Light influence on nematocyst firing in the Sea anemone *Haliplanella luciae*

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Abstract

Nematocytes, the cnidarians stinging cells, discharge nematocysts into prey to capture them. As nematocysts are expensive, the discharge is tightly regulated and occurs after proper chemical and mechanical stimulation. Here, I present evidence for nematocytes photosensitivity. I found that light decreases the propensity for nematocytes to discharge in the sea anemone *Haliplanella luciae*. Taken together with similar findings in cubozoan and hydrozoan, we believe that light modulates nematocysts discharge for all classes of Cnidaria. I also investigated the effect of light conditions, when combined with N-acetylated sugar Mucin, previously shown to sensitize nematocytes to discharge. Light and sugar seem to have separate and opposing effects on the propensity for nematocytes to discharge. We have preliminary evidences for a prevalence of the chemoreception over the light perception but further investigations should be conducted. Finally, our findings may suggest the involvement of an inhibitory G-protein in the sea anemone phototransduction pathway. While nematocytes chemo- and mechano-sensitivity have been extensively studied, we believe this is the first evidence for nematocytes sensitivity to light intensity.

INTRODUCTION

General introduction to Anthozoa

Phylum cnidaria and general biology

Cnidarians represent an ancient group of animals, which diverged with Bilaterians over 600 million years ago. The phylum contains over 9000 species, very diverse in form and found exclusively in aquatic, usually marine environments. The phylum Cnidaria is divided into four major living classes: Scyphozoa (true jellyfishes), Cubozoa (box jellyfishes), Hydrozoa (e.g. hydras), and Anthozoa (such as sea anemones and corals). Within the anthozoans, there are two contemporary orders, Octocorallia and Hexacorallia, the latter being divided into six contemporary families and one extinct family. *Haliplanella luciae*, commonly named the Orange-striped sea anemone, on which I have conducted my study, belongs to the family Actinaria, which includes all species of sea anemone.

Sea anemones, and more generally anthozoans, distinguish themselves from most other cnidarians by the lack of a medusa stage. They spend all their life as a polyp and tend to stay attached to the same spot until conditions become unsuitable. Their diet is mainly composed of small fish and zooplankton that they capture, even though many species also depend on a symbiotic relationship with zooxanthellar algae contained within their cells. Captured prey is typically moved by the tentacles to the mouth into the gastrovascular cavity, where digestion occurs. The tentacle motion, as well as the body motion, is achieved through the contraction of muscle fibers, which are longitudinal fibers in the tentacles and oral disc, and circular fibers around the body wall. Anthozoans possess a relatively simple nervous system, without centralization. Behind their apparent simplicity, anthozoans, like other cnidarians, display surprisingly complex traits, which make them truly exciting to study. One of those traits and distinguishing features is the so-called “stinging cells”, the

cnidocytes, where the phylum gets its name ("cnidos" in Greek means stinging nettle).

Cnidocysts: the hallmark of the phylum

Cnidocysts are organelles ejected from single-use cnidocyte cells. Cnidocyst ejection occurs after proper chemical and mechanical stimulation. They are comprised of three different classes with a diversity of functions and structures. First, ptychocysts are only found in Anthozoa in a special type of burrowing sea anemone that uses them to build the tube they are living in (Mariscal *et al.*, 1977). Second, spirocysts are also only found in Anthozoa. They represent about 66% of the cnidocysts present in the tentacle of a sea anemone (Thorington *et al.*, 1990). Spirocysts are volvent, they do not penetrate their target, but are thought instead to entangle the prey (Krayesky *et al.*, 2010).

The third group of cnidocysts, nematocysts, is found in all Cnidarians. They are very diverse morphologically, as over 30 types of nematocysts have been described so far (Fautin, 2009). Most nematocysts are penetrant and used for prey capture. Nevertheless, volvent nematocysts and glutinant nematocysts, used for defense and locomotion respectively, have also been described (Purcell, 1984). This also indicates that all nematocysts do not deliver venom, as commonly believed. Nematocyst venom has been extensively studied, and for good reason. Some of them are indeed extremely poisonous and can even be dangerous to humans, like the ones injected by the Australian box jellyfish *Chironex fleckeri*. No Cnidarian class displays all types of nematocyst. Hydrozoans for example, present the greatest diversity with 23 types of nematocysts, among which 17 are unique to the class (Fautin, 2009). Only three nematocyst types are known for Scyphozoans and Cubozoans. Anthozoans display 6 types of nematocysts, two of them exclusive to the class. But for most anthozoans and especially sea anemones, the tentacle presents only two types of nematocysts: microbasic p-magistophore and basitrichous isorhiza (Krayesky *et al.*, 2010)

Nematocyst structure, development and function

Cellular structure and organization

The structure of nematocysts, a bulb-shaped capsule enclosed in the nematocyte cell and containing a long coiled tubule, is central to their function (Fig.1). Nematocysts are completely enclosed in a vesicular membrane (Golz, 1994). This wall is composed of mini-collagens, which provides it great flexibility and the rigidity necessary for resisting the high pressure displayed inside the capsule: a 2M concentration of cations creates an osmotic pressure higher than 150 bar. The capsule wall has a lid, called *operculum*. The structure of the coiled tubule contained in the capsule differs depending on the species and the type of nematocyst. Nematocysts are surrounded by nematocyte

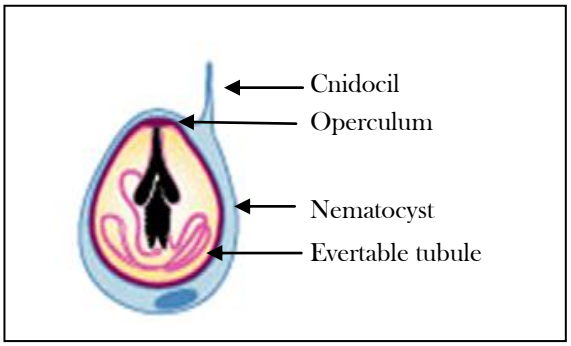

Fig.1. Nematocyst structure: Nematocyst inside the cnidocyte-nematocyte. Adapted from S. Ozbek *et al.*, Toxicon 54 (2009) 1038-1045

cytoplasm. They are anchored to the nematocyte at their pole, through a net of microtubules and intermediate filaments that link the cell membrane to the nematocyst vesicular membrane (Özbek *et al.*, 2009). The nematocyte is topped with a hair-like "trigger device", the *cnidocil*.

