008), ctenophores (Schnitzler et al., 2012), placozoans (Feuda et al., 2012) and bilaterians (reviewed by Terakita et al., 2012). Their division into subfamilies has been based on the type of G alpha protein coupled to them (Go, Gs, Gq, Gt), mediating different phototransduction cascades, but also on the photoreceptor cell type in which they are found, or the organ or animal clade in which they have been firstly discovered, sometimes leading to confusion (Nilsson et al., 2009; Terakita, 2005). Moreover, opsin gene molecular phylogenies have been controversial so far, with cnidarian opsins being the most problematic sequences, leading to debate about their true phylogenetic relationship with bilaterian opsins (Feuda et al., 2012; Feuda et al., 2014; Liegertová et al., 2015; Plachetzki et al., 2007; Ramirez et al., 2016; Suga et al., 2008). More specifically it is not clear whether the cnidarian opsin family is a single or a paraphyletic clade.

Light affects the physiological and behavioural activities of cnidarians, including diel vertical migrations, responses to changes in light intensity, reproduction and even vision (Martin, 2002; Wolken and Mogus, 1979). In theory these regulations could involve opsin and/or cryptochrome pigments (see section I of Introduction). While cryptochrome family members have only been described in two species of anthozoans so far (Levy et al., 2007; Reitzel et al., 2010), opsins have been studied in several cnidarians. Since the first opsin was identified in *Hydra* in 2001 (Musio et al., 2001), a broad range of cnidarian opsins have been described. Opsin family members have been identified and characterized in the complex lens eyes of the cubozoan jellyfish *Tripedalia cystophora* and *Carybdea rastonii* (Koyanagi et al., 2008; Kozmik et al., 2008). They have also been found in neuronal cells mediating cnidocyte discharge in *Hydra* (Plachetzki et al., 2012), and in the planula larvae of the coral *Acropora palmata* (Mason et al., 2012). The expression of opsins in diverse body regions of hydrozoan and cubozoan jellyfish, from eyes to gonads, strongly supports their role in both visual and non-visual photoreception (Liegertová et al., 2015; Suga et al., 2008).

The results described in the following manuscript document the identification and characterization of an opsin expressed in *Clytia* gonad ectoderm photoreceptive cells, and the demonstration by gene editing that it has an essential role in triggering MIH release in response to light.

Overview of the findings of the study

Briefly, the findings presented in this manuscript are as follows:

- The light spectrum optimal to trigger female *Clytia* gonads spawning was determined via monochromatic light stimulation. Blue-cyan light was shown to be the most active in triggering this process.
- A list of 10 opsins was compiled from *Clytia* transcriptome data, and RNA-Seq analysis showed that one opsin was strongly expressed in the gonad ectoderm (Opsin9) while two others were only moderately expressed in this tissue (Opsin4 and Opsin7).
- *In situ* hybridization analyses were unable to detect Opsin4 and Opsin7 mRNAs in the gonad, whereas Opsin9 mRNA was strongly detected in a scattered population of cells in the gonad ectoderm.
- Double fluorescent *in situ* hybridization demonstrated the co-expression of Opsin9 and PP4 (one of the two *Clytia* MIH precursors) mRNAs in the same gonad ectoderm cells.
- A mutant KO line of Opsin9 (*Opsin9* n1_4) generated via CRISPR/Cas9 gene editing produced jellyfish unable to undergo oocyte maturation and spawning upon dark-light transitions. These jellyfish displayed grossly inflated gonads full of immature oocytes due to spawning incapability.
- Oocyte maturation and spawning of *Opsin9* mutant isolated gonads could be restored using artificial MIH.
- Immunofluorescence of gonads fixed before and after light stimulation showed that *Opsin9* mutant gonad ectoderm cells fail to secrete MIH upon light stimulation.
- Opsin9-expressing MIH-secreting cells in *Clytia* gonad ectoderm were shown by immunofluorescence analyses to have a multipolar neurosensory morphology, with loosely connected neuronal projections on the basal side and a cilium on the apical side.

- Phylogenetic analyses showed that *Clytia* Opsin9 belongs to a well-established medusozoan opsins group and that Opsin9 is not orthologous to opsins previously described in medusozoan gonads.

PhD student contribution: Pascal Lapébie performed RNA-Seq data mapping that helped to find the molecular candidates in each of the steps of oocyte maturation initiation in *Clytia*. Spectral analyses were performed during a visit to G. Jékély's laboratory (MPI, Tübingen). CRISPR-Cas9 gene KO was performed with help and advice from Tsuyoshi Momose. I performed all the experimental work, prepared the figures and wrote a first draft of the submitted manuscript that follows.

A gonad-expressed opsin essential for light-induced spawning in the hydrozoan *Clytia*

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ABSTRACT

Across the animal kingdom, environmental light cues integrate with endogenous clocks and hormonal signals to synchronise gamete release, but the underlying photoresponsive mechanisms remain poorly understood. In hydrozoan jellyfish, spawning is triggered directly by dark-light transitions, a process mediated by oocyte maturation-inducing neuropeptide hormones (MIHs) released from the gonad ectoderm. Using the model species *Clytia hemisphaerica* we determined that blue-cyan light triggers autonomous spawning in isolated gonads. A candidate opsin (Opsin9) identified from gonad transcriptomes was found co-

expressed with MIH within specialised ectodermal neural cells. *Opsin9* knockout jellyfish generated by CRISPR/Cas9 were defective in spawning, a phenotype reversible by synthetic MIH. We conclude that gamete maturation and release in *Clytia* is regulated by gonadal photosensory-neurosecretory cells that co-express MIH and Opsin9. Similar photosensitive-neurosecretory cells in ancestral eumetazoans may have allowed tissue-level photo-regulation of diverse behaviours, a feature elaborated in cnidarians in parallel with expansion of the opsin gene family.

INTRODUCTION

Environmental light is a crucial regulator of physiological and behavioural activities across the animal kingdom. In some animals, responses to light involve image-forming visual systems composed of neuronal photoreceptor cell types, sculpted by evolution into ocelli or complex eyes (Nilsson, 2009). Much more widespread though is non-visual photodetection, which in animals can be mediated by a variety of neuronal and also other cell types including dermal cells and cardiomyocytes (Whitmore et al., 2000; Wolken and Mogus, 1979). Non-visual photoreception can elicit direct responses such as phototaxis, photoavoidance and skin colour changes, but also regulates more complex behaviours such as migration and sexual mating (Wolken and Mogus, 1979). In this regard, a major role of non-visual photodetection is in the entrainment of endogenous circadian clocks (Cermakian and Sassone-Corsi, 2002). Two families of photo-sensitive proteins (photopigments) contribute to light detection in non-visual photoreceptor cells: the evolutionarily ancient Cryptochrome/Photolyase family (CPF) (Gehring and Rosbash, 2003; Oliveri et al., 2014), as well as the eumetazoan-specific opsin family of light-sensitive G Protein-Coupled Receptors (GPCRs) (Terakita et al., 2012), which are highly adaptable through amino acid changes at specific positions to respond selectively to different wavelengths (Hope et al., 1997).

Sexual reproduction is also widely regulated by non-visual photoreception (Halford et al., 2009; Juntti and Fernald, 2016; Lambert and Brandt, 1967; Leclère et al., 2016; Tessmar-Raible et al., 2011). Monitoring changes in light intensity and spectral composition, along with endogenous clocks and other environmental factors, allow the reproductive rhythms of many animals to follow environmental cycles of different lengths (daily, monthly, and/or seasonal) (Tessmar-Raible et al., 2011). Hydrozoans, a subgroup of Cnidaria, often display light-regulated sexual reproduction. Sexually mature adult hydrozoans, either jellyfish or polyps, house simple gonads in which the germ cells are sandwiched between ectoderm and endoderm layers without any additional layers of somatic ‘follicle’ cells

surrounding the female oocytes (Amiel et al., 2010; Leclère et al., 2016). In hydrozoans, light-dark or dark-light transitions trigger the release of mature gametes into the sea water by rupture of the gonad ectoderm (Miller, 1979; Roosen-Runge, 1962; Takeda et al., 2006). Gamete release is coordinated with diel migration behaviours in jellyfish to ensure gamete proximity for fertilisation (Martin, 2002; Mills, 1983). Light-dependent swimming behaviour changes involve direct photosensitivity of neurons in the “inner nerve ring” coupled to the swimming muscles beneath the medusa bell (Anderson and Mackie, 1977). The photodetection systems that mediate hydrozoan spawning operate locally within the gonads. Even isolated gonads will spawn upon dark-light or light-dark transitions (Freeman, 1987; Ikegami et al., 1978). However, the molecular nature of the photodetection system is unknown.

In female hydrozoans, the regulation of spawning is tightly coupled to oocyte maturation, a universal animal process in which resting ovarian oocytes resume meiosis to be transformed into fertilizable eggs (Yamashita et al., 2000). Oocyte maturation is induced by “Maturation-inducing hormones” (MIHs), released from gonad somatic tissues upon reception of the appropriate light cue (Freeman, 1987; Ikegami et al., 1978). Recently, we revealed the molecular identity of MIH of several hydrozoan species as PRPamide family tetrapeptides, which are produced by scattered neural cells of the gonad ectoderm (Takeda et al., submitted). Nothing is yet known about the cells or the photopigments responsible for gonad photoreception, nor their relationship to the cells producing MIH.

Opsin proteins are clearly good candidates to mediate jellyfish spawning, with expression of particular opsins associated with gonads reported in the hydrozoan *Cladonema radiatum* (Suga et al., 2008), and the cubozoan *Tripedalia cystophora* (Liegertová et al., 2015). Here we identify a cnidarian opsin gene in the hydrozoan jellyfish *Clytia hemisphaerica* that is expressed in the MIH-secreting cells of the gonad, and show by gene knockout that it plays an essential role in spawning via MIH release.

RESULTS

Spawning of *Clytia* ovaries is induced by blue-cyan light

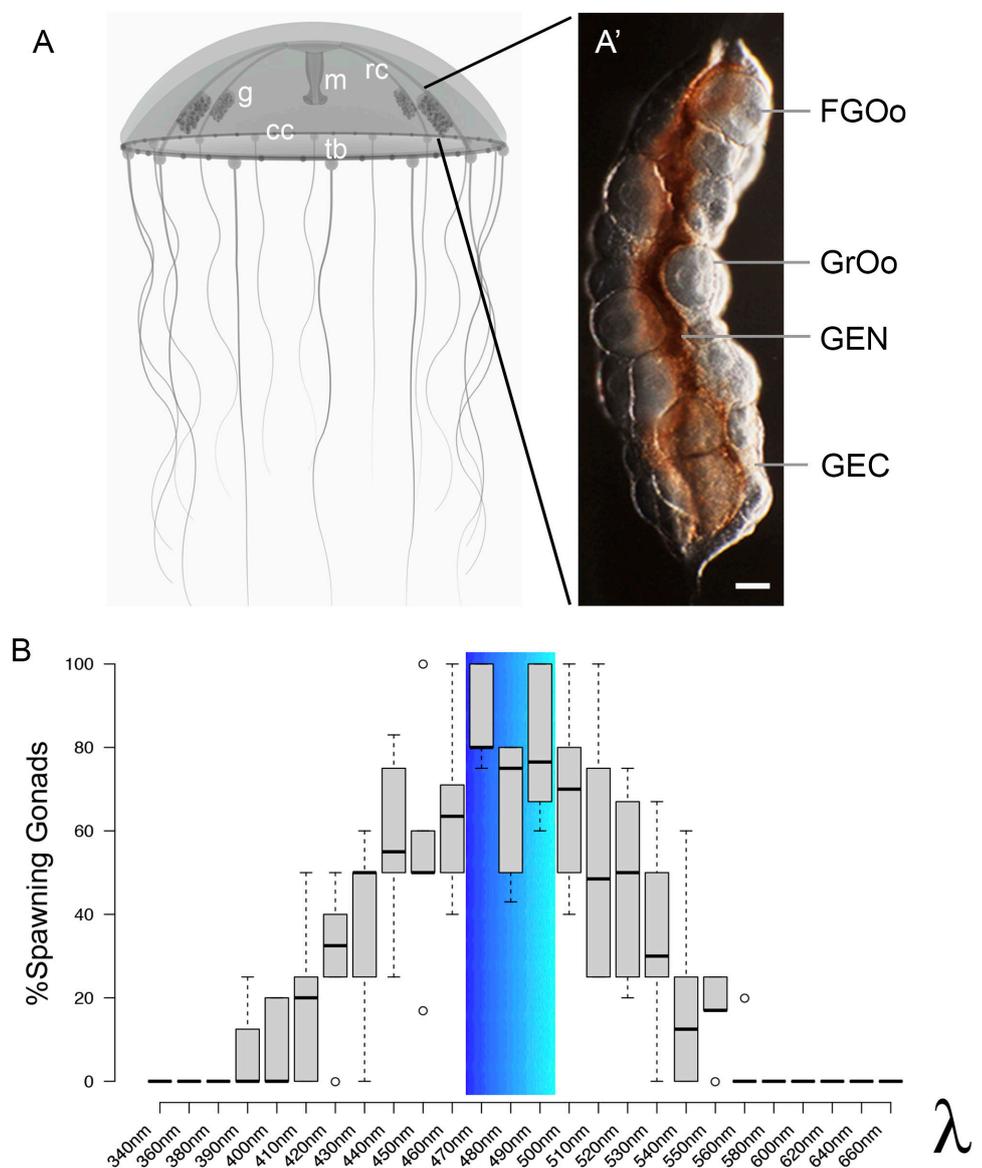
MIH release in *Clytia* gonads is triggered by a light cue after a minimum dark period of 1-2 hours, with mature eggs being released two hours later (Amiel et al., 2010). In order to characterise the light response of *Clytia* gonads (Fig.1A), we first assessed the spectral sensitivity of spawning. Groups of 3-6 manually dissected *Clytia* ovaries were cultured overnight in the dark and then stimulated from above with 10-second pulses of monochromatic light across the 340 to 660 nm spectrum. Stimulated

ovaries were returned to darkness for one hour before scoring oocyte maturation. Oocyte maturation is accompanied by the breakdown of the oocyte nucleus (Germinal Vesicle) in fully-grown oocytes, followed by spawning. We found that wavelengths between 430 and 520 nm provoked spawning in at least 50% of gonads, with 470-490 nm wavelengths inducing spawning of $\geq 75\%$ of gonads (Fig.1B). Oocyte maturation and subsequent spawning in *Clytia* ovaries is thus preferentially triggered by blue-cyan light (Fig.1B), the wavelength range which penetrates seawater the deepest (Gehring and Rosbash, 2003; Gühmann et al., 2015).

Figure 1. Spawning spectral characterization of *Clytia* ovaries

A) Schematic of a *Clytia hemisphaerica* female jellyfish: The four gonads (g) lie on the radial canals (rc) that connect the mouth/manubrium (M) to the circular canal (cc) running around the bell rim, adjacent to the tentacle bulbs (tb). **A')** Photo of a *Clytia* ovary. FGOo = fully-grown oocytes; GrOo = growing oocytes; GEN = gonad endoderm; GEC = gonad ectoderm. Bar = 100 μ m.

B) BoxPlot Graph showing spectral characterization of *Clytia* spawning. Groups of 3-6 isolated gonads were exposed to 10-second pulses of monochromatic light (see methods). Gonads were considered to spawn if at least one oocyte underwent maturation and release. Statistics were calculated based on percentage of gonad spawning in response to a specific wavelength obtained from 5-6 independent experiments. A total of 20-30 gonads were analysed per wavelength. Centre lines show the medians; box limits indicate the 25th and 75th percentiles (1st and 3rd quartiles) as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; outliers are represented by circles. Colour spectrum is shown only for wavelengths that exhibit medians of 75% or higher (470nm – 490nm). λ = wavelength.



Identification of a highly expressed opsin in *Clytia* gonad ectoderm

We identified a total of ten *Clytia* opsin sequences in transcriptome and genome assemblies from *Clytia* by reciprocal BLAST searches using known hydrozoan opsin sequences (Suga et al., 2008). All ten *Clytia* opsins possess the key lysine in the 7th transmembrane domain to which the retinal chromophore binds (Terakita et al., 2012) (Fig S4). We selected candidate opsins for a role in mediating MIH release by evaluating expression in the isolated gonad ectoderm, which has the autonomous capacity to respond to light (Takeda et al., submitted). Illumina HiSeq reads for gonad ectoderm, endoderm and oocytes at two different growth stages were mapped against each opsin sequence (Fig.2A). In the gonad ectoderm sample, two opsin mRNAs (Opsin4 and Opsin7) were detected at low levels and one was very highly expressed (Opsin9), while in the other gonad tissues opsin expression was virtually undetectable (Fig.2A). The high expression of Opsin9 gene in the gonad ectoderm made it a strong candidate for involvement in light-induced spawning.

