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TESI

"Nociception in the cephalopod mollusc Octopus vulgaris: a contribution to mapping putative nociceptors in the octopus arm"

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LIST OF ABBREVIATION

5HT	5-hydroxytryptamine
6TM	Six-transmembrane
acTub	Acetylated Tubulin
ADORA2A	Adenosine A2a Receptor
ano1	Anoctamin-1
ANOVA	Analysis of Variance
ASIC	Acid Sensing Ion Channel
AT	Axonal tract
BLAST	Basic Local Alignment Search Tool
BMP	Bone Morphogenetic Protein
Ca	Calcium
CadN	Neural-cadherin
CALCRL	Calcitonin Receptor Like Receptor
CaMKII	Calcium/calmodulin-dependent protein kinase II
CGRP	Calcitonin Gene-Related Peptide
CPEB	Cytoplasmatic Polyadenylation Element Binding protein
CPM	Counts Per Millions
Ct	Cycle Threshold
CtBP	C-terminal-binding protein
DEG-ENaC	Degenerin-epithelial sodium channel
DRG	Dorsal Root Ganglion
ECE1	Endothelin-converting enzyme 1
EFSA	European Food Safety Autority
F	Forward
FaRPs	FMRFamide-Related Peptides
FITC	Fluorescein isothiocyanate
FL	Frontal Lobe
FR	FMRFamide receptor
GDNF	Glial cell line-Derived Neurotrophic Factor
Glu	Glutamate
Gmr5	Metabotropic glutamate receptor 5
GO	Gene Ontology
HIER	Heat Induced Epitope Retrieval
IASP	International Association for the Study of Pain
IB4	Isolectin B4
MgCl2	Magnesium Chloride

MID	Middle
MME	Neprilysin
MRP	MIP-related peptides
Na	Sodium
NF200	Neuro Filament 200 kDa
NGS	Normal Goat Serum
OL	Optic Lobe
OPMR1	μ-type opioid receptor
Ov	Octopus vulgaris
PBS	Phosphate Buffer Saline
pCO2	Partial pressure of carbon dioxide
PCR	Polymerase Chain Reaction
PROX	Proximal
PTGER4	Prostaglandin E2 receptor EP4 subtype
R	Reverse
SDS	Sodium Dodecyl Sulfate
SEM	Supraesophageal Mass
SP	Substance P
SUB	Subesophageal Mass
TAC	Tachykinin
Tkp	Tachykinin related Peptide
tll1	Tolloid-like protein 1
trkA	Tropomyosin receptor kinase A
TRP	Transient Receptor Potential channel
TTX	Tetrodotoxin
VG	Ventral Ganglia
VGSC	Voltage Gated Sodium Channel
VL	Vertical Lobe
WPIME	Working Party of the Institute of Medical Ethics

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ABSTRACT

I studied nociception in the cephalopod mollusc Octopus vulgaris by using a transcriptome-analysis, gene expression and immunohistochemistry with the aim of identifying putative nociceptors and possible pathways within the octopus arm. The analysis of the transcriptome allowed me also to identify a number of selected transcripts (> 30) and I evaluated predicted expression levels in silico in central and peripheral nervous system. My data, allow to identify fibres and cells suggested to be involved in nociceptive neural pathways for the first time in the arm of O. vulgaris.

1. Aim and Prologue

Aim of this PhD project is to contribute to the knowledge on the presence of putative nociceptors and nociceptive pathways within the nervous system of the cephalopod mollusc Octopus vulgaris.

In order to achieve this goal, I applied immunohistochemical and biomolecular approaches as tools to investigate the presence of markers known to identify candidate nociceptors in other species.

I used as target structures, the octopus arm and parts of the central nervous system, i.e. supra-, sub-esophageal masses and optic lobes.

The study combined bioinformatic, real-time qPCR, and immunohistological methods.

Despite classic work carried out by Graziadei, Young (see below) before 1970, and by other few neuro-anatomists, studies on nociception (and pain) in O. vulgaris are still very limited.

In addition, nobody has never approached the topic by integrating transcriptome, morphological and gene expression studies, to the best of my knowledge.

Recent studies by Dr R. Crook and coworkers, greatly expanded the field, but limiting the approaches to a physiological characterization, supported by limited morphological analysis.

My PhD had the ambitious aim to integrate also neurophysiology and behaviour, thus to attempt to provide a comprehensive view on nociception in the common octopus. The time passed and many of the expected experiments have not being even attempted, and therefore I will not achieve the original plan.

Nevertheless, I believe to have contributed to some extent, and think I have also promoted interest in the study.

During these years, I had the possibility to experience an environment highly sensitive to the inclusion of cephalopods, as sole representatives among invertebrates, in the Directive 2010/63/EU.

Among the cardinal principles upon which the Directive stands, is the limitation and/or effective control and management of pain, suffering distress and lasting-harms in all animal species.
This prompted a specific and urgent need to better understand nociception and pain perception, also with the aim to optimize anaesthetic protocols and to identify analgesics, a field that is still very primitive for cephalopods.
Morphological and behavioural evidences although limited, supporting the capacity of cephalopods to experience pain, were utilized as the basis for the recommendation by EFSA (Andrews et al. 2013) for the inclusion. Although behavioural responses (review in Borrelli and Fiorito 2008) provide some support for higher processing of signals from nociceptors, studies need to be carefully designed as the isolated arm of octopus will withdraw from a potentially noxious stimulus (Hague et al. 2013).

It is clear that a study on nociception in octopus, was considered pivotal for the research group I belonged, and for the general international context I was exposed.

Recently, Sneddon (2015) reviewed the evidence for pain perception in aquatic animals covering fish, molluscs and crustaceans. Together with colleagues, I briefly discussed the principles stated by Sneddon in a short note (Di Cristina et al. 2015). In the note, we also discussed suggestions by Della Rocca et al. (2015) in promoting an in-depth analysis of behavioural responses in cephalopods with the aim to facilitate the assessment of painful status in animals; i.e. identifying potential physiological and behavioural indicators to characterize a painful condition in cephalopods, by analogy to criteria used for vertebrates.
Again, contributing towards this avenue was part of the original plan, and I am fully aware that this is also another ‘leftover’.

There is an outstanding need to identify the most appropriate behavioural indicators that may be indicative of pain, suffering, distress or lasting harm in cephalopods. These behavioural indices are only preliminary pointed out in Table 5 of the Guidelines (Fiorito et al. 2015), but their validity and utility for daily welfare monitoring will

require more accurate species-specific analysis and should be supplemented with biomarkers measured non-invasively (Di Cristina et al. 2015).

I hope that future studies may contribute in the above lines and that my work, will assist future students.

2. Pain and nociception: a general overview

The International Association for the Study of Pain (IASP) define pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP 1994, p. 210). According to the common view, 'pain' results from the activation of sensory neurons, so-called nociceptors, that occur in the peripheral nervous system.

On the other hand, nociception (from the Latin nocere, meaning "to hurt/harm") is the simple perception of a noxious event that originate a response, typically a reflex withdrawal, bringing the organism away from the source of damage. In physiological terms, nociception is the neural process that allows encoding and processing noxious 'feelings' induced by - for example - intense thermal, mechanical, or chemical stimuli detected, as mentioned above, by a subpopulation of specialized peripheral sensory neurons and fibres: the nociceptors (Basbaum and Jessell 2000). These transduce the signal originating from those sensory inputs into higher neural centers, for further processing and decision making, resulting in appropriate (possibly protective) behaviors.

The ability to detect dangerous and/or damaging stimuli is adaptive and there is evidence that a sort of 'warning' system appeared very early during evolution.

The nociceptive system has been identified in invertebrates through to humans (reviewed in Sneddon et al., 2014).

In humans, at the basis of this system there is a dedicated class of sensory afferents (nociceptors), defined by the IASP as, "a receptor preferentially sensitive to a noxious stimulus or to a stimulus which would become noxious if prolonged".

According to IASP, it is important to distinguish between nociception and pain, since the latter "always" involves an emotional component, and nociceptor activation does not represent itself "pain".

The fact that "pain" occurs also in animals has been (and still is) extensively debated. There are still Authors that consider that only Primates and humans can experience the

adverse affective component due to the fact that neocortex is a recent innovation in brain evolution (e.g. Rose et al. 2014). In contrast, the fact that negative experience that results from tissue damage alters the animal's subsequent behavior and its capability to perform protective and guarding reactions, including avoidance learning, is seen as a case in favour of the existence of "pain" in other (lower) species (e.g. Sneddon et al. 2014).

If these features have not been evolved, animals would continue to damage themselves repeatedly, resulting in disease, loss of limbs and possible death.

On the other hand, evolution, ecology and life history may have shaped (for example) nociceptive and pain systems in different species inhabiting different environments to meet the demands of their environment in quite a dissimilar way (Broom 2001, Rutherford 2002). This to better fit the requirements of the most adaptable and profitable system to challenge that particular biota.

In recent years, the view that other non-mammalian taxa may have evolved brain structures that differ from the mammalian cortex anatomically sensu stricto, but capable to perform similar functions, provides an example that can be considered as light motif in the analysis of nociceptive/pain systems of animals living in different environments and belonging to far distant phylogenetical roots.

The idea of sensory afferents capable of specifically detect noxious stimuli has been originally proposed by Sir Charles Scott Sherrington (Nobel Laureate), who suggested the "considerable evidence" of the existence in the skin of a set of "nerve-endings" possessing the specific "office" to respond to stimuli that cause injury to the skin and that he then proposed to be "preferably termed nocicipient" (Sherrington 1903).

As reviewed by Smith and Lewin (2009), Escherichia coli possesses mechanosensitive channels that open to release solutes upon an osmotic down-shock to prevent bacteria lysis. However, being E. coli a unicellular organism, its ability to react to osmotic shock does not constitute a nociceptive response per se, due to the lack of cells dedicated to the purpose of detecting noxious stimuli.

The nervous system seems to appear from the early evolution of Eumetazoa. Parazoa lack a nervous system, but genes associated with neuronal development have been identified in Porifera, and in some species globular cells are considered to be "proto-neural" cell . It is within the Cnidaria and Ctenophora that a basic nervous system appears and the monophyletic origin of the nervous system is thought to have occurred in their immediate common ancestor (Cavalier-Smith et al. 1996). According to Smith and Lewin, it is from this time point that evidence for nociceptors should be traced back. The diffuse nerve net of cnidarians is capable of modulating organismal responses, as those observed in sea anemones in cases where strong stimulations leads to a reflex response (the closure of the animal) that might be viewed as a nociceptive response (see Smith and Lewin 2009 and cited works therein).

A review of the evolution of "true" nociceptors and their specialization is out of the aims of this Thesis. However, it is noteworthy to underline that the acquisition of different capabilities by nociceptors from an evolutionary viewpoint, starting with Cnidaria with an ability to sense a noxious mechanical stimulus but not possessing defined nociceptors, and ending with mammals, which have both myelinated and unmyelinated nociceptors capable of detecting a wide range of mechanical, thermal and chemical stimuli is proposed in figure 4 by Smith and Lewin (2009). In the graph the Authors depict the distribution of the capability to respond noxious stimuli (mechanical, heat, chemical, cold) and the presence of myelinated and un-myelinated nociceptors among different taxa.

What is interesting for this Thesis is that un-myelinated nociceptors, together with the evidence of the capability to respond to some noxious stimuli, is reported for Mollusca (e.g. Aplysia; Smith and Lewin 2009).

On the other hand the absence of myelinated nociceptors in non-vertebrate taxa, is strictly linked with the existence and evolution of myelin (e.g. Hartline and Colman 2007, Roots 2008, Castelfranco and Hartline 2015). This is considered to be a vertebrate innovation, however functionally-equivalent "ensheathments" of axons appear to be present independently in other animal taxa such as annelids and some crustaceans (Hartline 2008). Future studies are required in order to establish whether

other invertebrates may possess analogous myelin-like, thus further challenging this view.

2.1. A short account on Nociceptors

The axons of nociceptors are distributed in skin, organs and muscles and are considered to be characterized by two major classes of fibres (Meyer et al. 1985): Aδ and C fibres (Fig. 1).

Aδ fibres are mid-size myelinated (diameter, 1.0 – 5.0 μm) afferents characterized by conduction velocity of 2.5 – 30 m/s, that mediate acute, well-localized, "first" or fast pain sensations. These myelinated afferents are different from the larger in diameter and rapidly conducting Aβ fibres, that respond to mechanical stimulations that do not elicit painful experiences.

The second class of nociceptors includes small diameter (0.2 - 1.5 μm) unmyelinated C fibres (conduction velocity: 0.5 – 2.5 m/s) that convey poorly localized, "second" or slow pain feelings (Basbaum et al. 2009). The regenerative capabilities of C fibers is very slow (Murinson & Griffin 2004), and they may still only regain incomplete function (resulting in abnormal sensory function). C fibres respond to stimuli which have strong intensities, and are the ones – as mentioned - to account for the slow, but deeper and spread out over an unspecific area second pain. They are considered as polymodal because they can react to various stimuli; there is also a lower percentage of C fibres responding only to specific noxious stimuli (e.g. heat).

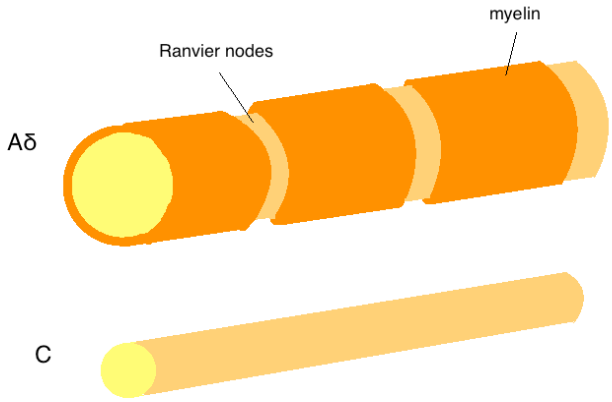

Figure 1. Aδ and C nociceptive fibers.

Muscle nociceptors are known to have the morphological appearance of free nerve endings, meaning that in the light microscope no receptive (corpuscular) structure can be recognized (Stacey 1969).

Nociceptors can also be distinguished according to their differential expression of proteins involved in the transduction and conduction of noxious signals, such as membrane channels and neurotransmitters. As schematized in Fig. 2, many ion channels confer to nociceptors sensitivity to chemical irritants, extreme temperatures (TRPs: transient receptor potential channels), acidic pH (ASICs: acid sensing ion channels), pressure (Julius & Basbaum 2001; Mense et al. 2010; Kim et al. 2012).

TRP channels are putative six-transmembrane (6TM) polypeptide subunits that assemble as tetramers to form cation-permeable pores. TRPV1 (transient receptor potential cation channel subfamily V member 1) is a Ca^{2+}-permeant ion channel activated by many exogenous and endogenous stimuli (high temperatures, capsaicin, acidic pH, allyl isothiocyanate), and inhibited by intracellular phosphatidylinositol-4,5-bisphosphate. $Trpv1^{-/-}$ mice are defective in nociceptive, inflammatory and hypothermic responses to vanilloid compounds, supporting the interpretation that TRPV1 contributes to acute thermal nociception and hyperalgesia after tissue injury (Caterina et al. 2000). TRPV1 immunoreactivity is frequently used as a neurochemical

marker for nociceptors. Many TRPV1-immunoreactive neurons contain the neuropeptides CGRP or SP, whereas others bind IB4 (Tominaga et al. 1998).

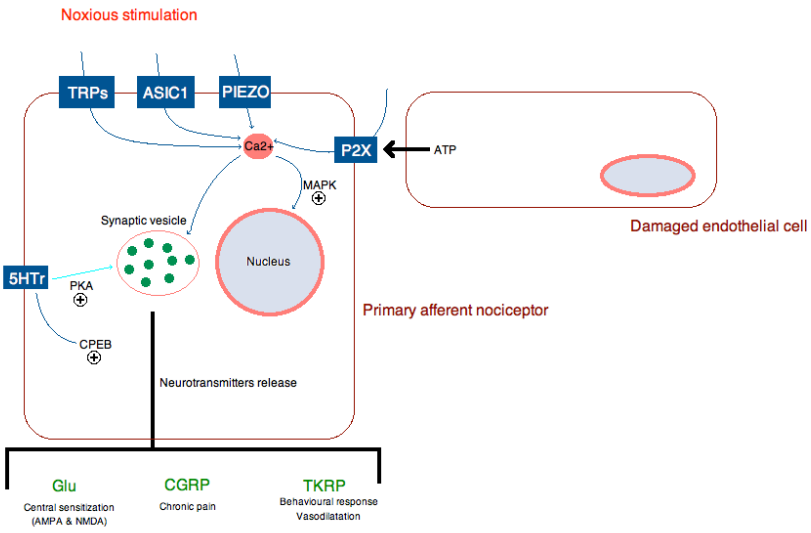

Figure 2. Nociceptive nerve endings and the underlying cellular machinery.

Nociceptive afferent fibres differ from other fibres in that they are equipped with a special type of sodium channel that cannot be blocked by tetrodotoxin (TTX), the toxin of the puffer fish (Matsutomi et al. 2006). Two TTX-resistant Na^+ channels important for nociception are the Nav (voltage-gated sodium) channels 1.9 and 1.8 (Akopian et al. 1999).

The isolectin IB4 (a glycoprotein isolated from the seeds of the tropical African legume Griffonia simplicifolia) is able to recognize the glycosylated extracellular domain of Ret receptor (Boscia et al. 2013) a transmembrane glycoprotein belonging to the receptor tyrosine kinase family. This lectin has been used in several researchers in order to identify population of nonpeptidergic neurons within the peripheral and central nervous system (Ruscheweyh et al. 2007, Price & Flores 2007). In mammals, IB4-positive neurons have small-sized cell bodies and primarily give rise to unmyelinated fibres, many of which are nociceptive. IB4-positive neurons comprise one of two broad classes of small-diameter, C-fibres sensory neurons. The other class (IB4 negative) typically expresses neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) and expresses trkA receptors for nerve growth factor

(NGF). IB4-positive neurons express receptors for glial cell line-derived neurotrophic factor (GDNF) and depend on GDNF for survival after birth. The nociceptive primary afferent fibres also contain peptidic transmitters, which are released from C fibres in response to noxious stimuli (e.g. Substance P, CGRP). Calcitonin-gene related peptide (CGRP) is a peptide produced in peripheral and central neurons (Rosenfeld et al. 1983) which act as a potent vasodilator and is involved in the transmission of pain. Most CGRP-expressing sensory neurons are small or medium size in diameter (many are nociceptors), but a few are large diameter. This is consistent with the finding that the afferent fibres of CGRP-positive neurons are primarily unmyelinated C fibres and thinly myelinated Aδ fibres and a few are large myelinated Aβ fibres (McCarthy and Lawson 1990).

Furthermore, nociceptors can be classified as peptidergic and not peptidergic.
During differentiation, non-peptidergic nociceptors stop expressing TrkA (a receptor to nerve growth factor) and begin expressing Ret, a transmembrane signalling component, which allows the expression of the glial cell-derived growth factor (GDNF). This transition is assisted by Runx1, a transcription factor which has proven to be vital in the development of non-peptidergic nociceptors.
Peptidergic nociceptors instead continue to express TrkA and completely different type of growth factor (Woolf & Ma, 2007).

Finally, most of mammalian nociceptors show nociceptive sensitization (nociceptor can change from being simply a noxious stimulus detector to a detector of non-noxious stimuli) after prolonged injury or inflammation (Gold & Gebhart 2010, Walters 2012). Sensitization shares conserved mechanism (i.e. behavioural, cellular and epigenetic) across phyla, and between pain and memory phenomena.
It is a non-associative conditioning response and the outcome appears enhanced following repeated stimulation (Rahn at al. 2013). In the gastropod Aplysia californica, long-term sensitization requires coordinated pre- and post-synaptic modifications (Bailey at al. 1996), such as synaptic facilitation and synaptic capture, which are mediated by new protein synthesis. The latter is regulated by an isoform of the

cytoplasmatic polyadenylation element binding protein (CPEB), that activates "sleeping" mRNA in the cytoplasm and has also a "prion-like" activity (Si et al. 2003).

3. Pain in fish and invertebrates

3.1. A general overview

In vertebrates, noxious information is relayed from primary nociceptors to brain structures as the thalamus and the somatosensory, insular and anterior cingulate, cortices passing through the dorsal horn of the spinal cord.

In fish, and of course in invertebrates, there are not known homologues to these brain structures.

As mentioned above, this actually does not prove that these species cannot feel pain, since independently derived neural structures might have assumed the same functions (Crook & Walters 2011).

The ability of an organism to detect, respond and avoid noxious stimulation is certainly a profitable trait and there is no reason to think that it is restricted just to higher vertebrates.

In teleosts both myelinated and a significant number of unmyelinated fibers are present, while in elasmobranchs do not seem to possess unmyelinated fibers (Sneddon, 2004). Electrophysiological evidences reported the presence of nociceptor classes similar to those in mammals (Sneddon, 2003; Ashley et al. 2007) in the trigeminal nerve of the trout Oncorhynchus mykiss, but the scientific community seems to have conflicting opinions on the ability of fish to feel pain, as emerged with the publication of Key's paper, and its numerous commentaries appeared in the first volume of the journal "Animal Sentience: An Interdisciplinary Journal on Animal Feeling" (Key 2016). A review is also available in Smith and Lewin (2009).

The first invertebrate in which nociceptors have been identified is the medicinal leech Hirudo medicinalis (Nicholls & Baylor 1968); these are slow adapting neurons that respond to capsaicine (Summers et al. 2014).

In the land snail Cepaea memoralis withdrawal response to the hotplate (~ 40°C) test is altered if treated with opiate agonists (Kavaliers et al. 1984). The animal also possess endogenous δ-receptor agonists in its peripheral nervous system (Sarakhov et al. 1993).