Within the epidermis, nematocyte cells form complexes with supporting cells. This organization differs among cnidarian classes and I will focus here on the one displayed in anthozoans. The structure of these complexes has been extensively described in the sea anemone *Haliplanella luciae* (Mire-Thibodeaux and Watson, 1993 and 1994; Watson and Hessinger, 1989). Two different types of ciliated complexes are observed, where projections outside the epithelium form hair bundles (the cnidocil of the sea anemone). The first complex, called the cnidocyte/supporting cell complex (CSCC), is composed of one cnidocyte and the adjacent supporting cells, which are very abundant epidermal cells surrounding nematocytes. The cnidocyte contributes a kinocilium to the hair bundle, referred to as the ciliary cone (Watson and Mire, 2004); the supporting cells contribute several stereocilia. There are approximately 41,000 CSCCs in each tentacle, among which 12,300 contain microbasic p-mastigophore nematocysts (Watson and Hessinger, 1994). The other complex, called the sensory cell /supporting cell complex (SNSC), is composed of one sensory cell and the surrounding supporting cells. The sensory cell contributes one central kinocilium and six large diameter stereocilia to the hair bundle; the supporting cells contribute several hundred small diameter stereocilia. Hair bundles arising from SNSC tune nematocyst discharge in response to mechanical stimulation and will thus be further described later. Sensory cells are also thought to synapse with the nerve net (Peteya, 1975).

Development of the nematocyst

Two events occur simultaneously: the development of the nematocyte (the cell) and the formation of the nematocyst within the developing nematocyte.

Differentiation of the nematocyte

The nematocyte originates from undifferentiated interstitial cells and undergoes morphological changes before migrating to its final location. First, one of the numerous undifferentiated interstitial cells located near the base of the epithelium of the body (the scapus in the case of anemones) differentiates into a nematoblast (Carré and Carré, 1973). The nematoblast in turn undergoes many changes that are not detailed here, while migrating through the ectoderm to its final destination: the tentacle, in our case. There, it becomes fully differentiated and develops the cnidocil (Slautterback and Fawcett, 1959; Carré and Carré, 1973).

Secretion of the nematocyst

Within the migrating nematoblast, the Golgi apparatus secretes a capsule, which grows into the mature nematocyst. An easy way to picture it is to consider the nematocyst as a "secretory product" of the nematoblast (Slautterback and Fawcett, 1959). Indeed, the nematocyst develops inside a giant vesicle in the nematoblast and is built up by spontaneous self assembly of proteins secreted by the Golgi apparatus (Slautterback and Fawcett, 1959). First, the capsule is secreted by the Golgi apparatus. Then, this capsule develops and the

operculum is formed. The nematocyst continues to grow within the migrating cnidoblast, and once it reaches its definitive dimension (length range from 20 to 200 µm), the tubule forms and invaginates in the capsule (Carré and Carré, 1973).

Cellular mechanisms of nematocyst discharge

Some steps have been identified **(Fig.2.)**, but the discharge mechanism is still debated. In addition, the mechanism is not exactly the same for all cnidarians. The following will thus present some common features and the most accepted hypotheses.

Discharge initiation

The main driving force of the discharge is the high intra-capsular pressure, which in conjunction with the structure of the capsule wall, allows the cell to invert. Just before the discharge, the capsule swells which presumably increases the intra-capsular pressure. Whether this change arises from a change in the ionic composition inside the capsule or by a swelling of the tubule coiled inside the capsule is still unknown (Özbek *et al.*, 2009). The reason may also differ from one cnidarian class to another, depending on how the cnidocyte is built. In addition to this swelling, a change in the *operculum* architecture has been reported: it seems that during this step, the proteins composing the *operculum* change their arrangement to form a more loose association (Özbek *et al.*, 2009). This observation helps understanding how the pressure breaks the capsule wall at a particular position, preventing the capsule from random rupture.

Ejection of the tubule

Once the operculum is open, the cell everts; the tubule is thus ejected and penetrates the prey. This is the critical step of the discharge and it is thought to be

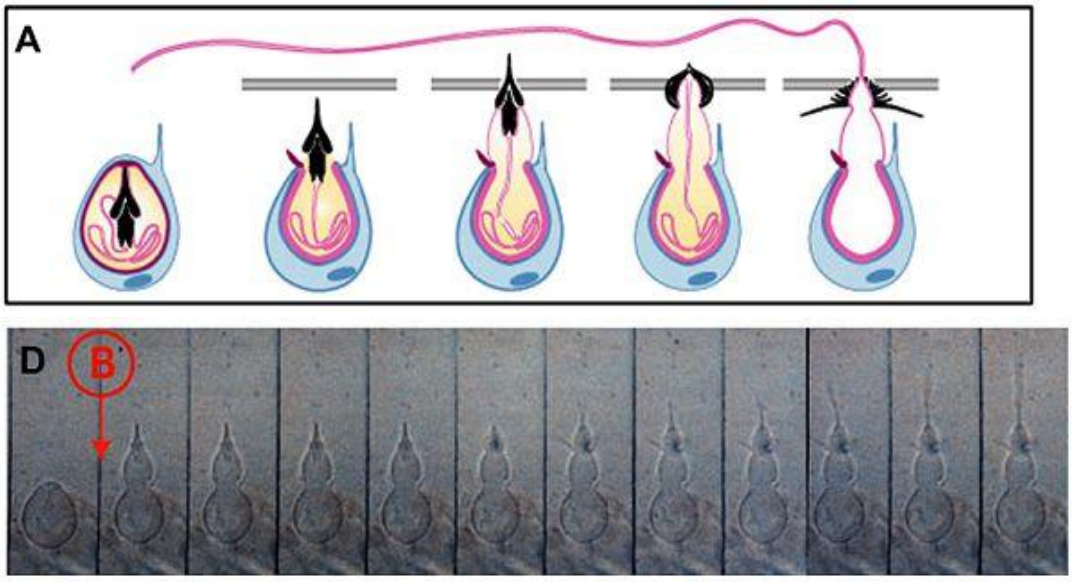

Fig.2. Discharge of a nematcoyst in *Hydra*. (A) depicted; (D) recorded with a rotary prism camera at 40,000 frames per second.

