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Caenorhabditis elegans Neuronal Calcium Sensor-1: From Gene To Behavior

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Introduction

As the subject of this thesis is the characterization and the analysis of Neuronal Calcium Sensor-1 protein in the *Caenorhabditis elegans*, I will first present an overview of: Ca^{2+} signaling, calcium binding proteins, neuronal calcium sensors, and of the nematode *Caenorhabditis elegans*.

Ca^{2+} Signaling

Ca^{2+} plays a critical role in many cellular processes including muscle contraction[1], neuronal plasticity[2], secretion, cell cycle, differentiation[3], apoptosis[4], and gene transcription[5]. It has been shown to be a key second messenger in a variety of regulatory and signaling processes.
The intracellular concentration of Ca^{2+} is tightly controlled. The level of free Ca^{2+} in non-stimulated cells is maintained at about 10-100nM. During stimulation, the level of Ca^{2+} can undergoes rapid and elevated changes (its concentration can reach several µM) frequently associated with complex temporal and spatial patterns. The elevated Ca^{2+} level will trigger the cell response to the stimuli and allow Ca^{2+} to function as an intracellular second messenger.
Eucaryotic cells maintain very low concentrations of free Ca^{2+} in their cytosol (10-100nM) whereas the extracellular Ca^{2+} concentration is much higher (1mM, 10 000-fold higher than in the cytosol). The gradient is maintained by Ca^{2+} extrusion from the cell and/or by its accumulation in intracellular Ca^{2+} stores. The large gradient tends to drive Ca^{2+} entry into the cytosol from both extracellular medium and internal stores. A stimulus can transiently opens Ca^{2+} channel present either in the plasma membrane or in intracellular Ca^{2+} store membranes, allowing the calcium to rush into the cytosol, dramatically and rapidly increasing the local Ca^{2+} concentration, and triggering the cell response.
Therefore, to use Ca^{2+} as an intracellular signal, cells must keep resting cytosolic Ca^{2+} levels low to take advantage of the gradient as a driving force to generate rapid Ca^{2+} level changes.

Ca^{2+} homeostasis

The low concentration of intracellular Ca^{2+} is maintained by a complex homeostatic mechanism[6, 7]. In the plasma membrane Ca^{2+} pumps actively transport Ca^{2+} out of the cell. Inside the cell, intracellular Ca^{2+} stores such as the Sarcoplasmic Reticulum (SR) in muscle cells and the Endoplasmic Reticulum (ER) in non-muscle cells can accumulate Ca^{2+}, thus lowering the cytosolic Ca^{2+} levels (see fig. 1).

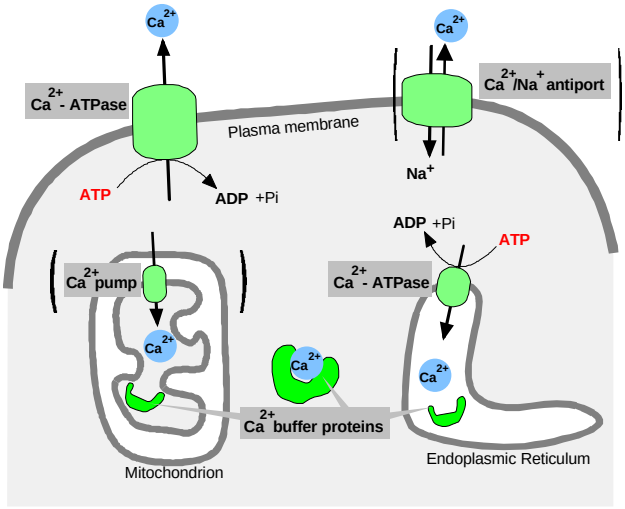


fig. 1.
Calcium homeostasis

All eucaryotic cells have a Ca^{2+}-ATPase in their plasma membrane that uses the energy of ATP hydrolysis to pump Ca^{2+} out of the cytosol. Cells which make extensive use of Ca^{2+} signaling such as muscle or nerve cells, have an additional Ca^{2+} pump in their plasma

membrane that couples the efflux of Ca^{2+} to the influx of Na^{+} (Na^{+}/Ca^{2+} antiport or exchanger). Due to its low affinity for Ca^{2+}, the Na^{+}/Ca^{2+} exchanger,operate only when cytosolic Ca^{2+} levels rise above 1µM (10 times the normal level), as it occurs after repeated muscle or nerve stimulation. In the ER membrane, a Ca^{2+}-ATPase pump also plays an important role in keeping the cytosolic Ca^{2+} concentration low: it enables the ER to take up and store the cytosolic Ca^{2+}. In the cytosol there are also several Calcium-Binding Proteins that buffers the free Ca^{2+} concentration.

Normally, even during stimulation, global Ca^{2+} levels never go beyond 5µM, but when a cell is damaged and cannot pump Ca^{2+} out of the cytosol efficiently, the free Ca^{2+} concentration can reach toxic level (>10^{-5}M). In this extrem situation a low-affinity, high capacity Ca^{2+} pump present in the inner mitochondrial membrane will take up excess Ca^{2+} from the cytosol using as a driving force the electrochemical gradient (generated during the oxidative phosphorylation) across this membrane.

Ca^{2+} as an intracellular messenger

Hormones, neurotransmitters, photons, odorants, or electrical activity can modulate the level of calcium within cells, either through the release of calcium from internal stores or via influx from outside the cell across the plasma membrane.

Two main pathways for Ca^{2+} signaling have been well defined: one present mainly in electrically excitable cells and the other in the majority of eucaryotic cells (see fig. 2).

In the first pathway (A), an electrical signal triggers the opening of voltage-gated Ca^{2+} channels in the plasma membrane allowing Ca^{2+} to rapidly enter the cytosol. In the second pathway (B), the binding of extracellular signaling molecules to surface receptors causes the release of Ca^{2+} from the ER in the cytosol through the IP_3 cascade, via IP_3-gated Ca^{2+} channels in the ER membrane.

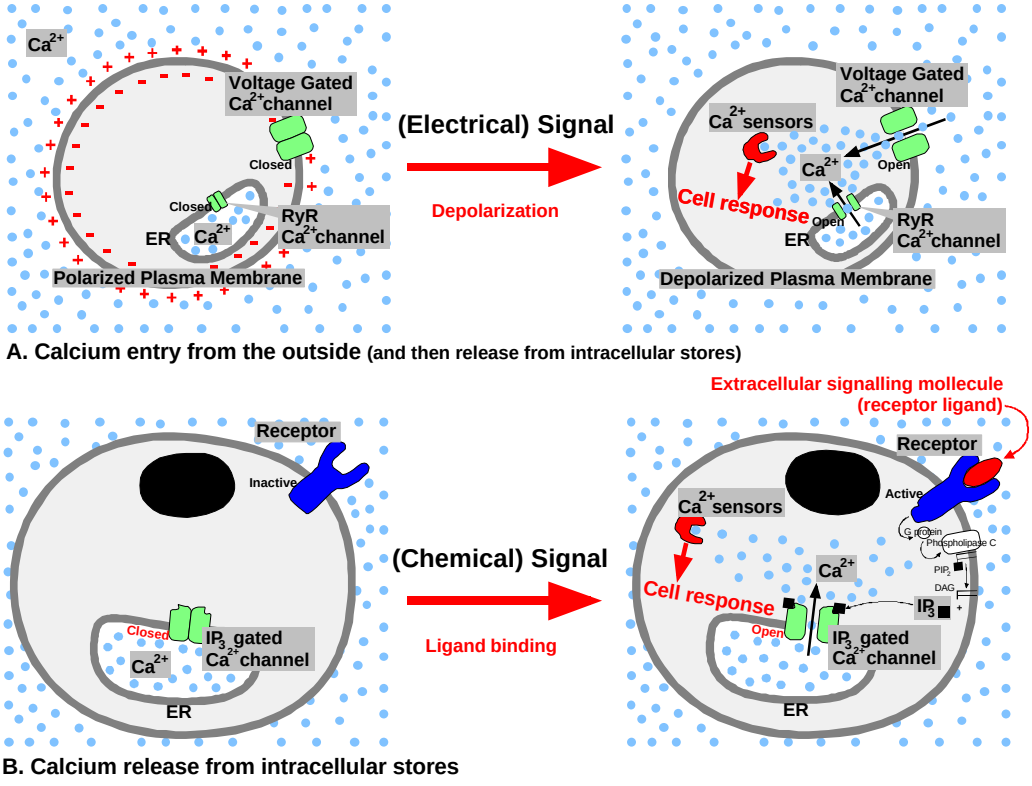


fig. 2.
Two common pathways for the entry of Ca^{2+} into the cytosol.

The IP_3 cascade[3] (fig. 2B) starts with the binding of a signaling molecule to some type of G-protein-linked receptor in the plasma membrane. The activated receptor stimulates a trimeric G protein called Gq, which in turn will activate a phosphoinositide-specific

phospholipase C named phospholipase C-β[8]. This enzyme will cleave the phosphatidylinositol-bisphosphate (PIP_2, a minor phospholipid in the plasma membrane) in inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3, a small water-soluble molecule will leave the membrane and diffuse through the cytosol to the ER where it can reach IP_3 stimulated Ca^{2+} channels (or IP_3 receptor, IP_3R)[9, 10]. These Ca^{2+} channels can then open, allowing the release of Ca^{2+} from the ER through the cytosol.

In skeletal muscle cell depolarization triggers a conformational change in a voltage sensor (dihydro-pyridin receptor; DHPR) interacting with a nearby ryanodine receptor (RyR, see just after) in the SR membrane. This conformational change in the DHPR will in turn (directly) promote the opening of the RyR allowing the efflux of the SR stored Ca^{2+} into the cytosol. In other electrically excitable cells (neurons, smooth/cardiac muscles), an electrical signal (depolarization) generally triggers the opening of voltage operated Ca^{2+} channels[11] (VOC) in the plasma membrane allowing the Ca^{2+} to enter the cytosol from the outside (fig. 2A). The Ca^{2+} will usually diffuse through the cytosol to the ER where it will stimulate the opening of RyR[12, 13] and/or IP_3R.

Ca^{2+} can also enter the cells from the outside by ligand gated calcium channels (receptor operated channels; ROC) and then may also activate RyR and/or IP_3R.

The ryanodine receptors (RyR)[12, 13] on the surface of the ER are Ca^{2+} stimulated / putative cyclic ADP-ribose stimulated Ca^{2+} channels (cADPR, a newly described cellular messenger promoting Ca^{2+} mobilization[14]). The locally released Ca^{2+} will stimulate these channels to release still more Ca^{2+} from the SR/ER into the cytosol (positive feedback). This phenomenon is called a Ca^{2+} induced release of Ca^{2+} (CICR). The IP_3 Receptor (IP_3R) is also influenced by Ca^{2+} in the same way. CICR is autocatalytic, meaning that a small local Ca^{2+} level increase could be amplified. Moreover the release of Ca^{2+} from one of these receptors could facilitate the opening of nearby receptors, propagating spatially the Ca^{2+} signal [15](waves, see below).

IP_3R and RyR are therefore under (at least) dual agonist control where IP_3/cADPR and Ca^{2+} itself act in synergy (the term “stimulated” is therefore more appropriated than “gated” to define their behavior toward these agonists). Moreover Ca^{2+} stimulate Ca^{2+} release, but high local Ca^{2+} concentrations are inhibitory (negative feedback), limiting in time Ca^{2+} release through a single store receptor (internal “off” switch). In many cells, including muscle and some neurons, both type of Ca^{2+} store receptors/channels are present allowing the generation of complex calcium signal patterns.

The intensity of the stimulus appears to determine the extend of the Ca^{2+} release (graded response). Fundamental release events come from the opening of single intracellular channels to give “blips” (from IP_3R) or “quarks” (RyR). Elementary events, named “puffs” and “sparks”, result from the concerted opening of small groups of IP_3R or RyR respectively, defining Ca^{2+} microdomains. Coordination (summation) and propagation by CICR of these elementary events can give rise to global Ca^{2+} signals. These events[16] seem to be elicited respectively by (null/)low, intermediate, high intensity stimuli. Calcium release can be sometime restricted to a small sub-cellular region even in the presence of a strong stimulus, probably by the availability of Ca^{2+}-stores and store receptors as well as by the presence of calcium buffer proteins preventing Ca^{2+} propagation.

To summary, Ca^{2+} channels both on the plasma membrane and on intracellular stores can generate localized Ca^{2+} pulses which can give rise to spatially restricted Ca^{2+} signals, or when coordinated/amplified, to global Ca^{2+} signals. Both type of signals can show complex spatio-temporal patterns (oscillations, waves; see below)**.**

Once the stimulation is over, the release of Ca^{2+} from internal store stops (and CICR is overcome by the negative feedback effect of Ca^{2+}). The remaining "excess" of cytosolic Ca^{2+} is pumped out of the cell and also pumped in the internal Ca^{2+} stores by the constituvely active "homeostatic" Ca^{2+} pumps (which can even be stimulated, in some cases, indirectly by the increase in cytosolic Ca^{2+} concentration).

Calcium oscillations/ waves:

Tonic Ca^{2+} level increases do not occur in many cells. Instead the initial transient increase in Ca^{2+} is often followed by Ca^{2+} level oscillations or "spikes"[15, 17], lasting for a few seconds to several minutes, as long as the cell surface receptors are activated. Ca^{2+} transient can also propagate across the cell in the form of calcium waves. Calcium level increase can show therefore complex temporal and spatial patterns. Calcium waves can even pass from cell to cell through gap junctions. The mechanisms responsible for the generation (and the propagation) of these oscillations are not well known, but these phenomenon probably depend on both Ca^{2+} positive feedback (CIRC, see above) and negative feedback. The waves may encode information in their frequency (giving a frequency dependent cellular response)[18]. Their frequency (but not their amplitude) can often depend on the concentration of extracellular signaling ligand. This will convert an initial amplitude signal into a frequency internal signal avoiding the toxic effects of a sustained rise in cytosolic Ca^{2+} with a highly concentrated signal ligand. These phenomenon are observed for example in hormonal stimulation of many nonexcitable cell types (e.g. vasopressin-induced Ca^{2+} oscillations in liver)[19].
Sometimes cytosolic calcium oscillations are observed without extracellular signaling molecules: for example, developing neurons can generate spontaneous Ca^{2+} transients which seems to implement an intrinsic developmental program[20, 21]. These spontaneous oscillations occur also with the repetitive Ca^{2+} spikes in the beating heart[22]. These phenomena probably arise from the amplification of spontaneous elementary Ca^{2+} release from IP_3R and RyR Ca^{2+} channels, generating a sort of internal oscillator[15].

Intracellular calcium store refilling (capacitive calcium entry)

When an internal calcium store is empty there is a depletion information promoting the refilling of this store. A putative calcium depletion signal (calcium influx factor; CIF, which is still unknown), may trigger the opening of specialized Ca^{2+} channels[23, 24] (store operated channels; SOC) in the plasma membrane, generally near the internal store. Alternatively the depletion information may be transmitted directly from IP_3R, which are store state sensitive[25], to the SOC. The Ca^{2+} will enter the cytosol through these channels, and then will be pumped into the calcium store.

Stimulus desensitization

In case of a prolonged “monotone” stimulus there is phenomenon called **adaptation or desensitization** in which the cell response will decrease despite the presence of the stimulus. In this way cells can reversibly adjust their sensitivity to the stimuli. Adaptation is achieved through a delayed negative feedback allowing the cells to respond to changes in the intensity (concentration) of stimuli rather than to their absolute intensity. Adaptation involves generally receptor phosphorylation[26] (rapid), and/or internalization[27] (slow) in which the Ca^{2+} also play a role. Long term adaptation can even involve changes in gene expression also induced by Ca^{2+} (see below).

Ca^{2+} in the nucleus

Ca^{2+} ion can freely diffuse to the nucleus, where it can elicit profound changes in gene expression[5, 28] giving rise to long term Ca^{2+} effect. These changes are thought to involve Ca^{2+} dependent phosphorylation of specific transcription factors that regulate expression of Ca^{2+}-responsive genes. Calcium effectors such as calmodulin (CaM, see later) as well as CaM kinases are found in the nucleus[29], moreover, some cytosolic activated CaM kinases can translocate to the nucleus, together transmitting the calcium signal to the nucleus.
For example the transcription factor CREB can be phosphorylated (at Ser^{133}) in response to a Ca^{2+} level increase, activating the transcription of the somatostatin gene[30, 31].

Alteration of Ca^{2+} metabolism

Disturbance of calcium homeostasis[32] (including mutation in Ca^{2+} binding proteins) has been found in several degenerative disorders of the central nervous[33] system such as Alzheimer's and Parkinson's diseases and in other disorders[34] like hypertension, familial hypercholesterolemia. Some cancer cells have been reported to have elevated levels of cytosolic Ca^{2+} that can be (partially) correlated with their elevated, "permanent" cellular activity (division, motility/invasiveness). In all these cases it is difficult to assert if the Ca^{2+} metabolism alterations are (part of) the cause or a consequence of the disease.
Nevertheless cytosolic Ca^{2+} is a crucial second messenger whose concentration must be tightly controlled to allow rapid and precise generation of (complex) signals. Any defect in Ca^{2+} "metabolism" could lead to aberrant cell response, cell over or under responsiveness and in case of cytosolic Ca^{2+} overload to cell death, as high calcium levels are toxic, over activating (Ca^{2+} dependent) degradative enzymes (proteases, lipases) leading to necrosis/ apoptosis and precipitating with the cellular phosphates.

Calcium binding proteins

The intermediary between the modification of intracellular Ca^{2+} concentration in response to a stimulus and the physiological response are some type of Calcium-Binding Protein (CaBP). They are either calcium dependent enzymes, channels, or calcium dependent modulating proteins. Other CaBP serve as Ca^{2+} buffers and do not participate in signal transduction.
Therefore, CaBP are **functionally** divided in two categories:

Calcium Sensors: calcium dependent **enzymes** (e.g. kinases, phosphatases, lipases, proteases), **channels** (e.g. Ca^{2+} gated channels, IP_3R, RyR) or calcium dependent **modulators** (activators, inhibitors) of the activity of target proteins (like calmodulin, troponin C). Calcium sensors are proteins that will transmit/ transduce the Ca^{2+} signal to other cellular components. Ca^{2+} binding to sensors is generally characterized by a fast kinetic and a relatively low affinity. Moreover with modulators, Ca^{2+} induces a conformational change resulting in the exposure of hydrophobic surface(s), allowing thereby interactions with other target proteins (ex: [35]).
Ca^{2+} sensors are characterized by an affinity for Ca^{2+} (K_D) in the range of 0.001µM – 1µM nicely poising to bind physiological Ca^{2+}.

Calcium Buffers: Ca^{2+} buffering proteins in the cytosol and in the intracellular Ca^{2+} stores (e.g. parvalbumin, calsequestrin). They help to "stabilized" Ca^{2+} concentration, and so play a role in the Ca^{2+} homeostasis (see fig. 1). Binding of Ca^{2+} to buffers proteins is generally characterized by a slow kinetic and a relatively high affinity (and capacity). Upon calcium binding they generally show no exposure of hydrophobic surfaces, and do not interact with target proteins. The buffer proteins play however an important role stabilizing Ca^{2+} concentration, allowing proper Ca^{2+} signal generation, and protecting cell from Ca^{2+} overload.

To bind Ca^{2+}, proteins possess different Ca^{2+} binding site structures (see below). The same Ca^{2+} binding motif can often be found, as well in sensor as in buffer proteins so there is no strict correlation between site structure and sensor/buffer partition.

The number of CaBP whose functions are known, such as calmodulin, troponin-C[36], calpain[37], Protein Kinase C(α,β,γ)[38], are far outnumbered by those whose roles are unknown, or elusive.

Different types of CaBP

On the basis of their cellular localizations and of the primary structure of their Ca^{2+} binding CaBP have been classified into different types:

Table 1. CaBP Classification

♦ **Extracellular CaBP**, where (generally) Ca^{2+} is a stabilizing ligand needed for proper folding, constitutively present in the protein. Ex: some proteases, phospholipases, amylases, clotting factors, and growth hormones.

♦ **Intracellular CaBP**,
Can be divided in 2 sub-types:

- **Cytosolic CaBP**, which can be divided in several groups depending on their calcium binding site structure:
  - CaBP with **EF-hand** Ca^{2+} binding motif (30 residues).
    A very large family, with a high affinity for Ca^{2+} and a high evolutionary diversity[39, 40]. They play versatile roles in Ca^{2+}-mediated cellular events and are mainly located within the cytoplasm and the nucleus although a few have been demonstrated to interact with membrane lipids when myristoylated (for references, see later).
    Ex: calmodulin, troponin C, recoverin, parvalbumin, calpain, ncs-1

  Ca^{2+}/ phospholipid-binding proteins:
  - CaBP with the **annexin fold** Ca^{2+} / phospholipids binding motif (80 residues).
    Also called the annexin proteins[41]. They have been suggested to mediate membrane fusion and to be involved in the control of cell proliferation and differentiation. Generally, they are localized in the cytoplasm and become translocated to the plasma membrane in response to an increase in the cytosolic Ca^{2+} concentration.
    Ex: lipocortin, calpactin, synexin.
  - CaBP with the **C2 key** Ca^{2+} / phospholipids binding motif (120 residues).
    The C2 region[42] was found originally in (some) Protein Kinase C (PKC) isoforms. The physiological functions of C2 proteins are relatively well elucidated[38, 43, 44] as compared with EF-hand or annexin proteins.
    They often show Ca^{2+} induced translocation from the cytosol to membrane (like the annexins), and interaction with common cellular receptors.
    Ex: PKC (α,β,γ), phospholipase C, synaptotagmins.
  - CaBP with **other** motifs
    - Some Ca^{2+}/ actin binding proteins that sever the actin microfilament show common structural motifs. ("**severin** motif"[45])
      Ex: severin, vilin.
    - We can also consider Ca^{2+} gated channels as CaBP, but their Ca^{2+} binding domains are not defined and their is a possibility that their sensibility to Ca^{2+} is mediated by intermediary proteins.
      Ex: Ca^{2+}-gated K^+ channels, IP_3R, RyR (see above).
    - Other unknown ?...
- **Intraorganellic CaBP**, in ER and mitochondria. **Calcium storage proteins.** They buffer the concentration of Ca^{2+}. They have no defined Ca^{2+} binding structural pattern and in general a low affinity and high capacity for Ca^{2+} binding.
  (few EF-hand CaBP have also been identified in the ER: e.g. ERC-55)
  Ex: calsequestrin, calreticulin[46].

So far, there is only one report of a protein containing two distinct Ca^{2+}-binding domain types: PLCγ that has the C2 domain and an EF-hand[47].

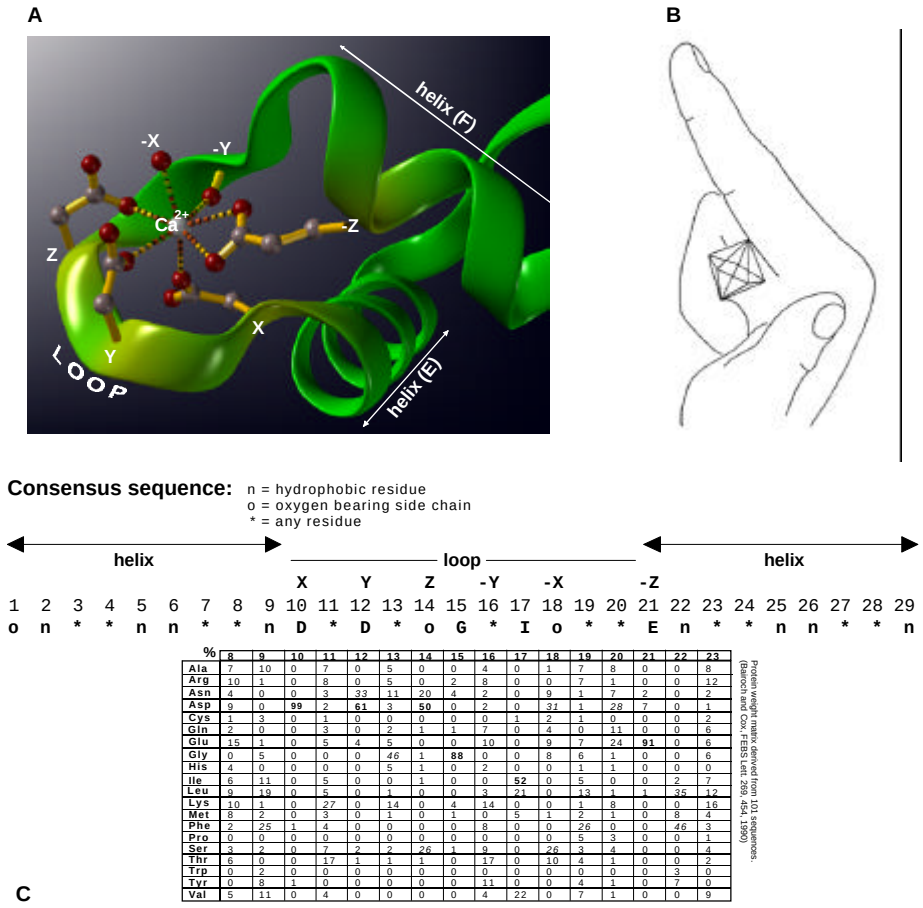
The calcium binding proteins with EF-hands represent the largest family of CaBP. *Caenorhabditis elegans*-Neuronal Calcium Sensor-1 (*Ce*-NCS-1), the object of this study, belong to this EF-hand CaBP family. Below I will present only EF-hand calcium binding proteins and then introduce neuronal EF-hand calcium sensors.

EF-hand calcium binding proteins

The superfamily of EF-hand helix-loop-helix Ca^{2+}-binding proteins[39] represents a very large group of proteins sharing a common structural motif, which consists of a Ca^{2+}-binding loop flanked by 2 α-helices. This motifs allows rapid and reversible binding of Ca^{2+}, with dissociation constants in the Ca^{2+} physiological range (0.01-10μM).
The EF-Hand superfamily can be divided in 39 subfamilies, comprising at the present times more than 250 proteins.
EF-hand CaBP are found in all eucaryotes cells. Generally they are localized in the cytosol (in few cases associated with membranes), although some are found within organelles and nucleus. In multicellular organisms, some are present ubiquitously (like calmodulin) or (more) restricted to specific cells (like recoverin in photoreceptors or troponin-C in skeletal and cardiac muscle).
For the vast majority of EF-hand CaBP, no specific function has been elucidated

The EF-hand Ca^{2+}-binding site

The canonical EF-hand site[48] consists of a α-helix (about 10 residues), a loop containing a β-pleated sheet (12 residues), and a second a-helix (11 residues) oriented almost perpendicularly with respect to the first.



%	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Ala	7	10	0	7	0	5	0	0	4	0	1	7	8	0	0	8
Arg	10	1	0	8	0	5	0	2	8	0	0	7	1	0	0	12
Asn	4	0	0	3	33	11	20	4	2	0	9	1	7	2	0	2
Asp	9	0	**99**	2	**61**	3	**50**	0	2	0	*31*	1	*28*	7	0	1
Cys	1	3	0	1	0	0	0	0	0	1	2	1	0	0	0	2
Gln	2	0	0	3	0	2	1	1	7	0	4	0	11	0	0	6
Glu	15	1	0	5	4	5	0	0	10	0	9	7	24	**91**	0	6
Gly	0	5	0	0	0	*46*	1	**88**	0	0	8	6	1	0	0	6
His	4	0	0	0	0	5	1	0	2	0	0	1	1	0	0	0
Ile	6	11	0	5	0	0	1	0	0	**52**	0	5	0	0	2	7
Leu	9	19	0	5	0	1	0	0	3	21	0	13	1	1	*35*	12
Lys	10	1	0	*27*	0	14	0	4	14	0	0	1	8	0	0	16
Met	8	2	0	3	0	1	0	1	0	5	1	2	1	0	8	4
Phe	2	*25*	1	4	0	0	0	0	8	0	0	*26*	0	0	*46*	3
Pro	0	0	0	0	0	0	0	0	0	0	0	5	3	0	0	1
Ser	3	2	0	7	2	2	*26*	1	9	0	*26*	3	4	0	0	4
Thr	6	0	0	17	1	1	1	0	17	0	10	4	1	0	0	2
Trp	0	2	0	0	0	0	0	0	0	0	0	0	0	0	3	0
Tyr	0	8	1	0	0	0	0	0	11	0	0	4	1	0	7	0
Val	5	11	0	4	0	0	0	0	4	22	0	7	1	0	0	9

Protein weight matrix derived from 101 sequences. (Barioch and Cox, FEBS Lett. 269, 454, 1990)

fig. 2.
A. 3D structure of an EF-hand from the parvalbumin[48] visualized using the ViSP software created by Edouard de Castro on a Silicon Graphics IRIX 4D/35 computer; B. Anthropomorphic representation of an EF-hand[39]; C. Consensus sequence of the EF-hand domain[49].

The Ca^{2+} is coordinated, in the loop, by 7 oxygen ligands, which are provided (for 5) by (acidic) side chains, (for 1) by a carbonyl group of the peptide backbone, (for 1) by a bridging water molecule (see fig. 2A). The spatial configuration of the ligands around the calcium is

pentagonal bipyramidal. The six residues involved in the binding are in relative positions 10, 12, 14, 16, 18, 21; these residues are named X, Y, Z, -Y, -X, -Z. From the sequence of the different EF-hand CaBP a consensus sequence for the EF-hand motif (see fig. 2C)[49] has been derived. The calcium coordinating residues X(10), Y(12), Z(14), -Z(21) provide oxygen directly with their side-chains for the coordination: Asp, Glu are the most frequent, but Asn, Gln, Ser, Thr are also found. Position X(10), Y(12), and -Z(21) are the most conserved. The invariant Glu or Asp at position 21 provides 2 oxygen for liganding Ca^{2+} (bidentate ligand). The -Y(16) residue just provide a carbonyl from the peptide backbone to coordinate the Ca^{2+} (therefore it can harbor almost any side-chain). The -X(18) residue frequently harbors an oxygen bearing side chain that helps to stabilize the coordinating H_2O molecule.
The three-dimensional structure of the EF-hand site can be represented by the right hand, with the index finger representing the 1st helix, the bent middle finger the loop, and the thumb the 2nd helix (see fig. 2B). Therefore the structure as been named "Hand". The designation EF derives from the C-terminal E and F helices in parvabumin, the first EF-hand CaBP that was crystallized. Parvalbumin[48] contains 6 α-helices, which were called A, B, C, D, E, F starting from N-terminus. Two Ca^{2+} ions were found in the loops joining helices C to D and E to F.
Although simple EF hand polypeptide have only a low affinity for Ca^{2+}, their presence in pairs is associated with high affinity binding. The EF-hand loop contains a B-pleated sheet, which allows the pairing of 2 EF-hands into a tandem domain (2 sites domain, see fig. 3)[50]. This tandem domain is a basic feature common to all EF-hand Ca^{2+}-binding proteins. It seems to be important for protein folding as well as for the functional properties of individual Ca^{2+}-binding sites.

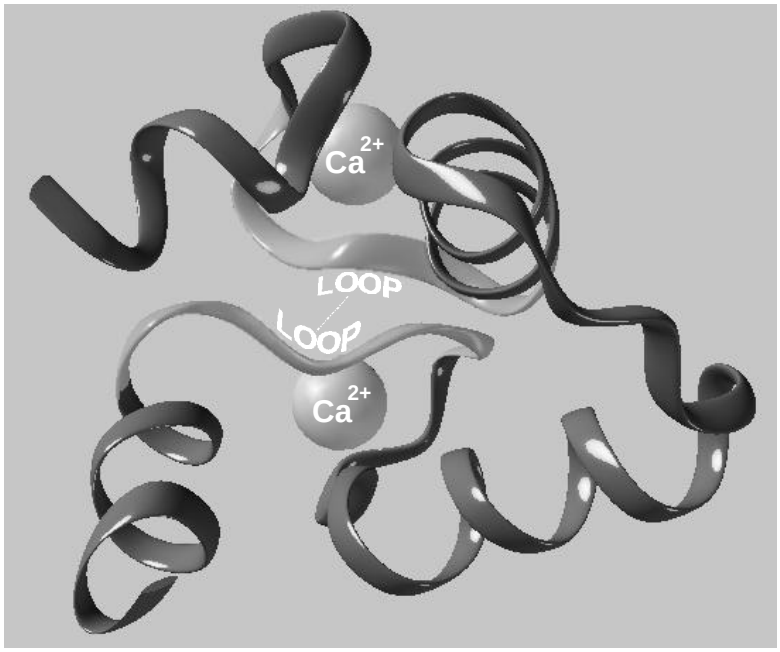


fig. 3.
3D structure of the 2-site (EF-hand) domain from parvalbumin visualized using the ViSP software (by Edouard de Castro).

This tandem domain can be described as a cup-shaped with the interior lined with hydrophobic side chains, the bottom of the cup containing the 2 Ca^{2+}-binding loop, and the rim, consisting of charged hydrophilic side-chains.
EF-hand proteins with degenerated EF-hand site(s) which can no longer bind calcium due to "incompatible" coordinating side chains are often found (resulting from divergent evolution).
It has also been observed that many EF-hand CaBP bind not only Ca^{2+} but also Mg^{2+} with a lower affinity (in the mM range)[51]. Therefore Mg^{2+} (which has a stable cytosolic concentration at ~0.5mM) is thought to occupy the sites in the "absence" of Ca^{2+} (in a resting cell) and to be displaced by Ca^{2+} when the cell is stimulated. The role of Mg^{2+} is though to be mostly structural.

The different classes of EF-Hand CaBPs

Phylogenetic studies suggest that the EF-hand CaBP superfamily may be derived from a 1 EF hand ancestor via a series of tandem gene duplications[52]. **EF-hands generally go by pairs (see above), and the majority of EF-hand CaBP posses 2 or 4 EF-hand**. Below I will present different examples of EF-Hand CaBP classified according to their number of EF-Hand.

2 EF hand proteins

S100 proteins[53] (see fig. 4) are members of the 2 EF-hand CaBP family. They are typically small, dimeric acidic proteins, though to be implicated in cell cycle progression, cell growth, and differentiation. Initially they were found in the nervous system, but now, are known to be more widely expressed (showing tissue-specific isoforms). Most S100 proteins have a mass of 9-12kDa. Their amino acid identity ranges from 25% to 60%. They can bind generally 2 Ca^{2+} ions (per monomer) with their 2 EF-hand. The first EF-hand is typical for S100 proteins and binds Ca^{2+} only with low affinity (200-500µM) as it is slightly different from the consensus. The second one is canonical with a better affinity for Ca^{2+} (10-50µM).

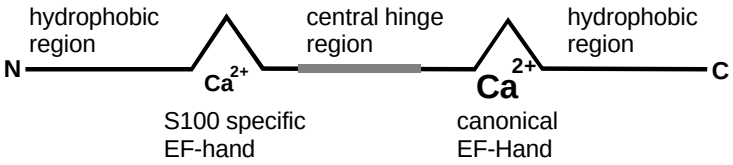


Fig. 4. Basic structure of S100 proteins

These S100 proteins are thought to be modulator proteins (see above) interacting with target proteins via exposed hydrophobic surfaces upon calcium binding. Some of these proteins are also know to oligomerize. Some S100 proteins can even be secreted in the extracellular space and have been shown to stimulate neurite extension, chemotaxis, and cell proliferation.
An interesting aspect of S100 proteins is their putative implication in cell cycle control and therefore in cancer. The deregulated expression of some S100 proteins seems associated with certain tumors. Moreover S100B is known to inhibit the phosphorylation of p53, (a tumor suppressor protein).

The spectrin, an actin binding protein (shaping the cytosqueleton), composed of an α and β-subunit was first identified in red blood cells. Now, other forms are found in a large variety of tissues. The brain **α-spectrin**[54] can bind calmodulin and also possesses two functional EF-hands allowing the binding of Ca^{2+}.

In the 2 EF-hand category we can also find for example non muscle **α-actinin**[55] (also an actin binding protein), **calsensin**[56], an invertebrate, small, nervous specific cytosolic Ca^{2+} binding protein; **ICaBP**[57] (Intestinal Ca^{2+} binding protein, a small S100 related protein) and **diacylglycerol kinase**[58]**,** a ubiquitous kinase.