Among molluscs, also in Aplysia californica nociceptive innervation is present in the siphon and the mantle (Castellucci et al. 1970, Illich & Walters 1997). In Drosophila the painless gene has been showed to be necessary for the detection of noxious heat in larvae (Tracey et al. 2003). The gene encodes for a TRP ion channel that is an evolutionary homolog of the mammalian TRPA1 (Al-Anzi et al. 2006).

So far, across invertebrates, polymodal and specialized nociceptors have been anatomically identified in four phyla (Table 1).

Functional similarities between nociceptive systems in invertebrates and mammals, such as the presence of high threshold primary sensory neurons (nociceptors) which possess specific membrane receptors (Pastor et al. 1996, Smith and Lewin 2009), may reflect the conservation of very primitive injury-related processes (Kumazawa 1998).

Table 1. List of invertebrate species in which polymodal nociceptors (P) or nociceptors responding to specific stimuli (M: mechanical, C: chemical, T: extreme temperature, and A: extreme pH) have been identified in a specific type of sensory neurons.

Phylum	Species	Neuron type[1]	M	C	T	A	P	References
Arthropoda	Drosophila melanogaster	MD-neuron					✔	Tracey et al 2003, Goodman 2003
Nematoda	Caenorhabditis elegans	Class IV neurons	✔		✔			Chatzigeorgiou et al. 2010, Zhong et al. 2010
Anellida	Hirudo medicinalis	N neurons		✔		✔		Nicholls & Baylor 1968, Pastor et al. 1996, Summers et al. 2014
Mollusca	Aplysia californica	ASH neurons					✔	Kaplan & Horvitz 1993
		PVD neurons	✔					Tobin & Bargmann 2004
		LE and VC neurons	✔					llich & Walters 1997, Walters et al. 2004

[1.] MD neuron: Multidendritic sensory neurons; Class iv neurons: dendritic arborization neurons (sensory); N: Nociceptive neurons; ASH neurons: Amphid single ciliated ending neurons (nociceptors); PVD neurons: mechanosensory neurons; LE: LE cluster abdominal ganglion; VC part of the pleural Ventro-Caudal cluster (mechanoreceptors).

3. 2. Criteria for pain in fish and invertebrates, summary of evidences

The Working Party of the Institute of Medical Ethics (WPIME) identified seven criteria that might provide evidence for pain experience in animals (Bateson 1991). If we choose to rely on those criteria, the situation for fish and two class of invertebrates (i.e. cephalopods and decapod crustaceans) maybe summarized as follows.

i. Possession of receptors sensitive to noxious stimuli: teleost fish possess nociceptors (Sneddon, 2003, Ashley et al.2007, Mettam et al. 2012), and they are likely to be present also in cephalopods (Hague et al. 2011, Crook et al. 2013, Alupay et al. 2014); they have not been identified in crustaceans, to the best of my knowledge (but see: Gherardi 2009, Puri & Faulkes 2010, 2015, Elwood 2012). Nevertheless, FMRFamide and substance P immunoreactive afferents have been identified in the nervous system of several species of decapod crustaceans (Mancillas et al. 1981, Sandeman et al. 1990, Schmidt and Ache 1996).

ii. Possession of brain structures analogous to the human cerebral cortex: analogous structures are the result of convergent evolution, the process by which two very genetically different species evolve structures having the same or a similar function. Therefore, an analogous of the cerebral cortex would be a nervous substrate in which sensitive, motor and associative areas are connected and work together to analyze and elaborate afferent stimuli.

In fish, multiple brain areas are active during noxious stimulation (Sneddon et al. 2015) and the telencephalon and pallium in fish may perform the same functions of mammalian cortex (Ng 2016).

In cephalopods, the vertical and frontal (superior and inferior) brain lobes are involved in learning and memory, and the cellular analogue (Long Term Potentiation) to the mammalian hippocampus has been characterized in the octopus (Shomrat et al. 2008). The same analogy of function appears in the lobula of crustaceans (Tomsic et al. 2003).

iii. Possession of nervous pathways connecting nociceptive receptors to higher brain structures: the connections between nociceptors and central nervous system have not yet been precisely identified nor in fish, neither in cephalopods and decapod crustaceans (but see discussion in Burrell 2017). Nevertheless, in Octopus vulgaris putative "pain" fibers rising from the receptors of the arm have been identified (Young 1965). The majority of these fibres make synapses in the ganglia of the axial nerve cord of the arm, where large and small neurons and their fibres form afferents that enter the brain from the brachial nerves. Their projections to the vertical lobe (i.e. the higher computational centers: Young, 1991) have not been revealed.

iv. Possession of receptors for opioid substances found in the central nervous system, especially the brain: opioid receptors and endogenous substances are present in the fish nervous system (Singh and Rai 2010). Two species of crustaceans (Squilla mantis and Carcinus mediterraneus) showed morphine-induced analgesia to noxious stimulation (Maldonado & Miralto 1982), and opioid receptors have been found in the thoracic and in the eye stalk ganglia of the crab Carcinus maenas (Hanke et al. 1996,

1997), and also in several tissues of the cephalopod Octopus ocellatus (Sha et al. 2007, 2012, 2013).

v. Analgesics modify response to noxious stimuli and are chosen by the animal when the experience is unavoidable: in fish analgesic drugs reduces adverse changes in complex behavioural responses after a painful event (Sneddon 2003; Mettam et al. 2011). Studies on the effects of analgesics on cephalopods and decapod crustaceans are not known to the best of my knowledge.

vi. Responds to noxious stimuli by avoiding them or minimizing damage to the body, and avoidance is relatively inelastic: fish, cephalopods and decapod crustaceans show behavioural response (avoidance, tail beating in fish, rubbing, hypersensitivity to touch and wound-directed behaviours) to noxious stimuli (see reviews for example, for fish: Sneddon et al. 2015, for cephalopods: Andrews et al. 2013, for crustaceans: Elwood 2011). Many species od crustaceans and cephalopods also respond to noxious stimulation with leg or arm autotomy (Wood & Wood 1932, Hanlon & Messenger, 1996), a defensive behaviour that can be interpreted as a reaction to severe pain (Coderre et al. 1986).

vii. Response to noxious stimuli persists and the animal learns how to associate neutral events with noxious stimuli: fish learn to avoid electric shocks usually in one or a few trials (e.g. Yoshida & Hirano 2010) and the avoidance persists for up to 3 days (Dunlop et al. 2006). This criterion is fully satisfied even in cephalopods (see also below) because of studies of learning and memory using an electrical shock as a negative reinforce (e.g.: Messenger 1973, Robertson et al. 1996) or the “prawn in the tube” protocol (Agin et al. 2006). Also Shore crabs (Carcinus maenas) do show avoidance learning to electric shock (Magee and Elwood 2013).
In conclusion, almost all criteria are satisfied in fish, but a few less in cephalopods and decapod crustaceans. This does not necessarily mean that those animals are capable to feel pain, but should at least be a starting point to better investigate the issue.

3.3. Pain and nociception in Cephalopods

As reviewed by Borrelli and Fiorito (2008) a long series of learning studies with Octopus vulgaris and other cephalopod species proven that these animals are fully capable of learning a large variety of tasks. In addition these studies also served to show that cephalopod brain regions are capable of storing memories of visual or tactile learning experiences (review in Young 1991).

In the overview by Wells (1978), while describing afferent and efferent tracts of the vertical and the median superior frontal lobe of octopus, the presence of 'pain' pathways is included. These accounts for sensory afferent signals from the arms, mantle and viscera that could reach the 'higher' levels of the nervous system. Nevertheless, he commented that they are "generally assumed to signal 'pain' though there is no proof of this and it might well carry 'pleasure' or any other signal" (Wells 1978, p. 364).

In addition, Young (1991) mentioned fibres "indicating pain" that are activated when trauma occurs and reach a small set of large neurons in the sub-frontal lobe. Young suggested that the activation of those fibres prevents the animal to touch an object if pain is perceived (Young 1991).

Pain signals create a tendency to reject the pattern of touch modifying synapses in the sub-frontal lobe. Indeed, after lesioning that area, the octopus fails to learn not to take objects from which electric shocks are obtained. The results of a long series of experiments reviewed by Wells (1978), Young (e.g.: 1971, 1991) also accounts for a residual capacity for learned discrimination which is suggested to lie in the suboesophageal mass or in the arms ganglia.

Most recently, Crook et al. (2013) identified putative nociceptors (high threshold mechanoreceptors) in the fin of the squid Doryteuthis (Loligo) pealeii. In the study, stimuli were delivered by series of four von Frey filaments (0.4, 2, 10, and 100 g) and the activity from neurons innervating the fin was recorded using a suction electrode on the cut end of the fin nerve. A significant reduction in threshold for activation by mechanical (von Frey filaments) and electrical test stimuli was observed measured five minutes after the application of the stimulus.

Furthermore, except for the 100 g von Frey filament stimulus, significantly more spikes were evoked by a second application of each force than the first one. The

application of an anaesthetic in the injury site ($MgCl_2$) suppressed almost all the activity evoked by the crush.

Putative nociceptors identified by Crook and his colleagues selectively encode noxious mechanical, but not thermal stimuli, and show long-lasting peripheral sensitization and spontaneous activity not only near the injury site but also on the contralateral side of the body.

Alupay et al (2014) carried out a similar procedure in the octopus Abdopus aculeatus. Semmes-Weinstein filaments were applied to two positions on the arm of the animals (proximal and distal to the tip) and recorded the evoked activity using a suction electrode applied to the axial nerve cord. Their results show that the crushes on the arm tip produce short-term sensitization (5 min) of the mechanosensory units in response to both light and heavy mechanical stimulation.

As mentioned above, there are still few published evidences to indicate whether or not cephalopods have opioid or other receptor/transmitter systems (e.g. cannabinoid) that could modulate pain perception (Martin et al. 1986, Voigt et al. 1981, Stefano et al. 1981, Sha et al. 2012).

This short summary of evidences and those based on the large body of knowledge available on learning in cephalopods, in avoidance or following negative reinforcements, seem to corroborate the view that cephalopods show reflex responses to the application of noxious stimuli (probably without reference to the brain), and that injuries evoke hypersensitivity to touch and wound-directed protective behaviour. The clear involvement of the central nervous system in these responses, and thereby the identification of the areas of the brain that receive and elaborate the nociceptive information is still missing.

4. The nervous system of the arms and the suckers of Octopus vulgaris

In this section I will overview the knowledge available on the nervous components identified in the arms and suckers of O. vulgaris. This with the aim to provide the background for the following description of my results.

4.1. General outline

The nervous system in the arms of the octopus is represented by nerve ganglia, assigned to motor and interconnecting functions, and by peripheral nerve cells, representing the sensory system (Young 1971). Most of the peripheral neurons are located in the axial nerve cords, which are organized into an extensive nervous system comprising both sensory and motor circuits (Young 1963, Rowell 1966, Graziadei 1971) and plays a major role in the control of arm behaviour. The main axial nerve cord is accounted to represent roughly one-fifth of the volume of the arm; two parts are described: an outer part, consisting of nerve cells of various shapes and size, presents special characters for each cephalopod; the other is a central region of fine argyrophil filaments usually interlocking, which constitute the neuropil (Rossi & Graziadei 1954).

It essentially consists of a chain of ventral ganglia and two dorsal axonal tracts. Since the axial nerve cord aligns on an axis of symmetry with the axis of the suckers, and since the suckers are alternately arranged on two longitudinal rows, the perineural space of the central cavity (where the axial nerve lies) forms ventrally a series of alternating extensions and shrinkage at right and left. At the larger side, vessels appear larger, with circular walls, and filled with a substance that is coloured by Azan's red; on the other side (the ventrolateral angle of the cavity), vessels are more flattened.

Cellular elements are arranged at the periphery of the cord as a coating, even if they lack on the dorsal side, wherein the two strands of longitudinal fibres are located. The other three sides are delimited by two edges, from which emerge nerve trunks that are mostly directed to the sucker. Nerve fibres which exit the ventral lateral side of the spinal cord are generally grouped into bundles of different volume. Rossi and Graziadei (1954) suggest that the larger trunks are those that come along both edges between the ventral and lateral faces, and head to the suckers.

Tracts emerging from the lateral side of the cord, close to each longitudinal one, also have a remarkable size. They head firstly dorsally, running on the same surface of the

cord, then pass through the space of the epidural cavity and they reach the intrinsic musculature at the union of the lateral longitudinal and dorsal longitudinal muscle groups. These beams then pass through a gap between these two muscle systems, distributing a large part of their nerve fibres; then they reach the skin where they disperse.

Nerve fibres that constitute the outgoing trunks bordering the nerve cord seem to come from the neuropil, especially from the most superficial part. During their passage in the musculature and in the conjunctival sheath, each of the beams that constitute the nerves flattens and becomes laminar. When they arrive in the peduncle, they regain their original cylindroid form and assume a very "tortuous" trend (Rossi & Graziadei 1958).

After crossing the membrane of the acetabular musculature, nerve bundles are then involved in the radial muscles between secondary sphincters. The presence of secondary sphincters against the conjunctival sheath that envelops all the sucker, limits the space required for the installation of radial fibres; therefore, they are forced to tighten closely against each other in the narrow interstices between the sphincter, thereby forming pillars (Fig. 3).

The deep ramifications that reach the membrane that envelops the secondary sphincters pass through it, more often going in a new direction; they are distributed in the elements of the secondary sphincters, forming the system of circumferential fibres.

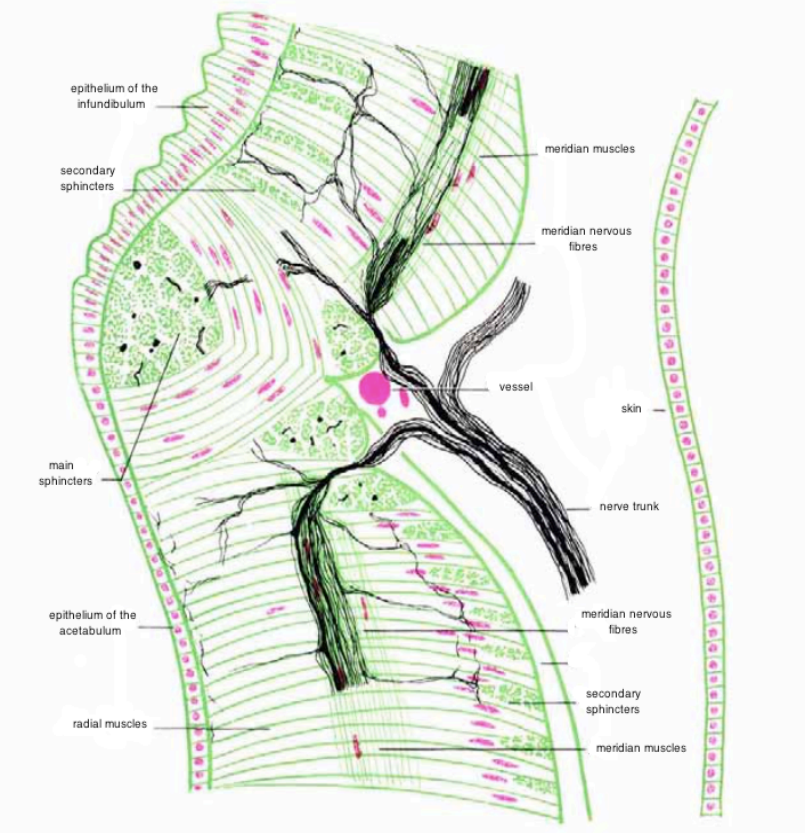

Figure 3. Diagram taken from a median sagittal section of the sucker of Octopus vulgaris (modified from Rossi & Graziadei, 1958).

Rossi and Graziadei (1958) identified, within the sucker muscles, abundant nerve fibres small in diameter (2-5 μm) and less abundant fibres with larger diameter (6-15 μm). The larger ones in cross sections appear provided with a thick surface sheath boundary; inside the sheath, there is often a vacuum or an amorphous substance, slightly or not at all argyrophil.

In the nodal points of the network there are elongated nuclei. These nuclei have the direction of the argyrophil filaments gathered in small bundles; they seem locked in the same filaments and should be putative elements of the Schwann sheath or the lemnoblastes of vertebrates.

There are also isolated filaments, relatively thick and apparently unbranched, especially visible in the portion of the infundibulum located closer to its epithelial surface and which appear to reach the muscle through the membrane implantation of the epithelium. They represent neurites of primary sensory cells that are widely distributed among the epithelial cells.

The coating epithelium of the cavity of the sucker is a monolayer, but with different characters in the two parties, acetabular and infundibular. The epithelium of the infundibular portion (Fig. 4) is much thicker than that of the acetabulum, is made of several rows of nuclei arranged at different heights: the superficial cells are cylindrical and towering, the deepest, which are interleaved with the first, are much less high and often rounded. The infundibulum epithelium is also covered by a thick (30 to 50 μm) chitinous cuticle that periodically detaches from the epithelium located below.

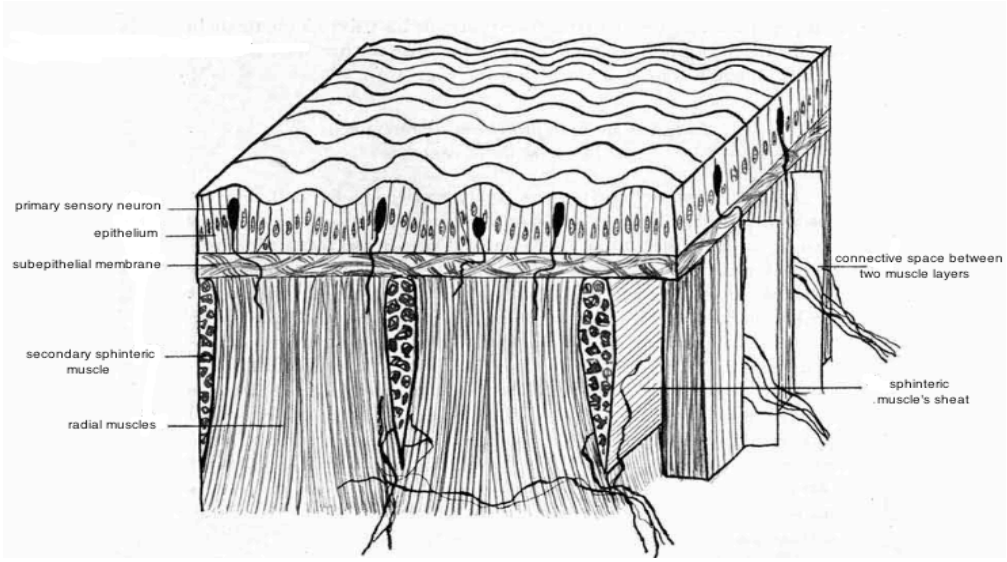

Figure 4. Reconstruction of the wall of the infundibulum of the sucker of Octopus vulgaris (modified from Rossi & Graziadei, 1958).

The pores on this outer cuticular surface are where the distal poles of the sucker sensory cells reach the external surface through the pore channels, so that they are able to enter into contact with the seawater (Graziadei 1964).

The marginal decline (or sucker rim, Fig. 5) is a soft and plastic epithelial bead that surrounds the opening of the sucker, which establish adhesion with the surrounding medium.

While the infundibular area serves for fixing, the marginal fold closes the communication between the cavity of the sucker and the external environment and allows the formation of the vacuum which cooperates to the successive expansion of the acetabulum.

Ball shaped neurons, whose distal part seems to be filled with a clear substance, are very frequent especially in the infundibular epithelium and they also extend to the epithelium of the marginal fold.

Girod (1884) considered the marginal decline as formed by a set of special nerve endings probably playing a particular role in some kind of sensory perception. Sensory cells are mainly located in the marginal decline, which lacks a cuticular layer.

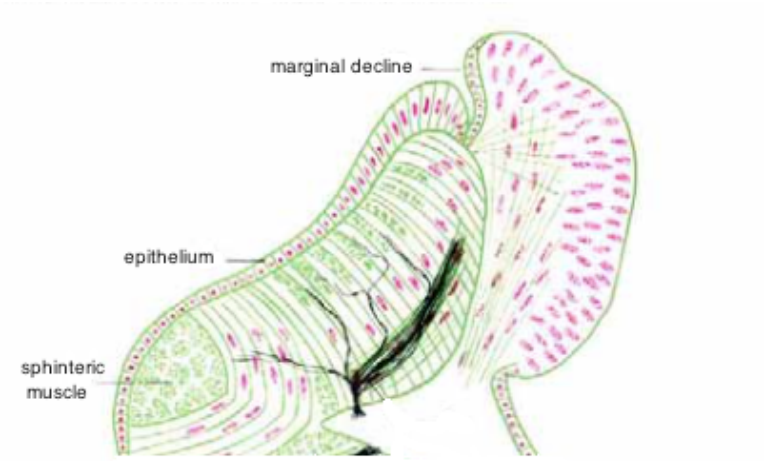

Figure 5. The marginal decline of the octopus sucker (modified from Graziadei & Rossi 1958).

4.2. Putative Receptors and sensory cells

Many primary receptors lie in the epithelium covering the surface of the arm.

The sucker, and particularly its rim, has the greatest number of these sensory cells, while the skin of the arm is rather less sensitive. Rough estimates of the receptors indicate that several tens of thousands of receptors lie in each sucker, bringing the total estimate of the receptors in all the arms of an octopus to as much as 2.4×10^{8}.

Three main morphological types of receptor are found in the arms, all with the cell body in the epithelium.

There are round cells, irregular multipolar cells, and tapered ciliated cells. All these elements send their processes centripetally towards the ganglia.

Ciliated receptors (putative chemo-receptors) are the most abundant, their axons meet encapsulated nerve cells lying underneath the epithelium and make synaptic contacts

with their dendrites. Round and multipolar receptors, on the contrary, send their axons straight to the ganglia where motor neurons lie.