Molecular phylogenies constructed from an alignment of the 10 *Clytia* opsin amino acid sequences and a dataset of Feuda et al., 2014 (Feuda et al., 2014), comprising a comprehensive subset of eumetazoan opsins, clustered all *Clytia* opsins within a clade containing only opsins from medusozoan cnidarians (Fig.S1). More detailed analysis of all hydrozoan and cubozoan opsin sequences available in GenBank reinforced the picture and expansion of opsins within the Medusozoa, with *Clytia* Opsin9 (along with the closely related Opsin10) being amongst the most divergent (Fig. S2, S3). Additional evidence for a distinct evolutionary history for these sequences came from analysis of draft genome sequences (not shown) revealing that *Clytia* Opsin9 and Opsin10, unlike all other described medusozoan opsin genes (Liegertová et al., 2015), contain an intron in a distinct position to the one predicted for the ancestral eumetazoan opsin. We confirmed, however, that the Opsin9 amino acid sequence exhibits all the hallmarks of a functional photopigment (Fig.S4), with conserved amino acids at positions required for critical disulphide bond formation and for Schiff base linkage to the retinal

chromophore (Fischer et al., 2013; Schnitzler et al., 2012), including acidic residues at the both potential ‘counterion’ positions, only one of which is largely conserved in cnidarian opsin sequences (Liegertová et al., 2015). The Glu/Asp-Arg-Tyr/Phe motif adjacent to the third transmembrane domain, involved in cytoplasmic signal transduction via G protein (Fischer et al., 2013; Kojima et al., 2000; Schnitzler et al., 2012), is also present in Opsin9.

The exact relationship of Opsin9/10 and other medusozoan opsins cannot be resolved with the available data, due to low branch support values, the variable topologies of Maximum Likelihood (ML) (Fig.S2) and Bayesian (Fig.S3) trees and unstable position of Opsin9/10 under different choices of evolutionary model (not shown). It was nevertheless clear from our analyses that *Clytia* Opsin9 is not orthologous to gonad-expressed opsin genes identified in *Cladonema* or in *Tripedelia* (Liegertová et al., 2015; Suga et al., 2008), indicating that the expression of any particular opsin genes in the gonad is unlikely to be a common ancestral feature of the medusozoans.

Opsin9 is expressed in MIH-secreting ectoderm cells

In situ hybridisation of *Clytia* ovaries revealed Opsin9 expression in a scattered population of gonad ectoderm cells (Fig.2B), but not anywhere else in the jellyfish (not shown). We were unable to detect either of the two minor ectoderm opsin mRNAs Opsin4 or Opsin7 by *in situ* hybridization in *Clytia* gonads (Fig.2D-E). The distribution of Opsin9-expressing cells was highly reminiscent of the expression pattern for PP1 and PP4 (Fig.2C), the two MIH neuropeptide precursor genes co-expressed in a common gonad ectoderm neural population (Takeda et al., submitted). Double fluorescent *in situ* hybridization using probes for Opsin9 and for PP4 revealed that these genes were expressed in the same cells (Fig.2F); 99% of Opsin9 mRNA positive cells were also positive for PP4 mRNA, and over 87% of PP4 mRNA-positive cells were also positive for Opsin9 mRNA. This striking result raised the possibility that spawning in *Clytia* might be directly controlled by light detection through an opsin photopigment in the MIH-neurosecretory cells of the gonad ectoderm.

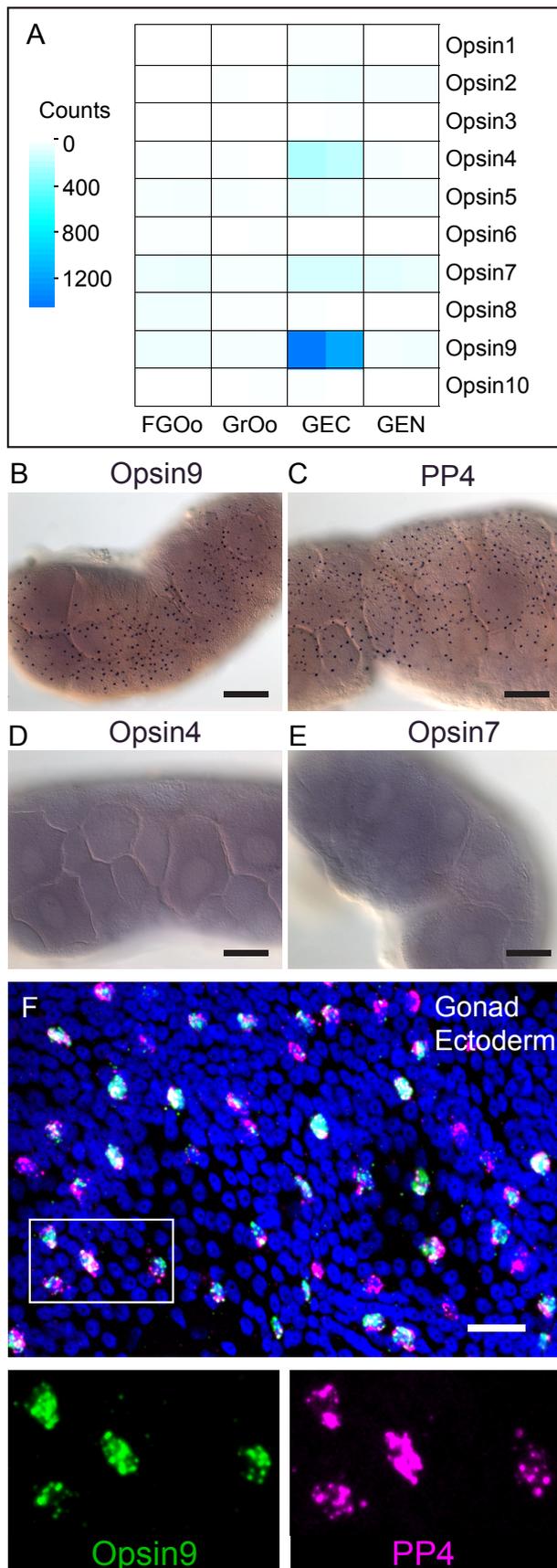


Figure 2. *Clytia* Opsin expression in gonad ectoderm cells

A) Heat map representing the expression of the ten opsin sequences from *Clytia hemisphaerica* in different gonad tissues. Illumina HiSeq 50nt reads from isolated endoderm (GEN), ectoderm (GEC), growing (GrOo) and fully-grown oocytes (FGOo) from mature female ovaries were mapped against the opsin sequences. Counts were normalized per total number of reads in each sample and per sequence length.

B) *In situ* hybridization (ISH) detection of Opsin9 mRNA in scattered ectodermal cells of female *Clytia* gonads.

C) ISH of the neuropeptide precursor PP4 mRNA in *Clytia* female gonads, also showing a scattered pattern in the ectoderm.

D-E) ISH of Opsin4 and Opsin7 mRNAs, respectively, in *Clytia* ovaries, showing no detectable localized expression of these two opsin genes.

F) Double fluorescent ISH showing co-expression of Opsin9 (green) and PP4 (magenta) mRNAs in gonad ectoderm cells; nuclei (Hoechst) in blue. Single channels are shown for the outlined zone in the top image. Of $n = 594$ randomly chosen cells expressing either gene counted in 10 different gonads, over 86% co-expressed Opsin9 and PP4 mRNAs. Controls with single probes were performed to validate a correct fluorescence inactivation and ensure that the two channels did not cross-react (not shown). Scale bars in B-E = 100 μm ; F = 20 μm .

***Opsin9* gene knockout prevents oocyte maturation and spawning**

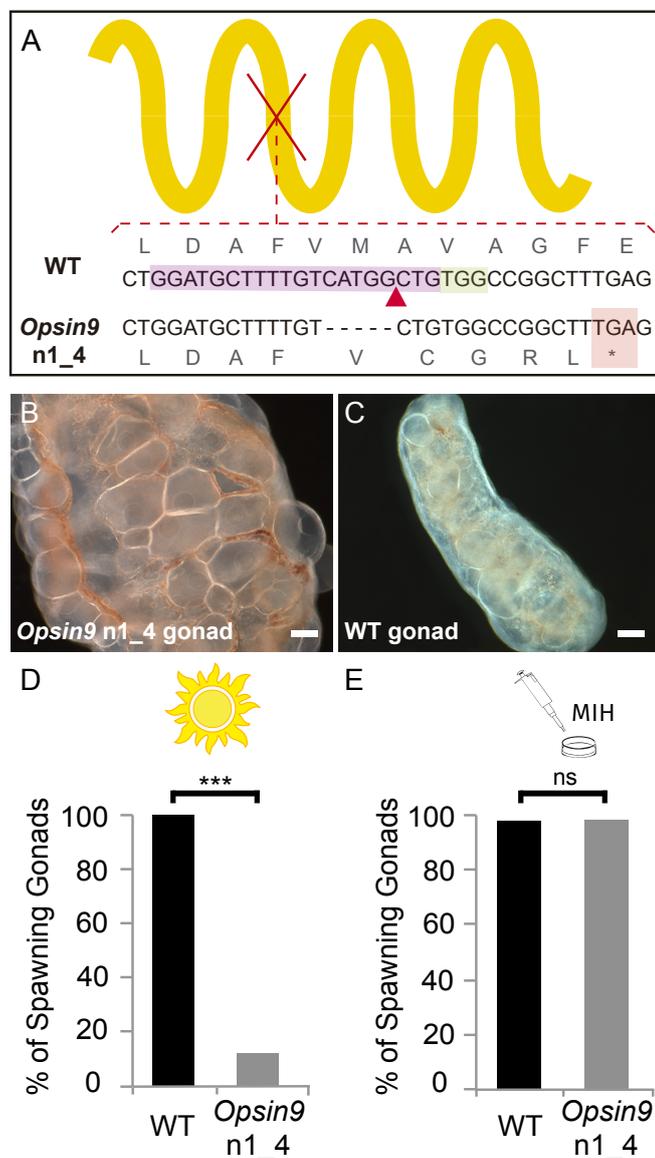
To test the function of Opsin9 in light-induced oocyte maturation and spawning, we generated a *Clytia Opsin9* knockout (KO) polyp colony using a CRISPR/Cas9 approach, which produces very extensive bi-allelic KO of the targeted gene in the F0 generation (Momose and Concordet, 2016) (see methods for details). This approach is favoured by the *Clytia* life cycle, in which larvae developed from each CRISPR-injected egg metamorphoses into a vegetatively-expanding polyp colony, from which sexual jellyfish stage bud clonally (Amiel et al., 2010; Leclère et al., 2016). CRISPR guide RNAs were designed to target a site in the first exon of *Opsin9* encoding the 3rd transmembrane domain, and one of the polyp colonies generated carried a predominant 5-bp deletion (*Opsin9* n1_4), corresponding to a frame-shift and premature STOP codon (Fig.3A). Larval development, metamorphosis and polyp colony expansion of this individual occurred normally.

For phenotypic analysis we collected *Opsin9* n1_4 jellyfish, which were all females, and grew them by twice-daily feeding for two weeks to sexual maturity. Although these *Opsin9* n1_4 mature medusae initially appeared normal, they did not spawn after the daily dark-light transition, and after a few days displayed grossly inflated ovaries due to an accumulation of unreleased immature oocytes (Fig.3B-C). In three independent experiments to test the light response of isolated gonads, over

85% of *Opsin9* KO gonads failed to undergo oocyte maturation and spawning upon light stimulation (Fig.3D).

Individual genotyping of gonads showed that the rare gonads from *Opsin9* n1_4 medusae which spawned after light had greater mosaicism of mutations, with a higher ratio of residual non-frameshift mutations and also a significant amount of wild type cells, whereas gonads that failed to spawn carried mainly the predominant 5-bp deletion, a second 21-bp deletion and no wild type cells (Fig.S5).

The spawning failure observed in *Clytia Opsin9* KO mutant jellyfish gonads strongly indicates an essential role for the Opsin9 photopigment in light-induced oocyte maturation.



Opsin9 is required for light-induced MIH secretion from gonad ectoderm

Since Opsin9 and MIH are co-expressed in the same cells, we reasoned that Opsin9 function was probably required for MIH secretion.

Two experimental approaches confirmed this hypothesis. Firstly, treatment of *Opsin9* mutant gonads with synthetic MIH peptides was able to rescue fully the mutant phenotype. Equivalent MIH concentrations to those effective for light-adapted isolated gonads (Takeda et al., submitted) reliably induced oocyte maturation and spawning in *Opsin9* KO isolated gonads (Fig. 3E). Spawned eggs from *Opsin9* mutants were competent to be fertilized and to develop into larvae, although the fertilization rate was lower than in the wild types (not shown).

Secondly, we showed by immunofluorescence that MIH release was compromised in *Opsin9* mutant gonads (Fig. 4). Quantitative immunofluorescence analyses based on anti-MIH staining and fluorescence levels were performed in both wild type and *Opsin9* mutant gonads, comparing light-adapted and dark-adapted specimens fixed 45 minutes after white light exposure. Whereas wild type gonads exhibited a significant decrease of MIH fluorescence values in MIH-secreting neural cells upon light stimulation (Fig. 4A-C), *Opsin9*

Figure 3. *Opsin9* n1_4 knock out line genetic engineering and phenotype

A) Scheme of Opsin9 GPCR showing part of the genomic region coding for the 3rd transmembrane domain targeted by *Opsin9* n1_4 CRISPR sgRNA. Corresponding amino acids are shown. Pink box indicates the target site of the sgRNA. Green box is the PAM sequence (NGG). The expected cleavage site of Cas9 is indicated by a red triangle. The predominant 5-bp deletion of *Opsin9* n1_4 is shown. This mutation leads to a frame-shift and an early STOP codon in Opsin9 (red box).

B) Representative inflated ovary of an *Opsin9* n1_4 mutant with an abnormal accumulation of oocytes due to spawning failure upon light stimulation.

C) Representative wild type *Clytia* ovary for comparison. Both images were taken 8 hours after the natural light cue (note the absence of fully grown oocytes in the wild type ovary). Scale bars all 100 μ m.

D) Oocyte maturation and spawning capability upon light stimulation of wild type (WT) and *Opsin9* n1_4 gonads. Percentage of spawning gonads combined from 3 independent experiments is shown in all cases; n = 92 for wild type and n = 154 gonads for *Opsin9* n1_4 mutants. The Fisher exact test showed significant differences (F = 0) between wild types and mutants.

E) Equivalent analysis for synthetic MIH treatment of wild type and *Opsin9* n1_4 gonads. Oocyte maturation and spawning were rescued by synthetic MIH treatment; n = 94 gonads for wild type and n = 80 gonads for *Opsin9* n1_4 mutants. Fisher exact test did not show significant differences (F = 0.595) between wild types and mutants.

mutant gonads maintained similar levels of MIH fluorescence in both conditions (Fig.4D-F). Moreover, average MIH fluorescence levels per cell were significantly higher (U test: $p < 0.001$) in *Opsin9* mutant than in wild type ovaries suggesting a progressive accumulation of MIH in *Opsin9* mutant gonads.

Taken together, these results demonstrate that Opsin9 is required for MIH-neurosecretory cells in the *Clytia* gonad to release MIH following dark-light transitions, and thus has an essential role in light-dependent reproductive control.