4 EF-hand proteins

The largest sub-family. Proteins showing 4 putative EF-hand domain generally have 4, 3 or 2 functional Ca^{2+} binding sites (some EF-hand can be degenerated; not functional). The 4 EF-hand are organized into two tandem regions.
The most well know proteins of this 4 EF-hand family is calmodulin which is a ubiquitous intracellular modulator, troponin C and myosin (light chain) which are involved in muscle contraction they belong to the calmodulin related proteins sub-family.

Presentation of some members of the ***calmodulin related proteins***:

Calmodulin (CaM)[59, 60] which is found in all eucaryotic cells is the best studied CaBP. It is a multipurpose intracellular modulator, mediating multiple Ca^{2+}-regulated processes. CaM is a highly conserved (through evolution) protein of about 150 amino acids, with four functional EF-hand Ca^{2+} binding sites arranged in pairs forming 2 globular lobes connected by a flexible tether[61-63]. The 4 Ca^{2+} binding sites have an affinity (K_D) ranging from 1μM (in the carboxy terminus lobe) to 10-100μM (in the amino terminus lobe). The CaM quite low affinity for Ca^{2+} is compensated by its high cellular concentration, above 1μM and its high affinity for its target proteins in the presence of Ca^{2+} (K_D= 0.1-1nM).
In some case CaM can serve as a permanent regulatory subunit of an enzyme complex or have Ca^{2+} independent functions, but generally it modulate the activity of target proteins in a Ca^{2+} dependent way .
Target proteins possess, recognition sites for CaM (CaM-binding domain) that are related[64, 65]. They comprise usually 18-20 contiguous residues, forming an amphiphilic, basic α-helix but with little primary sequence similarity to each other. These recognition sites are Ca^{2+}-dependent CaM binding site with a K_D of ~1nM (there is also a Ca^{2+} independent CaM binding site (K_D=200nM) called the IQ motif found for example in neuromodulin, brush border myosin 1 (see below).
Upon Ca^{2+} binding, CaM undergoes a conformational change (allosteric "activation")[35]. Structural studies with model peptide (from MLCK and CaM Kinase II) as synthetic targets[66] have shown that when (Ca^{2+}-) calmodulin binds to its target, it can undergo a further change in conformation, both of its lobes collapsing around part of the target (see fig. 5). The modulatory effect of CaM on a target enzyme often goes by the displacement of an autoinhibitory domain from the active site.

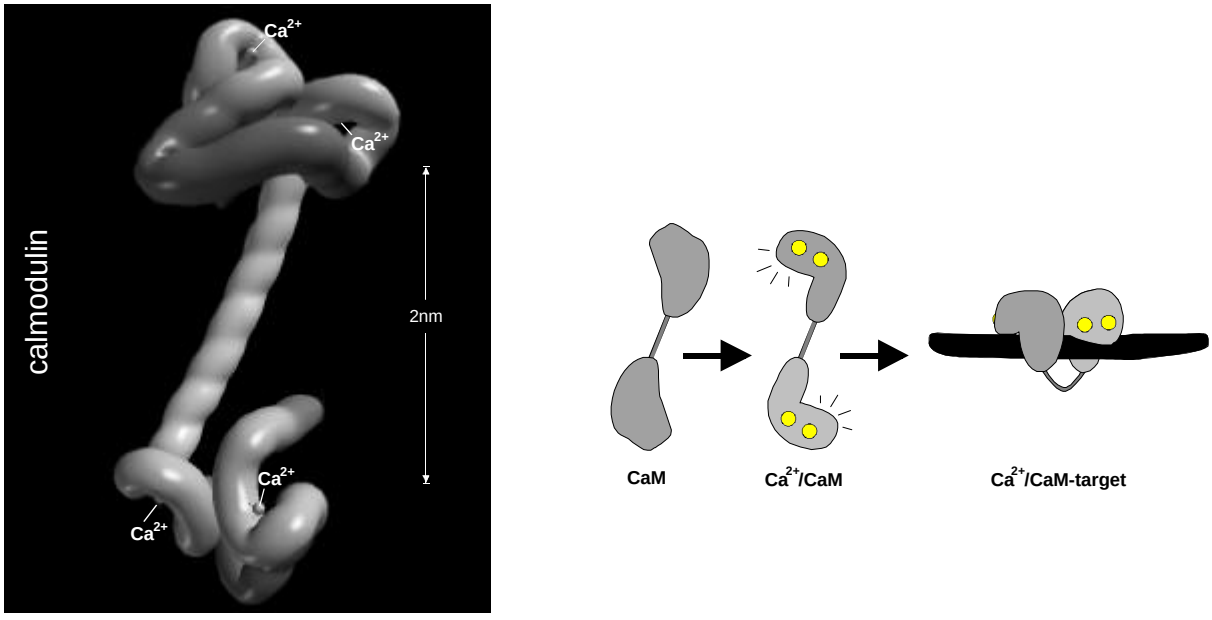


Fig. 5.
A. 3D structure of calmodulin (CaM) visualized using the ViSP software (by Edouard de Castro); B. Schematic representation of conformational changes in CaM when it bind Ca^{2+} and then when Ca^{2+}/CaM binds to its target

Although CaM is distributed ubiquitously, it has some tissue-specific functions partly because its target proteins are localized in a tissue-specific manner[67]. Among the **targets** regulated by calmodulin are various enzymes (mostly protein kinases) including *cyclic nucleotide phosphodiesterase, calcineurin, protein kinases/ phosphatases, adenylate cyclase, nitric oxide synthase*; and transport proteins like the plasma membrane *Ca^{2+}*-

ATPase pump, the RyR or the IP_3R. There are also Ca^{2+} independent targets (with IQ domain(s))[68] of CaM such as cytoskeletal proteins like non-conventional myosin, or CaM sequester like neuromodulin, neurogranin.
Most effect of Ca^{2+}/calmodulin are mediated by the Ca^{2+}/calmodulin dependent protein kinases[69] such as myosin light chain kinase (MLCK), which activate smooth muscle contraction, phosphorylase kinase which activates glycogen breakdown, CaM kinase II, etc. The multiple (pleiotropic) functions of calmodulin and Ca^{2+}/CaM-dependent protein kinases (CaM kinases) have been extensively studied. The best studied example of CaM target kinase is CaM kinase II, which is also ubiquitous (with tissue specific isoforms) but especially enriched in the nervous system. CaM-kinase II is a remarkable enzyme: it is a kind of molecular memory device[70, 71], activated by Ca^{2+}/CaM it can then remains active even after the end of the Ca^{2+} signal (via its autophosphorylation[72]). Therefore it can serve as a memory trace of a prior Ca^{2+} pulse and seems to play an important role in some type of memory and learning (in vertebrates).

Troponin C (TnC)[73, 74] in skeletal muscle cells is closely related to calmodulin. TnC is a part of the troponin complex found on thin actin muscle filaments. It has also 4 functional EF-hand Ca^{2+} binding sites. When TnC bind Ca^{2+} it relieves the inhibition of myosin (the force generating motor protein) binding to actin (due to the other troponin components) promoting muscle contraction.

Myosin light chain proteins: Essential (ELC) and Regulatory (RLC) Light Chains, part of the myosin complex[75] (formed by 2 heavy chains and 2 ELC, 2 RLC) show considerable sequence similarity with CaM and TnC but have lost during evolution most of their Ca^{2+} binding capacity (RLCs have only one Ca^{2+} binding site, ELCs in general do not bind Ca^{2+}) because most of their EF-hand are not functional (degenerated). In smooth muscle the phosphorylation of myosin light-chains by the enzyme myosin light-chain kinase (which is activated by Ca^{2+}/CaM) will trigger muscle contraction[76]. In striated muscle the functions of myosin light chains are poorly understood.

Together the CaM, TnC, myosin LC, and also calcineurin B[77] (the regulatory subunit of calcineurin, a serine/threonine phosphatase) show similar tertiary organizations with two lobs connected by a long tether.

The well known calcium buffer **parvalbumin**[48] is probably a 4 EF-hand protein, although only 3 are "visible", the 4th being to degenerated (see other EF-hand proteins).

In the 4 EF-hand category we can also find for example **aequorin**[78] (a bioluminescent protein in lower organisms used as Ca^{2+} indicator), **centrins**[79] (cytoskeletal Ca^{2+} binding proteins associated with centrosomes).

Still in this category there is also an emerging newly described family of neuron-specific EF-hand calcium binding proteins. They are all small (~200 amino acids) globular proteins with 4 identifiable EF-hands but generally bind only 2 or 3 Ca^{2+} ions because some of their EF-hand are not functional. They are found mainly in neurons and are though to be involved in the fine tuning of calcium-dependent processes in the nervous system. For convenience this family is named the ***Neuron specific Calcium Sensor (NCS) family.*** Caenorhabditis elegans-Neuronal Calcium Sensor-1 (*Ce*-NCS-1), the object of this study, belong to this family; so in the next chapter I will focus on these NCS proteins (for ref. See later).

Recently, a family of ***Ca^{2+} dependent Guanylyl Cyclase Activating Proteins*** ***(^{Ca}GCAP)***[80] has been described (the a.a. identity between NCS and GCAP range from 30 to 40%). The ^{Ca}GCAP are also small (~200 a.a.) proteins with probably 3 functional EF-hands out of 4 identifiable. In different species, these proteins are found in the retina photoreceptors where they activate the guanylyl cyclase (GC) in a Ca^{2+} dependent way (see fig. 7) participating in

the recovery of the photo-transduction machinery after stimulation by light. Remarkably these proteins are low calcium sensors, interacting with their target (the GC), activating it, at low Ca2+. They can be considered as "inverted" Ca2+ modulators, adapted to the inverted physiological system of the photoreceptors. The light signal decreases a high resting cytosolic Ca2+ level instead of increasing a low resting one as usual, induce hyperpolarization instead of depolarization, and gives a "no more" neurotransmitter release response instead of a transmitter release response(see fig. 8). So far these proteins are the only known CaBP modulators active at low Ca2+ concentration.

Some other members of the 4 EF-hand family posses enzymatic activities by themselves belonging to the ***high molecular weight Ca^{2+}-regulator proteins*** group; namely 4 EF-hand proteins with additional catalytic (or structural domain). For example, the **calpains**[37] are ubiquitous (with tissue-specific isoforms) Ca^{2+} dependent proteases which targets are cytoskeletal proteins (e.g. spectrin), membrane receptors, and enzymes, including calpain itself (autolysis of calpain renders it more active). We can also cite CaBP proteins with "internal" kinase activity like plants **calmodulin-domain protein kinases** (e.g. CPK1)[81].

6 EF-hand proteins

So far known 6 EF-hand CaBP are though to be just calcium buffer proteins such as calbindin D-28K[82], calretinin[83] (a cytosolic CaBP abundant in the nervous system), human ERC-55 protein[84] (found in the ER), some spec proteins (see below), Cab45 (found in the Golgi lumen)[85].

Other EF-hand proteins

Proteins with an odd number of EF-hand have also been described. For example SPARC (secreted protein acidic and rich in cysteine also named BM-40 and osteonectin) seem to have only one EF-hand, parvalbumin[48] three, and rdgC serine/threonine protein phosphatase five[86]. The missing EF-hand may be present but so degenerated that it is not recognized as a putative EF-hand (like for parvalbumin), or may have been lost during genetic rearrangements.
Spec/ spec like proteins[87] in see urchin embryos are calcium buffers with 4, 6 or 8 EF-hand. In some flagellate we can even find an ARP (buffer) protein[88] with 30 putative EF-hand!

To summary, EF-hand CaBP, the largest superfamily of CaBP, can have 3 different functional aspect: being **modulators** with a Ca^{2+} dependent modulator activity (such as calmodulin), **enzymes**, with a direct Ca^{2+} dependent enzymatic activity (such as calpain), and **buffers**, with a Ca^{2+} buffering function (such as parvalbumin).
They have generally an even number of the Ca^{2+} binding motif EF-hand (associated in pairs), which allows the (rapid) reversible binding of Ca^{2+} with a relatively high affinity, laying in the cytosolic Ca^{2+} physiological range (in resting and stimulated cells).
These EF-hand CaBP are found in all eucaryotes. In the cell they are mainly localized in the cytosol. For the vast majority of CaBP, their *in vivo* function(s) remains elusive.

The family of Neuron specific Calcium Sensors

Ca^{2+} plays an essential role in the nervous system. It is involved in neurotransmitter release where its entry in the presynaptic terminal is known to be the trigger of the transmitter release; in neuronal survival[89] (where activation promotes survival) and death[90]; in axon out growth[91], and in the regulation of neuronal plasticity[2] (via synaptic efficacy control). High frequency stimulation can induce potentiation or depression of neuron response. These phenomena called Long-Term-Potentiation[92] (LTP) and Long-Term-Depression[93] (LTD) are though to represent the molecular basis of memory and learning[70]. The induction of LTP and LTD requires the activation of (glutamate) NMDA receptors leading to Ca^{2+} influx into the postsynaptic cell. Calcium waves (see above) which encodes information in their frequency could be probably influenced by patterns of synaptic inputs, generating complex differential responses.
The effect of “neuronal” Ca^{2+} is often mediated via its binding to calmodulin which will modulate the activity of a variety of target enzymes (see above, calmodulin). Nevertheless some other Ca^{2+} modulator proteins are thought to plays important roles in the nervous system.

Proteins from a previously unknown family of neuron-specific EF-hand CaBP have been recently described. Members of this family are highly conserved through evolution. These small CaBP are though to be calcium modulators and upon Ca^{2+} binding should interact with target proteins and alter their activity. They are found mainly in neurons and are likely to be involved in the fine-tuning of calcium-dependent processes in the nervous system.

This emerging branch of the superfamily of EF-hand calcium binding proteins form the growing family of nervous system-specific calcium binding modulators often called the *recoverin or the vilip family*. For convenience this family was named the ***Neuron specific Calcium Sensors (NCS)family)***.

```
                                                                              (EF1)                                EF2
                        1                                                   *  *    *        *                          *  * 80
RECOVERIN (bovine)      MGNSKSGA.L SKEILEELQL NTKFTEEELS SWYQSFLKEC PSGRITRQEF QTIYSKFFPE ADPKAYAQHV FRSFDANSDG
S-MODULIN (Frog)        MGNTKSGA.L SKEILEELQL NTKFTQEELC TWYQSFLKEC PSGRISKKQF ESIYSKFFPD ADPKAYAQHV FRSFDANNDG
VISININ (Chick)         MGNSRSSA.L SREVLQELRA STRYTEEELS RWYEGFQRQC SDGRIRCDEF ERIYGNFFPN SEPQGYARHV FRSFDTNDDG
Ce-NCS-2 (C. elegans)   MGIKGSKPKL SKEDLEFLKK NTNFTEEQIK EWYKGFVQDC PKGHLTKEQF IKVYKDFFPS GSAEGFCEHV FRTFDTDNSG
VILIP (Rat/Chick)       MGKQNSK..L APEVMEDLVK STEFNEHELK QWYKGFLKDC PSGRLNLEEF QQLYVKFFPY GDASKFAQHA FRTFDKNGDG
VILIP-2 (Rat)           MGKNNSK..L APEELEDLVQ NTEFSEQELK QWYKGFLKDC PSGILNLEEF QQLYIKFFPY GDASKFAQHA FRTFDKNGDG
VILIP-3 (Rat)           MGKQNSK..L RPEVLQDLRE HTEFTDHELQ EWYKGFLKDC PTGHLTVDEF KKIYANFFPY GDASKFAEHV FRTFDTNSDG
NEUROCALCIN (Rat)       MGKQNSK..L RPEVMQDLLE STDFTEHEIQ EWYKGFLRDC PSGHLSMEEF KKIYGNFFPY GDASKFAEHV FRTFDANGDG
HIPPOCALCIN (Rat)       MGKQNSK..L RPEMLQDLRE NTEFSELELQ EWYKGFLKDC PTGILNVDEF KKIYANFFPY GDASKFAEHV FRTFDTNSDG
FREQUENIN (Droso.)      MGKKSSK..I KQDTIDRLTT DTYFTEKEIR QWHKGFLKDC PNGLLTEQGF IKIYKQFFPQ GDPSKFASLV FRFEDENNDG
NCS-1 (Rat/Chick)       MGKSNSK..L KPEVVEELTR KTYFTEKEVQ QWYKGFIKDC PSGQLDAAGF QKIYKQFFPF GDPTKFATFV FNVFDENKDG
Ce-NCS-1 (C. elegans)   MGKGNSK..L KSSQIRDLAE QTYFTEKEIK QWYKGFVRDC PNGMLTEAGF QKIYKQFFPQ GDPSDFASFV FKVFDENKDG
            consensus   MG---S---- -------L-- -T-------- -W---F---C --G------F ---Y--FFP- ---------- F---D----G

                                                                  EF3
                        81    *                                 *  *  *    *                                      160
RECOVERIN (bovine)      TLDFKEYVIA LHMTSAGKTN QKLEWAFSLY DVDGNGTISK NEVLEIVTAI FKMISPEDTK HLPEDENTPE KRAEKIWGFF
S-MODULIN (Frog)        TLDFKEYMIA LMMTSSGKAN QKLEWAFCLY DVDGNGTINK KEVLEIITAI FKMINAEDQK HLPEDENTPE KRTNKIWVYF
VISININ (Chick)         TLDFREYIIA LHLTSSGKTH LKLEWAFSLF DVDRNGEVSK SEVLEIITAI FKMIPEEERL QLPEDENSPQ KRADKLWAYF
Ce-NCS-2 (C. elegans)   FIDFKEFLLA INVTSSGTPE QKLEWAFRMY DIDGNGTIDE KEMIKIIEAI YEMLGPEVTK S...ADDSPR KRAKMIFEKM
VILIP (Rat/Chick)       TIDFREFICA LSITSRGSFE QKLNWAFNMY DLDGDGKITR VEMLEIIEAI YKMVGTVIMM KMNEDGLTPE QRVDKIFSKM
VILIP-2 (Rat)           TIDFREFICA LSVTSRGSFE QKLNWAFEMY DLDGDGRITR LEMLEIIEAI YKMVGTVIMM RMNQDGLTPQ QRVDKIFKKM
VILIP-3 (Rat)           TIDFREFIIA LSVTSRGKLE QKLKWAFSMY DLDGNGYISR SEMLEIVQAI YKMVSSV..M KMPEDESTPE KRTDKIFRQM
NEUROCALCIN (Rat)       TIDFREFIIA LSVTSRGKLE QKLKWAFSMY DLDGNGYISK AEMLEIVQAI YKMVSSV..M KMPEDESTPE KRTEKIFRQM
HIPPOCALCIN (Rat)       TIDFREFIIA LSVTSRGRLE QKLMWAFSMY DLDGNGYISR EEMLEIVQAI YKMVSSV..M KMPEDESTPE KRTEKIFRQM
FREQUENIN (Droso.)      SIEFEEFIRA LSVTSKGNLD EKLQWAFRLY DVDNDGYITR EEMYNIVDAI YQMVGQQP.. Q.SEDENTPQ KRVDKIFDQM
NCS-1 (Rat/Chick)       RIEFSEFIQA LSVTSRGTLD EKLRWAFKLY DLDNDGYITR NEMLDIVDAI YQMVGNTV.. ELPEEENTPE KRVDRIFAMM
Ce-NCS-1 (C. elegans)   AIEFHEFIRA LSITSRGNLD EKLHWAFKLY DLDQDGFITR NEMLSIVDSI YKMVGSSV.. QLPEEENTPE KRVDRIFRMM
            consensus   ---F-E---A ---TS-G--- -KL-WAF--- D-D--G---- -E---I---I --M------- --------P- -R--------

                             EF4
                        161                              203
                        * *  *     *
RECOVERIN (bovine)      GKKDDDKLTE KEFIEGTLAN KEILRLIQFE PQKVKEKLKE KKL
S-MODULIN (Frog)        GKKDDDKLTE GEFIQGIVKN KEILRLIQYE PQKVKDKLKE KKH
VISININ (Chick)         NKGENDKIAE GEFIDGVMKN DAIMRLIQYE PKK....... ...
Ce-NCS-2 (C. elegans)   DVNNDKELTL KEFVDGCLAD KELFQIL... .TN......E VKK
VILIP (Rat/Chick)       DKNKDDQITL DEFKEAAKSD PSIVLLLQCD .......... IQK
VILIP-2 (Rat)           DQDKDDQITL EEFKEAAKSD PSIVLLLQCD .......... MQK
VILIP-3 (Rat)           DTNNDGKLSL EEFIKGAKSD PSIVRLLQCD PSS......A SQF
NEUROCALCIN (Rat)       DTNRDGKLSL EEFIRGAKSD PSIVRLLQCD PSS......A GQF
HIPPOCALCIN (Rat)       DTNNDGKLSL EEFIRGAKSD PSIVRLLQCD PSS......A SQF
FREQUENIN (Droso.)      DKNHDGKLTL EEFREGSKAD PRIVQAL..S LGG......G ...
NCS-1 (Rat/Chick)       DKNADGKLTL QEFQEGSKAD PSIVQAL..S LYD......G LV.
Ce-NCS-1 (C. elegans)   DKNNDAQLTL EEFKEGAKAD PSIVHAL..S LYE......G LSS
            consensus   ---------- -EF------- ---------- ---------- ---
```

fig. 6.

Amino acid sequence alignment of 12 members of the NCS family. The consensus line indicates the amino acids (a.a.) conserved in all the sequences. EF hand loop domains (EF1-EF4) are underlined. In each member of the NCS family, EF1 is degenerated (indicated with brackets). * indicates the a.a. in a EF-hand that could directly coordinate to Ca^{2+} ion.

The members of the NCS family possess a high degree of homology (with some really conserved residues, see fig. 6), showing about 37-100% of amino acid identity. Members are

small acidic proteins of 190-200 amino acids with a molecular weight of about 22-24 kDa. They all contain 4 putative EF-hands, although some binding sites are clearly degenerated and do not bind Ca^{2+}. For all of them, their first EF-hand is clearly degenerated.
Some of these proteins like recoverin and hippocalcin are known to be myristoylated (covalently attached to a myristoyl moiety at the N-terminus) and show Ca^{2+} dependent binding to membrane (through Ca^{2+} dependent exposure of the myristoyl moiety allowing anchorage to the membrane). Other members of the family also display a consensus sequence for myristoylation but their myristoylation has not been demonstrated.

Based on sequence similarities (and on phylogenetic analysis) we can group these NCS proteins into sub-families (see figure 7)[94].

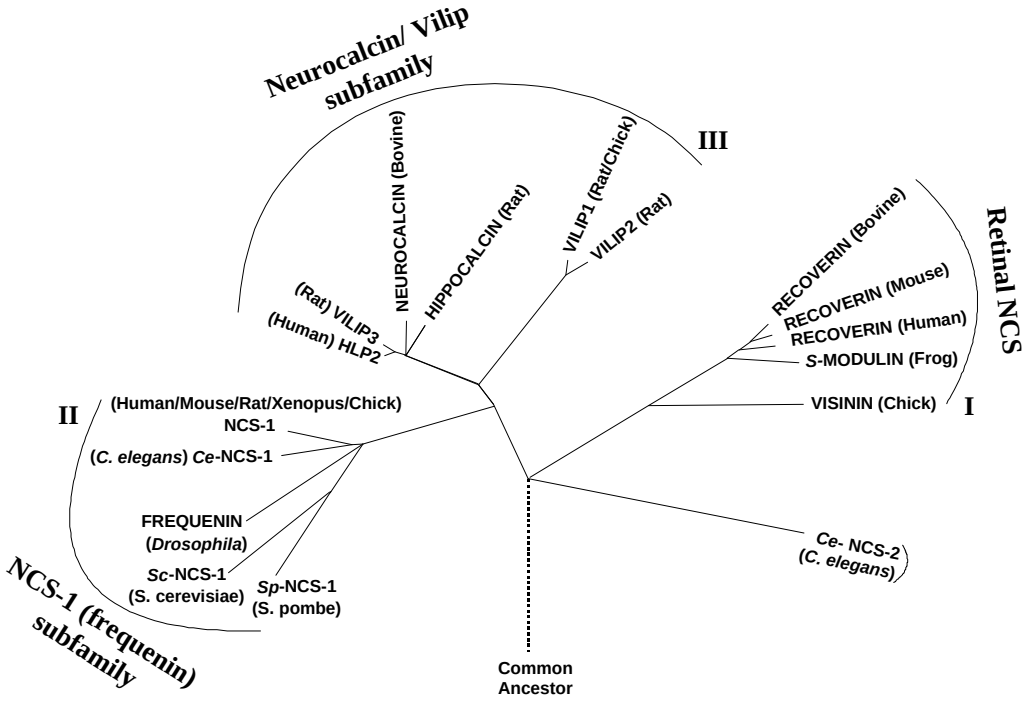


Fig. 7.
Phylogenetic tree[94] (using program TREE[95]) of some NCS proteins, representing evolutionary distances between the different sequences (branch length) assuming that they all evolved from a common ancestor. The topology of the tree (nodes, branches) allows the grouping of the sequences into different subfamilies (using only sequence identities the same grouping can be made).

One group named Retinal NCS represents the retina-specific NCS proteins (i.e. recoverin[96], visinin[97], S-modulin[98]). An other group named NCS-1 (frequenin) subfamily is composed of the NCS-1 proteins with highly homologous sequences isolated from rat, mouse, human, chick (NCS-1), *C. elegans* (*Ce*-NCS-1, the object of this study)[94], Drosophila (frequenin[99]), and even yeast. Other NCS proteins such as rat/chick Vilip-1[100],2,3[101], human/rat hippocalcin[102], neurocalcin[103] have been identified and grouped in the neurocalcin/ vilip subfamily. The most divergent sequence of the NCS family is *Ce*-NCS-2[94] (also described in this study) from *C. elegans*. Members of the NCS family can be considered as orthologues (species "equivalents") if the amino acid sequence comparison score is high, and their gene structures (intron/exon borders) are comparable.
All the members of the NCS-1 group seems to be orthologues. NCS-1 from human, rat, chick, *Xenopus* are 100% identical. *C. elegans* NCS-1, frequenin and even yeast NCS-1 are also very close to vertebrate NCS-1 (see fig. 10). The NCS-1 protein appears to be extremely conserved through evolution (like calmodulin).
In the retinal NCS group all the known members (S-modulin, bovine/mouse/human recoverins, chick visinin) are orthologues. For the neurocalcin/vilip group, the orthologs are: rat/chick vilip1; human/rat hippocalcin; rat vilip3, human HLP2.

Structure

Recoverin is so far the only NCS protein whose tri-dimentional structure is known[96, 104], but as the other members of this family are very similar (>37% a.a identity) they probably share a similar structure. Recombinant unmyristoylated recoverin was purified and crystallized. In this crystal, Ca^{2+} is bound only to EF3, but it does not mean that recoverin binds only one Ca^{2+} (biochemical studies have shown that recoverin binds 2 Ca^{2+} ions[104])

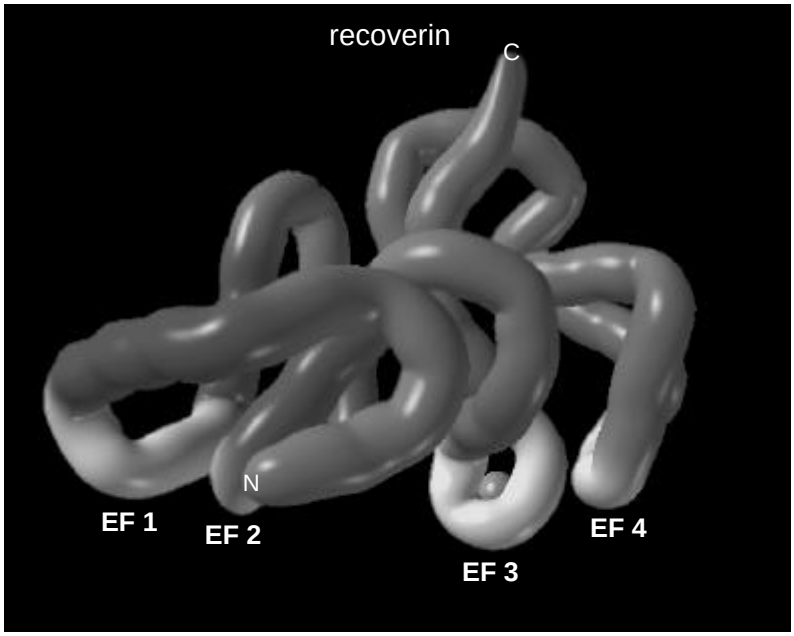


fig. 8.

3D structure of recombinant unmyristoylated recoverin (EF 1-4: identifiable EF-hand 1-4)[104] visualized using the ViSP software (by Edouard de Castro).

The 4 EF-hand of recoverin form two tandem domains that are connected by a short U-shaped bend so that they form a rather compact protein (see fig. 8) with all EF-hand located on one side Recoverin could be viewed as calmodulin with a shorter and bend tether, both lobs coming close each to another on the same side. An hydrophobic region is found around the N-terminal tandem EF-hand domain (EF1 and EF2). This hydrophobic "patch"/surface may be the docking site for the myristoyl moiety linked to the N-terminus or for target proteins.

Functional roles of NCS proteins

The functional role(s) for the majority of the NCS proteins remains elusive, except for retinal NCS proteins that are implicated in the modulation of light sensitivity (via Ca^{2+} dependent inhibition of rhodopsin phosphorylation)[105] and for frequenin, a *Drosophila* NCS, that is known to modulate the neurotransmitter release at the neuromuscular junction[99]. Based on *in vitro* assays some other NCS such as NCS-1, *Ce*-NCS-1, *Ce*-NCS-2, vilip1, and hippocalcin have shown a Ca^{2+} dependent inhibition of rhodopsin phosphorylation effect like the retinal NCS[94]. By extension, these data suggest a general role for the NCS proteins in the calcium-dependent regulation of G protein coupled receptor phosphorylation in the nervous system.
Our group has also shown that NCS-1 and *Ce*-NCS-1, both *in vitro* and *in-vivo*, can substitute for or potentiate calmodulin[106] (CaM) functions (see later).

The retinal NCS

Visinin[97] (chick), **Recoverin**[96] (bovine/mouse/human), **S-modulin**[98] (frog), were the first members of the NCS family to be isolated and sequenced. These orthologous proteins are predominantly expressed in photoreceptors. Recoverin is present in outer segments, cell bodies, and synaptic region of photoreceptors. It is also found in certain bipolar cone cells and in the pineal gland! Retinal NCS are known to be myristoylated (covalently attached to a myristoyl moiety at the N-terminus) and show a Ca^{2+} dependent association with membrane.
Both *in vivo* and *in vitro* experiments[105, 107-109] have shown that S-modulin and Recoverin prevent, at high calcium level, the quenching of the photoreceptor Rhodopsin by inhibiting its phosphorylation.

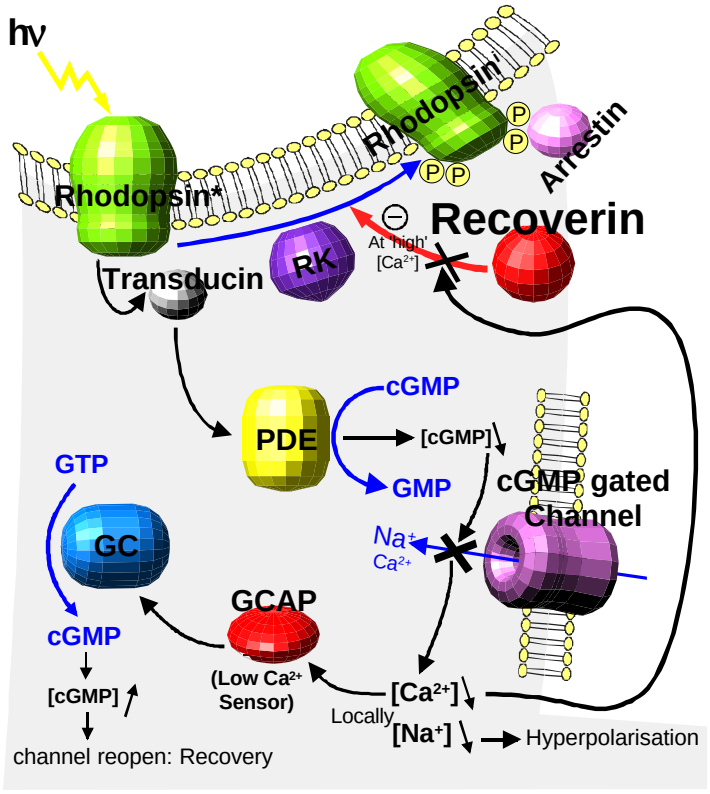


fig. 9.
The phototransduction cascade and functional role of recoverin in photoreceptors. PDE: cGMP phosphodiesterase; RK: rhodopsin kinase; GC: guanylyl cyclase; GCAP: guanylyl cyclase activating protein.

In the phototransduction (see fig. 9), the light receptor rhodopsin is activated by photons forming rhodopsin* (R*). R* then activates transducin (T), a G(TP binding)-protein promoting the exchange of bound GDP (T_{GDP}) for GTP (T_{GTP}). The activated transducin (T_{GTP}) will in turn activate a cGMP-hydrolyzing enzyme: the cGMP phosphodiesterase (PDE), leading to a decrease of the intracellular cGMP concentration. In the plasma membrane of photoreceptors there are cGMP gated Na^+, Ca^{2+} channels. The lowering of the cGMP levels resulting from the photontransduction cascade will therefore lead to the closure of these cGMP gated channels. Since the steady inward current carried by the channels is blocked, the cell will hyperpolarize, leading to the interruption of the steady transmitter release from the activated photoreceptor. The light signal is converted into a “no more transmitter release” signal which will be further processed by other retina neurons, and then send to the brain.
R* is partially inactivated by phosphorylation of its C-terminus region. The phosphorylated rhodopsin could then be recognized and bind by arrestin ensuring its inactivation.
Recoverin or S-modulin inhibits this phosphorylation reaction, namely the inactivation of R*, at high Ca^{2+} concentration[110]. Therefore it prolongs the lifetime of R* at high Ca^{2+} concentration[105]. It has been shown that these retinal NCS act by inhibiting in a Ca^{2+}

dependent way rhodopsin kinase (RK) the enzyme needed to phosphorylate R*[107, 109, 111].
Therefore in the photoreceptors retinal NCS proteins, by inhibiting rhodopsin kinase at high calcium concentration increase the photoreceptor sensibility to photons in dimness. In such a case, in the photoreceptors there is a high calcium concentration (the weak stimulation will not lead to a large cGMP level decrease, allowing the cGMP gated Na^+, Ca^{2+} channels to stay open) and the retinal NCS prevent the inactivation of the photon-activated rhodopsin (R*), prolonging the lifetime of the light response.
At the opposite, in steady light, where the physiological Ca^{2+} concentration is low (steady light stimulation makes the calcium level drop, because of the steady closure of cGMP gated Na^+, Ca^{2+} channels resulting from the drop in cGMP level), the quenching of rhodopsin is not affected by the presence of S-modulin or recoverin. The activated rhodopsin can therefore be readily inactivated, lowering the lifetime of the light response.
By this mechanism, recoverin regulates the light sensitivity of photoreceptors, adjusting it to light conditions[112].

As *Caenorhabditis elegans*-Neuronal Calcium Sensor-1 (*Ce*-NCS-1), the object of this study, belong to the NCS-1 group, I will present this group in more details:

The NCS-1 group

The NCS-1 subfamily is composed of highly homologous proteins (see fig. 10) probably all orthologues formed by NCS-1 from rat/mouse/chick/human/*Xenopus* (vertebrate), *Drosophila* frequenin, *C. elegans Ce*-NCS-1, and yeast NCS-1 (*Sc*-NCS-1 in *Saccharomyces cerevisiae*, *Sp*-NCS-1 in *Saccharomyces pombe*).

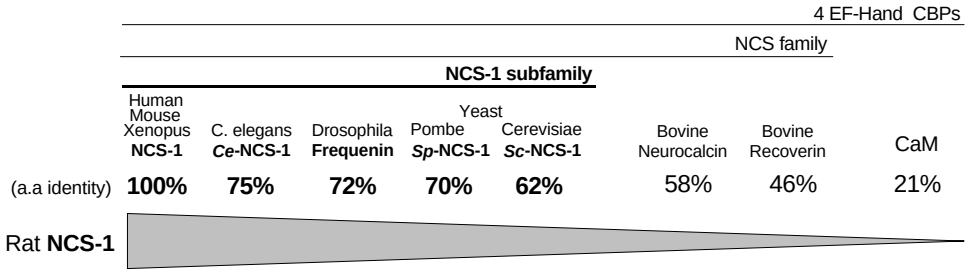


Fig. 10. Amino acid identity between rat NCS-1 and the other NCS-1 proteins (and with some other CaBP).

