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Tesi

Signal transduction and transmission in ASH nociceptive neurons of *Caenorhabditis elegans*

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1. INTRODUCTION

1.1) Pain and nociception

The sensation of pain alerts us to real or impending injury and triggers appropriate protective responses. Pain is a complex experience that involves the transduction of noxious environmental stimuli and cognitive and emotional processing by the brain. The perception of pain is initiated by signaling the presence of noxious stimuli through specialized primary sensory neurons (nociceptors) of the dorsal root ganglion (DRG), which innervate the skin. In humans, rodents and other vertebrates, painful thermal, chemical or mechanical stimulation activates nociceptors in the skin, which then convey this information to the first synaptic relays in the dorsal horn of the spinal cord.

1.2) Nociceptor neurons

Like all primary sensory neurons in the somato-sensory system, nociceptors have their cell bodies in dorsal root ganglia. They give rise to a single axon that bifurcates into a peripheral branch that innervates peripheral target tissue, and a central axon that synapse on nociceptive second order neurons in the spinal cord. The nociceptor in consequence has four major functional components, the peripheral terminal that transduces external stimuli and initiates action potentials, the axon that conducts action potentials, the cell body that controls the identity and integrity of the neuron, and the central terminal which forms the presynaptic element of the synapse with second order neurons (Figure 1) (Woolf and Ma, 2007). Nociceptors neurons have characteristic thresholds or sensitivities that distinguish them from other sensory nerve fibres. Fibres that innervate peripheral regions of the body originate from cell bodies in dorsal root ganglia (DRG), and can be grouped into three main groups based on anatomical and functional criteria (Figure 2). Cell bodies with the largest diameters give rise to myelinated, rapidly conducting Aβ primary sensory fibres. Most, but not all, Aβ fibres detect innocuous stimuli applied to skin, muscle and joints and thus do not contribute to pain (Djouhri et al., 1998). By contrast, small- and medium-diameter cell bodies give rise to most of the nociceptors, including unmyelinated, slowly conducting C fibres and thinly myelinated, more rapidly conducting Aδ fibres, respectively (Julius and Basbaum, 2001). There are two main classes of Aδ nociceptor; both respond to intense mechanical stimuli, but can be distinguished by their differential responsiveness to intense heat (Julius and Basbaum, 2001). Most C fibre nociceptors are also polymodal, responding to noxious thermal and mechanical stimuli. Others are mechanically insensitive, but respond to noxious heat. Importantly, most C-fibre nociceptors also respond to noxious chemical stimuli, such as acid or capsaicin, the pungent ingredient in hot chilli peppers (Julius and Basbaum, 2001). On the basis of histochemical criteria, unmyelinated C fibre are further grouped in two broad classes: one population, called peptidergic,

contains the peptide neurotransmitter substance P, and expresses TrkA, the high-affinity tyrosine kinase receptor for nerve growth factor (NGF); a second population does not express substance P or TrkA, but can be labelled selectively with the α-D-galactosyl-binding lectin IB4, and expresses P2X3 receptors, a specific subtype of ATP-gated ion channel (Julius and Basbaum, 2001).

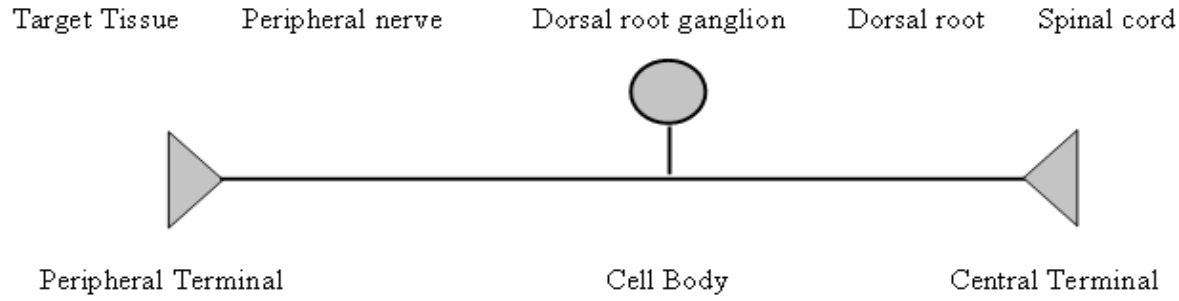


Figure 1. The operational components of the nociceptor include a peripheral terminal that innervates target tissue and transduces noxious stimuli, a cell body in the dorsal root ganglion, and a central terminal where information is transferred to second order neurons at central synapses. Adapted from (Woolf and Ma, 2007).

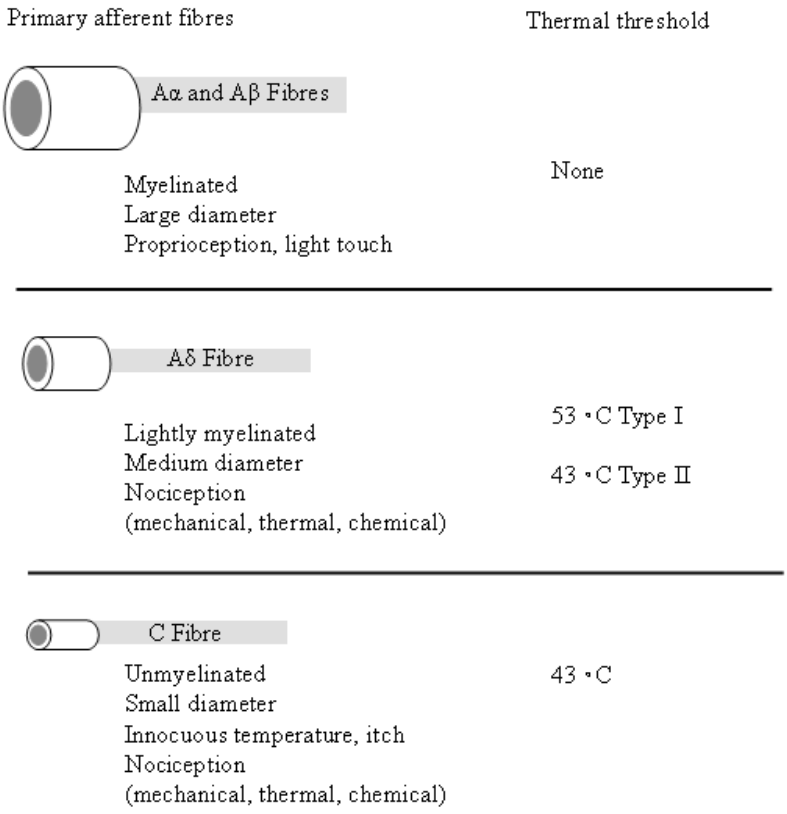


Figure 2. Different nociceptors detect different types of pain. Peripheral nerves include medium-diameter (Aδ) and medium- to large-diameter (Aα,β) myelinated afferent fibres, as well as small-diameter unmyelinated afferent fibres (C). Adapted from (Julius and Basbaum, 2001).

The fundamental role of nociceptors for organism integrity and survival is evident in patients with hereditary sensory and autonomic neuropathy type 4 which is due to mutations in TrkA.These individuals loose nociceptors in the early stages of development and show a congenital pain hyposensitivity such that as a result, they burn and chew their tongues and lips, loose the tips of their fingers and damage their joints (Woolf and Ma, 2007).

1.3) Diversity of nociceptor signaling

All sensory systems must convert environmental stimuli into electrochemical signals. In the case of vision or olfaction, primary sensory neurons need only detect one type of stimulus (e.g. light or chemical odorants) and use redundant and convergent biochemical mechanisms to do it (Figure 3a). In this regard, nociception is unique because individual primary sensory neurons have the remarkable ability to detect a wide range of stimulus modalities, including physical and chemical stimuli. This is the reason why nociceptors are referred to as polymodal. Compared with sensory neurons of other systems, nociceptors must therefore be equipped with different signal transduction machineries (Figure 3b). Through these different signal transduction pathways the nociceptors can discriminate and integrate information from different stimuli of a chemical (capsaicin and acid) or physical (heat) nature (Julius and Basbaum, 2001). Primary afferent nociceptors are also unique in the extent to which their activity can be modulated. In pathological pain conditions (allodynia) or in physiological inflammation states, pain can be produced by normally innocuous stimuli. These processes can result from two different conditions: decreased nociceptor activation thresholds (peripheral sensitization) or increased signal transmission and responsiveness of second order neurons in the spinal cord (central sensitization). Sensitization can be produced when nociceptor terminals, either central or peripheral, become exposed to products of tissue damage and inflammation (inflammatory soup). Such products include extracellular protons, arachidonic acid and other lipid metabolites, serotonin, bradykinin, nucleotides and NGF all of which interact with receptors or ion channels on nerve terminals perturbing signal transduction and signal transmission (Julius and Basbaum, 2001). Finally the release of peptides and neurotransmitters (for example, substance P, neuropeptides, ATP) from the peripheral terminals of nociceptors when activated by noxious stimuli, is able to induce production of the inflammatory soup from neighboring, non-neuronal cells and from vascular tissue, a phenomenon known as neurogenic inflammation (Woolf and Ma, 2007). In contrast to vision, olfaction and taste, sensory nerve endings that detect painful stimuli are not localized to a particular anatomical structure or organ, but are, instead, dispersed over the whole body, innervating skin, muscle, joints and internal organs.

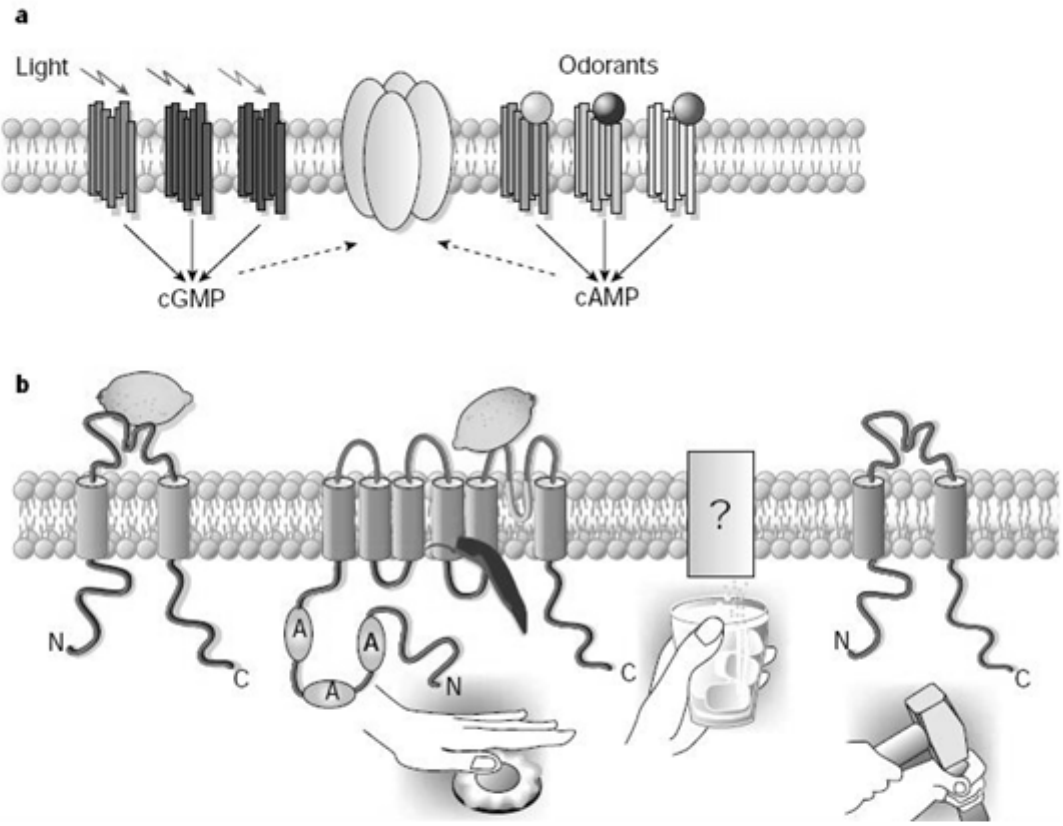


Figure 3. Polymodal nociceptors use a greater diversity of signal-transduction mechanisms to detect physiological stimuli than do primary sensory neurons in other systems. **a**) In mammals, light or odorants are detected by a convergent signaling pathway in which G-protein-coupled receptors modulate the production of cyclic nucleotide second messengers, which regulate the activity of a single type of cation channel. **b**) In contrast, nociceptors use different signal-transduction mechanisms to detect physical and chemical stimuli. Adapted from (Julius and Basbaum, 2001).

1.3.1) Peripheral terminal: Signal transduction

The peripheral terminal of the mature nociceptor is where noxious stimuli are detected and transduced into inward currents that, if sufficiently large, generate action potentials along the axon to the CNS, producing pain. The sensory specificity of the nociceptor is established by expression of ion channels tuned to respond with a high threshold only to particular features of the mechanical, thermal, and chemical environment (Ramsey et al., 2006). In the last ten years nociceptor ion channels have been identified. These ion channels are expressed in axon terminals as well as in cell soma. When these channels are activated, inward currents or outward currents are generated, which will lead to depolarization or hyper-polarization of the membrane causing increased or decreased excitability of sensory neurons. The main channels responsible for inward membrane currents in nociceptors are voltage-activated sodium and calcium channels, while outward current is carried mainly by potassium ions. In order to depolarize the membrane of nerve terminals, either inward currents should be generated or outward currents should be inhibited (Ramsey et al., 2006). In

addition, activation of non-selective cation channels is also responsible for the excitation of sensory neurons. Thus, excitability of neurons can be controlled by regulating the expression or by modulating the activity of these channels. Among non-selective cation channels involved in nociception the transient receptor potential channels (TRP) have a prominent role (Ramsey et al., 2006).

1.3.1.1) TRP channels

The transient receptor potential channels play important roles in in sensory physiology because they serve as molecular sensors that detect a variety of stimuli such as vision, taste, smell, hearing, mechanosensation, thermo-sensation and pain (Montell, 2005). The TRP protein superfamily consists of a diverse group of cation channels that are expressed predominantly in the nervous system. Mammalian TRP channel proteins form six-transmembrane (6-TM) cation-permeable channels that may be grouped into six subfamilies on the basis of amino acid sequence homology (TRPC, TRPV, TRPM, TRPA, TRPP, and TRPML). TRP channels are generally described as calcium-permeable cation channels with polymodal activation properties. Sensitivity to polymodal activation suggests that the physiologically relevant stimulus for any given TRP will be governed by specific characteristics of the cellular context (i.e. phosphorylation status, lipid environment, interacting proteins, and concentrations of relevant ligands). Most of the molecular mechanisms that activate TRP channels *in vivo* are still unknown. Most of the information on the modes of activation comes from studies based on TRP channels expressed in heterologous systems and may be divided into three general categories (Ramsey et al., 2006):

1. *Receptor activation.* G protein–coupled receptors (GPCRs)- and receptor tyrosine kinases-mediated signaling that activate phospholipases C (PLCs) can modulate TRP channel activity through: hydrolysis of phosphatidylinositol bisphosphate (PIP2), production of diacylglycerol (DAG), or production of inositol trisphosphate (IP3) and subsequent liberation of Ca2+ from intracellular stores.

2. *Ligand activation.* Ligands that can activate TRP channels may be broadly classified as exogenous or endogenous. Exogenous ligands are usually small organic molecules and include synthetic compounds and natural products (capsaicin, icilin, 2-APB); endogenous ligands include lipids and products of lipid metabolism (diacylglycerols, phosphoinositides, eicosanoids, anandamide) that can be produced also by receptor activation. They also include purine nucleotides and their metabolites, Ca^{2+} and Mg^{2+}.

3. *Direct activation.* Dangerous physical stimuli such as changes in ambient temperature, mechanical and osmotic stimulation can induce the opening of TRP channels through second

messengers or other unidentified mechanisms (Vriens et al., 2004).

Modulation of TPR. Moreover post-translational modification (i.e., phosphorylation) can regulate activation of TRPs channales. Indeed it has been demonstrated that protein kinases A, C, and G (PKA, PKC, and PKG, respectively) modulate TRP channel activity. Finally other cellular signaling mechanisms, such as regulation by Ca^{2+}/calmodulin (Ca^{2+}/CaM), have also been demonstrated to modulate TRP channel activity.

The TRPV subfamily. Within TRP superfamily the TRPV subfamily has vertebrate and invertebrate members with a prominent role in nociception physiology. Mammals express six TRPV channels TRPV-1, -2, -3 and -4,-5 and -6. Table 1 (Liedtke and Kim, 2005) summarizes molecular and physiological characteristics of mammalian TRPV channels.

Table 1. Mammalian TRPVs

Name	Expression	Agonists	Physiological roles
TRPV1	DRG, CNS, bladder, vessels	capsaicin, protons, heat (>42°C), anandamide 12-(S) HPETE(12-(S)hydroperoxyeicosatetraenoyl acid), 5-(S)-HETE, LTB4 (leukotriene B4)	thermal pain sensation, mechanosensation, vascular regulation, taste transduction
TRPV2	DRG, CNS, widelyexpressed	noxious heat (>52 °C), cell swelling, mechanical force	thermosensation, mechanosensation
TRPV3	DRG, skin, widely expressed	temperature (>31 °C) and diphenylboronic anhydride	thermosensation, pain sensation
TRPV4	DRG, kidney, skin, inner ear hair cells, inner ear stria vascularis, endothelium, brain, trachea, lung, fat, heart	cell swelling, mechanical force, 4- PDD (4 -phorbol-12,13 didecanoate), temperature (>27 °C), 5,6'-EET [5',6' epoxyeicosatrienoic acid]	osmotic regulation by the CNS, mechanically and osmotically mediated pain sensation, thermal preference
TRPV5	intestinal and renal epithelia, CNS	constitutively active in transfected cells	Ca^{2+} uptake in kidney, Ca^{2+} homeostasis and bone structure
TRPV6	intestinal and renal epithelia, CNS	constitutively active in transfected cells	Ca^{2+} uptake in kidney and intestine

TRPV1 is expressed highly in sensory neurons, preferentially in small sensory neurons. It is activated by capsaicin, noxious heat (> 43°C), acid and by various endogenous Poly Unsaturated Fatty Acids (PUFA) lipids including anandamide and N-arachidonyldopamine.

TRPV2 is mainly expressed in large diameter dorsal root ganglion (DRG) neurons and also in a wide range of tissues and cell types other than sensory neurons. TRPV2 is expressed in lung, spleen, intestine, brain, enteric neurons, and aortic smooth muscle cells. TRPV2 is activated at relatively high temperature and recently, TRPV2 has been shown to be sensitive to membrane stretch or hypotonic shock, suggesting its role in mechano-transduction (Ramsey et al., 2006).

TRPV3 is abundantly distributed to keratinocytes in the skin but with fairly low expression level in sensory neurons. TRPV3 is activated by warm temperature, camphor and 2-aminoethoxydiphenyl benzoate.

The nonselective cation channel TRPV4 is highly expressed in the kidney as well as in many other tissues, including skin, trachea, liver, lung, blood vessel, and brain. TRPV4 is gated by warm temperature ranging from 24 to 34°C, hypotonicity and by various chemicals including phorbol ester derivatives such as phorbol 12-myristate 13-acetate. TRPV4-/- mice showed hypoalgesic responses to pressures on the tail and application of acids suggesting that TRPV4 is important in thermal and mechanical nociception (Ramsey et al., 2006).

1.3.2) Central terminal: Signal transmission

The central terminals of nociceptors are located in the dorsal horn of the spinal cord (Woolf and Ma, 2007). These terminals connect synaptically to second-order neurons, transferring information, carried by action potentials, about the intensity and duration of peripheral noxious stimuli. Nociceptors use glutamate as transmitter, but also neuropeptides, and proteins like BDNF as synaptic modulators. As a consequence they are able to evoke fast and slow excitatory postsynaptic potentials that show considerable spatial and temporal summation. Transmitter release is regulated by multiple factors that control or modulate calcium influx in response to the action potentials that activate the vesicle-release machinery. In neurons, Ca^{2+} entry through Ca^{2+} channels is essential for synaptic transmission (Woolf and Ma, 2007). Voltage-gated Ca^{2+} channels (VGCC) are fundamental components of the presynaptic release machinery, through which neurotransmitter release can be modulated (Hille, 1994). Modulation of VGCCs by the activation of G-protein-coupled receptors (GPCR) critically controls presynaptic Ca^{2+} entry and hence neurotransmitter release. GPCR are present on the plasma membrane of nociceptor neurons and their terminals and are closely associated with the modulation of signal transmission. GPCR can affect ion channel function through two mechanisms: phosphorylation of ion channels through second messengers, such as protein kinase C and certain other kinases, and Gβγ binding to ion channels (Pan et al., 2008). Molecules that activate GPCR can inhibit signal transmission through an inhibition of VGCC to reduce excitatory neurotransmitter release from presynaptic terminals of nociceptive

sensory neurons (Pan et al., 2008).

1.3.3) GPCR and G-signaling

GPCR and heterotrimeric G-proteins have an essential role in nociception regulating both signal transduction and signal transmission in the primary afferent neurons.