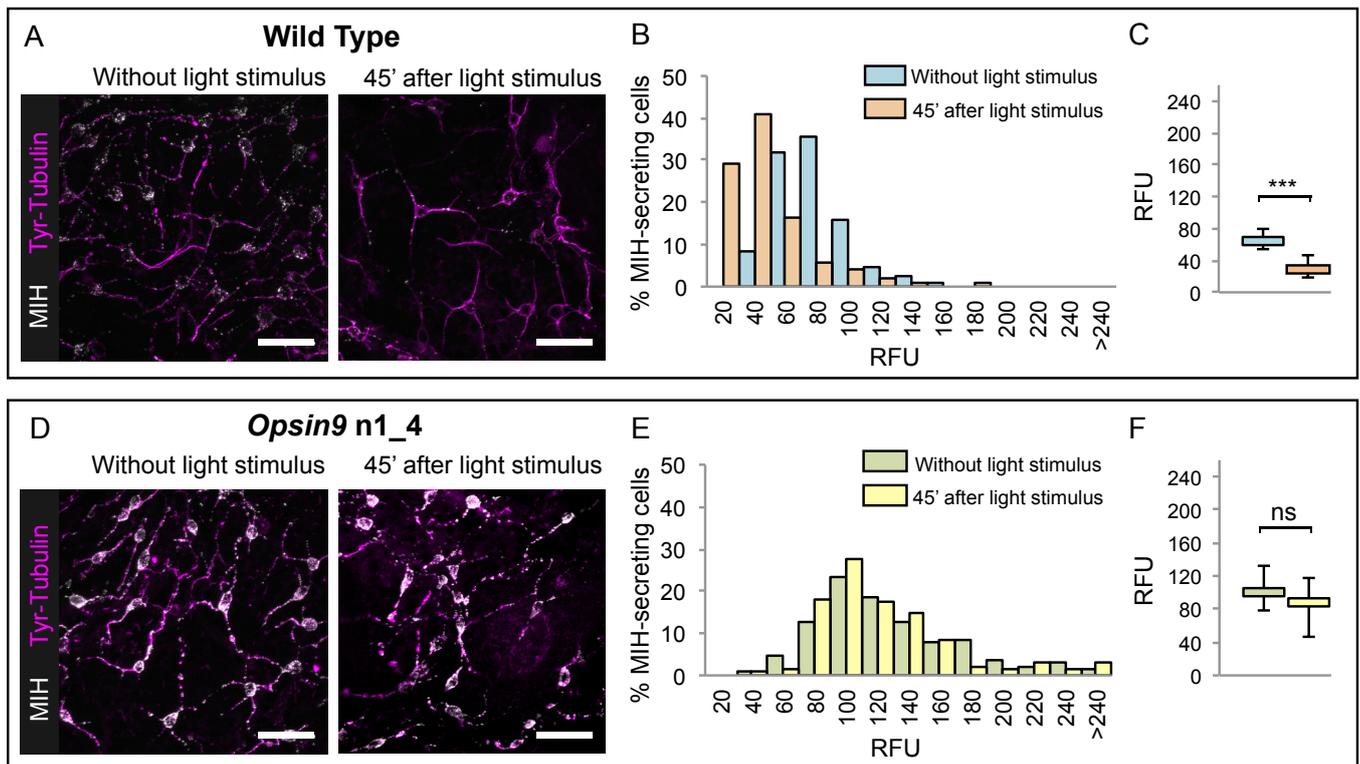


Figure 4. Impaired MIH secretion upon light stimulation in *Opsin9 n1_4* mutants

In all cases, the confocal images were taken during the same day, under the same scanning parameters (i.e. magnification, laser intensity and gain), and 10 confocal Z planes were summed over 4 μm depth at the gonad surface.

A) Representative immunofluorescence images of wild type *Clytia* gonad MIH-secreting cells before and after light stimulation. Anti-PRPa (MIH - white) and anti-tyrosinated tubulin (magenta) antibodies were used. The reduction in MIH fluorescence after light corresponds to MIH secretion.

B) Distribution of RFU (Relative Fluorescence Units) values after fluorescence quantification in wild type MIH-secreting cells in the two conditions (number of cells quantified = 226 and $n = 282$, respectively).

C) Graph showing the medians of the data in (B). Limits correspond to 1st and 3rd quartiles. The Mann-Whitney U test showed a significant difference ($p < 0.001$).

D) Representative immunofluorescence images of *Opsin9 n1_4* *Clytia* gonad MIH-secreting cells before and after light stimulation. MIH fluorescence is maintained upon light stimulation.

E) Distribution of RFU values after fluorescence quantification in *Opsin9 n1_4* MIH-secreting cells in the two conditions ($n = 183$ and $n = 201$, respectively).

F) Graph showing the medians of the data in (E). Limits correspond to 1st and 3rd quartiles. Mann-Whitney U test did not show a significant difference ($p = 0.976$). Scale bars all 20 μm .

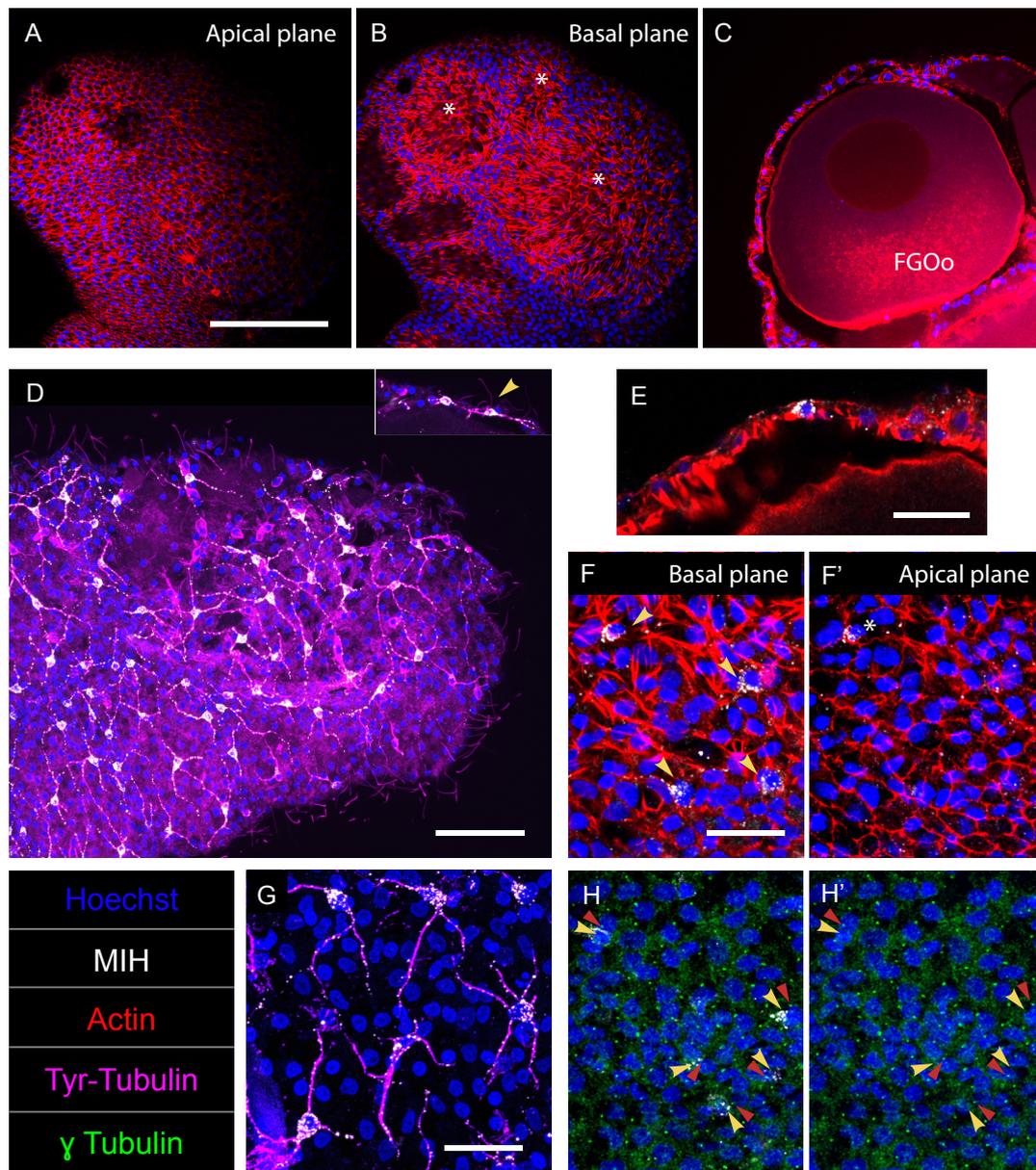


Figure 5. Morphology of *Clytia* MIH-secreting cells

Immunofluorescence of *Clytia* ovaries with focus on the gonad ectoderm epitheliomuscular cells and MIH-secreting cells. Staining with Hoechst (blue), anti-PRPa (MIH - white), phalloidin (red), anti-tyrosinated tubulin (magenta), and anti- γ tubulin (green).

A) Confocal plane taken at the gonad surface. Phalloidin decorates the apical junctions between epitheliomuscular cells.

B) Same gonad as in (A) in a plane corresponding to the basal level of the ectoderm in areas overlying large oocytes (asterisks); phalloidin strongly stains the epitheliomuscular muscle fibres.

C) Deeper Z plane from another gonad cutting through a fully-grown oocyte (FGOo) showing the monolayer of the thin gonad ectoderm and the relationship of the epitheliomuscular fibres to the underlying oocytes.

D) Confocal image of a gonad showing the distribution of MIH-secreting neural cells in the ectoderm (16 confocal Z planes were summed over 7 μ m depth at the gonad surface). Cilia were stained with anti-tyr-tubulin antibodies on epitheliomuscular cells, and could tentatively be detected on some MIH-secreting cells (Top right inset - yellow arrowhead).

E) Higher magnification image of a MIH-secreting cell inserted in the myoepithelial layer.

F, F') Two Z planes through the basal (F) and apical (F') planes of the epithelial layer. The cell bodies, nuclei and neural processes of the MIH-secreting cells (yellow arrowheads) are mainly in the basal plane, at the same level as the basal muscle fibres of the epitheliomuscular cells, however traces of MIH can be detected at the apical surface for some cells (asterisk).

G) Higher magnification image of MIH-secreting cells showing the lack of obvious connections between neural processes.

H, H') Gonad ectoderm surface Z plane showing ciliary basal bodies (stained for γ tubulin) of both epitheliomuscular cells and MIH-secreting cells (yellow arrowheads point to MIH-secreting cells and red arrowheads point to the basal bodies most probably associated with them - MIH staining shown only in H for clarity). Scale bars in A-C = 100 μ m; D = 50 μ m; E-H' = 20 μ m.

Neuronal morphology of *Clytia* gonad MIH-secreting cells

The functional studies described above indicate that *Clytia* gonad cells that co-express MIH and Opsin9 have both photosensory and neurosecretory functions. To characterize the morphology of these key cells we performed immunofluorescence to visualise microtubules and cortical actin in cells producing MIH (Fig.5). The *Clytia* gonad ectoderm consists of a monolayer of ciliated epitheliomuscular cells (Leclère and Röttinger, 2017), tightly coupled by apical junctions, and bearing basal extensions containing muscle fibres, which form a layer over the oocytes (Fig.5A-C, E-F). The MIH-secreting neural cells were found to be scattered among epitheliomuscular cells and to be much less abundant (Fig.5D, G). They possess a variable number of neural processes (Fig.5D, G) projecting from the basal side, which intermingle with the basal muscle fibres of surrounding epitheliomuscular cells (Fig.5F). Their cell bodies can reach the surface (Fig.5E, F'), although their

nuclei are generally located more basally (~1 μm) than those of the surrounding epitheliomuscular cells (Fig.5F-F'). In some cases, basal bodies and cilia could be identified at the apical pole of the MIH-secreting cells, (Fig.5D, H-H'). These neural, MIH-secreting cells appear loosely to intermingle but not to be directly connected in a network (Fig.5G). The peptide hormone (MIH) is probably secreted at a distance from the surface of fully grown oocytes where it acts, with gaps separating the gonad ectoderm and the large oocytes (Fig.6.C, E), likely corresponding to extracellular jelly components produced during the end of oocyte growth.

Taken together, these immunofluorescence analyses show that the MIH-secreting cells have morphological features characteristic of multipolar neurosensory cells in cnidarians (Saripalli and Westfall, 1996). Based on gene expression, morphology and biological function we propose that this cell type has a dual sensory-neurosecretory nature.

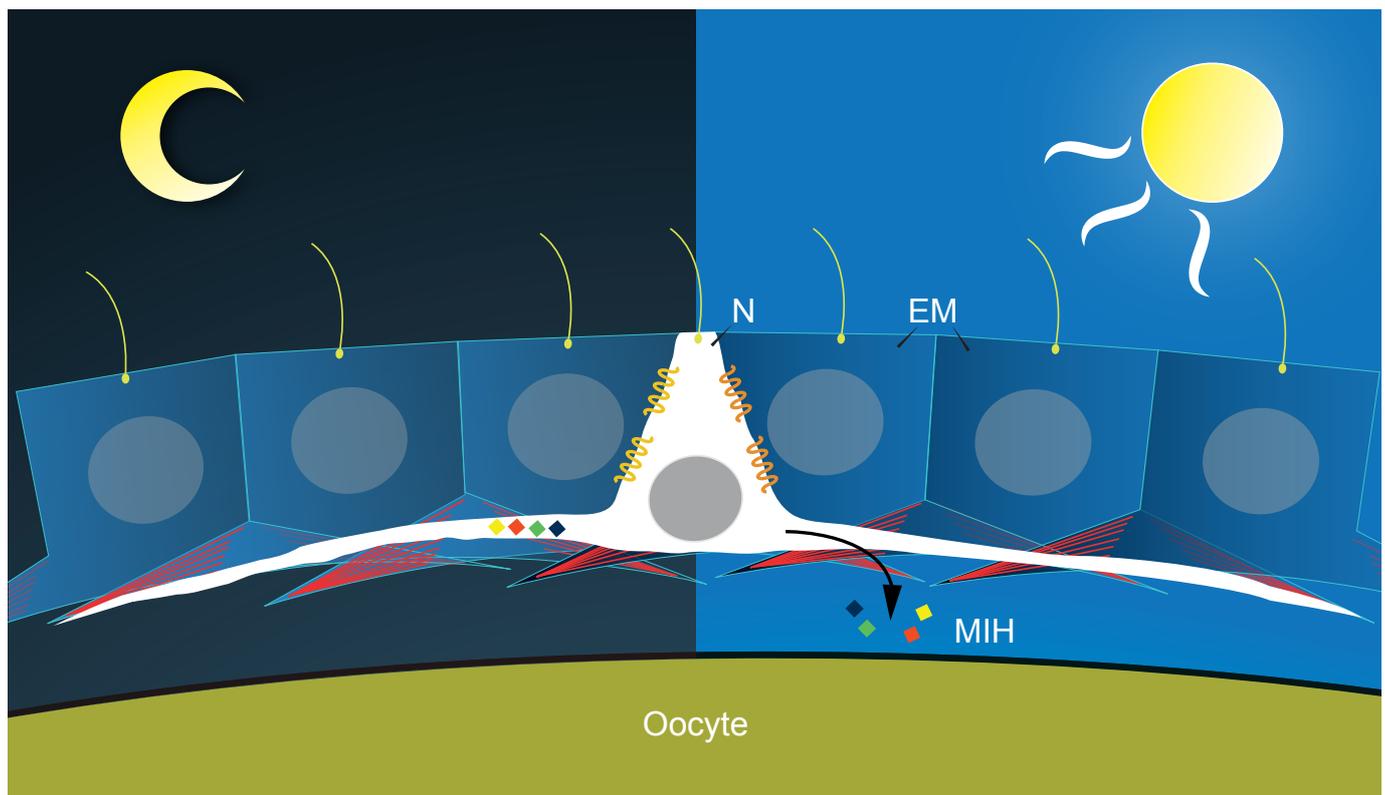


Figure 6. Model for Opsin9 function in *Clytia* oocyte maturation

Schematic representation of *Clytia* gonad ectodermal tissue depicting a MIH-secreting neural cell among myoepithelial cells. Light probably acts on Opsin9 in these cells, leading to MIH secretion, which in turn acts on the oocyte surface to trigger maturation. N = MIH-secreting neural cell; EM = Epitheliomuscular cell.

To conclude, we have provided the first demonstration, using CRISPR/Cas9-mediated gene knockout, of an essential role for an opsin gene in non-visual photodetection in a cnidarian. *Clytia* Opsin9 is an essential component of a direct light-response mechanism that acts locally in the gonad to trigger gamete maturation and release (Fig. 6). It

acts in specialized sensory-neurosecretory cells of the *Clytia* gonad ectoderm to trigger MIH secretion from these cells upon light reception. MIH in turn induces oocyte maturation in females, and also release of motile sperm in males (Takeda et al., submitted), efficiently synchronising spawning to maximise reproductive success.

DISCUSSION

Within Cnidaria, expansion of the opsin gene family has produced a large number of distinct non-visual opsins, whose precise evolutionary relationships are yet to be conclusively unravelled (Fig.S1-S3; Cronin and Porter, 2014; Feuda et al., 2014; Liegertová et al., 2015; Suga et al., 2008). This extensive cnidarian opsin diversity probably relates to individual species-specific spectral tuning optimised for both visual and non-visual photodetection (Liegertová et al., 2015), possibly involving co-expression in the same cells of opsins with different sensitivities to extend light responses over the broad range of wavelengths encountered in the marine environment (Gühmann et al., 2015). An additional factor favouring opsin diversification in Cnidaria is illustrated by our analysis of *Clytia* Opsin9 function in the gonad, namely the lack of a centralised nervous system and a corresponding localised regulation of many physiological processes and behaviours at the organ, tissue or even the cellular levels. Direct light-mediated responses in cnidarians, include light-sensitive discharge of cnidocyte-associated sensory-motor neurons expressing *hydra* HmOps2, (Plachetzki et al., 2012), diel cycles of swimming activity (Martin, 2002; Mills, 1983) controlled in *Polyorchis* by photoresponsive cells of the inner nerve ring (Anderson and Mackie, 1977) and tentacle retraction in corals (Gorbunov and Falkowski, 2002). *Clytia* Opsin9 is the first cnidarian opsin to have a demonstrated function. It is expressed in the scattered, MIH-secreting cells, which act autonomously in the gonad and appear to respond individually to light with no obvious coordination of MIH release, i.e. neighbouring MIH-secreting cells are loosely connected and do not release MIH in a coordinated way following light stimulation (Fig.5G; Fig.4B). Opsin9 is presumably tuned to respond to 470-490 nm light (Fig.1B) specifically to initiate the spawning response at depths where blue-cyan predominates (Gehring and Rosbash, 2003; Gühmann et al., 2015). We suggest that this “recent” cnidarian opsin has been co-opted in these cells and its characteristics honed for this environment-specific function.