Adapted (A) from Nütcher *et al.*, 2006 and (D) from Holstein and tardent, 1984

achieved in about 700ns (Nützcher *et al.,* 2006). The stylet – the tip of the tubule- is ejected with a great velocity (around 18.6 m/s), which allows it to hit the prey with a pressure comparable of the one exerted by a bullet (Özbek *et al.*, 2009). The stylet can then penetrate a crustacean prey, perforating its cuticle. Then, the rest of the tubule invaginates inside the prey and the toxin is released (if there is toxin).

Physiology - Electrical activity and ion currents underlying the discharge

The stimulation of the cnidocil induces a depolarization of the nematocyte membrane and sensitizes the surrounding nematocytes to discharge. When a prey touches the cnidocil, an action potential is generated that will depolarize the nematocyte membrane through the opening of Ca^{2+} ion channels (Gitter et *al.*, 1994). This influx of calcium inside the cell is also thought to induce the fusion of the nematocyst capsule with the nematocyte membrane, which is the last event before the discharge (Özbek et *al.*, 2009). Nevertheless, if it is accepted now that Ca^{2+} plays an important role in the initiation of the discharge, the mechanistic details of its influence are still unknown. Another electrical event occurs after the cnidocil has been stimulated. Thurm described an electrical coupling between nematocytes, in two species of hydrozoans (Thurm et *al.*, 2004). Thus, cnidocytes seem to communicate with each other: when one is stimulated, the surrounding cnidocytes receive electrical inputs which increase their probability to discharge.

Regulation of nematocyst discharge

Cnidocytes are single use cells, and expensive to produce. Therefore, nematocytes are sensitized to discharge under conditions corresponding to the presence of prey. This is accomplished by tuning discharge based on sensory input.

Mechanosensitivity

A mechanical stimulation is required to elicit the discharge. In *Haliplanella*, two types of mechanoreceptors are involved in triggering the discharge: a contact-sensitive mechanoreceptor (CSM) and a vibration-sensitive mechanoreceptor (VSM) (Watson and Hessinger, 1992; Watson et *al.*, 1997). Little is known about CSM but their existence is inferred by the fact that nematocysts discharge occurs only upon direct tactile stimulation of the tentacle. Without additional sensitization, sea anemones discharge relatively few nematocysts upon a simple touch. For convenience, the response induced by a single touch with a static probe will be referred to as baseline discharge. On the other hand, VSMs, called anemone hair bundles, have been extensively studied in *Haliplanella luciae* (review in Watson and Mire, 2004). VSMs comprise the previously described hair bundle arising from sensory cell/ supporting cell complexes. One of the most surprising findings is that the projections of the SNSC are organized in a way that is quite similar to vertebrate hair cells. The presence of lateral extracellular linkages between actin-based stereocilia, including tip links that interconnect the tip of adjacent stereocilia, is especially reminiscent of the vertebrate hair bundle (Watson et *al.*, 1997). VSMs are involved in the frequency-tuning of nematocyst discharge: nematocysts are preferentially discharged into targets vibrating at specific frequencies and amplitudes. These frequencies, referred to as preferred

frequencies, trigger a discharge twice as great as the baseline discharge. According to the model, vibrations that are carried through the water sensitize nematocytes to discharge in the event that the swimming prey touches the tentacle.

SNSC hair bundles detect vibrations and SNSC cells relay the information to the cnidocyte. In response to vibrations, the hair bundle oscillates which give rise to changes in the membrane polarization in the supporting cells (Watson and Mire, 2004). Supporting cells transduce the current to the sensory cells via gap junctions (Mire *et al.,* 2000). Sensory cells are thought to integrate and relay the information to the effectors, through a three cell neural pathway: the sensory cell communicates with a ganglion cell that acts as an interneuron. This ganglion cell has chemical synapses with the nematocyte cell (Westfall, 2004).

Chemosensitivity

Sensitizers

Two groups of naturally occurring substances sensitize nematocytes for discharge. It was early reported that chemical stimulation influences cnida firing (Parker and Van Alstyne, 1932, cited by Thorington and Hessinger, 1988). A wide range of chemicals have been tested (Thorington and Hessinger, 1988), among which two groups of sensitizers were identified. By sensitizer I designate a chemo-substance which sensitizes the cnidocyte to discharge upon mechanical stimulation, i.e. lower the threshold of mechanical stimulus required to trigger the firing. Thorington and Hessinger (1988) described two classes of chemoreceptor: the first is broadly specific to a variety of amino and imino compounds and is inhibited by antihistamines (e.g. proline). The second is specific for N-acetylated sugars or substance carrying terminal N-acetylated sugars such as mucin, a high molecular weight glycoprotein that is often used in research. According to the current model, chemoreceptors occurring at the surface of the supporting cells detect specific substances and sensitize mechanoreceptors. The number of these chemoreceptors changes over time and is regulated by endocytosis (Watson and Hessinger, 1989).

These compounds display a double action: they sensitize the CSMs to initiate discharge into static targets and tune the VSMs. Thus, the level of nematocysts discharged into static targets, in presence of these compounds at optimal doses is two to three times higher than the baseline discharge. The effect is maximal at 10^-7 M for N-acetylated sugars and 10^-8 M for proline. Interestingly, above this optimal concentration, discharge gradually decreases to baseline level (Thorington and Hessinger, 1988; Watson and Hessinger, 1989). But what is really exciting is that these compounds shift the VSM preferred frequencies, through morphological modifications of the hair bundle. Thus, N-acetylated sugars shift the preferred frequencies to lower frequencies and smaller amplitude, by triggering actin-polymerization-dependent elongation of the hair bundle of SNSC (Mire-Thibodeaux and Watson, 1993). Conversely, proline, which binds the other type of chemoreceptor, triggers an actin-depolarization-dependent shortening of the hair bundle, when associated to sugars. Interestingly though, proline alone does not affect VSM.