GPCR represent the largest and most diverse family of cell surface receptors and proteins. GPCR are widely distributed in the peripheral and central nervous systems and, together with the other components of their pathway, are the most important targets in pain therapy. All GPCR share a similar structure, which consists of 7 transmembrane domains linked by alternating intracellular and extracellular loops. Ligand recognition and binding depends on extracellular domains, whereas coupling to G proteins is determined mainly by intracellular domain (Kroeze et al., 2003; Lu et al., 2002). The G proteins consist of 3 subunits: α, β, and γ. Activation of G proteins by GPCR leads to dissociation of the Gα subunit from the Gβγ subunits. In the resting, inactive state, the α subunit binds GDP and is associated with the βγ subunits to form an inactive heterotrimer that is bound to the receptor. When a chemical or physical signal stimulates the receptor, the receptor becomes activated and changes its conformation. As a consequence the GDP-bound α subunit responds with a conformational change that leads to a replacement of GDP with GTP. The GTP-bound α subunit assumes its activated conformation and dissociates both from the receptor and from βγ. The dissociated subunits can activate downstream effectors. The activated state lasts until the GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the α subunit (Neer, 1995). The Gβγ subunits function as a dimer and can activate various effectors, such as enzymes and ion channels (Neves et al., 2002). The G_{α} subunits belong to four main families: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q/11}$, and $G_{\alpha 12/13}$, that differ primarily in effector recognition (Table 2).

On the basis of their G-protein-coupling preference, GPCR can be broadly classified into 4 major categories: Gαs-, Gαi/o-, Gαq/11- and Gα12/13-coupled receptors (Hur and Kim, 2002; Neves et al., 2002)). Almost all GPCR agonists that have an analgesic action, inhibiting signal transmission, are coupled to Gi/o proteins.

Table 2. G-alpha subunits

	Specific mechanisms
$G_{\alpha s}$	stimulates the production of cAMP from ATP. This is accomplished by direct stimulation of the membrane-associated enzyme adenylate cyclase. cAMP acts as a second messenger that goes on to interact with and activate protein kinase A (PKA). PKA can then phosphorylate a myriad of downstream targets.
$G_{\alpha i}$	inhibits the production of cAMP from ATP.
$G_{\alpha q/11}$	stimulates membrane-bound phospholipase C beta, which then cleaves PIP_2 (a minor membrane phosphoinositol) into two second messengers, IP3 and diacylglycerol (DAG).
$G_{\alpha 12/13}$	are involved in Rho family GTPase signaling (through RhoGEF superfamily) and control cell cytoskeleton remodeling, thus regulating cell migration.

Regulation of G-signaling. The cycle of G protein activation and deactivation that transmits the signal from receptor to effectors is illustrated in figure 4 [Gilman, 1987 #89]. The RGS proteins participate in this process as Regulators of G protein Signaling. RGS proteins bind to active, GTP-bound α subunit and accelerate its GTPase activity. These GTPase-activating proteins (GAPs) limit the lifetime of GTP-bound α subunit and terminate signaling event(s) [Hepler, 2003 #91].

More than 30 mammalian family members have been identified and classified into many (7 to11) subfamilies based on sequence identity and functional similarities (De Vries et al., 2000; Hollinger and Hepler, 2002). All family members contain a RGS domain responsible for GAP activity. The biochemical mechanisms with which RGS proteins stimulate the GTPase activity of G-alpha subunit has been studied in detail (Ross and Wilkie, 2000). However, much less is known about how RGS functions are regulated in living cells (Hollinger and Hepler, 2002). Several evidences suggest that RGS proteins act as modulators and integrators of G protein signaling and there are indications that specific RGS proteins regulate specific G protein-coupled receptor pathways. Most RGS proteins can regulate the activity of many members of the Gi or Gq alpha subunits (De Vries et al., 2000). The specificity is probably created by a combination of cell type-specific expression, tissue distribution, intracellular localization, post-translational modifications, and by the function of domains of RGS proteins, other than the RGS domain itself.

In the last few years the interest for RGS proteins as new drug targets has grown (Chasse and Dohlman, 2003). GPCRs and their GPCR- signaling pathways are the direct targets for a large number of currently used drug classes. The unique capacity of RGS proteins to modulate G protein signaling combined with their apparent specificity and localization within the nervous system makes them very attractive new drug targets. Small molecules that inhibit RGS protein/G interactions have been proposed as novel drugs to potentiate the actions of endogenous

neurotransmitters in various disease states (Chasse and Dohlman, 2003).

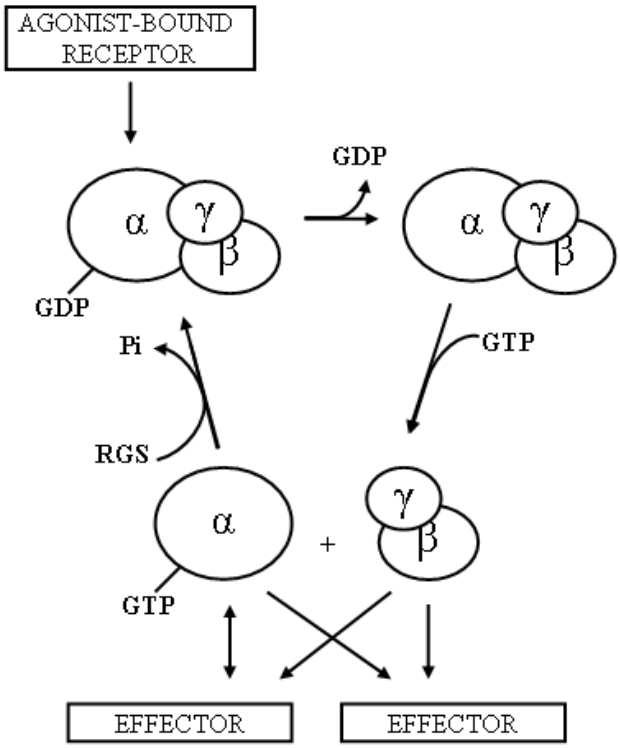


Figure 4. The Regulatory Cycle of Heterotrimeric G Proteins.

1.4) Aim of the project

The nociceptor neurons are specialized to respond to noxious stimuli and to transmit this information to the CNS. Their function is very important for organism integrity and survival and their loss poses in severe danger individual fitness (as an example see above, Autonomic Neuropathy type 4). In addition, persistent alterations of nociceptors can lead to pathological condition in which pain is generated in the absence of noxious stimuli. Some molecules important for nociceptor signaling have been identified but the molecular mechanisms by which they function are still largely unclear. The aim of my PhD project is to identify new molecules involved in nociceptor signaling and to try to elucidate the mechanisms of their action. To tackle this problem I decided to use a genetic approach using the nematode *Caenorhabditis elegans* as model.

1.5) *Caenorhabditis elegans* as model to study nociception

Progresses in our understanding of the molecular mechanisms of nociception signaling have arisen from the analysis of sensory systems in mammals as well as from studies of invertebrate nociception. Obviously, invertebrate organisms do not experience pain, but they do have transduction mechanisms through which they detect and avoid potentially harmful stimuli in their environment. These signaling pathways can be considered as the evolutionary precursors of

nociceptive processing in vertebrates, and genetic studies have led to the identification and functional characterization of molecules and signaling pathways that contribute to the detection of noxious stimuli in animals. Genetic analysis of nociceptive behaviors in the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* has led to the discovery of conserved sensory transduction channels and signaling molecules (Tobin and Bargmann, 2004).

In *Caenorhabditis elegans* the definition of nociception is based on characteristic behavioral responses (**avoidance**): in response to aversive cues, the animal ceases forward locomotion, moves backward briefly, and reorients to face away from the direction of the stimulus. Aversive cues include touch, certain odorants, high osmotic strength, acidic pH, heavy metals, and other molecules that are toxic to the animals or that signal an environment potentially dangerous for the animal (Bargmann, 1993; Chalfie and Sulston, 1981; Hilliard et al., 2002; Kaplan and Horvitz, 1993; Sambongi et al., 1999; Ward, 1973). Not all harmful compounds generate an avoidance response suggesting that avoidance is generated by sensory perception and not by general tissue damage.

1.6) Nervous system of *C.elegans*

Because of its compact nervous system and its amenability to experimental manipulation the nematode *Caenorhabditis elegans* is a favoured model for behavioral studies (Figure 5) (For more information on *C.elegans* as experimental model see Appendix). These advantages permit the study of behavior at the level of genes, individual neurons, and neural circuits. The natural environment of *C. elegans* is the soil, where it feeds on bacteria and other microbes. It moves by propagating bends along its body. *C. elegans* occurs in two highly dimorphic sexes, males and self-fertilizing hermaphrodites. Adults of both sexes are composed of a precise number of cells: Hermaphrodites have 959 somatic nuclei, and males have 1031 (Sulston and Horvitz, 1977; Sulston et al., 1983). These cells make up hypodermis, muscle, the digestive tract, gonad, and the nervous system. In hermaphrodites the nervous system consists of 302 neurons and 56 glial and support cells, whereas males have 381 neurons and 92 glial and support cells. About half the neuronal cell bodies are located in the head, surrounding a central neuropil called the nerve ring. The remainder is found along the ventral cord and in tail ganglia. In both sexes each neuron is uniquely recognizable in different individuals by its characteristic position and morphology (Sulston and Horvitz, 1977; Sulston et al., 1983) (White et al., 1986).

The complete structure of the hermaphrodite nervous system has been reconstructed from serial section electron micrographs (EM) so that the morphology of each neuron and its chemical synapses and gap junctions are known (White et al., 1986; http://www.wormatlas.org).

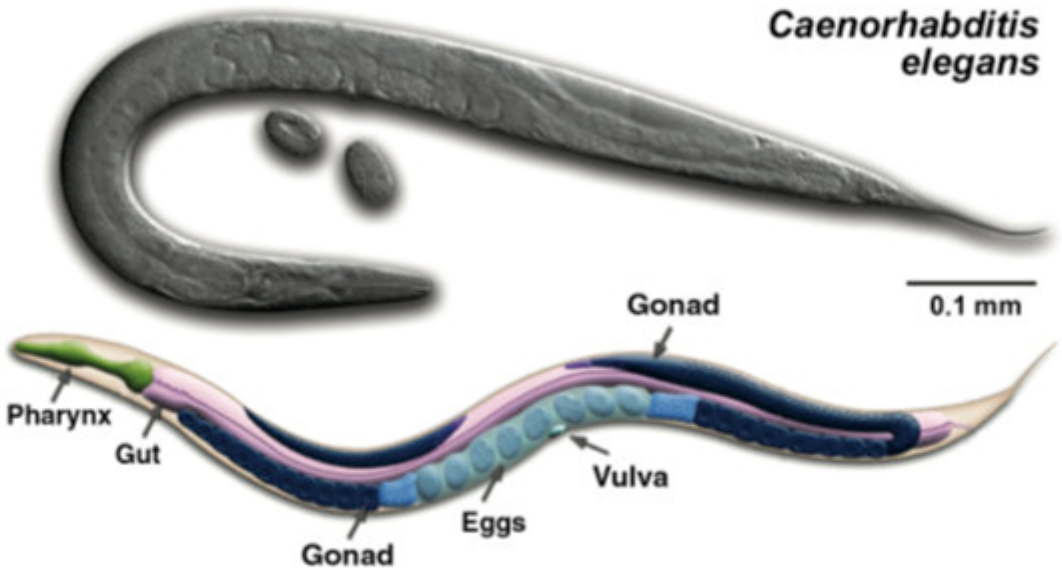


Figure 5. The nematode *C.elegans* - hermaphrodyte. (From http://www.wormbook.org).

The 302 hermaphrodite neurons can be grouped by anatomical criteria into 118 classes. These include 39 classes of sensory neurons, 21 of which have specialized sensory endings at the tip of the head, the sensory cilia (Bargmann and Kaplan, 1998; Sulston and White, 1980; Ward et al., 1975). Another 27 classes are motoneurons, and the remainder are classed as interneurons (White et al., 1986).

Most classes of sensory neurons and interneurons consist of two left/right homologs with similar synaptic connectivity. Together, the 302 neurons make approximately 5000 chemical synapses, 600 gap junctions, and 2000 neuromuscular junctions (White et al., 1986). *C. elegans* contains many of the classic neurotransmitters found in vertebrates, including acetylcholine, glutamate, gamma-aminobutyric acid (GABA), serotonin, and dopamine. Neurotransmitter assignments have been made for many *C. elegans* neurons (Rand, 1997). In addition to classic neurotransmitter vesicles, many *C. elegans* neurons also have dense core vesicles, characteristic of catecholamine- and neuropeptide-containing vesicles in other animals (White et al., 1986). The *C. elegans* genome sequence reveals numerous neuropeptides. For example, the Phe-Met- Arg-Phe-amide FMRFamide family of neuropeptides is represented by at least 23 genes.

1.6.1) Sensory system

With its sensory system *C. elegans* finds food, avoids noxious conditions, develops appropriately, and mates. Its chemical sensory organs are the amphids, the phasmids and the inner labial neurons ((Ward et al., 1975) and Figure 6). 32 putative chemosensory neurons have sensory cilia either directly or indirectly exposed to the environment through openings of the external cuticle generated

by glial cells called the socket and sheath cell. The amphid also contains an additional pair of thermosensory neurons, AFD**.** Generally, chemosensory neurons are bilaterally symmetric with the left and right members of each class structurally similar. Each left-right pair forms a class that can be distinguished from all other classes based on cilium morphology, axon morphology, and synaptic targets (White et al., 1986).

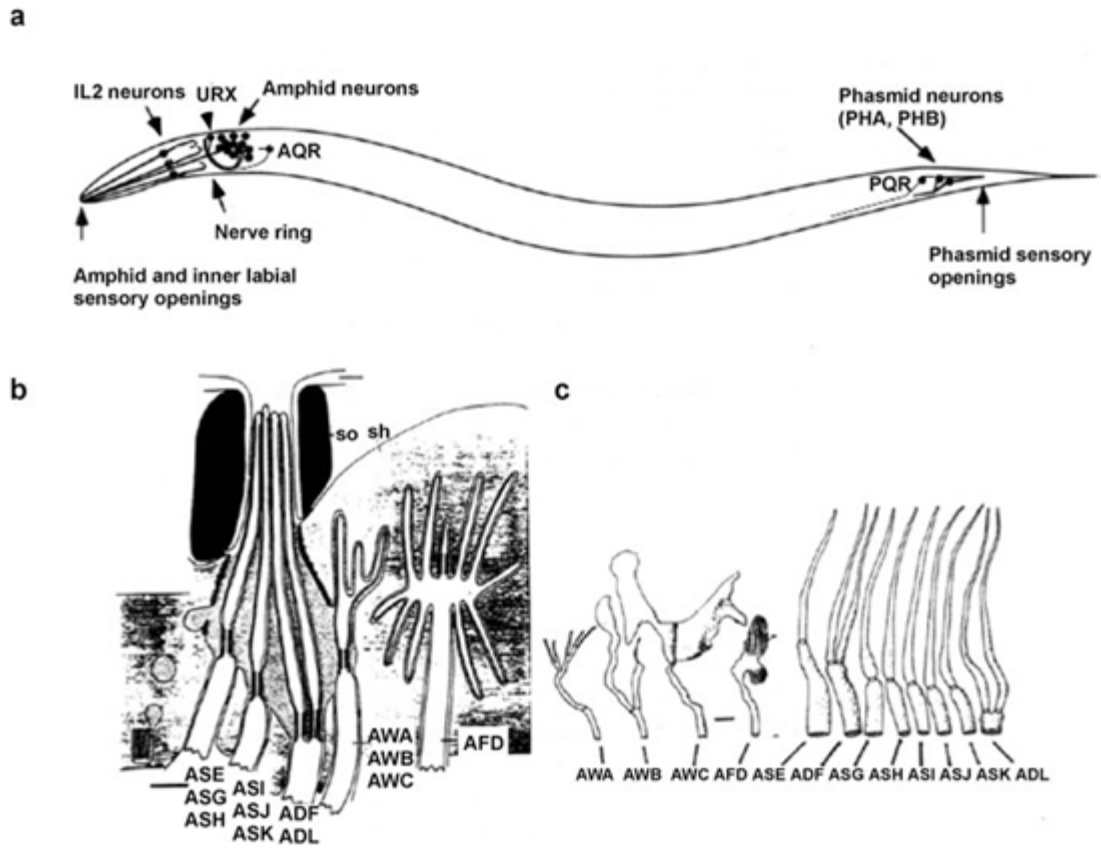


Figure 6. Structure of chemosensory organs. a. Disposition of chemosensory neurons in the animal. Each of the two amphids contains 12 associated chemosensory or thermosensory neurons. Each of the two phasmids contains 2 chemosensory neurons, PHA and PHB. There are six inner labial organs, each of which contains one IL2 chemosensory and one IL1 mechanosensory neuron. There are two URX neurons, the endings of these neurons are within the animal, and not exposed. b. Detailed structure of the amphid sensory opening showing the socket (so), sheath (sh), and ciliated nerve endings. AWA, AWB, AWC, and AFD endings are buried in the sheath and not exposed through the amphid pore. c. Detailed structure of the cilia in the 12 classes of amphid neurons. Adapted from http://www.wormbook.org.

1.6.2) Polymodal nociception by ASH and accessory neurons

C. elegans avoids a variety of noxious stimuli including high osmolarity (Culotti and Russell, 1978), touch to the nose (Kaplan and Horvitz, 1993), some odors (Troemel et al., 1995), heavy metals such as Cu^{2+} and Cd^{2+} (Bargmann et al., 1990; Sambongi et al., 1999), low pH (Sambongi et al., 2000), alkaloids such as quinine (Hilliard et al., 2004), and detergents (Bargmann et al., 1990;

Hilliard et al., 2002). These cues all elicit an escape response that involves the animal reversing rapidly and then resuming forward movement, usually in a different direction. The sensory neurons mediating these avoidance responses have been defined by laser ablation (Table 3). Remarkably, despite their physical and chemical diversity, these stimuli can all be transduced by the ciliated head neuron ASH. For these reason ASH is considered a polymodal nociceptive neuron similar to vertebrate nociceptor in the DRG. ASH has synaptic connections with AVA, AVB, and AVD, the forward and backward command interneurons (White et al., 1986). ASH neuron uses glutamate as neurotransmitter. Glutamate release from ASH activates the excitatory AMPA and NMDA-type glutamate receptors on the command interneurons (Hart et al., 1995; Lee et al., 1999; Maricq et al., 1995; Mellem et al., 2002) that control forward and backward movement through the A and B motor neurons (Figure 7).

Table 3. Sensory neurons mediating avoidance

Noxious stimulus	Sensory neuron
High osmolarity	ASH
Nose touch	ASH, OLQ, FLP
Odors	ASH, ADL, AWB
Heavy metals	ASH, ADL, ASE
Protons	ASH, ADF, ASE, ASK
Alkaloids	ASH, ASK
Detergents	ASH, ASK, PHA, PHB

1.6.3) Signal transduction in ASH

ASH detects chemical, osmotic, and mechanical signals. *In vivo* imaging of ASH sensory activity with the genetically-encoded calcium indicator cameleon suggests that each of these sensory signals directly depolarizes the ASH neuron (Hilliard et al., 2005). All of these signals are transmitted through the TRPV channels, OSM-9 and OCR-2. All ASH-mediated avoidance responses require the transient receptor potential vanilloid-related channel (TRPV) proteins OSM-9 and OCR-2, that are located at the ASH sensory cilia and appear to be the sensory transduction channels of these neurons (Colbert et al., 1997; Tobin et al., 2002). OSM-9 is a *C. elegans* ortholog of the mammalian TRPV4 channel, and the osmotic avoidance and nose-touch response defects of *osm-9* mutants can be rescued by expressing rat TRPV4 in the ASH neurons (Liedtke et al., 2003) confirming functional conservation. In contrast, expressing the rat TRPV1 channel in ASH does not restore native avoidance responses but is sufficient to induce an ectopic avoidance response to capsaicin, a chili pepper component and a TRPV1 channel agonist (Tobin et al., 2002). ASH sensory responses are also dependent on the Gα-protein ODR-3 (Hilliard et al., 2005) and on

biosynthetic enzymes necessary for the biosynthesis of polyunsaturated fatty acids (PUFA) (Kahn-Kirby et al., 2004). Mutations in these genes do not disrupt capsaicin avoidance in transgenic animals that express rat TRPV1 in ASH, which suggests that they are not required for signal transduction downstream of TRP channel activation.

