DOCTORAL SCHOOL IN BIOLOGY

SECTION "BIOMOLECULAR AND CELLULAR SCIENCES"

XXXI CYCLE

"THE ROLE OF TRP RECEPTORS IN THE SIGNALLING PATHWAYS IN *HYDRA VULGARIS*"

"RUOLO DEI RECETTORI TRP NELLA MODULAZIONE DELLE VIE DI SEGNALAZIONE IN *HYDRA VULGARIS*"

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Abstract

Transient Receptor Potential (TRP) receptors represent a newly identified superfamily of cationic membrane channels that show a great diversity of activation mechanisms and selectivity. Their structure consists of six transmembrane domains that form a central pore, in addition to different amino acid and carboxylic domains that impart differential sensitivity to various sensory stimuli, including temperature, touch and smell, making them able to promote more biological functions (Clapham, 2003). In mammals, 33 different TRP channels have been identified, while in the marine invertebrates, nematodes and fruit flies, 30, 24 and 16 different TRP channels were respectively spotted. This family of receptors is indeed present in different vertebrate and invertebrate cells, but the way in which the TRP channels have evolved over the years remains largely unknown.

Previously, our studies have demonstrated the expression of the TRPM3 receptor (a member of the melastatin subfamily) in *Hydra vulgaris*, a small freshwater polyp belonging to the Phylum of Cnidaria, Hydrozoa class, at the base of the evolutionary chain. Specifically, we found that TRPM3 was able to mediate the heat-dependent induction of HSP70, NOS, Nrf2 and SOD expression, genes usually involved in signaling of noxious heat also in mammals (Malafoglia., et al 2016).

The major aim of the PhD research was to demonstrate a direct involvement of TRPA1 receptor (a member of the ankyrin subfamily) in the modulation of the thermal shock response (*i.e.*, cold shock) and the primary immune response in *Hydra vulgaris*. Regarding the cold shock, polyps were treated with harmful cold 4°C at different time points. The cold-induced TRPA1 activation caused the up-regulation of NOS, Nrf2 and SOD gene expression, as measured by RT-qPCR analysis. In particular, we observed a significant increase of mRNA expression in all genes, between 90 min and 12 h. The direct involvement of the TRPA1 receptor was verified by using a specific TRPA1 inducer (Glybenclamide G0639), and a potent and selective TRPA1 inhibitor (HC-030031). The optimal concentrations of drugs were selected after repeated dose-response experiments. Overall, these results indicate an involvement of the TRPA1 receptor in the activation pathway of thermal shock response.

It has been demonstrated that *Hydra* presents molecular models of innate immunity; Toll-like receptors (TLRs) are membrane receptors and Nod-like receptors (NLRs) are cytoplasmic sensors. The functional interaction of TLR and NLR mediated by a ligand induces the innate immune response, with the

production of known antimicrobial peptides, such as periculin and hydramacin (Augustin et al., 2010). Some studies have shown a connection between TRP receptors (*e.g.*, TRPA1) and the primary immune response in mammals (Chul-Kyu Park et al., 2014). Furthermore, some authors support the idea of a specific role for TRPA1 channels in detection and response to harmful bacterial and viral products (Meseguer et al., 2014).

Thus, we decided to test TRPA1 involvement in primary immune response pathways in *H. vulgaris*, by analysing mRNA over-expression of genes (*e.g.*, NF-κB, NOS, periculin and hydramacin), after treatment of polyps with cell lysates of *P. aeruginosa* PA14 for 24 h. Again, the TRPA1 antagonist HC-030031 reverted PA14-induced mRNA expression of the above-mentioned genes, thus suggesting that TRPA1 receptor plays a role in modulating the immune response.

In a parallel research, we decided to address an evolutionary aspect of aging. *Hydra* represents a unique animal model for the absence of cellular aging and senescence as well as for its high regenerative capacities, properties that may be related to the fact that these animals have mainly stem cells (Bode, 2003). Furthermore, *Hydra* has unique properties including apparent biological immortality. In this respect, a study on individual lifespan in *H. vulgaris* provides evidence that members of this species are not subject to senescence (Martínez, 1998). For continuous growth, a telomerase activity is required; telomerase activity is repressed in most human somatic cells, while its activity is higher in immortal cell lines, germ cells, stem cells, activated lymphocytes and most of the tumor cells.

Thus, we decided to verify the presence of telomerase activity in *H. vulgaris*, using the TRAP (Telomere repeat amplification protocol) assay. Specifically, specimens were repeatedly cut in two parts and the telomerase activity were analyzed after body regeneration. Interestingly, we found that the constant treatment of polyps with epigallocatechin gallate (EGCG), the most abundant catechin in green tea, induced a considerable reduction of telomerase activity when compared with untreated polyps. Note that after two weeks from cut, a decrease in regenerative rate was also observed in samples treated with EGCG with respect to untreated specimens, thus suggesting a possible correlation between reduced telomerase activity and the induction of senescence in *H. vulgaris*.

However, this hypothesis, although intriguing, remains to be further investigated.

Sintesi

I recettori Transient Receptor Potential (TRP) costituiscono una superfamiglia recentemente identificata di canali di membrana cationici che mostrano una grande diversità di meccanismi di attivazione e selettività. La loro struttura consiste di sei domini transmembrana che formano un poro centrale, oltre a diversi domini amminoacidici e carbossilici che impartiscono sensibilità differenziale a vari stimoli sensoriali, tra cui temperatura, tatto e olfatto, che li rendono in grado di promuovere più funzioni biologiche (Clapham, 2003). Nei mammiferi sono stati identificati 33 diversi tipi di TRP, mentre negli invertebrati marini, nematodi e moscerini della frutta ne sono stati identificati rispettivamente, 30, 24 e 16. Questa famiglia di recettori è infatti presente in diverse cellule di vertebrati e invertebrati, ma il modo in cui si sono evoluti rimane in gran parte sconosciuto. Studi precedentemente condotti nel nostro laboratorio hanno evidenziato l'espressione di TRPM3 (un membro della sottofamiglia dei TRP melastatinici) in *Hydra vulgaris*, un piccolo polipo di acqua dolce appartenente al Phylum degli Cnidari, classe Hydrozoi, alla base della scala evolutiva. Nello specifico, abbiamo osservato come TRPM3 sia coinvolto nell'induzione dopo shock termico (34°C) dell'espressione di HSP70, NOS, Nrf2 e SOD, geni usualmente coinvolti nelle vie di segnalazione durante shoch termico doloroso nei mammiferi (Malafoglia., et al 2016).

Gli esperimenti condotti durante il percorso di dottorato hanno avuto come obiettivo primario quello di dimostrare un'associazione diretta del recettore TRPA1 (un membro della sottofamiglia dei TRP ankirinici) nella modulazione dei pathways coinvolti nella risposta allo shock termico (in particolare al freddo) e nella risposta immunitaria primaria in *Hydra vulgaris*. Per quanto riguarda lo shock termico, i polipi sono stati sottoposti a 4°C per differenti tempi. L'attivazione del recettore in seguito allo stimolo freddo ha provocato la modulazione dell'espressione dei geni NOS, Nrf2 e SOD, come verificato dall'analisi della RT-qPCR. In particolare, abbiamo osservato un incremento significativo di tutti i geni tra 90 minuti e 12 ore. Per valutare il coinvolgimento diretto del recettore TRPA1 è stato utilizzato l'agonista specifico Glybenclamide G0639, come controllo positivo, e l'antagonista HC-030031 (Babes et al., 2013). Le concentrazioni ottimali sono state individuate in seguito a ripetuti esperimenti dose/risposta. In particolare, l'aumentata espressione genica indotta da freddo o dalla Glybenclamide è stata riportata ai livelli di controllo in seguito al trattamento con l'antagonista selettivo di TRPA1, HC-030031. Nell'insieme questi risultati indicano un

coinvolgimento del recettore TRPA1 nella via di attivazione della risposta allo shock termico.
Recentemente, è stato dimostrato che *Hydra* presenta dei modelli molecolari di immunità innata; i recettori Toll-like (TLR) sono recettori di membrana e i recettori Nod-like (NLR) sono sensori citoplasmatici. L'interazione funzionale di TLR e NLR mediata da un ligando, induce la risposta immunitaria innata, con la produzione di peptidi antimicrobici, come ad esempio periculina e hydramicina (Augustin et al., 2010). Alcuni studi hanno dimostrato una connessione tra i recettori TRP, in particolare il TRPA1, e la risposta immunitaria primaria (Chul-Kyu Park et al., 2014). Altri autori supportano l'idea di un ruolo specifico per i canali TRPA1 nel rilevamento e nella risposta a prodotti batterici e virali (Meseguer et al., 2014).
Per queste ragioni, abbiamo voluto studiare il coinvolgimento del recettore TRPA1 nella modulazione della risposta immunitaria in *Hydra vulgaris*, analizzando l'espressione di NF-κB e dei geni NF-κB-dipendenti come ad esempio NOS, periculina e hydramacina, dopo trattamento delle idre con lisati cellulari di *P. aeruginosa* PA14 per 24 h. Anche in questo caso, l'antagonista HC-030031 è stato in grado di inibire l'espressione dei suddetti geni, suggerendo come il TRPA1 giochi un ruolo importante anche in questo meccanismo.
Parallelamente al precedente studio, abbiamo affrontato un aspetto evolutivo dell'invecchiamento e della senescenza cellulare.
L'*Hydra* rappresenta un modello sperimentale unico per l'assenza di senescenza cellulare e invecchiamento e per la sua elevata capacità di rigenerazione, proprietà legata al fatto che questi animali posseggono prevalentemente cellule staminali. Inoltre, l'*Hydra* possiede proprietà uniche tra cui un'apparente immortalità biologica. Uno studio sulla durata della vita individuale in *H. vulgaris* ha mostrato che membri di questa specie non sono soggetti a senescenza (Martínez, 1998). È noto che per una continua crescita, è richiesta l'attività della telomerasi per mantenere costante la lunghezza dei telomeri. Infatti, l'attività telomerasica è repressa in molte cellule somatiche umane, mentre è elevata in cellule germinali, staminali, in cellule immortali e in molte cellule tumorali.
Cosi, abbiamo voluto verificare la presenza di attività telomerasica in *Hydra vulgaris*, utilizzando il saggio TRAP (Telomere repeat amplification protocol). In particolare, gli animali sono stati ripetutamente tagliati e dopo la rigenerazione è stata evidenziata una intensa attività telomerasica. A questo proposito, abbiamo osservato che trattando le idre, precedentemente tagliate,

con epigallocatechina gallato (EGCG), la catechina più abbondante nel tè verde e potente inibitore della telomerasi, l'attività telomerasica era fortemente ridotta. È importante sottolineare che dopo due settimane dal taglio, è stato osservato un rallentamento della capacità rigenerativa delle idre trattate con EGCG, suggerendo una possibile associazione tra una ridotta attività telomerasica e l'induzione della senescenza nell'*Hydra*.
Ovviamente, questa intrigante ipotesi necessità di essere ulteriormente verificata e confermata.

Introduction

1.1 Transient Receptor Potential overview

Transient Receptor Potential (TRP) receptors represent a great group of membrane channels, capable of inducing multiple biological functions. Recently, several research areas including physiology, pharmacology and toxicology, have focused their attention on this channel family. TRPs are able to respond to a wide variety of stimuli and to form complexes with multiple proteins involved in different cellular processes (Clapham, 2003).

1.1.1 TRP family

TRP receptors have developed a large and functionally versatile family of cation-conducting channel proteins, expressed in different types of cells and tissue of both vertebrates and invertebrates, where they conduct a wide range of functions in the body, ranging from intracellular calcium homeostasis to signal transduction (Clapham, 2003; Moran et al., 2004; Montell, 2005; Voets et al., 2005; Nilius, 2007; Nilius et al., 2007). Several TRP channels are vital polymodal cellular sensors (*i.e.*, thermo, chemo, and mechanosensors) in both excitable and non-excitable cells (Ramsey et al., 2006; Colburn et al., 2007; Kim and Baraniuk, 2007; Venkatachalam and Montell, 2007; Rosenzweig et al., 2008; Mizuno, 2008). Cumulative reports demonstrate the role of TRP channels in the sensing of wide diversity of acute noxious physical, chemical and mechanical stimuli which render into nociceptive, neuropathic, and psychological pain (Levine and Alessandri, 2007) or in the perception of sensations, such as heat, cold, pain, touch, taste, smell and vision (Clapham, 2003; Voets et al., 2005).

A decisive first step in the discovery of these receptors can be traced back to the 1960s, when it was discovered that a mutant of Drosophila melanogaster showed a transient response to prolonged intense light (Huang et al., 2006).

Approximately, seventy TRP channels have been identified so far in both vertebrates and invertebrates. In the mammals, 33 different TRP channels have been identified, whereas in marine invertebrate animals, nematodes and fruit flies, 30, 24 and 16 different TRP channels have been identified, respectively (Figure 1.8). On the basis of amino acid homology sequence, the mammalian members of this family have been classified into 7 subfamilies. The TRPC (canonica) and TRPM (melastatin) subfamilies are composed of seven and eight different channels, respectively (*i.e.*, TRPC1-7 and TRPM1-8). The TRPV (vanilloid) subfamily currently comprises six members (TRPV1-6). The most recently identified subfamily, TRPA (ankyrin), has only one mammalian member (TRPA1). The TRPP (polycystin) and TRPML

(mucolipin) families, each containing three mammalian members, are relatively poorly characterized, but are attracting increasing interest due to their involvement in different human diseases. The TRPN subfamily (NOMP, No-mechanopotential) in the sensory neurons that support hearing in *Drosophila* and zebrafish (*Danio rerio*) has been detected only in worms, *Drosophila* and zebrafish and is likely to be a sensing channel mechanostimuli (Sidi et al., 2003; Walker et al., 2000). The currently available genome information indicates that mammals do not have TRPN orthologues (Nilius et al., 2007).