The biological explanation lies in the idea that the efficiency of the prey capture should be maximized. N-acetylated sugars are present on the prey coating and proline (as well as other amino compounds that binds the chemoreceptor) is present in the hemolymph of crustaceans, and thus likely to leak from wounded prey. The preferred frequencies displayed before chemosensitization are not biologically relevant, but those displayed with chemosensitization match vibrations generated by swimming prey (Anderson and Bouchard, 2009). The presence of N-acetylated sugars also broadens the range of frequencies that trigger maximal discharge (Krayesky et al, 2010). The discharge and the probability of the discharge are thus enhanced when it is more likely to lead to a successful prey capture. Presumably, an approaching prey generates a local increase of N-acetylated sugar and the sensory system responds by sensitizing mechanoreceptors to the corresponding frequencies. Once the prey is captured and wounded, proline is released and shifts the responsiveness to frequencies higher than those generated by the prey. This prevents additional discharge of nematocysts into a prey already captured (Watson and Hessinger, 1994). Another possibility is that proline tunes hair bundles so that the struggling movements generated by the wounded prey stimulate maximal discharge of nematocysts (Watson and Mire 2004). As the tuning of the hair bundle is not permanent, this cycle may happen again when a new prey approaches the tentacle.

Some aspects of this model are still debated. For instance, the time required to achieve the morphological changes of the hair bundle described above casts doubt on its biological interpretation. Indeed, actin polymerization occurs in a few minutes (Watson and Hessinger, 1991, cited by Krayesky et *al.*, 2010) and the vibrations cannot be detected from far away. The distance is also a concern when it comes to the chemosensitization. As the substances presumably released by the prey have to bind the chemoreceptor, the prey cannot be detected from more than a few cm. Nevertheless, as prey hunted by sea anemone and more generally cnidarians are planktonic and thus occurring in shoals, one can argue that this interpretation remains relevant when applied to the rest of the feeding period (after the first minutes).

Molecular chemosensory pathway

Agonist drug studies have begun to elucidate the molecular basis of the chemosensory pathway: they suggest that the transduction of the chemo-signal from the receptor is achieved via a G-protein, adenylate cyclase, cAMP pathway (Watson and Hessinger, 1992, 1994; reviewed by Ozacmak et *al.*, 2001). Typically, a ligand binds its surface chemoreceptor which activates the associated G-protein. Caged GTP- γ-S, which activates G-protein coupled signaling pathways, displayed a "mixed effect", tuning VSMs both to higher and lower frequencies (Watson and Hessinger, 1992). This suggests the possibility of two G-proteins with antagonist actions. Gs (stimulatory) agonists induce the same effect as N-acetylated sugars, both on CSMs and VSMs. On the other hand, pertussis toxin, which interferes with Gi (inhibitory), blocks the effect of proline on VSMs (Watson and Hessinger, 1994). Thus, N-acetylated sugar receptors may stimulate Gs whereas proline receptors stimulate Gi, both modulating adenylate cyclase levels, but in opposite directions. AC agonists induce the same tuning as N-

acetylated sugars. Moreover, binding of N-acetylated sugars to their receptors induced an AC activity in the apical membrane of the supporting cells. Binding of N-acetylated sugars also induces an increase of the intracellular cAMP level in the tentacle ectoderm. Reciprocally, agents that raise intracellular cAMP levels have the same effect as N-acetylated sugars, both on CSM and VSM. The relationship between hair bundle elongation and the increase of intracellular cAMP levels is also now established (Thibodeaux and Watson, 1992 cited by Watson and Hessinger, 1994). cAMP in turn activates the protein kinase A, involved in actin polymerization initiation and thereby in the tuning of the mechanoreceptor.

Photosensitivity

If the modulation of the firing by chemosubstances has been - and still is- extensively studied, no other types of sensitivity have been described. For example, light is of great importance for many natural processes and it seems that the light influence on nematocyst firing has never been studied before.

The discovery of light sensitivity genes (opsin) expressed in sensory cells associated with cnidocytes in *Hydra* (Plachetzki, 2010) raised the question of a possible involvement of light level in the modulation of the firing. David Plachetzki investigated this question on *Hydra magnipapillata* and found that different light levels modulate firing rates (unpublished). Indeed, the dim-light adapted animals fired significantly more nematocysts than bright-light adapted animals.

Also, interestingly, chemoreception and light perception seem to share the same G-protein, adenylate cyclase, cAMP signaling pathway. Phototransduction, i.e. the conversion of light into electrical signals, has been extensively studied in bilaterian and constitutes today one of the best understood signaling pathway. However, knowledge about the molecular basis of phototransduction in cnidarians is very recent and the pathway has not been fully established yet. In bilaterians, when light strikes the opsin-photoreceptor, it activates a G protein. Different opsins activate specifically a G protein belonging to one of the tree classes known so far: Gi/o/t, Gq or Gs. Koyanagi et *al.* (2008) identified most of the messengers involved in the phototransduction in the cubozoan *Carybdea rastonii*. They found a Gs protein, which co-localize with the opsin expressed specifically in visual cells. In hydrozoans, little is known to date. Plachetzki *et al.* (2007 and 2010) studied several opsins expressed in the nervous system of *Hydra magnipapillata*. Protein Gs is present in the *Hydra* genome but whether it is involved in photosensitivity has not yet been tested in hydra. Another G-protein, the inhibitory Gi, is also present in the *Hydra* genome and seems to co-localize with opsin (Plachetzki, unpublished). In bilaterians, the activation of the G protein triggers an increase of the level of Guanylate Cyclase (GC) or Adenylate cyclase (AC), which synthesizes cGMP or cAMP respectively. In a cubozoan, Koyagani reported specific abundance of Adenylate cyclase (AC) in visual cells (Koyanagi et *al.*, 2008). Adenylate cyclase is responsible for cAMP synthesis, and in cells expressing the cubozoan opsin gene, a light dependent cAMP increase was observed. Koyagani (2008) also found CNG cDNA transcripts from the eye of the cubozoan *Carybdea rastonii.* Opsin and CNG mRNA are co-

expressed in *Hydra* (Plachetzki *et al.* , 2010) and a CNG inhibitor ablates a specific photoresponse, which suggest a functional relationship between opsin and CNG ion channel in *Hydra*. Under the assumption that the phototransduction cascade is conserved among cnidarians, these findings indicate evidence for a Gs-protein, adenylate cyclase, cAMP, CNG phototransduction pathway in Cnidaria.