The NCS-1 protein seems extremely conserved through evolution (like calmodulin). The function of these NCS-1 proteins remains elusive except for the *Drosophila* frequenin in the neuromuscular junction (see below). NCS-1 and *Ce*-NCS-1 have been shown to have a recoverin effect and also a CaM effect (on some CaM targets). Nevertheless the exact in vivo physiological function of these proteins is not yet known.

Regulation of neurotransmitter release by frequenin

Drosophila frequenin[99] is expressed through all developmental stages and found predominantly in the fly adult nervous system. It appears to be concentrated in synaptic regions especially at the neuromuscular junction.
Clues on the frequenin function came from the analysis of a *Drosophila* mutant overexpressing the protein. Several *Drosophila* mutants have been described for their abnormal motor phenotype. Electrophysiological analysis of these mutants has shown altered action potential duration and/or frequencies as well as abnormal neurotransmitter release at neuromuscular junctions. In some mutants, the molecular basis of the altered motor activity has been discovered. In the Drosophila, the V7 mutant exhibits an enhanced potentiation of transmitter release (facilitation) at neuromuscular junctions (showing a “shaker” like phenotype) in response to repetitive nerve stimulation (>5 Hz) while basal synaptic transmission (< 1 Hz) is not affected. This V7 mutant was shown to have a 3 to 4 times increased expression of the gene

encoding frequenin. This result was confirmed with transgenic flies expressing higher amount (7-14 times) of frequenin than normal flies. The electrophysiological consequences of elevated frequenin levels are a 4- to 5-fold increase in the magnitude of facilitation of neurotransmitter release at motor nerve endings. **Therefore, frequenin seems to participate in the facilitation of neurotransmitter release (at the neuromuscular junction).**
It is known that the Ca^{2+} entry into presynaptic terminals is the trigger for neurotransmitter release. In many synapses this short-lived event is followed by a much longer period of facilitation during which a second nerve impulse is much more effective than the first in raising the probability of release. This process has been termed short-term facilitation (and also post-tetanic potentiation, augmentation or long term potentiation; depending on its duration and the stimulation frequency/duration required to elicit it). In the neuromuscular junction short-term facilitation usually decays in less than 1s. This facilitation is believed to be due to a prolonged increase in intracellular Ca^{2+} remaining at the transmitter release sites. Thus, in a background of this residual 'active' Ca^{2+}, calcium increments due to successive nerve impulses will trigger the release of more transmitter because of a (non-linear) relationship between release site Ca^{2+} concentration and transmitter release. Frequenin, as already said, seems to participate in the generation of this short-term facilitation. Nevertheless the precise mechanism of this neurotransmitter release modulation is not known. It is not yet clear how a neuronal calcium sensor such as frequenin could function at the (pre)synapse between a motor-neuron and a muscle. Electrophysiological data indicates that there is modulation of the Na^+/Ca^{2+} exchange by the Ca^{2+}-frequenin[113] through an increase in internal Na^+ concentration at the release site reducing (locally) Na^+ gradient and therefore Na^+/Ca^{2+} exchange. **The model is that at high (local) Ca^{2+} concentration after repetitive stimuli, frequenin increase the presynaptic terminus excitability by inhibiting Na^+/Ca^{2+} exchange therefore preventing Ca^{2+} expelling from the release site.** Does it inhibits Na^+ pumps and/or Na^+/Ca^{2+} exchanger (and/or modulates other pumps/channels)? Does it also modulate, like recoverin, the phosphorylation state of some presynaptic receptors? Moreover what about frequenin in the central nervous system; does it also modulate neurotransmitter release, does it also have other functions? What about frequenin functions in post-synapses. All these questions are still unsolved.

In vitro and in vivo biological activities of NCS-1

Rat/mouse/chick NCS-1 binds two Ca^{2+}, with a high cooperativity (n_H=1.95) and a high affinity, its apparent Ca^{2+} binding K_D being at ~0.3μM. This affinity is straight in the range of the Ca^{2+} transients in neuronal cells. With such Ca^{2+} affinity and high cooperativity a rise of Ca^{2+} concentration from 0.15 to 1.5 μM will provoke a shift from 10 to 90% in the NCS-1 calcium saturation (and likely in its activity)[114]. Therefore NCS-1 seems to be exquisitely fine-tuned to rapidly respond to Ca^{2+} transients occurring in synapses.
Chick NCS-1 was shown to be expressed in postmitotic neurons of several structures in the central and peripheral nervous system, and to be present like frequenin in motor-neurons. NCS-1 gene expression is turned on at very early stages during development (E3) and persists in newborn and adult animals. In the rat and mouse, NCS-1 is widely distributed within neurons in the peripheral and the central nervous systems[147], and is enriched in the retina, the cerebellum, and the hippocampus. In neurons the protein is preferentially localized in both axons and dendrites (neurites) but not in cell bodies[147]. Therefore NCS-1 appears to be found both pre- and post- synaptically and could play a role in both presynaptic excitability modulation and regulation of postsynaptic properties. Unlike some other NCS protein, NCS-1 is probably not myristoylated (as it shows no myristoylation consensus site). Nevertheless NCS-1 seems able to associate with membranes as it is preferentially localized in neuritic processes and often concentrated in post-synaptic densities (Nef *et al*, unpublished).

Our group has reported that NCS-1 (together with *Ce*-NCS-1, *Ce*-NCS-2, and vilip1) can, *in vitro*, inhibit rhodopsin phosphorylation in a calcium-dependent manner[94]. It act probably like recoverin, inhibiting rhodospin kinase, a G protein coupled receptor kinase (GRK). We must note that rhodopsin kinase is probably not the endogenous target of NCS-1 since NCS-1 is largely distributed in the nervous system and not only present in the photoreceptors. This suggests that other GRKs could be potential targets of NCS-1.
By extension, these data could suggest a general role for these NCS proteins in the calcium-dependent regulation of G protein coupled receptor phosphorylation in the nervous system which modulate receptor desensitization. For example it may potentiate a neuron response by inhibiting receptor desensitization at elevated cytosolic Ca^{2+} level after a high (frequency) stimulation.
We have also reported that NCS-1 and *Ce*-NCS-1, both *in vitro* and *in-vivo*, can substitute for or potentiate calmodulin[106] (CaM) (although there is only 21% of a.a. homology between CaM and NCS-1). We observed that *in vitro*, NCS-1 (together with *Ce*-NCS-1) directly activates two Ca^{2+}/CaM-dependent enzymes: 3':5' - cyclic nucleotide PhosphoDiEsterase (PDE) and calcineurin, and is also capable of potentiating the activity of nitric-oxyde synthase (NOS) -Ca^{2+}/CaM. Other NCS such as vilip1 and recoverin have no such effects. Furthermore, *In vivo*, NCS-1 alone partially restores the wild-type behavior in calmodulin-defective (in the interaction with a K^+ channel) *cam[1] Paramecium mutants*. Nevertheless NCS-1 can not activate the well-studied CaM target CaM kinase II, nor potentiate CaM kinase II activation by CaM (Hegi S. *et al*, submitted). Therefore NCS-1 can replace CaM or potentiate CaM effect in some cases but not all. NCS-1 represents an ideal "switch" for neurons that need to respond rapidly to slight variations in Ca^{2+} levels whereas CaM senses probably more sustained variations. Combined together NCS-1 and CaM could allow a differential range of Ca^{2+} sensing activities over common targets.
Together these results indicate that NCS-1 is probably a multifunctional modulator sharing some targets with CaM but also having its own 'proprietary' targets. As NCS-1 is widely distributed in the nervous system its function in several regions of the nervous system may depend on different targets. As they are likely orthologues, NCS-1 may have similar functions as *Drosophila* frequenin. Nevertheless the exact physiological functions of NCS-1 proteins in the various regions of the nervous system remain elusive.

***Ce*-NCS-1[94]** is likely the *C. elegans* orthologue of vertebrate NCS-1 and will be discussed in the 'Results and discussion' section.

Below, I will briefly describe the other members of the NCS family:

The neurocalcin/visinin NCS group

Neurocalcin[115-117]

Bovine neurocalcin exists as (at least) 6 isoforms. Their distribution seems to be restricted to the nervous system[103, 118]. Neurocalcin δ appears to be present in the cortex, adrenal gland, retina, olfactory bulb, cranial motor-neurons, and inner ear. In the cell it seems that neurocalcin is preferentially associated with microtubules, outer mitochondrial membrane, synaptic vesicles, and synaptic membranes. Neurocalcin δ (recombinant) binds 2 Ca^{2+} with high affinity (apparent K_D of 2.2μM)[119]. Like retinal NCS neurocalcin δ can be myristoylated[119].

Hippocalcin[102]

(Rat/human) Hippocalcin is mainly expressed in the hippocampus[102]. We found it abundantly it in hippocampal pyramidal cells, moderately in the dentate granule cells, cortical pyramidal cells and cerebellar Purkinje cells. During development (in the rat), hippocalcin expression

attains high levels early in development and decreases in the adult animal. In the cell hippocalcin seems to be localized in the cytoplasm and plasma membrane of cell bodies and dendrites. Hippocalcin as other NCS probably binds 2 or 3 Ca^{2+}. It can also be myristoylated[120]. This myristoylation is essential for its Ca^{2+} dependent membrane-binding properties: Hippocalcin bind to membrane in a Ca^{2+}-dependent manner showing a K_D at ~5µM of Ca^{2+}. Therefore hippocalcin should have a Ca^{2+} binding K_D of <=5µM.
Like NCS-1, *Ce*-NCS-1 and Vilip1, hippocalcin inhibits (in vitro) rhodopsin phosphorylation (see above, NCS-1 group)[94].
The hippocampus plays an important role in learning and memory; when it is destroyed, the ability to form new memories is largely lost, although previous established memories remain. Moreover, as Ca^{2+} is a key regulator of "molecular memory" phenomenon (like LTP, LTD, see above) this hippocampus almost specific NCS might be involved in memory formation processes, playing a role in synaptic efficacy control. A mouse knock-out mutant with no more hippocalcin expression might gives interesting insight into this question.

Vilip1[121]

Vilip1 (rat/chick Visinin-Like-Protein) is expressed in a sub-population of neurons throughout the brain and the retina. During development, in these neurons, vilip1 expression starts with the onset of terminal differentiation (therefore vilip1 is expressed in postmitotic neurons, like NCS-1)[122]. Vilip1 can bind two Ca^{2+} in a non-cooperative manner (unlike NCS-1) with a high affinity (K_D at ~2µM)[114]. Vilip1 is known to be myristoylated and show a Ca^{2+} dependent association with membrane. One of the identified binding partners of vilip is actin[123].
Like NCS-1, Vilip1 inhibits (in vitro) rhodopsin phosphorylation[94].

Vilip2[101]

Vilip2 (rat) which is closely related to (rat/chick) Vilip1 (89% a.a. identity) seems to be mostly expressed in the brain (cortex, hippocampus, hypothalamus, midbrain, olfactory bulb) but also in the testis (at a lower level). The highest amount of vilip2 mRNA is observed in the hippocampus. Only a small amount is detected in the cerebellum.

Vilip3[101]

Vilip3 from rat has 97% of amino acid identity with its human homologue, hlp2 (both proteins are therefore likely to be orthologues), and 69% with Vilip1. From phylogenetic studies vilip3 seems to be closer to neurocalcin than to vilip1 (see fig. 7). Vilip3 seems to be expressed mostly in the cerebellum; it is also found in the pons plus medulla oblongata, but not in other regions of the brain.

Other NCS proteins

***Ce*-NCS-2**[94] from *C. elegans*, is to date, the most divergent protein of the NCS family since it shares 45% of identity with *Ce*-NCS-1, the only other known (from the present study) *C. elegans* NCS protein, and only 37-49% of identity with other members of the NCS family[94]. *Ce*-NCS-2 seems to be a *C. elegans* specific NCS with no closely related sequences in other species. It forms a new 'group' in the NCS family phylogenetic tree (see fig. 7).

To summary, NCS proteins are highly related small EF-Hand CaBP predominantly expressed in neurons. They belong to the 4 EF-Hand CaBP group but they have generally only 2 or 3 functional EF-Hand Ca^{2+} binding motifs. Their exact *in vivo* function remains generally elusive. They are thought to be implicated in the fine modulation of neuron sensitivity and excitability.

As the object of this study is *C. elegans* Neuronal Calcium Sensor-1, predominantly expressed in the *C. elegans* chemo and thermo sensory system (see 'Results and discussion'), I will introduce bellow the *C. elegans* 'system', and then chemo/thermo-sensation in *C. elegans*.

The nematode *Caenorhabditis elegans*[124, 125]

***Caenorhabditis elegans* (*C. elegans*) is a small (adult is 1mm long) free-living soil nematode found commonly in many parts of the world. Its food source is bacteria.** There are two sexes, hermaphrodites that reproduce by self-fertilization (producing both oocytes and sperms), and males (arising spontaneously at low frequency) which can fertilize hermaphrodites. Hermaphrodites cannot fertilize each other. By self-fertilization a hermaphrodite can lay about 300 eggs during its life (by mating, 1000 eggs). *C. elegans* generation time (from the eggs to the adult form) is about 3 days under optimal conditions. Offspring hatch and develop through 4 stages also called larval stages (L1, L2, L3, and L4, see fig. 12B) although there is no metamorphosis. An adult is fertile for about 4 days and then lives for an additional 10-15 day. Therefore the total life span of a *C. elegans* is about 3 weeks.
The adult hermaphrodite has 959 somatic nuclei (+ ~1000 germ-line nuclei), and the adult male 1031. Its genome size is ~$100x10^6$ nucleotides, about 8 times that of the yeast *Saccharomyces* and one-half of the fly *Drosophila*. There are 5 autosome chromosomes and one sex chromosome (X). Hermaphrodites are diploid for all 6 chromosomes (therefore being XX for the sex chromosome), the males are diploid for the 5 autosomes but have only one X sex chromosome (XO). Males arise spontaneously (1 male for 500 animals) in hermaphrodite populations by X chromosome nondisjunction at meiosis.

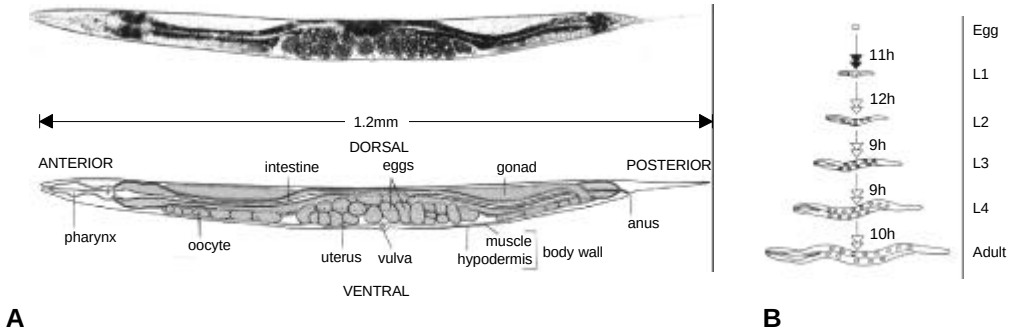


Fig. 11. A. General anatomy of the *C. elegans*, B. The different *C. elegans* developmental stages

Its general anatomy (see fig. 11A) like all nematodes can be described as 2 concentric tubes separated by a space, the pseudocoelom. The outer tube is formed by the external cuticle (the protective envelope), the hypodermis, muscles, and nerve cells surrounding a pseudocoelomic space that contains the intestine and (in the adult) the gonads. The inner tube is in the intestine, forming the digestive duct (enclosing a space topologically related to the outside).
The cuticle is made of 3 collagenous layers, secreted by the underlying hypodermis. The body-wall striated muscles of *C. elegans* are arranged into 4 strips running along the length of the animal. *C. elegans* feeds through the pharynx, that crushes and pumps food (bacteria) into the intestine. "Digested food" is excreted through the anus near its tail.
The hermaphrodite gonads are symmetrically arranged in 2 U-shaped lobs, where the ovary are formed, fertilized with the sperms (from the hermaphrodite itself: self-fertilization, or from a male during copulation: cross-fertilization) in the uterus, forming the eggs which will be then excreted through the vulva. The formation of the gonads is completed during the L4 stage. In the hermaphrodite, at this stage the gonads first produce sperm (~200), that are stored in the spermatheca (part of the uterus) then in the adult the sperm production ceases, and the gonads produce only oocytes (for only ~4 days). The male gonad is a arranged in a single U-shaped lob producing sperms which are stored in seminal vesicle and released during

copulation through a *vas deferens* to the *cloaca* in its tail. The male has a mating specialized fan-shaped tail with spicules, that are inserted into the hermaphrodite vulva during copulation, allowing the injection of male sperm in its uterus.
Most of its nervous system cells are located surrounding the pharynx, along the ventral midline, and in the tail. Many neuronal processes form a ring around the pharynx (called the nerve ring) or form process bundles running along the body (called nerve cords). Sensory neuron processes run anteriorly from the nerve ring and posteriorly from some tail neurons to sensory organs; sensilla. In *C. elegans* (and all nematodes), muscle cells send processes to motor-neurons in the cord (except pharyngeal muscles), rather than vice versa (neurons to muscle) as in other animals.
The *C. elegans* hermaphrodite entire nervous system is composed of 302 neurons and 56 glial and associated support cells (out of a total of 959 somatic cells, accounting for 37% of the somatic nuclei!). In the male there are 381 neurons and 92 supporting cells. The detailed structure and interconnections of the *C. elegans* nervous system are known at the electron microscopy level. **The *C. elegans* can move (forward or backward by undulatory movements), make exploratory movement with its head (as it feed), eat, defecate, lay eggs or mate. Adults exhibit sex-specific behaviors; egg-laying in the hermaphrodite, mating behavior in the male. *C. elegans* can also respond to a variety of external stimuli (touch, temperature, light, chemicals, osmotic pressure) by moving either toward or away from the stimulus and has the ability to modify its behavior on the basis of experience.** *C. elegans* has been shown to exhibit some behavioral plasticity, ranging from sensory adaptation and response fatigue to simple learning including habituation, sensitization, and classical conditioning. *C. elegans* also exhibits both short-term and long-term memory.

C. elegans as an experimental system[124, 125]

C. elegans is easily maintained in the laboratory, where it can be grown on agar plates or in liquid culture with *Escherichia coli* as a food source. A large number of worms can be grown in mass culture. Individual worms can be easily observed and manipulated under a dissecting microscope. **The *C. elegans* represents an excellent experimental organism especially for the study of metazoan nervous system and development. Its key features are its simplicity (anatomically, genetically and behaviorally), short life cycle, ease of cultivation in the laboratory, suitability for genetic analysis.** Moreover worms can be stored indefinitely in the frozen state.
A wide variety of mutant phenotypes has now been described. Mutants can be readily generated with chemical mutagens or radiation, allowing classical genetic screening. Reverse genetic can be done using mutagenesis by transposon insertion. A heterozygous mutation in a hermaphrodite will become homozygous in ¼ of that animal's self-progeny; therefore recessive alleles can be readily exposed. The hermaphrodite reproduction by self-fertilization has also the advantage to allow the generation of offspring even with severe behavioral or morphological defects that would make mating impossible. On the other hand, classical genetic crosses can be carried out using males. A great advantage of *C. elegans* genetic is also that large number of animals ($>10^6$) can be easily handled by routine laboratory methods. Transgenic worms are easily obtained by injecting DNA constructs in hermaphrodite gonad.
Moreover, there is a wealth of descriptive information available for the *C. elegans* such as the complete cell lineage (namely the timing, locations, and ancestral relationships of all cell divisions during development), the anatomy at the electron microscope resolution including the complete wiring of the nervous system, the description of (some) individual neuron function, the entire genome physical mapping and soon the complete genome sequence. ***C. elegans* is the first multicellular organism whose genome will be soon entirely sequenced (estimation: completion in 1998).** The *C. elegans* also shows extensive gene similarity with mammals.

Chemosensation and thermosensation in the *C. elegans*[126]

Chemosensation and thermosensation are two important sensory mechanism that *C. elegans* uses to interact with its environment. Chemosensation enables organisms to detect food, predators, potential mates, and other indicators of environmental quality. *C. elegans* is sensitive to numerous environmental chemical stimuli: it can chemotax to attractive compounds (mainly chemicals produced by bacteria, its food source), avoid noxious compounds[127], enters in an alternative development pathway (dauer larva) upon a pheromone signal[128], and find its sexual partner.

Thermosensation allows *C. elegan*s to track to a preferred temperature[129]. As a cold-blooded organism, *C. elegans* has a limited temperature range at which it is viable and fertile (~12-26°C). When grown at a temperature from 16° to 25°C and placed on a thermal gradient, the *C. elegans* will migrate to its growth temperature and then move isothermally. Behavioral adaptation to a new temperature takes several hours. In contrast, starved worms disperse from the growth temperature ('associated' with bad survival conditions).

C. elegans respond to a variety of chemical compounds[127, 130]

In the air-water interface where it lives, *C. elegans* can detect water-soluble chemicals ('tastants') in the micromolar range and volatile molecules ('odorants') in the picomolar concentration range. Many attractant compounds are known to be by-products of bacterial metabolism, allowing chemotaxis to food sources. Water-soluble chemicals (which diffuse slowly) are probably used for short-range chemotaxis to bacteria, and volatile chemicals can be used for longer-range chemotaxis to distant food sources. *C. elegans* can also avoid toxic or hazardous compounds (repulsive substances) and high osmotic strength (osmotic avoidance).

Among the 302 *C. elegans* neurons, 60 have dendritic endings that are ciliated and are therefore likely to be sensory. Most of the ciliated neurons are components of small sense organs called sensilla[131]. These sensilla are composed of one or more nerve endings and two or three non-neuronal support cells (a sheath cell and one or two socket cells). For 24 of these neurons (12 pairs of bilateral symmetric neurons), chemosensory/thermo sensory function has been directly demonstrated by observing behavioral deficits after laser killing of the cell[127].

Chemosensory/ Thermosensory organs and neurons[126]

In the hermaphrodite *C. elegans*, there are 3 types of sensilla 'opened' to the outside environment being potential chemo/thermo sensory 'organs' (with 26 neurons in direct contact with the outside): Amphids, phasmids, and inner labial sensory organs (see fig. 12)[131].

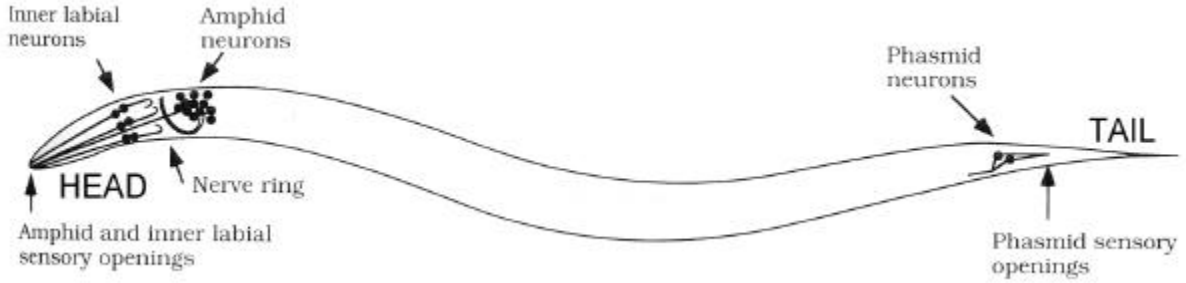


Fig. 12. Location of the major chemo/thermo-sensory organs. Dendrites extend from the sensory openings to neuron cell bodies; axons extend from cell bodies to enter the nerve ring (in the head) or the ventral nerve cord (in the tail). Only neurons on the left side of the animal are shown; all these neurons have symmetric homologues on the other (right) side.

The two bilaterally symmetric **amphids** in the worm's head contain the endings of 12 sensory neuron types (directly exposed to the outside, detecting mostly water-soluble chemicals: ASE, ADF, ASG, ASH, ASI, ASJ, ASK, ADL; embedded in the amphid sheath, detecting mostly volatile odorants: AWA, AWB, AWC, AFD[detecting thermal cues]). The two bilaterally symmetric **phasmids** in the tail contain each the endings of two neuron type (PHA, PHB both

exposed to the outside). The six bilaterally symmetric **inner labial sensory organs** in the head contain each the endings of two neuron types (IL1[,D,V] embedded, IL2[,D,V], exposed).
In addition to the amphid and inner labial organs, there are also other sensilla like organs found in the tip of the worm head: cephalic (with endings of neuron CEP [+ CEM in males]), outer labial quadrant (with endings of OLQ), and the outer labial lateral sensilla (with endings of OLL). Finally, there are also two classes of ciliated dendrites (from neurons BAG and FLP) that are not surrounded by support cells and are not part of a sensillum. Amphid neurons responsible for chemosensory/ thermosensory behavior and also for dauer formation have been identified through behavioral analysis of worms in which defined neurons were laser-ablated. Until now, cell ablations have not revealed functions for sensory organs other than amphids. Therefore **amphids seem to be the major chemo/thermo sensory organs**.
C. elegans males (adults) have 79 extra neurons compared to hermaphrodites. The mating structures in the mail tail contains 75 male specific neurons, out of the 79 extra neurons. Cell ablation experiments have revealed functions for many male-specific neurons in different steps of male mating. Over 20 male-specific neurons have exposed sensory endings and therefore are candidate chemoreceptor neurons that might detect pheromones during mating.

Flexibility of chemosensory and thermosensory responses

C. elegans displays flexibility in its chemosensory and thermosensory behavior based on its 'experience':

Sensory adaptation, sensory response modulation by starvation

Animals exposed to high concentration of an attractant odorant slowly lose their sensitivity to that odorant over a few hours[132]. These adapted animals still respond normally to other chemicals, including those that are detected by the same chemosensory neurons as the adapting chemical. This **sensory adaptation (fatigue of sensory response following prolonged exposure to sensory input)** is reversible; worms recover their sensitivity to the odorant (in few hours) after the complete removal of the adapting odorant.
The preferred temperature to which *C. elegans* will thermotax depends on the temperature at which it was raised Therefore thermotaxis intrinsically contains an experience-dependent component. Worms shifted from one temperature to another shift their preference to the new temperature over ~4hours. In contrast, starved (for 2-4 hours) worms avoid their cultivation temperature instead of approaching it, allowing the animal to disperse from its growth temperature ('associated' with starvation). Starvation also regulates chemotaxis responses. Starved worms appear to be suppressed in their responses to water-soluble attractants and some repellents but are enhanced in their attraction to some volatile attractants.
The molecular mechanisms of these behavioral flexibilities (adaptation, recovery, modulation by starvation) are (almost) unknown.

Learning and memory[126]

In the nonassociative forms of learning, an animal alters its behavior to a single stimulus. In associative learning, the animal learns to use (and remembers) a first stimulus to predict the presence (or the absence) of a second stimulus.
C. elegans has been shown to exhibit some behavioral plasticity, ranging from sensory adaptation and response fatigue (see above) to simple nonassociative learning including habituation (decrement in response to a repeated stimulus, distinct from adaptation), sensitization (increase in response to a wide variety of stimuli following a noxious stimulus), and even associative learning.
In response to chemosensory stimuli *C. elegans* has been shown to exhibit associative learning using classical conditioning paradigms. The worms can be conditioned to associate the presence of one ion to food and the presence of a second ion to the absence of food. They can be also conditioned to associate an attractive compound (diacetyl) to a noxious stimulus (acetic acid), avoiding the attractive chemical after this conditioning. The precise molecular mechanism of this phenomenon is unknown.

Aim of this work

C. elegans, a free-living nematode, is an excellent model organism to study neurobiology. The entire nervous system of (hermaphrodite) *C. elegans* is composed of only 302 neurons (out of 959 total somatic cells) whose detailed structures and interconnections have been characterized at the electron microscopy level. *C. elegans* can move, eat, defecate, lay eggs, and mate. It can respond to a variety of external stimuli and modify its behavior on the basis of experience. Moreover, *C. elegans* will be the first multicellular organism whose genome is completely sequenced. As this organism is also well suited for genetic analysis and often shows extensive gene similarity with vertebrates, it represents a very attractive system to study the function of NCS-1, a previously unknown member of the Neuronal Calcium Sensor (NCS) family isolated in our laboratory (initialy in the chick and then in the rat).

My objectives were:

- To isolate a *C. elegans* equivalent of NCS-1
- To study the distribution of this *C. elegans* NCS-1 homologue.
- To generate and to analyse the phenotype of a mutant *C. elegans* with the gene coding for this NCS-1 protein inactivated in order to get insights into the physiological role(s) of NCS-1

These studies together with my participation in other projects lead to the following publications:

1. Nef S, Fiumelli H, De Castro E, Raes M-B, Nef P (1995). **"Identification of a neuronal calcium sensor (NCS-1) possibly involved in the regulation of receptor phosphorylation."** J. Recept. Res. 15(1-4), 365-378. (1995)

2. De Castro E, Nef S, Fiumelli H, Lenz SE, Kawamura S, Nef P (1995). **"Regulation of rhodopsin phosphorylation by a family of neuronal calcium sensors."** Biochem. Biophys. Res. Commun. 216:133-140. (1995)

3. Nef S., Allaman I., Fiumelli H., De Castro E., Nef P. (1996). **"Olfaction in birds: differential embryonic expression of nine putative odorant receptor genes in the avian olfactory system."** Mechanisms of Development 55:65-77. (1996)

4. Schaad N., De Castro E., Nef S., Hegi S., Hinrichsen R., Martone M., Ellisman M., Sikkink R., Rusnak F., Sygush J., Nef P. **"Direct modulation of calmodulin targets by the neuronal calcium sensor NCS-1."** Proc. Natl. Acad. Sci. USA 93:9253-9258. (1996)

5. De Castro E., Nef P. Characterization and analysis of *Caenorhabditis elegans* Neuronal Calcium Sensor-1. In preparation.

Results and discussion

Cloning of *Caenorhabditis elegans* Neuronal Calcium Sensor-1 (*Ce*-NCS-1) cDNA

To determine whether Neuronal Calcium Sensor-1 (NCS-1) like sequences exist in the *C. elegans*, I screened at low stringency a *Caenorhabditis elegans* cDNA library using a chick NCS-1 cDNA probe. I was able to isolate several positive phages that contained inserts encoding a previously unknown protein highly related to chick/rat NCS-1. We named this protein *Caenorhabditis elegans*-Neuronal Calcium Sensor-1 (*Ce*-NCS-1). The *Ce*-NCS-1 deduced amino acid sequence has 75% identity with rodent/ avian NCS-1. This high homology suggests that these proteins are species homologues or orthologues.

To estimate the degree of gene diversity in the *C. elegans* NCS family, I screened at very low stringency the *Caenorhabditis elegans* cDNA library using *Ce*-NCS-1 cDNA as a probe. I isolated several positive phages that were shown to encode whether for *Ce*-NCS-1 or for a new unique protein related to the NCS family that we named *Ce*-NCS-2. This protein shows only 45% of amino acid identity with *Ce*-NCS-1, and 44% with rat/chick NCS-1. By this screenings no additional NCS genes were found in the *C. elegans*

Inhibition of rhodopsin phosphorylation

Both *Ce*-NCS-1 and *Ce*-NCS-2 proteins were overexpressed in *E. coli* and purified (see below enclosed article). Based on the fact that retinal NCS such as S-modulin, recoverin were shown to inhibit rhodopsin phosphorylation in a Ca^{2+} dependent way (see 'Introduction'), we wanted to test whether or not rat/chick NCS-1 and *C. elegans* NCS could act the same (*in vitro*). The purified proteins were used in a rhodopsin phosphorylation assay together with recoverin, S-modulin and vilip1. All six NCS proteins inhibit rhodopsin phosphorylation (and thereby inactivation) in a Ca^{2+}-dependent manner (controls with the ubiquitous calcium sensor calmodulin, or with ovalbumin had no effect on rhodopsin phosphorylation). It has been shown that the retinal NCS act by inhibiting in a Ca^{2+} dependent way rhodopsin kinase the enzyme needed to phosphorylate activated rhodopsin. By extension our results may indicate that other non-retinal NCS could act the same (although rhodopsin kinase is not their natural target) suggesting that other receptor kinase may be proprietary targets. Therefore these data suggest a potential role for NCS proteins in the calcium-sensitive phosphorylation of components of the signal transduction machinery.

These results were published in:

De Castro, E., Nef S., Fiumelli H., Lenz S. E., Kawamura S. and Nef P., **Regulation of rhodopsin phosphorylation by a family of neuronal calcium sensors.**, *Biochem. Biophys. Res. Commun.*, 216 (1995) 133-140.

See enclosed article starting next page:

Ce-NCS-1 and *Ce*-NCS-2 cDNA

Both cDNA, ranging from a 5' SL1 splice leader sequence (see below, legend of figures 13 and 14) to a poly(A) tail, are complete. They show a short 5' untranslated region (UTR) of about 50bp with part of the SL1 leader sequence. *Ce*-NCS-1 cDNA has a small 3' UTR of about 100bp. *Ce*-NCS-2 cDNA has a larger 3' UTR of about 750bp. Cloned cDNA total size (including cloning restriction enzyme linkers) for *Ce*-NCS-1 is 773bp and for *Ce*-NCS-2 1390bp (see fig. 13 and 14).

Sequences:

fig. 13. ***Ce*-NCS-1 cDNA**

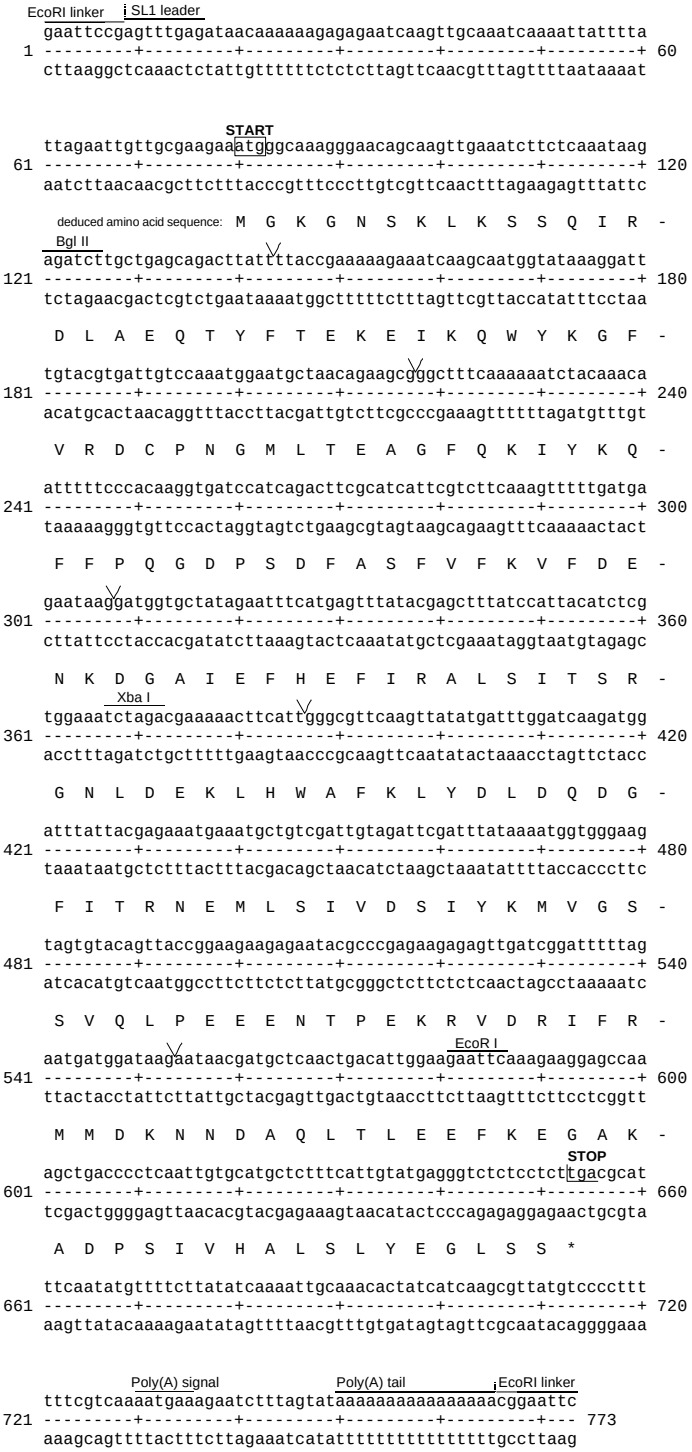


fig. 14. ***Ce*-NCS-2 cDNA**

fig. 13 and 14:

SL1 Leader: It has been shown that a large fraction (>= 40%) of RNA messenger in *C. elegans* are trans-spliced to a 22- nucleotide (nt) leader sequence named SL1[125] (obviously the conventional eucaryotic cis-splicings can also take place). Both *Ce*-NCS-1 and *Ce*-NCS-2 cDNA are long enough to show part (8 last nt for *Ce*-NCS-1, 9 last for *Ce*-NCS-2) of this leader sequence (just after the library Eco RI linker). Therefore both *ncs-1* and *ncs-2* messenger appears to be trans-spliced with SL1 leader. The function of the SL sequence on mRNA is unknown; there is no obvious pattern of commonality between messages that receive the SL1 sequence. SL sequences might stabilize the messenger RNA or enhance its translational efficiency. The SL1 leader originate from a small RNA transcribed (principaly) from the 1kb repeat (present at ~110 copies in the genome) that also contains the 5S ribosomal RNA genes. This SL1 RNA donates its 5-most 22nt to target messengers that have in their 5' UTR (untranslated region) a splicing 3' acceptor site lacking an (internal) upstream 5' splice donor (needed to allow a classical trans-splicing).