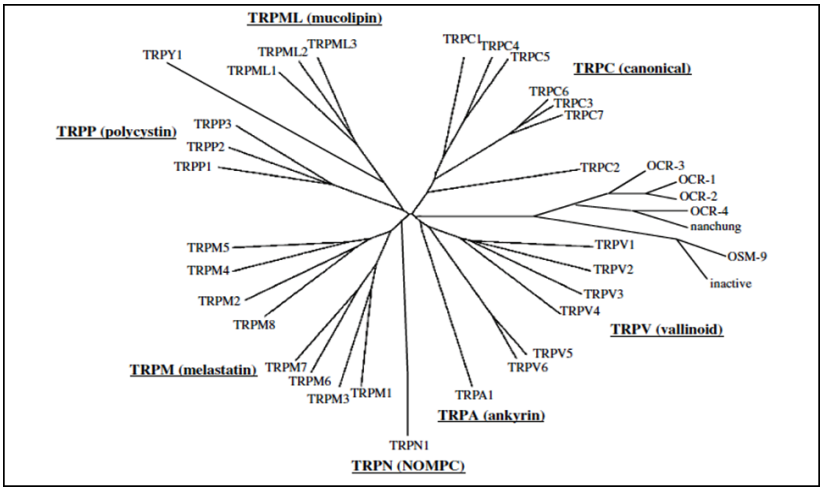

Figure 1.1. Unrooted phylogenetic tree generated by aligning the transmembrane domains of the TRP channels. The seven main branches are denoted with underline, the letters and numbers following TRP indicate TRP subfamily and member, respectively (Yin *et al*., 2010).

1.1.2 **TRP channel structure in Mammals**

Mainly located on the plasma membrane, the TRP mediate the transmembrane cation flow according to their electrochemical gradient, determining an intracellular increase of calcium/sodium ions. Sodium ion is involved in the formation of membrane potentials, while calcium ion is recognized as an activator of calcium-dependent mechanisms that control cellular events, such as regulation of gene transcription, cell proliferation and the induction of inflammatory mechanisms.

In general, each TRP channel subunit consists of six common transmembrane segments (S1-6), implanted with a pore-forming loop between S5 and S6. The assembly of channel subunits like a homo- or heterotetramers results in the formation of cation-selective channels (Figure 1.3). The terms intracellularly located cytoplasmic, amino (N) and carboxyl (C) vary considerably in length and amino acid sequence (Hoenderop et al., 2003) (Figure 1.2). These cytoplasmic regions contain various well-recognized domains and patterns that are likely involved in the assembly, activation and regulation of the channel through protein-protein and/or protein-ligand interactions (Minghui et al., 2015). All TRP channels are non-selective, with the exception of some being highly selective for Ca^{2+} or Mg^{2+}, like the monovalent-selective TRPM4 and TRPM5, and the Ca^{2+}-selective TRPV5 and TRPV6 (Venkatachalam et al., 2007). Cations are selected for permeation by the extracellular-facing pore loop, held in place by the S5 and S6 a-helices. The cytoplasmic ends of the S6 helices form the lower gate, which opens and closes to regulate cation entry into the channel. The S1–S4 domain may flex relative to S5–S6 in response to stimuli. All the elements outside the S5–S6 region provide means of either subunit association or act as linkers to elements that control gating (Clapham, 2003). The selectivity zone is formed by amino acids that dip into the bilayer (pore loops), one contributed from each of the four subunits. Depending on the TRP family, the N-terminus contains between zero and eight ankyrin repeats, a predicted coiled coil region, and a putative caveolin-binding domain. The C-terminus comprises a TRP signature motif (EWKFAR), a proline-rich motif, the calmodulin/inositol 1,4,5-trisphosphate (IP3) receptor-binding (CIRB) domain, and a predicted coiled coil region (Yin et al., 2010; Montell, 2001; Dohke et al., 2004) (Figure 1.3).

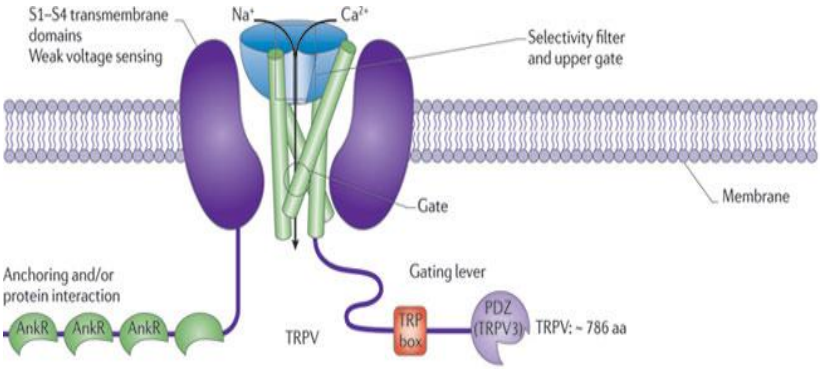

Figure 1.2. TRP channel architecture (Moran *et al*., 2011).

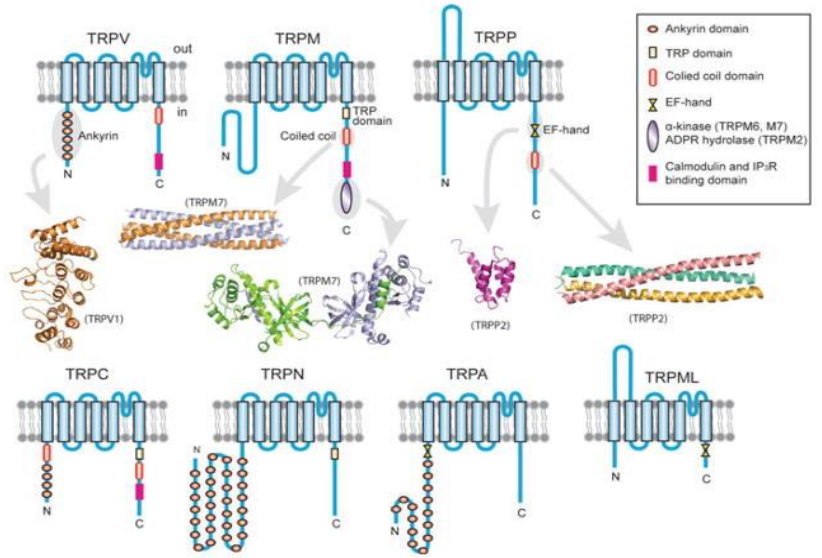

Figure 1.3. TRP channel subfamilies and the transmembrane topology and domain organization of their subunits (Minghui et al., 2015).

As previously mentioned, TRP channels are widely distributed throughout the body, present several splicing variants and they are expressed in a large

number of different cells where they assolve to a multitude of functions. While some TRP channels have been detected in neurons of the central nervous system, their expression is particularly abundant in the cells of sensory receptors, including cell-mediated vision, pheromone sensation and thermal sensation, taste, touch and cell volume regulation. The preferential distribution of TRP channels in sensory organs alludes to their critical and diversified role in signal sensation and transduction (Sukharev et al., 2004; Kung, 2005).

The activation and regulation mechanisms of the TRP channels are largely unknown and very different, based on their diversity of amino acid sequences. The activity of the channel is influenced by different physical parameters such as osmolarity, pH, mechanical resistance and biochemical interactions with external ligands or cell proteins. Sensitivity to polymodal activation suggests that the physiologically relevant stimulus for any given TRP will be governed by the specifics of cellular context (*i.e.*, phosphorylation status, lipid environment, interacting proteins, and concentrations of relevant ligands). Furthermore, cooperativity intrinsic to TRP channels may result in allosteric coupling of distinct activation stimuli, blurring the definition of activator versus modulator. However, it is possible to divide into three macro-categories the activation modalities established for the expressed TRP:

1. Direct activation. Changes in the environmental temperature are strongly coupled with the opening of TRPV1-TRPV3 and TRPM8 by little known mechanisms. Other putative direct activators include mechanical stimuli, conformational coupling to IP3 receptors, and channel phosphorylation. Cell warming and swelling may also act indirectly to activate TRP channels through second messengers or other unidentified mechanisms.
2. Activation of the receptor. G protein-coupled receptors (GPCR) and tyrosine kinase receptor activating phospholipases C (PLC) can modulate the activity of the TRP channel in at least three ways: (a) hydrolysis of phosphatidylinositol bisphosphate (PIP_2), (b) production of diacylglycerol (DAG) or (c) production of inositol trisphosphate (IP_3) and subsequent release of Ca^{2+} from intracellular reserves.
3. Activation by the ligand. The ligands that activate the TRP channels can generally be classified as (a) small exogenous organic molecules, including synthetic compounds and natural products (capsaicin, icilin); (b) endogenous lipids or lipid metabolism products (diacylglycerols, phosphoinositides, eicosanoids, anandamide); (c) purine nucleotides and their metabolites [adenosine diphosphoribose (ADP-ribose), βNAD^+]; or (d) inorganic ions, with Ca^{2+} and Mg^{2+} which are more likely to have physiological relevance. Although some TRP channels clearly function as chemosensors for

exogenous ligands (ie activation of the capsaicin of TRPV1), there are relatively few endogenous chemical ligands with the ability to activate TRP channels from the aqueous extracellular environment (2-AG, anandamide) (Ramsey et al., 2006).

1.1.3 TRP receptors and chemosensation

A wide variety of natural products derived from plants and other chemical agents evoke sensory responses with an infinite nuance of perceptual qualities. The perception of chemical stimuli with sensory means is referred to chemosensation or chemoreception. These sensory functions involve the activation of nociceptor and thermoreceptor endings and have a protective or defensive function, as many of these substances can be irritants or poisonous. However, the perception of chemical agents, like a perfume or the smell of a fruit, sometimes is strongly linked to memory and very often can evoke pleasant sensations. In fact, in time, humans have developed a liking for the distinct sharpness or pungency of many foods, beverages, and spices following activation of the same sensory afferents. In humans, the olfactory and gustatory systems are the main chemosensory systems and substrates for the sense of smell and taste, respectively (Fain, 2003). Albeit less well recognized, the trigeminal somatosensory system plays a fundamental role in chemosensation and the overall "flavor" of foods. Sensory endings of the trigeminal (V cranial) nerve innervate the skin covering the face, the mucous membranes of the nasal and oral cavities, and the cornea and conjunctiva of the eye (Figure 1.4).

Temperature and many chemical agents can stimulate chemosensitive channels directly. These channels are expressed in sensory nerve terminals and mucosal epithelial cells or skin keratinocytes. The opening of cationic channels (TRPs, ASICs) or the closing of potassium-selective channels (KCNK) generate a graded transduction current, depolarization, and the firing of action potentials. Voltage gated sodium (NaV), calcium (CaV), and potassium (KV) channels participate in action potential electrogenesis and propagation of the nerve impulse to the brainstem. Mechanical deformation of the skin can trigger the release of ATP from keratinocytes and the activation of purinergic (P2X) receptors from sensory nerve terminals (Viana, 2010).

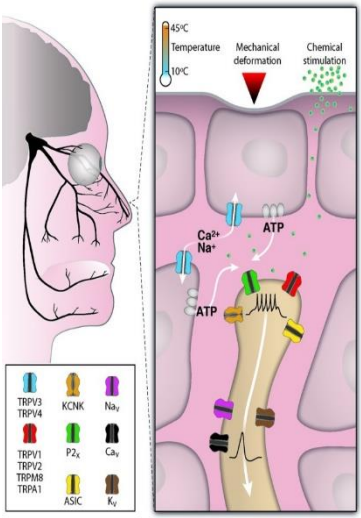

Figure 1.4. Molecular determinants of chemosensation in trigeminal nerve terminals (Viana, 2010).

These endings can be activated by physical stimuli (mechanical forces and temperature) and by a huge array of chemical agents (Bryant et al., 2000), and evoke sensations of touch, temperature, and pain. The capacity of the skin, including trigeminal endings, to detect chemicals is known as chemesthesis or cutaneous chemosensation (Green, 1996). Oral chemesthesis explains the pungent or sharp feel of many different foods and spices such as chili peppers, horseradish, wasabi roots, and Szechuan pepper, the coolness of peppermint, the tingle of carbonated drinks, and the irritation produced by substances such as nicotine or raw garlic extracts. Protective responses evoked by trigeminal stimulation include salivation, tearing, coughing, respiratory depression, sneezing, irritation and burning pain. Many chemical agents sensitize the perception of temperature (Green, 1985; Schafer et al., 1986). Menthol, for examples, sensitizes responses of trigeminal endings to cold temperature, while capsaicin sensitizes responses to warm temperatures. These interactions are easily explained by the allosteric gating of TRP channels by chemical and thermal stimuli. Thermosensory channels, also named “thermoTRPs”, define a subfamily of the TRP channels that are activated by changes in the environmental temperature, from noxious cold

(<15°C) to injurious heat (>42°C) (Figure 1.5) (Patapoutian et al., 2003; Viana, 2010; Ferrandiz-Huertas et al., 2014).

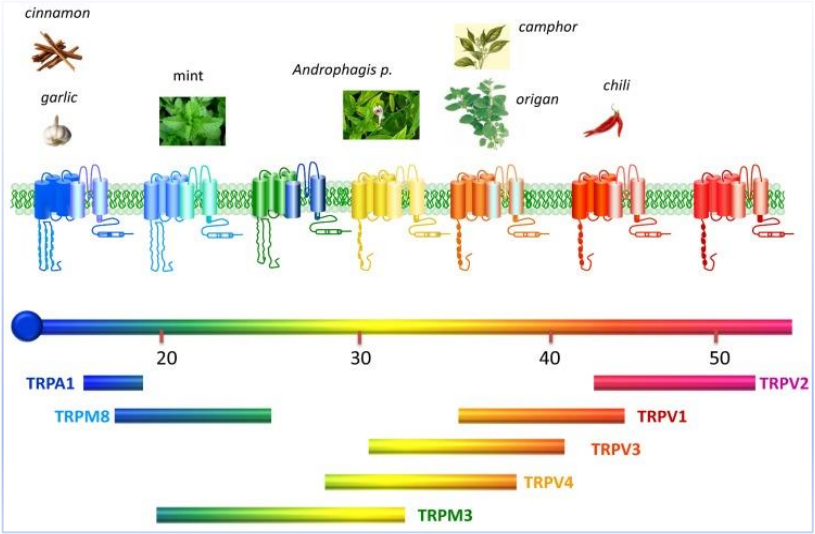

Figure 1.5. Thermotransient receptor potential (TRP) channels. ThermoTRPs display distinct thermal thresholds from very noxious cold (TRPA1) to harmful hot (TRPV2). Each thermoTRP is also activated by specific natural or synthetic compounds, known to induce the relevant thermal and pain sensations in humans (Ferrandiz-Huertas et al., 2014)

1.1.4 Evolution of TRP channel in different animal species

We have so far made considerations about the TRP receptors in mammals, but studies of the functions of the TRP channels have been mainly characterized in the model organisms within a limited evolutionary context. This family of receptors is present in different cells of both vertebrates and invertebrates, but the way in which TRP channels have evolved over the years remains largely unknown. Recent studies led to a characterization of the TRP channels in Choanoflagellate, Sponges, Cnidaria, Lophotrochozoa and Arthropods to understand how they emerged during the first evolution of the animals and how they changed during the diversification of various species (Peng et al., 2014). In particular, five members of the subfamily of TRP metazoans (TRPA, TRPC, TRPM, TRPML and TRPV) were identified in choanoflagellates, demonstrating that they evolved before the emergence of multicellular animals. The TRPN was identified in *Hydra magnipapillata*,

and then emerged in the last common ancestor of Cnidaria-Bilateria. A new member of the subfamily (TRPVL) was identified in Cnidaria and Capitella teleta, indicating that it was present in the last common ancestor of Cnidaria-Bilateria, but has since been lost in most bilateral. The characterization of the TRP channels of arthropods revealed that Daphnia pulex and insects specifically expanded the subfamily TRPA, which diverged from the ancient gene of the TRPA1 channel. The diversity of TRPA channels, with the exception of TRPA1, was also detectable within a single insect family. The study demonstrates the evolutionary history of the genes of the TRP channel, which can be divergent in concomitance with specific habitats and life histories of the individual species (Peng et al., 2014).