In other animals, opsin-mediated non-visual photodetection, has been linked to sexual reproduction via its role in clock entrainment, as exemplified by mammalian melanopsin (Hattar et al., 2003). In contrast, the essential and dominant function of Opsin9 in *C. hemisphaerica*, at least in our experimental setup, is to trigger immediate release of MIH at the gonad upon light stimulation. Intriguingly, other observations on hydrozoan jellyfish report spawning both at dawn and dusk (Roosen Runge, 1962), suggesting the existence of other opsins acting antagonistically to Opsin9 and/or of a parallel circadian clock. We thus propose that *Clytia* and many other hydrozoans might harbour an endogenous daily clock underpinning daily or twice-daily sperm and egg release, with more precise synchronisation being provided directly by opsins that detect the rising and/or setting of the sun. By extension, in corals, which can show monthly or yearly spawning, cryptochrome-entrained clocks may exist in parallel with the more precise dark-cued synchronisation via opsins at nightfall. Consistent with this idea, changes in cryptochrome levels have been correlated with both daylight and moonlight cycles in a reef coral (Levy et al., 2007), while levels of a key clock mediator, melatonin, show monthly oscillations in neural cells associated with the gonads in the sea pansy (Mechawar and Anctil, 1997). Coral opsins have not yet been linked to spawning responses but have been indirectly implicated in light-triggered tentacle retractions (Gorbunov and Falkowski, 2002) and characterised in association with putative sensorial cells in the phototactic larvae (Mason et al., 2012). Co-participation of ancient cryptochrome-based clocks and opsin-triggered immediate responses may well operate not only in cnidarians but in many other animals, for example the ascidian *Ciona*, in which dark-light transitions triggers immediately spawning of seasonally gravid animals (Lambert and Brandt, 1967).

Overall our findings support a scenario in which expansion of the opsin gene family within the hydrozoan clade accompanied the local deployment of new opsins with specific spectral characteristics optimised for regulation of a variety of physiological behaviours in response to light, epitomised by *Clytia* Opsin9. Thus, while one trend in animal evolution is for specialised cell types with distributed functions to evolve from ancestral multifunctional cell types (Arendt, 2008; Arendt et al., 2016), cnidarians may have accumulated new multifunctional cell types via secondary acquisition of photosensory ability by various populations of neural cells, muscle cells or nematocytes. Deep brain photoreceptor cells in vertebrates and equivalent cells in various

protostome species that regulate physiological responses through neurohormone release in response to ambient light (Fernandes et al., 2013; Fischer et al., 2013; Halford et al., 2009; Tessmar-Raible et al., 2007) bear many similarities with the *Clytia* gonad neural cells that we describe here. This similarity may reflect a common evolutionary origin from an ancestral multifunctional sensory-neurosecretory cell type (Arendt, 2008; Tessmar-Raible et al., 2007; Vigh et al., 2002), or multiple appearance of ‘new’ cell types (Porter, 2012) within both Cnidaria and Bilateria from pre-existing neuronal cells by integration of specialised opsins to fine-tune circadian entrainment.

METHODS

Animals

Sexually mature medusae from laboratory maintained *Clytia hemisphaerica* (“Z colonies”) (Amiel et al., 2010) were fed regularly with *Artemia* nauplii and cultured under light-dark cycles to allow daily spawning. Red Sea Salt brand artificial seawater (ASW) was used for all culture and experiments.

Monochromator assay

Manually dissected *Clytia* ovaries in small plastic petri dishes containing Millipore filtered sea water (MFSW) were maintained overnight in the dark and then stimulated with monochromatic light, provided by a monochromator (PolyChrome II, Till Photonics) installed above the samples, using the set-up described by Gühmann et al., (2015) (Gühmann et al., 2015), which delivers equivalent levels of irradiance between 400 and 600 nm ($3.2E+18$ to $4.3E+18$ photons/s/m²). Monochromatic light excitation was carried out in a dark room. 10 second pulses of different wavelengths, between 340 to 660 nm were applied to separate groups of 3-6 gonads, which were then returned to darkness for one hour before monitoring of oocyte maturation seen as loss of the oocyte nucleus (Germinal Vesicle) in fully grown oocytes, followed by spawning. A wavelength was acknowledged to induce maturation if at least one oocyte per gonad underwent maturation and spawning within the correct timing after monochromatic light excitation (Amiel et al., 2010). 10-second exposure times were chosen because initial trials (not shown) showed that these gave sub-saturating responses at all wavelengths. Gonads that spawned prematurely

due to manipulation stress were excluded from analysis.

Identification of *Clytia* opsin genes

BLAST searches were performed on an assembled *Clytia hemisphaerica* mixed-stage transcriptome containing 86,606 contigs, using published cnidarian opsin sequences or *Clytia* opsin sequences as bait. The ORFs of selected *Clytia* opsins were cloned by PCR into pGEM-T easy vector for synthesis of *in situ* hybridization probes. Sequences and accession numbers for the opsin sequences analyzed in this study are given in File S1.

Gonad transcriptome analysis

Illumina HiSeq 50nt reads were generated from mRNA isolated using RNAqueous micro kit (Ambion Life technologies, CA) from ectoderm, endoderm, growing oocytes and fully grown oocytes manually dissected from about 150 *Clytia* female gonads. The reads were mapped against the opsin sequences retrieved from a *Clytia* reference transcriptome using Bowtie2 (Langmead and Salzberg, 2012). The counts for each contig were normalized per total of reads of each sample and per sequence length. Opsins RNA read counts from each tissue were visualized as a colour coded heat map using ImageJ software.

Opsin molecular phylogeny

To assess the relationship of the *Clytia* opsin amino acid sequences to known opsins, they were added to the Feuda et al., (2014) (Feuda et al., 2014) dataset using the profile alignment option

in MUSCLE (Edgar, 2004) with Seaview v4.2.12 (Galtier et al., 1996). The alignment was manually trimmed to remove positions only present in the newly added *Clytia* opsin sequences. A subset of this alignment was highlighted by similarity group conservation (defined by GeneDoc and the BLOSUM62 matrix – FigS4). For more detailed comparison between medusozoan sequences, all cubozoan and hydrozoan currently available opsin protein sequences in GenBank were retrieved from NCBI and added to the 10 *Clytia* opsin sequences. Cd-hit (Fu et al., 2012) was run with 99% identity to eliminate sequence duplicates, obtaining a final dataset of 56 Cubozoa and Hydrozoa opsin protein sequences (FASTA file available in File S4). The alignment was performed using the MUSCLE algorithm (Edgar, 2004) with MEGA7 (Kumar et al., 2016), and was further adjusted manually to remove sites at the 3'- and 5'-ends as well as regions where gaps were created by one individual sequence. The trimmed alignments are in Files S2 and S3 (phylip interleaved format).

Both alignments (Feuda et al., 2014 + *Clytia* opsins, and Hydrozoa + Cubozoa opsins) were subjected to Maximum likelihood (ML) and Bayesian analyses using RaxML GUI1.5b (Silvestro and Michalak, 2012) and MrBayes 3.2.6 (Ronquist et al., 2012), respectively. The GTR+G model of protein evolution was used, since it provides a better fit for opsin sequences than other models (Feuda et al., 2012). Support for the branches in the ML phylogenies was estimated using non-parametric bootstrapping (500 replicates). Bayesian analyses were subjected to 2 independent runs of four chains, temp = 0.2 and 10000000 generations. Both datasets analyses were considered to have converged since average standard deviation of split frequencies dropped below 0.05. Consensus trees and posterior probabilities were calculated once the stationary phase was obtained. A ML tree was used as a starting guide tree for the Feuda et al., (2014) + *Clytia* opsins alignment. Melatonin receptors for the bilaterian + cnidarian + ctenophore tree were chosen as the outgroup for opsin phylogeny (Feuda et al., 2012). The resulting trees were visualized with FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>).

***In situ* hybridization**

For *in situ* hybridization, isolated gonads were processed as previously (Lapébie et al., 2014) except that 4M Urea was used instead of 50% formamide in the hybridization buffer. Details of this protocol optimization will be published elsewhere. Images were taken with an Olympus BX51 light microscope.

For double fluorescent *in situ* hybridization, female *Clytia* gonads were fixed overnight at 18°C in HEM buffer (0.1 M HEPES pH 6.9, 50 mM EGTA, 10 mM MgSO₄) 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 was performed using a DIG-labeled probe for Opsin9 and a fluorescein-labeled probe for PP4. A three hours 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 Opsin9 the fluorescence signal was developed using the TSA (Tyramide Signal Amplification) kit (TSA Plus Fluorescence Amplification kit, PerkinElmer, Waltham, MA) and Cy5 fluorophore (diluted 1/400 in TSA buffer: PBS/H₂O₂ 0.0015%) at room temperature for 30 min. After 3 washes in PBS-T, fluorescence was quenched with 0.01N 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 PP4 fluorescence signal was developed using TSA kit with Cy3 fluorophore. Controls with single probes were done to guarantee a correct fluorescence quenching and ensure that the two channels did not cross-react. 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.

Immunofluorescence

For co-staining of neuropeptides and tyrosinated tubulin, dissected *Clytia* gonads were fixed overnight at 18°C in HEM buffer 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 -20°C improved visualization of microtubules in the MIH-producing cells. Samples were rehydrated, washed several times in PBS-0.02% Triton X-100, then one time in PBS-0.2% Triton X-100 for 20 minutes, and again several times in PBS-0.02% Triton X-100. They were then blocked in PBS with 3% BSA overnight at 4°C. The day after they were incubated in anti-PRPa antibody and anti-Tyr tubulin (YL1/2, Thermo Fisher Scientific) 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 4°C, and nuclei stained using Hoechst dye 33258 for 20 min.

For co-staining of neuropeptides with cortical actin or γ -Tubulin, dissected *Clytia* gonads were fixed for 2-3 hours at room temperature in HEM buffer containing 80mM maltose, 0.2% Triton X-100 and 4% paraformaldehyde, then washed five times in PBS containing 0.1% Tween20 (PBS-T). Samples were further washed in PBS-0.02% Triton X-100, then one time in PBS-0.2% Triton X-100 for 20 minutes, and again several times in PBS-0.02% Triton X-100. They were then blocked in PBS with 3% BSA overnight at 4°C. The day after they were incubated in anti-PRPa antibody and anti- γ -Tubulin (GTU-88, Sigma Aldrich), or only anti-PRPa, in PBS/BSA at room temperature for 2 h. After washes, the specimens were incubated with secondary antibodies (Rhodamine goat anti-rabbit and Cy5 goat anti-mouse-IgG) or Phalloidin-Rhodamine and secondary antibody (Cy5 goat anti-rabbit) overnight in PBS at 4°C, and nuclei stained using Hoechst dye 33258 for 20 min. Images were acquired using a Leica SP8 confocal microscope and maximum intensity projections of z-stacks prepared using ImageJ software.

For MIH fluorescence quantification, 5-6 independent gonads for each of the two conditions (light-adapted and dark-adapted after light stimulation) and *Clytia* strains (WT and Opsin9 n1_4) were fixed as mentioned above and co-stained for MIH and Tyr-tubulin. All the fixations were done in parallel. Confocal images were acquired using the same scanning parameters (i.e. magnification, laser intensity and gain). In all cases, 10 confocal Z planes were summed over 4 μ m depth at the gonad surface using ImageJ software. With ImageJ, we separated the two channels (MIH and Tyr-tubulin) and selected the contour of MIH-positive cells using the Tyr-tubulin staining as guidance. Using the “Integrated Density” option, we recovered the “RawIntDen” values of the MIH-stained channel, which refer to the sum of the pixel intensity values in the selected region of interest. These values divided by 1000 correspond to the RFU (Relative Fluorescence Units) in Figure 4.

Generation of CRISPR-Cas9 mutant *Clytia* colonies

Opsin9 n1 small guide RNA (sgRNA) was assembled by cloning CRISPR RNA (crRNA) oligonucleotides into the vector pDR274 (42250, Addgene), which contains tracrRNA sequence next to a crRNA insertion site. sgRNA was then synthesized using Megashortscript T7 kit (Thermo Fisher Scientific) and purified with ProbeQuant G-50 column (GE healthcare). sgRNA was dissolved in distilled water

at 80 μ M and kept at -80°C until use. The crRNA *opsin9* n1 sequence is listed in FileS5. Purified Cas9 protein dissolved in Cas9 buffer (10 mM Hepes, 150 mM KCl) was kindly provided by J-P Concordet (MNHN Paris) and diluted to 5 μ M.

sgRNA was added to Cas9 protein in excess (2:1) prior to injection and incubated for 10 minutes at room temperature. The final Cas9 concentration was adjusted to 4.5 μ M and the sgRNA to 9 μ M. The mixture was centrifuged at 14,000 rpm for 10 minutes at room temperature. 2-3% of egg volume was injected into unfertilized eggs within 1 hour after spawning, prior to fertilization.

Injected embryos were cultured for 3 days in MFSW at 18°C. Metamorphosis of planula larvae into polyps was induced ~72 hours after fertilization by placing larvae (20-80/slide) on double-width glass slides (75 x 50mm) in drops of 3-4ml MFSW containing 1 μ g/ml synthetic metamorphosis peptide (GNPPGLW-amide), followed by overnight incubation. Slides with fixed primary polyps were transferred to small aquariums kept at 24°C, to favour the establishment of female colonies (Carré and Carré, 2000). Primary polyps and young polyp colonies were fed twice a day with smashed *Artemia* nauplii until they were grown enough to be fed with swimming nauplii. Following colony vegetative expansion, a single well-growing colony on each slide was maintained as a founder. After several weeks of growth, polyp colonies were genotyped to assess mutation efficiency and mosaicism, and medusae were collected from the most strongly mutant colony (n1_4) for further experimentation.

Mutant genotyping

Genomic DNA from *Clytia* polyps and jellyfish gonads was purified using DNeasy blood/tissue extraction kit (Qiagen). The *opsin9* target site was amplified by PCR using Phusion DNA polymerase (New England Biolabs). Primers used for genotyping are listed in FileS5. PCR products were sequenced and mutation efficiency was assessed using TIDE analyses (Fig.S5), which estimates the mutation composition from a heterogeneous PCR product in comparison to a wild type sequence (Brinkman et al., 2014).

We scanned *Clytia* genome for possible off-targets of *Opsin9* sgRNA at <http://crispor.tefor.net>. From 2 possible off-targets where Cas9 could cut, none was found in coding sequences nor were they right next to a PAM sequence.

Gonad spawning assays

Sexually mature wild type and *opsin9* n1_4 mutant medusae of the same age and adapted to the same day-night cycle were collected for gonad dissection. Individual gonads were transferred to 100µl MFSW in 96-well plastic plates. Plates were covered with aluminium foil overnight and brought back to white light the following day. For the rescue experiment with synthetic MIH, wild type and *opsin9* n1_4 mutant gonads adapted to light conditions were dissected and transferred to 96-well plastic plates and acclimatised for two hours. 100µl of a stock of 2x10⁻⁷M synthetic WPRPamide (MIH) in MFSW was added in each well to give a final concentration of 10⁻⁷M. Oocyte maturation was scored after one

hour. Spawning followed in all cases where oocyte maturation was triggered. Gonads that spawned prematurely due to manipulation stress were excluded from analysis. Gonad pictures in Fig.5 were taken with an Olympus BX51 microscope.

Graphs and statistics

Graphs and statistics for the monochromator assay were prepared using BoxPlotR (Spitzer et al., 2014). Fisher exact tests and Mann-Whitney U tests were performed at <http://www.socscistatistics.com>. Mann-Whitney U tests were chosen for MIH fluorescence quantification comparisons since the results did not follow a normal distribution following Shapiro-Wilk test.

ACKNOWLEDGEMENTS

We thank M. Khamla for the graphics in Figures 1 and 6, J-P Concordet (MNHN Paris) for generously providing Cas9 protein, M. Gühmann for monochromator training, and all our laboratory and

NEPTUNE colleagues for stimulating and critical discussions. Funding was provided by the Marie Curie ITN NEPTUNE, French ANR grant OOCAMP and core CNRS funding to the LBDV.