4.3. Connections with the nerve cord and higher neural centers

The ganglion of the sucker is a small assembly of nerve cells lying below the acetabular cup of each sucker, among connective tissue and peduncular muscles. Each ganglion consists of a few hundred neurons, the number depending upon the size of the sucker. Some of them are motor neurons and send axons to the muscles of the peduncle and the sucker. Other bipolar and multipolar neurons have been observed in this ganglion, but their function is still unknown.

Morphological studies prove that motor neurons for muscles of the sucker and peduncle lie in the ganglion, and that sensory receptors send axons towards this ganglion (Graziadei, 1965).

According to Young (1965) the nerve fibres entering or leaving the brain of O. vulgaris can be grouped into several types:

1. General somatic sensory fibres (for mechanoreception, chemoreception and nociception).
2. Special somatic sensory fibres (from eyes, statocysts, olfactory organs and chemoreceptor of the lips).
3. Proprioceptor fibres (in mantle, arm and lips).
4. Visceral sensory fibres (from the digestive system and other viscera).
5. General somatic motor fibres (from the central nervous system or peripheral ganglia, they reach the muscles of the mantle).
6. Chromatophore nerve fibres (from the chromatophore lobes, they seem to contain no afferents).
7. Visceral motor fibres (numerous and small, from peripheral ganglia).
8. Vasomotor fibres (from the subesophageal mass to the walls of the blood vessels).

Young also reports the presence of 30,000 post-ganglionic nerves in the arm and the suckers of the octopus, composed by 1.5×10^6 efferent fibres (the largest diameter being 6 μm), but does not describe the numbers and the appearance of the afferent nerves. Probably none of the efferent fibres run directly from the central nervous system to muscles of the arm.
About 25 nerves leave the axial nerve cord of the arm where it is swollen to form a ganglion opposite each sucker. Each nerve contains medium-small fibres (up to 6 μm) and some smaller ones (Table 2). In addition, each sucker sends 12 subacetabular nerves to the sucker muscles, with about 20 fibres in each, thus counting about 250 fibers per sucker.
Each arm bears approximately 200 suckers thus there are $2\text{-}5\times10^5$ motoneurons for the suckers in each arm.

Not all of the fibres reaching the brain arise from primary receptor cells with cell bodies in the skin. Indeed, for the arm nerves, there is evidence that most or all of the afferent pathways include at least one peripheral synapse.

There are four types of receptor cell in the margins of the suckers, though provisionally functions which has been assigned to them by Young (1965) as reported in Table 2. Some of these fibres make synapse with subepithelial nerve cells, of which there are about 300 in each sucker. Others end in the sucker ganglia, which are reflex centres, each containing some 300 cells.
However, the majority of the afferents probably end in the central ganglion of the main cord beneath each sucker. Here there are numerous large and small cells, and the fibres of some of them must compose the 140,000 or so afferent fibres that enter the brain from the brachial nerves.

Therefore, there is evidence for a reduction of about one hundred times in the number of afferent fibres between the periphery and the brain, and at least as great an increase on the efferent pathway, from $3\text{-}2\times10^4$ fibres in the brachial nerves to 3×10^6 final motoneurons.

Table 2. Abundance of receptors in the margins of the octopus suckers and their provisional functions according to Young (1965)

Receptors	number of receptors per sucker	Total number of receptors (one side)
Chemo-	8000	6.00×10^6
Touch	2000	1.60×10^6
Tension	1000	0.80×10^6
Pain	200	0.16×10^6

A special feature of the nervous system of cephalopods is the wide use of peripheral reflex centres, especially in the arms. This decentralization (e.g. Sumbre et al. 2001) presumably produces its own special requirements for the numbers and sizes of the pre- and postganglionic fibres.

The data available suggest that relatively few, larger preganglionic fibres control many, smaller postganglionic ones, these latter being also under the influence of local afferent fibres.

As mentioned, there is also a reduction by one hundred times in the number of fibres between the periphery of the suckers and the brain. The latter thus receives the essential information and serves to make general decisions as to types of action whose detailed execution is then controlled by the peripheral reflex centres.

Within the intrinsic musculature of the octopus' arm there are four smaller intramuscular nerve cords (Fig. 6), continuous all along the length of the arm, which lie in small canals and are linked to the main axial nerve cord by means of segmental connectives at regular intervals (Guérin 1908, Rossi & Graziadei 1956).

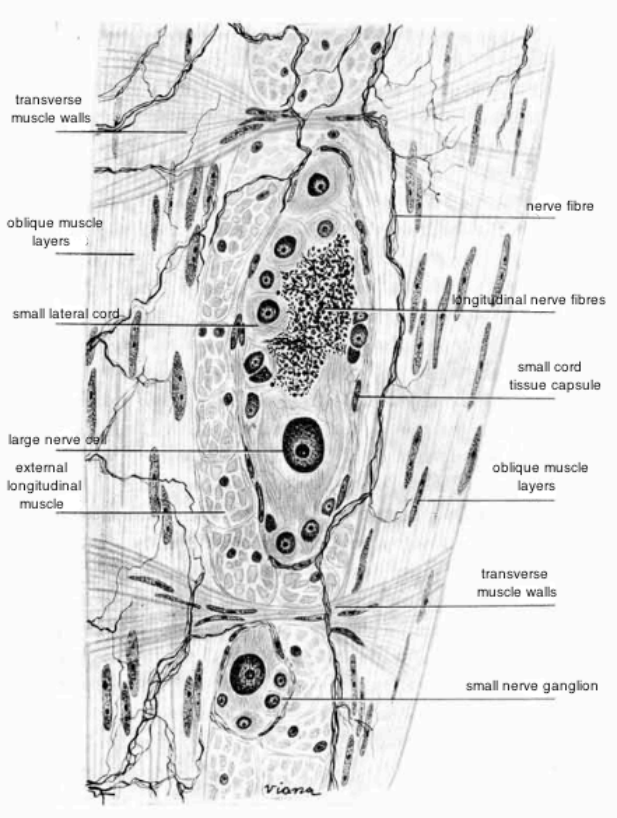

Figure 6. Transversal section of the octopus arm showing a small lateral nerve cord (modified after Rossi & Graziadei, 1956).

According to Guérin, they are formed by axons and cell bodies of motor neuron. Within the muscles there are also some small isolated ganglia, containing bipolar and multipolar neurons, but their function is still unknown. The mechanosensory system of the intrinsic musculature is preferentially located in the periphery where muscle strain is expected to be stronger during bending of the arm.

There is a widespread distribution of the signal from the arm to the supraesophageal mass: a first part of afferent fibres pass to the lateral and medial frontal lobe, a second to the lateral and median superior frontal lobe and a third to the subvertical lobe (Budelmann & Young, 1985), but fibres reaching the vertical lobe have not been identified yet (Fig. 7).

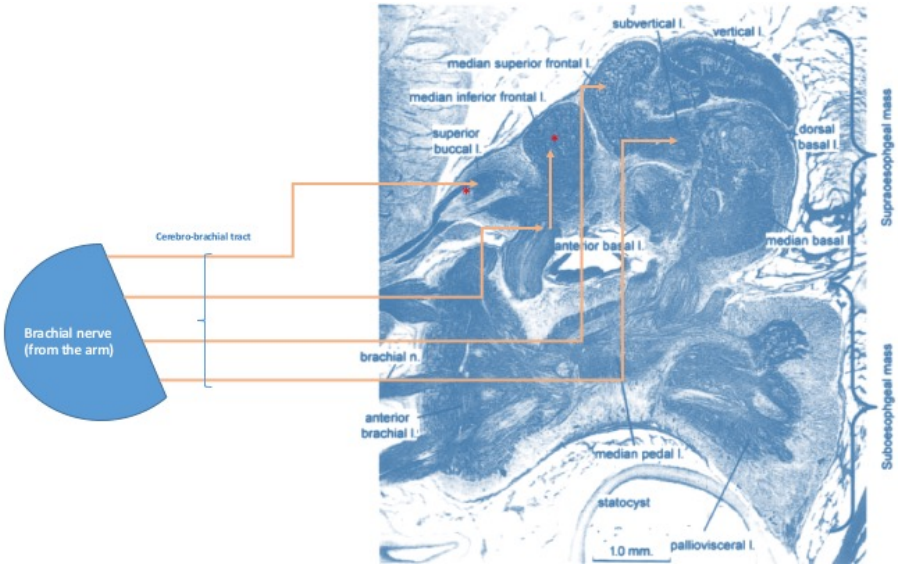

Figure 7. Schematic representation of the afferents from brachial nerve to the lobes of the supraesophageal mass (sagittal section of the brain of O. vulgaris, after Hochner et al., 2006). Red asterisks mark connections that reach also the contro-lateral side.

5. Searching for candidate 'pain' genes of interest: analysis of Octopus vulgaris transcriptome

5.1 Strategy and selection of transcripts

I based my study on the Octopus vulgaris transcriptome assembled by Petrosino (2015). This O. vulgaris transcriptome has been compiled from the collection and reannotation of the nucleotide sequences available from previous studies and from RNA-seq experiments carried out on RNA samples from different tissues (SEM: supraesophageal mass; SUB: subesophageal mass; OL: optic lobe; ARM) of three O. vulgaris.

For the purpose of this PhD, I analysed the available data using different two approaches, in order to identify pain related putative transcripts, and to compare their expression in the central and peripheral nervous system of the octopus. In both cases, I utilized a biased gene fishing approach.

I first search from literature, key molecules implicated in pain pathways as studied in in vertebrates and, whenever possible in invertebrates, and to check their presence within the O. vulgaris transcriptome. I searched candidates either by annotation or by Basic Local Alignment Search Tool (BLAST).
These key molecules mainly belong to three groups, depending on their possible site of involvement and function:

1. Molecules implicated in the primary activation of the nociceptive afferent and substances implicated in localised inflammatory reactions.
    1.1 Ion channels. Many ion channels have been linked to nociceptive pathways (see also above). Those of particular interest belong to the voltage gated sodium channel family (VGSC), the transient receptor potential channel (TRP) family, the P2X family of ion channels (in particular P2X3), acid sensing ion channels (ASICs). The TRP family has been extensively studied also in Drosophila where a "pain gene" (painless) has been identified in the TRPA subfamily (Reiger et al. 2010, Matsuura et al. 2009). The molecular biology

of the ASICS family is well defined (Chu et al. 2011) and there is functional evidence that O. vulgaris responds to acid pH (Rowell 1966, Wells 1963, Wells et al. 1965).

1.2 Ligands inducing pain, sensitization or inflammation. Is a potentially very long list but the molecules of most interest are: substance P, histamine, bradykinin, extracellular ATP, 5-hydroxytryptamine and prostaglandins.

2. Molecules implicated as neurotransmitters in pain pathways or in primary afferents in cephalopods.

2.1 FMRFamide has been found in afferents in the nervous system of cuttlefish, octopus and squid (e.g. Loi et al. 1997, Wollesen et al. 2008). There is evidence that ASICs can be modulated by FMRF-like peptides and probably there is still an unidentified endogenous ligand (Lingueglia et al. 2006; see also below).

2.2 Substance P. The neurokinin peptide family (SP, neurokinin A and B) is a family of peptides implicated in many biological processes, including pain pathways. There are three receptors in mammals: NK1, 2, 3 with the NK1 receptor most implicated in pain as it is the primary ligand for SP. Molecular biology of this family is well known in mammals and SP appears to be a highly conserved neurotransmitter (see also below).

3. Molecules shown to be capable of reducing pain or inflammation in vertebrate/mammalian systems.

3.1 Opioid neuropeptides and receptors. Leu-, met- and delta- enkephalin have been demonstrated by immunohistochemistry in Octopus ocellatus (Sha et al. 2012).

3.2 Endocannabinoids have been studied extensively in vertebrates and there is growing interest in their evolution and identification of endogenous ligands (Elphick et al. 2012). In mammals there are two receptors (CB1 and CB2), but we still know very little about invertebrates. CB1 is the one most relevant to pain. Apart from the receptor there is interest in endogenous ligands/modulators of the receptor. Many of the modulators identified primarily in mammalian studies are lipids synthesised from membrane

phospholipids and include the following: anandamide, 2-arachidonyl glycerol, N-arachidonyl serine. Endocannabinoids have been demonstrated to be endogenous and to affect leech's neurones (Meriaux et al. 2011).

The second approach was to look for sequences annotated with the specific Gene Ontology (GO, Ashburner et al. 2000) term for the biological process "sensory perception of pain".

The results of these two disting approaches produced more than 300 candidate transcripts. The transcriptome of O. vulgaris counts more than 64,477 uniquely expressed transcripts clustered in 39,220 putative genes (Petrosino 2015), and despite the large number I cannot exclude that this may extend further by applying a strategy based on the search of conserved domains and/or using different filtering strategies in the bioinformatic analysis of transcript.

However, during the course of this PhD I decided to finalize the analysis on a subset of potential list, and that resulted to be in 32 "pain related gene" products found in O. vulgaris (Table 3). Thirteen of these resulted to be annotated as involved in the biological process "sensory perception of pain" (GO:0019233). The others have been linked to nociceptive pathways by various authors (see references in Table 3).

Table 3. List of selected “pain” related nucleotide sequence transcripts selected from O. vulgaris transcriptome, their identifiers, including attribution to the Gene Ontology “sensory perception of pain” (GO:0019233) and reason for inclusion.

Description	Gene ID	HSPNameSP	GO:0019233	Molecular function	Involvement in nociceptive pathways	Reference
Tachykinin-related peptide	octtkrpre	Q6F6I8	No	Receptor binding	Modulates neuropathic and inflammatory pain	Kunde et al. (2013)
Opioid-binding protein/cell adhesion molecule	OPCML	P11834	No	-	Involved in opioid metabolism	Shark & Lee (1995)
MIP-related peptides	MRP	Q9NDE8	No	-	Precursor which produces opioid-like peptides known to be specific modulators in molluscan neurons	Moroz et al. (2006)
DNA polymerase delta catalytic subunit	DNApol-delta	P54358	Yes	Nucleotide binding	GO inferred by mutant phenotype	Neely et al. (2010)
Cytoplasmic polyadenylation element-binding protein 2	Cpeb2	Q812E0	No	Nucleotide binding	Generation of pain memory in primary afferent nociceptors	Bogen et al. (2012)
Proto-oncogene tyrosine-protein kinase receptor Ret	RET	G3V9H8	No	Nucleotide binding	Nociceptor signal transduction	Golden et al. (2010)
60S ribosomal protein L3	RpL3	O16797	Yes	Structural constituent of ribosome	Involved in local protein synthesis in neuron processes	Moroz et al. (2006) Neely et al. (2010)
Neprilysin	MME	P08473	Yes	Endopeptidase activity	Biologically important in the destruction of opioid peptides	Morisaki et al. (2010)
Calpain-B	CalpB	Q9VT65	Yes	Endopeptidase activity	GO inferred by mutant phenotype	Neely et al. (2010)
Neprilysin	Mme	Q61391	Yes	Metalloendopeptidase activity	GO inferred by mutant phenotype	Chen et al. (1998)
Endothelin-converting enzyme 1	ECE1	P97739	No	Metalloendopeptidase activity	Involved in endothelin metabolism	Khodorova et al. (2009)

Description	Gene ID	HSPNameSP	GO:0019233	Molecular function	Involvement in nociceptive pathways	Reference
Tolloid-like protein 1	tll1	Q8JI28	No	Metalloendopeptidase activity	Negatively regulated by stress and glucocorticoids	Tamura et al. (2005)
Prostaglandin E2 receptor EP4 subtype	PTGER4	Q8MJ08	No	G-protein coupled receptor activity	Lead to phosphorilation of TRPV1 (sensitization)	Wang & Woolf (2005)
5-hydroxytryptamine receptor	5htr	Q25414	No	G-protein coupled receptor activity	5HT4 increases TTXr small cell currents via PKA	Bogen et al. (2012)
μ-type opioid receptor	OPRM1	Q9MYW9	Yes	G-protein coupled receptor activity	Receptor for endogenous opioids such as beta-endorphin and endomorphin.	Wang et al. (1994)
Calcitonin gene-related peptide type 1 receptor	CALCRL	Q8WN93	No	G-protein coupled receptor activity	Vasodilation via cAMP pathway.	Li et al. (2008)
FMRFamide receptor	FR	Q9VZW5	No	G-protein coupled receptor activity	Involved in pain modulation	Askwith et al. (2000)
Metabotropic glutamate receptor 5	GRM5	P41594	No	G-protein coupled receptor activity	Pharmacological studies suggest a role in pain and anxiety states.	Schoepp (2001)
Adenosine receptor A2a	ADORA2A	P29274	No	G-protein coupled receptor activity	Knock-out studies in mice	Ledent et al. (1997)
Glutamate receptor ionotropic NMDA 2D	Grin2d	Q62645	No	Ionotropic glutamate receptor activity	Regulation of sensory perception of pain	Lima et al. (2003)
High-affinity choline transporter 1	CG7708	Q9VE46	Yes	Transporter activity	GO inferred by mutant phenotype	Neely et al. (2010)
Transient receptor potential cation channel subfamily A member 1	trpa1	Q7Z020	Yes	Ion channel activity	Present in 75% of mammal nociceptors	Nagata et al. (2005)
Anoctamin-1	Ano1	Q8BHY3	No	chloride channel activity	Heat sensor in nociceptive neurons	Cho et al. (2012)
Calcium/calmodulin-dependent protein kinase type II alpha chain	CaMKII	Q00168	Yes	ATP binding	GO inferred by mutant phenotype	Neely et al. (2010)

Table 3

Description	Gene ID	HSPNameSP	GO:0019233	Molecular function	Involvement in nociceptive pathways	Reference
Tyrosine-protein kinase Src42A	Src42A	Q9V9J3	Yes	ATP binding	GO inferred by mutant phenotype	Neely et al. (2010)
P2X purinoceptor 4	P2RX4	Q99571	Yes	ATP binding	Activation required in tactile allodynia under chronic pain	Inoue et al. (2004)
Neural-cadherin	CadN	O15943	Yes	Cell adhesion	GO inferred by mutant phenotype	Neely et al. (2010)
Piezo-type mechanosensitive ion channel component 2	PIEZO2	Q9H5I5	No	Mechanically-gated ion channel activity	Activated by mechanical noxious stimuli	Kim et al. (2012)
Acid-sensing ion channel 1	asic1	Q708S8	No	Ligand-gated sodium channel activity	Mediates glutamate-independent Ca2+ entry into neurons upon acidosis	Paukert et al. (2004)
Proto-oncogene c-Fos	FOS	P11939	No	Sequence-specific DNA binding	Marker for the activation of nociceptive neurons, binds with AP1 and activates the expression of prodynorphin gene.	Gao et al (2009)
Transcription factor AP-1	JUN	P18870	No	Sequence-specific DNA binding	Leading to increased steroidogenic and opioid gene expression upon cAMP signaling pathway stimulation.	Gao et al (2009)
C-terminal-binding protein	CtBP	O46036	Yes	NAD binding	GO inferred by mutant phenotype	Neely et al. (2010)

Table 3

On the basis of the RNA-seq experiments (Petrosino, 2015), I evaluated the predicted levels of expression for the selected transcripts (Figure 8).

The analysis show that the selected sequences belongs to different groups, depending on their in silico expression in the analysed tissues:

i. Two sequences annotated with the molecular function ATP-binding (P2X purinoceptor 4, tyrosine-protein kinase Src42A), together with the opioid-binding protein/cell adhesion molecule (molecular function unknown) appear highly expressed in the subesophageal mass (SUB). The expression level decreases in other tissues and is almost zero in the arm (and in TIP2).

ii. A group with heterogeneous molecular functions (CaMKII, ADORA2A, CG77080, Cpeb2) appears to be highly expressed in all tissues, but not the arm and the tip.

iii. Another group of transcripts, with heterogeneous functions, is highly expressed in the supraesophageal mass (SEM). This includes the G-protein coupled receptor CALCRL, the neuropeptide tkp (receptor binding activity), and the proto-oncogene tyrosine-protein kinase receptor RET (nucleotide binding).

iv. OPMR1, 5HTr, Gmr5 (G-protein coupled receptors), CtBP (NAD binding activity) and MME (endopeptidase) are highly expressed in the optic lobe (OL).

v. PTGER4 (G-protein coupled receptor), PIEZO2 (ion channel) and MRP are selectively expressed in SEM and SUB.

vi. Three ion channels (asic1, trpa1, ano1), two metallo-endopeptidase (ECE1, tll1), the transcription factor JUN and the structural component of ribosome RpL3 are selectively expressed in the arm TIP, with only exception of asic1, which is also expressed in SUB.

vii. Another heterogeneous group composed by FOS, CadN, FMRFamide and DNA pol-delta, is highly expressed in SEM and tip.