Overview of the project

The aim of this study was to investigate what factors modulate nematocyst firing. I addressed two main questions. The first one was whether light modulates nematocyst discharge in the sea anemone *Haliplanella luciae*. We predicted that animals under conditions of dim light fire more than those under conditions of bright light, and showed it was the case. We believed this is the first time that nematocyte photosensitivity has been described in Anthozoa. The second part of my project was inspired by the similarities between chemosensory and light perception. Previous knowledge on nematocyst firing modulation suggests that light decreases firing whereas the presence of chemosensitizer increases firing, both possibly acting through the same G-protein, AC, cAMP pathway. We wondered whether chemoreception and light perception interact together. To date, we believe they have a separate and opposite action on nematocyst firing, but we do not have enough evidence yet to determine whether Gi is involved, in addition to Gs.

MATERIALS AND METHODS

Experimental animals

Specimens of the sea anemone *Haliplanella luciae* were obtained from Glen Watson, University of Louisiana at Lafayette, LA, USA. I cultured the animals in glass dishes containing natural seawater held at room temperature (22°C ± 1°C). As the culture dishes were held in the lab, the animals were exposed to natural day light cycle. I fed the animals to repletion every three days with Selcon® enriched *Artemia* nauplii and changed the seawater at least three hours after feeding. I conducted all the experiments approximately 50 hours after feeding.

Assay of nematocysts capture

Under different light conditions

To investigate whether light influences the number of nematocysts captured on the test probe upon stimulation, I conducted the same assay under two different light conditions (bright light or dim light). The light source used was a blue LED (SuperBright LEDs) with a spectrum peak at 470nm. I measured the light intensity in Lux with a Smart Luxmeter (Milwaukee Instruments, SM 700). For each assay, animals were taken in their culture dish into a dark room and acclimatized for 4 hours to light (blue LED, 470 nm) of different intensities: less than 50 Lux for the dim light condition and more than 3500 Lux for the bright light condition. These illuminance levels correspond to those measured in a fully lighted street at night and on a sunny day, respectively. After the acclimation period, I touched the distal tip of a single tentacle of each animal using a gelatin-coated probe, to trigger nematocyst discharge. This probe consisted of a 10 cm 4lb fishing line segment, dipped three times into a 10% (w/v) gelatin mix (preheated to 70°C) and allowed to dry for 30 min before use. I used this gelatin concentration to match the protocol described in a previous study conducted on *Hydra* (Plachetzki *et al.*, unpublished). After probing, I mounted the probes on microscope slides with glycerol and counted the microbasic p-mastigophore nematocysts discharged into the gelatin under light microscopy (at 40X). I counted the number of nematocysts discharged into the whole probe, by searching the full length and the width of the probe by adjusting the focal plane of the microscope accordingly. Data for nematocyst counts excluded the other cnidocyst types (spirocysts and basitrich nematocysts) discharged into the probe. The temperature during each trial was recorded with a thermometer (Fisher Scientific Traceable RTD Platinum thermometer, accuracy ±0.1% plus 0.2°C) and varied from 21.78°C ± 0.80°C to 20.35°C ± 0.73°C throughout the experiments.

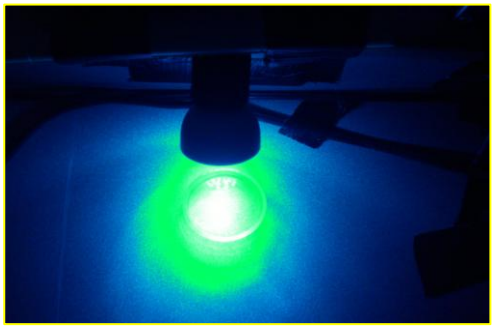

Fig. 1. Experimental setting. The blue LED was attached above the arena containing the animals. I adjusted the distance between the LED and the arena so that all the animals tested were subjected to the desired intensity. (i.e. less than 50 Lux for the dim light condition and more than 3500 Lux for the bright light condition.)

Under different light conditions and in the presence of sensitizer

To investigate whether light and sensitizer interact in the modulation of nematocyst discharge, I used a similar experimental setting but this time, I was adding the sensitizer before probing. The sensitizer used for this study was bovine submaxillary mucin (Sigma, Type I) at 10^{-7}M. This concentration has been shown to maximize nematocyst discharge upon stimulation (Watson and Hessinger, 1989). I prepared the mucin solution beforehand by dissolving 20 mg of mucin in 50 mL of filtered seawater. The solution was kept in the freezer between experiments and re-used twice. For each assay, I removed the animals from their culture dish and placed them into small glass arena filled with sea water (about 8 animals per arena). This step was to minimize the volume of mucin solution required in the experiments. The animals were then acclimated for 4 hours to different light intensities (blue LED, 470 nm), as previously described. I defrosted the mucin solution at room temperature just before use and preheated it to approximately 20 °C under running tap water, to prevent exposing the animals to temperature shock. Then, I replaced the seawater contained in the arena with the mucin solution by pipetting. After precisely 10 minutes, I probed the animals as previously described, and I recorded the number of microbasic p-mastigophore nematocysts captured on the probe.

Influence of physical disruption on the discharge level

In the experiment involving the use of mucin solution, the animals had to be placed in smaller arenas, in order to use as little solution as possible. The physical disruption of being detached from the culture dish surface and placed in a new arena might have some influence on the firing. Indeed, tearing sea anemone off their attachment point causes stress and therefore may affect the animal's reactivity. To test whether the transferring of the animals into the testing arenas had an effect on the nematocyst firing, I performed the first experiment again, but this time I removed the animals from their original dish and placed them in arenas before taking them to the dark room. Only the dim light condition was tested and the results obtained here were compared with the results obtained during the first trial.

Prey extract assay

To investigate whether the levels of discharge I measured here were comparable to those expected during prey capture, I conducted a prey extract assay. Animals were subjected to a light of intensity lower than 50 Lux for at least four hours. Then, I introduced a net containing living *Artemia* in the dish and one minute after, I probed the animals as previously described.

Speed of the light adaptation

The adaptation period of four hours was used to match a previous study (Plachetzki *et al.*, unpublished). To assess if such a long time is necessary or if the light adaptation occurs instantly, animals were taken from one light condition to the other and probed after different periods of time ranging from 1 minute to 10 minutes. For the first assay, I switched off the overhead lights (intensity around 400 Lux) and subjected the animals to a blue LED light of

an intensity lower than 50 Lux. I probed them as previously described after 1, 5 and 10 minutes. For the opposite assay, I exposed dim light adapted animals to a bright light (blue LED of an intensity higher than 3500 Lux) and probed them after 1, 5 and 10 minutes. Different animals were probed each time (i.e. an animal probed after one minute wasn't probed again during the same experiment).