1.1.4.1 TRP channel in Cnidaria

Phylum Cnidaria has about 9,000 species of organisms that live in aquatic environments (mainly marine). The phylum Cnidaria is an early branching of the evolutionary line of the metazoans and whose diversification took place over 500 million years ago and the subdivision of the same into five classes: Anthozoa, Hydrozoa, Cubozoa, Scyphozoa and Staurozoa (Cartwright et al., 2007). In more recent years, the interest in the use of cnidarians and other basal metazoa, as model organisms, has grown particularly following the sequencing of the genome of two species model; *Hydra magnipapillata* and *Nematostella vectensis*, with the aim of understanding the evolution of Bilateria. *N. vectensis* (sea anemone) and *H. magnipapillata* are two cnidarian species that diverged at least 540 million year ago, and have distinct body structures, habitats, and genome sequences. The genome apparently evolved more slowly in *N. vectensis* than in *H. magnipapillata*, due to the existence of the common ancestor of Cnidaria and Bilatio (Putnam et al., 2007; Chapman et al., 2010). *N. vectensis* shows 21 TRP channels, with the following subfamilies: two TRPs, three TRPs, three TRPs, two TRPMLs, eight TRPPs, two TRPVs, and one novel subfamily, TRPVL.

H. magnipapillata contained at least 34 TRP channels divided as follows: four TRPA, three TRPM, two TRPML, one TRPN, 14 TRPP, and five TRPVL. *H. magnipapillata* may also have five additional TRP channels; however, these were not phylogenetically characterized because their six transmembrane segments could not be completely annotated (Peng et al., 2014). Based on the presence of ARs and a canonical TRP domain, one of the five channels could be classified as a TRPC channel. Thus, *H. magnipapillata* likely has all TRP subfamily members except for TRPV. All *N. vectensis* and *H. magnipapillata* TRPA channels were clustered with mouse and D. melanogaster TRPA1 channels and were separated from other

D. melanogaster TRPA channels, demonstrating that they were derived from the ancient TRPA1 channel. *H. magnipapillata* shows a TRPN channel, suggesting this subfamily first emerged in the common ancestor of Cnidaria and Bilateral, although it has been found in many species including *N. vectensis* and mammals during evolution (Srivastava et al., 2010). It remains evident that cnidarians was characterized by most of the members of the TRP subfamily features compared to other animals analyzed in this study.

Despite the simplicity of the cnidarian nervous system (Technau & Steele, 2011), TRP channel genes have undergone a unique evolution in these organisms, in marked contrast to bilaterians. Understanding the physiological functions of these channels in the context of the life histories and habitats of cnidarians would be of significant interest.

1.2 *Hydra vulgaris*

Hydra is a small freshwater polyp belonging to the Phylum of the Cnidarians and to the Hydrozoi class, the order of the Hydroids, genus Hydra. Scrolling through the characteristics of this phylum, the Cnidarians are the simplest among the "true" metazoi (= Eumetazoi) or Metazoi in the strict sense: they lack true organs, a head and a true center of nervous coordination. It lives in fresh water and has a cosmopolitan distribution. It is considered the first model animal used in developmental biology (Tremble, 1744: Galliot, 2012). It reproduces mainly asexually by lateral budding of the polyps, but also has a sexual cycle. Polyps could be either hermaphrodites or with separate sexes, depending on the strain of the species. They can produce a new gem in 3-4 days. The conditions that induce the formation of gametes are not entirely clear, but in some species they involve temperature (for example *H. oligactis*) or fasting (*H. vulgaris*). In *Hydra* there is no larval stage between embryo and polyp. The embryo completes the development within a cuticle from which a fully formed polyp develops after a dormancy phase of weeks or months (Bottger et al., 2006). Their body is a sort of bag whose only cavity has the function of a bowel (celenteron), from which the term coelenterates is often used as an alternative to cnidarians. It is a sui generis gut because digestion is carried out within the cells that is made up, rather than in the cavity. Furthermore, there is only one "mouth", which is the only way to enter food and expel waste. The name of the phylum derives from the stinging (sticking) structures of which they are equipped, called precisely cnidocytes.
The main models of *Hydra* are *H. vulgaris* and *H. magnipapillata*. However, they are unlikely to be separate species; *H. magnipapillata* probably should be renamed *H. vulgaris* (Martinezet al., 2010). Other species are *H. littoralis*, *H. oligactis* and *H. japonica*. Belonging to different genera, *Chlorohydra viridissima*, which has a symbiosis with unicellular green algae called zoochlorella, and *Hydractinia*, which has a larval stage.

1.2.1 Morphological and biological aspects of the *Hydra*

From a morphological point of view, *Hydra* presents a cylindrical body about 5 mm long. The animal exhibits a radial symmetry. The basal side consists of a differentiated pedal disk that mediates an attachment to substrates. The apex, presents a crown of 6-10 tentacles rich in stinging cells, between which it extends into a short conical portion called hypostome. At the center of the hypostome there is an opening, the mouth, which is used both for the entry of nutrients and for the exit of waste substances (Figure 1.6A).
The body wall consists consists of two cell layers, endoderm and ectoderm which sandwich an acellular extra-cellular matrix layer named mesoglea. The

endodermal layer is made of endodermal, myoepithelial cells, gland cells and few nerve cells, whereas the ectodermal layer contains ectodermal myoepithelial cells, interstitial stem cells and their derivatives (nerve cells, nematoblasts, nematocytes) (Figure 1.6B).
Mesoglea, which in the hydra represents 2% of the weight of the body, consists of an optically transparent gelatinous matrix, devoid of structure, and plays a supporting role.

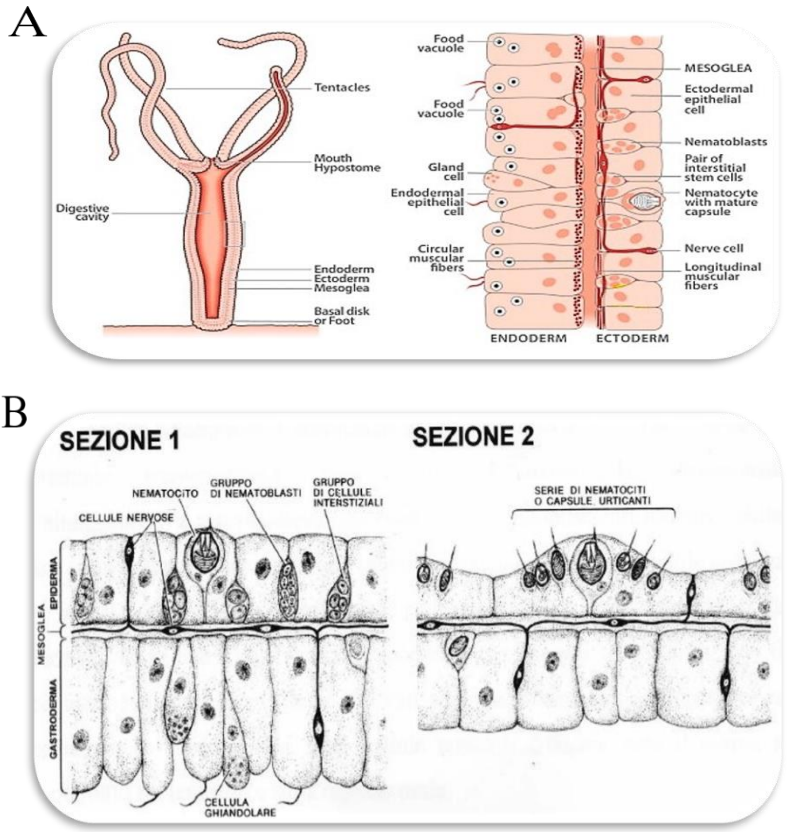

Figure 1.6. A. Anatomy of the cnidarian freshwater *Hydra* polyp. **B.** Section 1; cross-section of the hydra gastric region. Section 2; tentacle cross section (Wenger et al., 2014).

The nervous system is very simple but complete, composed of a nervous network that extends along the entire body (Figure 1.7). Nerve cells are bipolar or multipolar neurons. Neuronal bodies are found mainly near the basal sides of both tissue layers, while they emit their extensions between the epithelial cells; they form two nerve plexuses, one epidermal and one gastrodermal.

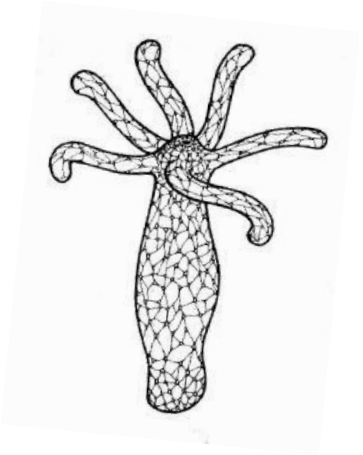

Figure 1.7. Nervous network of in *Hydra*. It is more concentrated around the mouth and the base (Spencer et al., 1982).

The density of neurons varies and is greater in the head and foot than in the rest of the body (Figure 1.7). A hint of centralized nervous system consists of the perioral ring at the base of the hypostome in the ectoderm. There are both electric synapses, in which the passage of the impulse takes place through communicating junctions or gaps, and chemical, in which the impulse is transmitted through the release of neurotransmitter substances. The presence of this latter type of synapse was demonstrated by electron microscopy observation of vesicles containing neurotransmitters (Westfall et al., 1980; Spencer et al., 1982). Among the neurotransmitters described above are dopamine, serotonin and noradrenaline (Venturini et al., 1984), as well as various neuropeptides.
The sensory cells are designed to perceive mechanical, chemical and perhaps bright stimuli and are numerous throughout the ectoderm, but particularly on the tentacles and in the region surrounding the mouth; they are also present in the gastroderma, but to a lesser extent. The sensory elements are nothing

more than nerve cells functioning also by receptors: they are mechanoreceptors equipped with a sort of scourge (not equipped with movement) that protrudes on the epidermal surface.
The musculature consists mostly of myo-epithelial cells resting on the central mesoglea. They have a flat base, prolonged in various directions and contain few muscle fibrils; these fibrils are oriented longitudinally in the epidermal cells and then contracting determine the shortening and dilatation of the body, while they are transversely oriented in the gastrodermal cells determining the lengthening and narrowing of the body. The muscular fibrils form, therefore, two homogeneous contractile layers that constitute a continuous contractile network. Within the epidermis, interstitial cells are found that represent a source of undifferentiated cells, potentially capable of producing other cell types including nerve cells, nematocytes and cnidocytes. The latter are stinging cells spread all over the body but more concentrated on the tentacles and the oral region, from which the name of the phylum derives. The cnidocytes are present in a variety of models in hydra (Figure 1.8A), and have allowed the representatives of this phylum to become efficient predators despite being poorly mobile. In addition to the prey capture function, they are used in defense, for locomotion and for adhesion to the substrate.

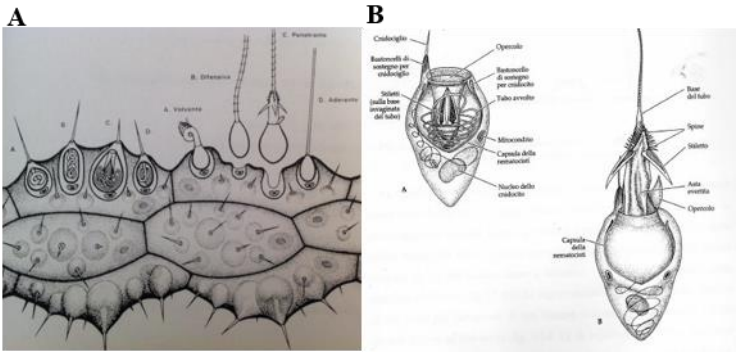

Figure 1.8. A) The four types of nematocysts present in *Hydra* are shown in the diagram of a small tentacle portion. B) Nematocysts. a) Before the discharge. b) After the discharge.

Each cnidocyte is produced from cells called cnidoblasts that develop from interstitial cells. When the cnidoblasts are completely formed, they have a cnidocyst inside them, a capsule with a diameter of 10-20 μm with a round or cylindrical shape. This urticating capsule is folded inside, at one end, and

is prolonged like a long hollow tube wound on itself, which refers to the poisonous liquid contained in the cyst itself. The capsule is covered by an operculum that opens snap when the cnide discharges; in the cell there is a bundle of contractile fibrils (Figure 1.8B). The urticating cell has, on the external side, a cnidociglio which, when stimulated by an animal, carries out the action of inducing an increase in pressure in the urticating capsule, which "shoots" the tubule outside, completely extracting it with a glove finger and determining the release of the poisonous liquid in the animal.

The cnidocyst can be considered as a sort of vesicle full of toxic liquid whose apical end, as already mentioned, is prolonged like a thin, long and hollow filament, variously armed with thorns and stilettos; the filament, due to the contraction of the fibrils or by osmotic phenomena, is suddenly extracted and inflicted on the prey, inside which the poisonous liquid contained in the capsule is injected, paralyzing or killing it. The urticant liquid is composed of a set of neurotoxic protein substances that can give respiratory paralysis, cardiocirculatory and sometimes also have haemolytic action. However, most Cnidarians, including *Hydra*, do not cause any harm to humans or cause only light skin reactions. The cubomeduses are an exception, the puncture of which can even be fatal. Although *Hydra* is devoid of complex sense organs, can recognize a fresh prey, from a debris or in any case from a non-living material. The cnidocytes, in fact, react to the slightest contact with a living prey by the recognition of a substance present only in living beings, the GSH (Loomis, 1955). The tentacles of the hydra, thanks to some specific receptor molecules, recognize the presence of GSH in the fluids released by the wounds produced by nematocysts (Venturini, 1987; Lenhoff, 1981). It is possible to consider the hydra response to GSH as a precursor of the sense of smell. In the induction of the alimentary answer, one of the most interesting RNS, the nitric oxide (NO), is also actively involved. NO induces the activation and coordination of the curling of the tentacles, determining the rapid diffusion of a primary stimulus in the neighboring tentacles, regardless of the presence of direct connections through the synapses (Colasanti et al., 1995, 1997).