Poly(A) signal: Like in mammals, *C. elegans* 3' end message maturation includes cleavage and polyadenylation at specific 3' end sites of the primary transcript[133]. Clivage and addition of a poly-A tail (polyadenylation) occurs usually 13nt downstream a poly(A) signal sequence matching a AAUAAA consensus {with frequent variations in the 1st and 4th (A being often replaced by a G) position}. Putative poly(A) signal sequences are; for *Ce*-NCS-1: AATGAA (14nt before poly(A) tail); and for *Ce*-NCS-2: AATAAA (15nt before poly(A) tail).

position of spliced introns are indicated by arrow heads ("V") in the cDNA nucleotide sequence (from comparison with the genomic sequence, see "cloning of *ncs-1* and *ncs-2* genes").

Some current **restriction enzyme cleavage sites** are over-lined (for EcoR I, Xba I, Bgl II, Nco I, Pst I, Sac I). **ATG** initiator codons and **stop** codons are also indicated.

Ce-NCS-1 and *Ce*-NCS-2 proteins

Both proteins are related to the NCS family. *Ce*-NCS-1 is highly related to rat/chick NCS-1 protein (with 75% amino acid identity). *Ce*-NCS-2 represents to date the most divergent protein of the NCS family since it shares only 37-49% of identity with other members of the NCS family, alone forming a new 'group' in the NCS family phylogenetic tree (see 'Introduction'). With no known equivalent genes in other species, *Ce*-NCS-2 is probably a nematode specific NCS. Some characteristics of both *Ce*-NCS-1 and *Ce*-NCS-2 proteins are shown in table 2:

Analysis	***Ce*-NCS-1 protein**	*Ce*-NCS-2 protein
Molecular Weight	22023.50	21986.80
Length	191	190
1 microgram =	45.406 pMoles	45.482 pMoles
Molar Extinction Coefficient	19180 ±5%	16860 ±5%
1 unit Absorbance at 280nm {1 A(280)}=	1.15 mg/ml	1.30 mg/ml
Isoelectric Point	5.02	5.00
Charge at pH 7	-6.59	-6.82

Table 2. Theoritical characteristics of *Ce*-NCS-1 and *Ce*-NCS-2 proteins (using GCG package).

Their amino acid sequence identity with some other NCS proteins are shown in table 3:

% a.a. identity with:	***Ce*-NCS-1 protein**	*Ce*-NCS-2 protein
***C. elegans* NCS-1 (*Ce*-NCS-1)**	100	44.62
Rat/chick NCS-1	75.26	44.09
***S. cerevisiae* NCS-1 (*Sc*-NCS-1)**	58.42	43.55
***S. pombe* NCS-1 (*Sp*-NCS-1)**	65.08	44.86
***Drosophila* Frequenin**	70.05	45.11
Bovine neurocalcin	58.42	48.92
Rat/chick vilip(1)	57.14	46.82
Rat vilip2	55.03	47.34
Rat vilip3	58.95	49.46
Rat hippocalcin	57.89	48.92
Chick visinin	38.10	35.98
Mouse recoverin	43.68	44.44
Frog S-modulin	40.53	43.39
C. elegans NCS-2 (*Ce*-NCS-2)	44.62	100
Human Calmodulin	20.95	23.65

Table 3. Amino acid identity of *Ce*-NCS-1and *Ce*-NCS-2 with some other NCS and calmodulin.

Amino acid sequence alignment / consensus site analysis

Ce-NCS-1 and *Ce*-NCS-2 deduced amino acid sequence were aligned (using the GCG package[134]) with some other NCS sequences. All these sequences were analyzed by the MOTIF program[134] to find known "motifs", including (consensus) putative "functional" sites, structural domains and putative post-transcriptional modification sites (see fig 15.).

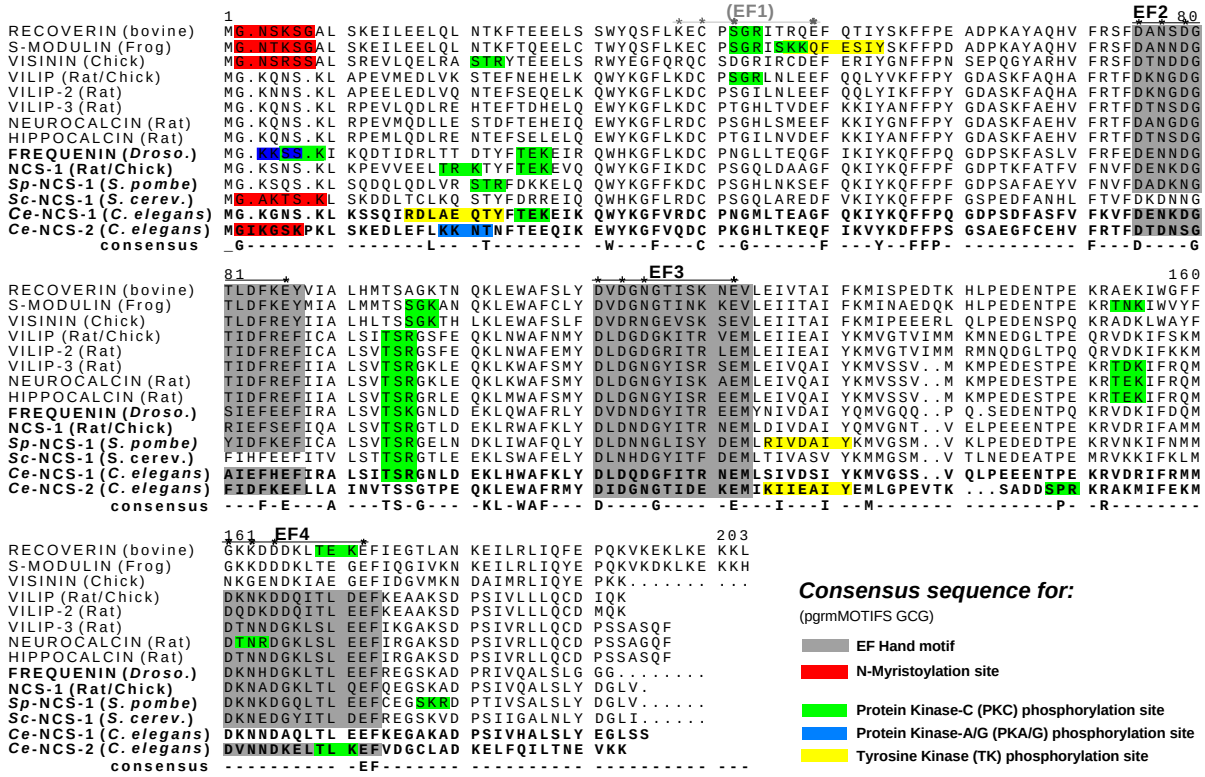


fig. 15. Alignment and analysis of *Ce*-NCS-1 and *Ce*-NCS-2 sequences together with some other NCS sequences. The consensus line indicates the amino acids (a.a.) conserved in all the sequences. EF hand loop domains (EF1-EF4) are underlined. In each member of the NCS family, EF1 is degenerated (indicated with brackets). * indicates the a.a. in a EF-hand that could directly coordinate to Ca^{2+} ion. MOTIF analysis; some consensus sites are boxed in colors, see legend "Consensus sequence for:".

These computer analyses are just indicative and the information they provide should be considered carefully. Moreover, found ASN-glycosylation sites and Casein kinase II phosphorylation sites were not taken in account as they are probably not physiologically relevant for those cytosolic proteins.

For *Ce*-NCS-1 the motif program find two putative sites for phosphorylation by protein kinase C (PKC) and one by tyrosine kinase (TK). The program also detect 2 consensus EF-Hand motifs (allowing the binding of Ca^{2+}, see "Introduction") although they are 4 identifiable EF-Hand motifs in all these NCS proteins (some of them being highly degenerated, see "Introduction"). EF1 is in all case degenerated. *Ce*-NCS-1 EF4 (the last EF-Hand) is not recognized by the motif program as a valid EF-Hand probably because of its alanine (A) residue at position 15 (relative to the EF-Hand), instead of a consensual glycine (G). Nevertheless all the Ca^{2+} coordinating side-chains are "compatible", and this EF-Hand may be functional. We must note that from biochemical studies it has been shown that rat/chick NCS-1 binds only 2 Ca^{2+}[114] although the motif program predicts 3 valid EF-Hands.

For *Ce*-NCS-2 the program find two putative sites for phosphorylation by protein kinase C (PKC), one by tyrosine kinase (TK) and one by cAMP/ cGMP protein kinases (PKA/G). The program also detect 3 consensus EF-Hand motifs, and a N-myristoylation site (myristoylation: see 'Introduction').

Genomic southern blot analysis

To verify that the genes of the isolated *Ce*-NCS-1 and *Ce*-NCS-2 cDNA really exist in the wild type worm genome and to estimate the number of *ncs-1* and *ncs-2* related gene, I performed a Southern blot analysis (probe hybridization on blots of digested genomic DNA) at medium stringency with genomic *C. elegans* DNA using *Ce*-NCS-1 and *Ce*-NCS-2 cDNA probes. From the data (show in fig. 16) both genes are present. They appear to be single copy genes, with no other closely related sequences (as the observed hybridation patterns can be assigned to single sequences).

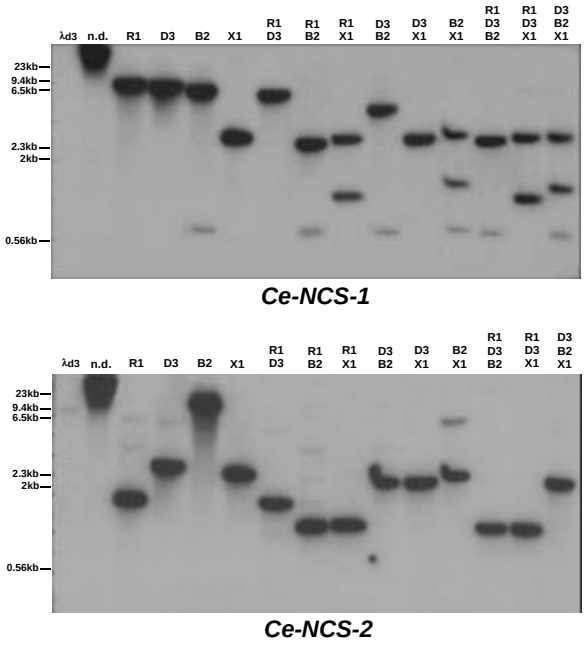


fig 16. Genomic southern blots with *Ce*-NCS-1 and *Ce*-NCS-2 cDNA as probes.
λd3: size marker; used restriction enzymes: R1: EcoRI, D3: Hind III, B2: Bgl II, X1: Xba I.

Experimental procedure

Genomic DNA isolation: ~1-2 grams of purified wt *C. elegans* (from a liquid culture[125]) were crushed with a cold mortar in presence of liquid nitrogen, resuspended in TE (10mM Tris-HCl pH 8. 0, 0. 1M EDTA pH 8. 0), incubated for 1 hr at 37°C in the extraction buffer (10mM Tris-HCl pH 8.0, 0.1M EDTA pH 8.0, 0.5% SDS, 20 µg/ml RNase), and digested for 3 hr at 37°C with 100 µg/ml of proteinase K, and extracted 2-3 times with phenol equilibrated in 0.5 M Tris pH 8.0. The genomic DNA was precipitated with ethanol and the pellet washed twice with ethanol 70%, and resuspended in TE.

Genomic southern blot: The DNA (10 µg/lane) was digested overnight with restriction enzymes, electrophoresed on 0.8% agarose gels in presence of 1 x Tris borate EDTA, depurinated with a HCl 0.25M solution, denatured and transferred overnight to nylon membrane (Positive Membrane, Appligene Oncor) in 0.4 M NaOH according to the manufacturer transfer protocol. Blots were prehybridized for 2 hr at 62°C in 5 x Denhardt's (0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 5 x SSPE (20x SSPE: 3.6M NaCl, 0.2M NaH_2PO_4, 20mM EDTA, pH adjusted to 7.4), 0.1% SDS, 100 µg/ml denatured salmon sperm DNA (sspDNA) and hybridized overnight at 62°C in 1 x Denhardt's, 5 x SSPE, 0.1% SDS, 100 µg/ml denatured sspDNA, and ~1 10^7 cpm/ml of denatured radioactive probe. Random labeling of EcoRI fragment from *Ce*-NCS-1 (580bp) and *Ce*-NCS-2 (560bp) cDNA spanning the 5' untranslated region and ~80% of the coding region were used (with ^{32}P-radionucleotides) to produce the radioactive probes. Blots were washed in SSPE solutions, the final wash for 30 min. at 65°C in 1 x SSPE, 0.2% SDS. Autoradiography was performed with an intensifying screen for 3 days at -70°C.

Northern blots

To verify that ncs-1 and ncs-2 genes are expressed in the wild type *C. elegans*, to compare the relative abundance of both messenger and to determine their size, I performed Northern blots analysis with *C. elegans* mixed-stage total RNA using both *Ce*-NCS-1 and *Ce*-NCS-2 cDNA probes. From the data (show in fig. 17) both genes appears to be expressed in wild type *C. elegans* giving single bands (therefore there is apparently no alternative splicing), *Ce*-NCS-2 messenger seems to be more abundant than *Ce*-NCS-1 messenger. *Ce*-NCS-1 messenger size is about 1kb, *Ce*-NCS-2 about 1.4kb (these approximate sizes correspond to the size of the cloned cDNA).

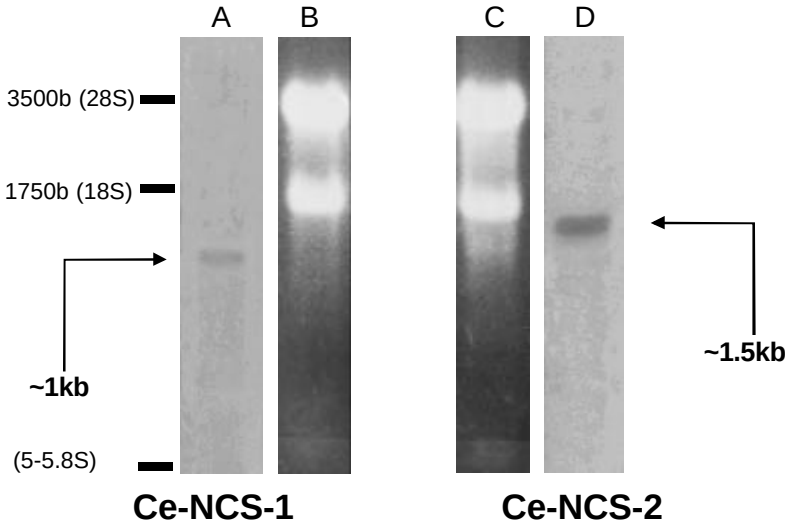


fig 17. Northern blots analysis of *ncs-1* and *ncs-2* transcripts
A,D: Autoradiography of northern blots visualizing *Ce*-NCS-1 and *Ce*-NCS-2 transcripts.
B,D: Corresponding total RNA with ethidium-bromide in the gel, visualized under U.V. light

Experimental procedure

Total *C. elegans* RNA isolation: 1 gr of purified mixed stage *C. elegans* (from a liquid culture) were resuspended in RNA Lysis Buffer (4M guanidinium isothiocyanate, 50 mM Tris-HCl (pH 7.5), 25mM ethylenediaminetetraacetic acid (EDTA), 1% N-lauryl sarcosine, 1% β-mercapto-ethanol), pellet was frozen in liquid nitrogen. Frozen worms were crushed (into a fine powder) with a cold mortar in presence of liquid nitrogen, resuspended in RNA Lysis Buffer, extracted with phenol (liquid with 10% H_2O; Fluka) equilibrated in 0.3 M sodium acetate pH 4.0, reextracted with phenol (same as before) / chlorophorm 1:1, precipitated with isopropanol. The pellet was resuspended in 10mM EDTA. The solution was reextracted with phenol, phenol/chloroform, chloroform and reprecipitated with ethanol and the pellet washed twice with ethanol 70%, and resuspended in 10mM EDTA.

Northern blots: 20 µg of total RNA /lane were denatured by heating 2 min at 95°C in 1x MOPS buffer (20mM 4-morpholinepropanesulfonic acid pH 7.0, 8mM sodium acetate, 1mM EDTA) in presence of 6.2% formaldehyde. Samples (with 1µg/ml ethidium bromide) were fractionated by electrophoresis in 1% agarose minigels in 1x MOPS buffer containing 2% formaldehyde. RNA was then blotted on Appligene Oncor positive membrane (transfer by capillarity in 3M NaCl, 8mM NaOH). Blots were prehybridized for 4 hr at 42°C in NaCl 1M, 5 x Denhardt's (0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 50% deionized formamide, 1% SDS, 100 µg/ml denatured salmon sperm DNA (sspDNA) and hybridized overnight at 42°C in, NaCl 1M, 5 x Denhardt's, 50% deionized formamide, 1% SDS, 10 µg/ml denatured sspDNA and ~1.10^7 cpm/ml of denatured radioactive probe. Random labeling of EcoRI fragment from *Ce*-NCS-1 (580bp) and *Ce*-NCS-2 (560bp)

spanning the 5' untranslated region and ~80% of the coding region were used (with ^{32}P-radionucleotides) to produce the radioactive probes. Blots were washed in SSPE solutions (20x SSPE: 3.6M NaCl, 0.2M NaH_2PO_4, 20mM EDTA, pH adjusted to 7.4), the final washes for 2x 20 min. at 65°C in 2 x SSPE, 2% SDS and then for 15 min. at RT in 0.1x SSPE. Autoradiography was performed with an intensifying screen for 15 days at -70°C.

Production and purification of recombinant *Ce*-NCS-1 and *Ce*-NCS-2 proteins

A basic feature of calcium modulators such as calmodulin and NCS-1 is that Ca^{2+} induces a conformational change resulting in the exposure of strong hydrophobic surface(s), allowing thereby interaction with other (target) proteins. This propriety greatly facilitate the purification of these proteins: In presence of Ca^{2+} the proteins with exposed hydrophobic surfaces will be readily retained on an hydrophobic column, which will play the role of an affinity column. These bound proteins are then eluted by removing the free Ca^{2+} from the media using a Ca^{2+} chelator such as ethylenediaminetetraacetic acid (EDTA). In presence of low Ca^{2+} concentration, these proteins show much less exposed hydrophobicity and are therefore eluted from the column.
I overexpressed *Ce*-NCS-1 and *Ce*-NCS-2 in *E. coli* using the complete open reading frame of both cDNA inserted in an inducible expression vector. The overexpressed proteins were purified (see fig. 18) following the principle described before.

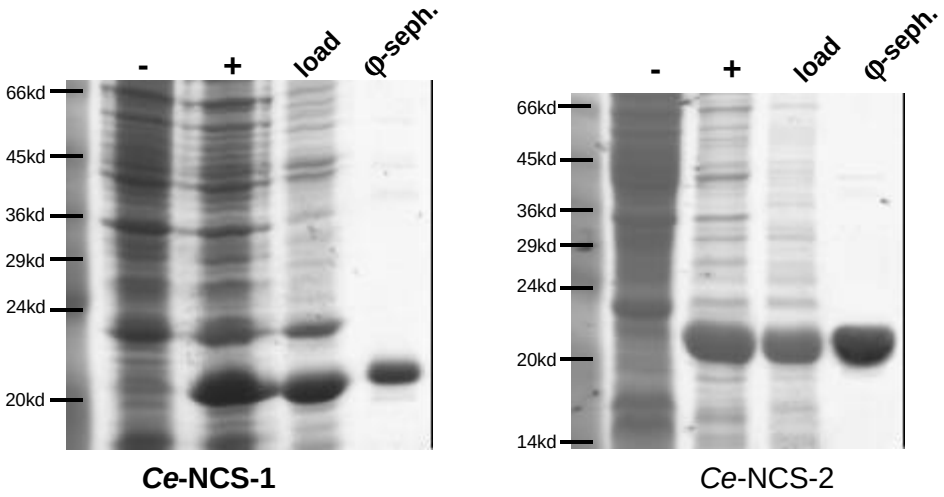


fig. 18. Purification of recombinant *Ce*-NCS-1 and *Ce*-NCS-2 proteins:
Sodium dodecyl sulfate-polyacrylamide gel electrophresis (SDS-PAGE) showing different steps in the purification of recombinant *Ce*-NCS-1 and *Ce*-NCS-2 proteins (gels are stained with coomassie blue).
Lanes: **-**: total E. coli extract, not induced; **+**: total E. coli extract induced; **load**: extract loaded on hydrophobic column (phenyl-sepharose) after ammonium sulfate and isoelectric precipitation; **φ-seph**: (phenyl-sepharose) purified eluted fraction after hydrophobic column.

The purified *Ce*-NCS-1 and *Ce*-NCS-2 proteins were used to generate antibodies (see below: "production of polyclonal antibodies"). They were also used in several *in vitro* assays: rhodopsin phosphorylation assay (see above: "Inhibition of rhodopsin phosphorylation" and enclosed article), 3':5' - cyclic nucleotide phosphodiesterase assay (see below: "Activation of 3':5' - cyclic nucleotide phosphodiesterase"). Moreover to prove that these proteins are really Ca^{2+} binding proteins, I performed a ^{45}Ca overlay assay (see below: "^{45}Ca overlay").

Experimental procedure

See above: enclosed article "*Regulation of rhodopsin phosphorylation by a family of neuronal calcium sensors.*"

^{45}Ca overlay

Both *Ce*-NCS-1 and *Ce*-NCS-2 sequences belong to the superfamily of EF-Hans Ca^{2+} binding proteins, they are retained on a hydrophobic column in presence of Ca^{2+} and released using a Ca^{2+} chelator (see above: "Production and purification of recombinant *Ce*-NCS-1 and *Ce*-NCS-2 proteins") suggesting that they are indeed Ca^{2+} binding proteins. Nevertheless to prove that *Ce*-NCS-1 and *Ce*-NCS-2 are really genuine Ca^{2+} binding proteins I performed a direct Ca binding assay. After a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE[135]) proteins can be transferred to a nitrocellulose membrane (like for western blots). Although the proteins are denatured by the electrophoresis, after the transfer they can retain (regain) part of their functional activities. It has been shown that Ca^{2+} binding protein after SDS-PAGE and transfer to nitrocellulose can still bind Ca^{2+}. By incubating the membrane with radioactive calcium (isotope ^{45}) it is possible to detect the Ca^{2+} binding proteins by autoradiography. This technique is named: ^{45}Ca overlay[136].

I performed this overlay assay with (see fig. 19) *Ce*-NCS-1, *Ce*-NCS-2 together with rat/chick NCS-1 (a positive control, as this protein has been shown by biophysical studies to bind 2 Ca^{2+} [114]), *Sc*-NCS-1 (yeast *Saccharomyces cerevisiae* -NCS-1), recoverin, hippocalcin and as negative controls, some proteins that do not bind calcium: bovine serum albumine, and molecular weight markers (Biorad) composed of: phosphorylase b (97.4kD), serum albumin (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), trypsin inhibitor, lysozyme (14.4 kD).

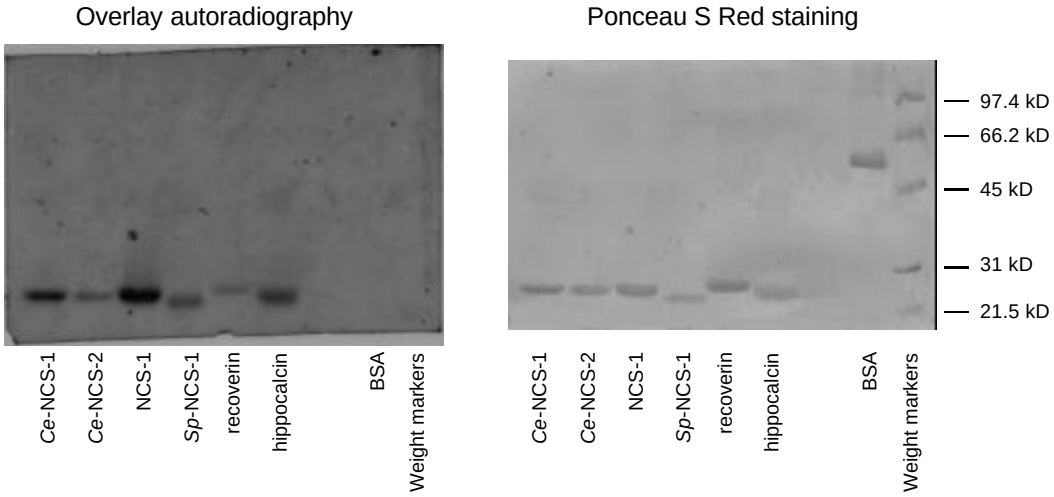


Fig. 19. ^{45}Ca overlay assay. Overlay autoradiography shows proteins that bind calcium. Ponceau S Red staining of the membrane (after autoradiography) shows all the loaded proteins (10µgr/protein loaded). BSA: bovine serum albumine; Weight markers: see text. Note that the negative controls are not marked in the overlay autoradiography (indicating that they indeed do not bind Ca^{2+})

From these data we can see that all the tested NCS can bind calcium: ***Ce*-NCS-1 and *Ce*-NCS-2 can therefore be considered as genuine Ca^{2+} binding proteins**.

Experimental procedure

The recombinant purified proteins; *Ce*-NCS-1, *Ce*-NCS-2, NCS-1, Sc-NCS-1, recoverin, hippocalcin together with bovine serum albumine (BSA) and weight markers (see above) were run on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE[135] with 13% polyacrilamide gel, in the presence of 2mM EDTA), bloted on nitrocellulose membrane. After transfer, the membrane was washed 3 times over an hour in a solution containing 60mM KCl, 5mM $MgCl_2$, and 19mM imidazole-HCl (pH 6.8). The membrane was incubated in the same buffer containing 2 mCi/liter $^{45}Ca(Cl_2)$ for 10min. The membrane was then washed in 50% ethanol for 5 min. The membrane was dried at room temperature for 3h then in a stream of hot air (from a hair drier) for 1 min. Autoradiography was performed for 2 days at room temperature. To visualize all loaded proteins the membrane was then stained in Ponceau S red for 10min. and washed in water 4 times 10sec.

Activation of 3':5' - cyclic nucleotide phosphodiesterase

Due to the rather limited number of Ca^{2+}-dependent pathways, NCS-1 targets could overlap with CaM targets. We therefore tested effect of NCS-1 on different CaM targets. For rat/chick NCS-1 we have shown that it can, both *in vitro* and *in vivo*, substitute for or potentiate calmodulin[106] (CaM) functions (There is only 21% of a.a. homology between CaM and NCS-1). We observed that *in vitro*, rat/chick NCS-1 directly activates two Ca^{2+}/CaM-dependent enzymes: 3':5' - cyclic nucleotide phosphodiesterase (PDE) and calcineurin, and is also capable of potentiating the activity of nitric-oxyde synthase (NOS) -Ca^{2+}/CaM. Furthermore, *In vivo*, NCS-1 alone partially restores the wild-type behavior in calmodulin-defective *cam*[1] *Paramecium mutants* (see 'Introduction'). Nevertheless NCS-1 can not activate the well-known CaM target CaM kinase II, nor potentiate CaM kinase II activation by CaM (Hegi S. *et al*. "Calcium signaling by the neuronal calcium sensor NCS-1: interaction with calmodulin antagonists and enzymatic specificity", submited). Therefore NCS-1 can replace CaM or potentiate CaM effect in some cases but not all.

Recombinant NCS-1 was able to substitute for CaM and to function as a direct PDE activator giving a 3.8±0.3 fold (n=7, 1μM NCS-1, 100μM Ca^{2+}) on PDE activity with a half-maximal effect (EC_{50}) at ~200 nM (CaM gives a ~6 to 7 fold increase with an EC_{50} of ~1nM). After these (published) studies[106] on rat/chick NCS-1, recombinant *Ce*-NCS-1 was also tested for PDE activation. *Ce*-NCS-1 shows the same effect on PDE activation as NCS-1, giving a 4.6±1.5 stimulation fold (n=5, 1μM *Ce*-NCS-1, 100μM Ca^{2+}) with the EC_{50} at ~200 nM.

Both NCS-1, *Ce*-NCS-1 – dependent activation of PDE were saturable and concentration dependent. Both proteins had no endogenous PDE activity by themselves. The effect of both proteins is slightly Ca^{2+} dependent. In the absence of Ca^{2+} their effect on PDE activity is low (~2 fold stimulation) but not negligible. Their maximum effect was observed at ~1μM free Ca^{2+} (~4 fold stimulation), whereas at higher Ca^{2+} concentration PDE stimulation fold was barely reduced.

Other NCS such as recoverin, hippocalcin, or vilip1 have no effects on PDE activity. *Ce*-NCS-2 was not tested. These *in vitro* data (together with the rhodopsin phosphorylation data) may suggest that *Ce*-NCS-1, together with NCS-1 (likely its orthologue) have multiple targets, being multifunctional (like CaM) modulators. Nevertheless the real in-vivo targets and the exact physiological functions of NCS-1 proteins remain elusive.

Experimental procedure

Done by N.C. Schaad. As described in article: "Schaad N.C., de Castro E., Nef S., Hegi S., Hinrichsen R., Martone M.E., Ellisman M.H., Sikkink R., Resnak F., Sygush J. and Nef P., Direct modulation of calmodulin targets by the neuronal calcium sensor NCS-1, *Proc. Natl. Acad. Sci. USA*, 93 (1996) 9253-9258."

Production of polyclonal antibodies

Recombinant *Ce*-NCS-1 and *Ce*-NCS-2 proteins were used to immunized New Zealand white rabbits. The rabbit serum containing specific antibodies (Ab) were purified by immunoaffinity chromatography on pure antigen (the recombinant proteins themselves). These highly specific purified polyclonal antibodies were used to determined the *in-situ* localization of *Ce*-NCS-1 proteins in the *C. elegans* (see below. "immunolocalization of *Ce*-NCS-1"). The antibodies tested by ELISA (enzyme-linked-immuno-assay) and Western blots (see fig. 20).
From **ELISA** data we observe that the titer of antibodies are high (mid-point for anti-*Ce*-NCS-1 Ab is at dilution 1:100'000, for anti-*Ce*-NCS-2 Ab at 1:20'000) and that they are selective (anti-*Ce*-NCS-1 on *Ce*-NCS-2 protein react slightly only at dilution 1:100, there is no cross reaction of anti-*Ce*-NCS-2 on *Ce*-NCS-1). W**estern blots** analysis showed that there is no cross reaction of Ab with antigens (anti-*Ce*-NCS-1 Ab doesn't recognize *Ce*-NCS-2 protein at the used dilution and *vice versa*) even with high amounts of purified antigens (1µgr). Both antibodies stain a band of the right size in *C. elegans* total extract. In the total extract *Ce*-NCS-1 protein seems to be less abundant than *Ce*-NCS-2 (both Ab recognize as efficiently purified proteins, data not shown), consistent with northern blot data were *Ce*-NCS-1 messenger is also less abundant than *Ce*-NCS-2 messenger.

ELISA:

anti-*Ce*-NCS-1 Ab on *Ce*-NCS-1 prot, dilution mid-point : 1:100'000

anti-*Ce*-NCS-2 Ab on *Ce*-NCS-2 prot, dilution mid-point : 1:20'000

Western Blots:

C. elegans total protein extract: **250µgr**
Ce-NCS-1 recombinant protein: **1µgr**
Ce-NCS-2 recombinant protein: **1µgr**

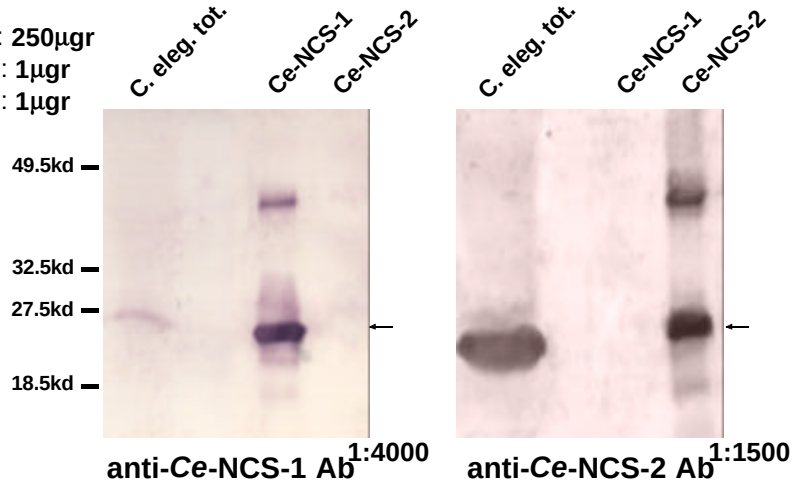


fig. 20. Immuno-affinity purified antibodies against *Ce*-NCS-1 and *Ce*-NCS-2 proteins analysed by ELISA and Western Blots. Both antibodies stain a band of the right size, around 22kD (see text). On the lanes loaded with purified recombinent proteins, the stained bands at ~42kD represent *Ce*-NCS-1 and *Ce*-NCS-2 dimers (as at high concentration these purified proteins tend to dimerize).

Experimental procedure

Production of polyclonal antibodies: The recombinant purified proteins were precipitated in 25% methanol. The suspension was dialyzed against phosphate-buffered saline (PBS) from Harlow and Lane[137] (10mM phosphate-buffered saline, 138mM NaCl, 2.7 mM KCl, pH 7.4) mixed with Freund's adjuvant. Immunization was performed by injecting a total of 250µgr of antigen to New Zealand white rabbits according to standard procedures[137]. The rabbit serums were purified by immunoaffinity chromatography on the corresponding purified antigens (~2mg of protein per ml of resin) coupled to vinylsulphone-activated agarose (according to the manufacturer protocol: Sigma Chemical Co); the highly purified polyclonal antibodies (Ab) were eluted with 0.1M glycine-HCl, pH 2.3, neutralized, dialyzed against PBS, concentrated and kept in 3mM NaN_3, 10mg/ml bovine serum albumin.

ELISA were performed as described[138]. The wells were coated with 500ng of recombinant protein and the assay were performed with antibody dilutions ranging from 1:100 to 1:1000000 in PBS- 0.5% dry-milk. The conversion of the phosphate substrate (SIGMA 104105) was determined with a Minireader II (Dynatech Microtiter Systems) at 415nm. The Ab dilution mid-point correspond to the Ab dilution giving a half-maximal response, representing the titer of the Ab.

***C. elegans* total protein extract** was made using ~1 gram of purified wt *C. elegans* [125](from a liquid culture). The worms were crushed (into a fine powder) with a cold mortar in presence of liquid nitrogen, resuspended in 5 vol. of 50mM Tris-HCl pH 7.4, 2mM EDTA, 2% SDS, 1% βME, 0.5µg/ml pepstatin, incubated 15 min at room temperature (RT), sonicated tremendously 8' (on ice) and centrifuged at 20000g for 30 min. The proteins in the supernatant were precipitated in presence of 3% trichloroacetic acid (TCA), resuspended in 500µl of PBS and neutralized with NaOH. The resulting protein extract concentration as determined by the method of Bradford[139] was about 5mg/ml.