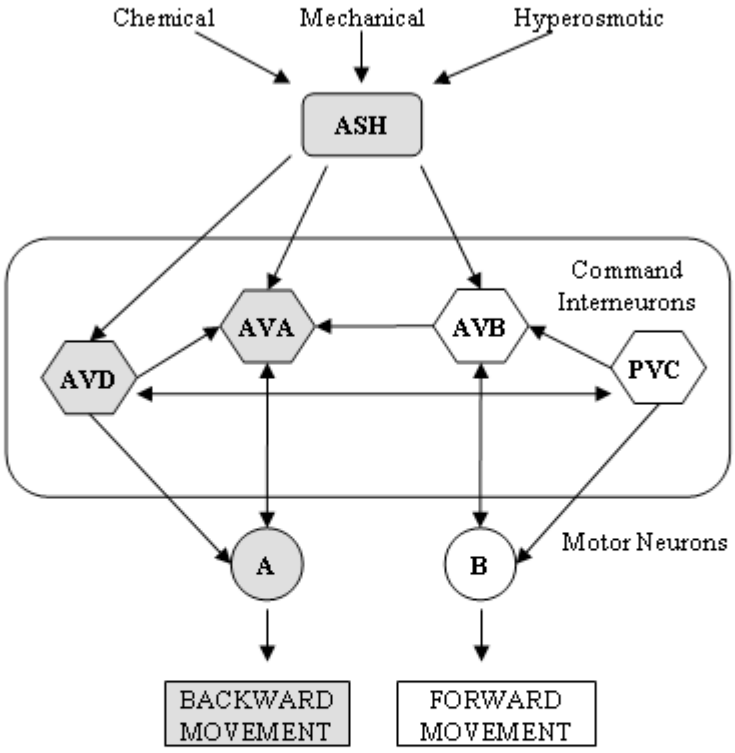


Figure 7. Avoidance motor neural circuit. Connectivities between ASH and the command interneurons and motor neurons are shown. Adapted from (de Bono and Maricq, 2005)

The most likely interpretation of these results is that transduction of noxious stimuli involves a G-protein cascade that activates OSM-9/OCR-2 by generating PUFA-containing lipids. Interestingly, the ASH neurons express at least eight Gα protein subunits (Jansen et al., 1999). At least one of these Gα subunits, GPA-3, is important for some nociceptive responses (Hilliard et al., 2004; Jansen et al., 1999) (Figure 8). The pathways by which different noxious stimuli are detected by ASH and that activate OCR-2/OSM-9 channels are still unknown. Different pathways detecting different noxious stimuli exist. Several genes have been identified that are required for the detection, by ASH, of some noxious stimuli but not others. For example, animals lacking the novel cytosolic protein OSM-10 fail to respond to hyperosmolarity but avoid normally nose touch, quinine, and 1-octanol (Hart et al., 1999; Hilliard et al., 2004). Conversely, *qui-1*, a novel protein with WD-40 repeats, is strictly required for quinine avoidance but has minor effects on osmotic avoidance (Hilliard et al., 2004). Loss of *grk-2*, G protein–coupled receptor kinase-2, attenuates the response

to hyperosmolarity, octanol, and quinine, but does not affect avoidance of nose touch (Fukuto et al., 2004) (Figure 8). Loss of *rgs-3*, a regulator of G-signaling, alters behavioral response to strong sensory stimuli but not to weak aversive (ASH-mediated) stimuli (Ferkey et al., 2007). In addition, *rgs-3* animals respond better than wild-type animals to the relatively weak stimulus of nose touch (Ferkey et al., 2007). In addition behavioral and imaging studies provide evidence for repellent-specific adaptation. For example animals subjected to repeated nose touch adapt, and decrease their response, to this stimulus but continue to avoid normally high osmolarity and the volatile repellent 1-octanol (Hart et al., 1999; Hilliard et al., 2005). Also, prolonged exposure to Cu^{2+} ions causes adaptation to this stimulus but not to hyperosmolarity (Hilliard et al., 2005). A key component for ASH adaptation is *gpc-1*, one of two G-protein gamma subunits encoded in the *C. elegans* genome (Hilliard et al., 2005; Jansen et al., 1999).

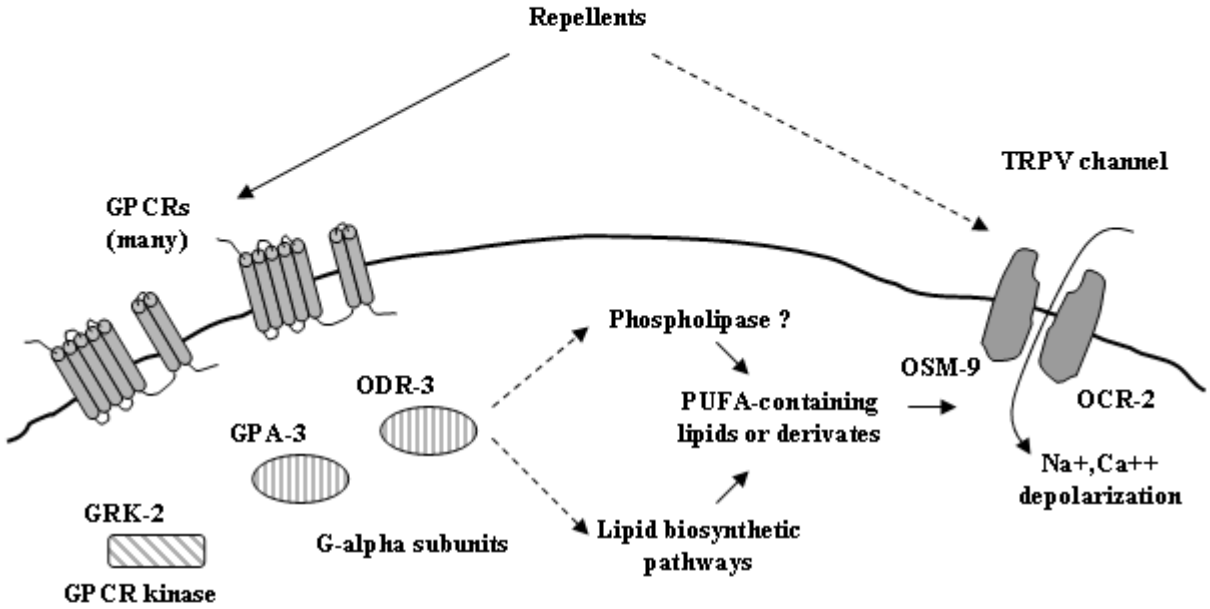


Figure 8. Potential signal transduction pathway for nociception in ASH cilia.
Genetic results support the importance of ODR-3, GPA-3, biosynthetic enzymes for polyunsaturated fatty acids (PUFAs), and TRPV channels for all forms of ASH nociception, and G protein-coupled receptors (GPCRs) for some forms of ASH nociception. A likely model is that repellents are detected by GPCRs and possibly by other molecules such as ion channels. GPCRs activate the G-alpha proteins ODR-3 and GPA-3, which regulate the production or consumption of phospholipids containing PUFAs (omega-3 and omega-6 polyunsaturated fatty acids). The GPCR kinase GRK-2 also promotes ASH activation. Lipid mobilization opens the TRPV channels encoded by OSM-9 and OCR-2 to depolarize the cell. TRPV channels may also be directly activated by mechanical, chemical or osmotic stimuli, perhaps with the assistance of accessory subunits such as OSM-10.

Mutants in *gpc-1* resemble wild type in their initial response to most repellents detected by ASH but adapt poorly to repeated noxious stimulation. The Ca^{2+}-dependent phosphatase calcineurin may also

be important to attenuate avoidance responses: *tax-6* mutants, which are defective in the calcineurin A subunit, take longer (i.e. adapt more slowly) to cross a hyperosmotic barrier (Kuhara et al., 2002). The Ca^{2+} influx evoked by different noxious stimuli varies in size depending on the type and intensity of the noxious stimulus (Hilliard et al., 2005; Kahn-Kirby et al., 2004). For example, the Ca^{2+} influx in ASH cell bodies evoked by osmotic shock is greater than that evoked by 10-mM quinine or nose touch, and stimulation by 10 mM Cu^{2+} for increasing lengths of time evokes longer-lasting increases in Ca^{2+} concentration.

1.6.4) Signal transmission of ASH

Genetic studies suggest that differential activation of ASH results in differential release of neurotransmitter and distinct patterns of downstream signaling (Mellem et al., 2002). The ASH neurons are glutamatergic: they express the EAT-4 glutamate vesicular transporter (VGLUT), which is required for ASH nociceptive responses (Lee et al., 1999). Differential release of glutamate from ASH may activate different types of glutamate receptors on command interneurons that are postsynaptic to ASH and that mediate the nociceptive escape response (Hart et al., 1995; Maricq et al., 1995; Mellem et al., 2002)(Figure 9). Weak activation, such as that elicited by nose touch, activates non-NMDA ionotropic glutamate receptor (iGluR) subunits GLR-1 and/or GLR-2 (Hart et al., 1995; Maricq et al., 1995; Mellem et al., 2002). Whereas stimuli that evoke higher levels of Ca^{2+} release, such as hyperosmolarity (Hilliard et al., 2005), can activate not only GLR-1/ GLR-2 channels but also NMR-1 and NMR-2-containing NMDA iGluR (Mellem et al., 2002). Thus, the intensity and type of aversive stimuli are decoded through differential activation of postsynaptic glutamate receptors (Hart et al., 1995; Maricq et al., 1995; Mellem et al., 2002)(Figure 9). ASH expresses the FMRFamide-related neuropeptides FLP-18 and FLP-21 as well as other neuropeptides (Li et al., 1999a; Li et al., 1999b; Nathoo et al., 2001; Rogers et al., 2003). This expression is an interesting parallel to the expression of neuropeptides like Substance P and CGRP by vertebrate nociceptors. Genetic and physiological evidence suggests that neuropeptides downregulate activity at the ASH interneuron synapse (Mellem et al., 2002).

1.6.5) Modulation of ASH signaling

C. elegans avoidance responses can also be modulated by food. ASH-mediated avoidance of nose touch and 1-octanol is stronger when food is present (Chao et al., 2004). The effect of food can be mimicked by serotonin (5-HT), which signals food abundance for many *C. elegans* behaviors (Horvitz et al., 1982; Segalat et al., 1995). 5-HT can modify ASH activity potentiating Ca^{2+} influx in ASH in response to nose touch (Hilliard et al., 2005). However, its effect is selective: Exogenous 5- HT does not stimulate Ca^{2+} influx evoked by Cu^{2+} or osmotic shock. 5-HT stimulation of octanol

avoidance is probably mediated by metabotropic receptors because it requires the Gα protein GPA-11, which is expressed in ASH and ADL (Chao et al., 2004; Jansen et al., 1999).

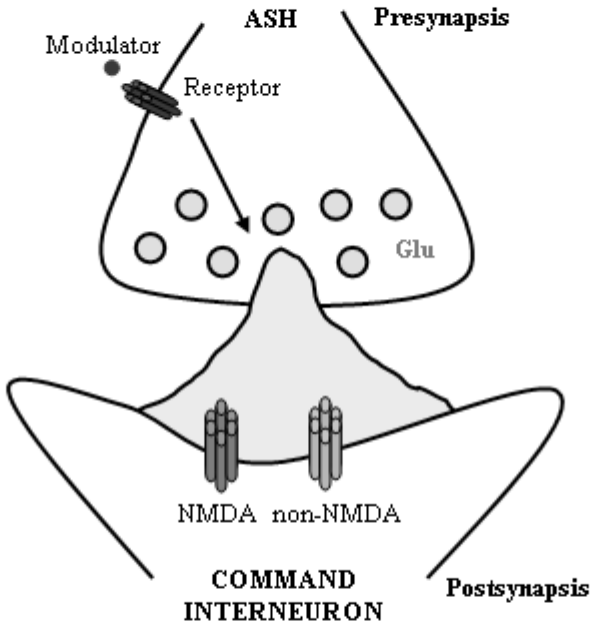


Figure 9. Potential signal transmission pathways for nociception in ASH.
Genetic studies support that differential activation of ASH results in differential release of neurotransmitter generating distinct patterns of downstream signaling. These patterns are decoded by activation of different type of glutamate receptors (NMDA, non-NMDA) on the target interneuron. A modulator (e.g., neuropeptides, serotonin) can regulate neurotransmitter release activating a signaling - linked receptor.

1.7) RNA interference

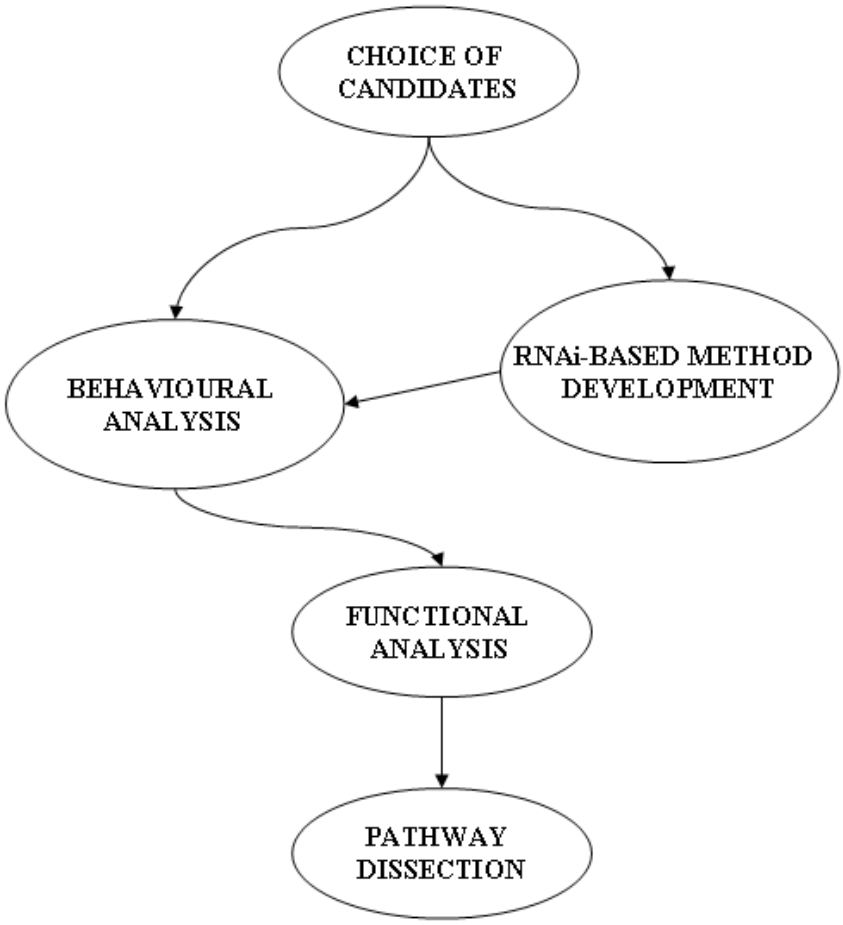
Reverse genetic analyses provide crucial contributions toward our understanding of how genes influence the behavior of multi-cellular organisms. However, many gaps still exist between our understanding of the functions of genes, which we describe in molecular and cellular terms, and our understanding of behavior, which we explain in terms of neural circuits and neurons. The nematode *Caenorhabditis elegans* has acquired a frontier role as a model in the analysis of behavior because its study promises to bridge some of these gaps, at least in a simple animal model (de Bono and Maricq, 2005). A critical step toward fulfilling this promise would be to find ways to determine the function that genes exert in each of the neurons/cells potentially responsible for a given behavioral trait (e.g. in each of the neurons composing a neural circuit). In *C. elegans*, beside traditional mosaic analysis, a commonly used approach to address this level of genetic analysis is the cell specific rescue of loss-of-function mutations (Fujiwara et al., 1999). Both approaches, although very useful, are generally laborious and time consuming and can only be applied to the study of genes for which true genetic mutants are available. The discovery of RNA interference (RNAi) has

provided a powerful reverse genetics tool that has increased enormously the range of *C. elegans* genes whose function can be rapidly analysed (Fire et al., 1998; Kamath et al., 2003). However, even in *C. elegans*, there are several limitations to the use of RNAi to address the function of genes in specific cells, especially in neurons (Tavernarakis et al., 2000). First, direct delivery of dsRNA to worms, by injection, feeding or soaking (Ahringer, 2006) results in systemic RNAi which affects many or all the cells of the organism. It is thus not possible, with this approach, to dissect the role exerted by the gene of interest in specific cells or groups of cells and essential genes are obviosly difficult to study because of lethality, sterility, etc.. Second, some late acting genes and most genes expressed in neurons are largely refractory to RNAi (Ahringer, 2006; Tavernarakis et al., 2000). In *C. elegans*, transgene driven genetic interference has been described (Tavernarakis et al., 2000) as a possible way to overcome some of the limitations of classic RNAi obtained by direct delivery of dsRNA to the animals. The method of Tavernarakis *et al*. (2000), is based on the generation of inheritable and inducible genetic interference with hairpin dsRNA encoded by transgenes. However, expression of the hairpin dsRNA is driven by a strong and ubiquitously expressed heat-shock promoter and thus the method cannot be utilized for cell specific knock-downs (Johnson et al., 2005; Tavernarakis et al., 2000). Modifications of this approach, using cell specific promoters, have also been described (Briese et al., 2006; Tavernarakis et al., 2000; Timmons et al., 2003) but the efficiency of gene function knock-down in neurons and the cell autonomy of the effects obtained have either not been addressed or have produced conflicting results. In addition, problems related to the stability of plasmids, and possibly also of transgenes, carrying inverted repeat genes (Briese et al., 2006; Tavernarakis et al., 2000) have hindered so far a wider use of the inverted repeat approach to study gene function in *C. elegans*. Inducible genetic interference has also been obtained with sense and anti-sense RNAs transcribed as separate molecules and not as a hairpin from inverted repeat sequences (Gupta et al., 2003). However, also in this case, an ubiquitously expressed heat-shock promoter was used and thus the knock-down of the gene function was not targeted to specific cells. In addition, the reduction of gene function in neurons was not analysed (Gupta et al., 2003). As part of the work on the PhD project I have also developed a method to silence/knock-down specific genes in chosen *C.elegans* neurons.

2. RESULTS

2.1) Experimental plan

The aim of my PhD project is to identify new genes involved in the signaling of polymodal nociceptive neurons. I decided to use as model the ASH neuron of the nematode *C.elegans*, a polymodal nociceptive neuron that, due its physiological and molecular features, is reminiscent of mammalian nociceptors in the dorsal root ganglion. For that purpose I used a reverse genetic approach choosing, on the basis of what is known in the literature and in data bases, candidate genes potentially involved in the transduction and transmission of signals in sensory neurons. Existing loss of function mutants of these genes were analysed by behavioral assays to measure their avoidance response to physical and chemical repellents. To analyse the genes of interest for which behavioral analysis was not possible (e.g. non existing loss of function mutants or lethal genes), I used RNA interference (RNAi) and also developed a new RNAi-based method to efficiently knock-down gene function specifically in the sensory neurons. Finally the genes, for which I could prove an involvement in the avoidance response, were studied to determine their role in the signal transduction and transmission cascades of the ASH neuron with the goal of identifying the mechanisms through which they function. The phases of the project are schematized below:



2.2) Best candidate genes

The choice of candidate genes was done on the basis of what is known on the sensory and nociceptive signal transduction and transmission mechanisms, in both vertebrate and invertebrate. Several studies indicate that G-proteins Coupled Receptors (GPCRs) and G-proteins play a central role in these processes (Hucho and Levine, 2007; Julius and Basbaum, 2001; Pan et al., 2008; Tobin and Bargmann, 2004; Zhang and Bao, 2006). In the last few years various studies have highlighted the role in G-signaling of a new class of proteins, the Regulators of G-protein Signaling (RGS), that are negative regulators of G-protein signaling pathways. Potential effectors acting downstream of G-protein signaling are the Phosfolipases C (PLC) that hydrolyses lipids in the membrane producing Inosytol-3-phosphate (I3P) and Diacyl-glicerol (DAG). These two second messenger molecules, by as yet unclear mechanisms, could in turn activate other effectors acting both in signal transduction and transmission. Molecules such as the PhosphoKinases-C (PKC) are potential effectors acting downstream of DAG. Others, like Diacyl-glicerol-kinases (DGK), control DAG levels in the cell and are also likely involved in these processes. Using this information I searched in the *C.elegans* database, WormBase (http://www.wormbase.org) the genes encoding for each class of the proteins mentioned above and for which a human homolog exists. The genes that satisfied these criteria are grouped in the table 3:

Table 3. Best Candidate Genes

Protein Classes	**Genes**
G-alpha subunits	*goa-1, egl-30*
G-beta subunits	*gpb-1*
RGS	*egl-10, eat-16*
Phospholipase C	*plc-1, plc-2, plc-4, egl-8*
Diaacylglycerol Kinase	*dgk-1, dgk-2, dgk-3*
PhosphoKinase C	*pkc-1, pkc-2, tpa-1,* T25E12.4

2.3) RNAi sas: a method for cell-specific gene silencing

In *C.elegans* the available approaches for identifying the function of a gene in specific cells are time consuming and restricted to non essential genes for which mutants are available. One goal of my project has been the development a simple reverse genetics approach for reducing, in chosen *C. elegans* neurons (particularly ASH) the function of genes potentially involved in nociception signaling. The method is based on the expression, under cell specific promoters, of sense and antisense RNA corresponding to a gene of interest.