Because of its regenerative capacities, the most developed of all the animal kingdom, hydra is one of the oldest scientific research model organisms. As already observed by Trembley, both the amputated head and foot grow back within a few days and even small segments of the trunk reproduce an entire individual in a short space of time (Trembley, 1774). In this way, a single hydra sectioned into several parts can produce numerous new individuals. The simplicity of this animal, the speed of reproduction, the ease of handling

and the ease of management in terms of breeding give the hydra unique qualities for an experimental model.

1.2.2 Hydra and TRP receptors

Recalling what has been considered up to this paragraph, therefore, the availability of the entire genomic sequence in the bank has allowed to verify the presence of TRP receptors also in the phylum of cnidarians and in particular on the genus *Hydra*.
Hydra magnipapillata and *Hydra vulgaris* find a very high homology of the genomic sequence, these two species can be considered as a unique onecontaining at least 29 TRP channels (Peng et al., 2014). In recent times, several studies have used *Hydra* as an experimental model to analyze the activation and involvement of TRP receptors in different molecular pathways. The choice of this animal, in addition to having an evolutionary aspect, also lies on the fact that despite their relatively simple anatomy present a surprisingly large number of conserved genes.

1.2.2.1 TRP thermosensation and Noxious Stimuli

The ability to detect temperature change enables mammals to find suitable thermal climes, maintain core body temperature, and perceive painful (nociceptive) stimuli. This important process begins in peripheral terminals of dorsal root (DRG) or trigeminal ganglia (TG) neurons where the intensity and quality of these stimuli are converted into neural activity and conveyed to the CNS. Thermosensory afferents fall into four subtypes based on their temperature response range; from innocuous warm (30°C – 43°C) to noxious heat (>43°C), innocuous cool (15°C – 30°C) to painful cold (< 15°C). Over the last decade and a half, ion channels of the TRP family have been recognized as the principle detectors of thermal stimuli in the peripheral nervous system (Vriens et al., 2014), yet recent genetic analyses in mice finds that some of these channels' roles in acute thermosensation are limited at best, suggesting that other molecular mechanisms underlying thermosensation are yet to be identified (Radhika et al., 2015).
The use of uncommon animal models, such as *Hydra vulgaris*, which has a simple nervous system, can provide interesting insights into the molecular mechanisms stored at the base of nociception at the primordial level of the animal kingdom. Earlier studies reported the critical role of oxidative stress during the development and maintenance of pain of several aetiologies, such as inflammatory central sensitization, hyperalgesia, chemotherapy-induced

peripheral neuropathy (CIPN) and tolerance to morphine (Muscoli et al., 2004: Doyle et al., 2012).
According to these data it has been hypothesized that the pathway of oxidative stress related to TRP could be a common defense through conservative evolution from primitive organisms to humans.
Recent studies conducted in our laboratory on *Hydra vulgaris* have demonstrated the expression of the TRPM3 receptor on whole body of polyp (Figure 1.9), and its role in the modulation of nociceptive/oxidative pathways following a thermal stimulus of 34°C (Malafoglia et al., 2016).

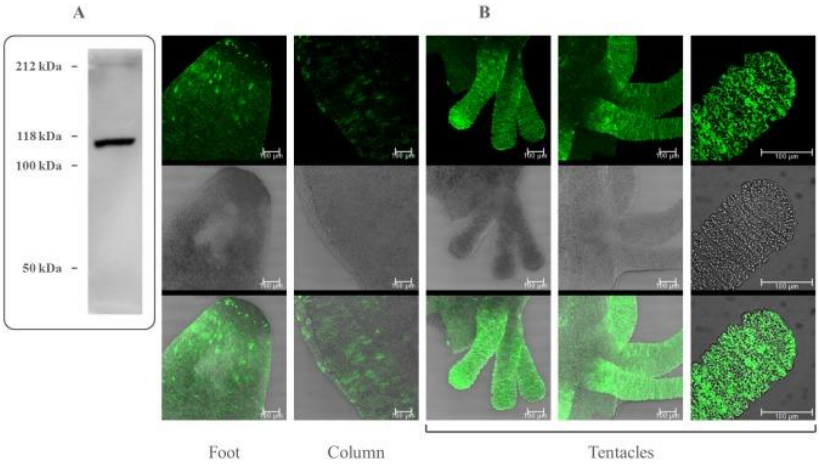

Figure 1.9. A) Expression of TRPM3 protein in whole body of *H. vulgaris*. Western blot is representative of six independent experiments. (B) Immunofluorescence of *Hydra* whole mounts with the anti-TRPM3 antibody. Signals for TRPM3 are clearly extracellular and appear mainly in tentacles and foot. Specimens were examined in the confocal microscope. Scale bars, 100 μm (Malafoglia et al., 2016).

For nociceptive response, it has been analyzed the expression of heat shock protein 70 (HSP70) and nitric oxide synthase (NOS) genes, considering their overexpression after heat-mediated activation of TRPs in mammals (Bromberg et al., 2013: Sung et al.,2004). For oxidative response, it has been analyzed the transcription of nuclear transcription erythroid 2-related factor (Nrf2), a known main regulator of the oxidative stress pathway, and superoxide dismutase (SOD), a Nrf2-dependent enzyme (Dash et al., 2007), also considering the involvement of TRPs melastatin subfamily in oxidative stress pathway (Forder et al., 2009; Miyake et al., 2014).

1.2.2.2 TRP and innate immunity

The immune system maintains the integrity of the organisms through a complex network of molecules, cells, and tissues that recognize internal or external antigenic substances to neutralized and eliminate them. Molecular genetic evidence has proven that the basic patterns of innate immune sensors were laid down in ancient animals, such as *Hydra*. Important functions of *Hydra*'s innate immune sensors and effectors include not only protection against pathogens but also controlling tissue-microbiota homeostasis. The deep evolutionary connections imply that invertebrate and mammalian immune pathways have evolved from a reduced number of common ancestral building blocks to their present configurations.

Although the majority of microbes appear to exist in peaceful coexistence with *Hydra*, identification of the sensors to detect infection and to trigger an innate immune response is critical in understanding the interaction between hydra tissue and microbes. During the past few years much has been learned about the molecular basis of innate immune perception in *Hydra* (Kasahara et al., 2003; Augustin et al., 2009). In general, the ability of the host to defend against invading pathogens is to a large extent mediated by germ-line encoded receptors known as pattern recognition receptors (PRRs) (Medzhitov et al.,1997; Beutler, 2004). Current concepts of the architecture of the hydra innate immune system is largely based on two distinct classes of PRRs (Bosch, 2008; Rosenstiel et al., 2009). One type of receptors comprises Toll-like receptors (TLRs) essential players of innate immunity, initially identified in Drosophila (Lemaitre et al.,1996) are a membrane-resident and detect widely conserved microbe-associated molecular patterns on the cell surface such as lipopolysaccharides (LPS), flagellin or not yet defined components of *P. aeruginosa*. The second type of receptors comprises intracellular immune receptors of the nucleotidebinding and oligomerization domain (NOD)-like receptor (NLR) family (Wenger et al., 2014). Analysis of the *H. magnipapillata* genome including about 170,000 Expressed Sequence Tags (ESTs), identified two genes whose inferred amino acid sequence contained a Toll/interleukin-1 receptor (TIR) domain, a transmembrane domain, and an extracellular domain lacking any specific domain structure (Hemmrich et al., 2007; Bosch et al., 2009). The authors have termed these *Hydra* genes Toll-receptor-related 1 (HyTRR-1) and Toll-receptor-related 2 (HyTRR-2), respectively (Hemmrich et al., 2007) (Figure 1.10). It has been recently shown that in *Hydra* it presents molecular models of innate immunity; Toll-like receptors (TLRs) are membrane receptors and Nod-like receptors (NLRs) are cytoplasmic sensors. The functional

interaction of TLR and NLR mediated by a ligand induces the innate immune response, with the production of known antimicrobial peptides, such as periculin or hydramacin (Augustin et al., 2010).

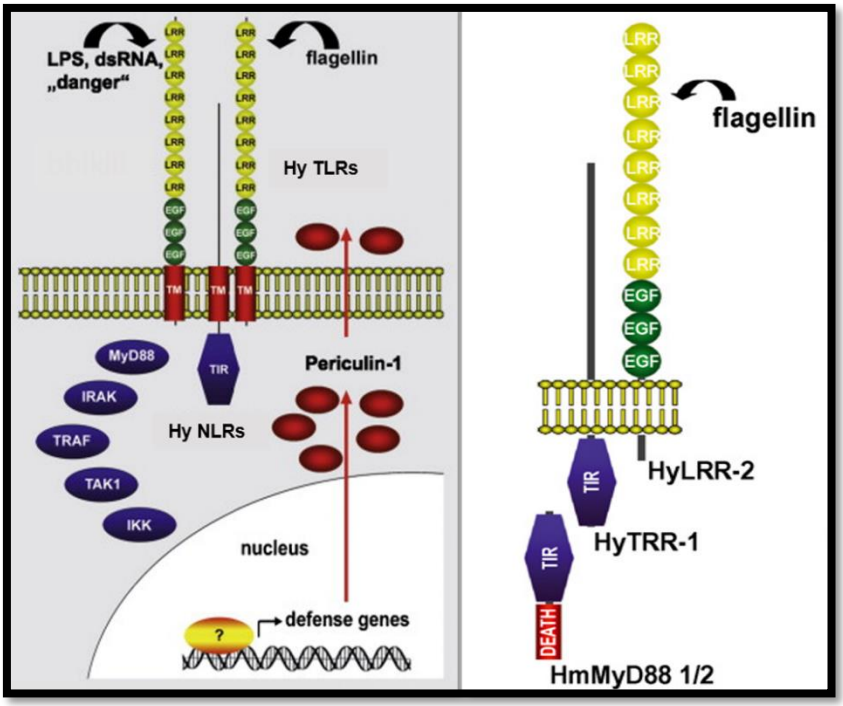

Figure 1.10. Molecular patterns of innate immunity in Hydra. Toll-like receptors (TLRs) are essential membrane receptors. Nod-like receptors (NLRs) are a cytoplasmic sensors. The functional interaction of TLR and NLR mediates by a ligand, induce the innate immune response, whith the production of antimicrobial peptides like periculin or hydramacin. Activation of pattern recognition receptors (PRRs) such as HyTRR-1 and HyLRR-2. Is evidence that in *Hydra*, leucine-rich-repeats (LRRs) and the canonical TIR domain are located on two separate proteins (Augustin et al., 2010).

Studies have shown a connection between the TRP receptors in particular the TRPA1 with the primary immune response (TLR7) (Chul-Kyu Park et al., 2014). In mammals, pathogenic infection or tissue damage initiates a defence response that is intended to contain the spread of invading pathogens, neutralize their activity, clear damaged tissues and promote their repair. This immune response is triggered by the local release of viral products (e.g. RNA,

envelope proteins) or pathogenic components of the bacterial wall, called pathogen-associated molecular patterns (PAMPs), which are recognized by pattern recognition receptors (PRRs) on leukocytes and macrophages and initiate an inflammatory response, including the release of cytokines and other mediators (for example NO). These PRRs include members of the Toll-like receptor (TLR) family (Janeway & Medzhitov, 2002), NOD-like receptors and RIG-I-like receptors. Similarly, tissue damage (e.g. mechanical wound, burn, freezing) also attracts and activates immune cells by certain intracellular products released from dying cells (Matzinger, 2002). Infection or sterile tissue damage recruits immune and glial cells to the site of injury. Activated immune cells release a variety of inflammatory mediators acting on multiple receptors on nociceptors, including TLRs and cytokine receptors, leading to their profound sensitization and exacerbated pain (Ren & Dubner, 2010). Recent studies support a specific role for TRPA1 channels in the detection and response to harmful bacterial and viral products. The most compelling evidence comes from the discovery that TRPA1 is activated by lipopolysaccharide (LPS) in mice (Meseguer et al., 2014) and in and *Drosophila melanogaster* flies (Soldano et al.,2016), or by endotoxin, the main immunostimulant in Gram negative bacteria, causing the rapid activation of nociceptors (Meseguer et al., 2014). Moreover, the activation of TRPA1 by LPS in vagal and somatic nociceptors led to the local release of neuropeptides (*e.g.*, CGRP), causing pain, neurogenic inflammation and vasodilatation (Félix Viana, 2016).

2. Aim of the work

Transient Receptor Potential (TRP) receptors are a large and diverse family of proteins expressed in different types of cells and tissues of both vertebrates and invertebrates. Mainly located on the plasma membrane, the TRP mediate the transmembrane cation flow according to their electrochemical gradient, determining an intracellular increase of calcium/sodium ions. They include a group of non-selective cationic channels with pleiotropic functions ranging from intracellular calcium homeostasis to the perception of a wide variety of sensations such as heat, cold, pain, touch, taste, smell and vision. The activation of these receptors may depend on multiple environmental factors or synthetic or naturally derived molecules, as active ingredients of the most known food products; capsaicin, allinine, menthol.

In recent years the study of these receptors has led to deepen different aspects. The study of the functions of the TRP channels has been mainly characterized in the model organisms within a limited evolutionary context and the conservation of these receptors on the ancestral level has made them more and more interesting. Recently, our group has provided clear evidence of the presence and role of the TRPM3 receptor in *Hydra vulgaris*, following a response to harmful heat and induced oxidative stress.

This doctoral project is fundamentally concerned with continuing to deepen the presence and a possible role of these receptors in the experimental animal model *H. vulgaris*, a small freshwater polyp belonging to the Phylum of Cnidaria, at the base of the evolutionary chain. Our attention fell on the TRPA1 receptor, a receptor that responds to different stimuli but is particularly involved in the response to the noxious cold and in the activation of the immune response. The first objective was to analyze the response and adaptation to the harmful cold of freshwater polyps treated at 4°C. The increase in expression of genes significant for this response as NOS, Nrf2 and SOD has been analyzed since the harmful cold implies a condition of nitro-oxidative stress in the organisms. The involvement of the TRPA1 receptor in the response has been verified, using a TRPA1 agonist (*i.e.*, Glybenclamide G0639) and a specific antagonist (*i.e.*, HC-030031). The second goal was to study the immune response in *Hydra vulgaris*, treating polyps with cell lysates of *P. aeruginosa* PA14. We then analyzed mRNA expression of NF-κB, a transcriptional factor involved in innate immunity, and significant NF-κB-dependent genes such as nitric oxide synthase (NOS) and two newly discovered antimicrobial peptides specific for *Hydra* (*i.e.*, periculin and hydramicin).