Western blots: SDS-PAGE[135] (13% polyacrilamide gel) was performed with total *C. elegans* extract (250µgr) and the two purified recombinant proteins (1µgr). Proteins were transferred to nitrocellulose membranes, blocked with 0.5% dry-milk in PBS for 2h at RT. The membranes were then probed for 1h with the primary antibodies (diluted at 1:4000 for anti-*Ce*-NCS-1 Ab and 1:1500 for anti-*Ce*-NCS-2 Ab) in PBS- 0.5% dry-milk. The primary antibodies were visualized by means of an alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody (Sigma A3687 used at a dilution of 1:32000) and the nitro-blue tetrazolium chloride (NBT)/ 5-bromo-4-chloro-3-indolyl phosphate [p-toluidine salt] (BCIP) substrates giving a purple-black signal.

Immunolocalization of *Ce*-NCS-1 (indirect immunofluorescence)

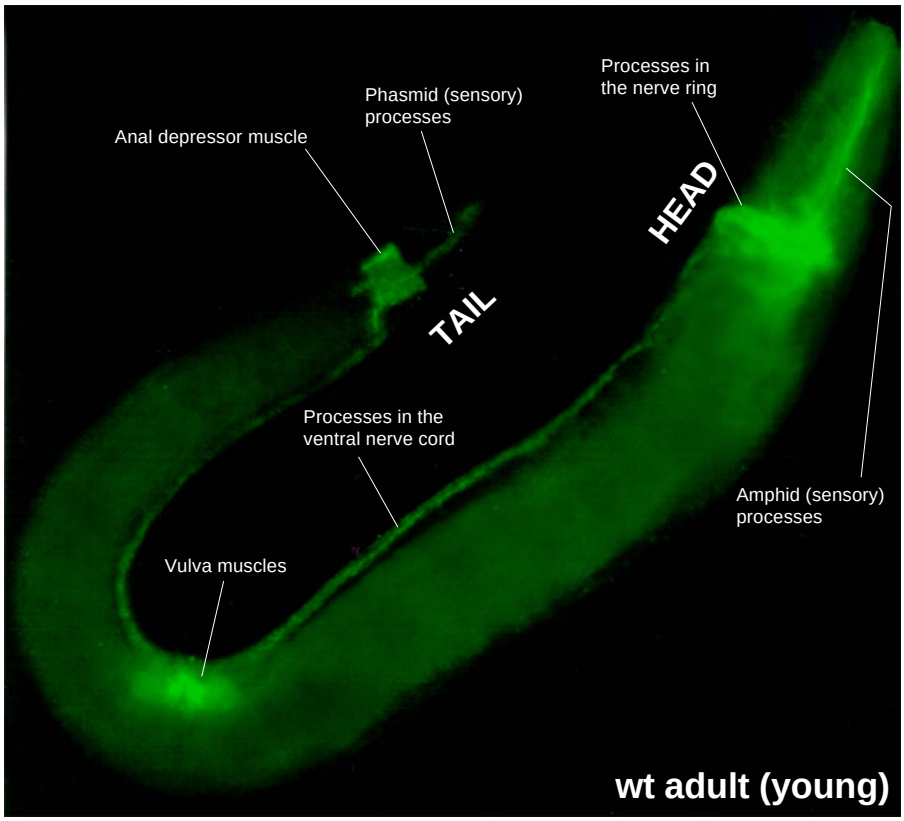
To localize *Ce*-NCS-1 protein in *C. elegans* I have used the anti- *Ce*-NCS-1 affinity purified anti-body (see above 'Production of polyclonal antibodies') to perform immunolocalizations studies. Because of the transparency and the small size of the animal, immunofluorescence staining can be clearly observed in whole mounts and does not require sectioning. Fixed (and permeabilized) worms were incubated with the rabbit anti-Ce-NCS-1 antibody; the antibody-antigen(*Ce*-NCS-1) association was then revealed by a second incubation using a fluorescently labeled antibody directed against rabbit antibodies. The resulting worms (see fig. 21) exhibited therefore fluorescently stained *Ce*-NCS-1 protein allowing the determination of *Ce*-NCS-1 localization. As controls I have also stained *ncs*-1 knock-out mutant worms (see fig. 21D,G,I and below, 'Isolation of a *ncs-1* knock-out mutant').

Note that no optimum condition of fixation was found to allow proper localization of the *Ce*-NCS-2 protein using anti-*Ce*-NCS-2 antibody. In addition there is (yet) no *ncs*-2 knock-out mutant as a good negative control. Anyway, the true object of this present study is *Ce*-NCS-1.

Fig. 21. In situ localization of *Ce*-NCS-1 using anti-*Ce*-NCS-1 antibody (indirect immunofluorescence) on wt or *ncs*-1 knock-out hermaphrodite C. elegans (see text).
A. whole wt young adult; B. whole wt L1 larva; C. wt adult head; D. ctrl⁻: *ncs*-1 knock-out mutant (XA403) adult head; E. wt adult trunc; F. wt adult vulva muscles close up; G: ctrl⁻: knock-out mutant adult trunc; H. wt adult tail; I. ctrl⁻: knock-out mutant adult tail.

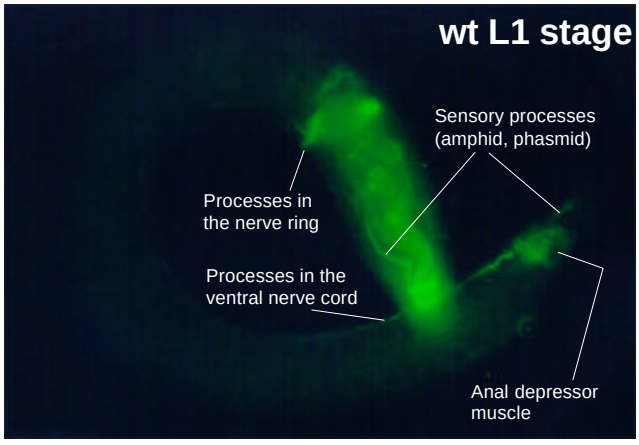
See next page:

Fig. 21; A,B



A

100 μm



B

100 μm

Fig. 21; C,D Head detail

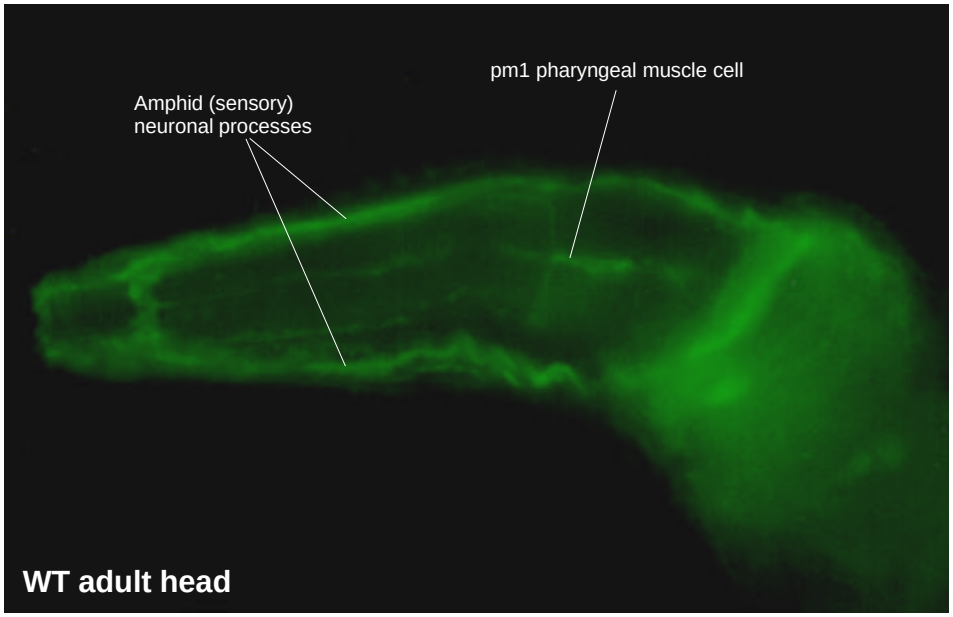
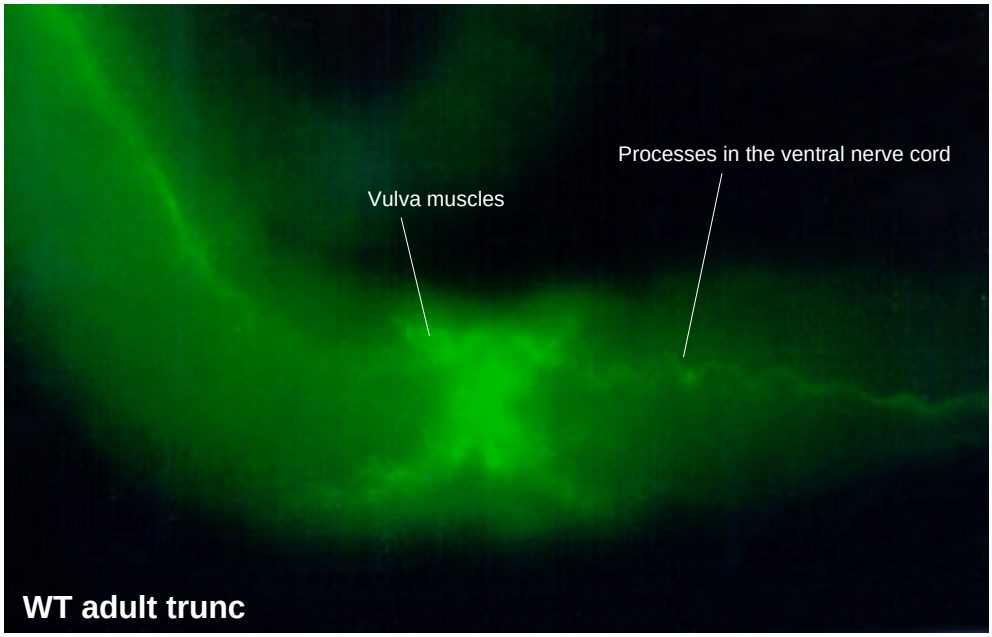
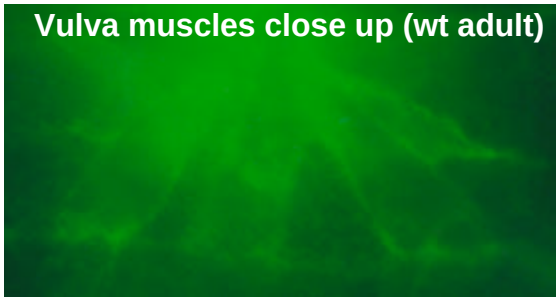


Fig. 21; E,F,G; trunc





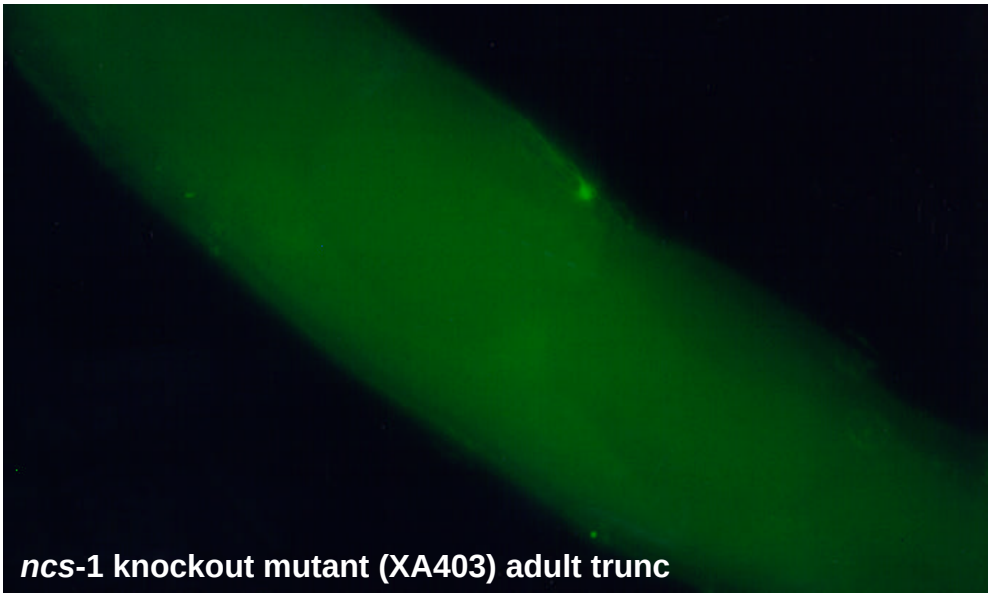
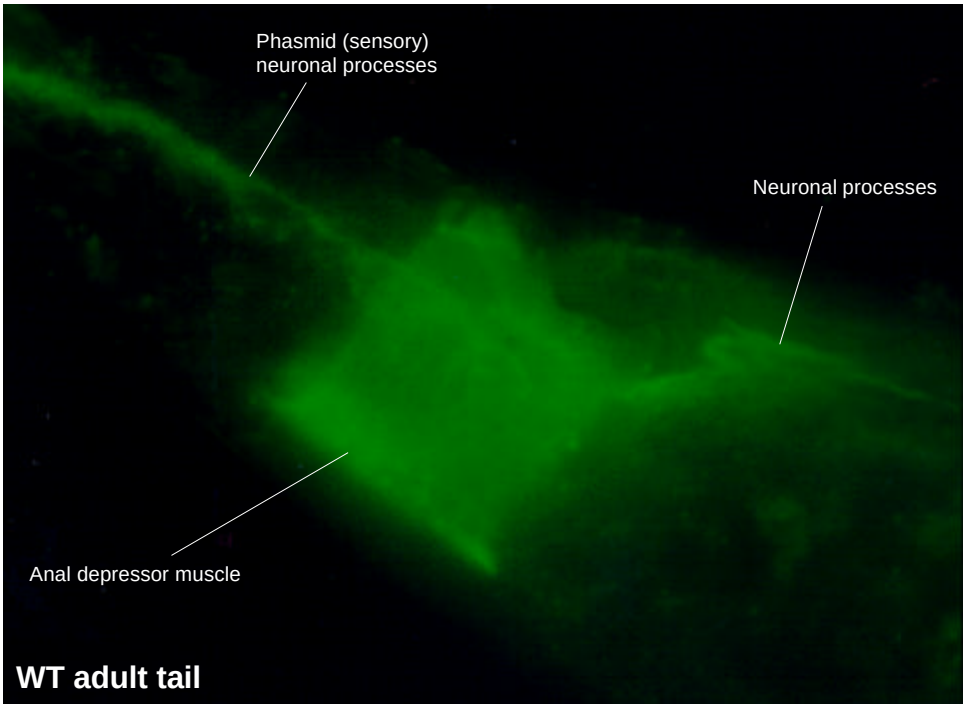
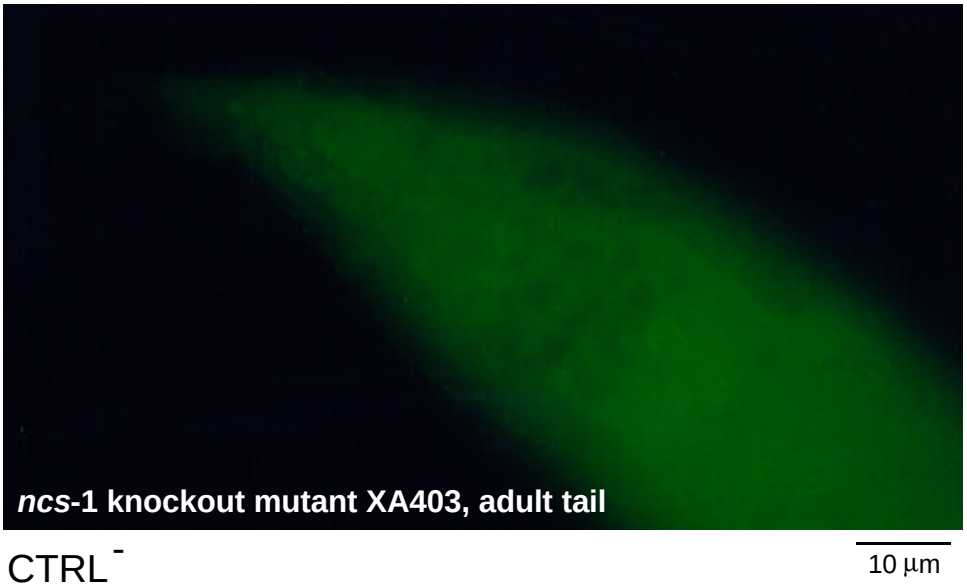


Fig. 21; H,I; tail





From these studies, *Ce*-NCS-1 appears to be localized in neuronal processes typical of sensory neurons (see fig. 12) in the head (amphid neurons) and in the tail (phasmid neurons) together with neuronal processes in the nerve ring (between the two bulbs of the pharynx) and in the ventral nerve cord. *Ce*-NCS-1 is also detected in some muscle cells: in the pharyngeal pm1 cell, in vulva muscles (in adults) that contract to expel the eggs, in the anal depressor muscle (see fig. 21) that contracts to open the anal canal. *Ce*-NCS-1 expression is found in all larva stages (e.g. L1 stage, see fig. 21B). The expression in males and in eggs was not examined.
In neurons *Ce*-NCS-1 protein is almost completely absent from cell bodies suggesting it is preferentially localized on neuritic (dendrites and axons) processes. Control using the *ncs*-1 knock-out mutant gives no signal (see fig. 21D,G,I) indicating that the observed staining is really specific for *Ce*-NCS-1 protein. In neurons, as the cell bodies and nucleus are not stained it is difficult to determine precisely the stained cells. Transgenic worms bearing gene-reporter construct for *ncs*-1 will be used to identify precisely cells expressing *Ce*-NCS-1 (see below 'GFP as a marker for *ncs*-1 gene expression').

Experimental procedure

Worms were fixed/permeabilized and stained using a modification of the wholemount Finney and Ruvkun protocol[125].

Fixation/permeabilization:

Starved (30' in M9) worms (~0.5gr) were resuspended in RFB 1x (80mM KCL, 20mM NaCl, 10mM Na_2EGTA, 5mM Spermidine HCl, 15mM Na PIPES, pH 7.4, 25% methanol) with 4% fresh paraformaldehyde. This emulsion was shacked 10 min. at room temperature (RT), then frozen in dry ice-ethanol and immediately defrosted under a stream of tap water in order to fracture the worms cuticle. The freeze-thaw cycle was repeated 2 more times. The emulsion was then incubated over-night on ice (fixation). The fixed worms were washed twice in Tris Triton buffer (TTB; 100mM Tris HCl, pH 7.4, 1% Triton X-100, 1mM EDTA). To further permeabilize their cuticle, worms were reduced (breaking cuticle disulfide linkages): The fixed specimens were incubated 1h at 37°c in TTB with 5% β-mercaptoethanol. This preparation was washed in 1x BO_3T buffer (50mM H_3BO_3, 25mM NaOH, 0.01% Triton X-100) and incubated in 1x BO_3T buffer with 10mM dithiothreitol (DTT) for 15 min. at 37°c. The worms were then washed once more in 1x BO_3T buffer. Finally, to prevent the reformation of cuticle disulfide linkages the worms were incubated (oxidized) in 1x BO_3T buffer with 0.3% H_2O_2 for 15 min. at RT and washed again in 1x BO_3T buffer. The fixed/permeabilized worms were then washed 1h in Antibody buffer B (AbB; 1xPBS, 0.1% Bovine serum albumin, 0.5% Triton X-100, 0.05% sodium azide, 1mM EDTA) and stored at 4°c in Antibody buffer A (AbA; 1xPBS, 1% Bovine serum albumin, 0.5% Triton X-100, 0.05% sodium azide, 1mM EDTA).

Staining:

The fixed specimens were incubated with the different anti-bodies (affinity purified polyclonal anti- *Ce*-NCS-1, diluted 1:200; anti- *Ce*-NCS-2, diluted 1:100) in AbA, 24h at 4°c (under mild agitation), each sample was then incubated o/n at 4°c with FITC (fluorescein isothiocyanate, giving a green signal {525nm} when excited at 495nm)-coupled goat anti-rabbit antibody (IgG, Sigma Immuno Chemicals; F-0511) diluted 1:200. The stained worms were then extensively washed with several changes of AbB for 6h at 4°c. 5μl of these worms were mounted on slides (cover with a 2% agarose-PBS pad) together with 5μl of mounting medium (1 mg/ml p-phenylenediamine, 10% PBS, 90% glycerol pH 8.) and examined on a Zeiss Axiophot microscope equipped with fluorescence capabilities.

Cloning of *ncs-1* and *ncs-2* genes

In order to make promoter-reporter gene constructs (see later: 'GFP as a marker for *ncs*-1 gene expression') and to isolate a *ncs-1* "knock-out" mutant (see below: "Isolation of a *ncs-1* knock-out mutant") I have isolated the *ncs-1* gene together with the *ncs-2* gene by screening a *C. elegans* genomic library at high stringency using either *Ce*-NCS-1 cDNA or *Ce*-NCS-2 cDNA probes. From both screening I was able to isolate several positive phages with large inserts containing (at least part of) the *ncs-1* and *ncs-2* genes. From restriction analysis and sequencing I was able to determine the general structure (some restriction site plus intron/exon boundaries sequences) of both genes (see fig. 22).

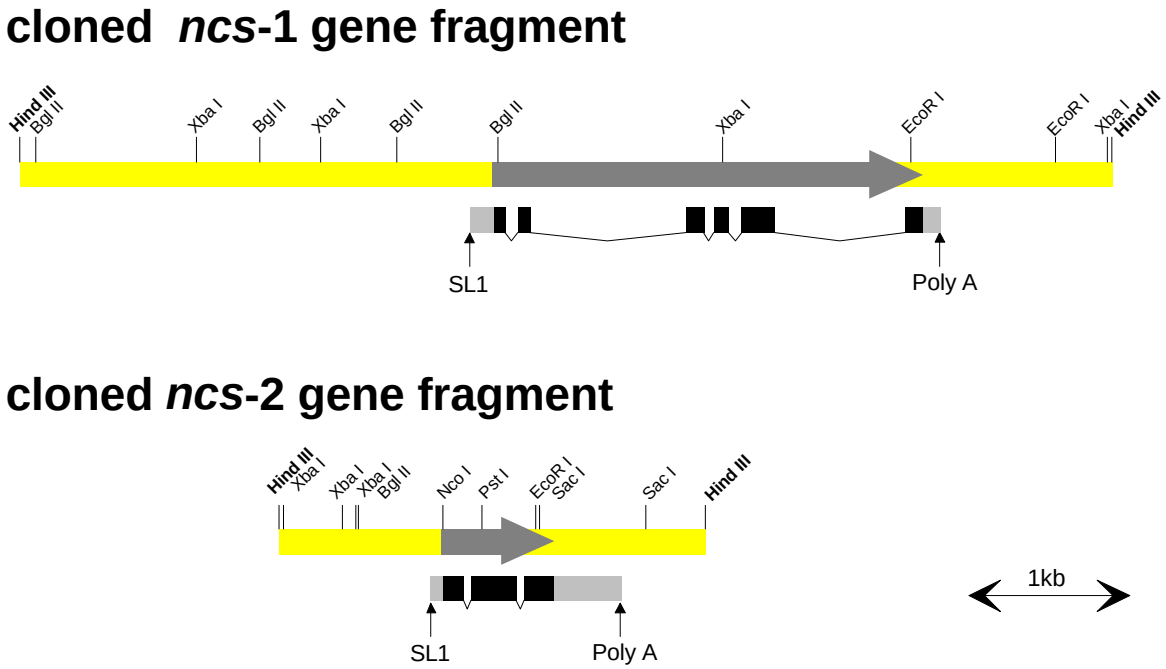


fig. 22. *ncs-1* and *ncs-2* gene structure,
Physical map of cloned *ncs-1* and *ncs-2* genes fragments with some restriction enzyme sites. Gray arrow represent region from ATG initiator to stop codon. Below gene map, alignment map of exons (black boxes represent translated exon sequences; gray boxes, untranslated sequences, introns are diagrammed as thin lines). Position of SL1 trans-splicing site and Poly A site are also indicated.

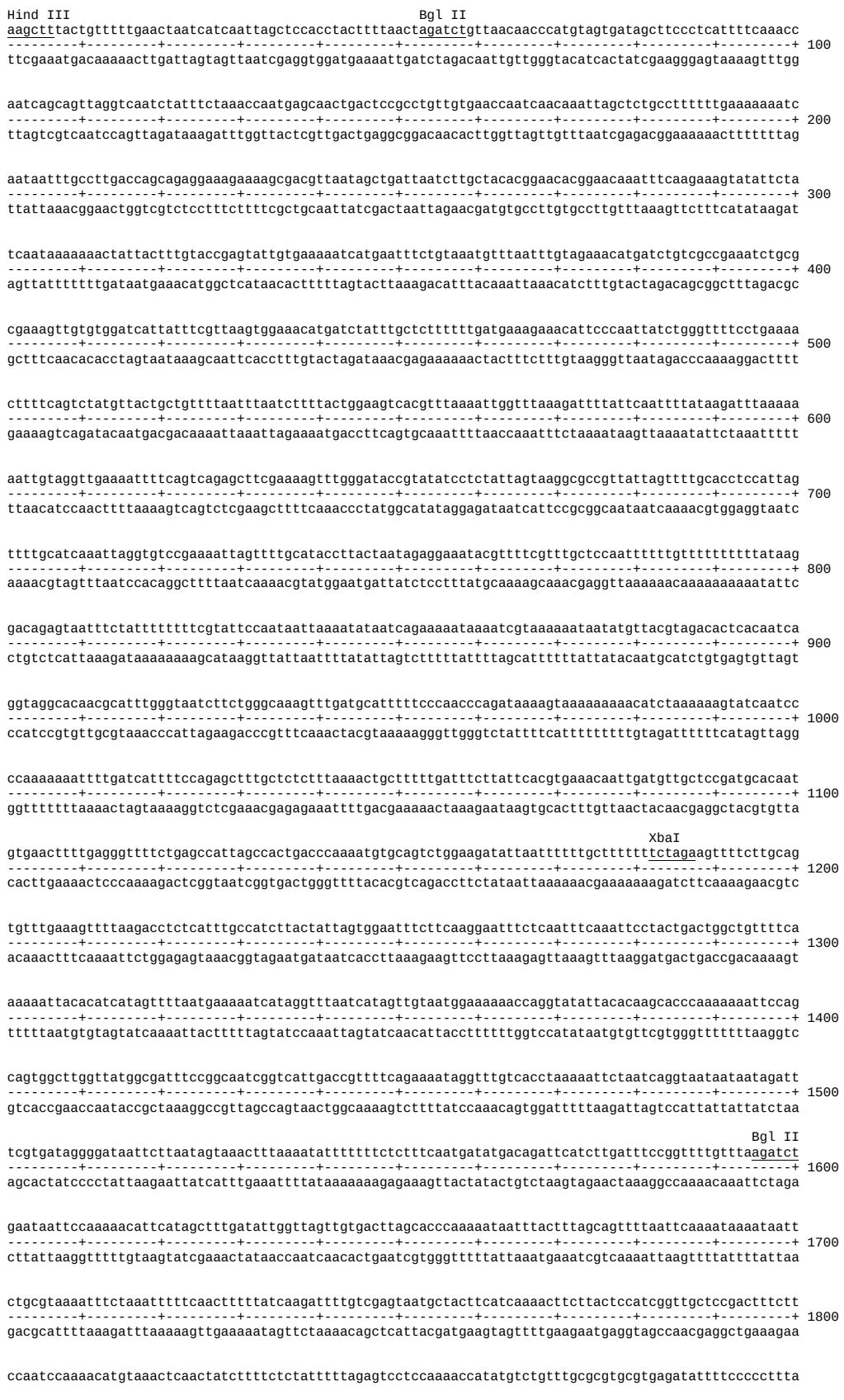
Both *ncs*-1 and *ncs*-2 genes have been recently sequenced in the scope of the *C. elegans* genome sequencing project (to date [June 1997] >70% of its genome is already sequenced). Therefore the complete sequence of the region enclosing these genomic fragments are known. The sequences of the cloned gene fragments are shown in fig. 23 and 24.

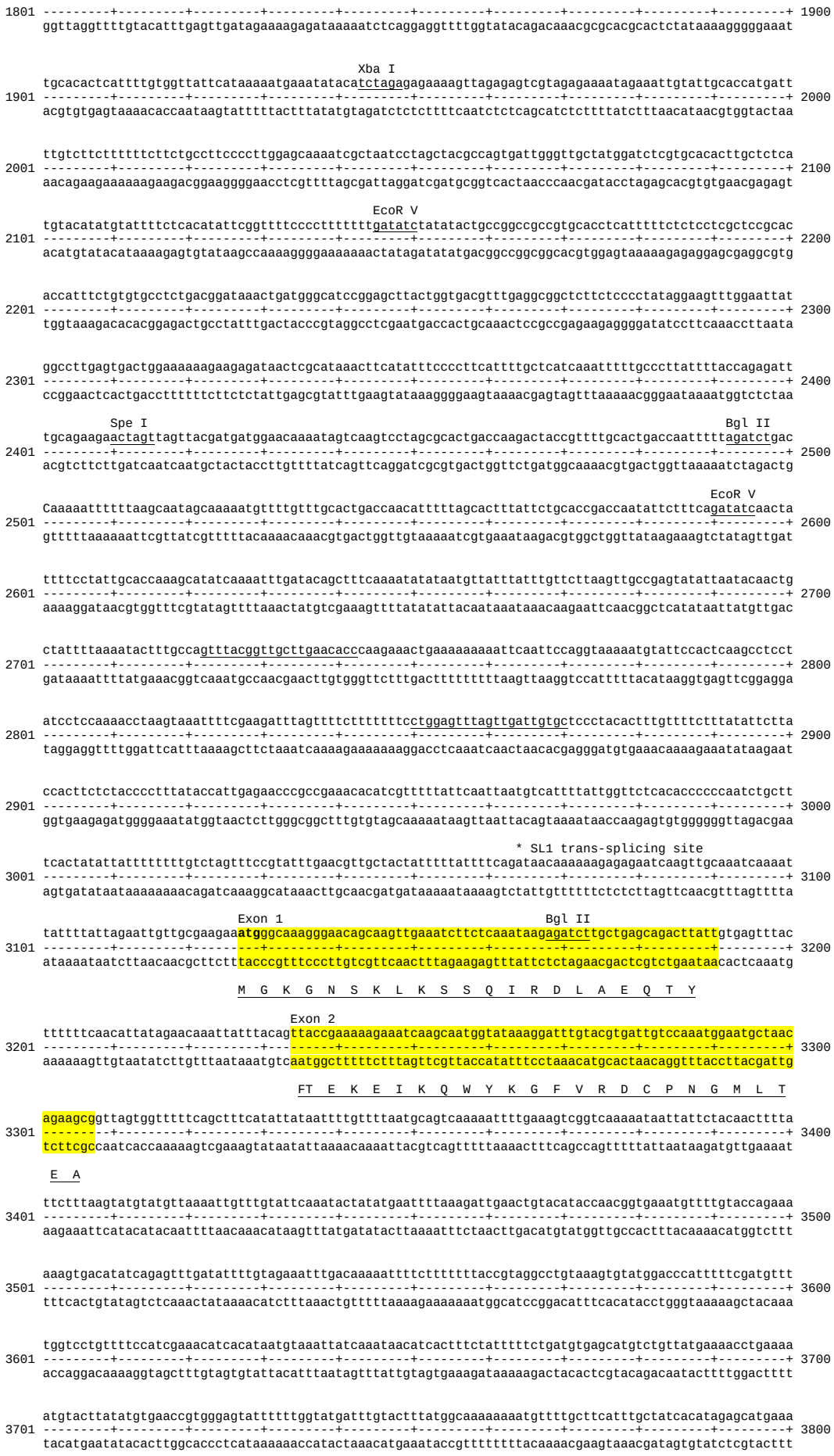
Comparison of cDNA and genomic sequences show that *ncs*-1 gene has five exons and *ncs*-2 three.

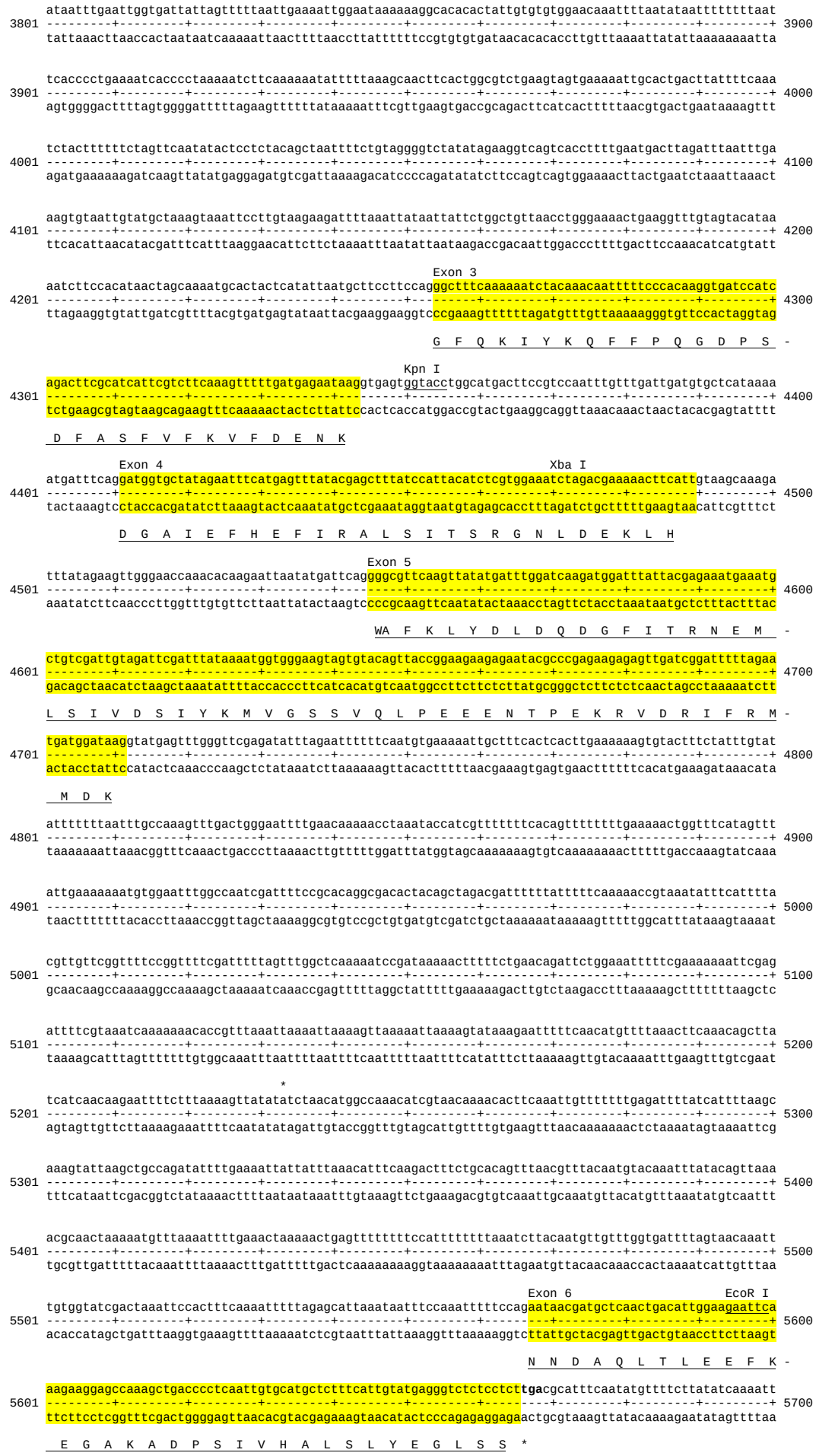
In addition to code for highly related proteins (with more than 70% amino acid identity between them) *C. elegans*, mouse ncs-1, and *Drosophila* frequenin genes (data not shown) **share almost the same structure (intron/exon borders) and are therefore probably orthologues (species equivalents).**

Fig. 23 and 24. Sequence of the cloned ***ncs*-1** and *ncs*-2 **gene** fragment
See next page:

Fig 23. Sequence of the cloned ***ncs-1* gene** fragment (plasmid p.*ncs-1*). Both DNA strands are shown. Position of some common restriction enzyme sites (for Bgl II, EcoR I, EcoR V, Hind III, Kpn I, Nco I, Pst I, Pvu II, Sac I, Spe I, Xba I) as well as other sites (SL1 trans-splicing, Poly(A), PCR primers, *etc*…) are indicated. Exons are boxed and their corresponding deduced amino acid sequence are shown bellow the boxes.