2.3.1) Construction of transgenes for cell specific knock-down

We tested whether a heritable reduction of gene function in chosen *C. elegans* neurons could be efficiently and simply achieved with transgenes from which sense and anti-sense RNAs,

corresponding to the gene of interest, are transcribed as separate molecules by cell specific promoters. If this were the case, a simpler and faster strategy for the construction of the appropriate transgenes could be followed. For each interference we fused, by co-amplification, an exon rich region of the gene under study to a cell specific promoter (Hobert, 2002) (Figure 10 and Material and Methods). Fusions of the promoter to the gene fragment in the sense and in the antisense orientation (sas) were obtained in separate amplification reactions. We also took advantage of the possibility offered by *C. elegans* of transforming animals directly with the products of PCR reactions, without the need to clone them (Evans et al., 2006; Hobert, 2002). The two amplification products were mixed in roughly equimolar amounts and injected into recipient animals together with DNA encoding a visible, selectable marker. Transgenic lines carrying the three components were obtained and analysed for the various phenotypes. Cell specific promoters were chosen on the basis of information present in the literature, in public databases and from personal communication. In each case we independently confirmed the pattern of expression of these promoters by fusing them to a *gfp* reporter gene. In addition we performed control experiments to show that these promoters had no effect *per se* on the phenotypic traits we intended to analyse (data not shown). In addition to being very rapid, the procedure depicted in Figure 10 also overcomes the technical problems that have limited the use of transgene driven gene silencing in *C. elegans* (Briese et al., 2006; Tavernarakis et al., 2000).

2.3.2) Efficient and specific knock-down of osm-10 function

To test whether our approach could be successful where conventional RNAi usually fails (e.g. genes expressed in sensory neurons), we first looked at the gene *osm-10*. *osm-10* codes for a novel cytoplasmic protein required for the avoidance response of *C. elegans* to high osmotic strength solutions. OSM-10 is expressed in the sensory neurons ASH, ASI, PHA and PHB and, for osmotic avoidance, its function is required in the main avoidance neuron ASH (Hart et al., 1999). We expressed, in the sense and antisense orientations (sas), an exon rich region of the *osm-10* gene under the *srb-6* gene promoter, which is active in the sensory neurons ASH, ADL, PHA and PHB (Troemel et al., 1995).

Two transgenic lines harbouring the p*srb-6*::*osm-10*(*sas*) transgene were obtained and osmotic and quinine avoidance responses of animals from these lines were compared to those of controls (Figure 11). While *osm-10* RNA interference administered by feeding was ineffective in reducing osmotic avoidance, animals from transgenic lines were indistinguishable from controls bearing the *osm-10* loss-of-function allele, *n1602*. In contrast, quinine avoidance, which also depends on the function of ASH but not on *osm-10*, was not affected. These results indicate that the sense and antisense (sas)

based knock-down is efficient, that it is *osm-10* specific and that it does not interfere with the general physiology of the ASH neuron.

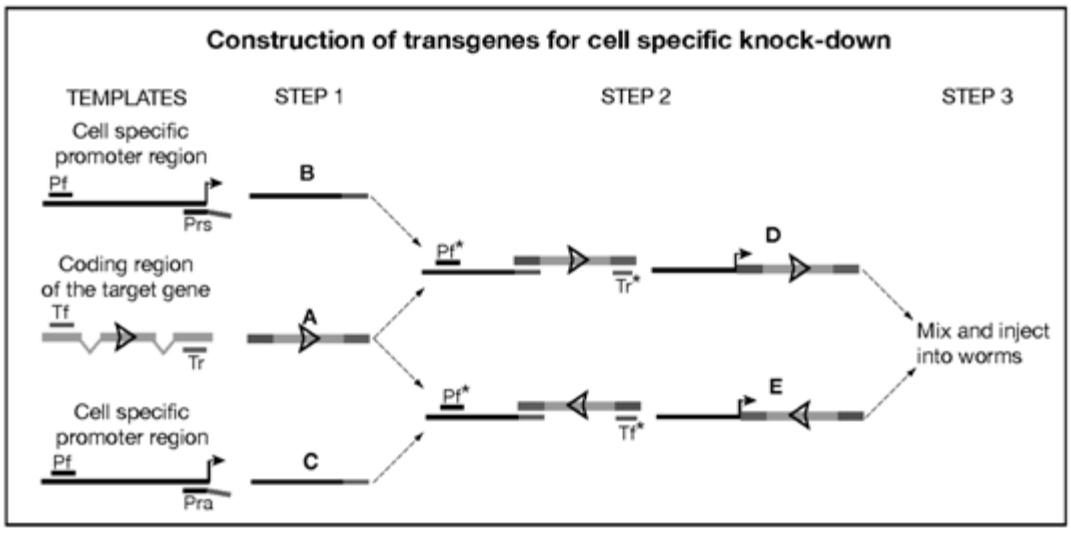


Figure 10. Construction of transgenes for cell specific knock-down of gene function includes three steps. In the first step, genomic sequences corresponding to the gene to be targeted and to the chosen cell specific promoter are amplified separately. An exon rich fragment of the gene to be targeted is amplified with primers Target forward (Tf) and Target reverse (Tr) to yeld product A. The cell-specific promoter is amplified, in two different reactions, with the Promoter forward primer (Pf) and with either one of two Promoter reverse primers (Promoter reverse sense, Prs or Promoter reverse antisense, Pra) to yield product B and C respectively. At their 3' end, primers Prs and Pra are identical and complementary to the promoter region but, at their 5' end, they differ for 25 nt which are complementary to one or the other of the extremities of A. In the second step, A is separately fused by amplification to B or C using the nested primers Pf* and either Tf* or Tr*. These two reactions yeld DNA fragments in which the target gene fragment can be transcribed by the cell specific promoter in the sense orientation in D and in the antisense orientation in E. In the third step D and E are mixed in equimolar amounts and injected, together with a visible selectable marker (not shown), in recipient animals. The large arrowhead on the target gene indicates the direction of the sense strand while the small arrow on the promoter indicates the direction of transcription.

2.3.3) The knock-downs obtained are cell autonomous

The purpose of our method is to determine the function of genes in specific cells. It is thus crucial that the knock-down effect that we trigger with a promoter active in one cell does not spread to nearby ones where the promoter is not active. To test if spreading were a problem we focused on two cell autonomous phenotypes. First we examined Dye filling (Dyf) in neurons exposed to the outside milieu. In wild type animals six amphid and two phasmid neurons (ASH, ASI, ADL, ASK, ASJ, AWB, PHA and PHB) take up fluorescent dyes from the environment through their sensory cilia and, as a result, become fluorescent (Hedgecock et al., 1985).

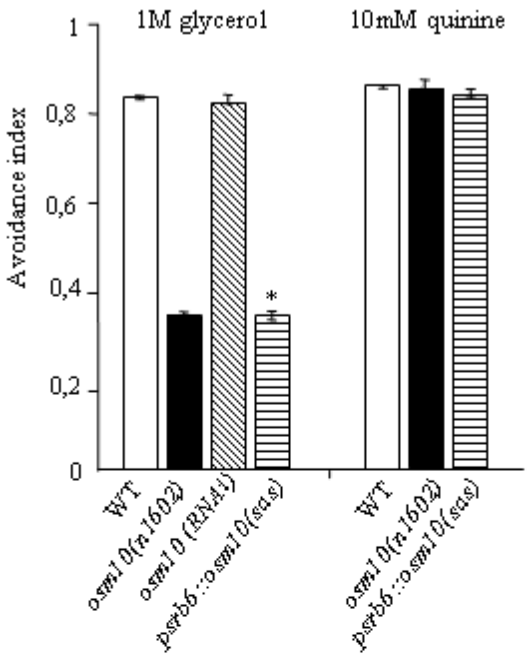


Figure 11. Knock-down of gene function in sensory neurons. Knock-down of *osm-10* function.
Osmotic and quinine avoidance (see Material and Methods) of N2, wild type animals; *osm-10(n1602)*, *osm-10* loss of function mutants; *osm-10(RNAi)*, animals fed bacteria that produce *osm-10* dsRNA; lines #1, #2 and #3, animals from three independent lines carrying the p*srb-6*::*osm-10*(*sas*) transgene. Bars indicate the mean number of animals staying within the ring after 15 min and the error bars show s.e.m. of at least five independent experiments. *, significantly different from wild type ($P < 0.001$).

The gene *osm-6* is required for ciliogenesis and loss-of-function mutants have ciliated neurons with shortened cilia that are not exposed to the environment, that do not take up the dye and hence, do not become fluorescent (Dyf$^-$). Mosaic analysis and cell specific genetic rescue experiments have shown that, with respect to the Dyf phenotype, OSM-6 acts cell autonomoulsy (Collet et al., 1998; Hilliard et al., 2004). In a first experiment we fused *osm-6*(*sas*) to the *srb-6* promoter which is expressed in ASH, ADL, PHA and PHB. We then tested the Dyf phenotype of each sensory neuron from transformed and from control animals. In wild-type animals, either untreated or after *osm-6* RNAi by feeding, almost 100% of each of the eight neurons took up the dye (Dyf$^+$). As expected, no neuron took up the dye (Dyf$^-$) in *osm-6*(*p811*) loss-of-function mutants. In transgenic animals bearing the p*srb-6*::*osm-6(sas)* construct, the neurons ASI, ASK, ASJ, and AWB (where *psrb-6* is not active) took up the dye (Dyf$^+$), whereas the neurons in which p*srb-6* is active became Dyf$^-$: almost 100% of PHA and PHB and about 50% of ASH and ADL (Figure 12a and Table 4). In a second set of experiments we fused *osm-6*(*sas*) to the *str-1* promoter which is strongly but selectively expressed in the AWB neuron (Troemel et al., 1995) and obtained transformed lines bearing p*str-1*::*osm-6(sas)*. In these animals, about 90% of AWB neurons were Dyf$^-$, while all the other sensory neurons were Dyf$^+$ (Figure 12b and Table 4). The AWB specific reduction of *osm-6* function also results in a behavioral phenotype: animals bearing p*str-1*::*osm-6(sas)* were defective for nonanone avoidance, a behavior which is known to be mediated by a functional AWB sensory neuron (Table 5). To extend our results we examined a second cell autonomous phenotype: expression of GFP. For this we used an integrated transgenic line in which GFP is expressed under

the *gpa-15* promoter in the sensory neurons ADL, ASH, ASK, PHA and PHB, and in the distal tip cell of the gonad (Jansen et al., 1999).

Table 4. Knock-down of *osm-6* function: Dye filling of sensory neurons

	% of cells taking up DiI for each neuron type				
	ASH	ADL	PHA/PHB	AWB	ASJ/ASK/ASI
N2	96 (n=208)	96 (n=208)	82 (n=368)	84 (n=214)	92 (n=330)
osm-6(p811)	0 (n=50)	0 (n=50)	0 (n=50)	0 (n=50)	0 (n=50)
osm-6(RNAi)	91 (n= 46)	91 (n= 46)	100 (n= 46)	83 (n= 46)	85 (n= 138)
p*srb-6*::*osm-6(sas)* #1	**52 (n=104)***	**53 (n=104)***	**5 (n=208)***	81 (n=124)	88 (n=340)
p*srb-6*::*osm-6(sas)* #2	**61 (n= 77)***	**65 (n= 77)***	**0 (n= 94)***	93 (n= 40)	90 (n=122)
p*str-1*::*osm-6(sas)* #1	98 (n=106)	94 (n=106)	98 (n= 84)	**5 (n=106)***	90 (n=126)
p*str-1*::*osm-6(sas)* #2	96 (n= 48)	96 (n= 48)	92 (n= 48)	**8 (n= 48)***	90 (n=116)

Number in parenthesis are the number of neurons observed. Asterisks (*) indicate significantly different from N2 with $p < 0.001$.

Table 5. AWB specific knock-down of *osm-6* function: nonanone avoidance

	Avoidance index
N2	-0.84 ± 0.007 (n=1036)
p*str-1*::*osm-6(sas)* #1	**-0.53 ± 0.004 (n=197) ***
p*str-1*::*osm-6(sas)* #2	**-0.45 ± 0.017 (n=595) ***

Avoidance indexes (Material and Methods) ± s.e.m. are reported. Number in parenthesis are the number of animals tested in at least three different experiments. Asterisks (*) indicate significantly different from N2 with $p< 0.001$.

We tested whether we could reduce the expression of GFP in some but not all of the expressing cells by driving the expression of *gfp(sas)* RNA with the *srb-6* promoter. In animals transformed with the p*srb-6*::*gfp(sas)* construct, GFP was efficiently silenced in ASH, ADL, PHA and PHB neurons where *srb-6* is expressed but not in ASK or in the distal tip cell where it is not (Figure 12b and Table 6). The experiments with *osm-6* and *gfp* confirm that our gene knock-down approach functions in the targeted neurons and strongly suggest that the effect does not spread from one neuron to nearby ones (Esposito et al., 2007).

Table 6. Cell specific reduction of GFP expression

Transgenes	Level of GFP expression in each cell type											
	ASH			ADL			PHA/B			ASK		
	–	+	++	–	+	++	–	+	++	–	+	++
In[p*gpa-15*::*gfp*] Control	0	2	98	0	0	100	6	2	92	0	0	100
In[p*gpa-15*::*gfp*] Ex[p*srb-6*::*gfp(sas)*] #1	**74**	**20**	**6***	**35**	**52**	**13***	**50**	**36**	**14***	0	0	100
In[p*gpa-15*::*gfp*] Ex[p*srb-6*:: *gfp(sas)*] #2	**92**	0	**8***	**35**	**53**	**12***	**49**	**45**	**5***	0	0	100
In[p*gpa-15*::*gfp*] Ex[p*srb-6*:: *gfp(sas)*] #3	**70**	**25**	**5***	**43**	**35**	**22***	**51**	**43**	**4***	0	0	100

Transgenes are described in the main text and in Material and Methods**.** Numbers represent percent of cells expressing GFP at different levels: no detectable GFP expression = – ; weak expression = +, strong expression = ++. ASH, ADL, PHA/B and ASK are the sensory neurons in which p*gpa-15*::*gfp* is expressed. ASH, ADL and PHA/B are the neurons in which *srb-6* is expressed. Asterisks (*) indicate significantly different from control with p <0.001.

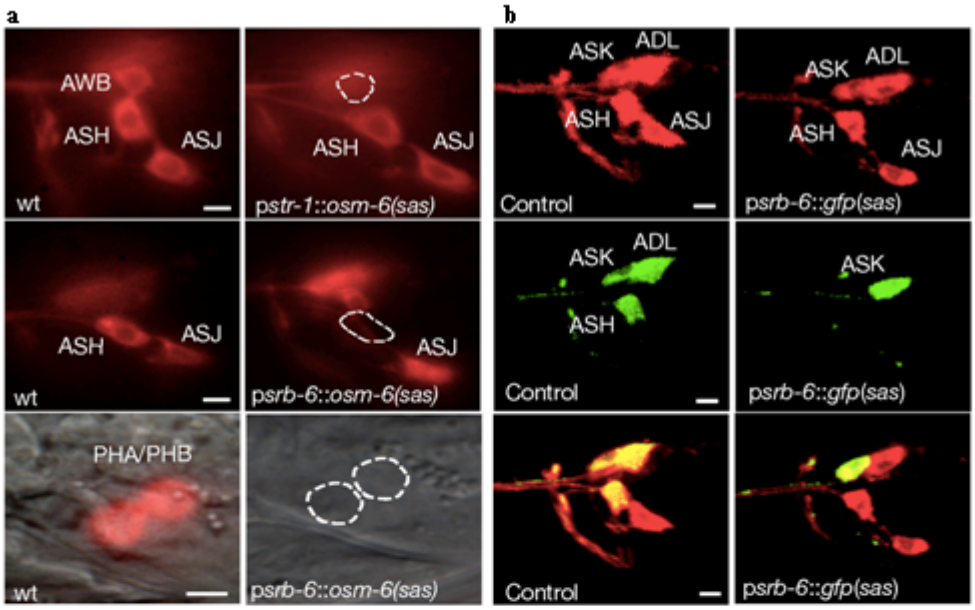


Figure 12. a) Knock-down of *osm-6* function. Dye filling of sensory neurons in wild-type animals (left panels) and in animals carrying either the p*str-1*::*osm-6(sas)* (upper right panel) or the p*srb-6*::*osm-6(sas)* (middle and lower right panels). Dashed lines outline the neurons in which knock-down of *osm-6* has prevented dye filling. **b)** Knock-down of GFP expression. Fluorescent confocal images of amphid neurons in a control animal, carrying the Is[p*gpa-15*::*gfp*] transgene (left panels), and in an experimental animal carrying, in addition to Is[p*gpa-15*::*gfp*], also the p*srb-6*::*gfp(sas)* transgene (right panels). Top panels, DiI dye filling (red). Middle panels, GFP expression. In the control animal GFP expression is strong in ASH, ADL and ASK while in the animal carrying the p*srb-6*::*gfp(sas)* transgene (right) only ASK expresses GFP. Bottom panels, merge.

2.4) Behavioral analysis

In order to valuate the role of the candidate genes in nociception we analysed the avoidance response to different noxious stimuli of animals in which the function of these genes had been reduced either by the RNAi(sas) cell specific knock-down method or because they carried a loss of function mutation. We tested them with a complete range of stimuli including: physical ones such as high osmolarity (1M glycerol) and nose touch (a mechanical touch on the nose); chemicals ones including alkaloids (10mM quinine, 50mM primaquine); heavy metals (1mM copper ions). To measure the avoidance response I used the assays described in Materials and Methods. In every case an avoidance index (A.I.) was measured. Animals that respond well to the repellents show an A.I. close to 1, whereas animals completely defective for avoidance show an A.I. close to 0.

2.4.1) Cell specific knock-down of the function of the essential gene, gpb-1

I sought to test if sas gene knock down could be used to study the function, in ASH, of the gene *gpb-1*. *gpb-1* is an essential gene encoding one of the two Gβ subunits of *C. elegans*. It is first expressed in early embryogenesis in many cell types and, later in the adult, in all neurons. *gpb-1* loss-of-function mutations are thus recessive lethal (Zwaal et al., 1996). Overexpression studies have suggested that *gpb-1* negatively regulates egg-laying, but the cells where its action is required for modulating this behavior could not be identified (Zwaal et al., 1996). A reasonable but unproven hypothesis is that these cells might be the two Hermaphrodite Specific Neurons (HSN). To test this hypothesis we used the *tph-1* promoter, which is active in the HSNs (Sze et al., 2000), to drive the expression of *gpb-1(sas)* RNA. In addition, we used the *srb-6* promoter to test whether GPB-1 is also required for avoidance responses, in the ASH sensory neuron. These responses are known to involve several Gα subunits (see Introduction) but the role of *gpb-1* could not be easily tested by conventional genetics because of its lethality and ubiquitous expression. Independent lines, carrying either p*tph-1*::*gpb-1(sas)* or p*srb-6*::*gpb-1(sas)* were obtained and assayed for egg-laying and for avoidance (Table 7). Animals in which *gpb-1(sas)* RNA was expressed in the HSN neurons were hyperactive for egg-laying but showed normal avoidance responses. Conversely, animals in which *gpb-1(sas)* RNA was expressed in the sensory neurons, were normal for egg-laying but defective for quinine and osmotic avoidance (Table 7). This experiment has provided new information on the function of *gpb-1* that could not easily have been obtained otherwise: i) the effect of *gpb-1* on egg-laying is mediated at least in part by its function in the HSN neurons or in the other serotonergic neurons in which *tph-1* is active (the ADF and NSM pairs in the head); ii) *gpb-1* function is required, in the avoidance sensory neurons, for proper response to repellent stimuli (Esposito et al., 2007).

Moreover the experiment demonstrated that with the RNAi (sas) gene knockdown approach it has been possible to overcome the problems due to the lethality of a mutation in an essential gene and to obtain viable transformant lines in which the function of an essential gene has been reduced specifically in certain cells but not in others.

Table 7. Cell specific knock-down of *gpb-1* function

	Avoidance		**Egg-laying**
			Number of Eggs in the uterus
	Quinine	**Osmotic**	
N2	0,8 ± 0,01 (n=110)	0,8 ± 0,03 (n=110)	14.3 ± 0.2 (n=129)
p*srb-6*::*gpb-1(sas)* #1	**0,4 ± 0,04 (n=70)***	**0,5 ± 0,03 (n=70)***	13.1 ± 0.2 (n=50)
p*srb-6*::*gpb-1(sas)* #2	**0,3 ± 0,03 (n=40)***	**0,5 ± 0,04 (n=50)***	13.3 ± 0.4 (n=47)
p*tph-1*::*gpb-1(sas)* #1	0,8 ± 0,03 (n=40)	0,8± 0,03 (n=40)	**8,8 ± 0,4 (n=40)***
p*tph-1*::*gpb-1(sas)* #2	0,8 ± 0,03 (n=40)	0,8 ± 0,03 (n=40)	**10,3 ± 0,03 (n=40)***

Mean ± s.e.m. of at least three experiments are indicated. Number in parenthesis are the number of animals tested. Asterisks (*) indicate significantly different from N2 with $p < 0.001$

2.4.2) Analysis of loss of function mutants

For the other candidate genes loss of function mutants already exists. I obtained these mutants from the Caenorhabditis Genetics Center (CGC) and analysed them for their avoidance responses. Table 8 summarises the results of this analysis.