Results and Discussion

3.1 *Hydra vulgaris* and transient receptor potential ankyrin type 1 (TRPA1) in noxious cold nociception pathways.

The transient receptor potential ankyrin type 1 (TRPA1) receptor channel, the only member of the transient potential receptor subfamily A, is a nonselective cationic TRP channel (1119 amino acids in humans) phylogenetically distant to other mammalian TRP proteins with high conductivity for sodium and calcium (Nilius & Voets, 2005). TRPA1 is expressed in small-diameter neurons of the trigeminal and dorsal root ganglia and has been attributed nociceptive and cold receptive functions (Kobayashi et al., 2005; Kim et al., 2010). In general, TRPA1 was identified as a receptor for noxious cold temperature (<17°C) (Stucky et al., 2009; Jaquemar et al., 1999; Story et al., 2003). Although controversy exists regarding its role as a thermosensor, its role in nociception is quite clear (Levine et al., 2007).

Here, we analyzed the involvement of the TRPA1 receptor in the modulation of nociceptive-like pathways after a cold shock (CS) at 4°C in *Hydra vulgaris*, an ancestral animal model.

Firstly, viability tests were performed for different weeks and animals were exposed to different temperatures, ranging from 17°C (*i.e.*, physiological temperature for *H. vulgaris*) down to 4°C (*i.e.*, extreme sublethal efficient temperature). Later, polyp morphology and integrity were observed at optical microscopy, using a 32X magnification objective.

Animals were collected in Petri dishes in Hydra medium and after a weak mechanical solicitation (needle) tentacles and body reactivity were analyzed as behavioural variables.

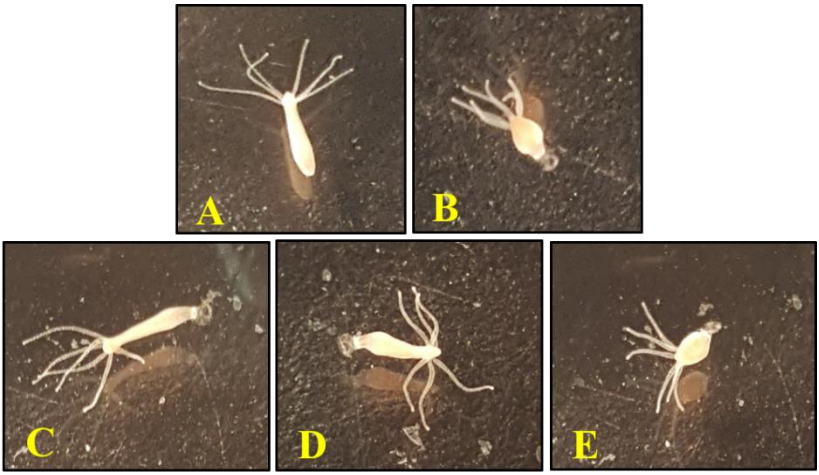

Figure 3.1. Response to mechanical solicitation in phisiological (17°C) and cold shock (4°C) conditions. A) natural behaviour of *Hydra vulgaris* at 17°C: tentacles are open and the body is eloganted. B) untreated specimen after mechanical solicitation (17°C): normal contraction of body and tentacles. C) cold-treated animal after mechanical solicitation: there isn't contraction. D) cold-trated animal put at 17°C for 5 minutes before mechanical solicitation: slowed movements and partial recovery of function. E) cold-trated animal put at 17°C for 10 minutes before mechanical solicitation: total recovery of functional features.

As shown in figure 3.1, polyps respond to a mechanical stimulus with the contraction of the body and tentacles, in physiological condition (17°C). When polyps are subjected to CS (4°C for 1 min), a delay in the response to the mechanical solicitation is observed. Infact, polyps seem to lose adhesion to the substrate and the ability to contract body and tentacles.

The next step was to detect the presence of TRPA1 protein in *Hydra vulgaris*, by western blot analysis. After specific interaction between a primary anti-TRPA1 antibody and hydra lysates, a single band has been evidenced, thus suggesting the presence of a TRPA1-like protein in the freshwater coelenterate (Fig. 3.2).

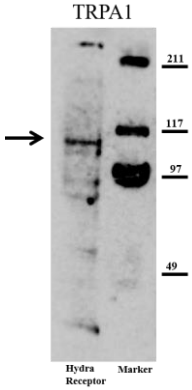

Figure 3.2. Western blot analysis to reveal the of TRPA1 proteins in *Hydra vulgaris*. This result is representative of different independent experiments.

To address the study of signaling pathways modulated by TRPA1 activation, agonists and antagonists have been used. Then, the expression of different genes (*e.g.*, NOS, Nrf2 and SOD) were analyzed, using Q-PCR as a semi-quantitative analysis.

The list of TRPA1 agonists a keep growing daily and includes many natural and synthetic irritants. Among the agonists we can find, the isothiocyanates occurring naturally in fruits and plants like allyl isothiocyanate or the cinnamaldehyde, for examples, (Vriens et al., 2008; Stucky et al., 2009) or natural fungal deterrents like isovelleral or oxidizing agents or chemical moleculars like hypochloritde (OCl^-) and the hydrogen peroxide (H_2O_2) (Babes et al., 2013; Bandell et al., 2004; Bautista et al., 2005, 2006; Kunkler et al., 2018). As regards the antagonists of TRPA1, the most used in the literature as a tool for studying TRPA1-mediated biology is HC-030031, a xanthine alkaloid discovered by Hydra Bioscience (McNamara et al. 2007; Eid et al. 2008), but there are others like A-967079 (Abbott), Compound 10 (Amgen), and Compound 31 (Novartis) (Bautista et al., 2005; Kunkler et al., 2018; Stucky et al., 2009).

In our experiments, we used both Glybenclamide G0639, a selective TRPA1 agonist, and HC-030031, a selective and potent TRPA1 antagonist (Babes et al., 2013).

Optimal concentrations of both agonist and antagonist were selected after dose- and time-response tests based on scalar concentrations (up to a sublethal but efficient condition) for 0-24 h. Thus, polyps were incubated with either the agonist G0639 (0.2, 2 and 20 µM) or the antagonist HC-030031 (0.1, 1 and 10 µg/ml) for 0, 0.5, 4, 8, 16, 24 h. In order to reveal

behavioural changes, animals were continuously monitored using an optical microscope. For each point, 15 specimens were processed for RNA extraction.

For CS, animals were moved, with a specific insert for co-culture cell system (TC insert for 6 well plate, PET membrane bottom, translucent, pore size 8 µm, sterile), from 17°C to 4°C Hydra medium beakers, for 1 minute. Then, specimens were placed again at 17°C and recovered in the incubator. Groups of 15 animals were collected at specific time points after the cold shock (0, 0.25, 0.5, 1.5, 4 and 12 h) and were processed for RNA extraction.

Recent studies have shown a link between the activation of TRP receptors and the nitrosidative stress in the mammalian nervous system. In general, the source of stress can be associated with several factors; an increase or decrease in temperature, the presence of substances that induce toxicity, absence of nutrients and situations that in some ways interfere with the maintenance of cellular balance. The TRPA1 receptor is sensitive for a large series of reactive nitro-oxidative stress products in models of different types of pain, including inflammatory pain, neuropathic pain and migraine (Nassini et al., 2014). The reactive oxygen and nitrogen species (ROS and RNS) can be induced from outside the cell or from the cell itself in response to external stimuli and when present in concentrations controlled by the biological system, they actively participate in a variety of complex processes to protect cells against oxidative stress and to restore homeostasis (Droge, 2002; Finkel, 2003; Esposito et al., 2004; Brigelius-Flohè, 2009).
The presence of NO pathway has been well demonstrated in Cnidaria, including *H. vulgaris* (Colasanti et al., 2010). Our previous studies have shown NO involvement both in the feeding response (Colasanti et al., 1995) and in the regeneration processes of the head (Colasanti et al., 2009). We have also shown the presence of an isoform independent of calcium but independent of calmodulin (Colasanti et al., 1997). Furthermore, there is a body of existing literature that characterizes the induction of the NO pathway from heat stress in several cnidarian species, including *H. vulgaris* (Safavi-Hemami et al., 2010; Malafoglia et al. 2016). The NO produced during exposure to high temperature averages a process known as cnidarian whitening (Bouchard et al., 2008; Perez et al., 2006), through a pathway mediated by caspases (Hawkins et al., 2013). However, nothing is yet known about the NO modulation from cold stress in cnidaria (*e.g., H. vulgaris*).
Here, we analyzed the expression of NOS mRNA after cold shock (4°C for 1 min) at different time points (0, 0.25, 0.5, 1.5, 4 and 12 h; T=0 was taken as

a control). As shown in figure 3.3A, CS is able to induce NOS gene expression, a peak being observed after 12 hours (~ 2 times, $p \leq 0.01$).

To investigate the TRPA1 involvement in the CS-induced NOS expression in *H. vulgaris*, the TRPA1 selective antagonist HC-030031 (0.1 μg/ml) has been used. In particular, we compared NOS mRNA modulation after CS (12 h) in two distinct groups of specimens: 1) CS and 2) HC-030031-preincubated CS polyps. We considered polyps at T = 0 as a control. Real time PCRs showed NOS ~2.2 folds of activation, as expected, in CS group ($p \leq 0.01$) and a reduction of expression, close to the control, in the second group treated with HC-030031 ($p \leq 0.01$) (Fig.3.3B).
To confirm TRPA1 involvement in the thermal noxious pathway, the specific agonist of the receptor, Glybenclamide-G0639, has been employed. In particular, we incubated polyps with Glybenclamide-G0639 (2 μM) and collected animals at specific time points (0, 1.5, 4, 8, 12 and 24 h; T = 0 as a control) for molecular analysis. According to the CS results, NOS mRNA higher expression has been detected at 12 h in the Glybenclamide-G0639-treated polyps (~2 folds; $p \leq 0.01$) (Fig. 3.3C). At the same time point, we repeated the test after Glybenclamide-G0639 and HC-030031 pre-incubation, to validate TRPA1 implication in the process. Real time data showed that pre-treatment with HC-030031 was able to revert Glybenclamide-G0639-induced NOS mRNA expresion ($p \leq 0.01$) (Fig. 3.3D).

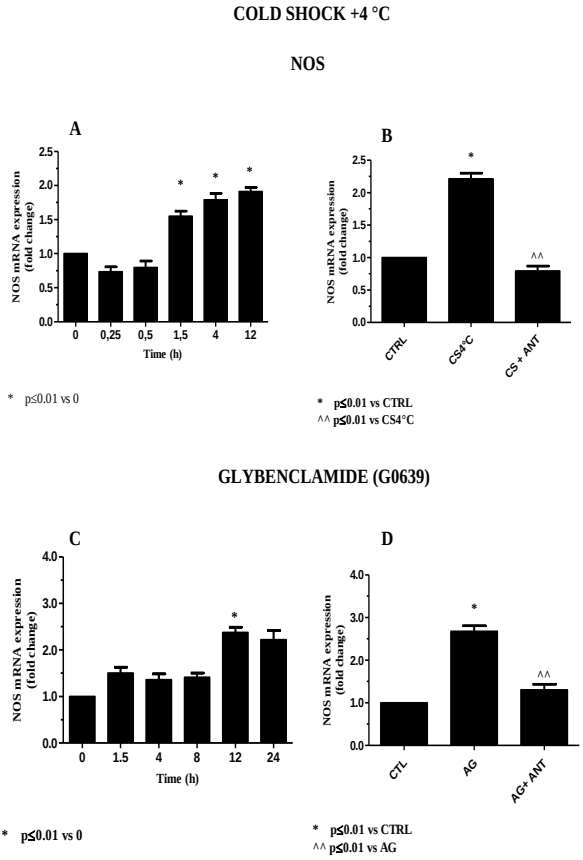

Figure 3.3. Cold shock (4°C) and G0639 (2μM) effect on NOS gene expression in *Hydra vulgaris*.
Both cold shock and the agonist of TRPA1 (A-C) induce over expression of NOS mRNA with a maximal effect at 12 h. This result is capsized when the polyps are pretreated with the specific antagonist HC-030031 (0.1 μg/ml) (B-D). The results of data are calculated respect to the internal housekeeping gene (β-actin) and are expressed as mean fold change compared with control (T = 0) ± SEM (n = 9). (A) * p≤0.01 vs 0; (B) * p≤0.01 vs CTRL and ** p≤0.01 vs CS 4°C; (C) * p≤0.01 vs 0; (D) * p≤0.01 vs CTRL and ** p≤0.01 vs AG.

Under conditions of stress or adaptation to extreme environmental conditions, vertebrates have developed a process of cellular stress resistance that involves the activation of transcription factors that in turn induce the expression of other downstream genes. A similar system is represented by Nuclear Factor Erythroid 2-related transcription factor (Nrf2) (Itoh et al., 1995), which induces the transcription of detoxifying and andoxidizing enzymes, such as superoxide dismutase (SOD) (Kim et al., 2003; Kobashi et al., 2005; Pietsch et al., 2003), migrating to the nucleus.