Data processing

For each assay, I considered about half of the probes as not relevant for different reasons. Indeed, the firing was induced by the contact between the probe and the tentacle. But despite efforts to keep things as constant as possible, this contact was not exactly the same each time, depending on the animal reaction, its position in the dish, the light condition, etc... Thus, when the tested animal had wrapped its tentacle around the probe, or touched the probe with another tentacle, the probe was not considered (about 22% of the probes). In addition, for about 13 % of the probes, I could not distinguish all the nematocysts (the gelatin was too dry, there were too many algae in the seawater ...). It was then hard to make a reliable count. These probes were not considered. Finally, even though I thought the probe did touch the tentacle, about 17 % of probes did not display any nematocysts. I did not take these "zero" values into account as they are likely to be due to a "false touch", caused by low light levels making distance between probe and tentacle harder to assess. The percentages displayed above are average for all the experiments. These proportions varied from one assay to another but for all of them the proportion of probes considered was similar (between 43% and 53%). The remaining data were compiled and subjected to one-way ANOVA test, using Excel software. P values of less than 0.05 were considered significant.

RESULTS

Assay of nematocysts capture

Significantly more nematocysts are captured under conditions of dim light relative to bright light

When the animals had been subjected to bright light (≥ 3500 Lux), test probes had a mean of 8.3 ± 0.5 (mean ± SE) microbasic p-mastigophore nematocysts adhering to them, based on n = 33 replicate experiments. When the animals had been subjected to dim light (≤ 50 Lux), test probes had a mean of 17.4 ± 1.0 (mean ± SE) microbasic p-mastigophore nematocysts adhering to them, based on n= 40 replicate experiments. As seen in Fig.2, there is a statistically significant difference between the firing level in a bright light environment and in a dim light environment: $p = 2.\ 10^{-11}$ (one-way ANOVA).

Under the same light conditions, animals which have been scraped off their original dish before the experiment display a lower level of firing: the average number of nematocysts discharged onto the test probe under conditions of dim light was 12.9 ± 1.2 (mean ± SE), based on n= 22 replicate experiments. This average is significantly lower than the one displayed when animals have not been transferred prior to the experiment: p = 0.03, one-way ANOVA.

Both Mucin and dim light enhance the number of nematocysts captured

Under conditions of bright light, discharge of nematocysts onto the test probe was significantly higher in 10^{-7} M Mucin /sea water than in sea water alone,

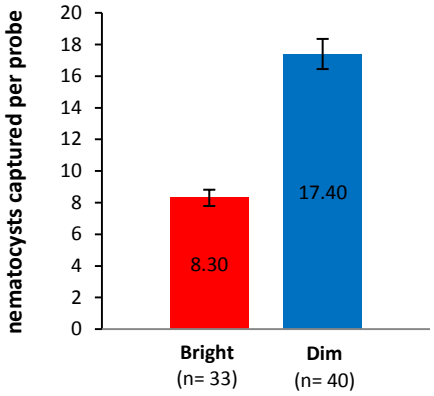

Fig. 2. Light intensity influence on the discharge level. The average number of nematocysts captured by the test probe is presented for two different light conditions: bright light (≥ 3500 Lux), referred as "bright" on the figure, and dim light (≤ 50 Lux), referred as "dim" on the figure. A one-way ANOVA reveals a highly significant difference: $p= 2.10^{-11}$.

with a mean of 15.6 ± 1.4 (mean ± SE) microbasic p-mastigophore nematocysts per probe, based on n= 29 replicate experiments (Fig.3). Under conditions of dim light, discharge of nematocysts onto the test probe was also significantly higher (p=0.03) in 10^{-7} M Mucin /sea water than in sea water alone, with a mean of 16.5 ± 1.0 (mean ± SE) microbasic p-mastigophore nematocysts per probe, based on n= 37 replicate experiments (Fig.3). Unlike what was observed in sea water alone, the discharge level did not differ significantly from one light condition to the other in the presence of 10^{-7} M Mucin: p = 0.588 (one-way ANOVA).

Prey extract assay

When a prey extract was introduced in the sea water prior to the probing, test probes had a mean of 14.2 ± 0.4 (mean ± SE) microbasic p-mastigophore nematocysts

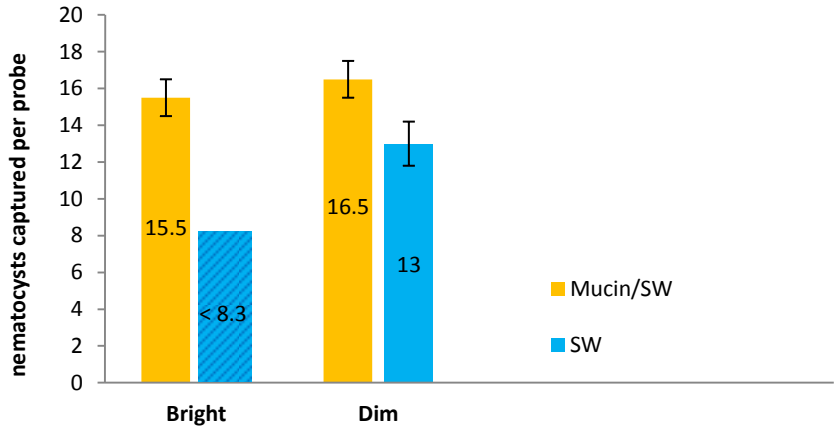

Fig. 3. Influence of light and mucin on the discharge level. The average number of nematocysts discharged onto the probe is presented for two different light conditions: bright light (≥ 3500 Lux) and dim light (≤ 50 Lux), and two different treatments: sea water alone (SW) and 10^{-7}M Mucin /sea water.
In sea water alone, only the dim light condition has been tested in these exact same experimental conditions, i.e. the animals were transferred before the light-adaptation period. Nevertheless, we observed that animals which have been scraped out display a lower level of discharge upon the same stimulation as non-transferred animals. We can thus reasonably infer that, in sea water alone under conditions of bright light, the average number nematocysts captured would be lower than 8.3 (average displayed by non-transferred animals under the same light conditions in sea water alone). The hatched rectangle represents this extrapolation.
In the presence of Mucin, the firing level did not differ significantly from one light condition to the other ($p = 0.588$; one-way ANOVA). Under conditions of dim light, discharge of nematocysts onto the test probe was significantly higher in 10^{-7} M Mucin /sea water than in sea water alone($p=0.03$).

adhering to them, under conditions of dim light, based on n = 13 replicate experiments. A greater number of trials would be required to assess whether this average significantly differs from the average number of nematocysts captured under the same light conditions, without prey extract ($p= 0.08$; one-way ANOVA). This would help us determine whether the levels of discharge I measured are comparable to the ones expected during prey capture.