Among the genes tested, mutants in *egl-10* and *egl-30* showed the most severe defects in the response to high osmolarity and to chemicals but were apparently normal for the response to mechanical stimuli. A similar pattern, but with a smaller effect was shown by *gpb-1*. A complementary pattern, instead was shown by mutants in *dgk-2*, *pkc-1* and *pkc-2* that have small but significant defects in the response to nose touch but appear normal for their response to chemicals and to high osmolarity. Mutants in *plc-2* and in *dgk-3* show more specific, but limited defects. Overall the results seem to indicate that the pathways and the genes involved in the response to mechanical stimuli are at least partially different from those involved in avoidance of high osmolarity and of noxious chemicals. This is particularly relevant as it has been shown that ASH is the main sensory neuron responsible for the detection of all these noxious stimuli (Bargmann et al., 1990; Culotti and Russell, 1978; Hilliard et al., 2002; Hilliard et al., 2004; Kaplan and Horvitz, 1993; Sambongi et al., 1999; Sambongi et al., 2000; Troemel et al., 1995)).

Table 8. Behavioral analysis

		Reppellents	
Mutants	**High osmolarity**	**Nose touch**	**Chemicals**
wt	0,9	0,8	0,8
dgk-1	0,8	0,8	0,7
dgk-2	0,8	**0,4***	0,7
dgk-3	1	0,6	**0,5***
plc-1	0,9	0,8	0,7
plc-2	**0,5***	0,8	0,7
plc-4	0,8	0,7	0,8
egl-8	0,8	0,7	0,6
pkc-1	0,9	**0,5***	0,9
pkc-2	0,8	**0,5***	0,9
tpa-1	0,9	0,7	0,8
T25E12.4(PKC)	0,9	0,8	0,9
egl-10	**0,3***	0,8	**0,3***
eat-16	0,9	0,7	0,8
goa-1	0,9	0,8	0,8
egl-30	**0,4***	0,8	**0,3***
gbp-1	**0,5***	0,7	**0,4***

Mean of at least three experiments are indicated. Asterisks (*) indicate significantly different from *wt* with $p < 0.001$

2.5) Functional analysis

Given the importance of RGS proteins in the regulation of G protein signaling in the sensory response and the opportunities that they offer for drug development (Chasse and Dohlman, 2003) I decided to focus further studies on the functional analysis of the *egl-10* gene. I also decided to analyse the function of *qui-1*, another RGS protein encoding gene, identified and isolated previously in the laboratory.

2.5.1) Functional analysis of *egl-10*

2.5.1.1) egl-10(md176) disrupts the response to noxious stimuli

egl-10 encodes a conserved RGS protein that affects egg-laying and locomotion behaviors (Koelle and Horvitz, 1996). Loss of *egl-10* function causes defective egg-laying behavior (Koelle and Horvitz, 1996). We found that *egl-10(md176)* animals also show an alterated avoidance response to various repellent stimuli ((Hilliard et al., 2002) and Materials and Methods). Although RGS proteins normally act to dampen G protein-mediated signaling, *egl-10*(*md176*) mutant animals were not hypersensitive to aversive stimuli. Instead, *egl-10(md176)* animals were defective in their response to multiple aversive stimuli detected by the ASH polymodal sensory neurons (quinine, high osmolarity and copper). *egl-10(md176)* mutant animals were defective in their response to the soluble repellent quinine (Figure 13a), which is detected primarily by the ASH neurons, with a

small contribution by the ASK and ADL neurons (Hilliard et al., 2004) and to copper ions (Figure 13a) which again is detected by ASH with a small contribution by ASE and ADL (Sambongi et al., 1999). *egl-10* mutant animals were also defective in the response to high osmolarity which is detected only by the ASH sensory neurons (Bargmann et al., 1990) (Figure 13a). Taken together these results suggest that loss of the EGL-10 function decreases sensory avoidance response, contrary to what might be expected for loss of a negative regulator of G protein signaling.

2.5.1.2) EGL-10 functions in the ASH sensory neurons

EGL-10 could affect avoidance behavior acting in one or more cell types of the neural circuit. The chemical avoidance neural circuit has been well characterized (Hilliard et al., 2002). It consists of the avoidance sensory neurons, ASH, ADL, and ASK, and the command interneurons, AVA, AVB, AVD and PVC that control forward and backward movement through motor neurons and muscle (de Bono and Maricq, 2005; Hilliard et al., 2002). The EGL-10 protein is expressed in the nerve ring, ventral nerve cord, and dorsal nerve cord, and appears to be localized exclusively to processes at the locations of the chemical synapses in the animals (Koelle and Horvitz, 1996). Expression of EGL-10 protein is also detected in muscle as spots arranged in linear arrays within the body–wall muscle cells (Koelle and Horvitz, 1996). To determine in which cells of the neural circuit the function of EGL-10 is required for avoidance we first measured the response of *egl-10* mutants to the anterior body touch. Body touch is sensed by the ALM and AVM sensory neurons, and not by ASH, but the behavioral response is mediated by the same interneurons and motor neurons that mediate chemical avoidance (Chalfie and Sulston, 1981; Chalfie et al., 1985; Kaplan and Horvitz, 1993). Avoidance of light anterior body touch is intact in *egl-10* mutants (Figure 13b), indicating that avoidance defects observed in *egl-10*(*md176*) animals are likely to result from a defect in the sensory neurons rather than in the neurons downstream in the circuit. Second, since the ASH sensory neurons are the primary neurons that detect quinine and high osmolarity (Bargmann et al., 1990; Hilliard et al., 2004; Troemel et al., 1995), we transforned *egl-10(md176)* mutant animals with *egl-10* cDNA driven by the *sra-6* promoter which is expressed only in ASH and in two other neurons, ASI and PVQ (Troemel et al., 1995), which, however, are not involved in avoidance. The p*sra-6*::*egl-10* transgene was sufficient to restore the behavioral responses to quinine and to high osmolarity of *egl-10(md176)* mutants (Figure 13a). As a control we also expressed the *egl-10* cDNA in the odorant sensory neurons AWA using the *ord-10* promoter (Sengupta et al., 1996). In this case we did not observe rescue of the avoidance phenotype (data not shown). Taken together these results suggest that, for avoidance of quinine and high osmotic strength, it is sufficient that EGL-10 functions in ASH.

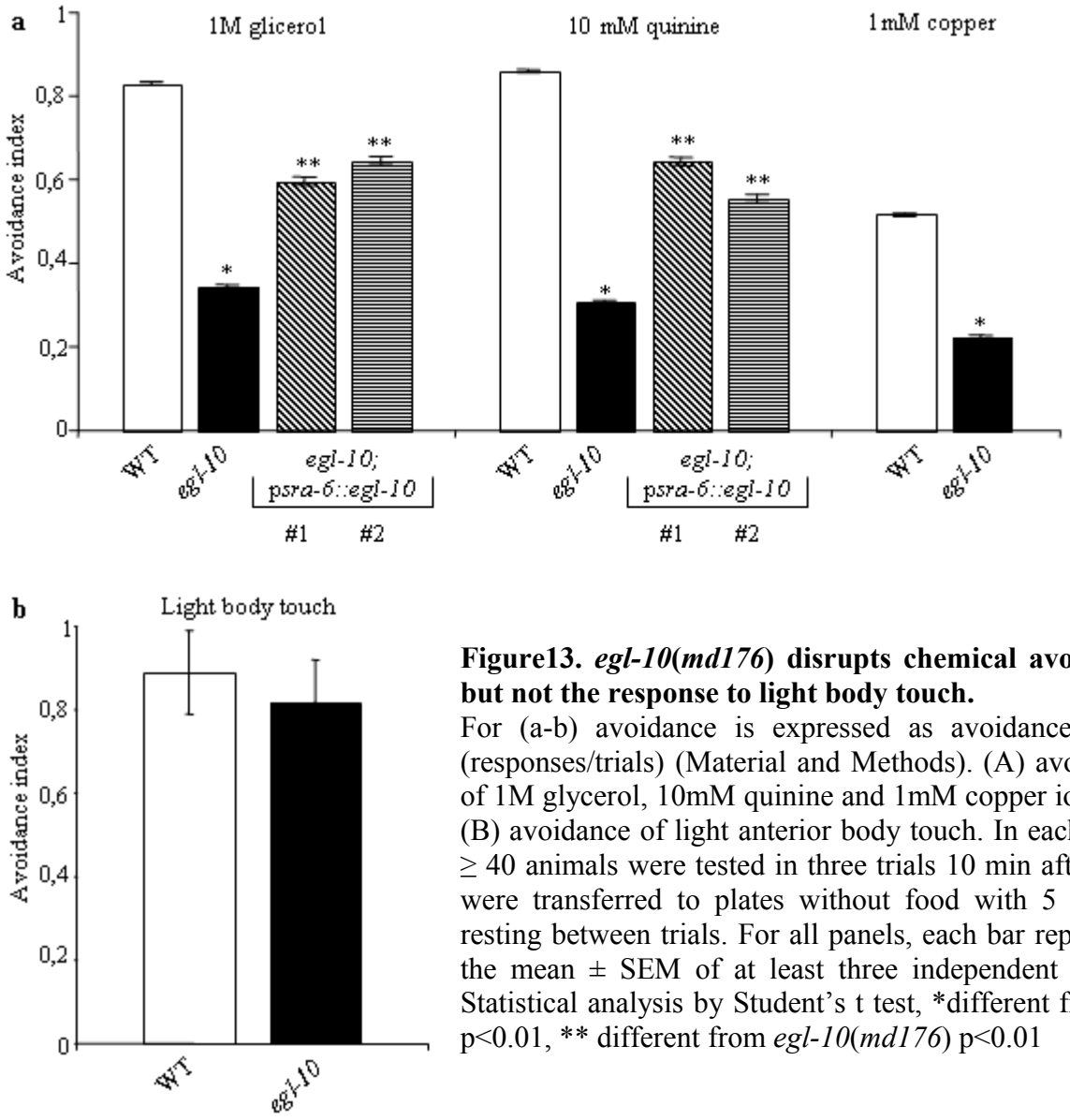


Figure13. *egl-10*(*md176*) disrupts chemical avoidance but not the response to light body touch.
For (a-b) avoidance is expressed as avoidance index (responses/trials) (Material and Methods). (A) avoidance of 1M glycerol, 10mM quinine and 1mM copper ions and (B) avoidance of light anterior body touch. In each assay ≥ 40 animals were tested in three trials 10 min after they were transferred to plates without food with 5 min of resting between trials. For all panels, each bar represents the mean ± SEM of at least three independent assays. Statistical analysis by Student's t test, *different from wt $p<0.01$, ** different from *egl-10*(*md176*) $p<0.01$

2.5.1.3) Stimulus-evoked Ca^{2+} transients mobilization in ASH is are retained in egl-10 mutants

The defects of *egl-10* mutants are consistent with a defect in ASH. The defect could be in signaling or in ASH differentiation (e.g. cilium development or morphology). To establish whether *egl-10* affects ASH differentiation we stained ciliated sensory neuron with the lipophilic dye DiI. The ASH sensory neurons take up this dye from the environment through their exposed sensory cilia, transport it retrogradely and become fluorescent. Uptake of DiI is disrupted by defects in cilia morphogenesis or in retrograde transport (Hedgecock et al., 1985; Perkins et al., 1986). The ASH neurons of *egl-10* mutants stained normally with DiI, suggesting that the sensory neurons and their cilia were structurally and functionally intact (data not shown).

Primary signaling in ASH requires the OSM-9/OCR-2 TRPV channel that acts downstream of

stimulus-evoked G protein-coupled signaling (Colbert et al., 1997; Tobin et al., 2002). Activation of the OSM-9/OCR-2 channel generates a calcium influx into the ASH neurons (Hilliard et al., 2005; Kahn-Kirby et al., 2004).

In ASH, EGL-10 could function either in primary sensory signaling or in the propagation of a Ca^{2+} signal from the cilium to the cell body. To ask directly whether EGL-10 affects primary signaling in ASH, we imaged Ca^{2+} fluxes *in vivo* using a transgenic calcium reporter protein. We used a strain expressing the genetically encoded Ca^{2+} sensor G-CaMP (Nakai et al., 2001) in ASH under the *sra-6* promoter (kindly provided by C.I. Bargmann). Ca^{2+} transients were measured in the ASH cell body as changes in background subtracted fluorescence intensity values relative to baseline (ΔF). We observed that Calcium fluxes evoked by high osmolarity, quinine and copper in *egl-10* mutants were similar compared to those of wt animals (Figure 14 a1-a3, b1-b3 and c1-c3). These results indicate that EGL-10 is not required in ASH for the stimulus-receptor interaction that generates the signal and for its transduction up to the first Ca^{2+} increase.

2.5.1.4) The efficiency of synaptic transmission of ASH is impaired in egl-10 mutants.

Since EGL-10 is not required in the generation and transduction of the signal then the behavioral and sensory defects of *egl-10*(*md176*) animals could be consistent with a defect in the propagation of a Ca^{2+} signal from the cilium where it is generated to the synapses of ASH. To determine whether the loss of EGL-10 function perturbs signaling downstream of OSM-9/OCR-2 channels, including the neurotransmitter release machinery at the synapse, we assessed avoidance of the chili pepper irritant capsaicin. *C.elegans* does not respond to the chili pepper irritant capsaicin, but animals expressing the rat TRPV1 channel in ASH respond to capsaicin with an escape behavior that is similar to endogenous ASH avoidance responses (Tobin et al., 2002). TRPV1-dependent capsaicin avoidance bypasses the upstream stimulus-evoked G protein-coupled signaling as well as OSM-9/OCR-2 TRPV channels but it is dependent upon the vesicular glutamate transporter EAT-4 (Tobin et al., 2002). Presumably capsaicin directly activates TRPV1 to depolarize ASH, activating calcium signaling and glutamatergic synaptic transmission (Tobin et al., 2002). Alterations in synaptic communication between ASH and downstream neurons result in a delay in the withdrawal response rather than in a lack of response (Mellem et al., 2002).

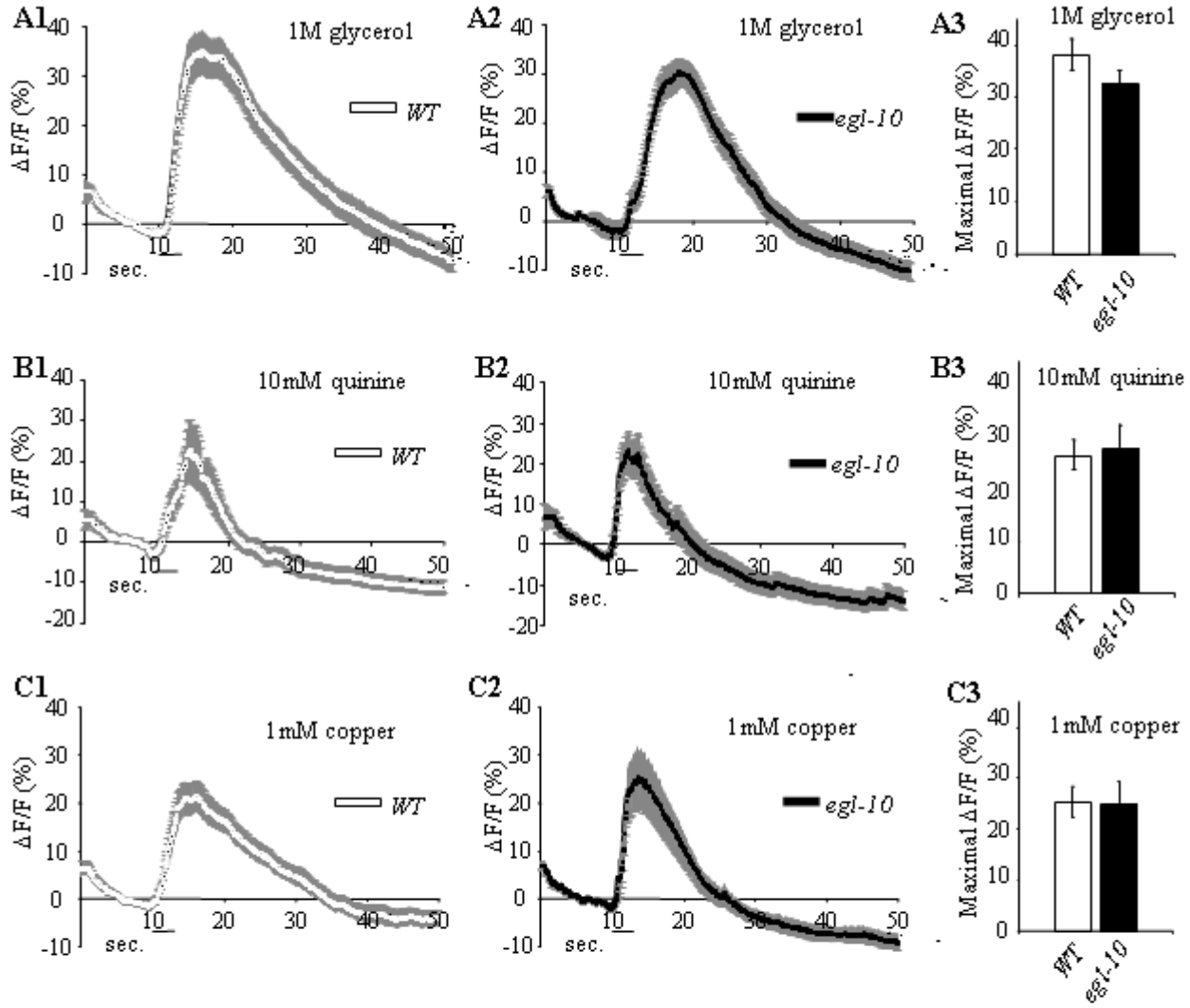


Figure 14. Stimulus-Evoked Calcium Transients in the ASH Neurons Are retained in *egl-10*(*md176*) animals.

A genetically encoded calcium indicator, G-CaMP, was expressed in the ASH neurons. Soluble stimuli were delivered to the nose of an adult animal for 3 s (black horizontal bar), and the change in GFP fluorescence intensities were recorded.

(a1, b1 and c1) Average calcium transients in ASH neurons of *WT* animals in response to (a1) 1M Glycerol, (b1) 10mM quinine and (c1) 1mM copper ions. (a2, b2 and c2) Average calcium transients in ASH neurons of *egl-10(md176)* animals in response to (a2) 1M Glycerol, (b2) 10mM quinine and (c2) 1mM copper ions. The change in GFP fluorescence intesities are shown relative to averaged pre-stimulus baseline (ΔF/F), the grey band represents SEM. (a3, b3 and c3) The maximum ΔF/F of individual imaging trials with *WT* and *egl*-10(*md176*) animals. The bar represents the mean ± SEM.

We thus measured the delay in avoidance response to capsaicin in *egl-10* and wild-type animals carrying the p*sra-6*::TRPV1 transgene (*egl-10(md176);*p*sra-6*::TRPV1 and *egl-10*(+)*;*p*sra-6*::TRPV1, respectively).

In the drop test assay, a small drop of 1 M glycerol (high osmolarity repellent stimulus) is placed in the path of a worm moving forward on an agar plate: upon encountering the repellent, the worms stop their movement and, after a brief delay, initiate a backing away response (Figure 15a). We found that compared to *egl-10*(+);p*sra-6*::TRPV1 control animals, the delay in the response was significantly increased in *egl-10(md176);*p*sra-6*::TRPV1 worms. On average, control animals took 2.6 s after the initial contact to respond to the stimulus whereas *egl-10(md176);*p*sra-6*::TRPV1 took 4.8 s (Figure 15b). We also measured the delay response of non-transformed *egl-10(md176)* when tested with 1M glycerol. We found that *egl-10* mutants showed an increased delay of the response to glycerol when compared to wild-type animals (Figure15c). In addition the delay is not due to the loss of the function of *egl-10* in cells other than ASH, because *egl-10* mutant animals in which *egl-10* is expressed only in ASH (*egl-10(md176);*p*sra-6*::*egl-10*) respond within 2.6 s like wild-type animals (data not shown**).** These results indicate that the delayed response to noxious stimuli of *egl-10* mutants is not limited to the capasaicin experimental paradigm and suggest that it probably is the origin of their avoidance defects. Taken together these results indicate that EGL-10 modulates synaptic communication between ASH and the neurons downstream to it in the avoidance circuit.