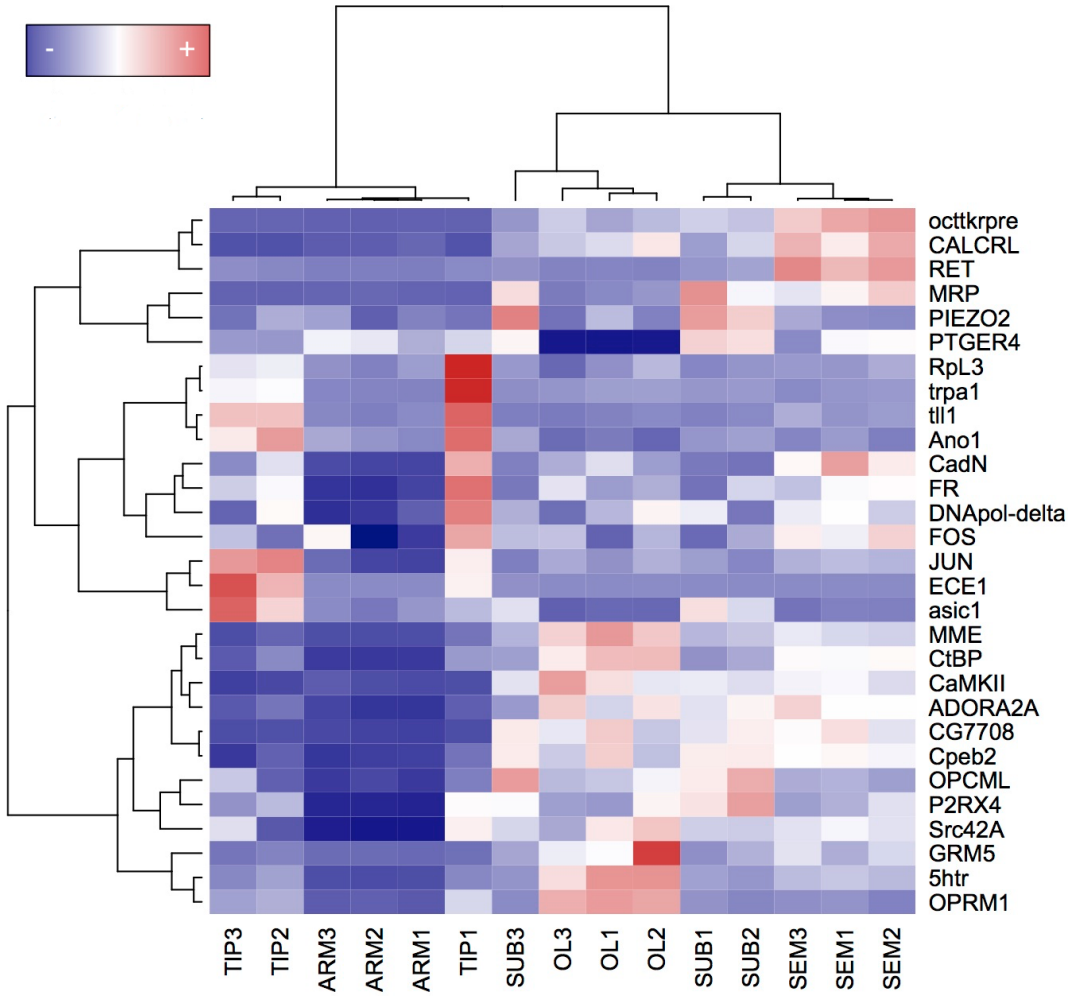

Figure 8. Heatmap (based on normalized RNAseq abundance data; see Petrosino, 2015) showing relative expression levels of selected (32) target genes in different tissues of O. vulgaris: supraesophageal mass (SEM), subesophageal mass (SUB), optic lobes (OL), arm (medial part, ARM) and arm tip (TIP). Biological replicates are indicated as digits for each tissue (i.e.: 1, 2, 3). Relative gene expression are color-coded from blue (lower) to red (high expression). Grouping is indicated by cluster branches as described in text.

5.2. Selection of genes of interest for gene expression analysis

From the 32 selected O. vulgaris putative pain related genes, I selected nine depending on their differential expression between the brain tissues, the arm and the arm tip, based on predicted in silico expression levels. In the following pages I will further describe these nine genes, also with the aim to discuss the reasons for inclusion.

Acid sensing ion channel 1

Acid-sensing ion channels are pH sensitive receptors producing acid-gated currents (Waldmann et al. 1997), closely related to the degenerin-epithelial Na^+ channel family (DEG-ENaC, Mano & Driscoll 1999). They are involved in many cellular functions and are diffused in peripheral and central neurons. In sensory terminals, they could have an important role for nociception and mechanosensation (Price et al. 2001) while is still uncertain how they are activated in the brain. Their activity is modulated by a lot of endogenous and exogenous modulators, such as dynorphin, FMRFamide and amiloride (for review see Wemmie et al. 2013).

We know almost anything about the presence of these channels in molluscs and invertebrates in general. Nevertheless, asic1 putative orthologous transcripts have been identified in C. elegans (NCBI accession number NM_058813) and in Octopus bimaculoides (Ocbimv22035600m; Albertin et al. 2015). O. vulgaris expression levels based on transcriptome data (Petrosino, 2015) are provided in Figure 9.

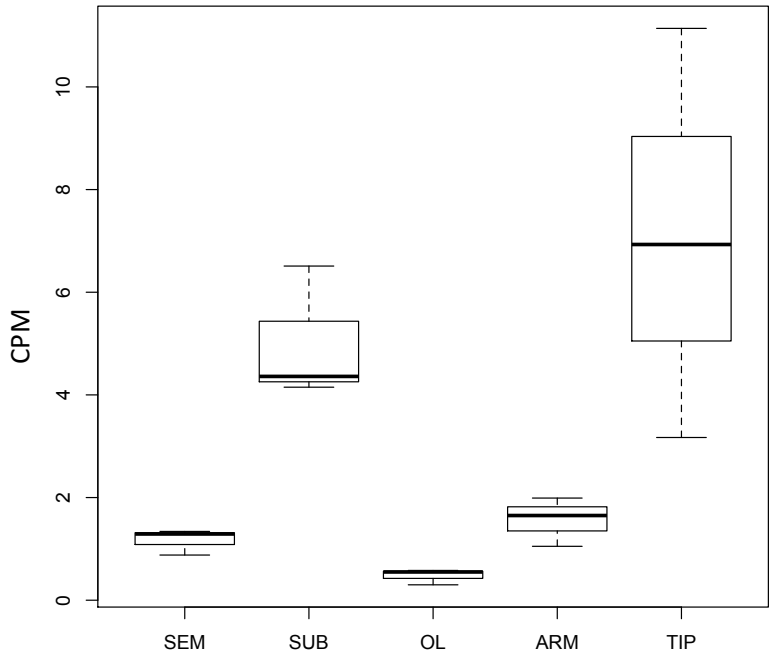

Figure 9. Ov asic1 expression levels (CPM: counts per millions) in O. vulgaris SEM, SUB, OL, ARM and TIP.

In O. vulgaris asic1 appears to be more expressed in TIP and SUB when compared with other parts considered (Fig. 9), but the analysis of the differential expression of the gene between them did not give significant results (data not shown).

The asic1 sequence identified in O. vulgaris transcriptome (c28071_g1_i1), the available sequences of invertebrates and those of the main vertebrate model species (NCBI Reference Sequence: NM_020039.3 for Homo sapiens, NM_024154.2 for Rattus norvegicus, NM_009597.1 for Mus musculus, NM_214791.1 for Danio rerio, XM_004911961.1 for Xenopus tropicalis) were aligned using the SeaView Software (Galtier et al. 1996).
Identity scores of the alignment were highest (>50%) with X. tropicalis, M. musculus and R. norvegicus; slightly lower (49%) with H. sapiens and C. elegans.
I found the lowest percentage (42%) score while comparing O. vulgaris with O. bimaculoides (for complete list of percentage scores see Table 4).

Transient receptor potential cation channel subfamily A member 1

The TRPA1 (transient receptor potential cation channel, subfamily A, member 1) channel is a non-selective cation channel that can be activated by a variety of molecules including bradykinin, formalin, anandamide, tetrahydrocannabinol, the reactive electrophiles AITC (component of mustard oil), and bradykinin (e.g. Bandell et al. 2004).
TRPA1 may also have thermosensitive properties, but whether it is directly activated by cold (< 17°C) or contributes to the development of cold hypersensitivity is still being debated (e.g. Laursen et al. 2014). Some authors consider TRPA1 as the central molecule for chemically induced pain (Tai et al. 2008).
Two groups have reported that TRPA1 is predominantly co-expressed with CGRP (Bautista et al. 2005); however, the majority of TRPA1+ neurons (> 90%) are co-labeled with IB4 and can thus be classified as non-peptidergic nociceptors (e.g. Barabas et al. 2012).
People with elevated pain sensitivity show differential DNA methylation in close proximity to the TRPA1 gene, thus making possible that such differences contribute

to individual differences in pain sensitivity. TRPA1 expression has been studied both in vertebrate and invertebrate species. Among invertebrates, in Drosophila an evolutionary homolog of the mammalian TRPA1 (NM_140006.5) is encoded by the painless gene, which has been showed to be necessary for the detection of noxious heat in larvae (Tracey et al. 2003). A TRPA1 homolog RNA has been identified also in C. elegans (NM_069848.4), the starlet sea anemone Nematostella vectensis (XM_001625230.1), the pacific oyster Crassostrea gigas (EKC35184.1), and in the octopus O. bimaculoides (Ocbimv22003285m).

The sequences of these invertebrate species were aligned, together with those of the main vertebrate model species (Y10601.1 for H. sapiens, AY496961.1 for R. norvegicus, AY231177.1 for M. musculus, AY677196.1 for D. rerio, BC166179.1 for X. tropicalis), to the TRPA1 sequence identified in O. vulgaris transcriptome (c31382_g11_i1), resulting in identity scores (Table 4) ranging from 42% (with O. bimaculoides) to 47% (with C. elegans and H. sapiens).

In O. vulgaris trpa1 appears to be more expressed in TIP and OL respect to the other tissues (Fig. 10), but the analysis of the differential expression of the gene between them did not give significant results (data not shown).

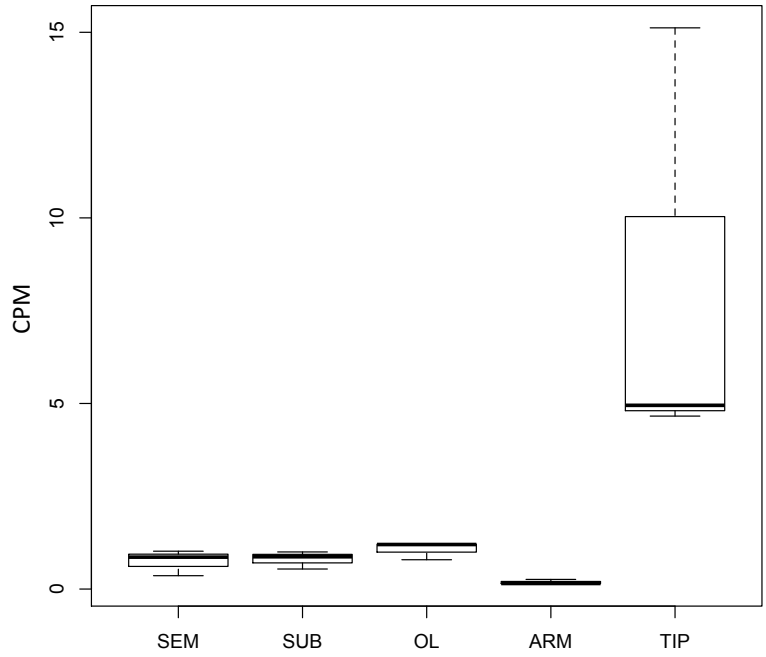

Figure 10. Ov trpa1 levels of expression (CPM: counts per millions) in O. vulgaris SEM, SUB, OL, ARM and TIP.

Anoctamin-1

Ano1 is a member of a 10-gene superfamily (anoctamins), encoding for a Ca^{2+} activated chloride (Cl^-) channels expressed in glands and flat epithelia.

Ano1 modulates cellular responses to various stimuli in smooth muscles, heart, endothelium, neuronal tissues, and epithelial organs (Kunzelmann et al. 2012). In mice ano1 is expressed in DRG neurons, highly co-localized with TRPV1, and a functional ablation of ANO1 in those neurons elicits a loss of thermal pain, suggesting a role in nociception (Cho et al. 2012). Ano1 also regulates the inflammation-induced membrane excitability in DRG neurons, suggesting that its phosphorylation by several kinases may account for the change of excitability (sensitization) of nociceptors caused by inflammation. The presence of anoctamins in invertebrates has not been extensively studied yet. According to Milenkovic et al. (2010) different number of anoctamins paralogs exists in invertebrates suggesting s a complex evolutionary history, but I did not find registered sequence for ano1 in invertebrates.

Ano1 sequence identified in O. vulgaris transcriptome (c31699_g1_i2) aligns to those of the main vertebrate model species (NM_018043.5 for H. sapiens, NM_001107564.1

for R. norvegicus, NM_178642.5 for M. musculus, NM_214790.2 for D. rerio, NM_001130327.1 for X. tropicalis) with an identity scores (seeTable 4) ranging from 47% to 55%.

In O. vulgaris the analysis of the differential expression of ano1 between tissues showed that it is significantly more expressed (ANOVA with Bonferroni post-hoc correction, $p < 0.001$) in TIP respect to the other tissues (Fig. 11).

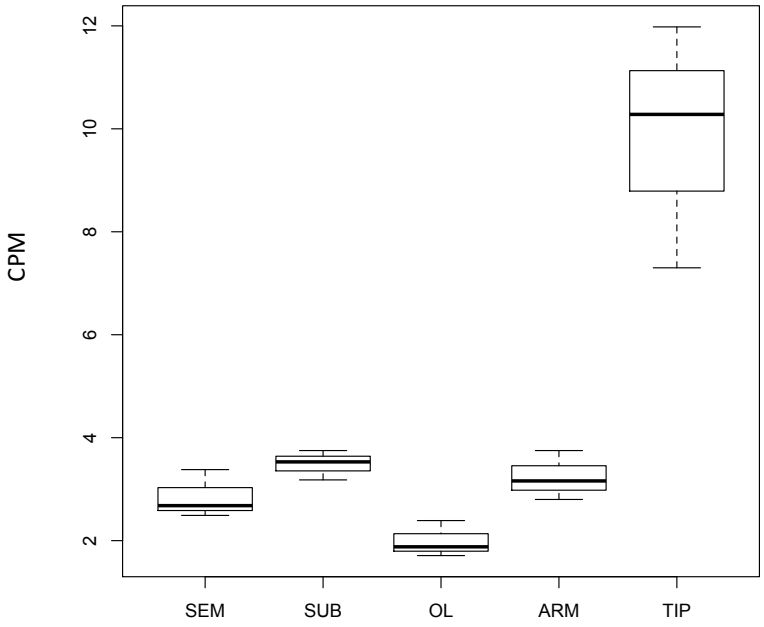

Figure 11. Ov ano1 levels of expression (CPM: counts per millions) in O. vulgaris SEM, SUB, OL, ARM and TIP.

Tolloid like protein 1

Mammalian tolloid-like 1 (tll1) belongs to a small family of structurally related proteases of which bone morphogenetic protein-1 (BMP-1) is representative, implicated in embryonic patterning in diverse species (Bond & Beynon, 1995).

In Drosophila Tolloid (NM_079763.4) affects dorsal–ventral patterning during development, but its role in invertebrates are still need to be investigated.

Moroz and co-workers performed a trascriptomical profiling and in situ hybridization study showing that tll1 transcript (U57369.1) is enriched in Aplysia sensory neurons (Moroz et al. 2006). It may have a role in structural changes associated with cell-cell communication.

Ov tll1 (c30328_g5_i1) alignment with invertebrate and vertebrate[1] species (Table 4) resulted in an identity spanning from 45% (with N. vectentis) to 55% (with H. sapiens). Interestingly, identity scores are higher with vertebrate tll1 sequences, than with invertebrate ones (Table 4).

Furthermore, the analysis of the differential expression of Ov tll1 between tissues showed that it is significantly more expressed (ANOVA with Bonferroni post-hoc correction, $p < 0.0001$) in TIP in respect to the other tissues (Fig. 12).

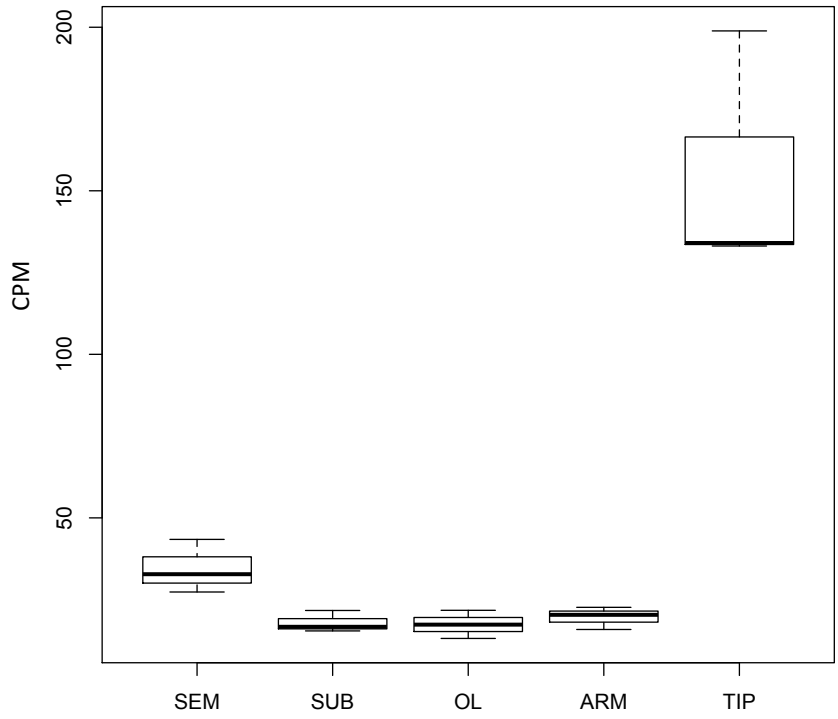

Figure 12. Ov tll1 levels of expression (CPM: counts per millions) in O. vulgaris SEM, SUB, OL, ARM and TIP.

Table 4. Pairwise identity percentage scores resulting from the alignment of O. vulgaris transcripts with those of listed species. Empty box: no sequence available for the relative species.

Species	asic1	trpa1	ano1	tll1	FR	tkp	camkII	piezo2	oprm1
Homo sapiens	49%	47%	53%	55%	38%	47%	44%	53%	49%
Mus musclulus	54%	45%	54%	54%	43%	49%	45%	53%	49%

[1] *D. rerio* - NM_131010.1; *H. sapiens* - NM_012464.4; *M. musculus* - NM_009390.2; *R. norvegicus* - NM_001106081.1; *X. tropicalis* - 001008038.1; *N. vectentis* - XM_001633796.1

Rattus norvegicus	50%	45%	54%	53%	37%	49%	45%	53%	51%
Xenopus tropicalis	56%	46%	55%	-	-	56%	48%	59%	50%
Danio rerio	46%	45%	47%	50%	-	46%	-	51%	47%
Drosophila melanogaster	-	42%	-	48%	53%	50%	51%	52%	-
Caenorbiditis elegans	49%	47%	-	-	52%	-	-	50%	-
Crassostrea gigas		43%	-	48%	44%	-	51%	54%	-
Octopus bimaculoides	42%	42%	-	-	49%	-	-	-	-
Nematostella vectentis	-	-	-	45%	-	-	52%	-	-

FMRFamide receptor

FMRF-amide is a member of the FMRFamide-related peptides (FaRPs) family, a group of neuropeptides all sharing an –RFamide sequence at their C-terminus.

FMRFamide was originally isolated from the ganglia of the clam Macrocallista nimbow (Price & Greenberg 1977). In the following years, a variety of FMRFamide, N- terminal extended, peptides were identified in other species of molluscs, as well as other invertebrate phyla.

These peptides, members of which have structural affinities with Met-enkephalin, have been implicated in the modulation of the activity of excitable tissues and sensory neurons in molluscs (Price 1986, Greenberg et al. 1988). In invertebrates, this class of neuropeptides plays a critical role in several biological functions and behaviors, including contributing to modulation of feeding, digestion, cardiac activity, reproduction and locomotion (review in Krajniak 2013).

Among vertebrates, exogenous FaRPs elicit a naloxone-sensitive antinociceptive effect and antagonize opioid and morphine-induced analgesia (Yang et al. 1985, Kavaliers & Hirst 1985, Raffa & Connelly 1992).

Despite numerous studies of the cellular effects of FMRFamide and related peptides, little is known about the receptors for these ligands. The FMRFamide receptor (FR) is a G protein-coupled receptor involved in many biological processes.

Ov FR (c31162_g13_i1) alignment with invertebrate[2] and vertebrate[3] species (Table 4) showed and higher identity with invertebrates (49-53%) than with vertebrates (37-43%) FR transcripts.

[2] *C. gigas* - EKC25570.1; *C. elegans* - NM_072603.1; *D. melanogaster* - NM_139501.4; *O. bimaculoides* - Ocbimv22024573m (Metazome)

[3] *R. norvegicus* - AB040103.1; *H. sapiens* - NM_022146.4; - NM_133192.3

In addition, Ov FR appears to be more expressed in all the tissues except ARM (Fig. 13), but the analysis of the differential expression of the gene between them did not give significant results (data not shown).

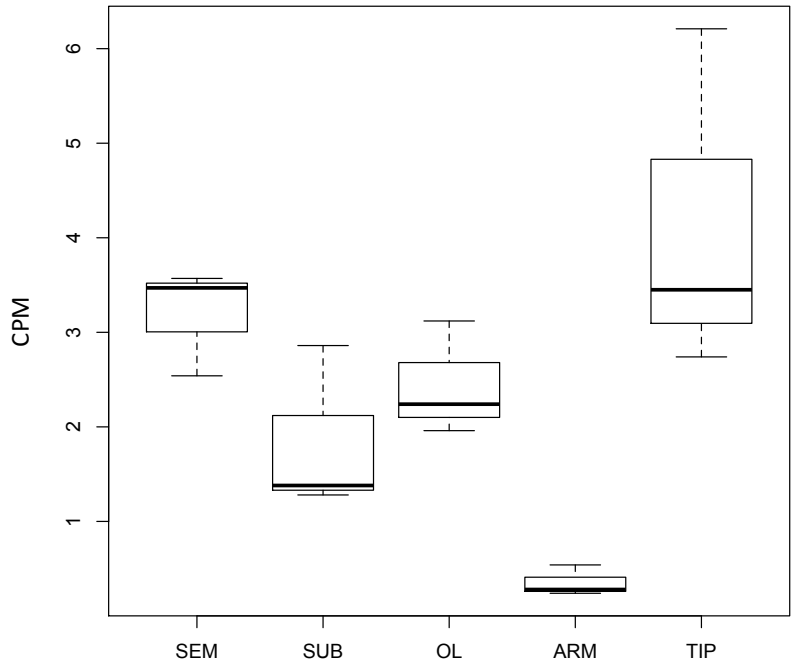

Figure 13. Ov FR levels of expression (CPM: counts per millions) in O. vulgaris SEM, SUB, OL, ARM and TIP.