Adaptation of the response to light intensity is not spontaneous

To determine whether the adaptation to a light condition was an instantaneous response, the light condition was changed and the animals were probed within one to two minutes of the change in light intensity. When the animals were dim light adapted (≤ 50 Lux) for four hours and then subjected to a bright light (≥ 3500 Lux) for one minute, the average number of nematocysts captured onto the test probe was 15.7 ± 0.4 (mean ± SE), based on n = 9 replicate experiments. For the opposite experiment (from a light of intensity around 400 Lux to an intensity < 50 Lux), the average number of nematocysts captured onto the test probe was 16.9 ± 0.6 (mean ± SE), based on n = 9 replicate experiments. However, this result should be warily considered as 400 Lux does not replicate the bright light condition used throughout this study, and we do not know yet how the discharge level changes within the two extreme intensities I tested. In fact, 400 Lux

is closer to the dim light condition I used than to the bright light condition, which is consistent with the high average number of nematocysts captured I observed. Both of these results display a significant difference with the firing level observed in a bright light environment ($p= 10^{-7}$ for both; one-way ANOVA). On the other hand, both are quite similar to the firing level observed in a dim light environment. The other results (i.e., when the animals were subjected to 5 or 10 min of light) are not presented here as the insufficient amount of data collected prevents us to draw any conclusion.

DISCUSSION

Different light levels modulate nematocyst firing rates

Light attenuates the firing in at least three of the four cnidarian classes

The main question in this study was whether light influences nematocyst firing in the sea anemone *Haliplanella luciae*. I have shown here that a bright light environment elicits a significantly lower propensity for nematocytes to be captured onto the test probe than a dim light environment. We assumed here and for the rest of this study that the number of nematocysts captured and counted on the probe is equal to the number of nematocysts fired. D. Plachetzki obtained the same result on *Hydra* (unpublished). C. Fong conducted some experiments on the Scyphozoan *Aurelia* and obtained indications that the same conclusion can be drawn for this species. Thus, light level modulates nematocyst firing for three of the four branches composing the Cnidarian phylogenetic tree: Scyphozoa, Hydrozoa and Anthozoa. Though no test was conducted on box jellyfish (class Cubozoa), the position of Anthozoa closest the beginning of the cnidarian phylogenetic tree allow us to infer that, for all cnidarians, the propensity for nematocysts to discharge is greater under conditions of dim light relative to bright light.

Biological explanation for nematocyte photosensitivity

Cnidarians feeding habits could explain this photosensitivity. Maximizing the efficiency of the discharge of energetically expensive nematocysts requires firing when a prey is most likely to be present. Now, Cnidarian diet is mainly composed of zooplankton (Ruppert and Barnes, 1994), described as being most active at dawn or dusk. Then, the dim light could be a signal to indicate the period when prey is likely to be most active.

A second hypothesis is that a prey, either swimming nearby or already caught by the tentacle, makes a localized shadow that predisposes the surrounding cells to discharge nematocysts. This implies that the adaptation to a change of light intensity occurs almost instantaneously. On the other hand, the results I obtained indicate that the adaptation to light lasts more than one minute. When dim light adapted animals are exposed to bright light for just one minute, the average number of nematocysts discharged is much higher than the one expected for bright light adapted animals. I did not get enough results to make strong conclusions, owing to time constraints. It is yet unlikely that the variance alone would explain the difference observed, and that with more replicates this average (15.7 nematocysts per probe) would become similar to the one expected under conditions of bright light (8.3 nematocysts per probe). Finally, we have not measured yet the size of the shadow created by an approaching prey, or how much it would change the light intensity. We can however presume that the prey must be no more than a few cm from the tentacle, or in direct contact, to create a significant shadow. In both cases, other clues such as activation of chemoreceptor or inter-nematocyte communication would be more efficient to sensitize surrounding nematocytes to discharge.

Another fairly different hypothesis is to consider the light influence on the firing as a side effect. As developed in the

introduction, chemosensation and light seem to signal through similar molecular pathways. If so, the light response could just be a by-product of the chemosensitivity that happens to trigger the same signaling cascade through a different sensor, but without any direct functional benefit. We do not have enough evidence yet to assess the likelihood of this hypothesis.

Light and sugar interaction in nematocysts firing modulation

Light and sugar have separate and opposing action

As developed in the introduction, N-acetylated sugars -including mucin- tend to increase the number of nematocysts fired, whereas light decreases firing. We hypothesized a separate contribution: when combined, each stimulus would have its own effect without interacting with the other. The results I obtained corroborate so far this idea. For the same conditions of light, mucin significantly increases the firing; and vice versa: in presence of mucin, bright light seems to decrease the firing relative to dim light.

Chemoresponse seems to prevail over light response

However, the difference in nematocyte firing between dim and bright trials with mucin present is less obvious than expected: the conditions of light seem not to have a strong influence on firing level when mucin is present in the medium. Indeed, I observed a highly significant difference between the two light conditions in sea water alone, whereas the average firing level is similar for both light conditions in mucin/sea water. This could be because I used the mucin concentration shown to maximize nematocyst discharge, while we do not know yet which light intensity induces the strongest response. Yet, the fact that mucin evened out such a difference in firing between dim and bright trials may suggest that it would still be the case, even if I had optimized the light intensity (i.e. conduct the experiment for the two light intensities respectively maximizing and minimizing the number of nematocysts discharged).