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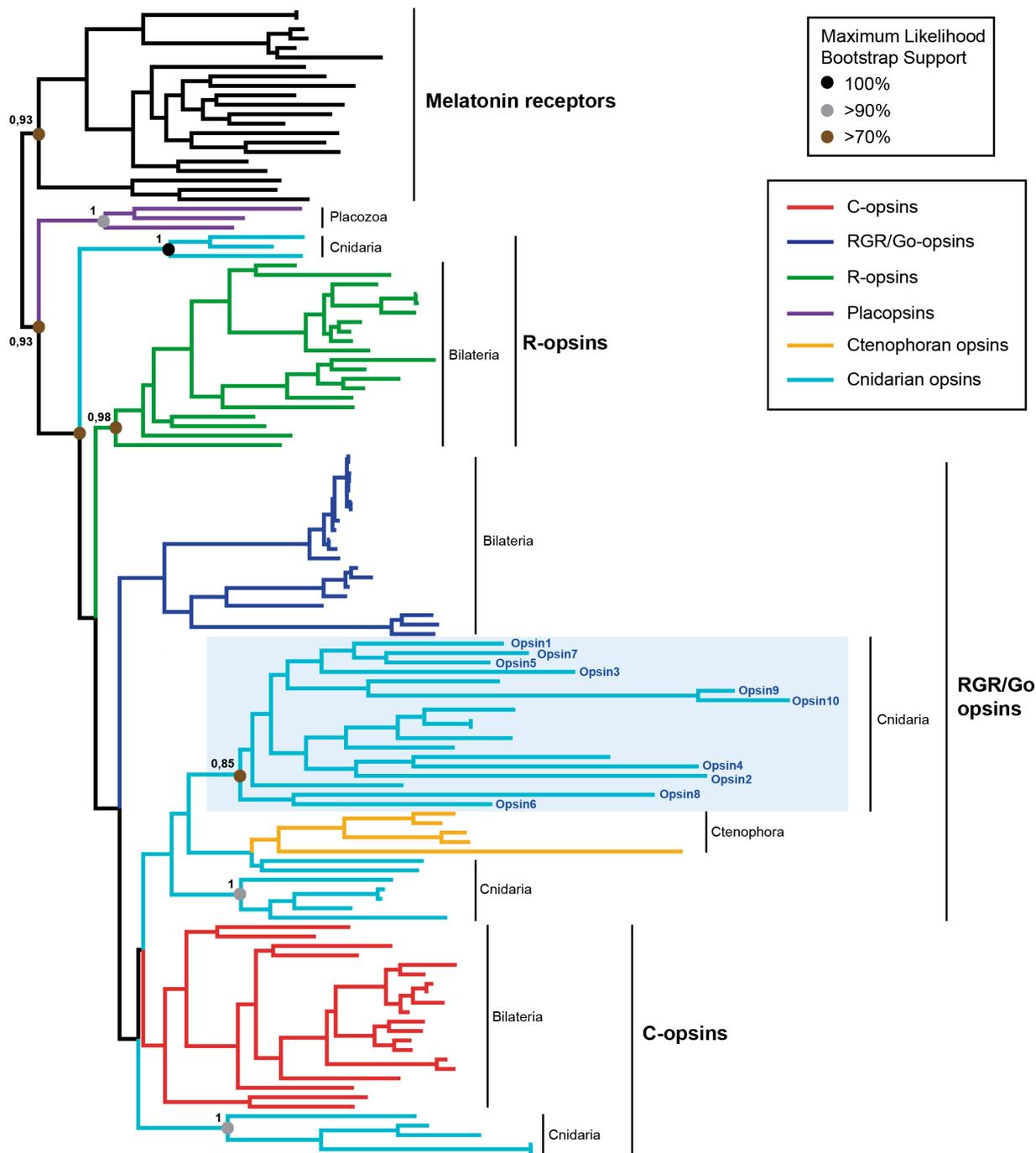


Figure S1. Phylogenetic position of *Clytia* opsins

Relationship of *Clytia* opsins to known opsin groups. Maximum likelihood (ML) phylogeny of opsin proteins based on the dataset of Feuda et al., 2014. Bayesian methods reconstructed a tree with very similar topology. ML bootstrap support is shown as coloured circles on the branch tips. Only those nodes separating major opsin clades and coming from branches with >70% bootstrap supports are displayed. Black circles indicate 100% support, gray circles indicate >90% support, and brown circles indicate >70% support. Bayesian posterior probabilities (PP) greater than 70% for these branches are denoted. ML tree rooted with Melatonin receptors as outgroup. *Clytia* opsin names (1-10) are shown. All of them cluster inside a well-supported clade of cnidarian opsins with representatives of Hydrozoa and Cubozoa (highlighted in cyan). Other cnidarian groups in the tree contain only Anthozoa species opsins. C-opsins = Ciliary opsins, RGR = retinal G protein-coupled receptor, R-opsins = Rhodomer opsins.

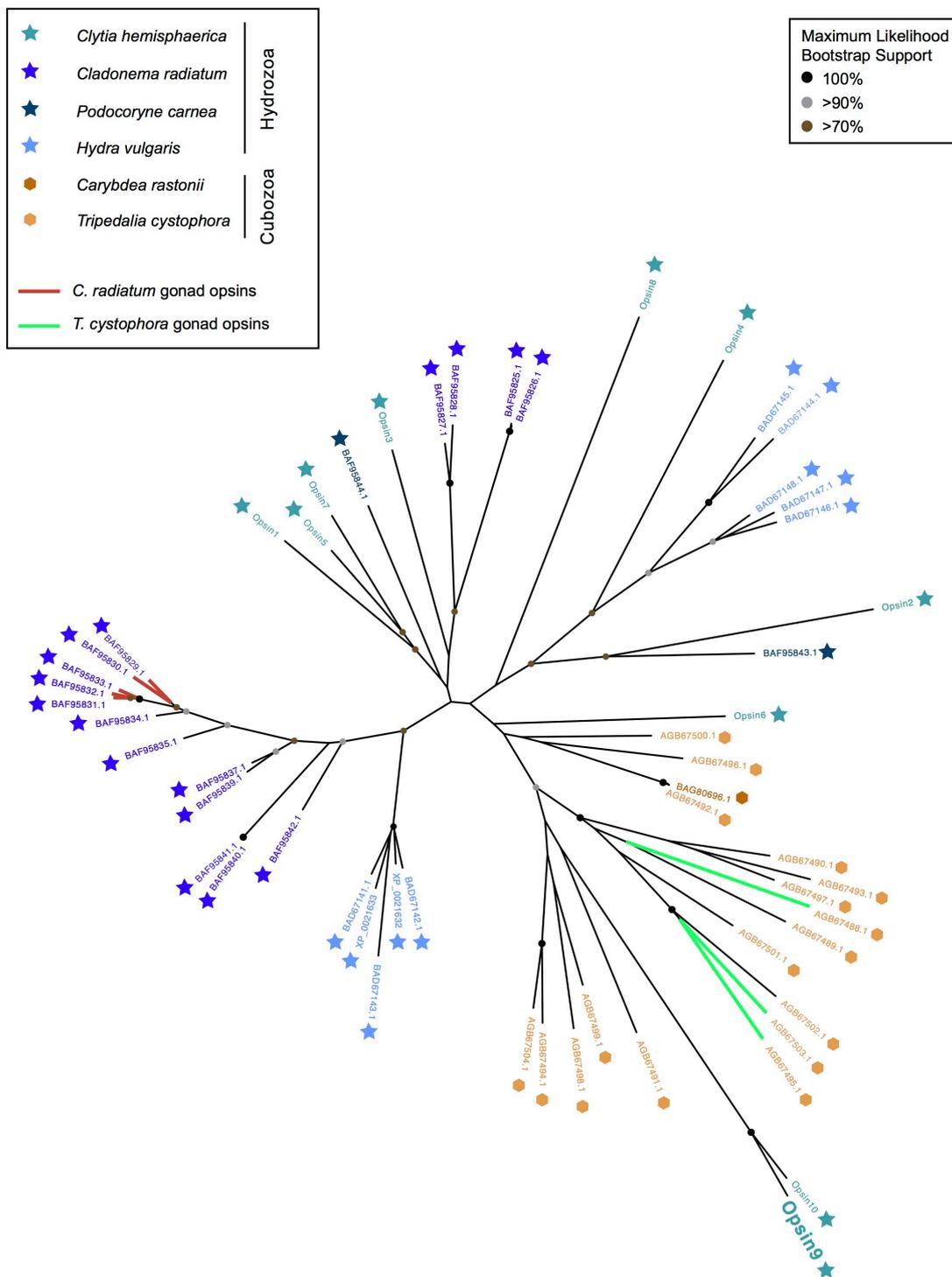


Figure S2. Maximum likelihood phylogeny of hydrozoan and cubozoan opsins

Maximum likelihood (ML) unrooted phylogenetic tree of all currently available cubozoan and hydrozoan opsins in GenBank. ML bootstrap support is shown as coloured circles on the base of the branches. Black circles indicate 100% support, gray circles indicate >90% support, and brown circles indicate >70% support. NCBI reference numbers and *Clytia* opsin names are shown at branch ends. Stars belong to hydrozoan opsins and hexagons to cubozoan opsins. Different symbol colours represent different species. Red and green branches indicate opsins expressed in the gonads of *Cladonema radiatum* and *Tripedalia cystophora*, respectively.

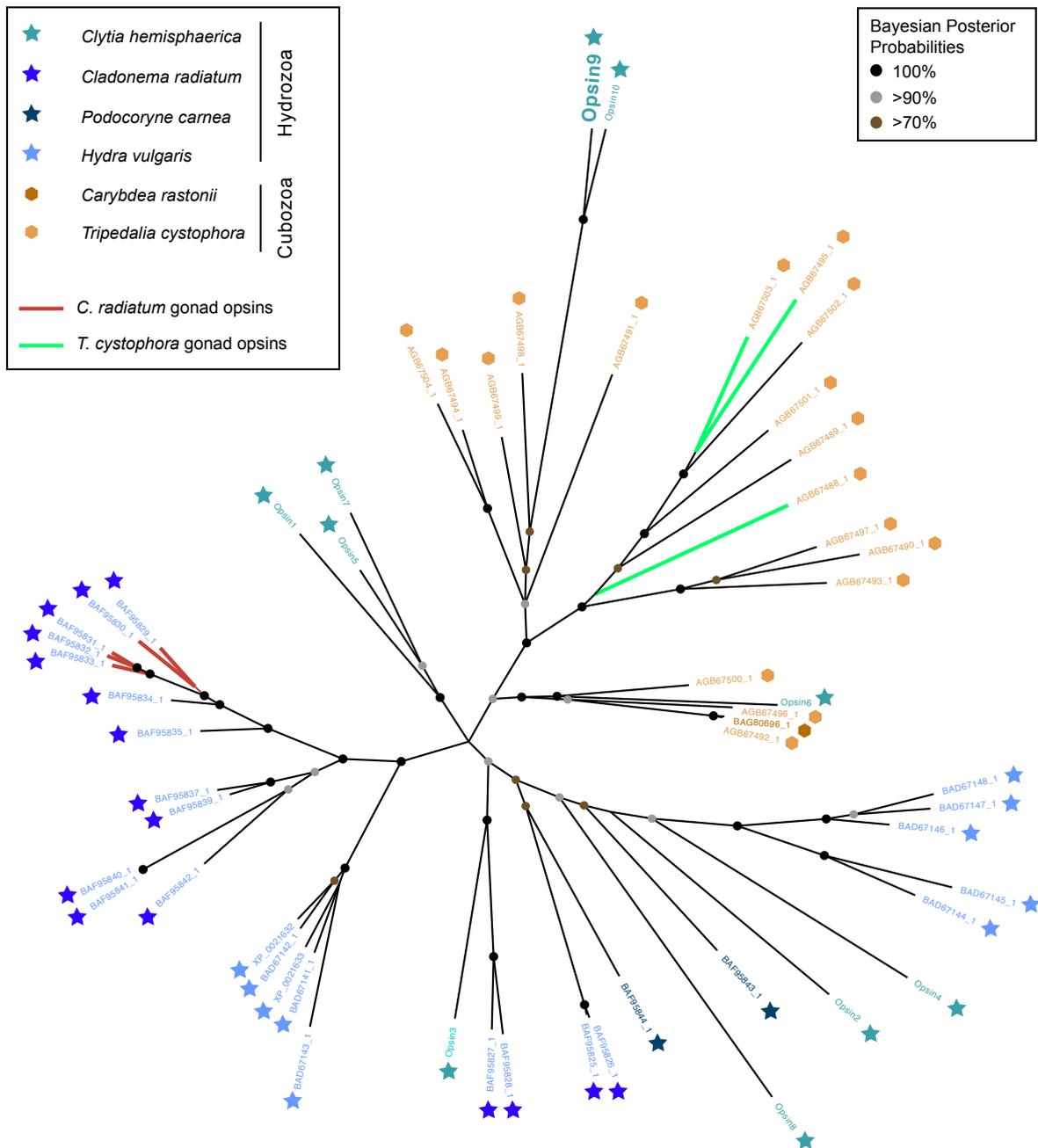


Figure S3. Bayesian phylogeny of hydrozoan and cubozoan opsins

Bayesian unrooted phylogenetic tree of all currently available cubozoan and hydrozoan opsins in GenBank. Bayesian posterior probabilities (PP) are shown as coloured circles on the tips of the branches. Black circles indicate 100% PP, gray circles indicate >90% PP, and brown circles indicate >70% PP. NCBI reference numbers and *Clytia* opsin names are shown at branch ends. Stars belong to hydrozoan opsins and hexagons to cubozoan opsins. Different symbol colours represent different species. Red and green branches indicate opsins expressed in the gonads of *Cladonema radiatum* and *Tripedalia cystophora*, respectively.

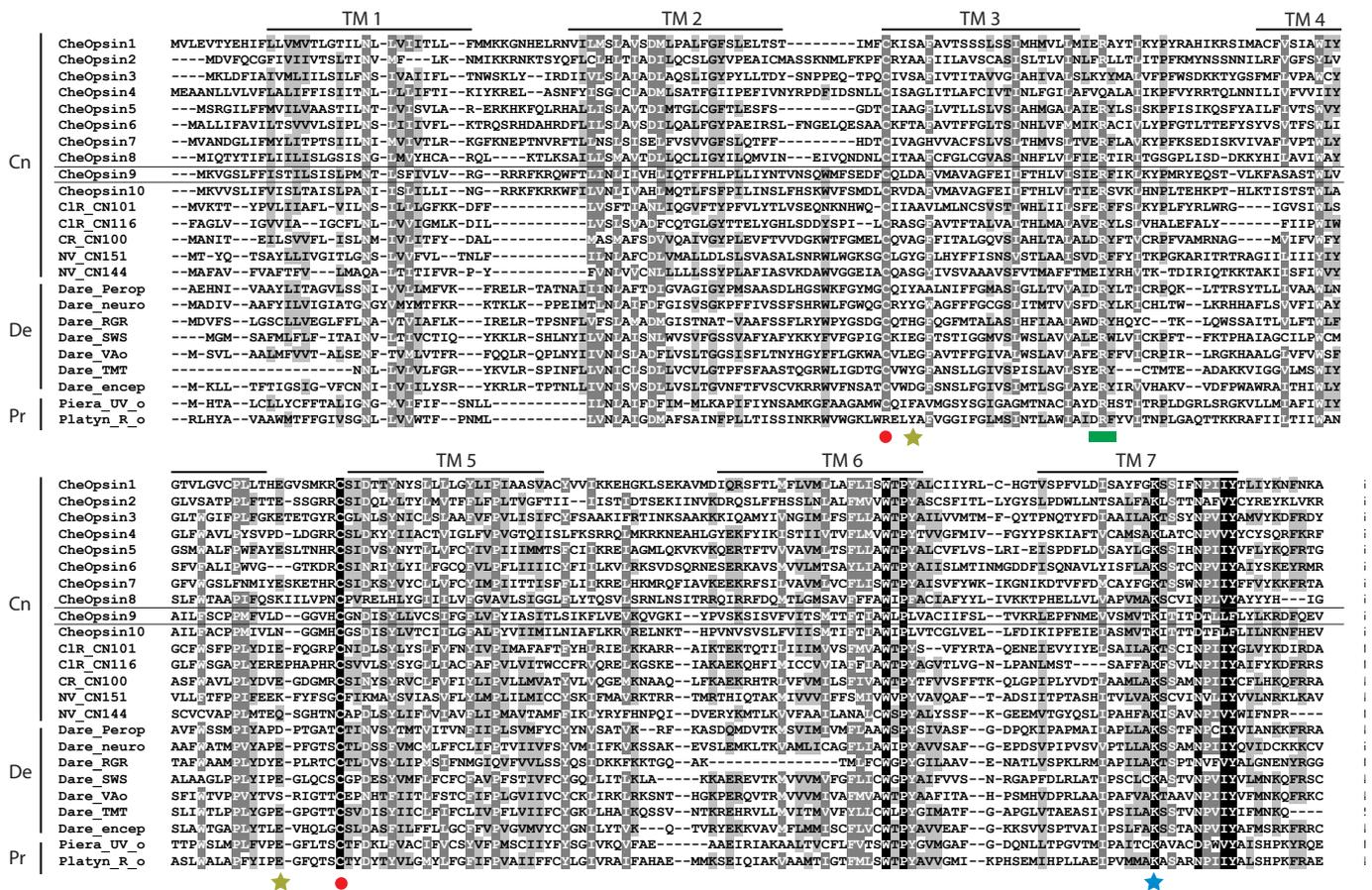


Figure S4. Alignment of selected opsin sequences, focussing on amino acid residues crucial for light detection and opsin function

Columns of residues are highlighted by similarity group conservation (defined by GeneDoc and the BLOSUM62 matrix). Black shows 100%, dark grey shows ≥80%, and light gray shows ≥60% residues similarity in each column. Amino acid position numbers used below correspond to the nomenclature used for bovine rhodopsin. Conserved sites are indicated for disulfide bridge formation between Cys110 and Cys187 (red circles), chromophore linkage at Lys296 (blue star), acidic counterions (which can be Glu/Asp113 and/or Glu/Asp181 – yellow stars) and G protein interaction for signal transduction (green square). Transmembrane domains are also indicated (TM1-7). Abbreviations: Cn = Cnidarians; De = Deuterostomes; Pr = Protostomes; Che = *Clytia hemisphaerica*; ClR = *Cladonema radiatum*; CR = *Carybdea rastonii*; NV = *Nematostella vectensis*; Dare = *Danio rerio*; Piera = *Pieris rapae*; Platyn = *Platynereis dumerilii*; perop = peropsin; neuro = neuropsin; RGR = retinal G protein-coupled receptor; SWS = short-wavelength sensitive; VAo = vertebrate ancient opsin; TMT = teleost multiple tissue opsin; encep = encephalopsin; UV_o = ultraviolet opsin; R_o = rhabdomeric opsin.