Activation of the Nrf2 transcription factor was demonstrated following a heat treatment in mice (Li et al., 2014) and in *H. vulgaris* (Malafoglia et al., 2016). Here, we analyzed Nrf2 modulation after CS (4°C for 1 min) at specific time points (0, 0.25, 0.5, 1.5, 4 and 12 h; T=0 as a control). During CS, Nrf2 mRNA expression increased, reaching the higher point of expression at 1.5 h, with ~2 folds of change ($p \leq 0.01$) (Fig- 3.4A). We demonstrated TRPA1 involvement by monitoring the inhibition of Nrf2 transcription by using 0.1 μg/ml HC-030031 ($p \leq 0.01$) (Fig. 3.4B). We went on in confirming TRPA1 role in the process, by treating polyps with the specific agonist Glybenclamide-G0639 (2 μM) for 0, 1.5, 4, 8, 12 and 24 h (T=0 as a control) and by finding increased Nrf2 mRNA expression ~2.3 folds of change at 1.5 h ($p \leq 0.01$) (Fig. 3.4C). This effect was reverted by pre-incubation of Glybenclamide-G0639-treated specimens with 0.1 μg/ml HC-030031 ($p \leq 0.01$) (Fig. 3.4D). Taken together, our findings are consistent with the emerging evidences showing the involvement of TRPs, especially those of the ankyrin subfamily, in the oxidative stress pathway during noxious stimuli

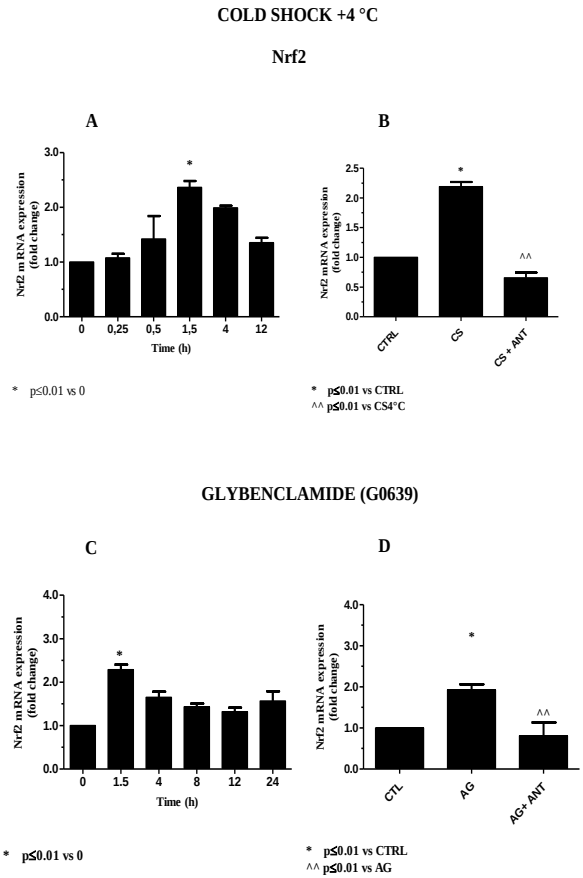

Figure 3.4. Cold shock (4°C) and G0639 (2µM) effect on Nrf2 gene expression in *Hydra vulgaris*.
Cold shock (A) and the agonist of TRPA1 (C) induce over expression of Nrf2 mRNA with a maximal effect at 1.5 h. When the polyps are pretreated with the specific antagonist HC-030031 (0.1 µg/ml), the expression is inhibited (B-D). The results of data are calculated respect to the internal housekeeping gene (β-actin) and are expressed as mean fold change compared with control (T = 0) ± SEM (n = 9). (A) * p≤0.01 vs 0; (B) * p≤0.01 vs CTRL and ** p≤0.01 vs CS 4°C; (C) * p≤0.01 vs 0; (D) * p≤0.01 vs CTRL and ** p≤0.01 vs AG.

SOD is an antioxidant, Nrf2-dependent enzyme. In vertebrates, three variants of this enzyme have been identified that differ from each other for the type of metal present in the active site: iron (Fe-SOD), manganese (Mn-SOD) and copper-zinc (CuZn-SOD); the last two are more frequent in eukaryotes, recent studies have shown an increase in the expression patterns of these two enzymes, in stress situations including heat treatment (Loomis, 1953).
In *H. magnipapillata* (Woo et al., 2012) and in corals (Downs et al., 2000), SOD has been used as a biomarker for oxidative stress induced by exposure to toxaphene, ultraviolet radiation (UV) and temperature variations.
Here, we analyzed the involvement of TRPA1 activation in CuZn-SOD mRNA expression induced by CS and Glybenclamide-G0639 treatment in *H. vulgaris*. In particular, the exposure of polyps to CS increased SOD mRNA expression at 12 h with ~3 folds of change ($p \leq 0.01$) (Fig. 3.5A) and this effect was abolished by 0.1 µg/ml HC-030031 ($p \leq 0.01$) (Fig. 3.5B), thereby suggesting that TRPA1 mediated CS-induced SOD expression. When polyps were incubated with 2 µM Glybenclamide-G0639, a time-dependent increase of SOD gene expression at 12 h was observed with ~4 folds of change ($p \leq 0.01$) (Fig. 3.5C) and this effect was inhibited by 0.1 µg/ml HC-030031 ($p \leq 0.01$), thus confirming an involvement of TRPA1 in these mechanisms (Fig. 3.5D).

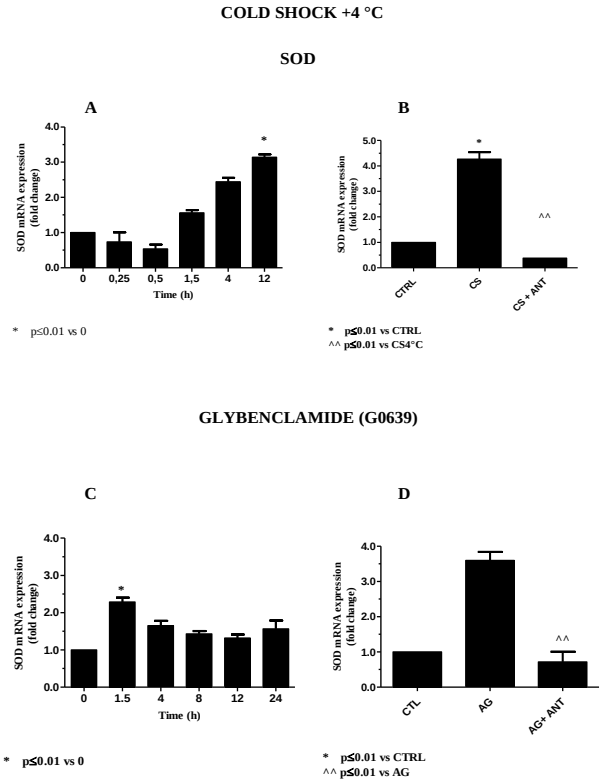

Figure 3.5. Cold shock (4°C) and G0639 (2μM) effect on SOD gene expression in *Hydra vulgaris*.
Cold shock (A) and the agonist of TRPA1 (C) induce over expression of SOD mRNA with a maximal effect at 12 h. When the polyps are pretreated with the specific antagonist HC-030031 (0.1 μg/ml), the expression is inhibited (B-D). The results of data are calculated respect to the internal housekeeping gene (β-actin) and are expressed as mean fold change compared with control (T = 0) ± SEM (n = 9). (A) * p≤0.01 vs 0; (B) * p≤0.01 vs CTRL and ** p≤0.01 vs CS 4°C; (C) * p≤0.01 vs 0; (D) * p≤0.01 vs CTRL and ** p≤0.01 vs AG

3.2 Transient receptor potential ankyrin type 1 (TRPA1) mediate the innate immunity pathways in *Hydra vulgaris*.

Cnidarians are essentially epithelial organisms. The epithelial cells play a fundamental role in immunity as they display phagocytic activities and secrete mucus, which acts as a physicochemical barrier preventing or slowing down the proliferation of potential pathogens (Augustin et al., 2011). An additional arm of immuno-defense in these organisms is awarded by the immense capacity to regenerate their tissues because of the continuous proliferation of stem cells (Fautin, 2002); the cells infected by intracellular parasites are quickly removed in a programmed way (apoptotic processes) and they are immediately replaced by non-infected cells (Augustin et al., 2011). It is known that Cnidarians are characterized by a complex set of symbiotic bacteria inhabiting the epithelial surfaces that compete with potential pathogens to colonize the tissues (Bosch, 2013). Alterations in the structure of the symbiotic bacterial communities due to environmental changes might promote the proliferation of opportunistic microorganisms that can cause disease (Cárdenas et al., 2012). Hence, bacterial communities associated to the epithelia can also be considered part of an efficient immune barrier in cnidarians.

Toll-like receptors (TLRs) are among the most conserved membrane pattern recognition receptors (mPRRs). TLRs are transmembrane proteins composed by an extracellular *N*-terminal domain having leucine rich repeats (LRRs), which is responsible for the recognition process, and an intracellular Toll/Interleukine-1 receptor (TIR) domain that initiates the transmission of intracellular signals leading to the translocation of transcription factors from NF-κB family. In *Hydra*, two transmembrane proteins have been characterized having an extracellular LRRs domain similar to those present in vertebrate TLRs (HyLRR-1 and HyLRR-2). However, these two proteins do not possess the intracellular TIR domain typical of vertebrate TLRs. In addition, *Hydra* expresses two other transmembrane proteins, HyTRR-1 and HyTRR-2, having an intracellular TIR domain with no recognizable extracellular domains (Augustin et al., 2011). An important study shows how silencing the HyTRR-1 and HyLRR-2 genes leads to a drastic reduction in the synthesis of different important antimicrobial peptides such as hydramacin-1, arminin-1 and periculin-1, indicating that the TLR pathway in this hydrozoan activates an antimicrobial state (Augustin et al., 2010).

Recent studies have shown a correlation between a primary immune response mediated by TRLs (*e.g.*, TLR7) and TRP receptors (*e.g.*, TRPA1) (Chul-Kyu Park et al., 2014). A recent discovery reports that TRPA1 is activated by lipopolysaccharide (LPS) in mice (Meseguer et al., 2014) and in *Drosophila*

melanogaster flies (Soldano et al.,2016), or by endotoxin (*i.e.*, the main immunostimulant in Gram negative bacteria), causing the rapid activation of nociceptors (Meseguer et al., 2014).

Here, we have analyzed the innate immune response in *H. vulgaris*, by treating the polyps with cell lysates from *Pseudomonas aeruginosa* PA14 strain. In particular, we have verified the expression of both transcriptional factor NF-κB and NF-κB-dependent genes, such as NOS, periculin and hydramacin. Subsequently, we have investigated the involvement of the TRPA1 receptor in these mechanisms.

Firstly, to exclude deadly conditions for animals, vitality tests were performed. In particular, polyps were incubated and kept under observation for two weeks at different PA14 lysate concentrations, ranging from 0.2 and 20 µl. At the end of the two weeks neither morphological changes nor stimulus response over time were observed (data not shown).

Optimal concentrations of the bacterial PA14 lysate were selected after dose-response tests based on scalar concentrations (0.2, 2 and 20 µl) for 24 h. Thus, polyps were treated with 20 µl PA14 lysate (*i.e.*, the optimal concentration) for 0, 4, 8, 16, 24 hours (T=0 was used as a control). For each sample, 15 animals were processed for RNA extraction. Then, mRNA expression for NF-κB, NOS, periculin and hydramicina were analyzed by real-time PCR analysis.

NF-κB ("nuclear factor kappa-light-chain-enhancer of activated B cells") is a pleotropic transcription factor, present in all cell types, which regulates the expression of a number of genes involved in the response to stimuli, such as stress, cytokines, free radicals, ultraviolet irradiation and attack from bacteria or virus antigens (Gilmore, 1999). In its canonical signaling pathway, NF-κB is a heterodimer composed of p50 and p65 (RelA) subunits (Baldwin, 1996; Ghosh et al., 2002). In unstimulated cells, this heterodimer is sequestered in the cytoplasm by p65 bound IKBα. Upon stimulation, IKBα is phosphorylated by the IKB kinase (IKK) and targeted for degradation via the proteasome pathway. This liberates NF-κB and promotes its nuclear translocation where it activates the transcription of target genes that influence the regulation on the cellular response in the immune response to infections, inflammatory processes, autoimmune diseases, cells proliferation, cells migration and in general oxidative stress (Hoffmann et al 2006). Studies conducted in other laboratories have shown that cnidarians have a developmental or stress response pathway regulated by NF-κB (Sullivan et al., 2007).

Here, we analyzed the modulation of NF-κB gene expression after treatment with *P. aeruginosa*, and a possible involvement of TRPA1 receptor. As

shown in Fig. 3.6A, the treatment with PA14 cell lysate (20µl) at specific time points (0, 4, 8, 16 and 24 h; T=0 as a control) induced a dose-dependent increase of NF-κB mRNA expression, reaching the higher point of expression at 24 h, with ~2.5 folds of change ($p \leq 0.01$). Furthermore, NF-κB gene expression was inhibited by pre-incubating the PA14-treated polyps with 0.1 µg/ml HC-030031 ($p \leq 0.01$) (Fig.3.6B), thus suggesting the involvement of TRPA1 in PA14-induced NF-κB gene expression.

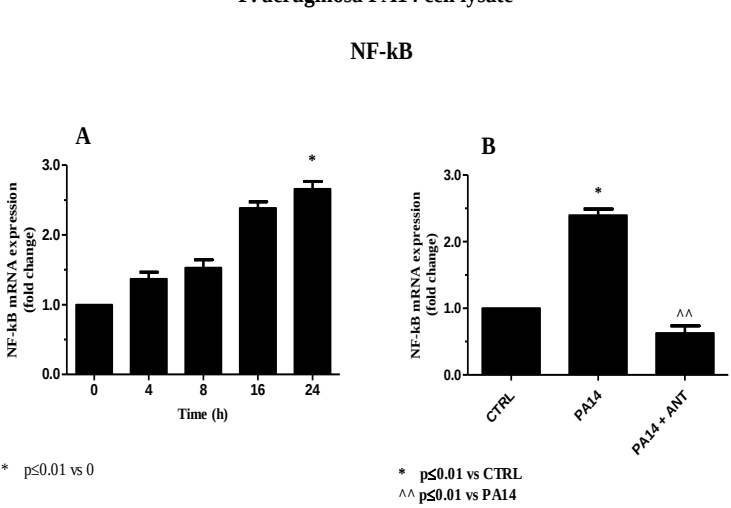

Figure 3.6. The effect of *P. aeruginosa* PA14 cell lysate (20µl) on NF-κB gene expression in *Hydra vulgaris*.
The PA14 cell lysate induces an increase of expression of NF-κB mRNA with a maximal effect at 24 h (A). The effect was inhibited by the TRPA1 receptor antagonist HC-030031 (0.1 µg/ml) (B). The results of data are calculated respect to the internal housekeeping gene (β-actin) and are expressed as mean fold change compared with control (T = 0) ± SEM (n = 9). (A) * $p \leq 0.01$ vs 0; (B) * $p \leq 0.01$ vs CTRL and ** $p \leq 0.01$ vs PA14.

Furthermore, we analyzed the expression of NF-κB-dependent genes (*e.g.*, NOS, periculin and hydramicina) after *P. aeruginosa* PA14 cell lysate treatment (20µl) at specific time points (0, 4, 8, 16 and 24 h; T=0 as a control). We observed that a treatment of polyps with PA14 cell lysate was able to up-regulate NOS gene expression, with ~3 folds of change at 24 hours ($p \leq 0.01$)

(Fig. 3.7A). This effect was reversed by the specific inhibitor of the TRPA1 receptor HC-030031 (0.1 μg/ml) ($p \leq 0.01$) (Fig. 3.7B), thereby suggesting a role of TRPA1 in this pathway.