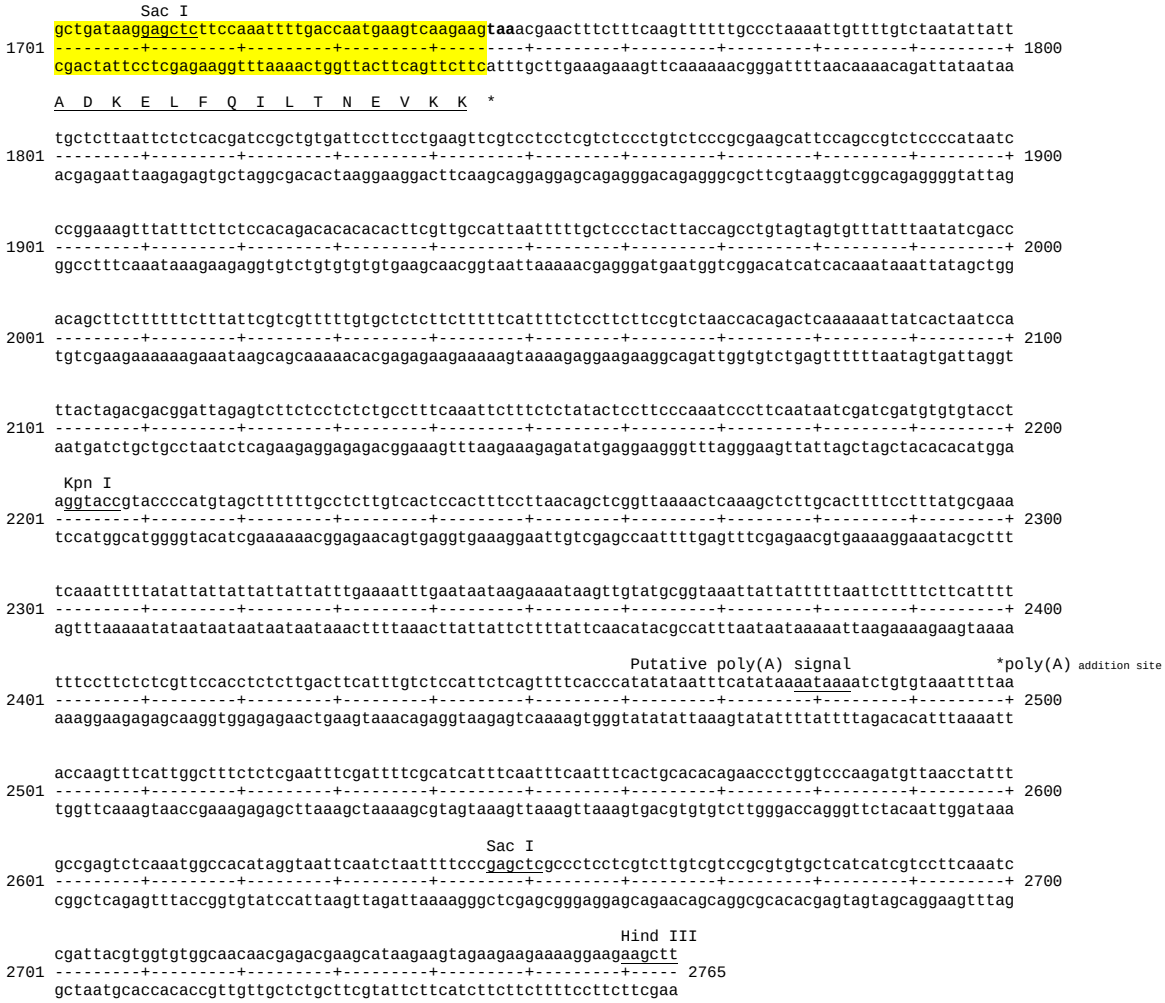



Xba I
EcoR V
Spe I
Bgl II
EcoR V
* SL1 trans-splicing site
Exon 1
Bgl II
M G K G N S K L K S S Q I R D L A E Q T Y
Exon 2
FT E K E I K Q W Y K G F V R D C P N G M L T
E A


Exon 3
Kpn I
Exon 4
Xba I
Exon 5
EcoR I
Exon 6


Putative poly(A) signal
*poly(A) addition site
EcoR I
EcoR I
Pvu II
Pvu II
Xba I
Sac I
Xba I
Hind III

Fig 24. Sequence of the cloned ***ncs-2* gene** fragment (plasmid p.*ncs-2*). Both DNA strands are shown. Position of some common restriction enzyme sites (for Bgl II, EcoR I, EcoR V, Hind III, Kpn I, Nco I, Pst I, Pvu II, Sac I, Spe I, Xba I) as well as other sites (SL1 trans-splicing, Poly(A), PCR primers, *etc*…) are indicated. Exons are boxed and their corresponding deduced amino acid sequence are shown bellow the boxes.



Experimental procedure

Genomic library screening. ~1x10^6 phages from a wild type *C. elegans* genomic library constructed in the phage vector λ2001 (from the Sanger centre, UK) were plated and screened[140] at medium stringency (60°C) with *Ce*-NCS-1 and *Ce*-NCS-2 cDNA as probes (separately). Random labeling of EcoRI fragment from *Ce*-NCS-1 (580bp) and *Ce*-NCS-2 (560bp) spanning the 5' untranslated region and ~80% of the coding region were used (with ^{32}P-radionucleotides) to produce the radioactive probes. Nitrocellulose membranes were first prehybridized for 2 hr at 60°C in 5 x Denhardt's, 5 x SSPE, 0. 1% SDS, 100 µg/ml denatured sspDNA, and then hybridized overnight at 60°C in 1 x Denhardt's, 5 x SSPE, 0. 1% SDS, 100 µg/ml denatured sspDNA, 10^6 cpm/ml of denatured probe (for detailed solution composition see above: experimental procedure in "Genomic southern blot analysis"). The membranes were then washed for 60 min. at 60°C in 4 x SSPE, 0. 2% SDS. Several positive phages were analyzed by Southern blot to determine which of them had a pattern of restriction-hybridization corresponding to the *C. elegans* genomic DNA Southern blot analysis (see fig. 13). Two phages, containing respectively the two *ncs* genes were further characterized. Large Hind III Inserts fragments hybridizing to the corresponding probes were subcloned in pBSK$^-$(Stratagene) and named p.*ncs-1*, p.*ncs-2*. Different sub-fragment spanning the complete Hind III fragments were also cloned in pBSK$^-$ and partially sequenced using the dideoxy method of Sanger[141]. The rest of the sequence come from the *C. elegans* genomic sequencing project data.

Chromosomal localization of *ncs-1* and *ncs-2* genes

Before both *ncs*-1 and *ncs*-2 genes were sequenced by the *C. elegans* genome project, I have performed experiments to localize *ncs-1* and *ncs-2* genes in the *C. elegans* genome. The *C. elegans* genome has been mapped into yeast artificial chromosomes (YAC). I could get from the Sanger center membranes with YACs spanning all the entire *C. elegans* genome. I probed YAC membranes with radiolabeled fragments of both the cloned *ncs-1* and *ncs-2* gene to determine which YAC will specifically hybridize to the probe. Positive YACs for *ncs-1* were: Y55B3, localized in the left arm of X chromosome; and for *ncs-2*: Y64B2 and Y62E9 (contiguous YACs), localized in the middle of the right arm of chromosome I. Knowing the positive YACs, we could get the cosmids spanning these YAC. The cosmids were directly bloted on a membrane (dot blots) and hybridized with the *ncs-1* and *ncs-2* probes. The positive cosmids for *ncs-1* were: F53H8, C44C1, localizing *ncs-1* to X: -18.85 (map unit); for *ncs-2*: C09D5, K09B7, AC2, localizing *ncs-2* to I: 4.46.

	***Ce*-NCS-1**	***Ce*-NCS-2**
Positive YACs	Y55B3	Y64B2, Y62E9
Positive cosmids	F53H8, C44C1	C09D5, K09B7, AC2
Chromosomal localization	X: -18.85 (between aex-3, unc-1 genes)	I: 4.46 (betweeþ tba-1, lin-11 genes)

Table 4. Information on chromosomal localization of *ncs-1*, *ncs-2* genes.

Experimental procedure

Random labeling of a ~2.5kb Xba I fragment from p.*ncs-1* and the complete Hind III fragment from p.*ncs-2* (~2.7kb) were used (with ^{32}P-radionucleotides) to produce the radioactive probes.
YAC Blot: YAC membranes were hybridized over-night at 65°c in hybridization mix (100g dextran sulfate, 160ml water, 30ml of sodium lauryl sarcosinate 30%, 300ml 20x SCP pH 6.2 {NaCl 2M, Na_2HPO_4 60mM, EDTA 20mM, 250mM HCl}) with 10^7 cpm/ml of denatured radioactive probe, 100 µg/ml denatured sspDNA, washed four times 20 minutes at 50°c cooling to room temperature (over the 20 minutes) in 0.5% SDS, 0.5x SCP. Autoradiography was performed with an intensifying screen for 5 days at -70°C.
Cosmid dot blot: 5µl of E. coli liquid culture transformed with the cosmid of interest were put directly on a nitrocellulose membrane. Membranes were incubated in 10% SDS for 5 min then treated in Benton-Davis I, II, III[140] solutions for 4 min and dried. Membranes were then prehybridized and hybridized (at 60°c over-night) and washed like southern blots. Autoradiography was performed with an intensifying screen for one day at -70°C. Tested cosmids for *ncs-1* (covering YAC Y55B3) were: T19F8, F53H8, C44C1, W07D7, C55D6, C04D1; for *ncs-2* (covering YACs Y64B2 and Y62E9): K07A5, C09D5, K09B7, AC2, B0379, T07H2.

GFP as a marker for *ncs*-1 gene expression

To determined the cellular distibution of *Ce*-NCS-1, besides immunolocalization (see above), I have also generated transgenic worms bearing a *ncs*-1 reporter gene construct. These transgene reporter system provide means for detailed and precise spatial (and temporal) characterization of expression patterns assuming that the transforming construct contains all relevant cis-acting control sequences, and that neither the construct itself nor the novel context of the transforming DNA alterates the expression pattern. Therefore the observed pattern should be confirmed by other means (antibody stainings in my case).
A putative *ncs*-1 promer fragment was used to drive expression of the reporter gene **green fluorescent protein** (GFP) in transgenic worms[142]. GFP is a 238 amino acid cytoplasmic protein found in *Aequorea victoria* (a bioluminescent jellyfish) converting blue light produced by the calcium activated photoprotein aequorin into green light. GFP absorbs blue light (maximally at 395nm) and emits green light (peak at 509nm). This fluorescence requires no exogenous substrates and cofactors, therefore GFP expression can be used to monitor gene expression in living *C. elegans* (as it is a transparent animal). The introduced construct express GFP without targeting signal allowing its diffusion (as it is a relatively small protein) throughout the cytoplasm facilitating the identification of positive cells.

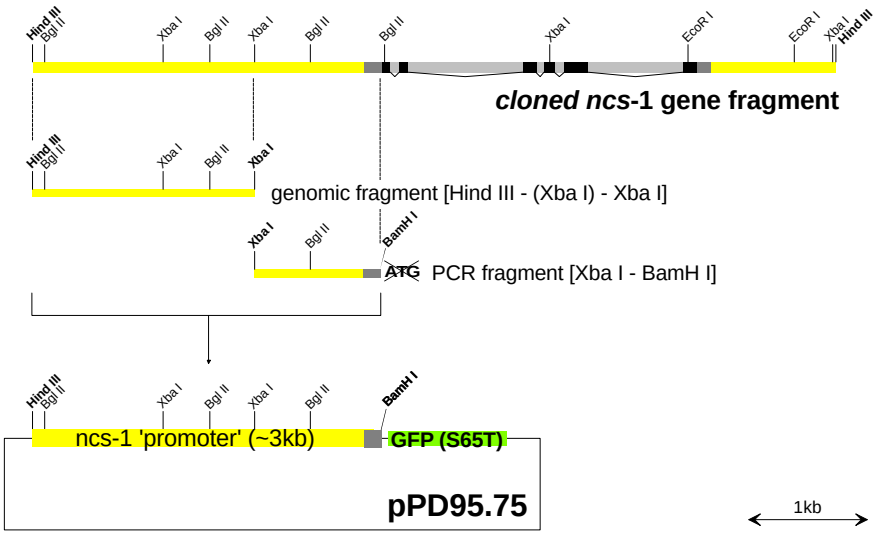


Fig. 25.
'promoter' *ncs*-1::GFP construct. 3kb of *ncs*-1 upstream region (up to the ATG, not included) were inserted in the pPD95.75 GFP vector to drive the expression of GFP.
Schematic representation of the construction; physical maps of used *ncs*-1 fragments with some restriction enzyme sites are shown. Black boxes represent translated exon sequences, dark gray boxes: untranslated transcribed sequences, introns are represented as light gray boxes.

I made a construct with GFP under the control of a *ncs*-1 fragment upstream of the coding region (~3kb up to the ATG $_{\text{not included}}$, see fig. 25). This construct was introduced into the germline of *C. elegans* to produce transgenic animals. By aligning GFP fluorescence with differential interference Nomarski images, expression could be attributed to individual cells (as the complete *C. elegans* anatomy is known at the cellular level).

Fig. 26. GFP expression under the control of a *ncs*-1 upstream 'promoter' fragment ('promoter' *ncs*-1:: GFP transgenic worms). Confocal microscope pictures of hermaphrodite XA411 worms (in red Normarski image, in green GFP signal).
A. whole L2 larva; B. whole L1 larva; C. adult head; D. adult head close up around the pharynx bulbs; E.;F. two adult head close up around the pharynx bulbs; G.;F. two adult head scan showing the multinucleated pharyngeal pm1 muscle cell; I.;J.;K. three close up of adult vulva; L.; M. close up of adult tail; N.;O. Schematic representation of neuronal and larger hypodermal nuclei in newly hatched L1 around: N. ring, ventral, and retrovesicular ganglia, O. preanal and left lumbar ganglia. left lateral aspect. GFP positive cells in XA411 worms are underlined; positive cells showing strong GFP signal are shown in dark gray. Positive cells with weaker and rarer signal are shown in light gray.
See next page:

Fig. 26; GFP expression; A,B

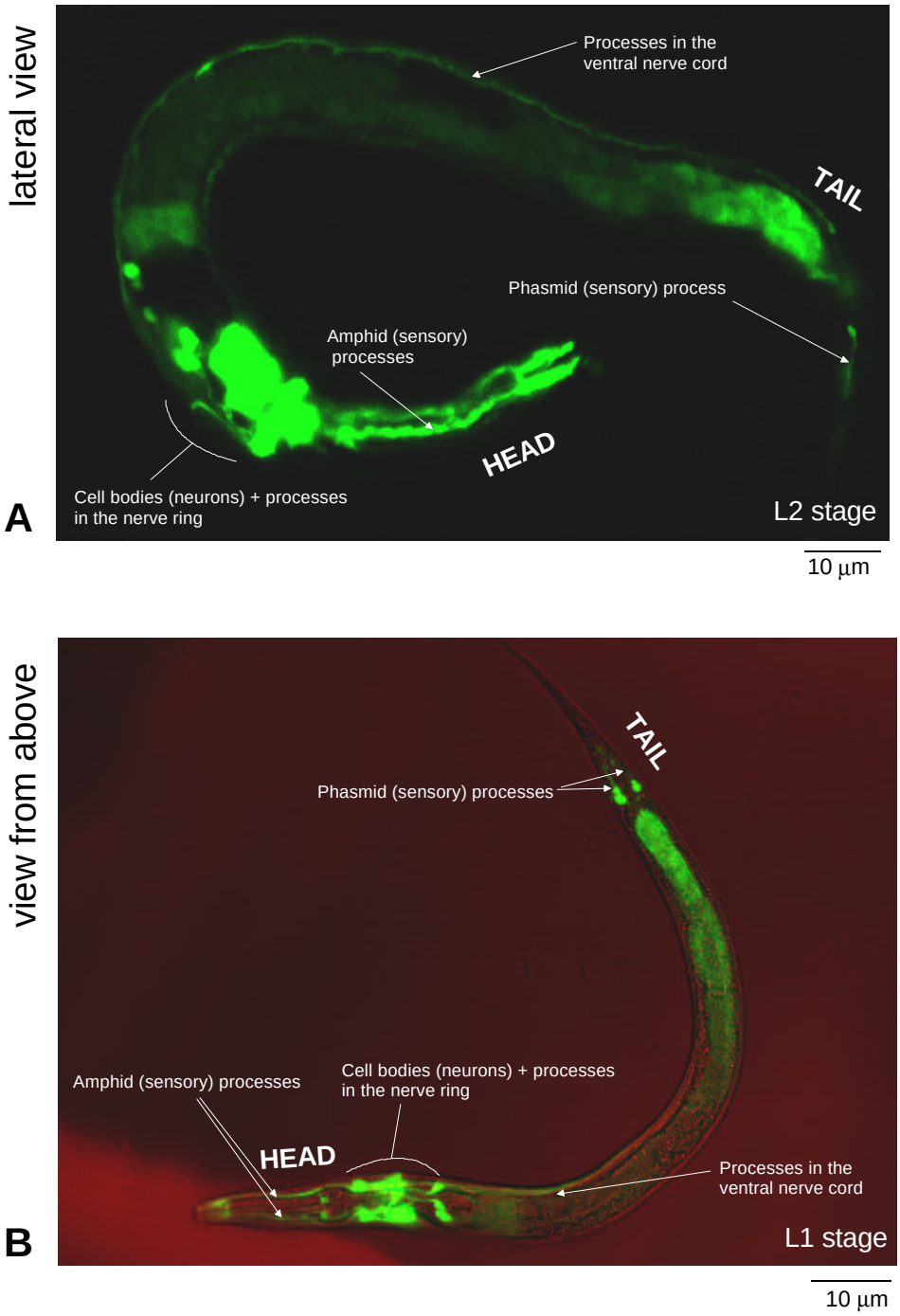
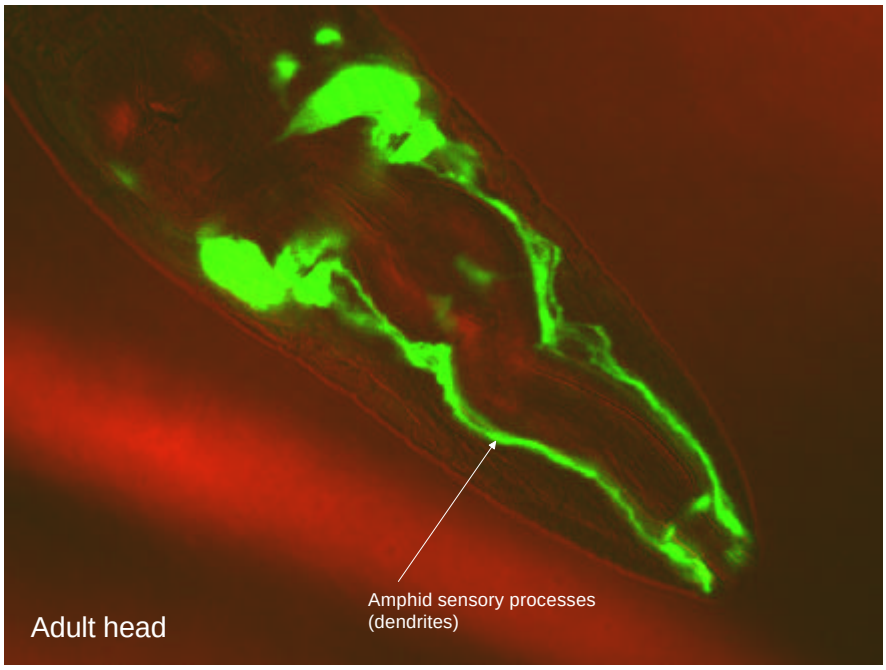
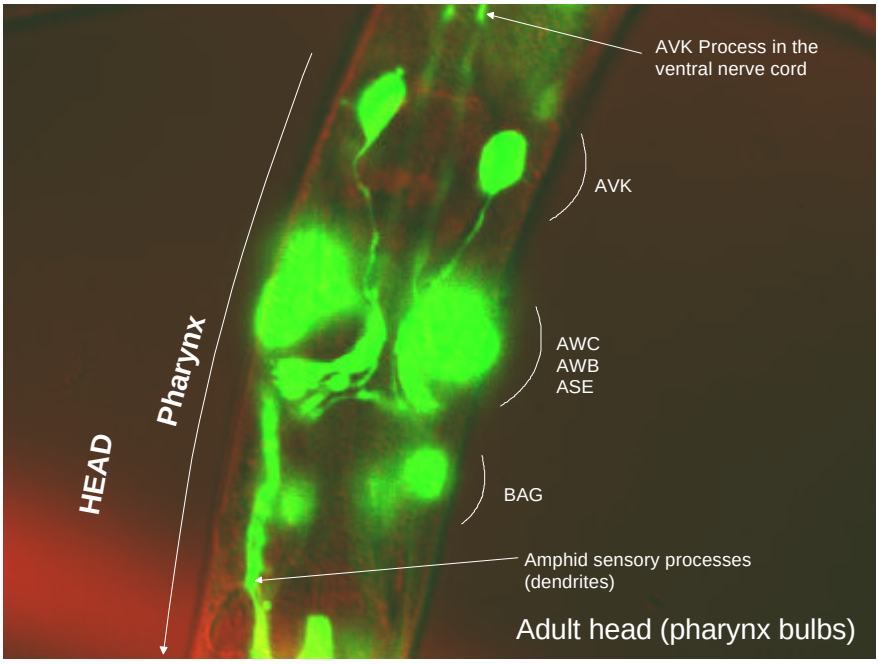


Fig. 26; GFP expression; C, D



C

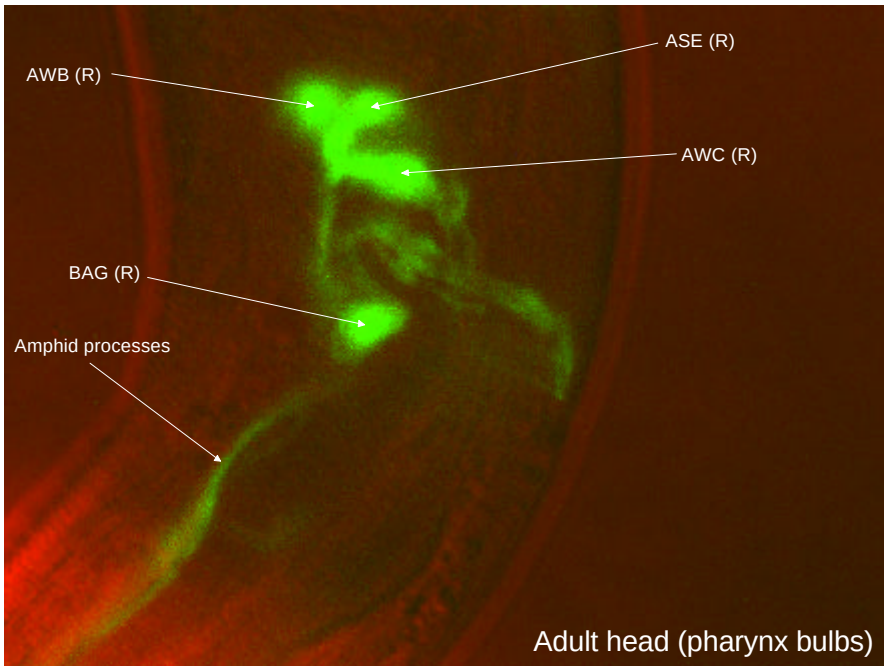
10 μm



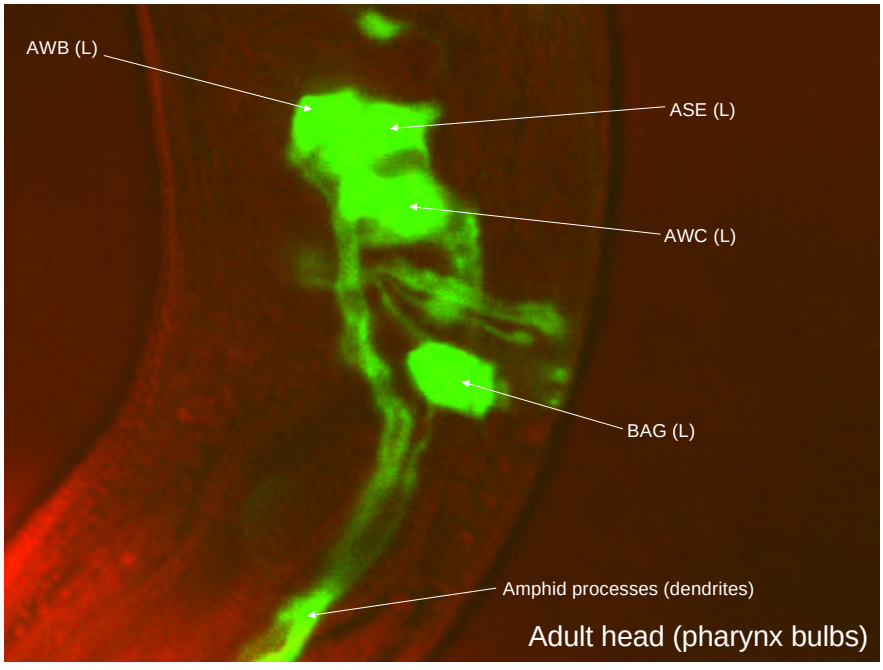
D

10 μm

Fig. 26; GFP expression; E, F



E



F

Fig. 26; GFP expression; G, H, I, J, K

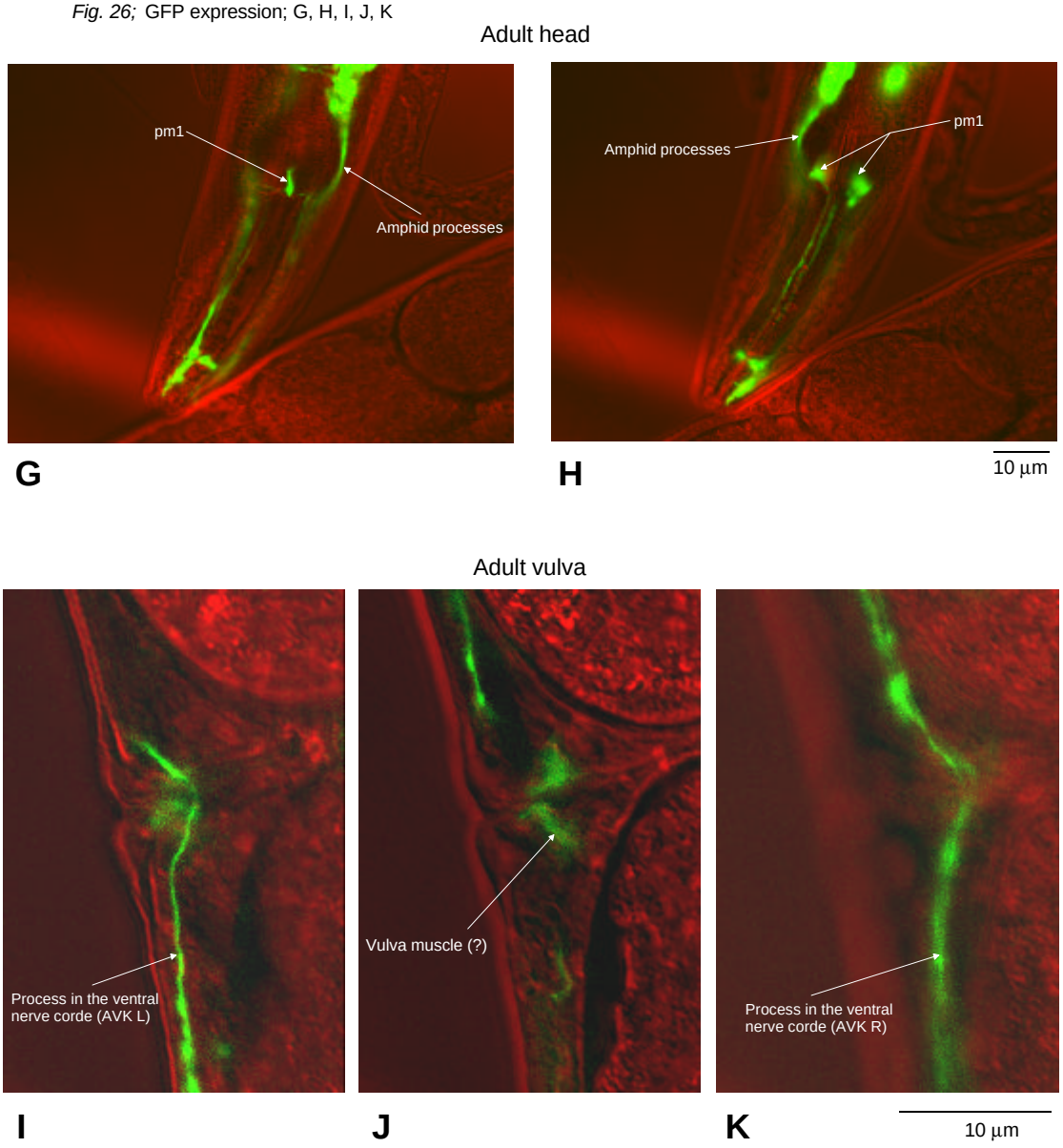
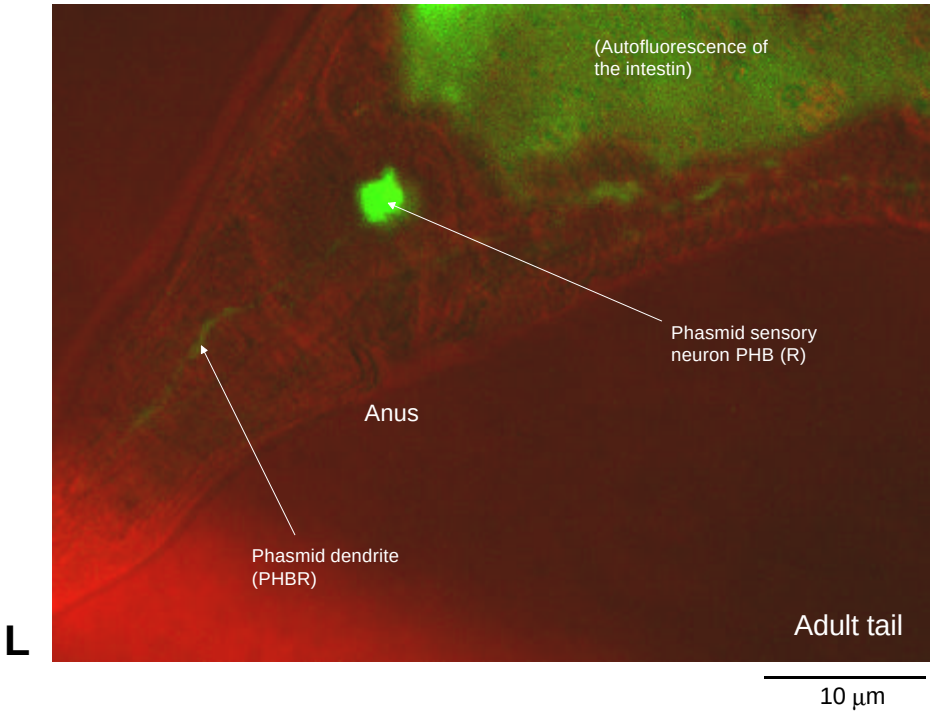
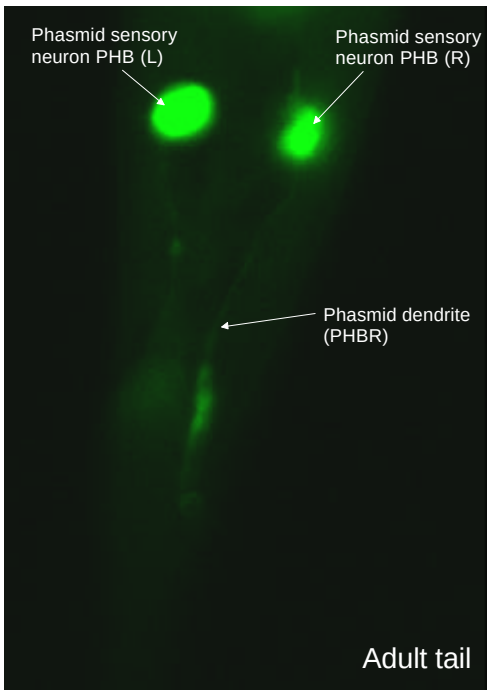


Fig. 26; GFP expression; L, M

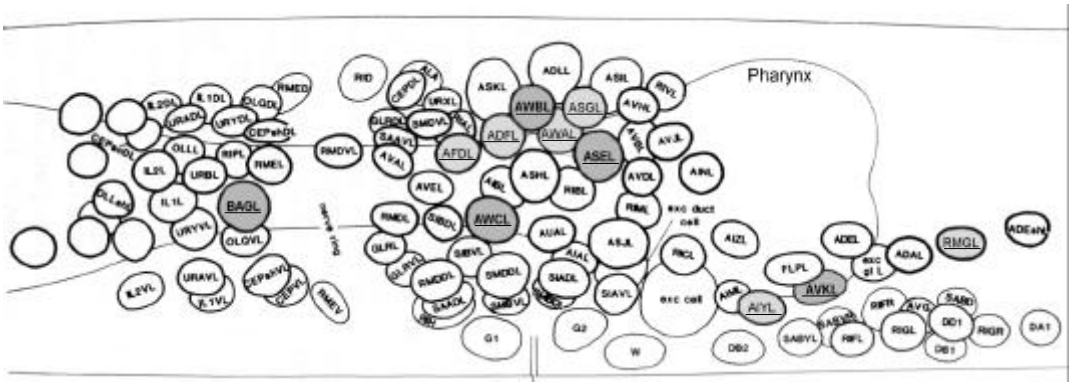


L

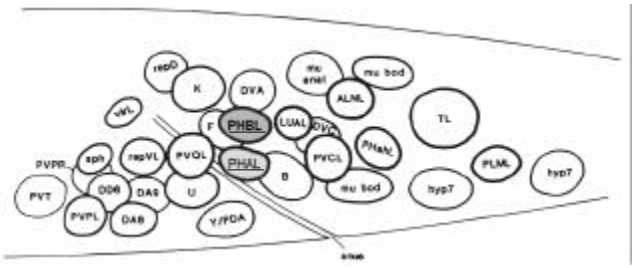


M

Fig. 26; GFP expression; N, O



N



O

ncs-1-GFP positive cells:

The determination was made for us by *Dr. Cori Bargmann (Dep. of Anatomy, University of California, San Fransisco, USA)* who is an expert in the *C. elegans* nervous system.

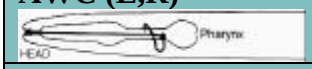
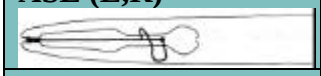
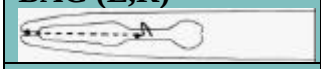
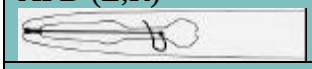
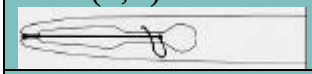
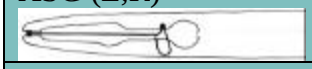
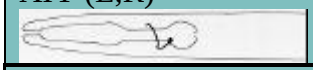
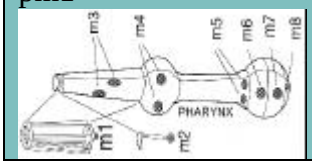
Positive cell	Known function(s), defined by laser ablation
Sensory neurons:	
AWC (L,R) 	**Amphid neurons.** Chemotaxis to volatile odorants (benzaldehyde, butanone, isoamyl alcohol)
ASE (L,R) 	**Amphid neurons.** Chemotaxis to soluble compounds (Na^+, Cl^-, cAMP, biotin, lysine), egg laying
AWB (L,R) 	**Amphid neurons.** Volatile avoidance
BAG (L,R) 	Sensory neurons
PHB (L,R) 	**Phasmid neurons.** [soluble compounds chemosensation?]
AWA (L,R) 	**Amphid neurons.** Chemotaxis to volatile odorants (diacetyl, pyrazine, 2,4,5-trimethylthiazol)
AFD (L,R) 	**Amphid neurons.** Thermotaxis
ADF (L,R) 	**Amphid neurons.** Dauer formation; chemotaxis to soluble compounds (minor)
ASG (L,R) 	**Amphid neurons.** Dauer formation (minor); chemotaxis to soluble compounds (minor)
PHA (L,R) 	**Phasmid neurons.** [soluble compounds chemosensation?]
Inter-neurons:	
AVK (L,R) 	
AIY (L,R) 	Thermotaxis
Motor-neuron:	
RMG 	Innervation of muscles in the head
Muscle cells:	
pm1 	Opening of the metastomal pharyngeal flaps (the function of these flaps is unknown)

Table 5. GFP positive cells in 'promoter' *ncs*-1::GFP XA411 transgenic *C. elegans*. Cells in bold: strong GFP expression; not bold: weaker (rarer) expression.