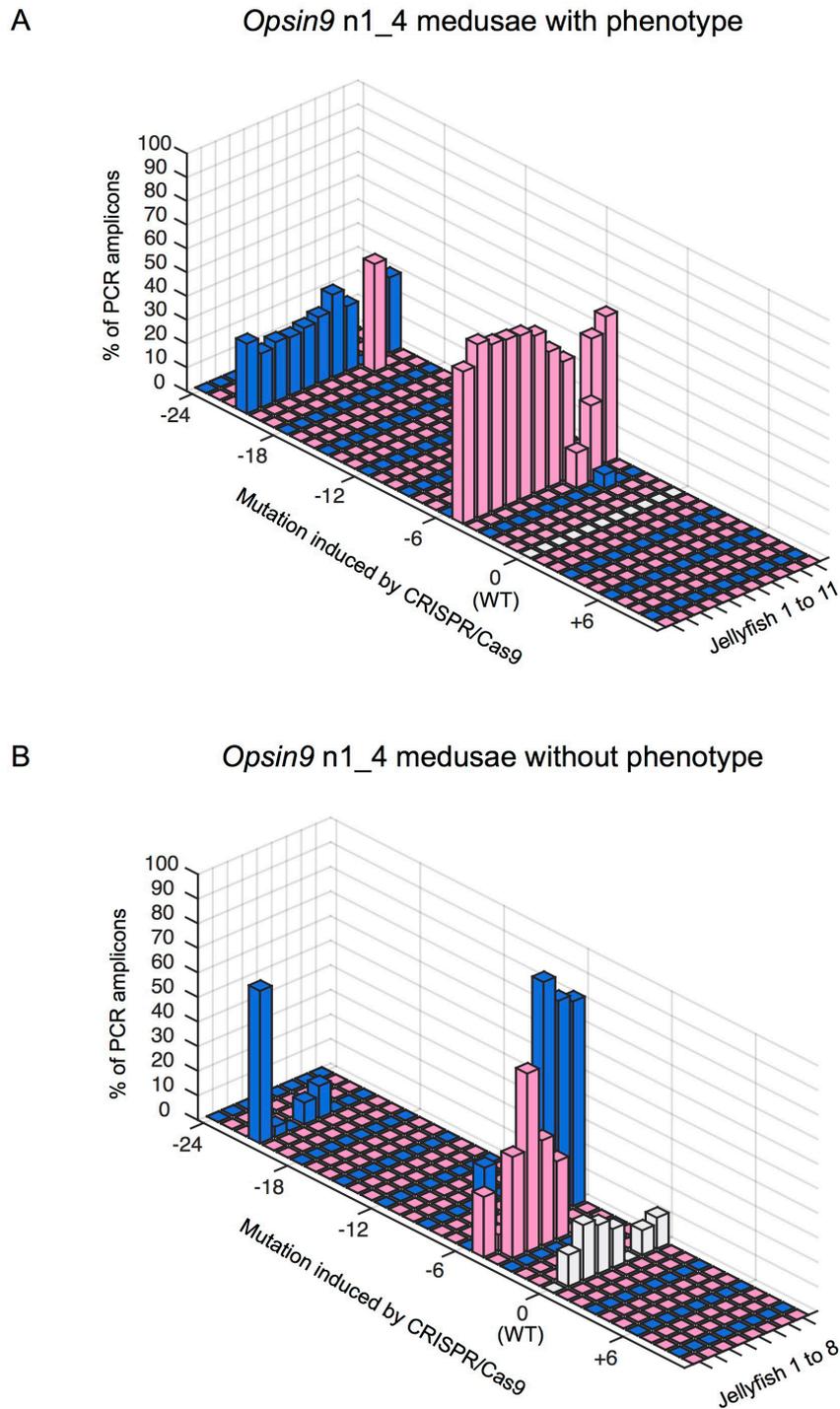


Figure S5. *Opsin9* n1_4 genotyping results

Mutant genotyping based on PCR amplification of target site genomic areas followed by sequencing and TIDE analyses (see methods). Frame-shift mutations are shown in pink, non-frame-shift mutations in blue and wild type (WT - no mutation) in gray.

A) 3D-graph showing the mutation rate and type of 11 independent *Opsin9* n1_4 jellyfish with phenotype. The dominant 5-bp deletion and a long 21-bp deletion were detected in 10/11 medusae.

B) 3D-graph showing the mutation rate and type of 8 independent *Opsin9* n1_4 jellyfish without phenotype. These medusae showed an increased proportion of sequences with no mutation, higher mosaicism and a lower ratio of frame-shift mutations.

Conclusions, Additional Results and Perspectives

The work described in this manuscript showed that *Clytia* Opsin9 is an essential photopigment for light-induced oocyte maturation and spawning in *Clytia*. Opsin9 mRNA is co-expressed with *Clytia* MIH precursors in the gonad ectoderm MIH-secreting cells, implying that these cells have a dual photosensitive-neurosecretory nature. Via CRISPR/Cas9 gene editing we generated *Opsin9* mutants unable to spawn upon dark-light transitions, and demonstrated the key role of Opsin9 in mediating MIH secretion upon light reception in the gonad ectoderm. We showed that *Clytia* female gonads were induced to spawn preferentially when stimulated with blue-cyan light, strongly suggesting that this is the action spectrum of Opsin9 photopigment. Phylogenetic analyses showed that *Clytia* Opsin9 sequence clusters inside a medusozoan opsins group, but is not orthologous to other opsins previously described in medusozoan gonads. Taken together, our results have provided the first characterization of a non-visual opsin in a cnidarian, and have demonstrated that it has an essential role in light-mediated reproductive control.

I mentioned in the Introduction to this thesis how light acts as a common synchronization cue for spawning in the animal kingdom, enhancing fertilization success and thus species survival (Juntti and Fernald, 2016; Martin, 2002; Mills, 1983; Tessmar-Raible et al., 2011). To achieve this light-mediated synchronization of spawning, however, light cues need to affect both males and females. Most of the studies I performed in *Clytia*, reported in the three chapters, were focused on female jellyfish gonads and oocytes. However, I also performed a set of experiments with male *Clytia* gonads to try to explore how light is involved in the release of active spermatozoa into the sea water, which is coordinated with egg release. I will describe these additional results in the following section.

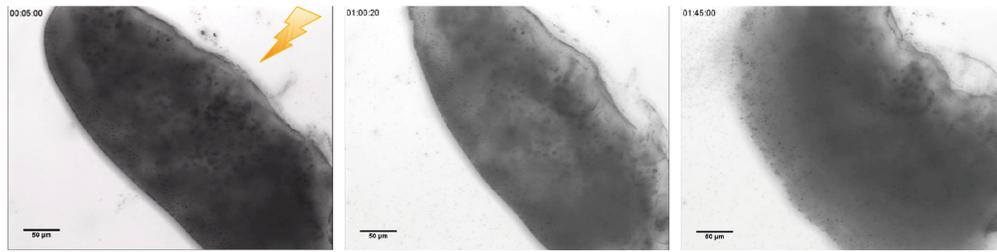
Opsin9 and spawning regulation in males

The developing germ cells in *Clytia* male gonads are arranged in five or six organized layers. Each layer represents a synchronously developing generation of germ cells. The youngest and largest spermatogonia are located in the innermost part of the testes, next to the gastroendodermal cavity, while the late spermatids are found right below the thin ectodermal surface (Roosen-Runge and Szollosi, 1965). Gamete release involves local rupture of gonad ectoderm for a few minutes and reassociation when the spermatozoa have been released from the testes (Roosen-

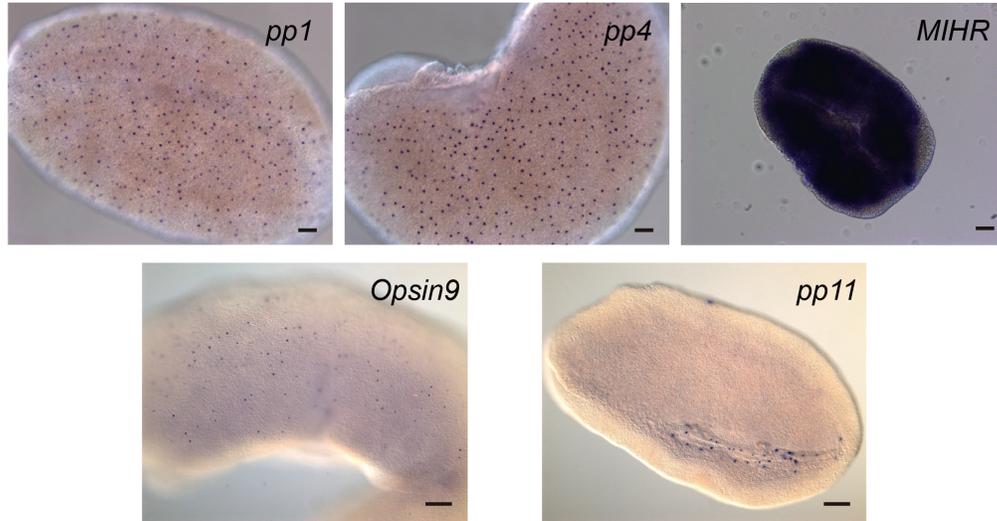
Runge and Szollosi, 1965). In our medusae culture conditions, *Clytia* males spawn upon dark-light transitions. Light stimulation after a period of darkness triggers gamete release in male *Clytia* isolated gonads, with male spawning generally commencing 15-20 minutes before female gamete release (i.e. around 100 minutes upon a light cue in males and 120 minutes in females at 18-20°C) (**Figure 23**).

To determine whether the molecular and cellular systems characterized in female gonads also control light-induced spawning in *Clytia* males, I performed *in situ* hybridization and immunofluorescence experiments. In male and in female gonads I found that the two *Clytia* MIH precursors pp1 and pp4 are expressed in scattered cells of the gonad ectoderm (see Chapter 1 manuscript). This is consistent with the finding that artificial MIH (PRPa) exposure triggers sperm release from isolated *Clytia* testes (Table 1 in Chapter 1 manuscript). Immunofluorescence with anti-PRPa antibodies confirmed the neural morphology of the MIH-secreting cells in the testes ectoderm (**Figure 23**), equivalent to that in female gonad ectoderm (Figure 4 in Chapter 1 manuscript). *In situ* hybridization in *Clytia* male gonads for the other two main core components of *Clytia* light-induced oocyte maturation (Opsin9 and CheMIHR) also revealed largely shared features between males and females. CheMIHR mRNA was detected strongly within the testes, indicating expression in the male germ cells at different stages of maturation. Opsin9 mRNA expression was localized in scattered male gonad ectodermal cells, although the amount of cells detected was much lower than that in female gonads (see Figure 2 in Chapter 3 manuscript) and that of pp1 and pp4 (**Figure 23**). These differences might reflect for a general lower expression of Opsin9 in male gonads, or might suggest the involvement of additional photopigments in mediating the light response. I did not verify that MIH precursors and Opsin9 are co-expressed in male gonads, although accumulated data predict that they would be expressed and synthesized in the same neural cells. Finally, mRNA for the GLWamide family proneuropeptide pp11 could be detected in a few sparse cells at the junction between the male gonads and the radial canals, as in female gonads (see chapter 1) (**Figure 23**). These results strongly suggest that essentially the same cellular and molecular partners are involved in light-induced gamete release in both male and female *Clytia* gonads.

A - Light-induced sperm release in isolated male gonads



B - Male gonads *in situ* hybridization



C - Male gonad immunofluorescence

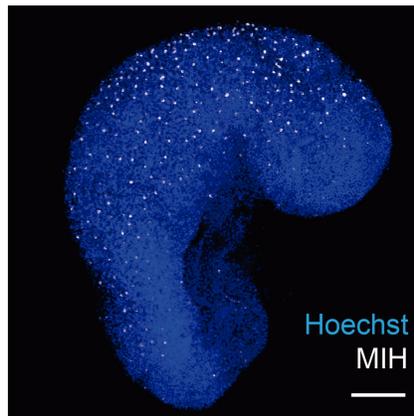


Figure 23. *Clytia* male gonads exhibit equivalent expression patterns of the core regulators of light-induced oocyte maturation in female gonads. (A) Three different time points showing the same male isolated gonad upon light stimulation. Note the massive sperm release accompanied by epithelial rupture at the top left corner of the last time point (i.e. 01h 45 minutes after the light cue). (B) *In situ* hybridization of male isolated gonads, detecting the three main neuropeptide precursors found in *Clytia* female gonads, as well as Opsin9 and MIHR. (C) Male gonad immunofluorescence. Staining with anti-PRPamide (white) and Hoechst (blue). Scale bars in A: 50µm; B: 50µm top row and 100µm bottom row; C: 100µm.

It is not clear precisely what process is triggered by MIH on the male gametes. Late spermatids are positioned in *Clytia* testes immediately below the ectodermal epithelia (Roosen-Runge and Szollosi, 1965). It is thus possible that MIH is detected by late spermatids and promotes the final steps of spermatogenesis into actively swimming, spermatozoa, similarly to the processes of spermiogenesis and capacitation of mammalian male germ cells (Georgadaki et al., 2016). It would be of interest to artificially isolate late spermatids from *Clytia* male testes to check whether they already show swimming activity or not. If they would not show swimming behaviour, addition of artificial MIH (PRPa) into the media might help the late spermatids to finish the spermatogenesis process, which could be checked by the observation of swimming activity after the right amount of time (i.e. at least 60-75 minutes) while compared to non-treated control spermatids. This experiment would be of particular interest since it could also reveal whether the last stages of spermatogenesis in *Clytia* need the presence of gonad somatic cells or not. Some preliminary observations with *CheMIHR* KO male mutants showed a less homogeneous surface of the male gonads when compared to wild types, suggesting global testes structural irregularities as part of the phenotype, and it seemed that lower amounts of sperm, which also swam less vigorously, were released upon light stimulation when compared to wild types (not shown). However, these assays had only been done once, with just a few gonads, and the male *CheMIHR* KO strain assessed exhibits a lower ratio of mutation than the female KO strain that showed a clear phenotype, as described in Chapter 2, rendering any evidence from these preliminary data tricky to use for the proposition of other complementary hypotheses. Further experimentation in this direction should provide a better understanding in how the three core molecular components of *Clytia* light-induced oocyte maturation are integrated in the male gonads to generate mature, fertilizing, spermatozoa upon light cue reception.

To conclude, the findings on Opsin9 function reported in this chapter provide a clear demonstration of how synchronisation of gamete maturation and release can be achieved using a direct light response. Based on the additional results found in *Clytia* males, it is probable that the same molecular intermediates are involved in light-mediated spawning in both male and female medusae. MIH release upon light reception into the gonads and into the seawater would allow direct synchronization of spawning in a population of grouped males and females.

Some evolutionary implications of these findings, put together with *Clytia* MIH and MIHR findings from chapters 1 and 2, will be discussed in the General Discussion section that follows.

GENERAL DISCUSSION

General Discussion

The work performed during my PhD resulted in the identification and characterization of 3 essential molecular regulators of light-induced oocyte maturation in *Clytia* (MIH, MIHR and Opsin9), along with a key neurosecretory cell type specific to the gonad ectoderm that contains the Opsin9 photopigment and secretes MIH. Together, the various findings reported in the preceding chapters provide a detailed picture of the molecular and cellular regulation of light-induced oocyte maturation initiation in *Clytia*, presented in **Figure 24**.