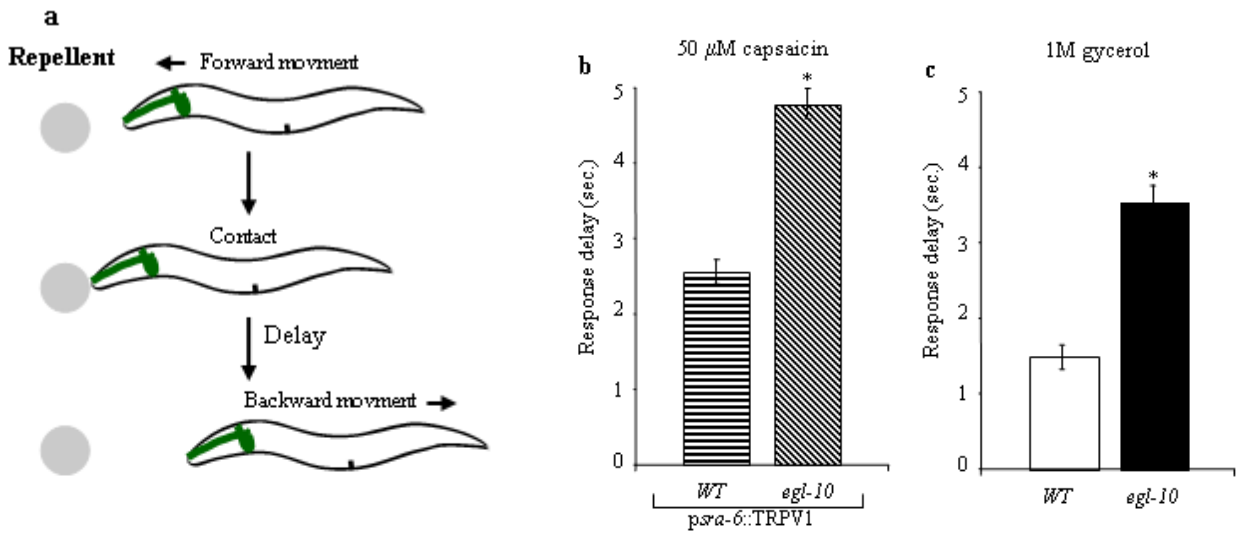


Figure 15. *egl-10*(*md176*) mutants show defects in synaptic function.
(a) The drop test assay (adapted from (Mellem et al., 2002)). Horizontal arrows indicate the direction of movement. (b) avoidance of 50μM capsaicin expressed as avoidance index (responses/trials). (c) The average time taken (response delay) for a worm to reverse direction after contacting the capsaicin. In each assay ≥ 40 animals were tested in three trials 10 min after they were transferred to plates without food, with 5 min of resting between trials. The bar represents the mean ± SEM of at least three independent assays. Statistical analysis by Student's t test, * different from control $p<0.01$.

The most important requirement for EGL-10 in ASH is thus downstream of the primary signal,

probably at the presynaptic level. This conclusion is also consistent with previous data showing that the EGL-10 protein localizes at chemical synapses (Koelle and Horvitz, 1996) and that, in animals overexpresssing *egl-10*, there is an increase in neurotransmitter release (Miller et al., 1999).

2.5.1.5) The egl-10 pathway

Previous results demonstrated that there are two opposing G protein signaling pathways that control egg-laying and locomotion behaviors in *C. elegans*. These two pathways involve the $G_o\alpha$ and $G_q\alpha$ proteins (GOA-1 and EGL-30, respectively). In particular GOA-1 activity inhibits egg laying and locomotion, whereas EGL-30 has the opposite effects on these behaviors (Chase et al., 2001). Genetic and biochemical experiments show that the RGS protein EGL-10 negatively modulates GOA-1 activity and that the RGS protein EAT-16 negatively modulates EGL-30.

The behavior exhibited by an individual animal is determined by the balance of GOA-1 and EGL-30 signaling which are modulated by EGL-10 and EAT-16 (Hajdu-Cronin et al., 1999; Koelle and Horvitz, 1996) (Figure 16). We asked if these two opposing pathways and the four genes involved, control also the avoidance response behavior. We measured the avoidance response of *goa-1(n363)* null mutants and of *eat-16(ad702)* and *egl-30(n68sd)* hypomorfic mutants.

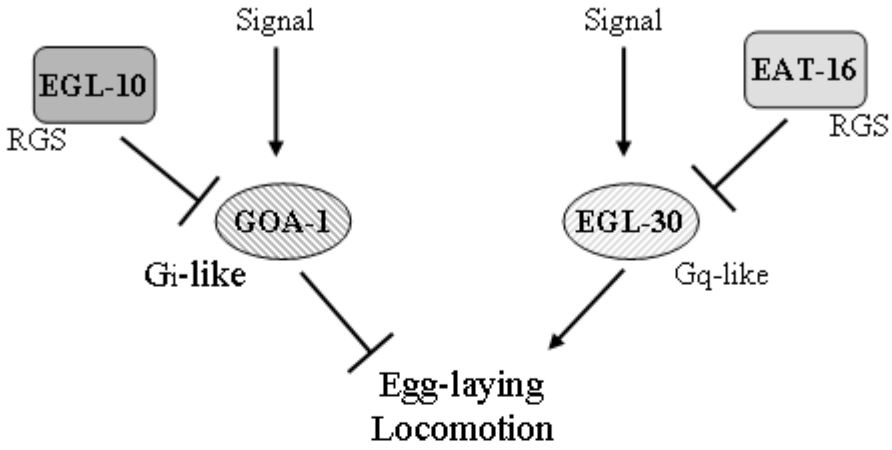


Figure 16. Opposing G proteins signaling control egg-laying and locomotion behaviors.
Schematic representation of the opposing G protein signaling pathways that control egg-laying and locomotion behaviors in *C. elegans*. Signaling through cell surface receptors activates the Gαo and Gαq proteins (GOA-1 and EGL-30, respectively). GOA-1 activity inhibits egg laying and locomotion, whereas EGL-30 has the opposite effects on these behaviors. Genetic experiments show that the RGS protein EGL-10 is a specific inhibitor of GOA-1 activity and that the RGS protein EAT-16 is a specific inhibitor of EGL-30 (from (Chase et al., 2001)).

As expected *egl-30* mutants showed a defective avoidance phenotype similar to *egl-10* whereas *goa-1* and *eat-16* single mutants showed normal avoidance response to the repellents tested at standard concentrations (1M glycerol, 10mM quinine, 1mM copper) (Figure 17a). On the basis of

the model described above, the loss of *eat-16* and the loss of *goa-1* function should result in hypersensitivity to repellents. However, the experiments described above assessed the response of *goa-1* and *eat-16* mutants to strong, saturated stimuli.

To determine whether *goa-1* and *eat-16* single mutants were in fact hypersensitive, we assessed their ability to respond to weaker stimuli, i.e. more diluted concentrations of glycerol. As expected *eat-16* animals responded better than wild type to dilute glycerol concentrations, whereas *goa-1* mutants did not (Figure 17b). A possible explanation is that the sensitivity of our test is not sufficient to detect the increase in signaling generated by the loss of GOA-1 function. However, we also carried out epistasis experiments analyzing the double mutants *goa-1(n363);egl-10(md176)* and *eat-16(ad702);egl-10(md176)* and found that both showed avoidance responses similar to wild type and significantly different from *egl-10* single mutants (Figure 17a). These results strongly support the hypothesis that the two opposing pathways that control egg-laying and locomotion also control the avoidance behavior and that, also for avoidance, EGL-10 functions together with EGL-30, GOA-1 and EAT-16.

2.5.1.6). For avoidance, the function of GOA-1 and EAT-16 is required in the ASH neuron

The genetic interaction of *goa-1*, *eat-16* and *egl-10* is compatible both with their function in the same cell or in different cells. We asked if, like EGL-10, also GOA-1 and EAT-16 act in the ASH sensory neurons. For GOA-1, we expressed the catalytic subunit (S1) of the pertussis toxin (PTX) in the ASH neurons using the *sra-6* promoter. PTX inactivates $G_o\alpha$ proteins by ADP-ribosylation of a conserved cysteine and, in *C.elegans*, the toxin has been used to specifically inactivate GOA-1 (Tanis et al., 2008). Inactivation of GOA-1 in the ASH neurons by PTX results in a complete rescue of the behavioral defects of *egl-10* mutants. *egl-10(md176)*;Ex[psra-6::PTX S1 subunit] transgenic animals had normal avoidance responses similar to those of *egl-10*;*goa-1* double mutants (Figure18a). For EAT-16, using the *sra-6* promoter we expressed, in ASH, a genomic fragment containing the *eat-16* coding sequence (CDS). In *eat-16 egl-10* double mutants, specific expression in ASH of the *eat-16* CDS restored the avoidance defects of *egl-10* single mutants (Figure 18b). These results indicate that EGL-10, GOA-1 and EAT-16 control avoidance behavior by functioning in the ASH sensory neurons.

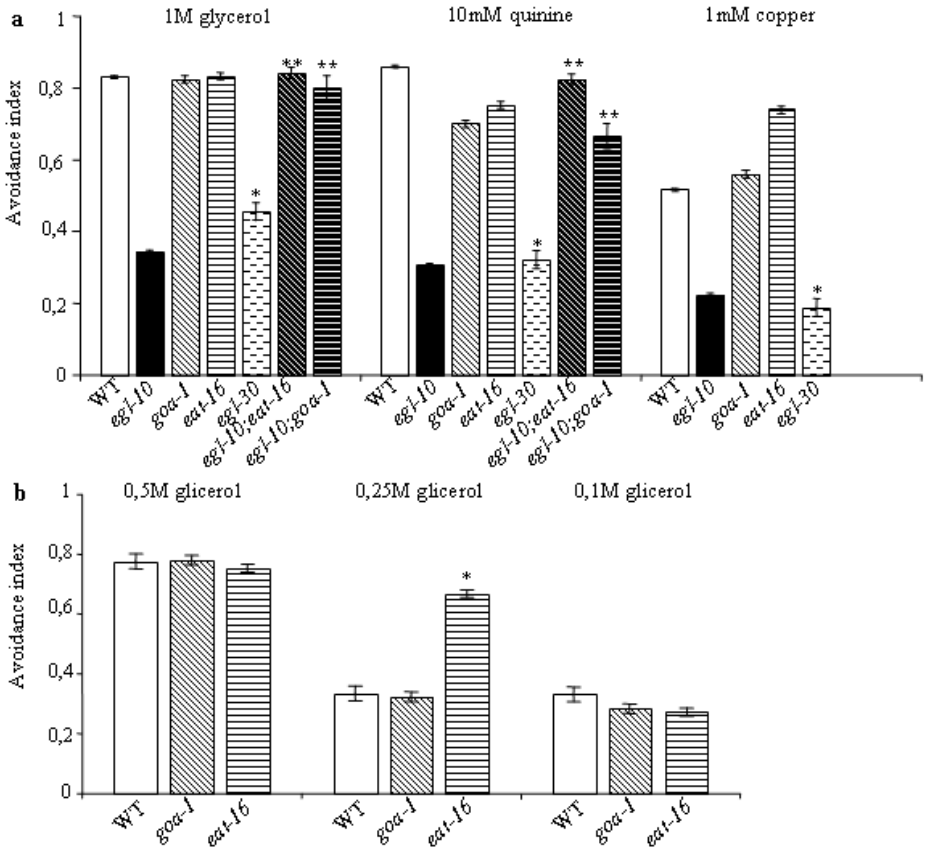


Figure 17. GOA-1 and EAT-16 function downstream of, or in parallel to, EGL-10.
For (a-b) avoidance is expressed as avoidance index (responses/trials). (a) avoidance of 1M glycerol, 10mM quinine and 1mM copper ions and (b) avoidance of dilute concentrations of glycerol 0,5M, 0,25M and 0,1M. In each assay ≥ 40 animals were tested in three trials 10 min after they were transferred to plates without food with 5 min of resting between trials. For all panels, each bar represents the mean ± SEM of at least three independent assays. Statistical analysis by Student's t test, *different from wt p<0.01, ** different from *egl-10*(*md176*) p<0.01

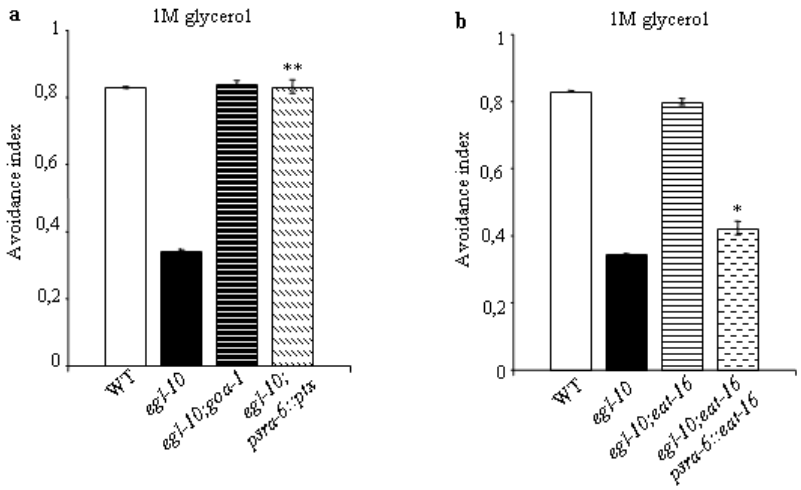


Figure 18. GOA-1 and EAT-16 act in the ASH sensory neurons.
For (a-b) avoidance of 1M glycerol is expressed as avoidance index (responses/trials). For all panels, each bar represents the mean ± SEM of at least three independent assays and combined data of two transgenic lines. Statistical analysis by Student's t test, *different from wt p<0.01, ** different from *egl-10*(*md176*) p<0.01

2.5.1.7) EGL-30 function is required for signal transduction of the ASH neuron

The genetic analysis, described above, indicates that EAT-16 and GOA-1 control avoidance behavior downstream or in parallel to EGL-10. Loss of *goa-1* or loss of *eat-16* function in the ASH neurons suppresses the avoidance defects of *egl-10* mutants whereas a reduction of *egl-30* function results in severe behavioral defects similar to those of *egl-10* mutants. Two lines of evidence are consistent with the hypothesis that, to control avoidance *egl-30* is functioning in ASH. One is that *egl-30* is expressed in the ASH neurons (BASTIANI ET AL., 2003) the other regards recent results demonstrating that, to control egg-laying behavior, both GOA-1 and EGL-30 function in the HSN motor neurons (Tanis et al., 2008).

The behavioral phenotypes of *goa-1(n363)*, *eat-16(ad702)* and *egl-30(n68sd)* single and double mutants are consistent either with their role in the generation and transduction of the signal or in its transmission. To determine whether these molecules have a role in ASH signal generation and transduction we measured stimulus-evoked Ca^{2+} transients in *goa-1 (n363)*, *eat-16 (ad702)* and *egl-30 (n68sd)* mutants. We found that in *eat-16* and *goa-1* mutants the calcium fluxes in response to 1M glycerol were indistinguishable from those of wild type whereas in *egl-30* mutant the Ca^{2+} transients were significantly reduced (Figure 19 a1-a3, b1-b3,c1-c3).

2.5.2) Functional analysis of the gene *qui-1*

2.5.2.1) Background information

qui-1 was identified as a gene involved in the avoidance response (Hilliard et al., 2004). The loss of function mutation *qui-1(gb404)* caused defective avoidance behavior (Hilliard et al., 2004). Studies conducted by Dr. Carmela Bergamasco in the laboratory, have revealed that *qui-1* encodes a member of a new class of RGS proteins and that the QUI-1 protein functions in the ASH neurons where it localizes to the sensory cilia.

2.5.2.2) qui-1 is necessary for the response to noxious stimuli and its function is required in ASH

We found that, as previously described (Hilliard et al., 2002), *qui-1(gb404)* animals were defective in their response to multiple aversive stimuli detected by the ASH polymodal sensory neurons including quinine, high osmolarity, and copper ions (data not shown).

We also confirmed previous results obtained in the lab regarding the function of QUI-1 in ASH. Behavioral analysis of *qui-1(gb404)* mutant animals expressing the cDNA of wild-type *qui-1* specifically in the ASH neurons confirmed that the expression of *qui-1* in these neurons is sufficient to rescue the behavioral defects of the *qui-1* mutants. These results suggest that, for the avoidance response, the function QUI-1 is required in ASH.

2.5.2.3) Stimulus-evoked Ca^{2+} transients in ASH are impaired in qui-1 mutants

The sensory and behavioral defects of *qui-1* mutants are compatible with a defect both in ASH sensory signaling or cilium development and morphology. To establish whether *qui-1* affects ASH development we stained ciliated sensory neuron with the lipophylic dye DiI. The ASH neurons of *qui-1* mutants stained normally with DiI, suggesting that the sensory neurons and their cilia were structurally and functionally intact (data not shown). Moreover localization of the QUI-1 protein at the tip of the sensory cilia, where signal transduction of stimuli occurs, suggests that QUI-1 functions in ASH primary sensory signaling or in the propagation of a Ca^{2+} signal from the cilium to the synaptic terminal.

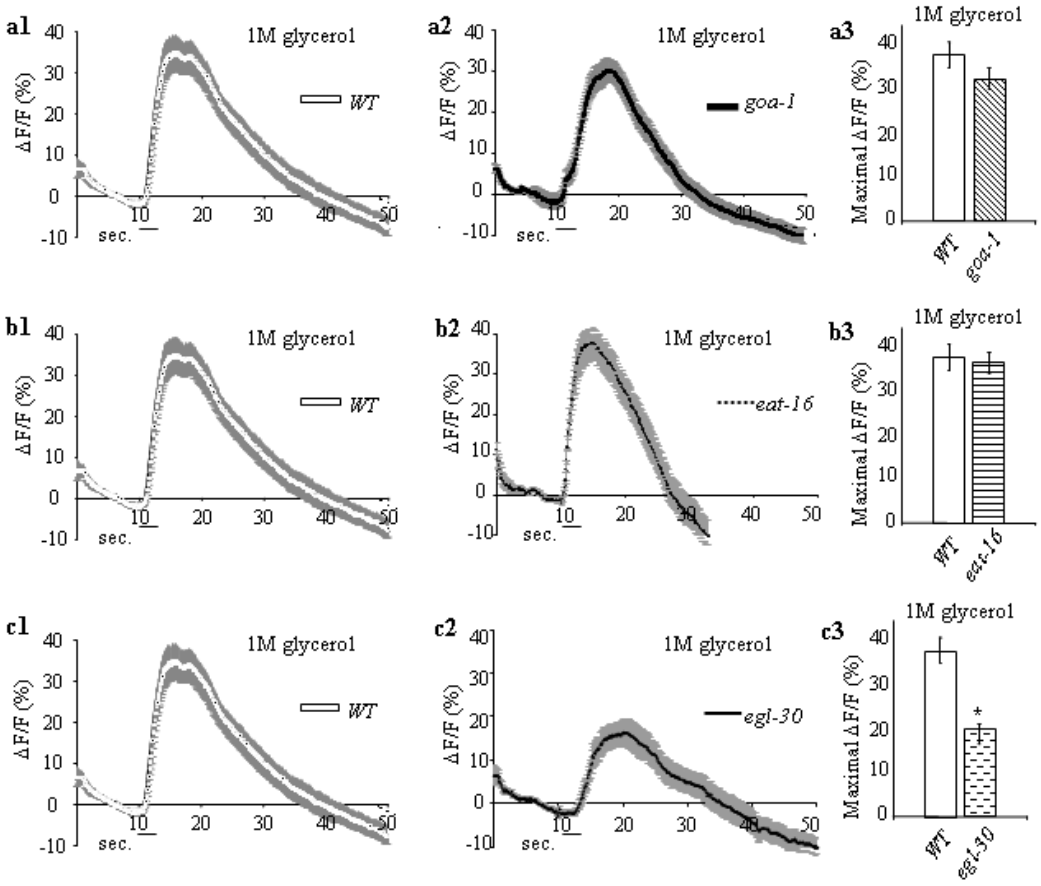


Figure 19. Stimulus-evoked Calcium transients in the ASH neurons are impaired in *egl-30(n68sd)* animals. Soluble stimuli were delivered to the nose of an adult animal for 3 s (black horizontal bar), and the change in GFP fluorescence intensities were recorded. (a1, b1 and c1) Average calcium transients in ASH neurons of *WT* animals in response to (a1) 1M Glycerol. (a2) Average calcium transients in ASH neurons of *goa-1(n363)* animals in response to 1M Glycerol. (b2) Average calcium transients in ASH neurons of *eat-16(ad702)* animals in response to 1M Glycerol. (c2) Average calcium transients in ASH neurons of *egl-30(n68sd)* animals in response to 1M Glycerol. The change in GFP fluorescence intensities are shown relative to averaged pre-stimulus baseline (ΔF/F), the grey band represents SEM. (a3, b3 and c3) The maximum ΔF/F of individual imaging trials with *WT* , goa-1, eat-16 and egl-30 animals. The bar represents the mean ± SEM. *different from wt $p<0.01$.

To ask directly whether QUI-1 affects primary signaling in ASH, we imaged, *in vivo*, stimulus-evoked Ca^{2+} transients using a transgenic strain expressing in ASH, under the *sra-6* promoter (kindly provided by C.I. Bargmann), the genetically encoded Ca^{2+} sensor, G-CaMP (Nakai et al., 2001). Ca^{2+} transients were measured in the ASH cell body as changes in background subtracted fluorescence intensity values relative to baseline (ΔF). We observed that Calcium fluxes evoked by high osmolarity, quinine and copper were strongly reduced or completely absent in *qui-1* mutants compared to wild-type animals (Figure 20 a1-a3, b1-b3 and c1-c3). These results directly demonstrate that QUI-1 is required in ASH sensory signal generation or transduction.