Piezo-type mechanosensitive ion channel component 2

Piezo2 is a rapidly adapting, mechanically activated ion channel expressed in a subset of sensory neurons of mammal dorsal root ganglion (DRG) and in cutaneous mechanoreceptors known as Merkel-cell–neurite complexes (Coste et al. 2010, Woo et al. 2014).

A relatively recent study (Ranade et al. 2014) showed that the deletion of piezo2 in mice leads to a strong decrease in the ability to sense innocuous, but not noxious, touch. Authors also observed no differences to noxious temperature sensitivity between control and Piezo2^{CKO} mice.

On the contrary, in Drosophila Piezo is required for mechanical nociception, and not for gentle touch sensation. Indeed, behavioural responses to noxious mechanical stimuli are severely reduced in piezo knockout Drosophila larvae, whereas responses to another noxious stimulus or gentle touch are not affected (Kim et al. 2012).

Zebrafish piezo homolog piezo2b shows a specific neural expression pattern, appearing in the trigeminal and Rohon–Beard neurons (Faucherre et al. 2013) that innervate the skin and are involved in sensing external stimuli (Prober et al. 2008).

Ov piezo2 alignment with sequences of invertebrate[4] and vertebrate[5] species resulted in an identity spanning from 50% (with C. elegans) to 59% (with X. tropicalis< see Table 4).

[4] *C. gigas* - EKC42880.1; *C. elegans* - NM_001268458.2; *L. gigantea* - XM_009061322.1; *D. melanogaster* - NM_001201790.2

[5] *D. rerio* - XM_017352447.1; *H. sapiens* - NM_022068.3; *M. musculus* - NM_001039485.4; *R. norvegicus* - XM_017601114.1; *N. vectentis* - 86759 (Metazome); *X. tropicalis* - 455908 (Metazome)

The analysis of the differential expression of Ov piezo2 between tissues showed that it is significantly more expressed (ANOVA with Bonferroni post-hoc correction, $p < 0.001$) in SUB respect to the other tissues (Fig. 14).

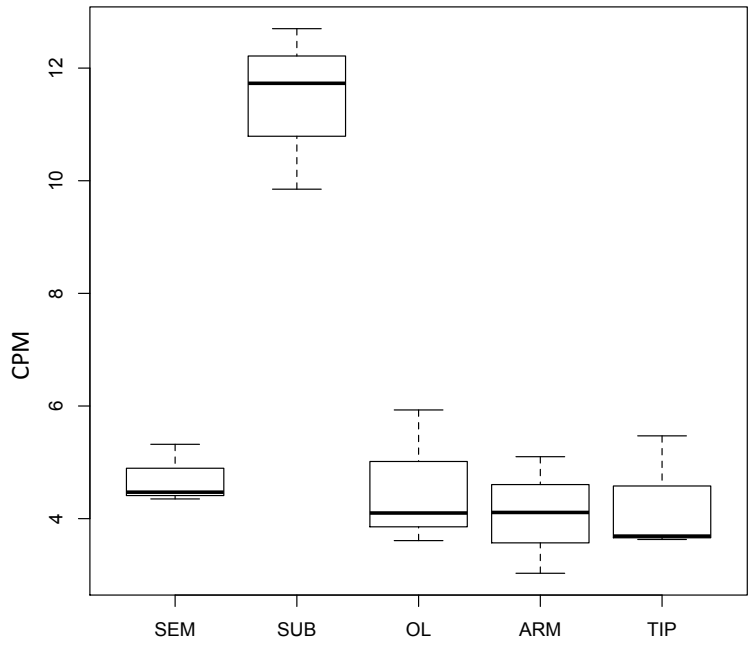

Figure 14. Ov piezo2 levels of expression (CPM: counts per millions) in O. vulgaris SEM, SUB, OL, ARM and TIP.

Calcium calmodulin-dependent protein kinase type II alpha chain

This is a serine/threonine-specific protein kinase that is regulated by calcium/calmodulin complex. CaMKII is involved in many signalling cascades, including learning and memory (e.g. fear, fear conditioning; Rodrigues et al. 2004). Analysis of the molecular mechanisms underlying the generation and maintenance of central sensitization, LTP and other phenomena indicates that, although differences between the synaptic plasticity contributing to memory and "pain" exists, striking similarities emerge (Ji et al. 2003).

Ov CamKII alignment with invertebrate[6] and vertebrate[7] species showed and higher identity with invertebrates (51-52%) than vertebrates (44-48%) FR transcripts (Table 4).

The analysis of the differential expression of Ov CaMKII between tissues resulted in being significantly more expressed (ANOVA with Bonferroni post-hoc correction, $p < 0.001$) in SEM, SUB and OL when compared to ARM and TIP (Fig. 15).

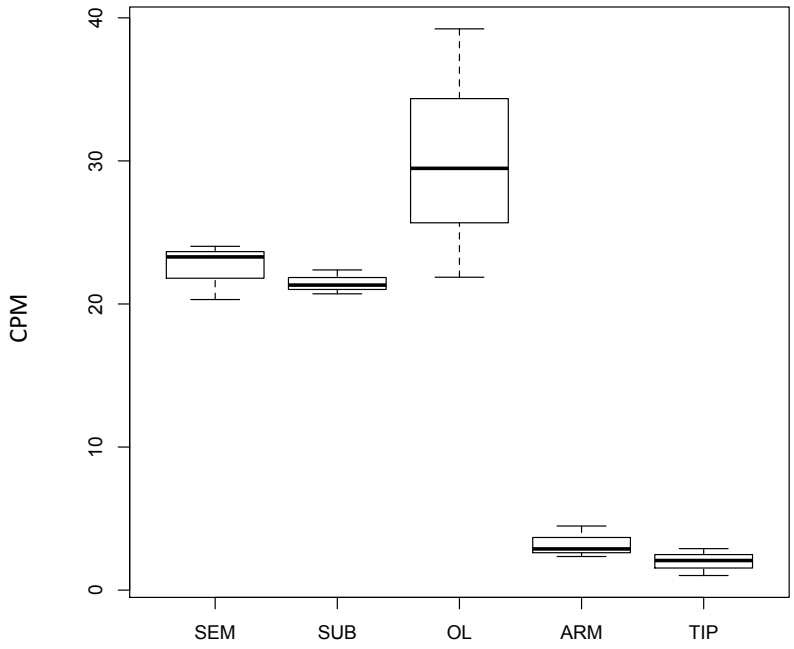

Figure 15. Ov CaMKII levels of expression (CPM: counts per millions) in O. vulgaris SEM, SUB, OL, ARM and TIP.

Tachykinin-related peptide

Tachykinin peptides are traduced from different transcripts encoded by the protachykinin genes.

Substance P (SP) is a neuropeptide belonging to the tachykinin family that acts as neurotransmitters and neuromodulator. It derives from a polyprotein precursor (protachykinin) and it is stored in vesicles of sensory neurons for axonal transport (Harrison & Geppetti 2001). Its receptor is the G-protein coupled receptor NK1

[6] *C. gigas* - XM_011439571.1; *L. gigantea* - XM_009061772.1; *D. melanogaster* - NM_001169361.2; *O. bimaculoides* - ocbimv22026165m (Metazome)

[7] *D. rerio* - BC093372.1; *M. musculus* - NM_177407.4; *R. norvegicus* - NM_012920.1; *H. sapiens* - NM_171825.2; *N. vectentis* - 157808 (Metazome); *X. tropicalis* - NM_001100267.1

(neurokinin 1), and is distributed over cytoplasmic and nuclear membranes of many cell types. It plays an important role as mediator in the processing of nociceptive information and is released from primary sensory neurons (mostly polymodal nociceptors) following noxious stimulation (Zubrzycka & Janecka 2000). It has be reported to be present predominantly in nociceptive sensory fibres (Mense & Gerwin 2010).
Individually identified SP-expressing dorsal root ganglia neurons have either C fibre or Aδ fibre axons and exhibit nociceptive response properties (Lawson et al. 1997).

Substance P is highly co-localized with CGRP in that nearly all SP-expressing dorsal root ganglia neurons also contain CGRP, although only half of the CGRP-expressing neurons also contain SP.

Ov tkp (c31437_g10_i1) alignment with sequences of invertebrate (D. melanogaster, NM_141884.4) and vertebrate[8] species (Table 4) resulted in an identity spanning from 47% (with H. sapiens) to 56% (with X. tropicalis).

The analysis of the differential expression of Ov tkp among tissues showed that it is significantly more expressed in SEM when compared with other brain parts (SUB and OL; after ANOVA with Bonferroni post-hoc correction, $p < 0.0001$), and that it is significantly more expressed in the brain than in ARM and TIP (data not shown, but see Fig. 16).

[8] *D. rerio* - NM_001256391.1; *H. sapiens* - NM_013996.2; *R. norvegicus* - NM_012666.2; *M. musculus* - XM_006505027.1; *X. tropicalis* - OCA18896.1

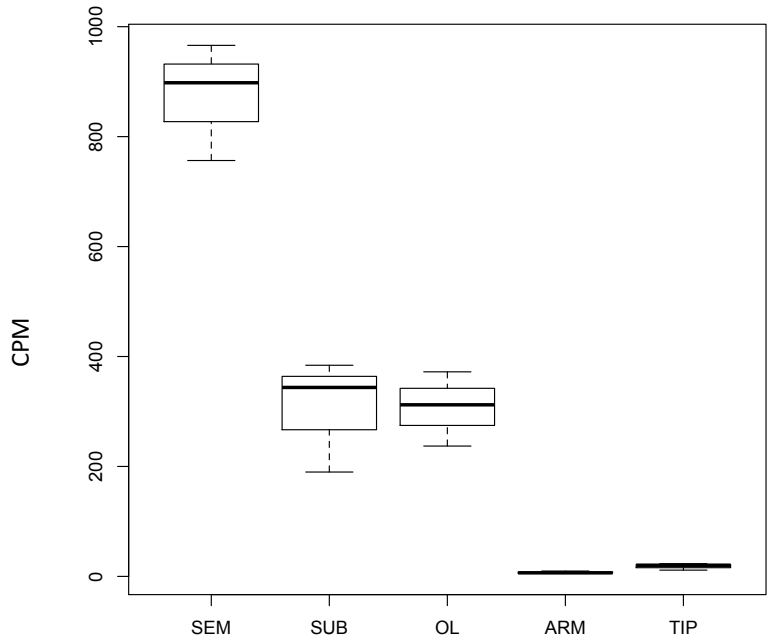

Figure 16. Ov tkp levels of expression (CPM: counts per millions) in O. vulgaris SEM, SUB, OL, ARM and TIP.

μ-type opioid receptor

Receptors for opioids can be divided into three classes, depending on the ligand and of the effects at the cellular level:

1. μ receptors (MOR or OPRM)
2. κ receptors (KOR)
3. δ receptors

All three are coupled to protein G (Janecka et al. 2004) activated by endogenous peptides and by exogenous compounds (Waldhoer et al. 2004).

The action of opioids is involved in several biological processes, including stress-induced analgesia, reinforcement and reward phenomena, affiliative adaptive behaviours (e.g. allo-grooming), the mother-infant bond during the early days postnatal and copulatory analgesia. They are also responsible for defeat-induced analgesia, caused by an increase of the nociceptive threshold, which is inhibited by opioid antagonists (e.g. naloxone).

OPMR mRNA increases in the ventral tegmental area of “defeated” mice, and endogenous dynorphin is released (D'Amato & Peacock 2012). Opioids may also affect the withdrawal reflex that animals manifest as a result of painful stimulation. In mice, this phenomenon is regulated by the action of two cell types present in the medial-rostroventral medulla: “off” neurons, which are normally active and stop before the reflex; and “on” neurons, which are normally silent and fire just before the reflex (Fields 2004).
OPMR agonists seem to inhibit the reflex eliminating "off" neurons’ activity break.

So far, oprm1 transcripts have been identified only in vertebrate[9] transcriptomes. The alignments of those sequences with Ov oprm1 (c35350_g9_i3) resulted in an identity spanning from 47% (with D. rerio) to 51% (with R. norvegicus; Table 4).

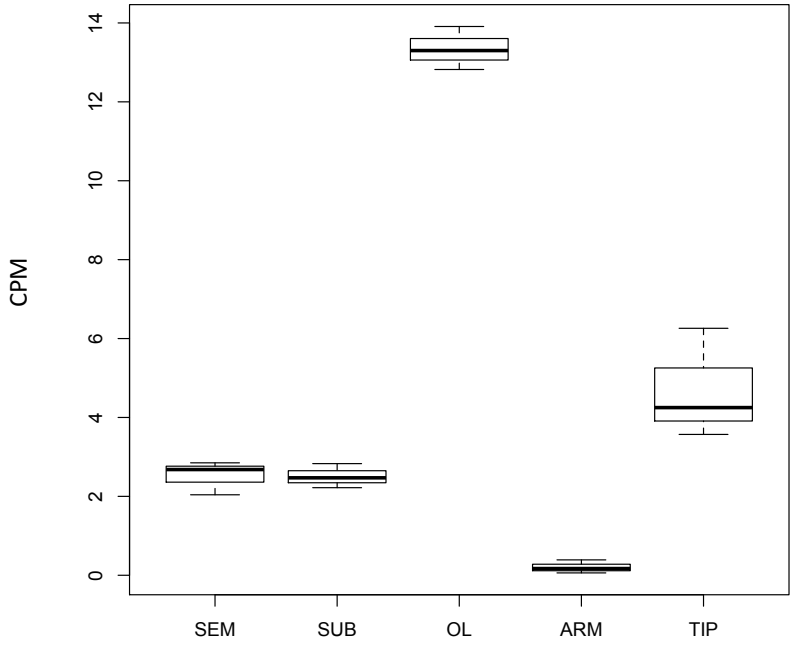

Figure 17. Ov oprm1 levels of expression (CPM: counts per millions) in O. vulgaris SEM, SUB, OL, ARM and TIP.

The analysis of the differential expression of Ov oprm1 between tissues showed that it is significantly more expressed (ANOVA with Bonferroni post-hoc correction, p <

[9] *D. rerio* - BC163729.1; *R. norvegicus* - NM_001304740.1; *H. sapiens* - L25119.1; *M. musculus* - NM_001302793; *X. tropicalis* - XM_002933863.2

0.0001) in OL respect to the other brain parts (SUB and OL). The expression in TIP is again higher than in SUB and OL, but still lower that in OL (Fig. 17).

5. 3. Observed gene expression of transcripts

Pain related transcripts resulted to be expressed at different levels in various parts of the nervous system of O. vulgaris.

I found that in the optic lobe (OL) of octopus there are mostly G protein-coupled receptors (5HT-receptor, μ-opioid receptor 1, glutamate metabotropic receptor 5, adenosine A_2 receptor), or enzymes (Ca^{2+}/calmodulin-dependent protein kinase II, tyrosine-protein kinase Src42A, neprilysin, C-terminal-binding protein 1, cytoplasmic polyadenylation element-binding protein).

In the supraesophageal mass (SEM), I found more expressed genes encoding for neuropeptides (tachykinin, the Mytilus inhibitory peptide) a G protein-coupled receptor (the calcitonin gene related peptide receptor), the proto-oncogene tyrosine-protein kinase receptor Ret and a neural cadherin.

Finally, in the subesophageal mass (SUB) I found mainly receptors: two ion channels (P2X purinoceptor 4 and Piezo-type ion channel 2) and G protein-coupled receptor (Prostaglandin E2 receptor EP4 subtype).

Also, the opioid-binding protein/cell adhesion molecule, whose precise function is still unknown, resulted to be highly expressed in SUB.

The majority of ion channels (trpa1, asic1 and ano1) appeared highly expressed in the more distal part of the arm (TIP), together with two metallo-endopeptidases (endothelin-converting enzyme 1 and tolloid-like protein 1), the G protein-coupled receptor for FMRFamide, and the 60S ribosomal protein L3.

None of the transcripts considered in this study, appeared to particularly expressed in the medial part of the arm (ARM).

6. Analysis of pain related genes expression in the octopus arm

The above listed selection of target genes, have been validated and the expression studied in the arm of O. vulgaris.

6. 1. Methods

Octopus vulgaris of both sexes (two males and one female) were obtained from local fishermen (Bay of Naples, Italy) as freshly killed animals and tissues immediately obtained from octopuses.

Tissue removal do not require authorization from National Competent Authority according to the principles stated in the Directive 2010/63/EU and National transposition.

From freshly killed octopus, two arms were dissected and from them parts of the left anterior arm (L1) further isolated, i.e. the distal (TIP), central (MID) and proximal (to the mouth, PROX) segments (Fig. 18), each one with the same number of suckers, and therefore, the same number of ganglia. A piece of the gill, as non-nervous control tissue was also collected from each animal.

Samples were immediately preserved in RNA-later, and upon freezing ice. In the laboratory, they were immediately processed for RNA extraction.

Total RNA was extracted using SV Total RNA Isolation System (Promega™, Z3100) according to manufacturer instructions.

RNA optical density measurements at 230, 260 and 280 nm were read using the Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies). The absence of DNA contamination was verified by running a PCR with ubiquitin primers and analysing the sample by gel electrophoresis.

For cDNA synthesis, 500 ng of RNA from each sample were processed with SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen™, 11904018).

Figure 18. O. vulgaris arm and the three segments (TIP: distal, MID: central, PROX: proximal) utilized in this study for gene expression experiments.

Prior to use in RT qPCR experiments, cDNA was diluted 1:10 with sterile H_2O and stored at -20°C until use.

One µl of diluted cDNA was used in a SYBR Green PCR for each reaction. Polymerase chain reactions were carried out in an optical 384-wells plate Applied Biosystems (Life Technologies) ViiA7, using FastStart SYBR Green Master mix (Roche, Indianapolis, IN) to monitor dsDNA synthesis.

Reactions (total volume: 10 µl) contained: 1 µl cDNA, 5 µl SYBR Green Master mix reagent, 4 µl of forward and reverse primers mix (0.7 ρmol/µl each).

The following thermal profile was used: 95°C for 10 min; 95°C for 15 sec and 60°C for 1 min, 40 cycles for amplification; 72°C for 5 min; one cycle for melting curve analysis, from 60°C to 95°C to verify the presence of a single product.

Results were analyzed using the With ViiA™ 7 Software (Life Technologies) to determine cycle threshold (Ct) values. Each assay included a no-template control for every primer pair.

The efficiency (E) of each pair of primers has been calculated according to standard method curves with the equation $E = 10^{-1/slope}$ (Pfaffl et al. 2002, Radonic et al. 2004). Primers are listed together with amplicon size and efficiency in Table 5 (reference genes, selected by Normfinder, are highlighted).

Five serial dilutions (1:5, 1:10, 1:20, 1:40, 1:80) of a standard sample were made to determine the efficiency of reactions conducted with each pair of primers. Standard curves were generated for each sample/gene combination using the Ct value versus the logarithm of each dilution factor. Efficiency values were taken into account in all subsequent calculation. The melting curve of each sample was analysed to confirm the specificity of the primers and to be sure of the nature of PCR products.

Table 5. Primer sequences (F: forward; R :reverse), amplicon size and amplification efficiency of reference (bold) and target genes.

Gene	GenBank	Primer	Sequence 5'-3'	Amplicon size (bp)	E
asic1	Q708S8	F	TTTGTCCCTTTGTGGGATGT	143	2.04
		R	TCGTCCTGTGAAGCAGAATG		
trpa1	Q18297	F	AAACGTAGAGCCATGCAGGT	115	2.08
		R	CGGTACACAGCATTCGGATA		
piezo2	Q9H5I5	F	GGACGCTTTGCTCGTATGTT	117	2.00
		R	AGCATTCGCGAACAAGGTAG		
tkp	Q6F6I8	F	CGTTTAGTTGGGGCTTTTCA	118	2.92
		R	GGGTTCCCGAGGTAAAAGAG		
c2k	K1RLP1	F	TGACCACCCGAATGTAGTGA	147	2.09
		R	GTACTGCCATGCCTGTTCCT		
hct	Q9VE46	F	TGTTGGAACAAGCAGAGGTG	159	2.04
		R	AATCAACCCACCGACAAAAG		
ano1	Q8BHY3	F	GAACTCATTCGCCAAGAAGC	171	2.00
		R	AGGCAGTTTCGTCTGCAGTT		
FMRF amide	Q9VZW5	F	CACCAGCAAATCACAAATGG	189	1.76
		R	CTCCCAGCAACAGGATTCAT		
tll1	Q8JI28	F	TGAGAAATACCGCAACCACA	196	1.74
		R	CTGCAACACACGGAGAGAAA		
mor	Q9MYW9	F	CGTCCTTCTCCTTCGAACTG	133	1.89
		R	ATCTTTCGACCGAAACACCA		
fmr1	Q9VF87	F	GCATTGGTCCACCACTGAAA	117	2.00
		R	CCTGAAGTCAAGCGATGCTG		

The combination of best reference genes was selected using Normfinder (Andersen et al. 2004) and relative genes expression was calculated using the method described in Sirakov et al. (2009).

Differentially expressed genes were identified using the omics tool "Differential Expression" in XLSTAT (https://www.xlstat.com/en/) with the following settings: Test type: parametric, Significance level: 5%, Multiple pairwise comparison: Tukey, Post-hoc correction: Bonferroni.

6.2. Results

The real time qPCR experiments analysis showed a significative differential expression for three out of the seven target genes considered for these experiments. In particular, tolloid-like protein 1 (tll1) resulted to be significantly less expressed in TIP respect to the other proximal parts of the octopus arm (MID and PROX) and when compared to the gill. On the contrary, the tachykinin related peptide (tkp) resulted to be more expressed in TIP than in the other samples.