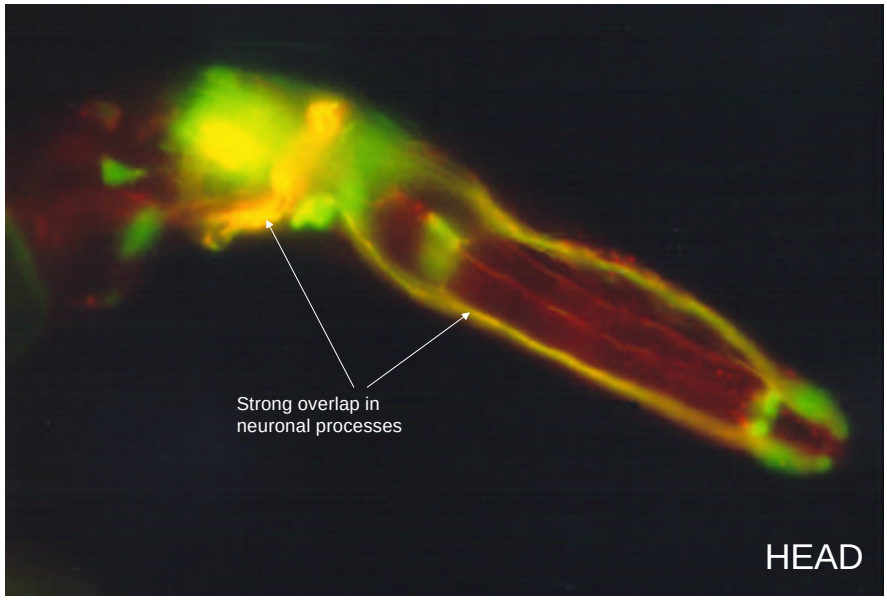
Isolated transgenic *C. elegans* lines (generally) show no-mendelian inheritance of the marker (and therefore the construct) meaning that the transformants bear extrachromosomal arrays of the injected DNA that are occasionally lost during meiosis and mitosis being (moderately) mosaic animals. Therefore to allow proper determination of the expression pattern many worm have to be examined and their signal integrated.
In the adult hermaphrodite, GFP expression was observed (see table 5) in 26 (13 pairs of symmetric bilateral [left, right]) neurons. Expression was detected in 20 *sensory neurons* (in the *C. elegans* there is 60 putative sensory neurons out of 302 neurons) including head sensory neurons (amphid neurons + BAG) **AWC, ASE, AWB, BAG** (strong expression), AWA, AFD, ADF, ASG (weaker and rarer expression), and tail sensory neurons (phasmid neurons) **PHB** (strong expression), PHA (weak). Expression was also observed in 4 *inter-neurons*: **AVK** (strong expression), AIY (weak and rarer expression) and in the RMG *motor-neurons* pair (weak). There is also expression in the **pm1** pharyngeal muscle cell. With the GFP we do not observe staining in the anal depressor muscle (unlike in immunofluorescence) and in the vulva muscle (although a really faint staining may be observed on some worms, see fig. 26J).
The GFP signal was present in all larva stages (like in IF). We do not examine expression in males and in the embryos (eggs).
The function of some GFP positive cells is indicated in table 5.

Ce-*NCS-1 double staining: GFP + Immunofluorescence*

To see if the GFP and the Ab signals overlap, I performed immunofluorescence with anti-*Ce*-NCS-1 Ab on 'promoter' *ncs*-1::GFP transgenic worms. These transgenic worms were stained using a TRITC (tetramethylrhodamine isothiocyanate) conjugated secondary antibody giving a RED signal. **Superposition of both GFP (green) and TRITC (red)- antibody signals shows that there is complete overlap (giving a yellow signal) in all neuron processes and in pm1 cell** (see fig. 27)**.** The antibody signal was (almost) absent in cell bodies of positive neurons confirming that *Ce*-NCS-1 protein is predominantly localized in dendrites and axons (neurites) but excluded from cell bodies. The signals in the anal depressor muscle and the in vulva muscles are observed only with antibody stainings. As this signal is absent in the *ncs*-1 knock-out mutant (see above 'Immunolacalizations' and after 'Isolation of a *ncs-1* knock-out mutant') it seems not to be an artifact suggesting that there is probably some missing control sequence(s) in the used GFP reporter constructs.

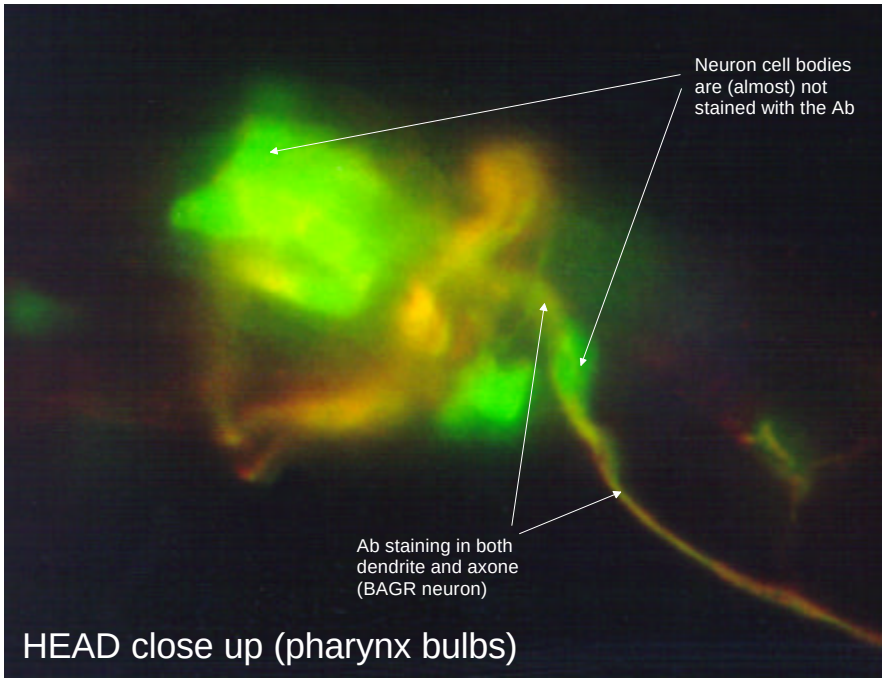
Fig. 27.
Immunofluorescence with anti *Ce*-NCS-1 Ab on 'promoter' *ncs*-1::GFP worms; in green GFP signal, in red IF signal, in yellow/orange overlap of both signals.
A. head; B. head close up; C. tail; D. tail (anal region) close up.
See next page:

Fig. 27; A,B **GFP + IF**



A

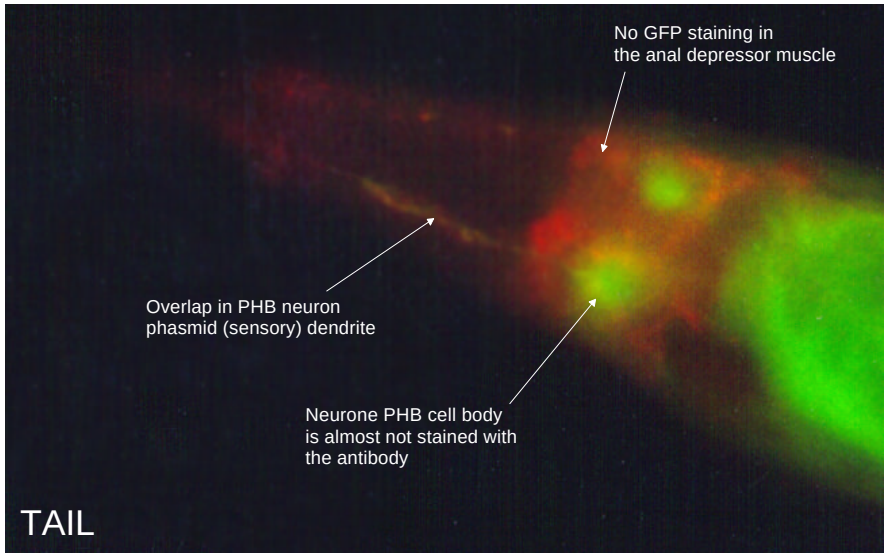
10 μm

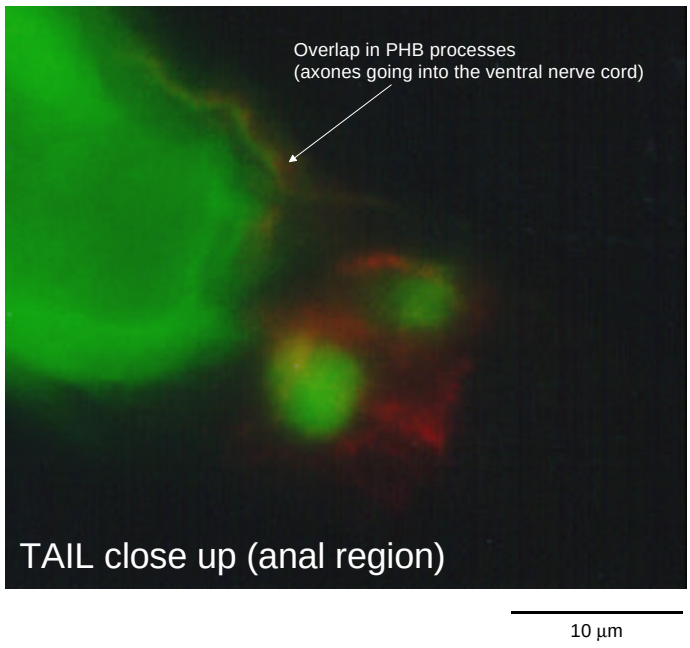


B

10 μm

Fig. 27; C, D


No GFP staining in
the anal depressor muscle
Overlap in PHB neuron
phasmid (sensory) dendrite
Neurone PHB cell body
is almost not stained with
the antibody
C
TAIL
10 µm


Overlap in PHB processes
(axones going into the ventral nerve cord)
D
TAIL close up (anal region)
10 µm

The chosen 'promoter' *ncs*-1 region drives the expression of GFP in the correct cells (except for the anal and vulva muscles), therefore *Ce*-NCS-1 protein is expressed in the aforementioned GFP positive cells.

Localization of *Ce*-NCS-1 (conclusion)

***Ce*-NCS-1 is expressed in certain excitable cells including: different types of neurons (mainly sensory but also inter- and motor- neurons) representing 1/10 of the total neuron population, and in few muscles. In neurons *Ce*-NCS-1, is preferentially localized in neurites (axons and dendrites), being almost excluded from cell bodies. This axon and dendrite localization indicates that *Ce*-NCS-1 can be found both pre- and post-synaptically.** The uniform preferential localization of *Ce*-NCS-1 in axons and dendrites may indicate that this protein is associated with membranes **(**or with some cytoskeleton components**)**. As the surface to volume ratio is higher in thin cylindrical neuritic processes than in the 'flat' cell body, a protein associated with plasma membrane is more spatially concentrated in neuritic processes than in cell body. It is know that some NCS proteins such as recoverin are associated to membranes via a myristoyl moiety anchored at their N-terminus. Yet *Ce*-NCS-1 (like rat/mouse/chick NCS-1) do not show a consensus sequence for myristoylation. Nonetheless, vilip1 is known to be myristoylated although it does not shows the classical consensus site. Therefore we can not rule out the possibility that *Ce*-NCS-1 may be myristoylated. Anyway, ***Ce*-NCS-1 seems to be associated with plasma membrane by a yet unknown mechanism**.
Ce-NCS-1 distribution in the *C. elegans* nervous system seems to correspond to NCS-1 distribution in other organisms: Both *Ce*-NCS-1 and mouse NCS-1[147] are found pre- and post-synaptically being localized predominantly in axons and dendrites. *Ce*-NCS-1 and other NCS-1 proteins (*Drosophila* frequenin, rat/mouse/chick NCS-1) are found in many but not all sensory, inter-, and motor- neurons.
Expression of NCS proteins in muscles has not yet been observed in other species (neither has been carefully examined).

Experimental procedure

Generation of GFP construct:
The *ncs*-1 promotor::GFP fusion construct was made (see fig. 25) by ligating a ~1950bp Hind III - (Xba I) – Xba I (partial digestion) *ncs*-1 genomic fragment together with a ~1180bp Xba I – BamH I PCR fragment into the GFP expression vector pPD95.75 (from A. Fire labs, Carnegie Institution of Washington, department of Embryology, Baltimore, USA). The PCR fragment was obtained by performing PCR amplification on p.*ncs*-1 (see 'Cloning of *ncs-1* and *ncs-2* genes') plasmid using 5'prom1 primer (CGC AAG CTT CTA GAG AGA AAA GTT AGA GAG TCG) including a genomic Xba I site together with a Hind III linker, and 3'prom1 primer (CGG GAT CCT TCT TCG CAA CAA TTC TAA TAA) engineered to contain a BamH I site in place of the start ATG. The amplification product was first sub-cloned in pBSK$^-$ as a Hind III - BamH I fragment, sequenced and then digested/purified to form the used Xba I – BamH I (PCR) fragment. All junctions were verified by sequencing.
In the pPD95.75 vector, the GFP gene contains five engineered synthetic introns (found to increase expression) and the S65C mutation (giving a stronger fluorescent signal, faster folding of the fluorochrome and improved resistance to fading).

Generation of transgenic worms (germline transformation):
Germline transformation was carried out as described[143]. Marker rol-6 (plasmid pRF4 carrying the dominant collagen mutation rol-6(su1006)), which make the worms roll and move in circles, at a concentration of 200 µgr/ml and GFP construct at a concentration of 50 µgr/ml were co-microinjected into the cytoplasm of hermaphrodite syncytial gonads. Transgenic animals (in the progeny of injected worms) were identified by their roller phenotype.
5µl of these worms, fixed 2h in PBS with 1% paraformaldehyde, were mounted on slides (cover with a 2% agarose-PBS pad) together with 5µl of mounting medium (1 mg/ml p-phenylenediamine, 10% PBS, 90% glycerol pH 8.) and examined on a Zeiss Axiophot microscope equipped with fluorescence capabilities. 6 lines were obtained (named: XA410, XA411, XA412, XA413. XA414, XA415) with high and low expressors (giving high and low intensity signals) but all showing the same expression pattern. 3 high expressor lines (XA410, XA411, XA412) were kept and frozen.

Identification of positive cells:
The positive cells in the XA-410 line were identified for us by Cory Bargman (UCSF, San Francisco, USA) comparating GFP fluorescence (under a microscope equipped with fluorescence capabilities) with differential interference Nomarski images. The XA-411 strain, the highest expressor also shows a clear signal in the pm1 pharyngeal muscle cell (in XA-410 this signal was to weak to allow identification of the positive cell).

Immunofluorescence on transgenic worms:
XA-411 ‘promoter’ *ncs*-1::GFP worms were labeled with the anti- *Ce*-NCS-1 Ab as described above (see ‘Immunolocalizations’) except that the secondary antibody was a TRITC (tetramethylrhodamine isothiocyanate) conjugated giving a RED signal.

Isolation of a *ncs-1* knock-out mutant

In order to get insight into the *in vivo* function of *Ce*-NCS-1, I have isolated a *C. elegans* mutant line with the *ncs-1* gene inactivated. The phenotype of this *ncs-1* knock-out may help us to determine the physiological processes in which *Ce*-NCS-1 is involved (see "Phenotypic analysis of *ncs-1* knock-out"). In the *C. elegans* to inactivate a gene, the only method that works in practice is **target-selected mutagenesis**; the mutagen being deletions induced by the excision (jumping) of an inserted transposon (Tc1 transposon). Therefore gene inactivation *per se* is not directly targeted, but selectively isolated from randomly mutagenized worms. This inactivation (deletion) has to be performed in two steps. *First step:* isolation of a *C. elegans* line with a transposon in the gene of interest. *Second step:* from this first line, isolation of progeny with a deletion in the gene of interest resulting from an improper repair after the spontaneous excision of the transposon. These isolations are performed by polymerase chain reaction (PCR) screenings.

Isolation of a Tc1 Transposon insertion allele

This first step is routinely performed by Ronald H. A. Plasterk's laboratory for colleagues as a "public" service. Starting with a *C. elegans* strain (MT3126) where worms contains several copies of the Tc1 transposon (and is permissive for jumping of this Tc1) as a "library", they are able to isolate by PCR screening a strain that contains a Tc1 transposon (1610bp) in a gene of interest (Tc1 insertion allele).

A typical library consists of a set of (frozen) *C. elegans* cultures and a set of corresponding crude lysates. The lysates can be pooled and screened by PCR in such a way that insertions of Tc1 transposon into a gene of interest are visualized. PCR are performed using a primer specific for Tc1 and the other primer specific for the gene of interest. PCR products are obtained only if Tc1 sequences are near and properly juxtaposed to the sequences of the gene of interest, namely if a Tc1 is inserted into this gene. Once a positive pool has been found, the screening can be refined up to the individual lysates to find a positive lysat (corresponding to a positive culture). Once a positive culture has been found, a positive line has to be isolated from this culture.

Worms from the positive culture are separated into several sub-cultures. After a few days growth, part (12) of the worms of each sub-culture are sacrificed to make lysates that will be analyzed again by PCR. Once a positive lysat has been found, the screening can be refined up to individual worms by successive fractionations/sub-culturing to find a single positive homozygote worm giving rise to a (homozygous) Tc1 insertion line (obviously when single worms are sub-cultured they are sacrificed/ analyzed only after they had lay eggs so that one can recover their progeny).

One can determine the precise sequence of the insertion site by sequencing the amplified PCR product. Homozygousity can be determined by testing progeny which should be 100% positive (and/or by southern blots and/or negatively confirmed by PCR using primers giving rise to products only on the wt allele). In general homozygous lines can be easily obtained unless the homozygote has a selective disadvantage, which is rare as Tc1 insertion are usually not harmful.

Tc1 alleles are generally not null alleles: insertions in introns (which occur predominantly) are removed from mature RNA together with the rest of the intron by normal splicing, and even insertions in exons can be removed by an aberrant splicing process that seems to be rather good at removing Tc1 sequences. However, such insertion can be used as a first step toward gene inactivation, allowing the isolation of Tc1 deletion derivatives (see below).

For our *ncs-1* gene, R. Plasterk's laboratory isolated a (homozygous) line with a Tc1 insertion in the middle of the last intron (see "Tc1 insertion allele" fig. 28), which is unlikely to result in a loss of function. We determined the exact insertion site (see above) which is at position 5231 relative to the cloned *ncs-1* gene fragment (in p.*ncs-1*, see fig. 22).

Experimental procedure

Done for us by Ronald H. A. Plasterk (Division of Molecular Biology, Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands) as described in R.H.A. Plasterk "Reverse Genetics: From Gene Sequence to Mutant Worm" pages 59-80 in *Caenorhabditis elegans, Modern Biological Analysis of an Organism*, METHODS IN CELL BIOLOGY, VOL. 48. Ed. H.F. Epstein, D.C. Shakes. Academic Press.

Used *ncs-1*-specific primers (for nested PCR):

5':
EdC1: 5'-GTTTACGGTTGCTTGAACACC-3' (at position 2722 relative to cloned *ncs-1* gene # (see fig. 22))
EdC2: 5'-CTGGAGTTTAGTTGATTGTGC-3' (at position 2850)
3':
EdC3: 5'-ACGCTTGATGATAGTGTTTGC-3' (at position 5721)
EdC4: 5'-ACAGGGACTCGACTATTTCAC-3' (at position 5885)

Used Tc1-specific primers (5' R1, R2; 3' L1, L2, pointing outside the transposon) are shown at page 69 in the previously referred book.
The isolated (homozygous) Tc1 strain was named NL748 (the *ncs-1* allele with the Tc1 insertion was named pk242). The primers that allow the detection of this insertion by nested PCR were: R1, EdC 4 for the 1st PCR and R2, EdC3 for the second, nested PCR.

Isolation of deletion derivatives

As already said, in general transposon insertions do not result in a loss of function. In our case, the transposon is located in the last intron and is very unlikely to give loss of function. Nevertheless Tc1 has been shown to induced deletions allowing the isolation of deletion derivatives. Therefore the Tc1 insertion mutant is the starting point to get a strain with a deletion in the *ncs-1* gene. Such deletions can arise spontaneously (at a low frequency) probably after an improper DNA repair following the jumping of the inserted Tc1 transposable element. Any event taking place in the germ line will be transmitted to the progeny. Therefore progeny may be selected in which the transposon plus part of the interrupted gene has been deleted. This selection is again a PCR screening. PCR is used to visualize deletions: the primers are chosen 3kb apart in the genomic DNA, which implies that the amplicon is too large to be efficiently amplified, and thus one selectively visualizes rare deletion derivatives were a large enough DNA segment has been removed nearing the 2 prime sites (allowing efficient PCR amplification). The chosen primers can be the same as the gene specific primers used for the isolation of the insertion strain. Note that the choice of the primers bias the search for deletions, allowing only the detection of deletion enclosed by the prime sites.
Many cultures of the Tc1 insertion strain are started. After the cultures are full grown, half of the worms are sacrificed to make PCR analyzable lysates. The lysates can be pooled. The different pools are analyzed by PCR, visualizing the deletion (see just before). Once a positive pool has been found, the screening can be refined up to the individual lysates to find a positive culture and then up to the individual worms from a positive culture (same screening principle as for the isolation of a Tc1 insertion strain) to get a single positive worm giving rise to a deletion line. Once a deletion derivative is obtain, one can analyze whether it is homozygous, and if not, if the homozygote is lethal or sterile (in this case one can induce a balancer allowing the lethal mutation to be stably maintained in a heterozygous strain; see "Genetic Balancer" again in "*Caenorhabditis elegans, Modern Biological Analysis of an Organism"*[125]).

I performed this deletion screening on the Tc1 insertion mutant and was able to isolate a deletion derivative strain with 4/5 of the *ncs-1* gene removed. This initial homozygous strain named XA401 is viable (and was crossed 3 times with wt, see below "crossings"). The deletion starts in the non-translated region of the first exon (just after the SL1 site) including the initiator ATG up to the middle of the last intron. Therefore the translated region of the 5 first exons (out of 6) are completely removed knocking out *ncs-1* gene (see fig. 28).

As the knock-out mutant is isolated from a Tc1 rich strain (permissive for transposon jumping) it bears a lot of transposons (which may be sometime harmful) in its genome and it also may have or may get deletions in many other genes, showing defects unrelated to *ncs-1* loss of function. Therefore to analyze the phenotype of *ncs-1* loss of function the genetic background of this initial knock-out strain as to be cleaned by crossing it with wild type (wt). **Crosses** are carried by putting together a hermaphrodite (homozygous) knock-out mutant together with several wt males. Half of the resulting progeny should be males if the crossing was successful. The hermaphrodite cross-progeny is heterozygous for *ncs-1* deletion and will give rise to ¼ of homozygotes self-progeny. These second generation worms are isolated and their progeny tested by PCR to isolate again a homozygous *ncs-1* deletion strain.
I performed such crossing 3 times. The resulting cleaned homozygous strain named XA403 is viable, healthy and looks normal in the standard laboratory conditions.

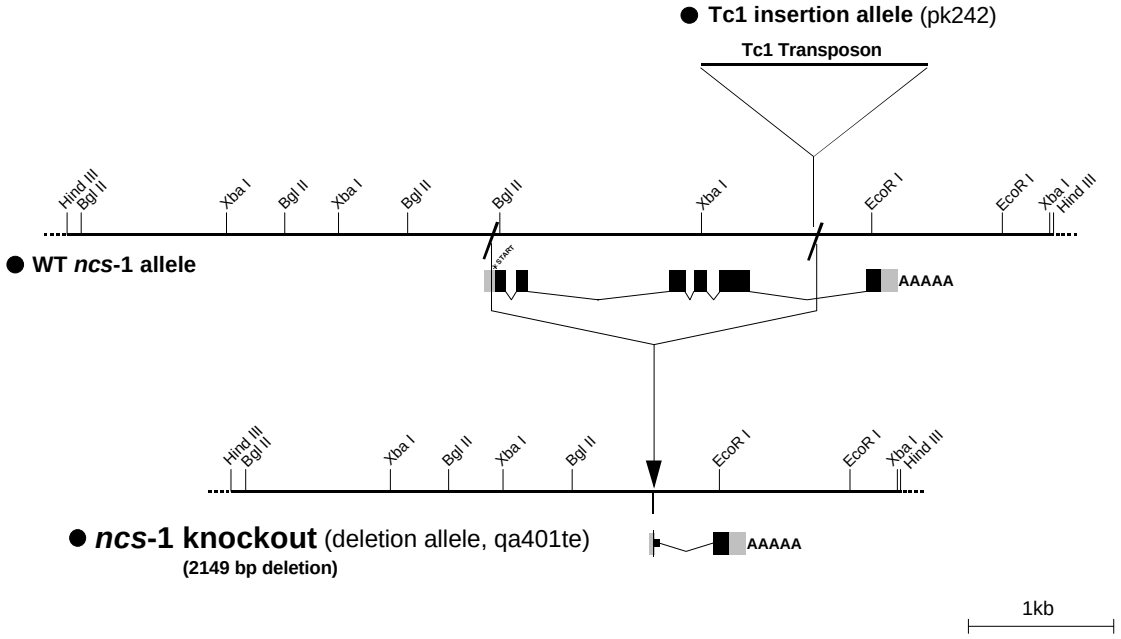


fig. 28.

Physical map of the *ncs-1* gene for the wt allele, for the Tc1 insertion allele, and in the knock-out deletion allele. Bellow WT and *ncs-1* knock-out maps, alignment map of exons (black boxes represent translated exon sequences; gray boxes, untranslated sequence, introns are diagrammed as thin lines; see below: "RT-PCR" for the exons in the deletion allele). The deletion starts in the non-translated region of the first exon (just after the SL1 site) including the start ATG up to the middle of the last intron, removing 2149bp.

Experimental procedure

PCR screening for deletion derivatives: as described in "Isolation of Deletion Derivatives" page 76-78, from R.H.A. Plasterk "Reverse Genetics: From Gene Sequence to Mutant Worm" in *Caenorhabditis elegans, Modern Biological Analysis of an Organism,* METHODS IN CELL BIOLOGY, VOL. 48. Ed. H.F. Epstein, D.C. Shakes. Academic Press.
Used *ncs-1*-specific primers:

5':
EdC1: 5'-GTTTACGGTTGCTTGAACACC-3' (at position 2722 relative to cloned *ncs-1* gene # (see fig. 22))
EdC1+: 5'-TCCGTATTTGAACGTTGCTAC -3' (at position 3028, just before SL1 site)
EdC2+: 5' GAGAGAATCAAGTTGCAAATC-3' (at position 3075)
3':
EdC3: 5'-ACGCTTGATGATAGTGTTTGC-3' (at position 5721)
EdC4: 5'-ACAGGGACTCGACTATTTCAC-3' (at position 5885)
NODEL3': 5'-GCTCTATGTGATAGCAAATG-3' (at position 3794, in the 2nd intron)

The PCR program used 40'' denaturation at 94°c, 50'' annealing, 1' elongation at 72°c. The primers that allow the detection of the deletions by nested PCR were: EdC1, EdC 4 for the 1st

PCR (with 35 cycles PCR, at an annealing temperature, T_{ann}, of 58°c) and EdC1+, EdC3 for the second, nested PCR (with 35 cycles PCR, at T_{ann}=58°c). For single worm analysis, only one round of PCR was performed using primers EdC1+, EdC3. (with still 35 cycles PCR, at T_{ann}=58°c). Without deletion the amplicon using EDC1+, EdC3 primers should be 2694bp long (+1610bp if the Tc1 is still present) and is not efficiently amplified in the used conditions. 80 culture plates of the Tc1 insertion strain were used to start the screening. Culture lysats (made of half of the culture worms) were first pooled 5 by 5. Deletions were visualized in many pools. The cultures corresponding to the pool showing the biggest deletion (giving the smallest PCR product) were further analyzed. A single positive culture was found, and fractionated in 8 sub-cultures. One of these sub-culture showed the deletion. 20 worms from this positive sub-culture were isolated. Out of these 20 worms, 14 made progeny (these worms originating from the mutator strain MT3126 are often sterile) and were analyzed for the deletion. One of these 14 worms was positive and homozygous (showing only positive worms in its progeny) for the deletion. The resulting deletion (knock-out) strain was named "XA401", the mutated *ncs-1* allele was named "qa401te" (where "te" stands for transposon excision, indicating that this deletion allele has been generated by imprecise loss of the transposon).
The precise extend of the deletion was determined by sequencing the amplified EdC1+, EdC3 PCR product cloned in a pBSK$^-$ plasmid (using standard molecular biology procedures[140]). The deletion start (relative to the cloned *ncs-1* gene fragment, see fig. 22) at position 3090 up to position 5238, removing 2149bp (EDC1+, EDC3 amplicon was 545bp long).
Crosses were carried out by placing a single L4 hermaphrodite knock-out mutant together with 5 young adult wt males on different small seeded culture plates (basic *C. elegans* culture methods[125]). In a plate where the cross was successful (showing many males in the progeny) 6 hermaphrodite worms from the resulting progeny were sub-cultured. The ones that had lay eggs (5), were tested for (*ncs-1* alleles) heterozygousity ensuring they result from a cross (all 5 tested worms were heterozygotes). The heterozygousity was tested with single worm PCR using EdC1+, EdC3 primers giving only amplification products on *ncs-1* deletion allele (knock-out) and EdC2+, NODEL3' primers giving only amplification products on wt allele. Heterozygotes should be positive in both tests (meaning they bear both alleles). The single worm lysat were divided in two to make both PCR tests at the same time (with 32 cycles PCR, at T_{ann}=55°c). 3 heterozygote hermaphrodites were sub-cultured (to be sure at least one will give progeny). 10 worms from a resulting progeny were sub-cultured. The progeny of this second generation offspring worms was then tested for the presence of the deletion allele (PCR with EdC1+, EdC3). If the mother is homozygous its tested progeny should be 100% positive for the deletion, giving rise to a crossed homozygous line. Homozygousity was also checked using the same double PCR test as described before: homozygotes should be positive using EdC1+, EdC3 primers, and negative with EdC2+, NODEL3' primers.
Successive crossing were then performed 2 more times, leading to a homozygous *ncs-1* knock-out strain crossed 3 times with wt, named XA403.

Knock-out controls

In order to prove that I have isolated a real homozygous *ncs-1* knock-out mutant I performed several control experiments: genomic southern blots to confirm that part of the *ncs-1* gene is deleted; RT-PCR to reveal the absence of *ncs-1* messenger, immunolocalizations (see above "Immunolocalization of *Ce*-NCS-1") to show that the *Ce*-NCS-1 protein is not produced.

Southern blot controls

I have compared the *ncs-1* knock-out strain to a wt strain by genomic southern blot analysis to confirm that the mutant has really a deletion in its *ncs-1* gene. Using either *Ce*-NCS-1 cDNA or a fragment of *ncs-1* gene as probes the resulting data show that my mutant really bears two (same) *ncs-1* alleles corresponding to the expected deletion alleles. With the cDNA probe the mutant shows reduced signal (as most of its exon sequences have been removed) compared to the wt and a hybridization pattern resulting from the expected deletion. With the genomic probe, again the hybridization pattern shows the deletion (see fig. 29). These data, together with the PCR data coming from the isolation of the mutant itself (see above), indicate that the isolated mutant is really a *ncs-1* homozygous deletion mutant.

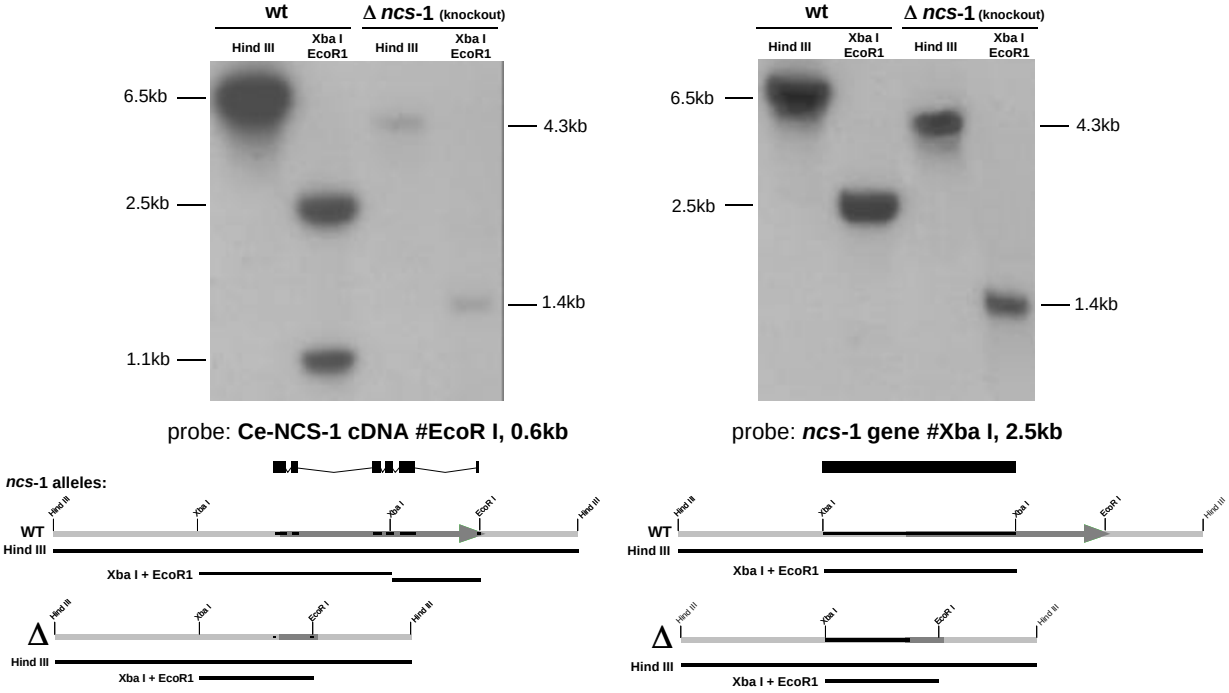


fig. 29. Genomic southern blot analysis of knock-out mutant compared with wt.
Both wt and mutant (Δ*ncs-1*) genomic DNA were digested by Hind III and Xba I/EcoR I restriction enzymes; hybridized with a cDNA and a genomic probe against *ncs-1* gene. Below autoradiography, used probes (cDNA or genomic) are shown in black over the physical maps (with used restriction enzymes) of wt and knock-out (deletion: Δ) *ncs-1* alleles. In physical maps dark gray lines represent region between ATG (start) and stop codon, black line regions that hybridize with the used probe. Bellow each physical map, the restriction fragments (produced by the Hind III and Xba I/EcoR I digestions) hybridizing with the used probe are shown in black and aligned with the corresponding physical map.

Experimental procedure

Genomic DNA isolation: as described before for "Genomic southern Blot analysis". Genomic DNA was isolated from both wt and *ncs-1* knock-out mutant worms.