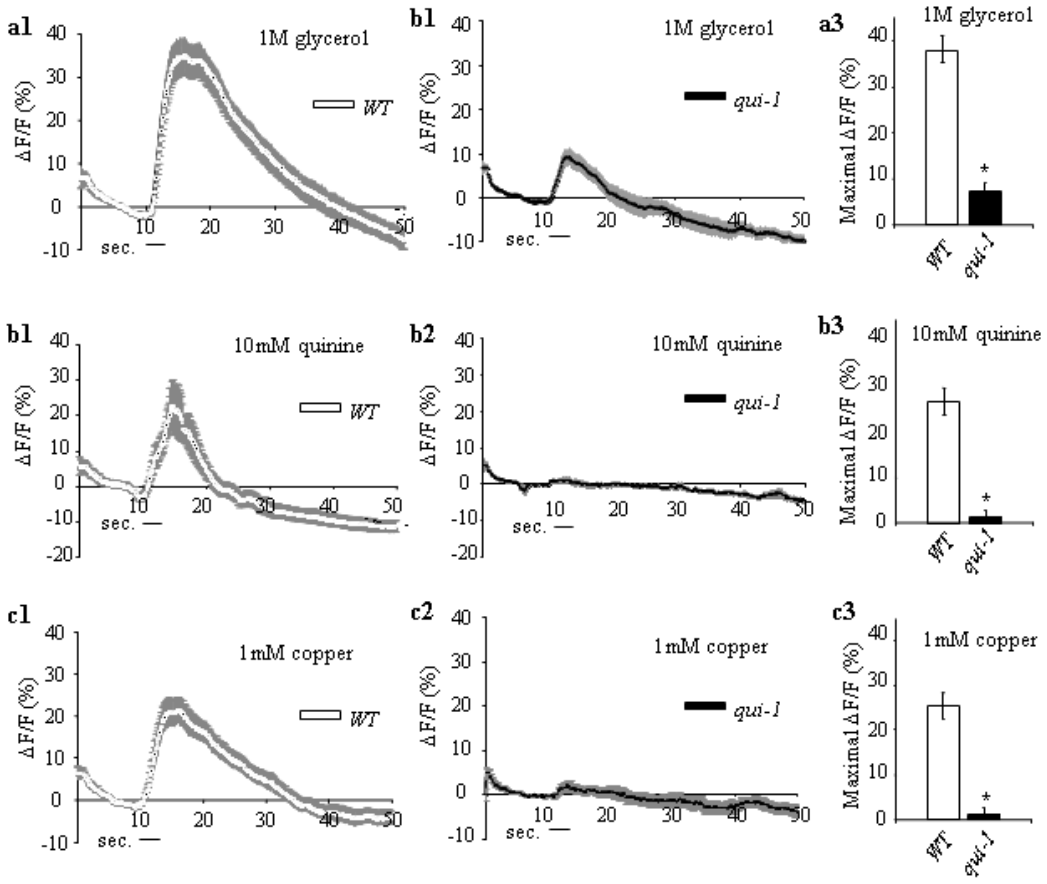


Figure 20. Stimulus-Evoked Calcium Transients in the ASH Neurons Are impaired in *qui-1(gb404)* animals. Soluble stimuli were delivered to the nose of an adult animal for 3 s (black horizontal bar), and the change in GFP fluorescence intensities were recorded.
(a1, b1 and c1) Average calcium transients in ASH neurons of *WT* animals in response to (a1) 1M Glycerol, (b1) 10mM quinine and (c1) 1mM copper ions. (a2, b2 and c2) Average calcium transients in ASH neurons of *qui-1(gb404)* animals in response to (a2) 1M Glycerol, (b2) 10mM quinine and (c2) 1mM copper ions. The change in GFP fluorescence intesities are shown relative to averaged pre-stimulus baseline (ΔF/F), the grey band represents SEM. (a3, b3 and c3) The maximum ΔF/F of individual imaging trials with *WT* and *egl*-10(*md176*) animals. The bar represents the mean ± SEM. *different from wt $p<0.01$.

3. DISCUSSION

3.1) Summary

Using a reverse genetic approach we found that *egl-10*, a gene encoding for a RGS protein, is involved in most ASH-mediated avoidance responses. Further analysis revealed that, for avoidance responses, the function of EGL-10 is required in the ASH neuron where it contrasts the function of GOA-1, a G_o alpha subunit that negatively regulates several *C.elegans* behaviors. We demonstrated that the GOA-1/EGL-10 pathway functions in ASH by acting not on signal generation and transduction, but on neurotransmitter release at the synapse. The G_q alpha subunit EGL-30 stimulates several *C.elegans* behaviors and we showed that, in ASH, it acts by contrasting the function of GOA-1. The RGS protein EAT-16 negatively regulates EGL-30 in many districts and we showed that it does so also in ASH. Our results suggest that, in this neuron, the EGL-30/EAT-16 pathway contrasts the GOA-1/EGL-10 pathway not by stimulating neurotransmitter release but by enhancing signal generation or transduction. Moreover, we found that the previously identified *qui-1* gene, enconding an RGS protein containing several WD40 domains (Hilliard et al., 2004), is required in ASH neurons for signal transduction in response to repellents. Finally we also developed a method to reduce the function of selected genes in chosen *C.elegans* neurons. Our RNAi-based method results in an efficient gene knock-down in sensory neurons and makes it possible to analyze the role in behavior of essential and ubiquitously expressed genes.

I will now discuss some aspects of our finding and their possible interpretation, including models of ASH functioning that can fit our data

3.2) RNAi sas

The goal of the method presented here is to determine the function of a gene in specific cells, including neurons. The procedure results in the efficient knock-down of gene function in chosen *C.elegans* neurons. The effect observed is cell autonomous and heritable. The method can also be usefully applied to ubiquitously expressed essential genes and possibly to other cell types. We do not know whether the reduction of gene function obtained is due to post-transcriptional or transcriptional interference and further investigations are necessary to establish this point. In any case, in using this approach, a safe assumption is to consider the gene knock-downs obtained more similar to hypomorphs than to null alleles. Since some phenotypes can be observed even when the reduction of gene function is limited while others require a much greater reduction, the choice of the promoter(s) to use and of the phenotype(s) to analyse is crucial. Different promoters should be tried until those with the appropriate combination of strength and cellular and temporal specificity are found. It should also be possible to further restrict the range of targeted neurons by combining,

for the sense and for the antisense strand, two promoters active in different but partially overlapping sets of cells. The procedure involved is such that trying various promoters is relatively easy and rapid and thus this approach is well suited for taking full advantage of various ongoing projects aimed at identifying large number of cell and time specific promoters in *C.elegans*. Finally, the potential relevance of this approach for the study of the function of genes in other cell types and of their role in other processes (e.g. development) is evident. Especially important is the fact that with this method it will be possible to reduce the function of essential genes in restricted number of neurons and study their effect on behavior, on development or on the survival of neurons.

3.3) *gpb-1* essential gene functions in ASH to control avoidance behavior

We used RNAi sas to study the function, in ASH neurons, of a ubiquitously expressed essential gene *gpb-1* encoding one of the two Gα subunits of *C. elegans.* ASH-mediated avoidance responses are known to involve several Gα subunits (Bergamasco and Bazzicalupo, 2006) but the role of *gpb-1* could not be easily tested by conventional genetics because of its lethality and ubiquitous expression. Animals in which *gpb-1(sas)* RNA was expressed in the sensory neurons, were defective for quinine and osmotic avoidance. Thus, using the RNAi sas method it has been possible to overcome the problem of *gpb-1* lethality and to obtain viable transformant lines in which the function of an essential gene has been reduced specifically in certain cells but not in others. In addiction these experiments have also provided new information on the function of *gpb-1*, in the avoidance sensory neurons, for proper response to repellent stimuli.

3.4) EGL-10 affects signal transmission of ASH nociceptive neuron

To better understand the physiological effect of mis-regulated G-protein coupled signalling in polymodal sensory neurons, we analysed the role of *C.elegans* EGL-10. EGL-10 is an RGS protein member of an evolutionarily conserved family of regulators of G protein signaling (Koelle and Horvitz, 1996). Eleven mammalian EGL-10 homologs that regulate many or all G protein signalling pathways have been identified (Koelle and Horvitz, 1996).

EGL-10 is expressed throughout the nervous system and localise exclusively to processes at the locations of the majority of the chemical synapses in the animals (Koelle and Horvitz, 1996). Previous studies in *C.elegans* reveal that EGl-10 function is required in motor neurons to control locomotion behaviours and in the HSN motor neurons to control egg-laying (Koelle and Horvitz, 1996). Loss of EGl-10 function results in defective egg-laying and locomotion behaviours. EGl-10 over-expression decreases acetylcholine release at the neuromuscular junctions (Miller et al., 1999). Our results provide, for the first time, evidence of a role of EGL-10 in sensory neurons. Molecular genetics manipulations, genetic and behavioural analysis reveals that loss of EGl-10 affects many

ASH-mediated avoidance responses and that, for this behavior, its function is required in ASH neurons. Loss of EGL-10 function does not alter ASH development and signal generation and transduction whereas it decreased neurotransmitter release at the ASH pre-synaptic terminal. This function of EGL-10 in ASH neurons is also supported by immunolocalization experiments carried out on *egl-10* mutant animals that express EGL-10 cDNA specifically in ASH. In these experiments the EGL-10 protein localized at the neural processes of ASH (Bergamasco C., unpublished data).

3.4.1) EGL-10 may function upstream of G_0 alpha subunit GOA-1.

$G_o$and G_q have opposing effect on egg-laying and other *C.elegans* behaviours apparently through opposing effect on neurotransmitter release (Lackner et al., 1999; Miller et al., 1999; Nurrish et al., 1999). Two opposite G-signalling pathways that involve GOA-1 (G_o alpha subunit) and EGL-30 (G_q alpha subunit) tightly regulated egg-laying rate (Chase et al., 2001). GOA-1 inhibits whereas EGL-30 stimulates egg-laying behaviour. The RGS proteins EGL-10 and EAT-16 negatively regulate GOA-1 and EGL-30 respectively (Hajdu-Cronin et al., 1999; Koelle and Horvitz, 1996).

Our results suggest that GOA-1 and EGL-30 pathways also control avoidance behavior. As expected loss of EGL-30 function disrupts many ASH-mediated responses. Loss of GOA-1 does not affect avoidance responses whereas loss of EAT-16 results in hypersensitivity to weaker aversive stimuli. In addition molecular genetic manipulations showed that EGL-10 and GOA-1 function both in the ASH neuron. Loss of GOA-1 can rescue behavioral defects of *egl-10* mutants suggesting that EGL-10 acts upstream or in parallel of GOA-1. Combined with evidence from biochemical experiments that EGL-10 interacts directly with GOA-1, these results support a molecular model in which EGL-10 may function as a negative regulator upstream of a GOA-1-mediated signalling pathway that inhibits neurotransmitter release, possibly in response to a modulator molecule (e.g. serotonin, neuropeptide) (Figure 21).

3.4.2) EGl-30 and EAT-16 may function in signal transduction of ASH

Genetic analysis reveals that also loss of EAT-16 function can rescues behavioral defects of *egl-10* mutants indicating that EAT-16 probably function in parallel to EGL-10 controlling negatively the opposite EGL-30-mediated pathway. Although previous results suggested that EAT-16 and EGL-30 control egg-laying behavior through regulation of neurotransmitter release in HSN motor neurons, we surprisingly found that calcium fluxes induced by high osmotic strength are reduced in *egl-30* mutants suggesting a role of EGL-30 in primary signal generation and transduction in ASH.

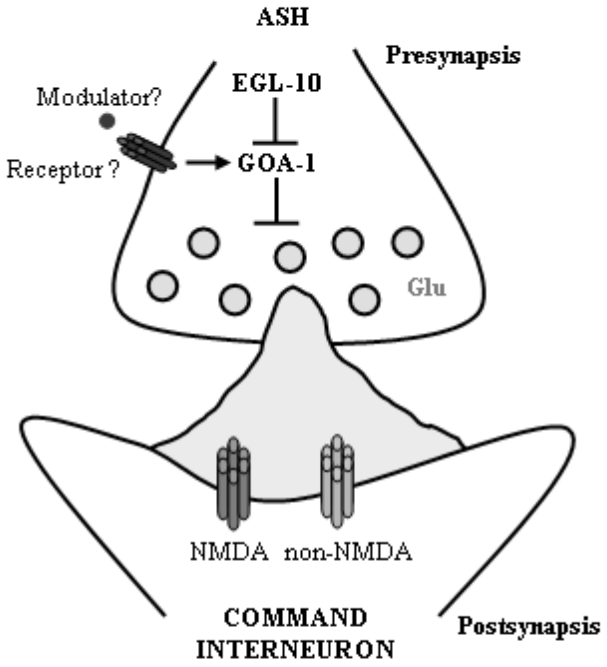


Figure 21. EGL-10 and GOA-1 regulate neurotransmitter release in ASH presynapsis. GOA-1 signaling inhibits neurotransmitter realease, possibly in response to the activation of a receptor by a modulator (e.g., neuropeptides, serotonin). EGL-10 negatively regulates GOA-1 activity dampening GOA-1 mediated signaling.

We think that EGL-30 may act downstream of a GPCR activating OSM-9 TRPV channel probably through phospho-lipase gamma (PLC) (Figure 22).

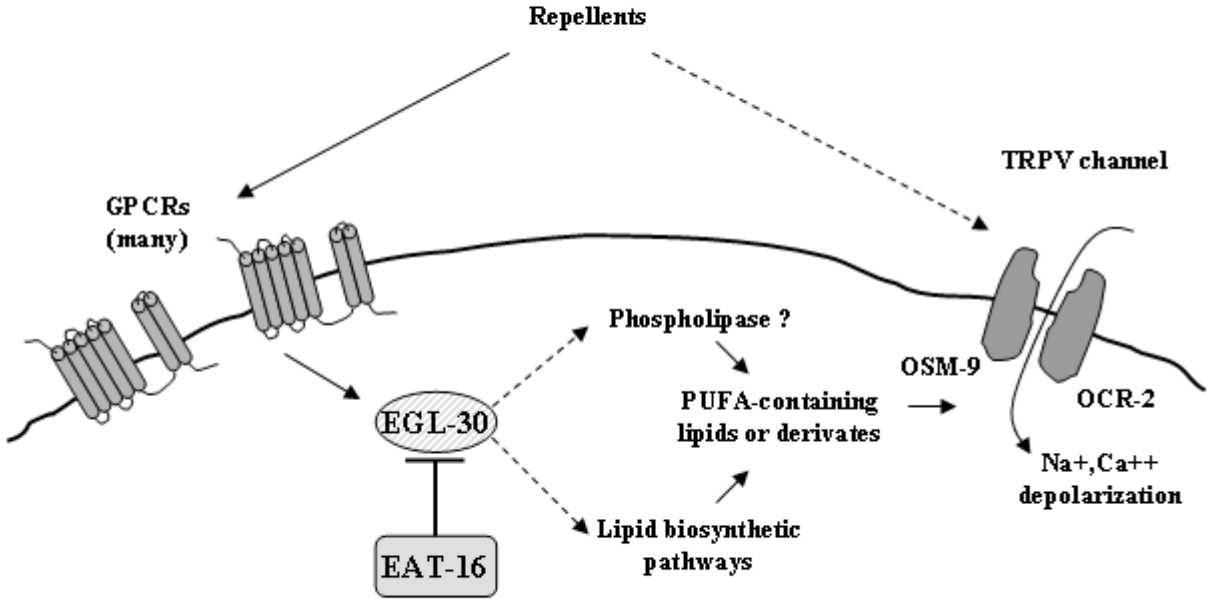


Figure 22. EGL-30 and EAT-16 regulate ASH signal transduction.
GPCR activates EGL-30 – mediated signaling, which regulate the production or consumption of phospholipids containing PUFAs. Lipid mobilization opens the TRPV channels encoded by OSM-9 and OCR-2 to depolarize the neuron. EAT-16 negatively regulates EGL-30 activity dampening EGL-30-mediated signaling.

Two lines of evidence support this model. First G_q and PLC activate a TRP channel (oppure TRP

channels) in Drosophila photoreceptors and in other contexts (Montell, 2005). Second, PLC, by hydrolizing lipids of the membrane, can produce Poly Unsaturated Fatty Acids (PUFA) that have been demonstrated to activate the TRP channel OSM-9 in *C.elegans* (Kahn-Kirby et al., 2004). We analyzed behaviorally loss of function mutants in four PLCs coding genes but none appeared to have a role in the avoidance. It is possible that another, not yet identified, PLC exists. Alternatively EGl-30 may activate multiple PLCs and thus mutation in single PLC genes did not result in avoidance behavior defects. In our model EAT-16 also acts in ASH signal generation and transduction regulating negatively EGL-30. A role of EAT-16 in signal transduction is supported also by recent experiments demonstrating that EAT-16 function is required in the AFD neuron for thermo-sensation signal transduction (Kuhara et al., 2008). On the basis of this model, as suggested by the hypersensitivity to weak aversive stimuli of *eat-16* mutants, loss of EAT-16 function can compensate the decreased neurotransmitter release of *egl-10* mutants through a sustained signal transduction. However our experiments do not allow us to exclude the possibility that EGL-30 and EAT-16 may also function in the regulation of neurotransmitter release.

3.5) QUI-1 affects primary signal generation and or transduction in ASH

Previous results demonstrated that ASH mediated avoidance responses require the *qui-1* gene. QUI-1 encodes an RGS protein with several WD40 domains (Hilliard et al., 2004). QUI-1 function is required in the ASH neurons and the protein localized at tip of the cilia where primary signal generation and transduction occurs (Bergamasco, unpublished data). Our results indicate that QUI-1 function is important in ASH for signal transduction. Indeed in *qui-1* null mutants ASH calcium fluxes evoked by many repellents are abolished. Several WD40 proteins are involved in Intra Flagellar Transport (IFT) and it has been proposed that QUI-1 is a component of IFT machinery (Burghoorn J., personal communication). Mutations in genes encoding for IFT components, in general, result in an alterated cilia structure. However our results indicate that cilia structure is normal in *qui-1* mutants. There are two possible explanations of these results. One is that QUI-1 functions in the ASH signal transduction as an RGS protein, thus modulating a G alpha signaling, and a second one is that QUI-1 functions as a necessary component of the IFT machinery for the localization to the ASH cilia of proteins important for signal transduction. Further experiments are needed to distinguish between these two possibilities.

4. MATERIALS AND METHODS

Strains

Wild-type animals were *C. elegans* variety Bristol strain N2. Alleles used in this work included *osm-10(n1602)III, osm-6(p811)V, dgk-1(nu62), dgk-2(gk124), dgk-3(gk110), plc-1(tm753), plc-2(ok1761), plc-4(ok1215), egl-8(md1971),goa-1(n363), egl-30(n686sd), egl-10(md176), eat-16(ad702), pkc-1(nj1), pkc-2(ok328), tpa-1(k530),* T25E12.4*(ok1704)* and were provided by the Caenorhabditis Genetics Center (CGC), which is funded by the NIH National Center for Research Resources (NCRR). A strain carrying an integrated transgene in which the *gpa-15* promoter is fused to the gfp gene Is[pgpa-15::gfp], was kindly provided by G. Jansen (Rotterdam, The Netherlands) , strains in which the *sra-6* promoter is fused to G-CaMP gene Is[p*sra-6*::g-camp] and to TRPV1 gene were kindly provided by C.I. Bargamnn. The name of the strains and the genotype of the transgenic lines indicated in the main text in Figures and in Tables were as follows:

p*srb-6*::*osm-10(sas)* (line #1) = [p*R09E10.7*::*gfp* p*srb-6*::*osm-10(sas)*]

p*srb-6*::*osm-10(sas)* (line #2) = [p*R09E10.7*::*gfp* p*srb-6*::*osm-10(sas)*]

p*str-1*::*osm-6(sas)* (line #1) = [p*R09E10.7*::*gfp* p*str-1*::*osm-6(sas)*]

p*str-1*::*osm-6(sas)* (line #2) = [p*R09E10.7*::*gfp* p*str-1*::*osm-6(sas)*]

p*srb-6*::*osm-6(sas)* (line #1) = [p*elt-2*::*gfp* p*srb-6*::*osm-6(sas)*]

p*srb-6*::*osm-6(sas)* (line #2) = [p*elt-2*::*gfp* p*srb-6*::*osm-6(sas)*]

p*srb-6*::*gpb-1(sas)* (line #1) [p*elt-2*::*gfp* p*srb-6*::*gpb-1(sas)*]

p*srb-6*::*gpb-1(sas)* (line #2) = [p*elt-2*::*gfp* p*srb-6*::*gpb-1(sas)*]

p*tph-1*:: *gpb-1(sas)* (line #1) = [p*elt-2*::*gfp* p*tph-1*:: *gpb-1(sas)*]

p*tph-1*:: *gpb-1(sas)* (line #2) = [p*elt-2*::*gfp* p*tph-1*:: *gpb-1(sas)*]

p*srb-6*::*gfp(sas)* (line #1) = Is[p*gpa-15*::*gfp*]; [p*elt-2*::*gfp* p*srb-6*::*gfp(sas)*]

p*srb-6*::*gfp(sas)* (line #2) = Is[p*gpa-15*::*gfp*]; [p*elt-2*::*gfp* p*srb-6*::*gfp(sas)*]

*egl-10;*p*sra-6::egl-10* (line #1) = *egl-10(md176);* Ex[p*elt-2::gfp* p*sra-6*::*egl-10(cDNA)*] ,

*egl-10;*p*sra-6::egl-10* (line #2) = *egl-10(md176);* Ex[p*elt-2::gfp* p*sra-6*::*egl-10(cDNA)*] ,

p*sra-6::TRPV1* = Is[p*elt-2::gfp* p*sra-6*::*TRPV1(cDNA)*] ,

*egl-10;*p*sra-6::TRPV1 = egl-10(md176);* Is[p*elt-2::gfp* p*sra-6*::*TRPV1(cDNA)*] ,

egl-10;p*sra-6*::*PTX = egl-10(md176);* Ex[p*elt-2::gfp* p*sra-6*::*ptx*] ,

egl-10;eat-16; p*sra-6::eat-16 = egl-10(md176);eat-16(ad702); Ex*[p*elt-2::gfp* p*sra-6*::*eat-16*] ,

The strains and the genotype of the transgenic lines generated in this work for *calcium imaging* are:

egl-10(md176); Is[p*sra-6*::*G-CaMP*],

goa1(n363); Is[p*sra-6*::*G-CaMP*],

eat-16(ad702); Is[p*sra-6*::*G-CaMP*],

egl-30(n686sd); Is[p*sra-6*::*G-CaMP*],

qui-1(gb404); Is[p*sra-6*::*G-CaMP*],

The strains and the genotype of the double mutants generated for genetic analysis are:

egl-10(md176);goa-1(n363),

egl-10(md176);eat-16(ad702).