Finally, anoctamin 1 (ano1) resulted to be more expressed in MID respect to TIP, PROX and GILL.

Table 6. Results of the differential gene (n = 9) expression analysis in the octopus arm between the three segments considered and the gill, considered as control tissue. Two tissue sharing the same letter are not significantly different. Two tissues having no letter in common are significantly different. Letters in parenthesis group statistically similar expression levels.

Gene	p-value	Significant	Tip	Mid	Prox	Gill
tll1	0.003	Yes	15.831 (a)	26.668 (b)	23.777 (b)	27.934 (b)
tkp	0.005	Yes	0.494 (b)	0.287 (a)	0.123 (a)	0.105 (a)
ano1	0.009	Yes	0.361 (a)	0.551 (b)	0.395 (a)	0.311 (a)
ck2	0.127	No	0.027 (b)	0.025 (b)	0.015 (a)	0.018 (ab)
asic1	0.237	No	0.009 (ab)	0.002 (a)	0.003 (a)	0.018 (b)
piezo2	0.986	No	0.674 (a)	0.583 (a)	0.560 (a)	0.474 (a)
trpa1	1.000	No	0.023 (a)	0.025 (a)	0.015 (a)	0.014 (a)
fmrf	1.000	No	3.049 (a)	4.087 (a)	3.056 (a)	4.933 (a)
mor	1,000	No	0.238 (a)	0.166 (a)	0.227 (a)	0.232 (a)

I considered the gill as control tissue and therefore further expressed gene expression values as ratio (R), as:

$$R = \frac{(E_{\ target})^{\ \Delta Ct\ _{target}\,(control-sample)}}{(E_{\ reference})^{\ \Delta Ct\ _{reference}\,(control-sample)}}$$

Figure 19 summarize the observed variations in the expression of the target genes considered for this experiment considering the three parts of the arm and the gill.

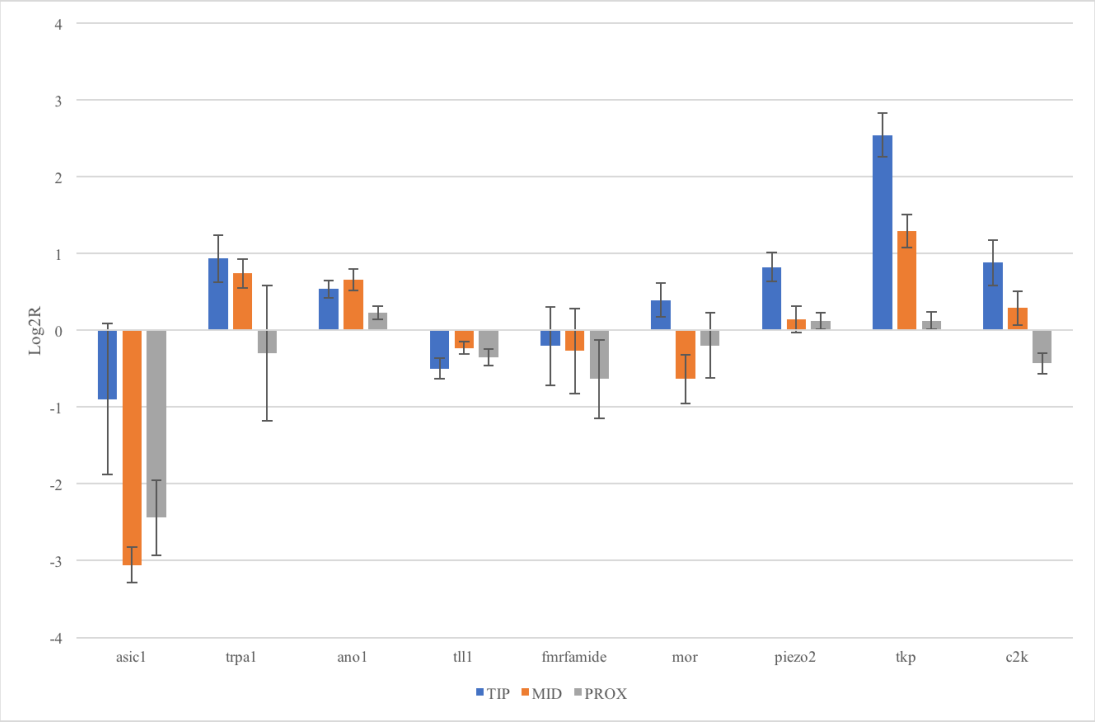

Figure 19. Log_2 expression ratio of observed gene expression values for the octopus arm segments (TIP, MID, PROX) relative to the gill.

The differential pattern of gene expression along the arm, highlights that trpa1, mor, piezo2, tkp and c2k are more expressed in octopus' tips (see also Table 6).

Asic1, tll1, FMRFamide appears expressed less than in the gill.

These data need further analysis and experiments.

7. Contribution to the identification of putative nociceptors in O. vulgaris arm

To further provide an analysis of the pattern of expression of putative nociceptors in the O. vulgaris arm, I utilized an immunohistochemical approach using antibodies for potential markers of nociceptors.

7. 1. Methods

In analogy to the gene expression studies, Octopus vulgaris samples were obtained from local fishermen (Bay of Naples, Italy) as freshly killed animals and tissues

immediately taken and preserved. Segments of the tip and the middle part of the anterior and posterior arm (L1 and L4) were dissected and immediately fixed in 4% paraformaldehyde in filtered sea water for 3 hours.
Samples were washed three times in PBS 1X (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2mM KH2PO4, pH 7.4) for one hour.
Samples were cryo-protected by overnight immersions in ascending concentrations (10%, 20%, 30%) of sucrose in PBS 1X, then included in Killik cryostat embedding medium (Bio-Optica, 05-9801) and stored at -80°C until use.
Sections (25 μm) using transversal and longitudinal planes were cut using a Leica CM 3050S cryostat and mounted on Superfrost Ultra Plus® slides (Thermo Scientific™, J3800AMNZ). Samples were dried at room temperature for one hour before immunohistochemistry.

For paraffin section, samples were dehydrated (one hour each in 30, 50, 70, 95 and 100% ethanol), cleared in xylene and embedded in Bio Plat Plus paraffin (Bio-Optica, 08-7920). Sections (10 μm) in transversal and longitudinal planes were obtained using a microtome (Leica, RM 2245); sections were mounted on Superfrost Ultra Plus® slides and dried at 57°C overnight.
Different antigen retrieval methods were used on slides in order to obtain the best staining result: 40 minutes of heat induced epitope retrieval (HIER) with citrate buffer (pH 6), or 5 minutes pretreatment with 1% sodium dodecyl sulfate (SDS).

For both paraffin and cryo-sections the following staining protocol for immunohistochemistry was utilized: slides were washed 3 times (5 minutes each) in PBS 1X containing 0.1% Tween-20 (PBT), then incubated for one hour in PBT containing 5% Normal Goat Serum (NGS, Sigma® G9023) prior incubation overnight with primary antibody (see Table 7 for a full list) in PBT containing 2% NGS.
The next day, slides were washed 5 times (5 minutes each) in PBT and incubated for one hour with secondary antibody 1:250 in PBT. After several washes with PBT slide were stained for 15 minutes with DAPI nuclear staining (Sigma® D9542) 1:1000 in PBS, washed with PBS and mounted with Fluoromount™ Aqueous Mounting Medium (Sigma® F4680).

In some case slides were also stained with the isolectin IB4 FITC conjugated (Sigma® L2895, diluted in Ca^{2+} PBS, 1:100), prior to DAPI staining.
Slides were finally observed and photographed using a Leica DMI 6000B microscope. Measures were made with ImageJ software (http://imagej.nih.gov/ij/).

Table 7. List of primary antibodies utilized in this study. Antibodies utilized as reference as in the consolidated practice are marked in blue

Target	Clonality	Host	Dilution	Producer	Product N°
TAC1	Monoclonal	Rat	1:250	NovusBio	NB100-65219
Nav1.8	Monoclonal	Mouse	1:250	NovusBio	NBP1-47615
TRPV1	Polyclonal	Rabbit	1:500	NovusBio	NBP1-97417
CGRP	Polyclonal	Rabbit	1:500	Cloud-Clone Corp	PAA876Ra01
acTub	Monoclonal	Mouse	1:1000	Sigma-Aldrich	T6793
TRPA1	Polyclonal	Rabbit	1:200	Atlas Antibody	HPA026630
NF200	Monoclonal	Mouse	1:2000	Sigma-Aldrich	N5389

The use of commercial antibodies have been validated through western-blot analysis or by testing the similarity of the sequences of the epitopes characterizing the some of them against the predicted protein sequence available in the O. vulgaris transcriptome I utilized as reference (data not shown; see also below).

Some paraffin slides were also treated using Mallory trichrome stain (Beccari & Mazzi, 1966). Briefly, paraffin was removed with two washes in xylene (10 minutes each), followed by ethanol series (3 minutes in 100, 95, 70, 50% ethanol) and distilled water. Then they were stained for 2 minutes in Solution A (Acid Fuchsin 1% in distilled water), washed in distilled water, differentiated for 2 minutes in Solution B (1% phosphomolybdic acid in distilled water), washed twice in distilled water, stained 15 minutes in Solution C (Orange G 2%, Aniline Blue 0.5%, Oxalic acid 2% in distilled water), washed, dehydrated in ethanol, cleared in xylene and mounted.

As a result, collagen and reticular connective tissue appears light-blue, nuclei red, smooth musculature is violet, striated musculature orange-red and mucus is blue (Figure 20). The figure serves also as reference for identify parts of the octopus's arms and suckers.

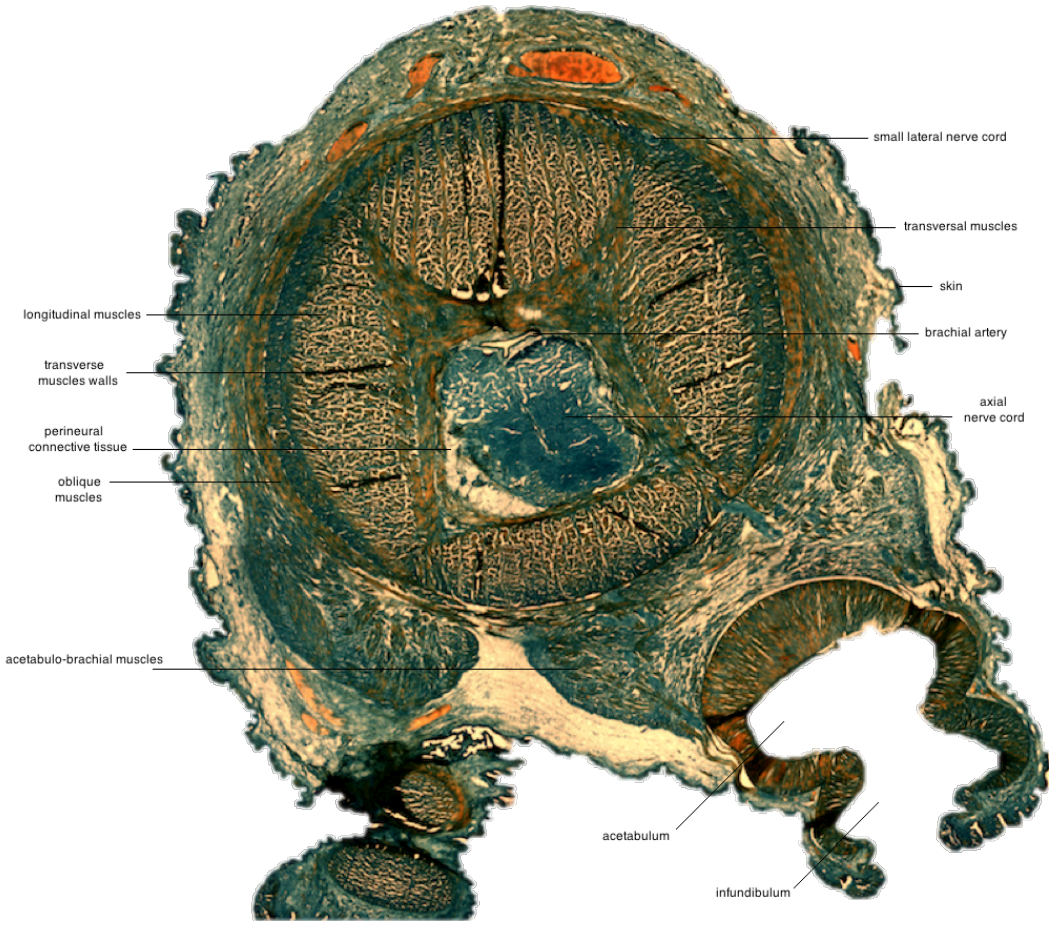

Figure 20. Transversal section of Octopus vulgaris arm, after Mallory trichromatic staining.

7. 2. Results

For sake of clarity, results of the immune-histochemical assays are presented here for area of interest and target.

Infundibulum

I found a diffuse positivity, possibly aspecific, of TRPV1+ at the level of the epithelium covering the infundibulum (Fig. 21B). Among the epithelial cells there are many acTub+ and TRPA1+ primary sensory neurons (cell body diameter: 7.067 ± 0.398 µm, n = 50, Fig. 21A).

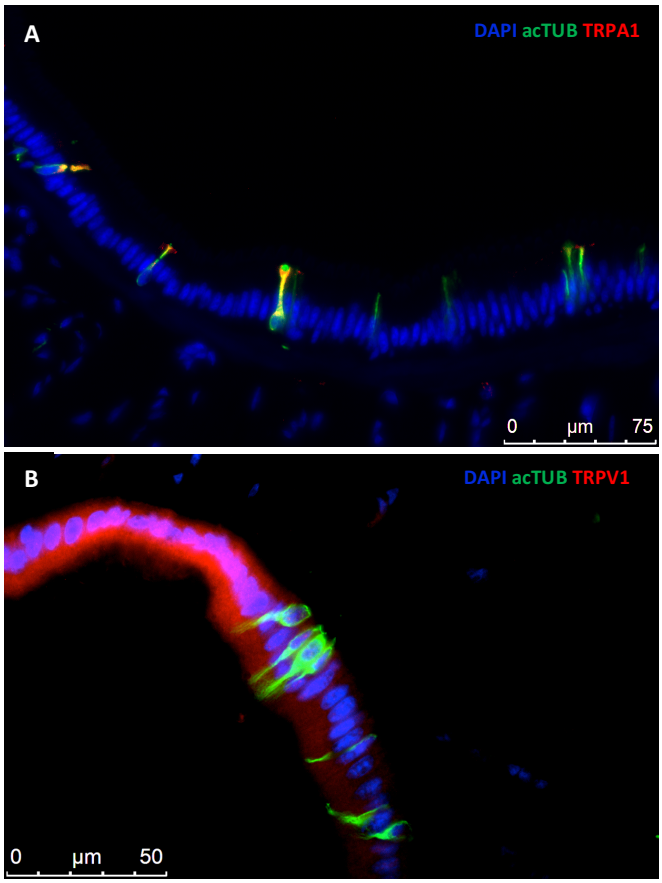

Figure 21. Primary receptor cells within the epithelium covering the infundibulum stained with anti-acetylated tubulin and TRPV1 (B), anti-acetylated tubulin and anti-TRPA1 antibody (A).

Sucker rim

The sucker rim (or marginal fold) is a soft and plastic epithelial bead that surrounds the opening of the sucker. It appears highly innervated by acTub+ and TRPA1+ fibers (Fig. 22). Also, IB4, TRPV1 and Nav1.8 positive structures have been identified (Fig. 23).

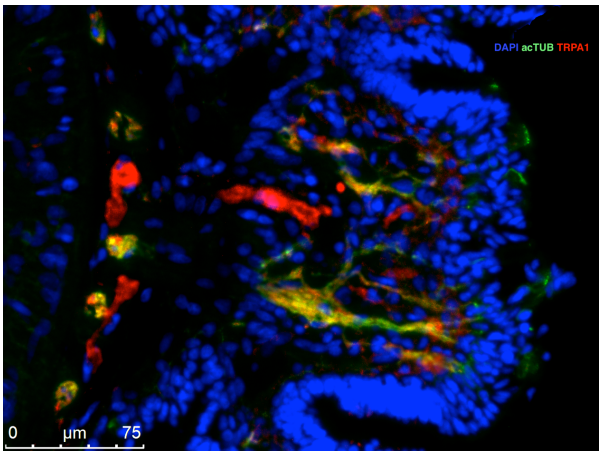

Figure 22. AcTub+ and TRPA1+ fibers innervating the rim of the octopus sucker.

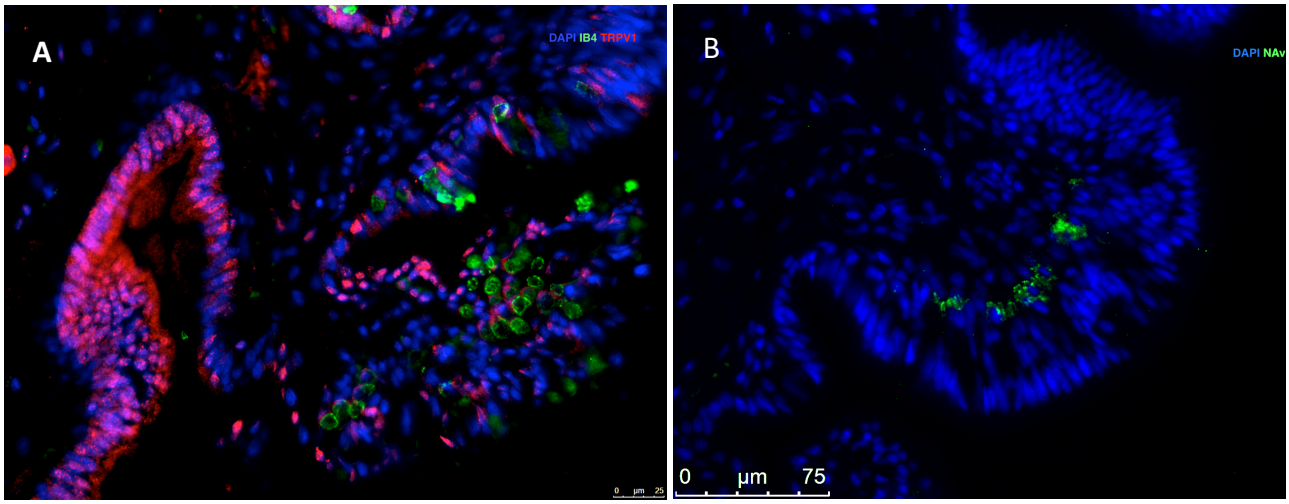

Figure 23. IB4, TRPV1 (A) and Nav1.8 (B) positive structures in the rim of the octopus sucker.

Sucker muscles

In the musculature of the sucker many fibers and cell bodies positive to acetylated tubulin and TRPA1 are present (Fig. 24A). The fibers rise from the musculature towards the gangliar portion of the axial nerve cord of the arm (Fig. 24B).

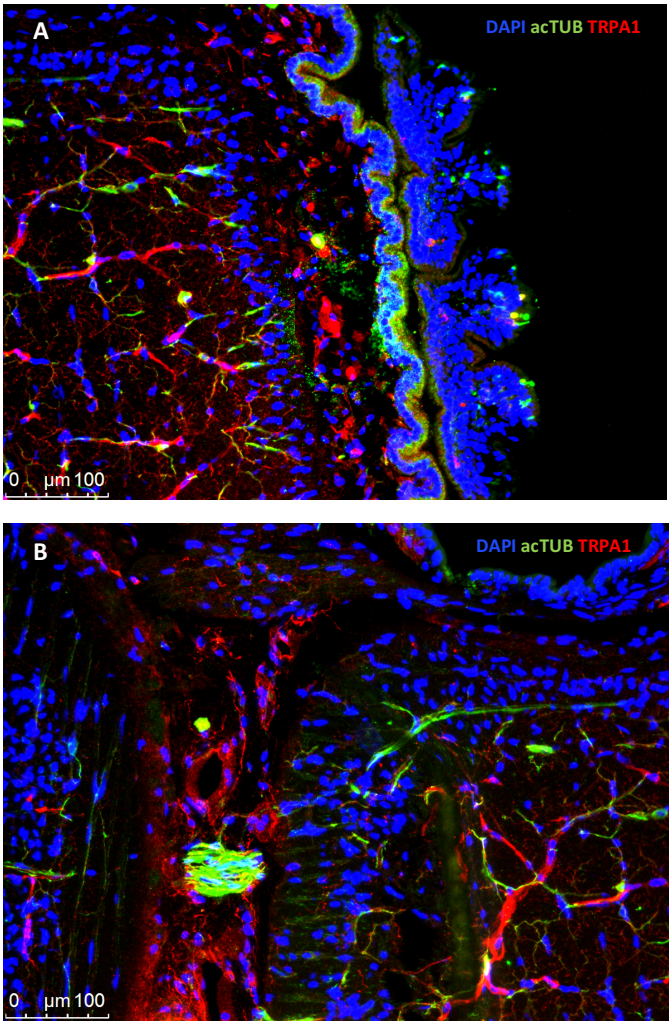

Figure 24. AcTub+ and TRPA1+ fibers innervating the muscle of the octopus sucker and proceeding towards the axial nerve cord (B).

Axial nerve cord

The axial nerve cord of the arm (Fig. 25, see also Fig. 20) consists in a chain of ventral ganglia and two dorsal axonal tracts.