The response seems to reach a maximum

It is also very interesting to see that a firing maximum seems to appear. Indeed, mucin is reported to double the number of nematocysts fired (Watson and Hessinger, 1989) and we have seen that dim light has a similar effect relative to bright light. But we do not find the same ratio when the two stimuli are combined. Both stimuli increase the firing but they do not seem to add up. It suggests the existence of a maximal discharge limit: there is a maximum number of nematocysts that can be fired upon one stimulation. This is rather consistent with the fact that nematocysts are very expensive and poisonous so a small number only should be discharged in one prey, and not more than necessary.

Possible mechanistic relationship between light and chemosensation

At the organismal level, both N-acetylated sugars and light modulate the propensity for nematocysts to discharge. Now, from a molecular perspective, light and sugar also seem to signal through similar pathways, using many of the same messengers. We have designed three possibilities for the mechanistic

relationships between light and chemosensation, illustrated in Fig.4. There are two main unknowns: are there different G-proteins involved or do photosensory and chemosensory pathways share one? Second, are the two sensors (chemoreceptor and opsin) in the same cell or in different cells?

G protein

It is quite accepted now that modulation of intracellular cAMP levels plays a significant role in the regulation of nematocysts discharge. Both photosensory and chemosensory pathways use cAMP as a messenger, but light and mucin display opposing influence on the firing level. As different G-proteins lead to different effects on cAMP level, this may suggest that light and N-acetylated sugars do not signal through the same G-protein. Now, I found the highest firing level under conditions of dim light. There are two ways to look at this: either the light inhibits the firing, or the dim light stimulates the firing. The latter seems less likely as it is hard to consider the dim light, which is basically an absence of stimulus (photons), as an excitation signal. The light would thus reduce the level of intracellular cAMP, which suggests the activation of an inhibitory G-protein, in addition to the stimulatory G-protein activated by N-acetylated sugars.

This hypothesis is supported by the co-localization of Gi and opsin in *Hydra* sensory cells (Plachetzki, unpublished). Gi belongs to the same family as the vertebrate visual transducin, the G-protein involved in vertebrate phototransduction. Besides, Watson and Hessinger (1992) suggested the presence of two chemotransduction pathways: one involving Gi and one involving Gs protein. Thus, Gi seems to be a good candidate, though the involvement of Gs in box jellyfish phototransduction cascade first led us to assume otherwise. Nevertheless, the aforementioned findings are preliminary results and have not been investigated in details. Since there are more than one opsin, and that these opsins are

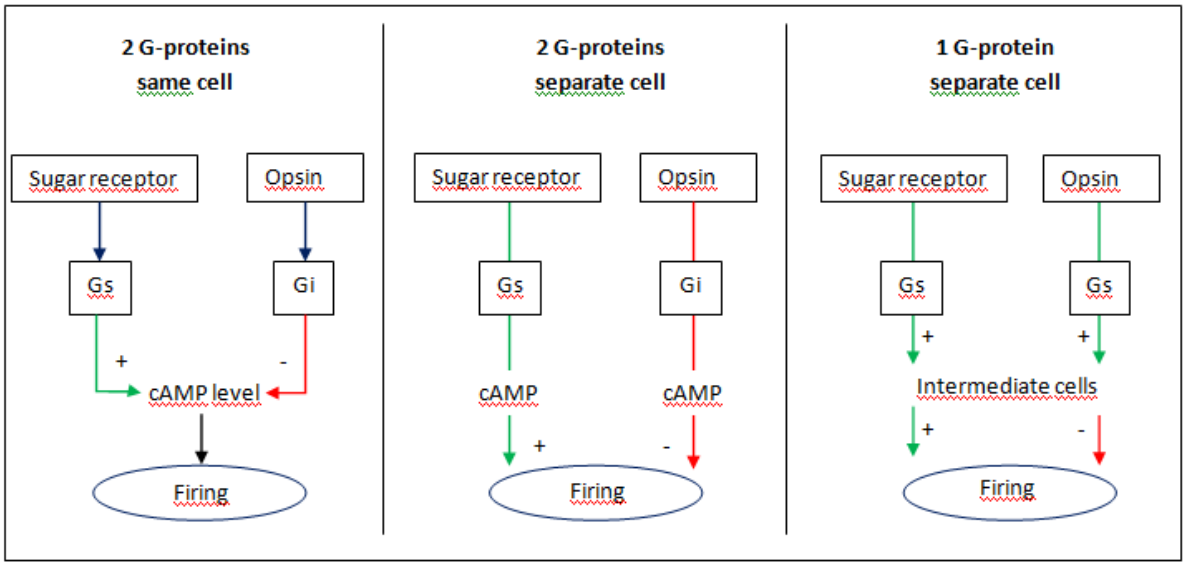

Fig.4. Possible molecular mechanism for the competitive influence of light and sugar on the discharge level.

There are two main questions: are there two G-proteins involved? And are the two sensors in the same cell? We do not have enough data yet to favor any of these hypotheses.

not expressed in the same location, it is also not impossible that different opsins use different G-proteins.

Involvement of intermediate cells

The involvement of intermediate cells would explain how the same signaling pathways lead to a different outcome. Chemoreceptor and photoreceptor would thus signal through the same G-protein but in different cells, and the information would be relayed by an interneuron-like cell to the nematocyte. As detailed in the introduction, the frequency-tuning of nematocysts discharge is achieved through a three cell neural pathway, involving sensory cells, ganglion cells and nematocyte cells. A similar neural organization could be involved in the light modulation of firing level. However, we do not have enough evidence yet to favor any of these possibilities; a combination of the above is not ruled out either.

Summary

In conclusion, I demonstrated that light decreases the propensity for nematocytes to discharge in *Haliplanella luciae*, and probably in all Cnidarians. Experimentally, I found a highly significant difference between the average number of nematocysts captured onto test probes under conditions of dim light relative to bright light. I also investigated the effect of light conditions, when combined with N-acetylated sugar Mucin, previously shown to sensitize nematocytes to discharge. I found that light and sugar seem to have separate and opposing effects on the propensity for nematocytes to discharge. There is preliminary evidence for a prevalence of the chemoreception over the light perception but further investigations should be conducted. Finally, our findings may suggest either the involvement of an inhibitory G-protein in the sea anemone phototransduction pathway, or the involvement of a stimulatory G-protein and multiple cells. While nematocyte chemo- and mechano-sensitivity have been extensively studied, we believe this study to be the first evidence for nematocyte sensitivity to light intensity.

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