Worms were grown under uncrowded conditions at 20°C on NGM agar plates seeded with *Escherichia coli* strain OP50.

Molecular Biology

DNA genomic extraction

For long PCR reactions up to 20 Kb, genomic DNA from wild type animals was extracted by the following protocol: Starved worms were washed and harvested from NGM plates; 1mL of lysis buffer (0.1M Tris-Cl pH 8.5, 0.1M NaCl, 50 mM EDTA pH 8.0, 1% SDS) with 20 mg/mL of Proteinase K was added to harvested worms and incubated at 62 °C for 1 hour. Three phenol/chloroform extractions were performed on the lysates and the DNA was precipitated with ethanol.

For short PCR reaction up to 5 Kb, genomic DNA was extracted by a simplified version of the method described above. 1-20 worms were washed and harvested from bacteria. 5 µL of lysis buffer solution with 20 mg/mL of Proteinase K was added to harvested worms and incubated at 62°C for 1 hour. Proteinase K was then inactivated by incubation for 15 min at 90°C and 5 µL of the lysate were directly used for PCR reactions.

PCR fusion

Construction of transgenes for cell specific knock-down were obtained by fusion of a cell specific promoter to exon rich regions of the genes to be silenced following the strategy depicted in Figure 10. Construction of transgenes for cell specific rescue were obtained by fusion of a cell specific promoter to genomic fragment containing the coding sequences region (CDS) or the cDNA of the gene studied following the strategy depicted in Figure 10 (A,B and D)

Three promoters were used in this work: p*srb-6*, p*str-1,* p*sra-6*.

For the *srb-6* promoter a 1,3 Kb fragment just upstream of the ATG was amplified using the following primers:

srb-6 Pf = AGTTTGGTCAGATCTTTGCC

srb-6 Pr = TTTTATTTCTTCTGTAGAAATTTCA

nested primer for the second step was:

srb-6 Pf* = GACGCATGACTTTCATTCTTTGCG

For the *str-1* promoter a 4,3 Kb fragment just upstream of the ATG was amplified using the following primers:

str-1 Pf = CAACATTTTCCTTTTTTATCATTGAAGG

str-1 Pr = TAGTCAAATGATATGAAGTTTGTGT

nested primer for the second step was:

*str-1*Pf* = GAAGTGCTTTATTATGGTATTTGG

For the *sra-6* promoter a 3 Kb fragment upstream of and including the ATG was amplified using the following primers:

sra-6 Pf = AGTGAGCATGAAGAAGGTAGAGGTTTTC

sra-6 Pr = GGCAAAATCTGAAATAATAAATATTAAATTCTGCG

nested primer for the second step was

sra-6 Pf* = CATGTTAGATAGTATGCTGCACTATAAGG

For the *tph-1* promoter a 1,7 Kb fragment upstream of and including the ATG was amplified using the following primers:

tph-1 Pf = GTCCACAAAATATGCCGATTCAC;

*tph-1*Pr = CATATTTTACCTGGAATTTAG

nested primer for the second step was

*tph-1*Pf* = CGTCTTAAGTTCTTGATATCTCTG

Because these promoters were used to drive transcription of different target genes, as depicted in Figure 10, the Pr series of primers had, at their 5' end, 25 additional nucleotides complementary to either one of the two extremities of the amplified target gene fragment. They are indicated in Figure 10 as Pra and Prs (Primer reverse antisense and sense, respectively). For the cell specific rescue experiment only the Prs primer were used.

Construction of transgenes for cell specific knock-down

Cell specific promoters were fused to exon rich regions of the genes *osm-10*, *osm-6*, *gpb-1* and *gfp*. For the *C. elegans* genes we amplified, from genomic DNA, the same exon rich region that was used as an insert for the *E. coli* plasmid library prepared to obtain RNAi by feeding (Ahringer, 2006). For *gfp* we amplified the insert from A. Fire plasmid pPD128.110.

Primers for the *osm-10* region (1,7 Kb) were:

osm-10 Tf = TAGCGGTGTTAAGAATGGGGCTTCA

osm-10 Tr = CGACAACTTCGATTATTTCCCG

osm-10 Tf* = GTGTTAAGAATGGGGCTTCA

osm-10 Tr* = AACACCGTAGTCAATACG

Primers for the *osm-6* region (2,2 Kb) were:

osm-6 Tf = AAGACATCATTTGGAACTGGAGAGG

osm-6 Tr = TTTCAATTGTGCCCACTAAAATCC

osm-6 Tf* = TCATTTGGAACTGGAGAGG

osm-6 Tr* = TTGTGCCCACTAAAATCC

Primers for the *gpb-1* region (2,1 Kb) were:

gpb-1 Tf = AATCAGCAAATGACACAACACTGGC

*gpb-1*Tr = CTCGGTGACTCCTAGACAAGAAACT

gpb-1 Tf* = GCAAATGACACAACACTGGC

gpb-1 Tr* = GACTCCTAGACAAGAAACT

Primers for the *gfp* coding region (890 bp) were:

gfp Tf = GGCCGATTCATTAATGCAG

gfp Tr = GTTGTAAAACGACGGCCAGT

gfp Tf* = TCACTATAGGGAGACCGGCA

gfp Tr* = TCACTATAGGGCGAATTGGG

Construction of transgenes for cell specific rescue

Cell specific promoters were fused to cDNA of the *egl-10* and *ptx* genes, and to genomic fragment containing *eat-16* gene. For *ptx,* we amplified the insert from plasmid pjT40 (kindly provided by Dr. Koelle, Yale University, New Haven, CT). For *egl-10* we amplified from cDNA (kindly provided by Dr. Koelle, Yale University, New Haven, CT) from 5µg total RNA using Superscript and protocol suggested by Invitrogen For the *eat-16* genes we amplified, from genomic DNA, the region containing *eat-16* gene.

Primers for the *egl-10* cDNA (3Kb) were:

egl-10 Tf = ATGGCTCTACCAAGATTGAG

egl-10 Tr = GTTCAGAGAAGACAATAGCAG

egl-10 Tr* = GAGCAAACAGTCTCAAAGATG

Primers for the *ptx* cDNA (1,5Kb) were:

ptx Tf = ATGGACGATCCTCCCGCCACC

ptx Tr = GCCGACTAGTAGGAAACAGT

ptx Tr* = CAGTTATGTTTGGTATATTGG

Primers for the *eat-16* genomic fragment (2,4Kb) were:

eat-16 Tf = ATGATGCCACCGTTGACCAAG

eat-16 Tr = ATTGAACATCAACGCCTACA

eat-16 Tr* = TTATGTAACAACTCCGGTTCTG

The conditions for PCR-fusion were as described [Hobert, 2002 #60]. The fragments for the sense and antisense expression of the target gene RNA were injected at 100 ng/μL each, together with 50 ng/μL of one of the selection markers: p*R09E10.7*::*gfp* which is expressed in vulval muscle and the canal cell (our unpublished data); p*elt-2*::*gfp* (pJM67), which is expressed in intestinal cells and is a gift from Jim McGhee (Calgary, Canada). Injection were performed according to standard *C. elegans* procedures and transformed animals were obtained at the usually observed frequencies.

Behavioral Assays

Well-fed, young adult animals were used for behavioral assays. Ring assays for osmotic and quinine avoidance were performed as described (Culotti and Russell, 1978) on NGM agar plates 6 cm in diameter. The ring shaped barrier was 8M Glycerol for osmotic avoidance and 50 mM quinine for quinine avoidance. Quinine was dissolved in a buffer containing 30 mM Tris-HCl pH7.0, 100 mM NaCl, 10m M KCl and 1.5% ethanol. Ten young adult animals were transferred to the center of a plate where 45 μL of repellent had been deposited in the shape of a ring (1.5 cm in diameter) and allowed to soak for 5 min. After 15 min, the animals that had remained within the ring were counted. The avoidance index (A.I.) was measured as number of animals within the ring out of total number of animals.

Drop tests for osmotic, quinine, copper and capsaicin avoidance were performed as described (Hilliard et al., 2002) on NGM agar plates 6 cm in diameter. A single drop of repellent (1M Glycerol, 10mM Quinine, 1mM Copper) was placed directly in front of the animal. At least 40 animals were assayed with three drops/each. The avoidance index (A.I.) is the number of positive responses divided by the total number of trials. The animal usually starts a backward motion within 1 second of the delivery of the drop. The response to each drop is scored as positive if the animal reacts within 3 seconds. To determine delay in the response to osmotic stimuli, we used an alternative assay to that described above (Hilliard et al., 2002). Capsaicin was dissolved in a buffer containing 30 mM Tris-HCl pH7.0, 100 mM NaCl, 10m M KCl and 1.5% ethanol. A young adult worm was transferred without food to an agar plate and allowed to recover for at least 2 min. A small drop of repellent (1M Glycerol or 50μM Capsaicin) was then placed in the path of the worm as it moved forward. The time interval (delay) between the initial contact with the solution the response (backward movement) was determined.

Nose touch response was performed as described (Chalfie and Sulston, 1981) on a very thin bacterial lawn (on food). Animals were assayed by laying a hair on the surface of the plate in front

of the animal. As an animal moves forward, it contacts the hair with the tip of the nose perpendicular to the direction of movement. Light touch response was performed as described (Chalfie and Sulston, 1981). The response was tested by stroking an animal with an eyelash at the posterior bulb of the pharynx. In both cases a trial was scored as a success when animals either halted forward locomotion or initiated backward movement following the stimulus. Each animal was usually subjected to 30 total trials, administered in three sets of 10 consecutive trials. The avoidance index (A.I.) was measured as number of positive responses out of total number of trials. Between sets of 10 trials, animals were given a rest of at least 10 min.

Nonanone avoidance was assayed essentially as described in (Troemel et al., 1997) except that round, 9 cm plates were used and the media contained agar at 2%. To calculate the avoidance index (A.I.), the number of animals in sectors close to the repellent minus the number of animals in sectors away from the repellent was divided by the sum of these numbers. The A.I. of wild-type animals approaches -1, that of animals not sensing the repellent approaches 0 and that of animals attracted by nonanone approaches +1.

Egg-laying was assayed as described in (Koelle and Horvitz, 1996). To test precisely staged adults, all assays were carried out on animals selected as late L4 larvae and grown at 20°C for 30 additional hrs at which time the eggs contained in the uterus were counted.

Dye-Filling

DiI (Molecular Probes) filling of amphid and phasmid sensory neurons was tested as previously described (Starich et al., 1995). Animals were washed twice with M9 buffer and several hundred animals were incubated in 0.5 mL of M9 buffer containing DiI 2 µg/L for 1 hour at room temperature. Animals were then washed once with M9 buffer and transferred to a NGM plate seeded with bacteria to chase excess staining. After 1 hr the animals were mounted on slides and viewed with fluorescence or confocal microscopy.

Microscopy

Worms were viewed using a Zeiss Axioskop equipped with epifluorescence and DIC, and images were collected with an Axiocam digital camera. The expression of GFP in animals transformed with the p*srb-6*::*gfp(sas)* transgenes was analyzed using a Leica TCS SP2 confocal microscope. The identification of sensory neurons expressing GFP was under DIC optics and was aided by staining with DiI.

***In vivo* Ca^{2+} Imaging and Data Analysis**

Young adult hermaphrodites with *sra-6::G-CaMP* expression were removed from plates with food and immediately glued with 2-octyl cyanoacrylate adhesive onto a chilled, hydrated 2% agarose pad

on a glass coverslip. The coverslip was attached with silicone speculate to a laminar flow chamber (Warner Instruments, RC-26GLP) perfused with saline buffer (NaCl 80 mM, KCl 5 mM, D-glucose 20 mM), at a rate of 1.0 ml/min.

Glycerol, quinine and copper were dissolved in saline buffer to final concentrations 1M, 10mM and 1mM respectively. Stimulants dissolved in saline buffer were delivered under light pressure through a borosilicate glass needle to the tip of the animal's head. Movement of the needle was controlled manually. Each imaging trial lasted for ≈40 s with the following temporal sequence: ≈10 s baseline, ≈3 s stimulation, ≈35 s recovery. To minimize neuronal adaptation from repeated stimulus exposure, the inter-trial interval was 3–5 min, and the stimulation needle was moved ≈1mm away from the animal. Animals were stimulated for a maximum of three trials and were healthy throughout the experiment.

Optical recordings were performed on a Leica TCS SP2 confocal microscope. Fluorescence images were acquired so as to be able to choose the region of interest (ROI) from which the photomultiplier data on the intensity of the G-CaMP fluorescnce could be calculated. Changes in fluorescence intensity over time were calculated by subtracting background intensity (i.e. averaged prestimulus baseline), $\Delta F/F$. Both average of incremental ratios over time and the maximal incremental ratios from each trial were calculated.

To minimize signal variations due to G-CaMP expression levels, only animals within a narrow window of expression were used. Noise artefacts such as movement and bleaching were minimal relative to signal.

Statistical Analysis

Mean, standard deviation and standard error of mean values were calculated for each data set. The statistical significance was determined using z statistic tests comparing each sample against the control. For egg-laying assays, the significance was determined using ANOVA analysis followed by Bonferroni tests for multiple comparisons of samples against control.

5. APPENDIX

5.1) *C. elegans* as an experimental system

Caenorhabditis elegans is a small free-living soil nematode found commonly in many parts of the world. It feeds primarily on bacteria and reproduces with a life cycle of about three days. One criterion for selecting a genetic organism is that it should be the simplest organism that has the traits of interest. Brenner thought that the simplicity of *C. elegans* would make the worm the metazoan equivalent to T4 phage studies (Jorgensen and Mango, 2002). A second issue in the selection of model organisms is the ease of manipulation. *C. elegans* is small transparent and can be grown either in small Petri dishes or in liquid culture if large numbers are required. It has a three-day generation time at room temperature, and strains can be kept as frozen stocks. Furthermore it is suitable for genetic analysis, has a small genome and it can be easily mutagenized generating viable worms, with a large variety of visible phenotypes (e.g. uncoordinated or rolling locomotion; small, long or dumpy bodies; blistered cuticles; twitching muscles; forked or bent heads).

An unusual feature of this nematode is that it can be maintained as a hermaphrodite (Figure A1), which means that an interesting mutant can be transferred to a fresh plate and, in three days, the self-progeny can be observed to see if the phenotype breeds true. However, males (Figure A1, bottom panel) are also produced and are essential for moving mutations between strains (Jorgensen and Mango, 2002).

Hermaphrodites and males are each about 1 mm in length but differ in appearance as adults. Hermaphrodites produce both oocytes and sperm and can reproduce by self-fertilization. The adult hermaphrodite has 959 somatic nuclei and the male 1031. Males, which arise spontaneously al low frequency, can fertilize hermaphrodites; hermaphrodites cannot fertilize each other.

C. elegans has the typical nematode body plan with an outer tube that consists of cuticle, hypodermis, neurons and muscles surrounding a pseudocoelomic space that contains intestine and gonads (Edwards and Wood, 1983). A basement membrane separates hypodermis from muscles. The shape of the worm is maintained by internal hydrostatic pressure.

The nervous system consists in the hermaphrodite of 302 neurons, which with the 56 glial and associated support cells account for the 37% of the somatic nuclei. In males the nervous system is more extensive with 381 neurons and 92 glial and supporting cells. Most of the cells of the nervous system are found in the space surrounding the pharynx, along the ventral midline, and in the tail. Processes from these neurons form an external ring around the pharynx (the nerve ring) or contribute to process bundles running the entire length of the body, the most prominent being the dorsal and ventral nerve cords.

C. elegans feeds through a bilobed pharynx that pumps food into the intestine, crushing it as it passes through the second lobe. The intestine connects to the anus near the tail. The simple excretory system is probably also responsible for osmoregulation. It consists of a pair of excretory canals, which are processes of a single cell that run the length of the animal, connecting to the exterior through the anteriorly located excretory pore.

The hermaphrodite reproductive system consists of a symmetrically arranged bilobed gonad, with one lobe extending anteriorly and the other posteriorly from the center of the animal. Each lobe is U-shaped, comprising a distal (to the uterus) ovary and a proximal oviduct and spermatheca (Figure A1). The two lobes are linked in correspondence of the vulva, which is also the exterior opening of the uterus; the vulva opens visibly on the ventral surface of the adult.

The male gonad is a single lobed, U-shaped structure, extending anteriorly from its distal end and then looping posteriorly and connecting with the cloaca near the tail. The male copulatory organ is the tail: it is fan-shaped, with nine sensory rays for each side and is endowed with two spicules that are used during copulation (Emmons, 1997).

The worm has many of the tissues and organs of more complex animals (such as muscles, nervous system, gonad, epidermis and gastrointestinal tract), but each is radically simplified.

C. elegans is the first metazoan where the sequence of the entire genome has been determined (The C.elegans Sequencing Consortium, 1998).

In addition all the information on the genome, EST libraries, Microarrays experiments, wide range screenings are easy to consult on the web (http://wormbase.org).

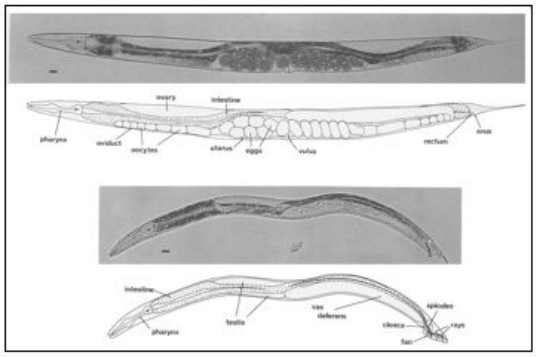


Figure A1. Photomicrographs and schematic drawing showing major anatomical features of C.elegans adult hermaphrodite (above) and male (below). Lateral views, anterior is to the left. Bar represents 20 μm (adapted from (Sulston and Horvitz, 1977)).

5.2) Life Cycle

The life cycle of *C. elegans* lasts three days and consists, after hatching, of four larval and an adult stage. Mature oocytes pass trough the spermatheca and become fertilized, either by the

hermaphrodite's own sperm or by male sperm; the latter are introduced into the uterus during mating and stored in the spermatheca. Within 30 minutes after fertilization, the zygote develops a chitinous shell and a vitelline membrane that enable it to survive outside the uterus. Normally eggs are laid three hours after fertilization, during gastrulation.

The embryo develops through a series of invariant cell divisions that occur during the first 5 h of embryonic development at 25 °C. After about 14 h of development in the egg case, the larva hatches from the eggshell. At this stage his length is 250 μm and the hermaphrodite consists of 558 cells. The animal then passes through four larval stages (L1–L4) that are separated by periods of lethargy, during which the animal sheds its old cuticle (Figure A2). Under crowded conditions and with limited food, the L1 larvae can enter an alternative developmental program called the dauer stage, in which the animal does not feed, is resistant to desiccation and can survive for months under harsh conditions. If food becomes available during this period, the dauer larva moults to become an L4, which resumes normal development. L4 larvae moult into the sexually mature adults, which will reproduce. In standard condition, with no food restriction during life cycle, worms live for about 17 days after reaching adulthood.

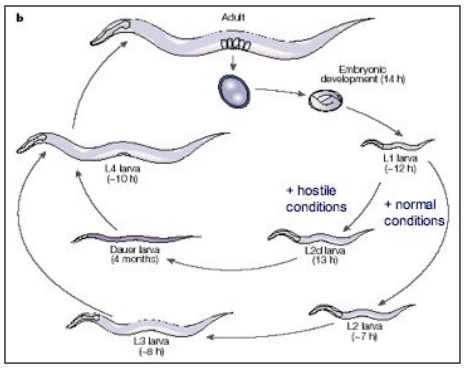


Figure A2. Life cycle of *C. elegans* at 25°C. After about 14 h of development in the egg case, the larva hatches from the eggshell. The animal then passes through four larval stages (L1–L4). Under crowded conditions and with limited food, the L1 larvae can enter an alternative developmental programme called the dauer stage, in which the animal do not feed, is resistant to desiccation and can survive for months under harsh conditions. If food becomes available during this period, the dauer larva molts to become an L4, which resumes normal development. L4 larvae molts into the sexually mature adult which will reproduce. (adapted from (Jorgensen and Mango, 2002)).

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