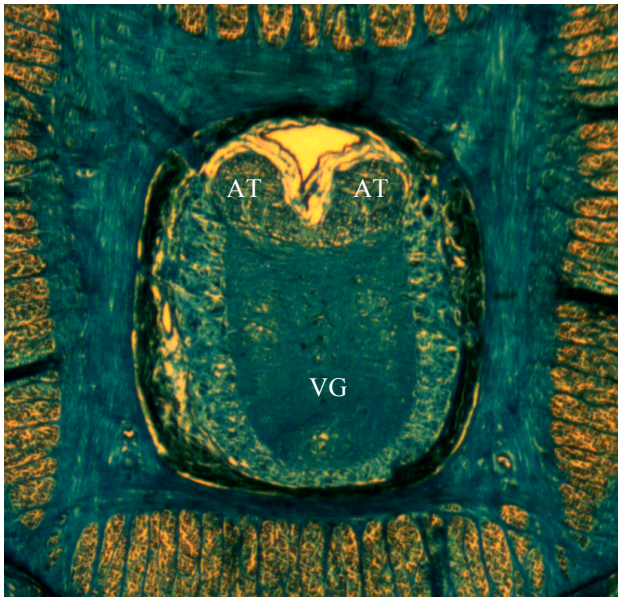

Figure 25. The axial nerve cord in transversal section (Mallory trichrome stain). VG: Ventral Ganglia; AT: axonal tract.

I identified TAC1 positive fibres (diameter: 2.11 ± 0.09 µm, n = 16) running along the axonal tracts, and TAC1 and IB4 positive rounded cell bodies (diameter: 14.78 ± 0.58 µm, n = 20) within the ganglia (Fig. 26).

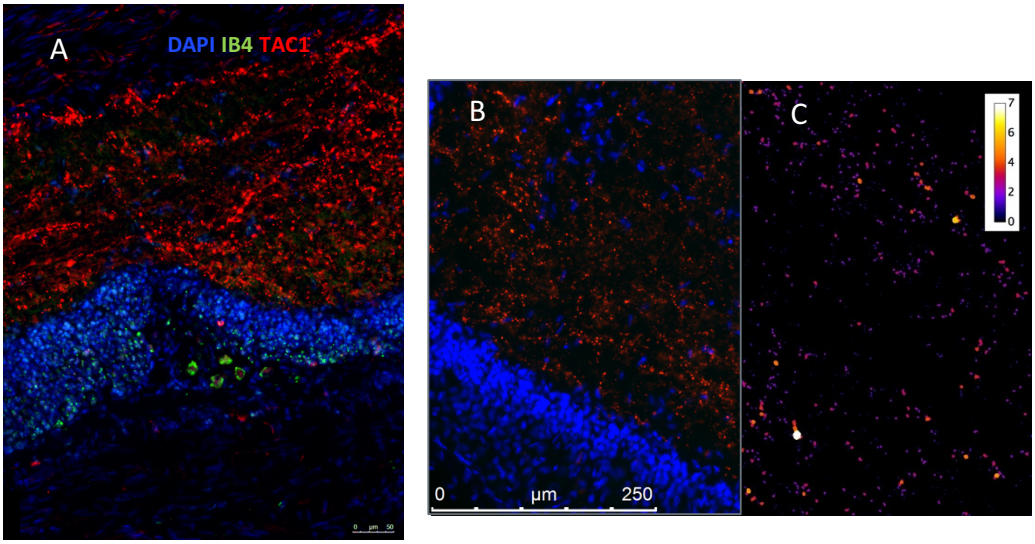

Figure 26. Sagittal (A) and transversal (B) sections of the axial nerve cord, with TAC1 positive fibres and IB4 positive cell bodies. (C) fibres' diameter is illustrated following coloured scale (in µm).

Figure 27 shows TRPA1 and NF200 positive fibres running along the axonal tract of the nerve cord. The cell bodies in the sucker ganglion appear also positive to TRPA1 and NF200. Examination of details of the axonal tract reveal TRPA1 positive cells and axons (Fig. 28).

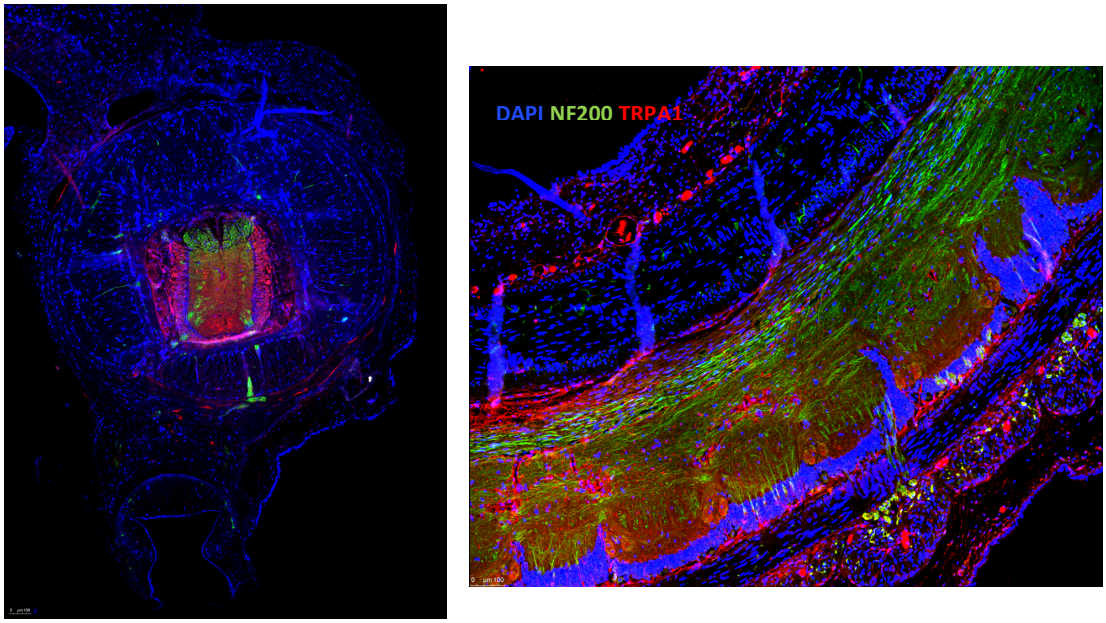

Figure 27. Longitudinal section (right) and the corresponding transversal section (as reference, left) at the same level of the arm of O. vulgaris. TRPA1 positive fibres and cell bodies appears in the axonal tract of the nerve cord and in the sucker ganglion, respectively.

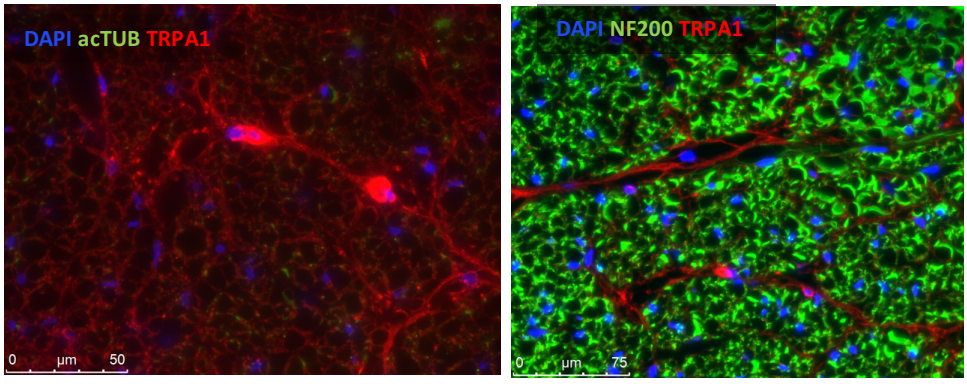

Figure 28. Details of the axonal tract of the nerve cord in transversal section to show TRPA1 positivity in cells and fibres.

Radial arm muscles

Fibres positive to TRPA1 are also present within the radial muscles of the arm (Fig. 29).

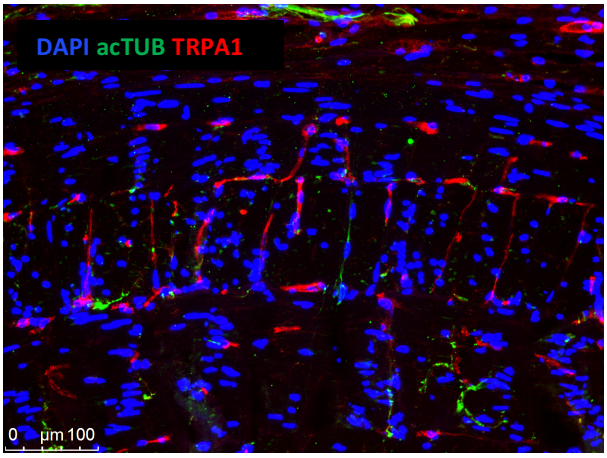

Figure 29. Transversal section of the arm to reveal details of the radial muscles in which acTub and TRPA1 positive fibres are visible.

Skin

Receptors-like structures are presents all over the skin covering the arm: all resulted positive to acetylated tubulin in my study.

The great majority of them showed also CGRP reactivity, and IB4 (Fig. 30A, B).

A fewer number of structures in the skin of the arm show TRPV1 positivity (Fig. 30C).

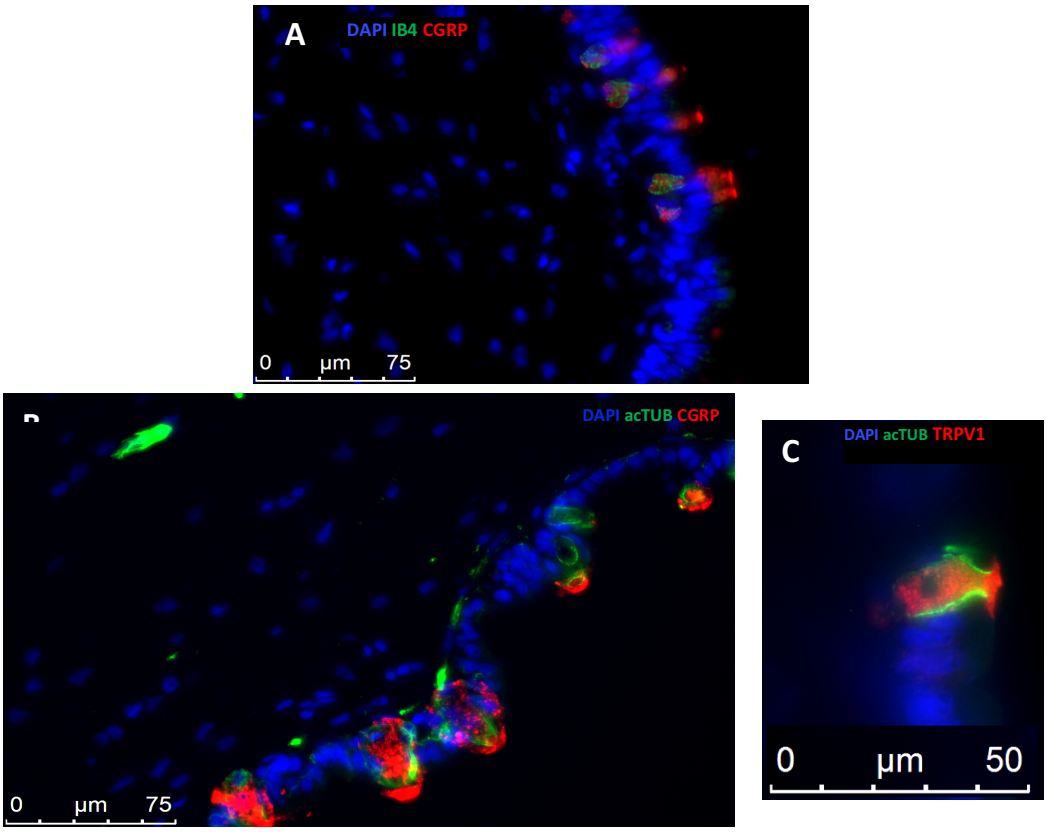

Figure 30. IB4, CGRP and TRPV1 positive structures on the skin covering the octopus arm.

I found that the epitope sequence of the antibodies for TAC1, Nav1.8 and TRPA1 blast (tblastn) with the corresponding annotated mRNA with an E-value < 0.0001. On the other hand, I found no annotated sequences for TRPV1 and CGRP, and these two antibodies were also utilized in western blot analysis on arm protein extract: only TRPV1 appeared to identify a protein corresponding to the expected one. Results of validation and of immune histochemistry experiments are summarized in Table 8.

Table 8. Summary of results for validation and immunohistochemistry assay (Exp) with the listed antibodies.

Protein	Validation experiments	IHC
Sub P	AB immunogen aligns with Ov Sub P (c31437_g10_i1).	Specific signal in NC fibers
TRPA1	AB immunogen aligns with Ov TRPA1 (c31382_g11_i1).	Spread signal (cells and fibers in the whole arm)
Nav 1.8	AB immunogen aligns with Ov Nav (c32552_g1_i2).	Specific signal in sucker rim cells
TRPV1	Western blot: Dimer (100 Kda, 50 Kda) in the arm,	Spread signal, more specific on the skin
CGRP	-	Structures on the skin and in minor lateral nerve cords

8. Discussion

One of the major benefits coming from the ability to "feel" pain, is the capability of organism to avoid to experience anything that elicits "pain". This is something that in the brain is commonly achieved by "tagging" a memory event as painful, hence implying a process of learning (e.g. avoidance learning); it requires a certain degree of self-consciousness (Bateson 1991, Metzinger 2017).

8.1 Candidate "pain" related genes in O. vulgaris nervous system

The learning system of cephalopods has been well described by Young in 1991: he reported the presence of two memory systems: one for visual and the other for tactile learning. The latter relies on information obtained from receptors around the rim of the suckers (like the ones identified by Graziadei) and ensures that the arms provide food to the mouth and reject the rest.

The signals start in the receptors of the sucker and from those of the lip, and reaches the frontal lobes (FLs) of the supraesophageal mass (SEM).

One of the first centers is the lateral inferior FL, which may function as a competitive learning matrix for the incoming stimuli. Then part of the information rise to the lateral superior FL, while other fibers move to the median inferior FL.

From the latter, the signal reaches the subfrontal lobe, which is composed by large cells with complex dendritic fields that, according to Young (1991), can be activated also by "pain" fibers.

On the basis of this description, the subfrontal lobe modulates the behavioral response (draw or rejection), that is mediated by the neurons of the posterior buccal lobe. When signals coming from "pain" fibers reach these centers and rejection happens, the activated synapses in the subfrontal lobe are consolidated by the action of many amacrine cells (which have short axons connected to the dendrites of the larger cells). According to Young, the basic function of this first circuit within the SEM's lobes is to take touched objects unless signals of "pain" are perceived. The other pathway that goes from the lateral inferior to the lateral superior FL proceeds towards the subvertical

lobe (and then back to the posterior buccal lobe), but a branch of fibers rise from the median superior frontal lobe to the vertical lobe (VL). It is from the Vertical Lobe that fibers pass down to the subvertical and posterior buccal lobe. The re-excitation through this second circuit maintains the conditions necessary for a Hebbian type of learning.
The VL is involved in the tactile memory system, but is also the main component of the visual learning system, and may thus be a center in which the two different information are integrated, making possible to discriminate visually between something "painful" or not. Indeed, together with the median superior FL, the VL provides a system that prevents visual attack when trauma occurs; the optic lobes and the basal lobes are the regions where the memory that connects a stimulus to shock is set up (Boycott & Young 1955).

My results show that in the SEM the calcitonin gene related peptide mRNA is highly expressed, suggesting that in this mass some CGRP carrying fibers branches with other neural centers thus bringing nociceptive information.
Another gene highly expressed in the SEM is the one that encode for a neuronal cadherin (N-cadherin). N-cadherins are transmembrane proteins expressed in the majority of CNS synapses (Yagi & Takeichi 2000). The intracellular tail region forms complexes with intracellular catenin proteins, thus facilitating a "link" between synaptic activity and plasticity, and and in learning and memory (Tang et al. 1998, Raduolovic et al. 2007). N-Cadherins are reported to stabilize the connection between the presynaptic and postsynaptic terminals (Arikkath & Reichardt 2008).

The abundance of N-cadherins in the SEM is then consistent with Young's description of the first circuit in the SEM. We can hypothesize that N-cadherins could be involved in the stabilization of synapses in the subfrontal lobe mediated by the amacrine cells.
The proto-oncogene RET is also highly expressed in the SEM: RET is a tyrosine-protein kinase receptor for GDNF (glial cell derived neurotrophic factor) involved in neurogenesis and then in learning and memory consolidation (Huguet et al. 2009).

My results suggest that the OL could act as a center where "nociceptive" signals are modulated by the activity of ligands (Glu, 5HT, peptides with opioid-like activity) on the G protein-coupled receptors.

Neprilysin is a zinc-dependent metalloprotease that inactivates several neuropeptides such as enkephalins and substance P (Oefner et al. 2004). Then, the presence of a high number of opiod receptor oprm1 transcripts in OL, together with the evidence of highly expressed tachykinin and Mytilus inhibitory peptides in the SEM, suggests that this two brain areas of the octopus nervous system, are somehow involved in the modulation of "pain" mediated by tachykinins and opioid-like peptides. Some kind of transcriptional, translational and regulative activity connected to nociception may also take place in the OL (possibly connected to the consolidation of memory).

The lobe also appears to have significant high expression of the C-terminal binding protein 1 (a regulator of DNA regulated transcriptional activity), the cytoplasmic polyadenylation element-binding protein (a regulator of translation) and kinases (Ca^{2+}/calmodulin-dependent protein kinase II, tyrosine-protein kinase Src42A).

SUB is enriched in P2X purinoceptor 4 (P2RX4), Piezo-type ion channel 2 (piezo2) and the prostaglandin E2 receptor EP4 subtype (Ptger4). Piezo2 is the major mechanotransducer required for touch sensation in mammals (Ranade et al. 2014). P2RX4 is a receptor for ATP that acts as a ligand-gated ion channel, involved in many biological processes, whose activation is required in tactile allodynia after nerve injury in rats spinal cord microglia (Inoue et al. 2004).

Ptger4 is one of the four receptors identified for prostaglandin E2; in humans, its activation suppresses the release of cytokines and chemokines from macrophages and T-cells and its involvement in cellular response to mechanical stimuli has been inferred by sequence or structural similarity.

The abundance of these transcripts in the SUB shows that this brain mass has a role in processing mechanical (noxious and innocuous) stimuli. The TIP is enriched with transcripts of ion channels involved in the detection of noxious chemical, thermal and mechanical stimuli (trpa1, asic1, ano1) that in vertebrate sensory terminals have a critical role for nociception. This result suggests that primary nociceptors equipped with these specialized receptors, if present, are probably located in this area. Moreover,

tll1 transcript, which resulted enriched in Aplysia sensory neurons (Moroz et al. 2006), is relatively very highly expressed in the TIP, strengthening the hypothesis that this tissue has an important role in sensation.
In the TIP also the FMRFamide receptor (FR) and the endothelin-converting enzyme 1 (ECE1) are highly expressed, therefore is likely that also some kind of nociceptive modulation may take place in the foremost distal part of the octopus arm (i.e. TIP ganglia).

A residual capacity for learned discrimination is present after cutting the cerebro-brachial tract (then removing the influence of the inferior frontal and the vertical system on the arm), and it probably relays in the SUB or in the arm itself (a circuit in the sucker ganglia may be an explanation; Young 1991). The arms, that all together contain around 350 million neurons (compared to about 170 million in the brain, Young 1963, Giuditta et al. 1971), are then likely to possess a certain degree of autonomy (Hochner 2012).
Bellier et al. (2017) found 5HT (serotonin) immunoreactive neurons in the gangliar part of the nerve cord, sending axons to the neuropil that are not connected to the axonal (cerebro-brachial) tract. Their observations suggest the presence of two distinct type of 5HT innervation in the arm: a group of efferents running from the brain to the arm by the cerebro-brachial tract, and an intrinsic circuit of fibers that arises from the neurons of the gangliar part of the nerve cord and innervates the arm, including the receptors of the sucker rim.
This circuit may have a sensory function in the octopus, probably modulating sensory transmission via presynaptic inhibitory control and maybe integrating the inputs from more than one receptor. Then, also a local modulation of nociceptive afferents mediated by 5HT is possible.

None of the transcripts considered in this study, appeared to particularly expressed in the medial part of the arm (ARM).
I cannot provide any other explanation, apart from the consideration of the fact that in this region, the relative area/volume of the nervous/muscle tissue ratio is lower than in the tip (e.g. Margheri et al. 2011), and therefore the muscular tissue-component may

have provided a bias during annotation considering the cutting-off filtering of the original study (Petrosino 2015).

8.2 Gene expression data

In order to contribute to the knowledge of the distribution of pain related genes in the arm of O. vulgaris a quantitative analysis (through real-time qPCR) of the differental expression of nine Octopus putative 'pain-related' genes was carried out: asic1, trpa1, ano1, oprm1, tkp, piezo2, tll1, camkII, FR. The study have been conducted comparing three different segments of the octopus arm, i.e. PROX: proximally to the head-mouth; MID: middle part, TIP: distally to the head. The gill was used as control/reference tissue (as non-nervous tissue, Capano et al. 1986).

Tolloid like protein 1 (tll1), which appeared to be highly expressed in the tip from the in silico study, resulted to be significantly more expressed in the parts closer to the head (MID and PROX), but the difference was not significant, when expression values were normalized to those of the gill (where tll1 expression is comparable to that of MID and PROX).

The tachykinin related peptide (tkp), enriched in SEM (see in silico data), resulted to be significantly more expressed in TIP respect to the other two more proximal part of the arm.

A possible explanation is that substance P (encoded from tachykinin mRNA) is present in the axial nerve cord (see above) and, in the tip the nervous/muscle tissue ratio higher than in the other two segments. Indeed, when the expression were normalized to those of the gill, the difference resulted to be not significant.

Anoctamin 1 (ano1) appeared expressed in MID respect to the other two segments but, again, the difference is not statistically significant when considering the relative expression as fold changes in comparison with the gill.

Asic1 is highly expressed in the TIP, but even more, doubling the expression values, in the gill. This is actually not surprising, since in marine organisms gills are considered as being the predominant site of osmoregulation and acid-base regulation

(Wheatly & Henry 1992; Lignot & Charmantier 2001). Ion-transport systems located in highly developed gill epithelia form the basis for efficient compensation of pH disturbances during exposure to elevated environmental pCO_2 (Melzner et al. 2009).