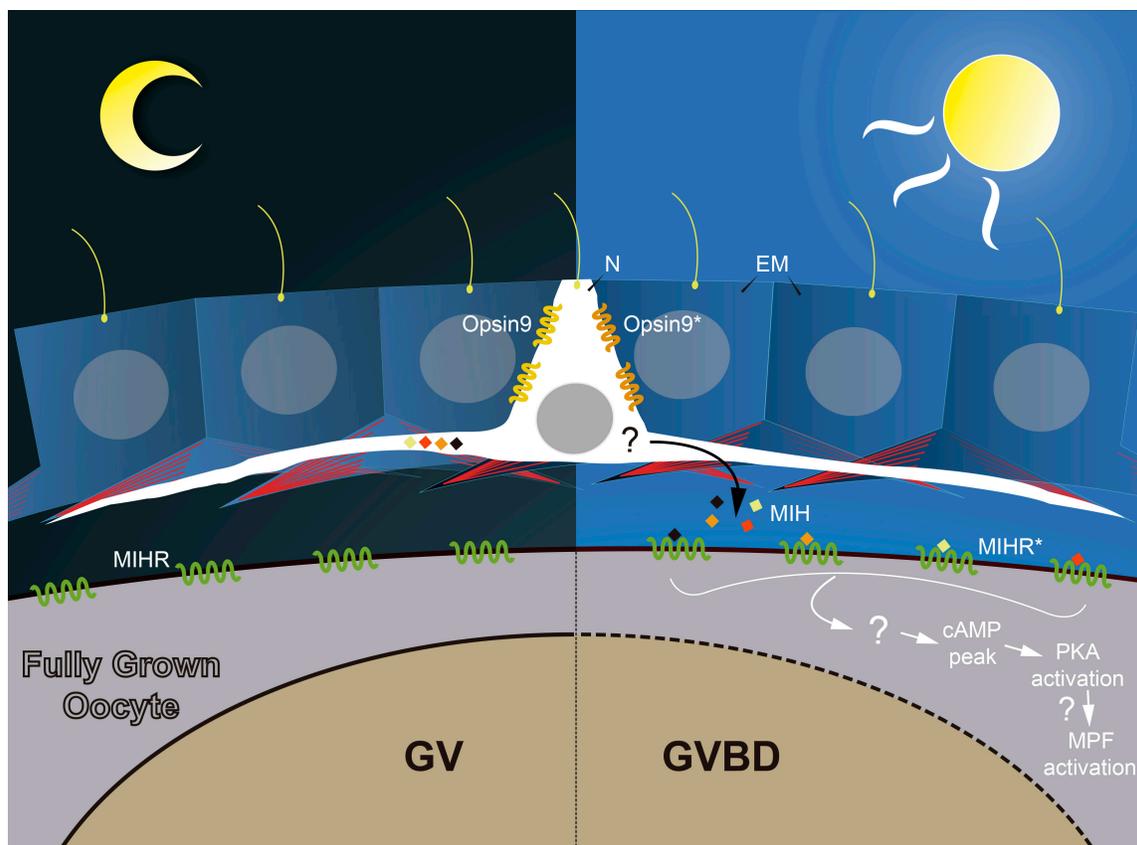


Figure 24. Current model for light-induced oocyte maturation in *Clytia hemisphaerica*. A dark-light transition activates Opsin9 in gonad ectoderm photosensitive-neurosecretory cells, which causes the secretion of neuropeptidic MIHs by a yet unknown mechanism. MIHs interact with *Clytia* MIHR on the oocyte plasma membrane and activate it, starting an as yet uncharacterized signalling cascade, which promotes an immediate rise in cytoplasmic cAMP. High levels of cAMP activate PKA, which in turn activates MPF, leading to oocyte maturation initiation. Opsin9* = activated Opsin9; MIHR* = activated MIH receptor; PKA = cAMP-dependent protein kinase; MPF = M-phase promoting factor; GV = germinal vesicle; GVBD = germinal vesicle breakdown.

The main findings contributing to this model can be summarised as follows: In collaboration with Drs. Noriyo Takeda and Ryusaku Deguchi, we identified *Clytia* MIH from candidate neuropeptides as PRPamide related tetrapeptides that are released from gonad ectoderm upon light stimulation. I then identified *Clytia* MIH receptor (MIHR) from oocyte transcriptome data, using a cell culture based GPCR deorphanization assay, as a neuropeptide GPCR closely related to a subset of bilaterian “FMRFamide-like family” receptors, and showed by CRISPR-Cas9 gene knockout that this receptor is essential for MIH-mediated meiosis resumption. Finally, I determined the nature of gonad light reception in *Clytia* through the identification of an opsin photopigment, Opsin9, co-expressed with MIH in the same gonad ectoderm neural cells. Again I was able to achieve CRISPR-Cas9 gene knockout of the *Opsin9* gene, which revealed an essential role in the light-mediated secretion of MIH.

On the basis of these results we propose that Opsin9 triggers downstream signalling pathways when stimulated by light after a dark period. These ultimately cause MIH release inside the gonad, and also into the surrounding seawater. In female gonads, MIH triggers fully grown oocytes to initiate meiotic maturation, most likely via G α S-adenylate cyclase signalling (to be confirmed), resulting in a cytoplasmic cAMP increase, PKA activation and then MPF activation. Consistent with this model, oocytes from *Opsin9* but not *MIHR* mutant jellyfish could be induced to mature using synthetic MIH, while *MIHR* mutant oocytes mature upon stimulation with cell-permeable cAMP analogues. MIH also acts on males to provoke release of active sperm in response to light. Since it is released and diffuses in the external seawater, it may contribute directly to coordinating spawning in the natural environment between gonads and even between animals, grouped together at the sea surface at dawn.

Many questions remain unanswered to complete this basic model. For instance, the phototransduction pathway of Opsin9 in *Clytia* gonad ectoderm MIH neural cells mediating MIH release upon light reception is yet to be unravelled. It was shown in a cubozoan (box jellyfish) that an opsin expressed in ocellus photoreceptor cells triggers a light-dependent G α S-adenylyl cyclase-cAMP cascade, and the same authors suggested that cnidarian opsins might be generally associated to this pathway (Koyanagi et al., 2008). Cyclic nucleotides such as cAMP or, more commonly, cGMP are widely used as second messengers in opsin-mediated phototransduction pathways, although not exclusively, and cyclic nucleotide-gated (CNG) channels respond to these, generating cellular responses (Koyanagi et al., 2008; Terakita et al., 2012). One immediate reaction is the hyperpolarization or depolarization of the cell membrane (Terakita et al., 2012). Based on the cnidarian

nature of *Clytia* Opsin9 and the neural identity of the MIH-secreting cells in which it is expressed, it is reasonable to think that a similar phototransduction pathway might occur to induce MIH release in *Clytia* gonads. As a hypothetical scenario to test, Opsin9 coupled to G α S would activate the G protein upon light reception, which in turn would activate adenylyl cyclases inside the cell, raising the cAMP cytoplasmic levels. Among other possible downstream reactions, CNG channels might open or close, leading to a change in the membrane action potential of MIH-secreting cells that could lead to MIH secretion. To test whether Opsin9 binds to G α S, pharmacological inhibition experiments with G α S activators (e.g. cholera toxin) or inhibitors (e.g. NF449) could be undertaken with *Clytia* isolated gonads (Stricker and Smythe, 2001). MIH secretion quantification (see Chapters 2 and 3) after the appropriate treatment would help to assess the involvement of G α S in this process. However, drug experiments would be insufficient to confirm the identity of the G protein. Raising an antibody against Opsin9 and against one of the two G α S proteins found in *Clytia* transcriptome (or expression of fluorescence-tagged proteins) would allow assessment by immunofluorescence of co-localization with the photopigment and would help to define more precisely Opsin9 localisation within the MIH-secreting cells. Although we can assume that it is located in the cell membrane, as all GPCRs are, it would be of interest to see whether it is confined in specific parts of the neurons, like the cilium (see Chapter 3), or rather distributed homogeneously in the neural cell.

Another important question that needs to be resolved is the nature of the signalling events that coordinate oocyte maturation completion with spawning in *Clytia*. I showed that not only oocyte maturation but also spawning itself, i.e. release of the mature oocytes, was abolished in *Clytia* MIHR KO isolated gonads and rescued by addition of a cAMP analogue into the media (Chapter 2). Since in the gonad MIHR is expressed almost exclusively in the oocytes, these findings suggest that MIH and MIHR regulate gamete release in *Clytia* gonads indirectly via signals produced by the maturing oocytes, although we cannot rule out the possibility that cAMP signalling in each of the gonad tissues could contribute to spawning. It appears likely that changes to all three tissues are coordinated to achieve the spawning process. An endodermal mechanical push of the maturing oocytes towards the ectoderm involving whole gonad inflation (personal observation), and an ectodermal weakening of the epithelial junctions leading to its temporary rupture, facilitating the release of the eggs, may contribute to the spawning process. It is tempting to propose, however, that spawning is directly mediated by factors secreted from the oocytes during their maturation, and that their synthesis and/or secretion is induced upon MAPK or MPF activation, after the onset of oocyte maturation. These factors could act on the gonad endoderm to induce a partial reorganization of the

tissue that might help as a mechanical push to the oocytes, and/or on the gonad ectoderm to induce its partial rupture, possibly only at a very local level. To address some of these questions we could inhibit the MAPK pathway in maturing oocytes, since this pathway has been implicated in the regulation of sperm chemo-attractant secretion from unfertilized eggs of *Cladonema* and *Cytaeis*. Sperm attraction, mediated by factors secreted at the animal pole during maturation (Freeman, 1987; Miller, 1978), is reversibly inhibited by MAP kinase inhibitors (Arakawa et al., 2014; Kondoh et al., 2006). Furthermore, inhibition of translation of one of the two *Clytia* Mos genes (*Mos2*) in *Clytia* oocytes within the ovary does not block maturation but can result in a failure to spawn (Amiel et al., 2009). If MAPK-inhibited oocytes consistently fail to spawn, we could conclude that secretion of a MAPK-dependent factor synthesized or activated during oocyte maturation is necessary for the spawning process, as it is for sperm attraction. Isolation of fully grown oocytes in small drops, followed by cAMP-maturation induction and media collection after maturation completion, could be analysed by mass-spectrometry to check if any peptidic molecule is present in the media. This would be informative to assess what kinds of molecules, if any, are secreted from the oocytes during the maturation process. Treatment of isolated gonads with protease inhibitors, followed by cAMP analogue or artificial MIH stimulation of oocyte maturation and consequent spawning, would help to discriminate whether proteases are involved in the rupture of the gonad ectoderm epithelium during egg release or if this process is rather underpinned mainly by mechanical pressure.

The findings of my studies also raise questions of a more general nature. For instance, how (if at all) does local Opsin9-based regulation of spawning at the level of the gonad integrate more globally into the neurophysiological regulation of the jellyfish? The data shown in chapter 3 strongly suggest that MIH-secreting cells, through Opsin9 light reception, can act independently from the rest of the jellyfish to trigger light-induced oocyte maturation and consequent spawning in *Clytia*. However, as discussed in chapter 1, we cannot rule out the possibility that other factors, such as nutrient availability or GLWamide family neuropeptides, among others, could also play a facultative role in this regulation at the organismal level, to help fine-tuning spawning events in correlation with the changing environment. Another interesting avenue for future investigation, discussed more fully below, is that direct opsin-mediated triggering of spawning may integrate in some hydrozoan species, including perhaps *Clytia*, into a more complex and ancient system of light control involving circadian clocks.

Light-mediated reproductive control. Direct responses and entrained clocks

In the marine environment, where animal life started, an enormous variety of reproductive strategies can be found which are influenced to a certain level by ambient light (Lambert and Brandt, 1967; Levy et al., 2007; Oliveri et al., 2014; Tessmar-Raible et al., 2011; Zantke et al., 2013). The involvement of light in reproductive regulation within cnidarians, protostomes and deuterostomes (Tessmar-Raible et al., 2011) suggests that this environmental reproductive control strategy may have had an ancient origin followed by different specializations and the use of different molecules throughout animal evolution (see section I of introduction). Alternatively, the strategy to use light cues to coordinate sexual reproduction may have appeared many times in different evolutionary lineages, perhaps using different cellular and molecular mechanisms. To our knowledge, this work reveals the first opsin (i.e. *Clytia* Opsin9) whose function has been demonstrated to be essential for the light-mediated regulation of sexual reproduction in an animal. This finding thus gives a first concrete example concerning the molecular actors involved in light-mediated reproductive control in the animal kingdom, and their mode of action. Previously, the main role proposed for cryptochromes and opsin photopigments in reproduction had been as components of light-entrained molecular clocks (Halford et al., 2009; Levy et al., 2007; Tessmar-Raible et al., 2011; Zantke et al., 2013).

In *Clytia*, Opsin9-mediated MIH release and the consequent oocyte maturation and spawning appear to represent a direct and autonomous response to a light cue after dark. Thus oocytes of a sufficient size, achieved by about 15 hours of growth after the previous spawning in our aquarium conditions (Amiel and Houlston, 2009), mature with constant timing in response to a light cue. There are, however, various indications that spawning regulation in other hydrozoans and perhaps even in *Clytia hemisphaerica*, might involve more molecular partners. Firstly, some other species of hydrozoans, including *Cladonema pacificum* (see chapter 1), have been observed to spawn after light-dark rather than dark-light transitions (Mills, 1983), or indeed both at dawn and dusk. This could be explained by involvement of a different type of opsin working in an antagonistic manner (i.e. triggering MIH release when in the inactive rather than the active state) in these species. Consistently with this idea, phylogenetic analyses showed that no *Cladonema radiatum* opsins are orthologous with *Clytia* Opsin9 (Figure S2, S3 in chapter 3). Alternatively, the downstream signalling pathways in the opsin-expressing cells may integrate with additional regulatory elements. One possibility would be that circadian clock genes might be

involved in the process of MIH release. Supporting the idea of such a regulatory possibility operating in *Clytia*, individuals collected at Friday Harbor (NW Pacific coast of the USA), unlike the Mediterranean ones, spawn both at dawn and dusk (Roosen-Runge, 1962). The response of these animals to a dark-light cue shows characteristics of a direct triggering effect, but in contrast precocious light-dark transitions advance the spawning time in a variable, cycle-dependent, manner. This observation suggests involvement of an entrained circadian (or semicircadian) clock in the regulation of spawning in certain hydrozoan species and environments. One hypothesis would be that particular levels of clock genes targets, such as melatonin, predispose the MIH cells to secrete the MIH when peaks of melatonin synthesis occur inside these cells. Circadian regulation of spawning has also been described in the coral *Montastraea franksi*, in which spawning precisely occurs each year, at sunset during a particular time of the lunar cycle. It has been shown that the precise timing of spawning can be shifted in accordance to the time of sunset (Brady et al., 2009). These observations in *M. franksi* suggest a fine-tuned control of spawning by interacting endogenous circannual and circalunar clocks, which appropriately mark the time of the year and the lunar month, as well as a more direct response to changing environmental light when the corals are gravid and ready to spawn, possibly mediated by opsins. These examples of light-mediated spawning in different cnidarian species, together with our findings, suggest that the regulation of this process in cnidarians might be mediated by both molecular clock components and opsins, possibly interacting with each other.

Based on these observations, I propose that in *Clytia* Opsin9 might collaborate with an endogenous molecular clock in the MIH-secreting cells. Unidentified factors necessary for MIH synthesis or secretion would be transcribed following a circadian rhythmicity, and the phototransduction cascade mediated by Opsin9 upon light reception would induce MIH release conditional on the presence of this factor. This regulation might also be aided by GLWamide family peptides integrating metabolism and reproduction (see Chapter 1). A similar but more complex scenario has been proposed for the avian photoperiodic response, involving VA Opsin (vertebrate ancient opsin) expressed in deep brain photoreceptors, a circadian clock and hypothalamic neuropeptide hormones activating reproductive physiological responses (Halford et al., 2009). To understand better the light-induced spawning behavior in *Clytia* it would be of interest to study the possible involvement of cryptochromes and circadian clock genes (e.g. *pdp1*, *period*, *clock*, or *timeless*) in this process. If orthologs of these genes were indeed expressed in *Clytia* gonads, assessing their expression levels under different dark-light regimes would be informative. Further experimental work in this direction, using also other cnidarians

and a wide range of phyla known to have light-mediated spawning behaviours, would test the involvement of molecular clock components and opsins in light-mediated reproductive control, as well as their evolutionary relationships.