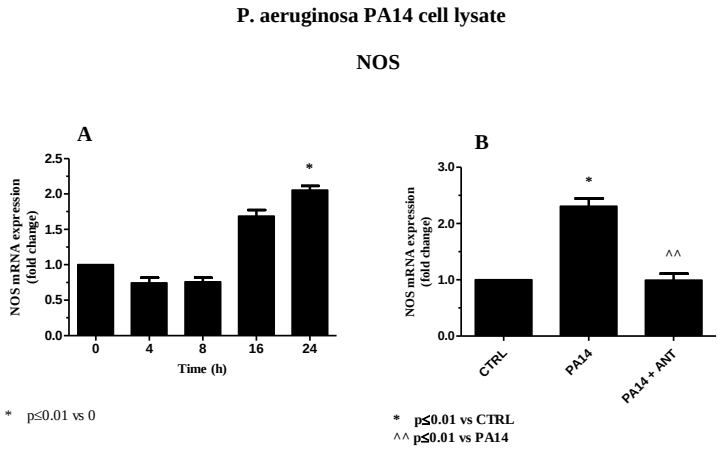

Figure 3.7. Effect of *P. aeruginosa* PA14 cell lysate treatment (20μl) on NOS gene expression in *Hydra vulgaris*.
The lysate induces an increase of expression of NOS mRNA with a maximal effect at 24h (A). The effect was inhibited by the TRPA1 receptor antagonist HC-030031 (0.1 μg/ml) (B). The results of data are calculated respect to the internal housekeeping gene (β-actin) and are expressed as mean fold change compared with control (T = 0) ± SEM (n = 9). (A) * $p \leq 0.01$ vs 0; (B) * $p \leq 0.01$ vs CTRL and ** $p \leq 0.01$ vs PA14.

Recently, a number of specific anti-bacterial peptides, including hydramacin and periculins, were detected in *Hydra* and characterized as NF-κB dependent genes (Jung et al., 2009; Fraune et al., 2010).
Here, we analyzed both periculin and hydramacin mRNA expression after treatment with *P. aeruginosa* PA14 cell lysate at 0, 4, 8, 16 and 24 h (T=0 was used as a control). In this respect, we observed a dose-dependent increase of both gene expression, with a peak at 24 h of ~2.5 folds for the periculin gene ($p \leq 0.01$) (Fig. 3.8A) and ~3 folds for the hydramacina gene ($p \leq 0.01$) (Fig. 3.9A), respectively.
Both effects were reversed by 0.1 μg/ml HC-030031 ($p \leq 0.01$) (Fig. 3.8B and Fig. 3.9B, respectively), thus suggesting a role of TRPA1 in PA14-induced anti-bacterial gene expression.

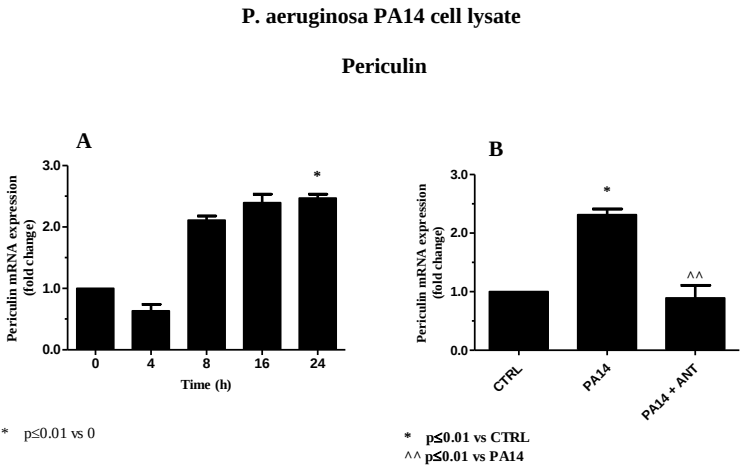

Figure 3.8. Effect of *P. aeruginosa* PA14 cell lysate treatment (20µl) on periculin gene expression in *Hydra vulgaris*.
The PA14 cell lysate induces an increase of expression of periculin mRNA with a maximal effect at 24h (~ 2.5 times, p≤0.01) (A). The effect was inhibited by the TRPA1 receptor antagonist HC-030031 (0.1 µg/ml) (B). The results of data are calculated respect to the internal housekeeping gene (β-actin) and are expressed as mean fold change compared with control (T = 0) ± SEM (n = 9). (A) * p≤0.01 vs 0; (B) * p≤0.01 vs CTRL and ** p≤0.01 vs PA14

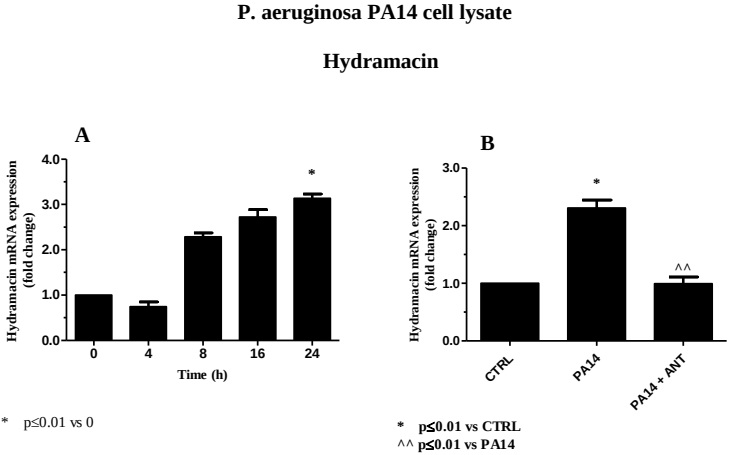

Figure 3.9. Effect of *P. aeruginosa* PA14 cell lysate treatment (20µl) on hydramacin gene expression in *Hydra vulgaris*.
The PA14 cell lysate induces an increase of expression of hydramacin mRNA with a maximal effect at 24h (~ 3 times, p≤0.01) (A). The effect was inhibited by the TRPA1 receptor antagonist HC-030031 (0.1 µg/ml) (B). The results of data are calculated respect to the internal housekeeping gene (β-actin) and are expressed as mean fold change compared with control (T = 0) ± SEM (n = 9). (A) * p≤0.01 vs 0; (B) * p≤0.01 vs CTRL and ** p≤0.01 vs PA14

4. Conclusions

Hydra vulgaris is one of the most primitive species of animal kingdom in terms of biological evolution and molecular complexity. In this respect, *Hydra* represents a good model to study the mechanisms involving the large and diverse family of TRP receptors. The data obtained in the first part of research indicate that exposure of *H. vulgaris* to harmful cold induces an increased expression of genes usually induced by TRP-mediated cold noxious stimuli in mammals (Bouchard et al., 2008; Perez et al., 2006). These genes are for example NOS, Nrf2 and SOD, known as markers of the nitro-oxidative stress during pain conditions (Nguyen et al., 2009; Rosa et al., 2008). Interestingly, the regulation of gene expression appears to be mediated by TRPA1 receptor, which is usually involved in the cold response in mammals. These results suggest a nociceptive-like response at the primordial level of the animal kingdom that involves evolutionarily conserved pathways. However, whether and how the proposed mechanisms can cause nociception in *H. vulgaris* remains to be further investigated.

In the second part of the research, we studied the involvement of TRPA1 in the innate immunity response in *Hydra vulgaris*. In particular, we observed that cell lysates of *Pseudomonas aeruginosa* PA14 strongly increased expression of genes usually involved in the anti-inflammatory and immune response. Specifically, we analyzed the transcriptional factor NF-κB and NF-κB-dependent genes, such as NOS as well as periculin and hydramacin, two antimicrobial peptides recently discovered in *Hydra*. In addition, our results suggest that the modulation of this immune response is mediated by TRPA1. It remains to investigate the specific pathways in which the TRPA1 receptor is involved in this response.

As whole, the presence of TRPs and its modulation, showed here, are intriguing and revolutionary data demonstrating the importance of evolutionarily conserved pathway in nociceptive-like system and innate immunity in low invertebrates.

5. Parallel research: Telomeres and Telomerase in *Hydra vulgaris*

5.1 Introduction

Hydra represents an ancestral experimental animal model for many studies. Among others, *Hydra* is used for the study on cellular senescence (Anthony et al., 2015) or for they high capability to regenerate completely their body (Trembley, 1744; Holstein et al., 2003; Bosch, 2007; Galliot, 2012; Gierer et al., 1972), a property which is likely related to the fact that these animals have an intense activity of stem cells (Bode, 1996; Bode, 2003). In mammals, self-renewal and differentiation of at least some somatic stem cells become abnormal with age, and the ability of stem cells to regenerate diverse tissues declines with age in both humans and mice (Sahin & DePinho, 2010). Invertebrate models like *Hydra* are critical for the understanding of aging for their unique properties including apparent biological immortality and inducible senescence. A study on individual lifespan in *H. vulgaris* provides evidence that members of this species are not subject to senescence (Martínez, 1998). Cellular senescence is a state in which the cell is no longer able to proliferate, characterized by a loss of physiological functions, a resistance to apoptosis (the senescent cell goes into apoptosis at a lower rate) and various cellular modifications, such as cytoplasmic volume increase and the presence of double nuclei in some cell types.
Senescence is a possible response of the cell, in place of apoptosis, to events that alter DNA. Among the possible stimuli for the senescent phenotype is telomere consumption due to numerous duplications (Daniel et al., 2013).

5.1.1 Telomeres and Telomerase

Telomeres are the DNA-protein complexes located at the ends of eukaryotic chromosomes. With a few exceptions, including plant and insect species, telomeric DNA sequences are usually rich in guanine and consist of a repetition of six bases. The sequence is TTAGGG/CCCTAA in all vertebrates underlining that is highly conserved to protect genome (Armstrong & Tomita, 2017) (Fig. 5.1). The genome instability events can include degradation of the terminal regions of chromosomes, fusion of a telomere, either with another telomere or with a broken DNA end, or inappropriate recombination and these processes could be potentially catastrophic (Fig. 5.1). Telomeric DNA consists of tandemly repeated, simple, often G-rich, sequences specified by the action of telomerase. The

tandem repeats form a molecular scaffold containing many binding sites for telomeric proteins, which in turn nucleate the coalescence of a higher order, although still ill-defined, complex of protective telomeric proteins, including the telomeric DNA-sequence-specific binding proteins. The resulting DNA-protein complex at the telomere is dynamic; during interphase, the telomerebound proteins are exchanged on and off individual telomeres at rates of the order of minutes or less, depending on which protein components are examined (Mattern et al., 2004). All chromosomes lose a small amount of telomeric DNA every time a somatic cell divides because of the noted "end replication problem". To avoid continuous sequence losses from the telomeres in dividing cells, special mechanisms evolved. In most eukaryotic organisms, the solution to the problem involves a ribonuclear complex, called telomerase based on an enzymatic part (transcriptase, TERT) and a RNA component (TR) (Lin et al ., 2004; Prescott & Blackburn, 1997). This last functions like a template for the de novo synthesis of telomeric DNA sequences. However, telomerase activity varies across taxa and differs in mortal and immortal cells (Armstrong & Tomita, 2017). For continuous growth, continuous telomerase activity is required; telomerase activity is repressed in most human somatic cells, while its activity is higher in immortal cell lines, germ cells, stem cells, activated lymphocytes and most of the tumor cells analyzed (Schmitt et al., 1994; Bolzan et al., 2000). Loss of telomerase enzymatic function leads to progressive telomere shortening over time, eventually resulting in the disappearance of detectable telomeric DNA. Although losses of chromosomal end capping have consequences in a wide range of cellular processes, including senescence, apoptosis, and carcinogenesis (Harley et al., 1992; Morin, 1996)

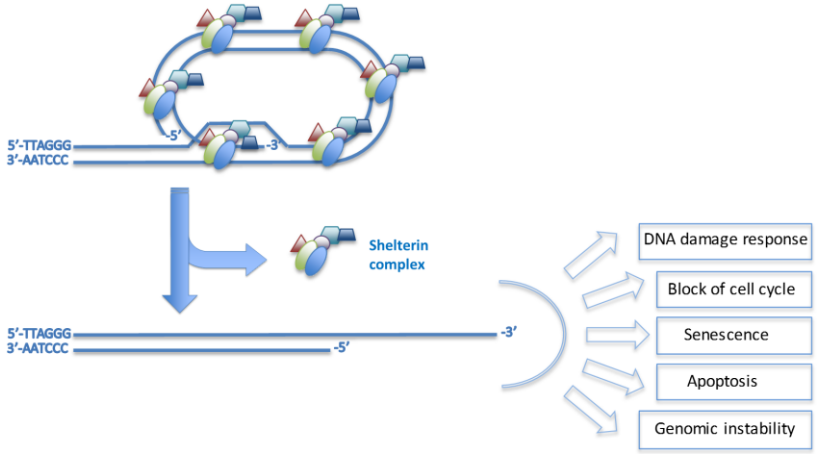

Figure 5.1. Telomeric dysfunction/shortening. Extensive telomeric erosion or telomere dysfunction activate several biological processes including activation of DNA damage response, block of cell cycle, apoptosis and senescence (Udroiu et al., 2017)

5.1.2 Telomeres and telomerase in Metazoa

Important discoveries made in recent years have shown that the TTAGGG sequence, common in the telomeric DNA of eukaryotes, is conserved in almost all Metazoa (Fig. 5.2) and since this motif is shared also by their unicellular sister-group Choanoflagellata (Fairclough et al., 2013), it can be inferred that it represents the ancestral telomeric repetition. Different studies investigated the presence of telomerase in basal Metazoa.

Applying the telomeric repetition amplification protocol (TRAP) assay, telomerase activity has been detected in *Cassiopea sp.* (Ojimi et al., 2009), *Galaxea fascicularis* (Nakamichi et al., 2012), *Madracis auretenra* and *M. decactis* (Zielke & Bodnar, 2010), *Suberites domuncula* (Koziol et al., 1998). A few studies demostrated that the gene encoding telomerase reverse transcriptase (TERT) is present in *Trichoplax adhaerens* (Robertson, 2009b), *Hydra vulgaris* and *Nematostella vectensis* (Steele et al., 2011). From all these evidences, and from the detection of the TERT gene also in Choanoflagellata (Robertson, 2009a), we deduce that telomerase is present in all basal Metazoa.