The normalized (to gill) expression of trpa1 seemed to decrease from TIP to PROX in my data, and this could confirm the higher expression in TIP as emerged from in silico data, that suggest that this area is specialized in perceiving stimuli.

The expression of oprm1 is very low and homogenous between all the tissues, while that of FR (FMRFamide receptor) is still homogeneous but relatively high. Also Piezo2 expression appeared homogeneously distributed in different parts. Finally, CamkII appeared expressed in TIP and MID when compared to PROX and gill, but all those comparisons resulted to not achieve statistical significance.

8.3 Distribution of putative nociceptors in the arm of the octopus.

TRPA1 and acetylated tubulin (acTub, marker for neurons) immunoreactivity resulted to provide a pattern that resemble for position and morphology the putative chemoreceptors identified in O. vulgaris suckers by Graziadei (1962).

From my data, is not always completely clear if the two types of immunoreactive cells and fibers identified ($TRPA1^+$ and $acTub^+$) overlap or not.

I have found them within the epithelium covering the infundibulum of O. vulgaris sucker. The presence of fibers stained with the same two antibodies in the muscles of the acetabulum, climbing up to the ganglionic part of the axonal nerve cord suggest that the signals perceived by those putative $TRPA1^+$ and $acTub^+$ receptors travel to that part of the nerve cord.

Some of the information carried from those fibers also reach the axonal tracts of the nerve cord.

Indeed, there are $TRPA1^+$ cell bodies within that area (and many others in the immediately underlying ganglionic area), and $TRPA1^+$ fibers running longitudinally along the nerve cord.

It is not clear if those fibers partially correspond to the neurofilament (NF200) immunoreactive-fibers that also run along the nerve cord.

The mRNA coding for TRPA1^{+} in O. vulgaris has been identified and its expression has been studied in silico and in vitro and it resulted to be relatively more expressed in the tip of the arm. The immunogen of the antibody I utilized blasts (tblastn in the trascriptome assembled by Petrosino, 2015) to the ovTRPA1 with an E-value of 9E-10, then is likely that it recognizes the product of the identified mRNA.

Moreover, TRPV1^{+} structure have been found diffusely in the whole arm, especially in the rim of the sucker and on the skin.
TRPV1, in mammals, is expressed in many neuronal tissue (dorsal root ganglia, trigeminal ganglia, nodose ganglia), predominantly in small and medium peptidergic neurons, but it has also been detected in non-neuronal tissue as, glial cells, macrophages and keratinocytes of the epidermis.
Blasting the immunogen sequence of the antibody against O. vulgaris transcriptome I did not obtained any result. However, its specificity has been verified by western blot (data not shown). I found positivity in the rim of the sucker (where the most of tactile receptors should be located according to Young 1991).
I found also IB4^{+}, Nav1.8, acTub and CGRP positive structures with different morphology, whose function should be investigated.
TAC1^{+} (substance P) fibers appeared to run – in my data - along the axial nerve cord of the arm, and preliminary measurements of their diameter suggest that these correspond to that of nociceptive C-fibres.

9. Conclusions and future perspectives

Pain related genes have been identified in O. vulgaris brain and arms and their expression has been studied, revealing an abundance of genes encoding for protein involved in the sensory perception of pain in the arm (especially in the distal part), while genes related to the consolidation of memory and to the modulation of pain appear to be more expressed in the supraesophageal mass and in the optic lobe.

In the subesophageal mass, genes involved in the processing of mechanical (noxious and innocuous) stimuli are abundant.
Although interesting, these results need to be validated in order to better understand the distribution of genes within the brain lobes (especially those of the supraesophageal mass). A possible approach may be to perform in situ hybridization experiments using RNA probes on brain slices.
It would be also interesting to study the expression of the genes coding for the receptors (trpa1, asic1, anoctamin) involved in the detection of noxious (chemical, thermal and mechanical) stimuli in the arm. Also in this case, in situ hybridization experiments would help to understand if there are specialized areas (e.g. within the skin or the parts of the sucker) for the detection of specific kind of stimuli. On the other hand, a strategy for utilizing specific antibodies in immunohistochemistry experiments may facilitate an in depth analysis of the possible intricate patterning between different cellular types and their modulatory role and the nociceptive pathways in the octopus brain.

This study also showed the presence of transcripts annotated as opioid receptors and opioid-like peptides in the brain of O. vulgaris for the first time, suggesting the existence of a system for the modulation of pain.

Fibres and cells involved in nociceptive pathways are shown for the first time in the arm of O. vulgaris.
Several kinds of receptors positive to nociceptors molecular marker (such as TRPA1, CGRP, TRPV1, IB4) are present on the skin covering the arm. They are particularly abundant on the rim of the sucker, which is innervated by many small caliber fibers. These results are consistent with observations by Graziadei (1962; 1965). These fibers project towards the sucker ganglion, where TAC1, TRPA1 and IB4 positive cells are present.
Within the epithelium covering the radial muscles of the infundibulum I identified cells that resemble the ciliated primary receptor cells identified by Graziadei. I also observed putative nociceptive fibres running along the axial nerve of the arm of the octopus, with a diameter matching that of nociceptive C-fibres.

Many TRPA1-like immunoreactive receptors and fibres are present in the suckers and in the arm of octopus. This is consistent with the evidence of a relatively high number of trpa1 transcripts in the tip.

Since TRPA1 is probably the central molecule for chemically induced pain (Tai et al. 2008), its presence in the arm (tip and suckers, in particular) support the hypothesis that this marks chemoreceptors.

Graziadei (1962, 1964) counted about 10,000 ciliated putative chemosensitive cells (Fig. 31) in each sucker, that resemble the TRPA1/acTub and TRPV1/acTub receptors identified in the same area in this study (but note aspecificity of the TRPV1 I obtained in some experiments).

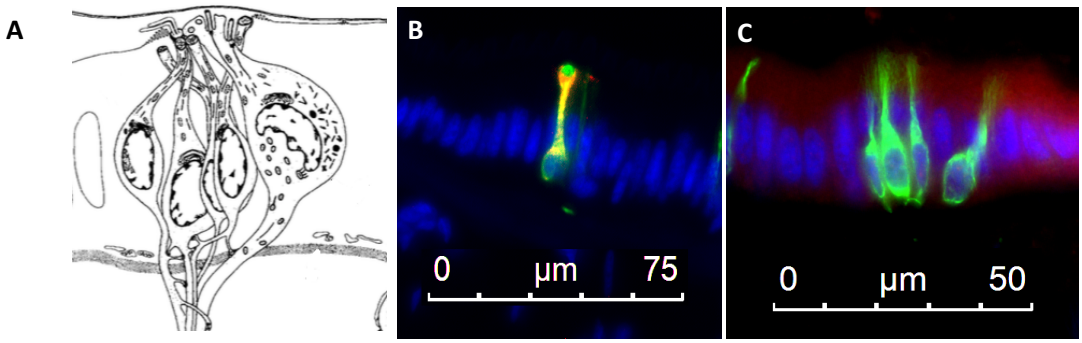

Figure 31. (A) Putative chemoreceptors identified in the infundibulum epithelium of the octopus sucker (from Graziadei, 1964). (B) Acetylated tubuline (green) and TRPA1 positive (red) immunoreactive putative receptors located in the same area. (C) Acetylated tubuline (green) and TRPV1 (red) immunoreactivity (possibly aspecific).

TRPA1 is also present in the fibers of the axial nerve cord of the arm, and would be very interesting to trace those fibers to check where they project, and if they follow one or more of the directions identified by Budelmann and Young (1985).

For that purpose, would be very useful and interesting using nerve tracers on the receptors of the sucker and controlling their diffusion in the arm and in the brachial nerve.

Probably, the best way to understand if the TRPA1 and TRPV1 positive structures here identified in the octopus arm are putative thermo- and chemoceptors would be to use some agonist of those two channels and to record the electrical activity of the afferents.

Moreover, from the study of Alupay et al. (2014) in the octopus A. aculeatus emerges that a potentially noxious mechanical stimulus (Semmes-Weinstein filament ≥ 2 g), applied to the arm or the mantle of the animal, lead to the activation of more units in respect to a considered innocuous stimulus (Semmes-Weinstein filament < 1 g). The authors suggest that those units could be represented by nociceptors. To better investigate this theory it would be interesting to check if the noxious mechanical stimulation induces also a change in pain related genes expression, also because both studies (in squid: Crook et al. 2013; in octopus: Alupay et al., 2014) observed long-term sensitization of the mechanosensory units after stimulation, that may be the result of the activation of early response genes (possibly in arm ganglia).

In vertebrates, nociceptors have been identified using a wide variety of approaches. Key (2016) argues that fish are not able to feel pain: since these animals lack cerebral cortex, and that this is the single and unique substrate capable of processing "pain" in animals, therefore they also lack the ability to suffer.
This article has sparked different reactions in the scientific community: many commentators strongly disagree with Key's arguments. Some of them have noted that evolution has often provided convergent innovation with the same function achieved by different organs/structures (Edelman, 2016; Jones, 2016; Merker, 2016; Seth, 2016; Striedter, 2016). Others others have pointed out that pain perception has a fundamental evolutive role, hence it is very unlikely that it is completely absent in organisms that do not possess a cerebral cortex.
On the contrary, the "ability to suffer" would be a feature of invertebrates and even plants (Baluška, 2016; Elwood, 2016; Walters 2016). Dinets (2016) stated that the presence of nociceptors in fish nervous system (Sneddon, 2003) must be considered an "irrefutable proof" that the animals can feel pain.
However, pain perception requires the presence of a nociceptive system, but the latter does not implicate the ability of pain perception.

More studies are required to demonstrate the ability of feeling pain in many taxa including cephalopods and other invertebrate species, where preliminary evidences commence to appear. Adding information would also help us to better understand how

a conservative physiological process such as nociception may function in an organism phylogenetically so distant from the mammalian lineage. In a broader context, these may also assist to figure out how it has evolved through the animal kingdom.

In conclusion, combined electrophysiological, morphological and molecular studies may help revealing the presence, characteristics and function of the receptors in the octopus (e.g. in the arm), and also help in drafting possible connection with higher neural centers (i.e. for octopus SEM or OL).

The combination of different approaches, including a detailed analysis of behavioral responses, that are here only attempted preliminary, may provide future support to the study of nociception in octopus and other cephalopods.

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APPENDIX

List of selected pain related transcripts, with corresponding name, ID (black: Octopus_uniref_2013, blue: octopus_global_2015) and nucleotidic sequence.

Tachykinin-related peptide comp33729_c11_seq3 c31437_g10_i1

TTTTTTTTTTTAGTTCATGATTCCTTAACGAATGATTTATTTCAAAAGAAAACAGTTTTTTTTATTTAT
TGTCATACAACCTAAAGTAATCGCAGGATCCATTATGGAAAACACTGGAAAATTATAATTAAAAAA
AAACGACAAAAACATTCTGTAAATTACACGCACAAGTTTTTGTGTAACATGAGATTATTACGTCAAA
CAGTTAATACGCGAAGCGTTTTTAAAGCAGACTATATGTTGTCGAATCTCTTCGAATCTCATTGTAT
CTGGTCGTAACCACTGGACAATTTGCAAGATGGCAGAAATCACGGTTGATTAGTTTGTTTGGTTAAT
TGGTTAATCAGTGGTGGTAATAGTGATGTGAGTGGTAGTGGTAGTGGTAGTTGGGAGGAATGTAAT
GGTTCAGTGTCGTTTATTGTCACTCAAGCTCAAGTATTTTGATTGGTTGCTCGGGTCTCTTTATGTGG
ATCGCTTACCTCGCGAAGGTATGAATGTGTTGTGTAAAGACGCCCGCCTTTCGTAGTTCTCCTTTGGT
GAATCGTTTTGGGTATTTAACACATAATCTTCAAAAGTACTTTTCTTGCCACGCGATCCAATGAAGT
CAAGTGGACTGTATTTTTTGCCTTTTGGGGGACCAAACGATAAGGAATTCATTCTTCGGCCTTCTCTC
AATAACATCGGACCCGTGTAAGCAAGTTCGTCATTGGTTGATTGTGATCTGCCTCTTGTTGGGACGA
ATGCTAAAGCATCTGATTTTTTGTCAATGACTCCCGTAATATCGTTTAGTTGGGGCTTTTCACCGAGT
AAGGTAACCAGACGTTTACCCCTCGATCCGAGGAAAGCGTTAGCACTGACAGTCCGTTTGTCATTAA
CGAGGACTCTTTTACCTCGGGAACCCATGAAACTGTTTGCGTTTAATCTTTTGCCTCTGGTACCTTGG
AAACTGTAGGGGTTAACTTTTTTCATTCTTTCAGCAGATTCTGAGTCGGTGTCGTCAATTGAAGCTCT
ACTTTCGATAGTGTTGGCCAGGAAACCCAGTTTGCGTAAAAATTCCATAATTTCTTCATGGCCTGGG
TACCTACCACTGTTGACGTTATTACTGTCCAACTCATAGTTCTTCTGCTGTGGTTCCAATTGCTTGGG
TAGATAACTGGCAAACGCACCCCATTGCAAAGTGTTCAAAAGTCCGGAAAGTAGCAAGGATACTTG
TAAAAAACAGCTTATTCGCCTTGAGCTTGATACTCTAATTGCATACATGAGGGAAGAAGAAGAAAG
ACGAAAAGAAAGAAAGAAGGAATGCTAGAAAGAAAGAAAACTGGGGTGTGTGTGCACGTAAATAT
ATGTGTGTGTTGATTAAATAGACAGACAGACAGACAGATAAACAGAAAGATACATTGAGTAGGTAG
AGAGATAGCTAGATAGGTAGATGATGTAGAATAAAACAAAGGAAAAGCAGAGCTAAGTGGAGACA
GGGATGAAAGTGAAGAAAGTTATTAAATAAATATGAAAATATGCAGTTCTTTAAAAAAGTGTTTAA
GTTTTTCTTTGTTCTCTCTCTCTACATATATATCTTTTTTTTTTCAATAAATGTAACTGCTTGTATGTTC
GGAAAAAAAGATCGTCTGCAGAAATTAGAGTTGTAGAGGTTGTCTGCTTACTGTCCAAATGATCAG
ATCTGGTCCTGCATTGCAAATATGCATGTATATATACATATATACGCATATACACACTCGTATATAG
CTTTATTTATATCTTAATGTGTATGTATATTTTGTATATATATATATAAGTACATCTATGACTGTCGTT
ACAACTATATGTCTATATATTCACGCGTATATACTTTCTGTGTAGGTCTTATATATTCTCAACACCCC
CTTTATATGTATGCCCATGCACAGACATACATATATAAACCAACACACGCATATATATATGTATGAC
ATATATAGAT

Opioid-binding protein/cell adhesion molecule comp32654_c2_seq1 c29681_g1_i3

ATATAATTTGGTTGCTTATACCAAATATATAGTATTTTTATATCTTAAAGCTGTAATTTCGCATTGAT
AAGATCCTTCTGAAAATATTTTCCTTTTTTTTTTTTATTATTCACATTTTTTTTTTATTATTCACAGTTTTC
CAACAAAGCCAGGGGCAATGAACTATTTAAACATAAGATAAAGTTTTATTTTTCATGTATCATGGCT
TGTGTTTTATTTCATAAGTCAATTTTCGCCCACTTTAAAAACAAAAAAATTGTTGATACCAAGAAAC
AATCCAAATGTTTTTGTCATTATTTGTTAAGCATTGGTATGTCATATACATGTATGTATATATACATA
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TAATGCTATCACCACAATAATCATCATCATCATCTTCACCCTTAGCATTAACTTCATCTTCCTCT
ACCTCCTCATAATCAGCCATCAGCACAACTGTCACAACTATCACAAACGATTCCAGGGAATGTTACT
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TACGTCTACTTTTACCACACCTTCCTTGAAACTTTTCTCCAAATACGAACATTTGAGAGACAGAGGG
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GTGTTATTATGCCGAGCATCATGTCGACACAAATACCGAGATTTCTTCCGACCCCAACCAATGTCAC
ATACAGACGAGGACAATTAGCAACGTTGCGGTGTTCCGTAGATAAATTGGGAACAAAATTTGTTGT
GTGGCGGAGGGCGAGTGACCCGAACCCTCTAACCGTCGGGAGAGAAAAATTCGCTGATGATCCGTA
TATAGGGGTCAGTCATCAGGCAACTGACTGGAACCTAATCATAAAAAATGTGCAACCACGGCATGC
CGGTGTGTACGAATGTCAAGTCTCTTCTGTCCATCAGCTAATACGACATATCATGTTACGAGTTGTA
GATAACACAGTAAAAAAGAACGTTCTCACGATATCGGGAAGCAGTTCTGTCACACGAGGTTCTGCC
ATTTCTTTGATGTGTAACTCATCAGGAGGTGACAGCCGGTCTCGTGATATTGGCTGGTATAAAGAGG

GAACTAAGATATATTCGAATCGAGACAATCGAATTTTGACGACTAAATACACATCGGCAGATAAAG
TTACAGTCAGTTCTTTAAGAATTGAAAACAGTCGGTTAGAGGATGGAGGTGTGTACGTCTGCCGTAC
GCCCAACAAAGCGTCCATTACTATTGATGTTAAGGTACATAATAGTTCGGGAACCACTGTGAATGG
AACTTTCGTTAAAAGAGAAGAACACAAAGCCGGCTCTGAGCAGGCTTACAGGGGTGATAATAATCT
TTTTGAGTCGTCTGCTGATAATGCAAATGATATTCAAATTCCTGGAATAGAACCAACACAAACGACC
AACGGACAAATATCTAACTCACAGTTCACGCCACAGTTGCCTTGTTTCCTTCTACTATTTTACTTACT
TATATATTCAACAGAACATCTGTAGCAACGGACAAGAGTGCGTGAGCAAATATGGTGGCCTTTTTG
ATTAATATTATCAGATTTGGTCTGAAGATCAAATTTCAGCCAGACTGCTTAACTTGCAGGGGGAATT
CCACAAGCAGGAGGACCTGGCAGATGGTTTGATACAATGATGGAAGTCCGCTTGAGCCGTAAATCA
AGTCTTAAGTCATTAGAAACTTTGACTGGGCTGAAATGAATCCAGTCAAAGATCATTTGATCCGTTA
AGAAGTTGTCGCTTCGATACGACCAGAATGTTAACGCTAAGACTGACAGCAAAAGAACGAAAATGT
AACACACACATAACATTTATGTAAGCGTAGATATATGCATATATATATATATA

MIP-related peptides comp35771_c6_seq9 c32005_g3_i5

AAACATGGGATCCTCTCTTTTACCGACAAACATAAGATCTCTTTTACCAACGAACATAGGATCTTCT
TTTTTACCGATAAACATAGGATCTTCTCGTTTCCTGACAAACATAGGGTTCTTCTCTCGTTTACCCAC
AAACATTGGATCGTCTCGTTTACCTACAAACCTAGGATTTCTTTTACCGACAAACATAGGCTCTTTCC
CCCGTTTACCGATAAACATTGGATCTTCACGTTTACCAACAATCATAGGCTCTTTCTCTCTTTTACCA
ACAAACATTGGATCTTCATCACGTTTACCAACAAACATAGGTTCTTTCCCCCGTTTCCCGACAAACA
TAGGATCTTCTCGTTTACCGACAAACACAGGATCATCCTCTCGTTTTCCAACAAACATGGGATTTTC
CTCACGTTTACCGACAAACATTGGATCCTGCTCTCTTTTACGACCAAATACAGTCTCCCTGGAAAAT
GGTGCAACGAAAGCTGAGGGAAATGACGCCGGCTTTTGTTTTCCCGTTAAAAACGTATCGTTATTTC
GTAAAGAACGAAATATTAGATCCGGTTCATGTATATTAGTGTATACAGATCTTGAATTTCGCTTTCT
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DNA polymerase delta catalytic subunit comp8274_c0_seq1 c33532_g6_i1

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Proto-oncogene tyrosine-protein kinase receptor Ret comp36619_c5_seq26 c32554_g1_i1

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60S ribosomal protein L3 comp21925_c1_seq1 c33927_g12_i3

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Neprilysin comp35164_c8_seq1 c33714_g6_i1

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Endothelin-converting enzyme 1 c36036_g2_i2

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Tolloid-like protein 1 comp32880_c0_seq2 c30328_g5_i1

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Calpain-B comp35975_c4_seq1 c33923_g6_i4

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Prostaglandin E2 receptor EP4 subtype comp33852_c1_seq1 c32689_g5_i1

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5-hydroxytryptamine receptor comp30193_c2_seq1 c31475_g4_i1

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Mu-type opioid receptor comp31894_c1_seq1 c35350_g9_i3

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Calcitonin gene-related peptide type 1 receptor comp29572_c0_seq1 c31238_g13_i1

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FMRFamide receptor comp33243_c14_seq1 c31162_g13_i1

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Metabotropic glutamate receptor 5 comp35240_c0_seq1 c33380_g1_i2

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Adenosine receptor A2a comp33777_c3_seq1 c32628_g1_i1

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Glutamate receptor ionotropic NMDA 2D comp24794_c2_seq1 c27203_g4_i1

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High-affinity choline transporter 1 comp32586_c0_seq2 c29445_g1_i2

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Transient receptor potential cation channel A1 comp29969_c1_seq2 c31382_g11_i1

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Anoctamin-1 comp31949_c0_seq1 c31699_g1_i2

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Calcium/calmodulin-dependent protein kinase type II alpha chain comp35471_c10_seq2 c33522_g14_i2

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Tyrosine-protein kinase Src42A comp22449_c0_seq1 c35335_g9_i1

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P2X purinoceptor 4 comp32424_c1_seq1 c33163_g11_i3

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Neural-cadherin comp36071_c11_seq1 c34787_g10_i1

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