The proposition that opsin-mediated direct responses, such as Opsin9-mediated spawning in *Clytia*, may have been introduced during animal evolution into neurons previously regulated by an older eukaryote cryptochrome clock system is of interest in view of the proposition that cnidarian-type multifunctional sensory-neurosecretory cell types have an ancient evolutionary origin (Arendt, 2008; Fischer et al., 2013; Tessmar-Raible et al., 2007). Cells that combine sensory functions with neurosecretion (i.e. multifunctional sensory-neurosecretory cell types) are hypothesized to have been largely replaced by more specialised cell types during bilaterian evolution, but to have been retained in both vertebrate and invertebrate brains (Arendt, 2008; Tessmar-Raible et al., 2007; Vigh et al., 2002). In vertebrates, these cells are found in the pineal organ, in brain ventricles in contact with the cerebrospinal fluid, and in the hypothalamus (Tessmar-Raible et al., 2007; Vigh et al., 2002). For instance, in the vertebrate hypothalamus, a region of the brain intimately related to reproductive control through neuropeptide hormones (Halford et al., 2009; Shahjahan et al., 2014), vasotocinergic non-visual photoreceptors have been suggested to coordinate reproduction according to light cycles (Tessmar-Raible et al., 2007). These dual photosensitive-neurosecretory cells inside the brain, which likely regulate vital physiological responses through neurohormone secretion in response to changing environmental light, are called deep brain photoreceptors (Fernandes et al., 2013; Fischer et al., 2013; Halford et al., 2009; Tessmar-Raible et al., 2007). Deep brain photoreceptors share similarities with *Clytia* MIH-secreting cells (see chapter 3), since both regulate physiological responses through neurohormone secretion in response to changes in ambient light. It is thus conceivable that *Clytia* MIH-secreting cells and vertebrate deep brain photoreceptors might have a common evolutionary origin. Under this scenario, the integration of novel opsins during evolution to equivalent cell types in both cnidarian and bilaterian clades could reconcile the distant phylogenetic position of Opsin9 to any known bilaterian opsin. Alternatively, *Clytia* Opsin9 (and the distinct gonad-expressed opsins in *Cladonema* and *Tripedalia*) may have been co-opted into previously non-photoreceptive, MIH secreting cells in the gonad during medusozoan evolution.

Given the complexity of reproductive regulation in animals with centralised nervous systems, involving various organs, cell types and hormones (Halford et al., 2009), the first hypothesis, implying an evolutionary relationship between *Clytia* MIH-secreting cells, acting locally in the gonads, and sensory-neurosecretory cell types located deep inside bilaterian brains, is difficult to imagine, but clues to reinforce this hypothesis are offered by the intriguing nexus between bilaterian neurohormonal regulation of sexual reproduction and *Clytia* MIHR phylogenetic position, discussed in the next section.

***Clytia* MIHR and the neurohormonal regulation of sexual reproduction**

In vertebrates, reproduction is coordinated by the hypothalamic-pituitary-gonadal axis (HPG axis) in both males and females (Herbison, 2016; Meethal and Atwood, 2005; Nozaki, 2013; Shahjahan et al., 2014). The integrating centre of the HPG axis is the hypothalamus. In the hypothalamus, a subset of neural cells called GnRH (gonadotropin-releasing hormone) neurons synthesize and release in a highly regulated, pulsatile manner, the neuropeptide GnRH into the hypophyseal-portal system, a network of blood vessels that connects the hypothalamus to the anterior pituitary. GnRH interacts with endocrine cells in the pituitary to induce the secretion of neuropeptidic gonadotropin hormones (i.e. LH for luteinizing hormone and FSH for follicle-stimulating hormone) into the bloodstream. LH and FSH bind to receptors in the gonads and stimulate spermatogenesis and oogenesis, as well as sex steroids (e.g. oestradiol, testosterone or progesterone) and inhibin production. These, in turn, negatively feedback to the brain and pituitary, resulting in a decrease in gonadotropin release to complete the HPG axis and regulate the reproductive cycle (Herbison, 2016; Meethal and Atwood, 2005; Nozaki, 2013; Shahjahan et al., 2014). The activity of GnRH hypothalamic neurons can thus tune complex hormonal signalling and plays a key role in the regulation of reproduction in vertebrates.

In vertebrates, the neurohormonal control of sexual reproduction is even more complex, since it exhibits yet another layer of regulation at the level of the central nervous system (CNS). Cells of the vertebrate hypothalamus secrete a number of neuropeptide hormones besides GnRH, such as neuropeptide Y (NPY), neuropeptide FF (NPFF), QRFPamide, orexin, galanin, kisspeptin and gonadotropin-inhibitory hormone (GnIH), among others. All of these have been described to affect reproductive control in different vertebrate models (Kriegsfeld, 2006; Navarro et al., 2006; Osugi et al., 2015; Pasquier et al., 2014; Shahjahan et al., 2014). As an

illustration, orexin is known to stimulate the HPG axis via GnRH secretion in mammals (Campbell et al., 2003). NPY can stimulate GnRH and LH release *in vitro* in several fish species (Shahjahan et al., 2014), and regulate gonadotropic hormone secretion at the CNS levels in mammals (Wójcik-Gładysz and Polkowska, 2006). In rats, QRFamide stimulates LH and FSH secretion *in vivo* and in pituitary culture *in vitro* (Navarro et al., 2006), and intraperitoneal injections of QRFamide highly increase plasma LH levels in the goldfish (Shahjahan et al., 2014). In fish and mammals, the release of some of these hypothalamic neuropeptides can be regulated by metabolic signals, suggesting a link at the level of the CNS between reproductive control and nutrition levels (Shahjahan et al., 2014; Wójcik-Gładysz and Polkowska, 2006). Notably, hypothalamic GnRH neurons have been shown to express receptors for several of these neuropeptide hormones. For example, orexin, kisspeptin, galanin and NPY receptor mRNAs have been found in GnRH neurons in mammalian and fish models (Campbell et al., 2003; Herbison, 2016; Klenke et al., 2010; Shahjahan et al., 2014). Moreover, the hypothalamic neurons synthesizing some of these neuropeptides are positioned close to GnRH neurons in several fish species and mammals (reviewed by Shahjahan et al., 2014). All these elements contribute to a very complex neuroregulatory network in the hypothalamus involved in sexual reproduction regulation.

Neuropeptide hormones such as kisspeptin, NPY, GnRH and gonadotropic hormones, as well as their receptors, which belong to the neuropeptide class-A GPCR family, play a crucial role in reproductive control throughout the animal kingdom. In mice, high follicular-phase oestradiol levels during female ovarian cycles and circadian inputs are integrated by kisspeptin neurons in the hypothalamus, which project to GnRH neurons and lead to a preovulatory GnRH surge and consequent LH surge (Herbison, 2016; Le Tissier et al., 2016). LH in turn binds to its receptor in the membrane of mural granulosa cells in the ovarian follicle leading to a decrease in intracellular cGMP levels, which triggers diffusion of second messengers that ultimately trigger oocyte meiotic resumption and ovulation (Shuhaibar et al., 2015) (see section II of introduction). In fish and amphibians, LH surges produce the same outcome, although an intermediate step is the release of MIHs by follicle cells (Haccard et al., 2012; Nagahama and Yamashita, 2008). In contrast, outside the vertebrates, the neurohormonal control of reproduction, although present, is less well characterized. Vertebrate counterparts of GnRHs and their receptors have been found in most protostome and non-vertebrate deuterostome lineages, and they are referred to as adipokinetic hormones (AKH) in ecdysozoans (Jékely, 2013; Lindemans et al., 2011). Outside vertebrate animals, the involvement of GnRH and its receptor in reproductive control has only been

functionally proven in *C.elegans*, where knockdowns of the GnRH precursor and the GnRH receptor lead to delayed egg-laying and decreased amount of progeny (Lindemans et al., 2009). Gonadotropic cycles are clearly present in invertebrates, although they are poorly characterized. To illustrate a few examples, insect parsins and neuroparsins have been assumed as the physiological counterparts of FSH and LH, and several peptide hormones are described to regulate ovarian functions (reviewed by Kuczer et al., 2008). An insulin-like peptide released from neurosecretory cells in the brain of the mosquito *Aedes aegypti* was shown to stimulate egg production (Brown et al., 2008). The best-described gonadotropin hormone in any invertebrate is the gonad-stimulating substance (GSS) in starfish, a relaxin-like peptide secreted from radial nerves, which stimulates MIH (1-MeAde) secretion from follicle cells (Mita, 2013). A recent study has demonstrated that knockdown of NPYR-1, a NPY GPCR in the planarian *Schmidtea mediterranea*, results in loss of germ cells differentiation and sexual maturity (Saberi et al., 2016). These examples highlight the importance of neuropeptide-GPCR interactions in the regulation of reproductive control across the bilaterian clade.

Until now, no cnidarian neuropeptide GPCR had been characterized functionally. Our finding that *Clytia* MIH-MIHR couple is indeed a ligand-neuropeptide GPCR pair, representing the first example of this kind in a cnidarian, has allowed evolutionary comparisons of their relationships to other characterised bilaterian neuropeptidergic systems (see Chapter 2). Assessing evolutionary connections between neuropeptides is difficult, and requires analysis at the level of the neuropeptide precursor sequences, due to the short amino acid lengths of the cleaved peptides, but even so allows for only rather restricted conclusions (Jékely, 2013; Mirabeau and Joly, 2013). Neuropeptide GPCR amino acid sequences are more conserved and give better material for phylogenetic analyses, although this is still challenging because of wide sequence divergence outside the TM domains. Broad phylogenetic screening of metazoan neuropeptides and neuropeptide GPCRs suggested a coevolution of neuropeptide-GPCR pairs and the existence of a deep conservation of neuropeptidergic regulation between different metazoan lineages (Jékely, 2013). However, this hypothesis could only be applied to bilaterian peptidergic systems, due to the lack of information of neuropeptide GPCRs in cnidarians. The uncovering of *Clytia* MIH-MIHR couple, their biological function in the direct control of oocyte maturation, and the phylogenetic position of MIHR, strongly suggest the involvement of similar neuropeptidergic systems in the regulation of important physiological functions in the eumetazoan ancestor.

Our phylogenetic analyses of *Clytia* MIHR (see Figure 4 in Chapter 2) indicate that it belongs to a large group of cnidarian GPCRs related to a small subset of bilaterian neuropeptide GPCR families (specifically the NPY/NPF, NPFF/GnIH, QRFP and RY/Luqin receptor families). Intriguingly, members of these four neuropeptide GPCR families in various bilaterian species are expressed in the CNS and peripheral tissues, and are involved in reproductive control as well as in other physiological functions, notably feeding. For instance **NPY**, synthesized in vertebrate hypothalamus neurons, can stimulate GnRH and LH secretion as well as act as an orexigenic factor (i.e. appetite enhancer) in fish and mice (Shahjahan et al., 2014; Wójcik-Gładysz and Polkowska, 2006). Hypothalamic GnRH neurons are known to express NPY receptors (Klenke et al., 2010). **QRFPs**, produced by the hypothalamus also have orexigenic activity and can lead to increased LH plasma levels in rats (Chartrel et al., 2003; Navarro et al., 2006). The mouse QRFP receptors are expressed in the hypothalamus, as well as in the pituitary gland and peripheral tissues such as heart, kidney, retina and testis (Jiang et al., 2003; Navarro et al., 2006), suggesting a broad range of functions in rodents. **NPFF/GnIH** in birds and mammals seems to act at various levels of the HPG axis, since it inhibits GnRH secretion from GnRH neurons as well as gonadotropin release from the pituitary (Tsutsui et al., 2010). Moreover, NPFF/GnIH hormones stimulate water intake, increase arterial blood pressure and inhibit food intake in rodents. These hormones are also strongly implicated in pain modulation based on their ability to attenuate the analgesic effects of morphine, and on the expression sites of the corresponding receptors in the CNS (Bonini et al., 2000; Parhar et al., 2012). NPFF/GnIH receptors have been found in the CNS, notably in hypothalamic GnRH neurons, as well as in pituitary gonadotropes and several peripheral tissues in vertebrates (Bonini et al., 2000; Tsutsui et al., 2010). Concerning the **RY/Luqin receptor family**, only characterized in protostomes for the moment, there is little functional evidence available but RYα peptides suppress appetite in *Drosophila* (Ida et al., 2011).

Putting this together, we can conclude that *Clytia* MIHR is evolutionarily related to a group of bilaterian neuropeptide GPCRs produced in the CNS and involved in various physiological functions, notably reproduction and feeding. Certainly it is closer to this NPY/NPF-NPFF/GnIH-QRFP-RY/Luqin receptor cluster than it is to any of the GPCRs functioning at other levels of ovulation control in vertebrates such as mouse GPR3 or fish mPR expressed in oocytes, gonadotropin GPCRs expressed in follicle cells or GnRH GPCRs in endocrine cells of the pituitary. As mentioned in chapter 1 and 2, we could detect *Clytia* MIH and MIHR mRNA expression in non-gonadal sites in the jellyfish (Figures S5 in Chapter 1 and 2), as well as MIH-immunopositive neural cells in different body parts of the jellyfish and also polyp

stage, notably the tentacles and manubrium (Figure S6 in Chapter 1 and Figure S4 in Chapter 2). Moreover, *Clytia MIHR* KO exhibited polyp growth and jellyfish swimming problems (see Chapter 2). These observations suggest that *Clytia* MIH-MIHR interactions might regulate wider aspects of *Clytia* physiology besides oocyte maturation initiation, similarly to how the bilaterian neuropeptidergic systems described above are involved in more than one physiological pathways, including reproductive control. It could be actually possible that physiological functions other than spawning, may relate more closely to the roles of the ancestral form of CheMIHR (and all the cnidarian peptides ligands of this receptor group) and the bilaterian NPY/NPF-NPFF/GnIH-QRFP-RY/Luqin peptide and receptor families. Further experimentation focused on *Clytia* MIH-MIHR functions outside the gonad would be useful to explore this possibility.

The common ancestry of *Clytia* MIHR and the related groups of bilaterian neuropeptide GPCRs allows us to provide a speculative evolutionary scenario based on receptor family expression. *Clytia* MIH-secreting cells could be equated to hypothalamic neurosecretory cells such as NPY neurons, and *Clytia* oocytes expressing CheMIHR would be comparable to GnRH neurons. Under the cell type evolution concept (Arendt et al., 2016), these *Clytia*-vertebrate pairs of cells could have derived from cell types in the common eumetazoan ancestor that already participated in regulating spawning (for instance a photosensitive-secretory cell and a germ cell). During bilaterian evolution, reproductive control could have gained layers of complexity through the acquisition of follicle cells, endocrine organs and the centralization of their nervous systems (see Figure 6 in Chapter 1 and Deguchi et al., 2011). In contrast, cnidarians present non-centralized nervous systems, which are strongly peptidergic, contain relatively few cell types, and do not have follicle cells, suggesting that they may have remained more similar to the ancestral situation, involving only local regulation of gamete activation. The alternative hypothesis, alluded to above, is that in primitive eumetazoans the ancestral “CheMIH-MIHR-like” system had a different or wider neural function, and only relatively late in hydrozoan evolution did gonad MIH neurons in hydrozoans acquire opsins to become directly light sensitive. This latter scenario is consistent with the large expansion of opsins in cnidarians, which may have acquired specialized functions by co-option in pre-existing neural cells (see Chapter 3), and also with the localization of *Clytia* MIH-immunopositive neural cells at diverse sites in the jellyfish and also in the polyp stage (see above).

To finish I will link these two possible evolutionary scenarios more explicitly to the regulation of oocyte maturation. Under the first scenario, the eumetazoan ancestor would have expressed an ancestral form of *Clytia* MIHR in the oocytes, which would trigger oocyte maturation in response to neuropeptidic MIHs produced by nearby cells, possibly under direct light regulation. In this case, cnidarians would have retained this feature, whereas in bilaterians, during the evolution of neuroendocrine systems, expanding families of CheMIHR-related GPCRs would have been retained in neurons located in the CNS, which would have acquired less direct roles in the control of sexual reproduction (as well as additional functions in regulating related physiological processes). They would continue to regulate gamete maturation and release via the secretion of 'relay' hormones produced by bilaterian-specific cells, such as neuroendocrine and follicle cells. Under the alternative scenario, the eumetazoan ancestor would have possessed an ancestral form of *Clytia* MIH and MIHR in neural cell types and their responding tissues outside the gonad, involved in the regulation of various physiological processes, for example diel behaviours including feeding and swimming. In this case, bilaterians would have retained ligands and GPCRs, diversified from the ancestral form, in a variety of neural cell types, becoming specialised in functional regulation of different physiological processes including reproduction. In parallel, during cnidarian evolution, particular GPCRs of this family would, at least in some hydrozoan lineages, have been co-opted into the oocytes to directly control oocyte maturation. Regardless of which of these scenarios is closest to the truth, MIHR in hydrozoan oocytes today allows a simple, fast, and localized way of triggering oocyte maturation initiation that has enhanced their reproductive success in marine ecosystems.

To help discriminate between these two evolutionary scenarios, it would be of great interest to locate the expression site of other medusozoan and anthozoan orthologs of *Clytia* MIHR (see Chapter 2), and to test their functions. The results would strengthen one of the two intriguing evolutionary scenarios we have proposed for the possible function of an ancestral form of *Clytia* MIHR, and would give more insight on the current understanding of how oocyte maturation initiation is regulated in different animals.

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