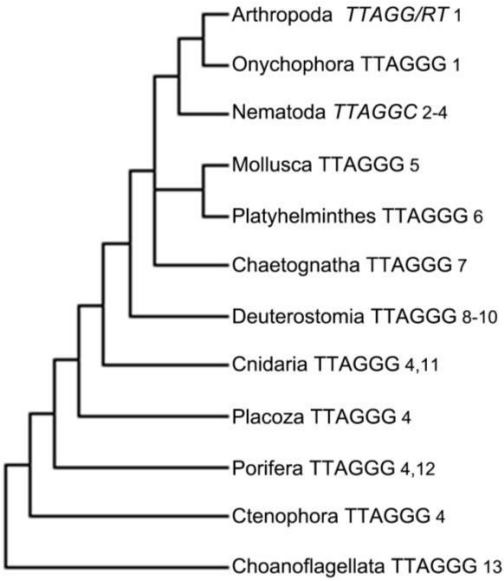

Figure 5.2. Phylogenetic tree showing telomere motifs in Metazoa (Udroiu I. et al., 2017). Tree after Dunn et al. (2014). Length of branches is arbitrary. RT: retrotransposons. References: 1: (Vitkova et al., 2005); 2: (Wicky et al., 1996); 3: (Niedermaier and Moritz, 2000); 4: (Traut et al., 2007); 5: (Nomoto et al., 2001); 6: (Bombarova et al., 2009); 7: (Barthelemy et al., 2008); 8: (Meyne et al., 1989); 9: (Castro and Holland, 2002); 10: (Li et al., 2007); 11: (Sinclair et al., 2007); 12: (Sakai et al., 2007); 13: (Fairclough et al., 2013).

Some of the studies on Cnidaria above quoted, investigated also possible differences in telomere length and telomerase activity in the different tissues and/or life stages of the studied species. The presence of telomerase activity in these phila is very important both from the point of view of conservation of the pathways and because some species have peculiar characteristics.

5.2 Aim of the work

Hydra represents a unique animal model for the absence of cellular aging, cellular senescence and for its high regenerative capacities, properties that may be related to the fact that these animals have high concentrations of stem cells (Bode, 2003). A goal of this work was to study the presence of very high telomerase activity in *Hydra vulgaris*. In addition, the activity of telomerase

has been analyzed in the presence of epigallocatechin gallate (EGCG), a known inhibitor of telomerase activity.

5.3 Results and discussion

Here, we found an intense telomerase activity in *Hydra vulgaris*. To verify the specificity of the telomerase activity, we used the inhibitor epigallocatechin gallate (EGCG).

EGCG is a type of catechin and is the most abundant catechin in tea, and particularly in green tea. This active ingredient is quite known due to its many beneficial properties. Among the most important the EGCG is an antioxidant that helps protect the skin from damage caused by UV radiation (Katiyar S. et al., 2007), is a water-soluble antioxidant (Mitscher LA, et al 1997), has antiviral activity and anti-inflammatory: inhibits interleukin 6 and 8, pro-inflammatory cytokines, useful in Chron's disease and in ulcerative colitis, and in the prevention of colon cancer (Shin HY et al., 2006).

In this preliminary study, animals were divided into 3 groups; 1) uncut specimens 2) cut specimens and 3) cut specimens treated with EGCG (10 μg/ml). Each group was composed of 15 polyps. In groups 2 and 3, polyps of *Hydra vulgaris* were cut in two parts at regular intervals (4/5 days) over a period of about seven weeks. Animals were monitored every day, using a stereoscope, to highlight possible death or malformations or delays in the regenerative rate. The experiment was repeated in biological triplicate. Up to seven cuts were made, so the experiment went on for at least seven weeks. Towards the end of the experiment, malformations began to appear.

Fig. 5.3 shows the telomerase activity present in *Hydra vulgaris*.

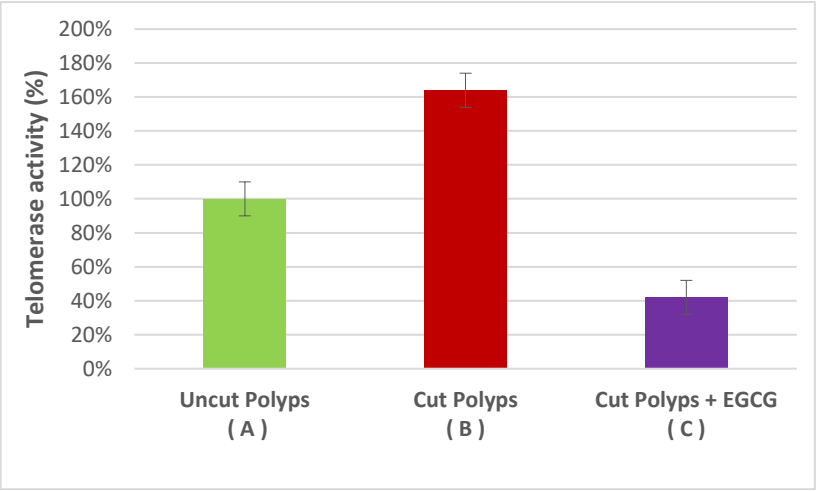

Fig. 5.3. Comparison of telomerase activity using the TRAP (Telomeric Repeat Amplification Protocol) assay.
A) untreated specimens were cut. B) uncut and untreated specimens. C) specimens were cut and treated with the EGCG.

After seven weeks of treatment, we found an increase of telomerase activity in cut hydras (Fig. 5.3B) with respect to control (*i.e.*, uncut specimens) (Fig. 5.3A). When cut specimens were co-incubated with EGCG, telomerase activity was strongly reduced (Fig. 5.3B).
Note that after two weeks from cut, a decrease in regenerative rate was observed in samples treated with EGCG with respect to untreated specimens, thus suggesting a possible correlation between reduced telomerase activity and the induction of senescence in *Hydra vulgaris*.
However, this hypothesis, although intriguing, remains to be further investigated.

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Material and Methods

Hydrae colture and conservation

In all the experiments, hydra of the species *Hydra vulgaris* from a line supplied by the university were used. The animals were kept at a constant temperature of 17 ° C according to a light / dark cycle of 16h-8h. Raised in glass tanks in a medium hydra culture medium consisting of CaCl2 1mM and NaHCO3, the animals were fed once a week, at regular intervals, with Artemia salina nauplii by standard methods (Lenhoff and Brown, 1970) (Artemia cysts hatched in artificial sea water - 35 g / liter non-iodized cooking salt) carefully washed with fresh water before adding them to the tank containing the hydro. The day after feeding the tanks containing the hydro are washed to remove the non-ingested artemia and the hydro are moved into tanks containing new hydra medium. For in vivo experiments, polyps were selected without gems or in development. The animals are treated according to the guidelines of the Roma Tre University. For each well a number of 15 hydro was taken and every effort was made to minimize the number of idra used.

Morphological and behavioral analysis

The polyps morphology and integrity behavioural it is continuously monitored before, during and after treatments. For optimal observation we used an optical microscope with a 32X magnification lens. The behavioral variables analyzed were the reactivity of the tentacles, the reactivity of the body and the adhesion of the substrate.

Cold Shock Treatment

To perform the molecular response following a temperature change, Hydra vulgaris animals were moved, with a specific insert for co-culture cell system (Sarstedt: TC insert for 6 well plate, PET membrane bottom, translucent, pore size 8 µm, sterile), from 17°C to 4°C Hydra medium beakers, for 1 minute, then placed again at 17°C and recovered in the incubator. The samples placed at 4 ° C are then selected at specific time intervals after the test (numbers expressed in hours after the stimulus): T0.25, T0.5, T1.5, T4, T12. T = 0 'is considered as a negative controll. For each time interval, groups of 15 Hydre are processed to perform RNA extraction and quantitative analysis of Real Time PCR. During treatment, the animals were continuously monitored using an optical microscope to analyze behavioral or morphological changes.

Pseudomonas a. PA14 cell lysate treatment

Single colonies of *Pseudomonas aeruginosa* PA14 (Rahme *et al*., 1995) were cultured for 18 h (O/N) in Tryptic Soy Broth (TSB, Acumedia) at 37 °C. Bacteria were centrifugated for 15' at 5000 rpm and washed in saline. The bacterial suspension was then diluted to an OD_{600} equal to 0.5 and sonicated three times for 5' in ice. The cell lysate was filtered through a Millipore membrane (pore size 0.45µm) and stored at 4 °C. The selected polips we incubated wit the lysate 20 µl, for the useful time.

Agonist and Antagonist of TRPA1

The selected hydres were incubated with the selective agonist of TRPA1, Glybenclamide G0639 (Sigma-Aldrich) (Babes A. et al., 2013) 2 µM, and with the selective antagonist HC-030031 (Sigma-Aldrich) (Babes A. et al., 2013), 0,1 µg / ml for 24h. Samples were collected at specific time intervals to perform RNA extraction and Real time PCR.

EGCG treatment

The selected hydres were treated with Epigallocatechin gallate (EGCG) (Sigma-Aldrich) molecule (10 µg/ml).

Western Blot Analysis

Samples were loaded in 7.5% SDS-PAGE minigels (Bio-Rad Laboratories, Mi, Italy). After separating by SDS/PAGE proteins were transferred electrophoretically to nitrocellulose membranes (Bio-Rad).
Ponceau red (Sigma) staining was used to ensure successful proteins transfer. Membrane was blocked (2 h, room temperature) with 1% Bovine Serum Albumin (BSA)/1X TBS/0.1% Tween-20. Membrane was incubated with rabbit polyclonal anti-TRPM3 antibody (O/N, 4°C, 1:100 dilution; Alomone Labs, Jerusalem, Israel). After washing with TBS/T, the membrane was incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1 h, room temperature, 1:20000 dilution; Amersham, GE Healthcare Europe, Mi, Italy) and the specific complex was detected by an enhanced chemiluminescence detection system (ECL, Amersham). Quantitation of protein levels was then performed by densitometry using Image Quant 5.2 software by Molecular Dynamics.

Evaluation of telomerase activity (RTQ-TRAP assay)

Telomerase activity was measured on 1 µg of protein by the SYBR green real-time quantitative telomerase repeat amplification protocol (RTQ-TRAP) assay, which was conducted as described elsewhere, Berardinelli et al., 2010, with minor modifications. The reaction was performed with protein extracts and anchored return primer mixed with SYBR Green PCR Master Mix (Biorad, Hercules, CA, USA). The reaction was performed using the Agilent AriaMx Real-Time PCR system (Agilent Technologies, Palo Alto, CA, USA). The threshold cycle values (Ct) were determined from semi-

log amplification plots (log increase in fluorescence as a function of cycle number) and compared with standard curves generated from serial dilutions of telomerase-positive (tel+) U251MG cell extracts. Each sample was analyzed in triplicate in at least three independent experiments. Telomerase activity was expressed relative to the telomerase-positive (tel+) sample.

RNA Extraction and cDNA Synthesis

The RNA was extracted using the commercial TRIzol® Reagent (Life technologies Italia-Invitrogen, Monza, Italy). After the addition of chloroform and the separation of two distinct phases, the RNA present in the aqueous phase was precipitated by the addition of isopropanol. Subsequently two washes were carried out in an aqueous solution of 75% ethanol and finally resuspended in distilled H2O. For each time interval 15 hydres were used, 1 μg of total RNA was reverse transcribed to cDNA in 20 μl of total reaction by using a protocol system GoTaq 2 Step RT qPCR System Protocol (Promega Italia Srl, Milan, Italy). The retrotranscription reaction provides in a first step the incubation of mix1, containing RNA at 70 ° C for 5min; after a 5-minute ice break, mix2 is added and incubated at 25 ° C for 5 minutes, 42 ° C for 60 minutes. The reaction was terminated with an incubation of 70 ° C for 15 minutes

Real time PCR (qPCRs)

PCR product quantification was calculated by applying the SYBR-Green method. The cDNA obtained by the retrotranscription was diluted 1:90 in H2O and for each sample technical triplicates were made. The reaction was carried out in 20μl of mixture composed of: 4μl of cDNA, 10μl of SYBR-Green, 5.2μl of H2O and 0.4μl of "sense" and "antisense" primers of the gene of interest. We used the Master Mix from Promega. Reactions were performed in a Agilent AriaMx Real-Time PCR system (Agilent Technologies, Palo Alto, CA, USA) using the following program: pre-incubation 95°C, amplification at 95 °C for 15 sec, 56 °C for 60 sec, 72 °C for 20 sec for 45 cycles, melting at 95°C-65°C-97°C, cooling at 37°C. The data are calculated relative to the internal housekeeping gene (β-actin) according to the second derivative test, delta–delta Ct (2-ΔΔCT) method, choosing control samples to normalize our data.

The primers pairs have been chosen so as to amplify the ~ 100bp fragments, and they are:

1) Actin
Fwd: 5’- TCC TTG TAT GCT TCT GGT CG - 3’
Rev: 5’- ATA ATG GCA TGG GGA AGA GC - 3’
2) NOS
Fwd: 5'- TAT CAA GCA GCA GGT GTG AC -3'
Rev: 5- TAC AGA TCC AGA AAG CGG AG -3
3) Nrf2
Fwd: 5’- CTA GTA GAG TCA TTA TCT CC -3’
Rev: 5’- AAA CTT GAA TCT GAC CTC TG -3’
4) SOD
Fwd: 5'- TCA GTT TGG GGA TTA TTC AGG TG -3'
Rev: 5- CAA AAC CAC CGG AAA TGC TGG A -3
5) NF-κB
Fwd: 5’- ATA ATT TTC CGC AAC CCG GC- 3’
Rev: 5’- AAA ATC AGG AAT CGC CGG AG - 3’
6) Periculin
Fwd: 5'- TGG ATA CAA ACC CAA GAA GG -3'
Rev: 5- CTA TAT AAC CAG CTC TGG GC -3
7) Hydramacin
Fwd: 5'- GAT GCA CGA AAT GGA GTC AG -3'
Rev: 5- TTA GTA ACA GAT GCA AGC CC-3

(Seonock, 2012).

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM) of n observations. We statistically analyzed mRNA expression level for each gene through three technical repeats from three biological replicates, n = 3 (3 triplicates for each gene in a single experiment, for a total of 3 experiments) with one-way ANOVA (and nonparametric) assay, followed by Bonferroni's Multiple Comparison Test.

Appendix

Manuscript in preparation during Ph.D.:

Russo V., Malafoglia V., Persichini T., Colasanti M.
"*Hydra vulgaris* and transient receptor potential ankyrin type 1 (TRPA1) in noxious cold nociception pathways".

Russo V., Persichini T., Colasanti M.
"Transient receptor potential ankyrin type 1 (TRPA1) mediate the innate immunity pathways in *Hydra vulgaris*"

Publications during Ph.D.:

I. Udroiu, **V. Russo**, T. Persichini, M. Colasanti, A. Sgura. (2017). Telomeres and telomerase in basal Metazoa. ISJ 14: 233-240.