Southern blot: like before in "Genomic southern Blot analysis". Two blots were made: one used with a *Ce*-NCS-1 cDNA probe, the other with a *ncs-1* genomic probe. For both blots wt and mutant DNA were digested once by Hind III, once by Xba I and EcoR I. *Ce*-NCS-1 cDNA probe was produced by random labeling (with ^{32}P-radionucleotides) of the ~580bp EcoRI fragment from *Ce*-NCS-1 cDNA. Ncs1 genomic probe was produced by random labeling (with ^{32}P-radionucleotides) of the ~2500bp XbaI fragment from the *ncs-1* gene. Autoradiography was performed for 4 days for the blot with the cDNA probe and 2 days for the blot with the genomic probe.

RT-PCR

In order to determine what appends in the knock-out mutant at the level of the messenger RNA, I performed PCR amplifications on reverse-transcribed total RNA (first strand cDNA) coming from the mutant (and the wt as a control). The PCR was done using primers against sequences enclosing the deleted region of *ncs-1* transcript. Amplification using primers against Ce-NCS-2 messenger was also performed as a RT-PCR positive control showing that the RNA of the mutant was efficiently reverse-transcribed giving a strong signal (like with wt RNA). PCR reactions were also performed on non-reverse transcribed RNA as negative controls to show that they were no contamination. With the *ncs-1* primers, a single round of PCR amplification on reverse-transcribed RNA from both wt and mutant give respectively little and no amplification products. Therefore nested PCR was used: a first PCR reaction is used as template for a second round of PCR amplification using more internal primers. With nested PCR, amplification of *ncs-1* messenger was efficient. Reverse-transcribed RNA from the mutant yielded a smaller amplification product than reverse-transcribed RNA from the wt worms. These PCR products were cloned and sequenced. The sequence of the "wt product" corresponded to *Ce*-NCS-1 cDNA, showing that the amplification was specific. The "mutant product" was *ncs-1* messenger showing the expected deletion plus the removal of most of the remaining part of the last intron. The sequence jumps before the ATG up to middle of the last intron and then after few bases the rest of this last intron is excised (from a new 5' donor site) up to exon 6. Before exon 6 sequence, there is no new ATG in the same reading frame.

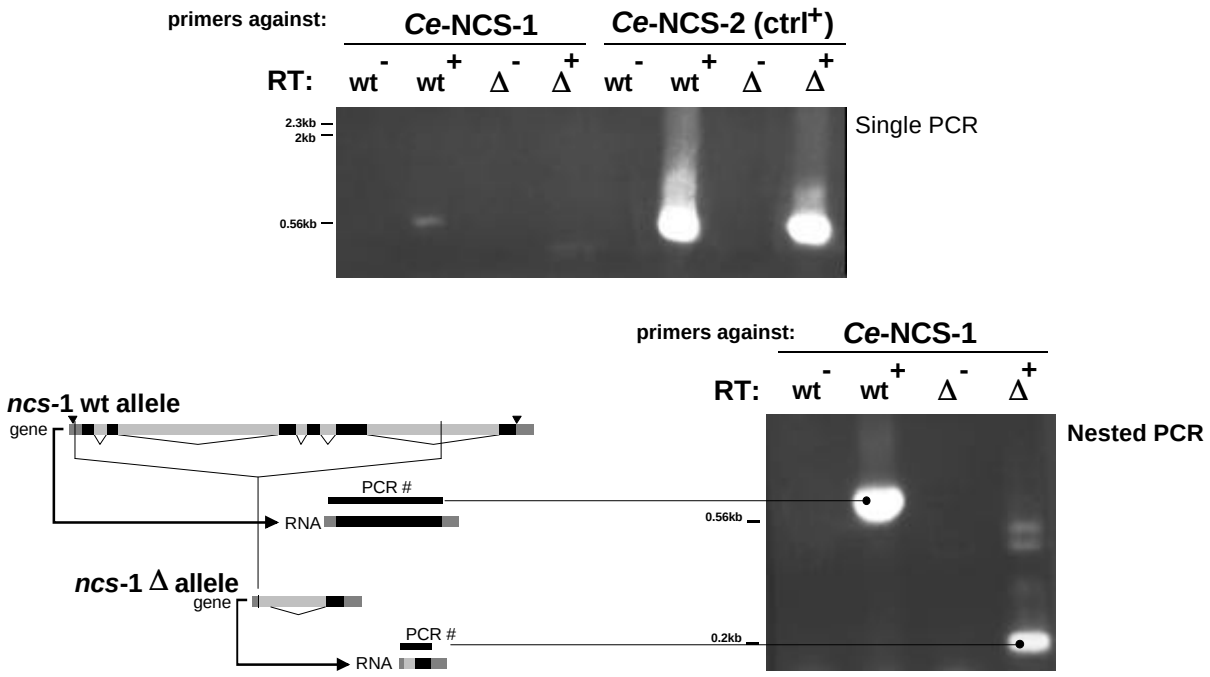


Fig. 30. PCR on reverse transcribed RNA from mutant and wt worms.
Both wt and knock-out mutant (deletion: Δ) reverse transcribed {+} total RNA as well as non-reverse transcribed total RNA {-} (as negative controls) were used as templates for PCR reactions. Resolution by electrophoresis of the resulting reaction products are shown for the single PCR *Ce*-NCS-1/*Ce*-NCS-2 and for the nested PCR *Ce*-NCS-1. Physical map of the *ncs-1* gene for the wt allele and for the knock-out deletion {Δ} allele (black boxes represent translated exon sequences) are represented. Bellow WT and *ncs-1* knock-out maps, schematic representation of the corresponding transcribed messenger RNA (black boxes represent translated exon sequences; dark gray boxes, untranslated exon sequences; light gray intron sequences). Over representation of the messengers, aligned derived amplified PCR products are depicted in black together with arrows pointing to their corresponding product in the electrophoresis

These data indicates (see fig. 30) that in the mutant, *ncs-1* messenger may be still made but that no part (even the one coded by the remaining exon 6) of *Ce*-NCS-1 protein can't be translated anymore.

Experimental procedure

RNA isolation: as described before for "Northern Blots". RNA was isolated from both wt and XA403 mutant starting from 1gr of purified worms.

Reverse transcription (RT): was performed with the Boerhinger-Manheim RT-kit (ref. 1483188) using 5μgr of total RNA by reaction according to the manufacturer protocol.

PCR: was performed on reverse transcribed RNA from both wt and knock-out mutant worms using 1/10 of the RT reaction. The PCR program used 40'' denaturation at 94°c, 50'' annealing, 1' elongation at 72°c.

Used primers were:

non hybridizing bases (cloning linkers) are shown between brackets

Against SL1 sequences (for the first round of nested PCR):

5': 5'SL1: 5'-(CGCAAGC)TTACCCAAGTTTGAG-3'

Against *ncs-1* messenger:

5': 5'X*Ce*-NCS-1: 5'-(CGCAAGCTT)CAAAAAAGAGAGAATCAAG-3'

3': EdC3: 5'-ACGCTTGATGATAGTGTTTGC-3'

3': 3'*Ce*-NCS-1: 5'-(CGCAAGCTTCC)ATGGGCAAAGGGAACAGCAAGT-3'

Against *ncs-2* messenger:

5': 5'*Ce*-NCS-2: 5'-(CGCAAGCTTCCC)ATGGGAATCAAGGGTTCCAAGC-3'

3': 3'*Ce*-NCS-2: 5'-(CGGGATCC)TTACTTCTTGACTTCATTGGTC-3'

For the single PCR (with 40 cycles, at an annealing temperature, T_{ann}, of 58°c) the used primers were: for *Ce*-NCS-1: 5'X*Ce*-NCS-1 / 3'*Ce*-NCS-1; for *Ce*-NCS-2: 5'*Ce*-NCS-2 / 3' *Ce*-NCS-2. In the nested PCR against *ncs-1* messenger, the used primers were: 5' SL1 / EdC3 for the first round (with 40 cycles, at an annealing temperature, T_{ann}, of 56°c) and 5'Xce-NCS-1 / 3'*Ce*-NCS-1 for the second round (with 30 cycles, at T_{ann}= 58°c with 1/100 of the first PCR reaction). The PCR products were resolved by electrophoresis, cloned (in pBSK$^-$) and sequenced using standard molecular biology procedures[140, 141]

Immunolocalization control:

See figure 21D,G,I.

As shown before in 'Immunolocalization of *Ce*-NCS-1', the knock-out mutant shows no more expression of *Ce*-NCS-1 protein (there is no more signal in immunolocalization with the anti-*Ce*-NCS-1 antibody).

Together all these controls show that **the isolated mutant is a real knock-out mutant, showing no more expression of the *Ce*-NCS-1 protein**.

Analysis of *ncs*-1 knock-out mutant phenotype

The homozygous *ncs*-1 knock-out mutant is viable and looks healthy. Its responses to touch is apparently normal and it can move. Knowing the cells where *Ce*-NCS-1 is expressed (mainly sensory neurons, see table 5, together with the anal depressor muscle and vulva muscles), the *ncs-1* knock-out should be tested for chemo/thermo-taxis defects and for dauer formation, egg-laying and defecation defects (see below).
Frequenin (see 'Introduction'), the *Drosophila* orthologue of *Ce*-NCS-1, has been shown, in the neuromuscular junction, to participate in the facilitation of neurotransmitter release (in response to repetitive stimulation) enhancing neuron excitability.
Recoverin (see 'Introduction'), regulates the light sensitivity of photoreceptors (in vetebrates), adjusting it to light conditions by inhibiting in a Ca^{2+} dependent way rhodopsin kinase the enzyme needed to phosphorylate active rhodopsin.
By extension these data could suggest that *Ce*-NCS-1 plays a similar role in *C. elegans*.
Working hypothesis: In some neurons and some muscles *Ce*-NCS-1 is involved in the modulation of cell excitability (like frequenin) and/or sensitivity (like recoverin) in function of stimuli frequency/intensity. It may therefore also play a role in behavioral plasticity (adaptation, learning). The *ncs*-1 knock-out is currently analyzed for sensory sensitivity/plasticity defects together with muscle contraction defects.

The analysis of the *ncs*-1 knock-out mutant behavior might gives insights into the *in vivo* function of *Ce*-NCS-1 in the *C. elegans* nervous/muscular system, and by extension of this novel NCS-1 sub-class of evolutionary highly conserved proteins.

In progress:

Chemosensory analysis[126, 130]

As some (pairs of) neurons where *Ce*-NCS-1 is expressed are known to be involved in the chemotaxis (attraction) to soluble compounds ('taste') such as cAMP, biotin, Cl-, Na+ (**ASE**, ADF, ASG neurons), in chemotaxis to volatile compounds ('olfaction') such as alcohols, ketones (**AWC** neurons: benzaldehyde, butanone, isoamyl alcohol; AWA neurons: diacetyl, pyrazine, 2,4,5-trimethylthiazole), in avoidance to volatile compounds (**AWB** neurons: e.g. octanol, nonanone, high conc. of benzaldehyde) and in detection of a pheromone inducing dauer larva formation (**ADF**, ASG neurons, see below 'Egg laying, defecation, dauer formation analysis'). The *ncs*-1 knock-out mutant is tested for "defects" in chemosensation.
C. elegans is sensitive to numerous environmental chemical stimuli (both water soluble and volatile compounds): e.g. it can chemotax to attractive compounds (mainly chemicals produced by bacteria, its food source), avoid noxious compounds. Animals exposed to high concentration of an attractant odorant slowly lose their sensitivity to that odorant over a few hours, showing odorant-specific adaptation (see 'Introduction'). Therefore *ncs*-1 knock-out mutant is currently analyzed (in our laboratory) for defect in sensitivity and adaptation to several chemicals attractant.
Moreover, this mutant will also be analyzed for defect in associative learning related to chemosensation (see 'Introduction' and 'Conclusions and prospects').

Basic Chemosensory Assays

Accumulation assay

The standard chemotaxis assay measures the ability of *C. elegans* to track to the point source of a chemical gradient on an agar plate. Chemotaxis of populations is measured by determining the proportion of worms at the attractant source (over time) relative to all worms, giving a chemotaxis index. *C. elegans* adapts to an attractant after prolonged exposure (see 'Introduction' and below), so the steady-state number of worms at an attractant will reflect

both attraction and adaptation processes. To minimize the effect of adaptation, an anesthetic can be used to capture animals at the attractant area.
Chemotaxis of individual worms can be measured by watching the tracks that they leave on the agar plate in the chemical gradient. Time needed for proper orientation in the gradient, threshold sensitivity for the attractant (minimum concentration start position in the chemical gradient), speed toward the attractant can be measured. The *ncs*-1 knock-out mutant is currently compared to wt regarding these behaviors.

Adaptation
Following continuous exposure for varying amounts of time, the worms' attraction to the adapting odorant can be measured (chemotaxis index) in standard accumulation chemotaxis assay. The extend of adaptation was dependent on the adapting odorant, the time of exposure to that odorant, and to the amount of odorant to which the worms were exposed. The *ncs*-1 knock-out mutant is currently compared to wt regarding odorant adaptation (kinetic, amount, selectivity).

Results (preliminary, data not shown):

The mutant react (chemotax) to isoamyl alcohol and benzaldehyde both sensed by the AWC pair of neurons indicating that these AWC neurons are (likely) still present and functional. We can conclude that *Ce*-NCS-1 expression is probably not required for the proper development of AWC neurons and not required for 'basic' sensory abilities (consistent with the putative fine-tuning/ modulatory function of NCS proteins).
Treshold sensitivity and adaptation to benzaldehyde and isoamyl alcohol together with chemotaxis to soluble compounds are currently tested.

Egg laying, defecation, dauer formation analysis[145, 146]

As *Ce*-NCS-1 is strongly expressed in the neuron ASE neuron, know to play a role in the control of egg laying[126] and as *Ce*-NCS-1 is also found in vulva muscles, the egg laying behavior of the *ncs*-1 knock-out mutant is analyzed.
The uterine and the vulva muscles contract to expel the (fertilized) eggs produced by the hermaphrodite. Environmental stimuli can affect the rate of egg laying. Hermaphrodites lay more eggs in the presence than in the absence of food (bacteria), probably via sensory neuron ASE connected to the egg-laying neuronal circuitry. *ncs*-1 knock-out mutant is analyzed for defect in these phenomena (egg laying frequency, sensitivity to food, etc…).

As *Ce*-NCS-1 is expressed in the anal depressor muscle needed to open the anal canal (involved in defecation expulsion step).The defecation behavior of the *ncs*-1 knock-out mutant is analyzed.
When assayed at temperatures from 19°C to 30°C in the presence of plentiful food, defecation occurs every 45 seconds (standard deviation < 3sec. at 20°C). In the hermaphrodite, defecation is carried out by 3 distinct motor steps (forming the defecation motor program; DMP): posterior body muscle contraction (pBoc), anterior body muscle contraction (aBoc), and expulsion muscle contraction (Exp or EMC). *ncs*-1 knock-out mutant is analyzed for defect in defecation frequency, volume, and defecation muscle excitation.

As *Ce*-NCS-1 is expressed in the neurons ADF and ASG know to play a role in the control of dauer larva formation the dauer formation 'behavior' of the *ncs*-1 knock-out mutant is analyzed.
Dauer larva is an alternative, stress-resistant arrested developmental stage (starting after L2 molt) assumed in 'hard times', specialized for dispersal and long-term survival. Dauer larva are capable of active movement but do not feed. They can survive at least four to eight times the normal 2-week life span of *C. elegans*. They are relatively thin and dense compared to other larval stages. When growth conditions improve the dauer larva will recover, molt, and resume the life cycle at L4 stage. The dauer larva formation is induced by increased population density and limited food supply via sensory cue integrating presence of food,

temperature, and a dauer inducing pheromone. The concentration of this pheromone, constitutively released by the worms, reflects nematode population density. The ratio of pheromone to food signal influence dauer larva formation (and recovery). Temperature modulates the response to the food/pheromone balance, with higher growth temperatures favoring the dauer state. The 'decision' whether or not to enter the dauer state is made during the L1 and L2 stages assessing the aforementioned environmental cues. *ncs*-1 knock-out mutant is analyzed for defect in this process (dauer formation/recovery, sensitivity to dauer pheromone, food and temperature).

Basically, we observe that *ncs*-1 knock-out mutants lay eggs. Therefore *Ce*-NCS-1 expression is not required for the proper development of vulva muscles (in which it should be expressed normally) nor for 'basic' vulva contraction.

These analysis (egg laying, defecation, dauer formation) of ncs-1 knock-out mutant are currently performed for us by ***Dr. James H. Thomas*** *(department of Genetics, University of Washington, Seattle, USA) an expert of C. elegans egg laying, defecation, and dauer larva formation.*

Thermotaxis analysis[126, 129]

As the neuron AFD and AIY, where *Ce*-NCS-1 are expressed, are known to be involved in thermotaxis[144] (only 3 pairs of neurons are involved in thermotaxis: AFD, AIY, AIZ), the thermosensory behavior of the *ncs*-1 knock-out mutant is analyzed.
When wt worms are grown at a temperature from 16° to 25°C (in presence of food) and placed on a thermal gradient (without food), they will migrate to their growth temperature and then move isothermally. Behavioral adaptation to a new temperature takes several hours (worms shifted from one temperature to another shift their preference to the new temperature over ~4hours). In contrast, starved worms disperse from the growth temperature. *ncs*-1 knock-out mutant was analyzed for defect in these phenomena.
This thermotaxis analysis of ncs-1 knock-out mutant was performed for us in the group of ***Dr. Yasumi Ohshima*** *(department of Biology, Kyushu University, Hakozaki, Japan) by* ***Dr. Ikue Mori****.*

Results

The thermotaxis of *ncs*-1 knock-out mutant to its cultivation temperature is abnormal. The mutant does not "remember" its growth temperature or does not sense temperature (this should be checked), as it does not move isothermally in a radial gradient (see fig. 31).

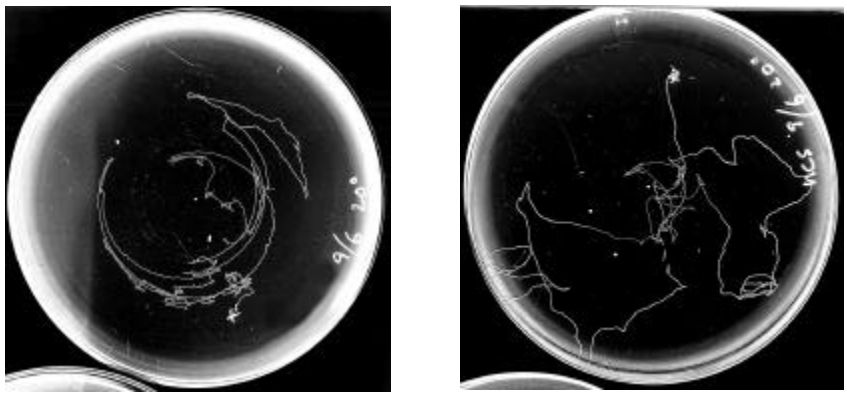


Fig. 31. Tracks of worms (wt and *ncs*-1 ko mutant) grown at 20°c in a radial temperature gradient (center: 17°c – border: 25°c)

Experimental procedure

Done by Ikue Mori in the Yasumi Ohshima laboratory. As described in article [144]

Conclusion and prospects

Conclusion

In the present study, I have characterized a *C. elegans* Ca^{2+} binding protein, *C. elegans* Neuronal Calcium Sensor-1 (*Ce*-NCS-1), member of the recoverin/neuronal calcium sensor-family (NCS-family), highly related to NCS-1 sequences[94] found from yeast to mammals. ***Ce*-NCS-1 belong to the NCS-1 sub-family that is formed by highly conserved species equivalents (orthologues)** found in chick/rat/mouse (NCS-1) in *Xenopus* (Xfreq), *C. elegans* (*Ce*-NCS-1), Yeasts (*Sc*-NCS-1, *Sp*-NCS-1) showing more than 60% of amino acid identity between them (see 'Introduction'). NCS-1 is the only protein from the recoverin/NCS-family that has a homologue in yeast. It represents therefore the most conserved 'member' of this family.

To estimate the degree of gene diversity in the *C. elegans* NCS family, I looked for other *C. elegans* NCS. I characterized a unique protein related to the NCS family that was named *Ce*-NCS-2. This protein represents the most divergent protein of the NCS family since it shares 45% of identity with *Ce*-NCS-1 and only 37-49% of identity with other members of the NCS family being likely a nematode specific NCS. By my cDNA screenings I didn't find any additional NCS. These results, together with genomic Southern blot data indicate that *Ce*-NCS-1 is probably the only NCS-1 like protein in the *C. elegans*.

***Ce*-NCS-1 is a *C. elegans* Ca^{2+} binding protein** as purified recombinant *Ce*-NCS-1 can bind Ca^{2+} in ^{45}Ca overlay assay.

Purified recombinant *Ce*-NCS-1 (as well as *Ce*-NCS-2) was shown *in vitro* to inhibit rhodopsin phosphorylation in a Ca^{2+} dependent way[94] like recoverin by inhibiting in a Ca^{2+} dependent way rhodopsin kinase, the enzyme needed to phosphorylate activated rhodopsin. By extension these results indicate that *Ce*-NCS-1 like other non-retinal NCS, could regulate rhodopsin kinase, although is not its natural target, suggesting that other receptor kinase may be endogenous targets. Therefore *Ce*-NCS-1 could play a potential role in the calcium-sensitive phosphorylation of components of the signal transduction machinery (leading to adaptation or desensitization).

Purified recombinant *Ce*-NCS-1 was also shown *in vitro* to directly activates 3':5' - cyclic nucleotide phosphodiesterase (see 'Activation of 3':5' - cyclic nucleotide phosphodiesterase'), a well known target of calmodulin, acting like chick/rat NCS-1 which can, in some cases, replace CaM or potentiate CaM effects[106].

Together these *in vitro* data suggest that *Ce*-NCS-1, and by extension other NCS-1 proteins, may have multiple targets, being multifunctional (like CaM) modulators. Potential targets of (*Ce*)-NCS-1 are: pumps/channels, G-proteins coupled-receptor kinases, calmodulin target enzymes. Nevertheless the real in-vivo targets and the exact physiological function of NCS-1 proteins remain unknown.

In the hermaphrodite *C. elegans*, *Ce*-NCS-1 is expressed in all larva stages and adults (expression in embryos and males has not been examined). ***Ce*-NCS-1 is expressed in some neurons (mainly sensory neurons, but also inter-neurons and in a motor-neuron) and in few muscles**. Among the total 302 *C. elegans* neurons (in the hermaphrodite), 26 (13 pairs of bilateral symmetric neurons) express *Ce*-NCS-1: 10 pairs of sensory neurons, 2 pairs of inter-neurons, and a single pair of motor-neuron (see table 5).

In neurons, *Ce*-NCS-1 is preferentially localized in both axons and dendrites (neurites), being almost excluded from cell bodies. This subcellular localization indicates that *Ce*-NCS-1 can be found both pre- and post-synaptically and that it is probably associated with plasma membrane (or some cytoskeleton components).

The homozygous *ncs*-1 knock-out mutant is viable, fertile and looks healthy. As *Ce*-NCS-1 is expressed in the anal depressor muscle and vulva muscles together with some sensory neurons known to be involved in chemotaxis to volatile odorants, chemotaxis to soluble compounds, volatile avoidance, thermotaxis, egg laying regulation, and dauer larva formation control (see table 5) the ***ncs-1*** **knock-out mutant is currently tested for chemo/thermo-taxis defects and for dauer formation, egg-laying and defecation defects.**

ncs-1 knock-out mutants can still lay eggs and sense the volatile odorants isoamyl alcohol and benzaldehyde. Therefore **it seems that *Ce*-NCS-1 expression is not required for 'basic' sensory or motor abilities** nor for the proper development of cells expressing it.

Ce-NCS-1 is highly related to *Drosophila* Frequenin (see 'Introduction'), which has been shown, in the fly neuromuscular junction, to increase the neurotransmitter release in response to repetitive stimulation, enhancing neuron excitability. Apparently, frequenin functions as a Ca^{2+} sensor that is involved in facilitation of the neurotransmitter release. Frequenin seems to modulate excitation-secretion coupling and thus enhance the sensitivity of the synapse to an incoming stimulus. Electrophysiological data suggest a possible modulation of the Na^+/Ca^{2+} exchange by the Ca^{2+}-frequenin[113] through an increase in internal Na^+ concentration at the release site reducing (locally) Na^+ gradient and therefore Na^+/Ca^{2+} exchange. The modulation of this exchange is probably mediated via the inhibition of Na^+ pumps and/or Na^+/Ca^{2+} exchanger (and/or the modulation of other pumps/channels). Moreover, rat/chick NCS-1 has been shown to partially restore the wild-type behavior in calmodulin-defective *cam[1] Paramecium* mutants[106]. These *cam[1]* mutants have lost their Ca^{2+}-dependent K^+ current as their mutated calmodulin can not activate a K^+ channel anymore. The (partial) restoration of wt behavior in these mutants by NCS-1 indicates that there is probably an interaction of NCS-1 with this K^+ channels. Together these data are clues for a possible interaction of NCS-1 proteins with channels.

Recoverin, an other member of the NCS family, is known, in mammal eyes, to regulates the light sensitivity of photoreceptors, adjusting it to light conditions.

By extension *Ce*-NCS-1 may play similar roles in *C. elegans*. Cellular and subcellular localization of *Ce*-NCS-1 in neurons indicates that it is not restricted to presynaptic terminal and axons where it could modulate neurotransmitter release like frequenin, but is also presents in the postsynaptic dendrites where it could modulate cell sensitivity like recoverin.

Our working hypothesis is the following: in some neurons and some muscles *Ce*-NCS-1 is involved in the modulation of cell excitability/sensitivity in function of stimuli intensity/frequency likely by modulating, in a Ca^{2+} dependent way, channels/pumps and/or the phosphorylation of signal transduction machinery components. It may therefore play a role in behavioral plasticity (adaptation, learning). **We are expecting to see, in the knock-out mutant, sensory sensitivity/plasticity defects together with muscle excitability defects.**

We observed that the *ncs*-1 knock-out mutant show abnormal thermotaxis. The mutant does not "remember" its growth temperature (or does not sense temperature). **This result may indicate that *Ce*-NCS-1 is involved in some memory processes.**

The *in vivo* functional role of NCS-1 proteins is still elusive. The presented studies shed light on possible functions of *C. elegans* NCS-1. The currently undertaken analysis of the *ncs*-1 knock-out *C. elegans* mutant that I have generated may give insight into the function of *Ce*-NCS-1 in the *C. elegans* nervous/muscular system, and by extension into the function of the highly conserved NCS-1 sub-family of neuronal calcium sensors.

Results summary

- ***Ce*-NCS-1 is a *C. elegans* Ca^{2+} binding protein highly related to other NCS-1 proteins**
- *Ce*-NCS-1 seems to be the only *C. elegans* NCS-1 sequence.
- Ce-NCS-2 was the only other NCS sequence found in *C. elegans*.
- *In vitro, Ce*-NCS-1 can inhibit rhodopsin phosphorylation in a Ca^{2+} dependent way.
- *In vitro, Ce*-NCS-1 can directly activate 3':5' - cyclic nucleotide PDE.
- **In the hermaphrodite, *Ce*-NCS-1 is expressed in all larva stages and adults**
  (expression in embryos and males has not been examined)
- ***Ce*-NCS-1 is expressed in certain 'excitable' cells including:**
  Different types of neurons:
  - **10 pairs of sensory neurons**:
    involved in chemotaxis to volatile odorants, chemotaxis to soluble compounds, volatile avoidance, thermotaxis, egg laying regulation, and dauer larva formation control.
  - **2 pairs of inter-neurons**
  - **1 pair of motor-neurons**

  Muscles:
  - Vulva muscles
  - Anal depressor muscle
  - pm1 pharyngeal muscle cell
- **In neurons, *Ce*-NCS-1 is find both in axons (pre-synaptically) and dendrites (post-synaptically) but is significantly excluded from cell bodies.**
- *Ce*-NCS-1 is probably associated with membranes **(**or with some cytoskeleton components**)**.
- ***ncs*-1 knock-out mutant is viable, fertile, can move, and looks healthy.**
- ***Ce*-NCS-1 seems not to be required for 'basic' sensory or motor abilities nor for the proper development of cells expressing it**, but should be rather implicated in the modulation, the fine-tuning of such sensory/motor processes.
- ***Ce*-NCS-1 is possibly involved in the modulation of cell excitability/sensitivity and plasticity** (likely by modulating, in a Ca^{2+} dependent way, channels/pumps and/or the phosphorylation of signal transduction machinery components).
- **The phenotype of ncs-1 knock-out mutant is currently analyzed.** We are expecting to detect, in this knock-out mutant, sensory sensitivity/plasticity defects together with muscle excitability defects.
- **The *ncs*-1 knock-out mutant show abnormal thermotaxis. The mutant does not "remember" its growth temperature (or does not sense temperature). This result may indicate that *Ce*-NCS-1 is involved in some memory processes.**

Prospects

C. elegans provides an opportunity to examine behaviors at a number of levels. Because the nervous system is so compact and well-described, the contributions of individual neurons to complex behavior such as chemo/thermo -taxis, egg laying, dauer formation can be studied in detail. Even the contribution of individual genes to such behaviors are analyzable. *C. elegans* represent a very attractive system to study the function of NCS-1 proteins. The putative 'subtle' sensory defects of the *ncs*-1 knock-out mutant that I have generated might represent a special opportunity to gain new insights into the function of the NCS-1 proteins.
In addition to the requisite (currently undertaken) analysis of the *ncs*-1 knock-out mutant phenotype:

Analysis of the ncs-1 knock-out mutant phenotype (still in progress…)

See above ('Results', 'Analysis of *ncs*-1 knock-out mutant phenotype').

Testing chemosensory associative memory

To see if other (than thermosensory) "memory" related phenomena are affected by the ncs-1 mutation, we could try to test associative memory related to chemosensation (e.g. conditioning with an attractive odorant associated to a noxious stimuli, leading to repulsion toward this odorant). We must note that this kind of chemosensory associative memory is still under controversy and that reliable tests are not yet published.

Rescuing of the ce-ncs1 -/- mutant phenotype

To prove that the observed *ncs*-1 knock-out mutant phenotype is due to the knock-out of *ncs*-1 gene and not to other unrelated mutation in the starting strain, we will have to rescue the mutant with the *ncs*-1 wt gene. The wt behavior should be restored to prove that mutant behavior was solely due to the knock-out of the *ncs*-1 gene. Transgenic *ncs*-1 knock-out worms bearing a wt *ncs*-1 gene copy should be made and analyzed for rescuing.
(In order to see if the different *ncs*-1 genes are really interchangeable, we can test the rescuing of the *ncs*-1 knock-out mutant with *ncs*-1 genes from other species)

The results I have obtained serve as an excellent starting point for numerous investigations. Below, I have listed some points which I believe should be the next stages in furthering our knowledge on NCS protein using *Ce*-NCS-1 (and Ce-NCS-2) as model(s):
(list is neither exhaustive nor listed by order of priority)

Localization of Ce-NCS-1 in embryos and males

Ce-NCS-1 is expressed in all larva stages and adult hermaphrodite. Localization of the protein in embryos and in males has not been examined. It would be interesting to determine if there is any sexual dimorphism in *Ce*-NCS-1 expression (sex specific patterns), if some additional male specific neurons (which probably detect pheromones during mating) express *Ce*-NCS-1 and, in embryos, at which stage the expression of *Ce*-NCS-1 starts.

Precise sub-cellular localization of Ce-NCS-1

It would be interesting determine if *Ce*-NCS-1 is localized in synaptic vesicles, in postsynaptic densities, associated with membranes or freely diffusing in the cytoplasm using electron microscopy. Moreover we could determine whether *Ce*-NCS-1 is myristoylated *in vivo*.

Ca^{2+} binding properties of Ce-NCS-1

We could determine the Ca^{2+} binding properties of *Ce*-NCS-1 (and *Ce*-NCS-2) such as affinity, number of binding sites, cooperativity , kinetic.

Over expression of* Ce-*NCS-1

Like what was made in *Drosophila* with frequenin[99] (motor-neurons expressing excess amount of frequenin exhibit enhanced synaptic transmission, leading to a shaker phenotype),

we can generate transgenic worms overexpressing *Ce*-NCS-1 (bearing additional copies of *ncs*-1 gene). Such mutant may show more severe defects than the loss of function knock-out mutant.
Phenotypes of both *ncs*-1 over expressing and *ncs*-1 knock-out mutants might tell us more about *Ce*-NCS-1 functions.

Targets of Ce-NCS-1

The further step toward the understanding of *Ce*-NCS-1 physiological function is to find its target(s). This can be done using the two-hybrid screen system in yeast, that allows the direct characterization of physically interacting partners for a protein of interest (in progress for rat/chick NCS-1 which may have similar targets).
One can also look for proteins co-localizing with *Ce*-NCS-1. These co-localizing proteins may be targets/partners of our *Ce*-NCS-1.
In an other hand, purified recombinant *Ce*-NCS-1 can be directly used in *in vitro* and/or *in vivo* reconstitution assays to see if it has any physiological effect. As *Ce*-NCS-1 may interact with pumps/channels it can be added in recordings of reconstituted channels. For example the *C. elegans* K^+ channel egl-36 (expressed in some neurons and muscles) is analyzed in *Xenopus* oocytes (by the group of James H. Thomas) and may be a putative *Ce*-NCS-1 target. Purified recombinant *Ce*-NCS-1 will be added in such recording to see if it modifies the electrophysiological behavior of the channel (indicating a modulation of the channel properties by *Ce*-NCS-1 and thereby an interaction of *Ce*-NCS-1 with that channel).
Obviously the phenotype of the *ncs*-1 knock-out mutant might give us clues about *in vivo* function of *Ce*-NCS-1 and therefore about possible *Ce*-NCS-1 targets.

Among potential candidates, true direct targets should:

- At least partially co-localize with *Ce*-NCS-1.
- Have their activity/properties modified by *Ce*-NCS-1 (for enzymes and channels/pumps).
- Show interaction with *Ce*-NCS-1 in two hybrid assay.

In vitro assays have shown that purified recombinant *Ce*-NCS-1 can inhibit rhodopsin phosphorylation[94] in a Ca^{2+} dependent way, probably like recoverin, by inhibiting rhodopsin kinase and also can directly activates purified 3':5' - cyclic nucleotide phosphodiesterase (like chick/rat NCS-1[106]). These results indicate that there is an *in vitro* interaction of *Ce*-NCS-1 with both rhodopsin kinase and PDE. What about *in vivo*? Obviously frog photoreceptor rhodopsin kinase is not *Ce*-NCS-1 natural target nor bovine brain PDE, but particular *C. elegans* receptor kinase/ PDE may be proprietary targets. Anyway *in vitro* interactions may not be relevant of true *in vivo* mechanisms and should be confirmed with the aforementioned criteria.

In vivo analysis of mutated Ce-NCS-1

If there is a clear phenotype for the *ncs*-1 knock-out mutant, mutated forms of *Ce*-NCS-1 protein could be expressed in this knock-out mutant (generation of transgenic *ncs*-1 knock-out worms bearing mutated *ncs*-1 genes, analysis of the phenotype) to determine which part of the protein is essential for a particular function. For example *Ce*-NCS-1 Ca^{2+} binding sites could be inactivated to see, *in vivo*, if there is '*Ce*-NCS-1 core' dependent but Ca^{2+} independent functions of *Ce*-NCS-1.

Analysis of Ce-NCS-2

It would be also interesting to determine *Ce*-NCS-2 function although it seems to be a *C. elegans* specific NCS with no closely related sequences in other species. This protein seems to be expressed both in many neurons and in the gonads (preliminary, data not shown).
The same kind of analysis as with *Ce*-NCS-1 could be undertaken: analysis of the expression, generation of a knock-out mutant, overexpression of *Ce*-NCS-2, characterization of its targets.

Résumé en français

But de ce travail

Dans notre laboratoire, nous avons pu isoler, chez le poulet, un membre de la famille des senseur de calcium neuronaux, jusqu'alors inconnu (« neuronal calcium sensor » NCS, voir « Introduction ») qui fut nommé NCS-1. Par la suite, j'ai caractérisé l'homologue de NCS-1 chez le rat qui s'est révélé avoir une séquence absolument identique au niveau des acides-aminés. Comme NCS-1 apparaissait être très conservé au cours de l'évolution, et comme nous cherchions un organisme modèle simple pour analyser la fonction de NCS-1, nous avons alors décidé d'utiliser le nématode *Caenorhabditis elegans* (*C. elegans*) pour l'étude de cette protéine. Le *C. elegans* est un excellent organisme modèle pour la neurobiologie. Il possède environ 300 neurones sur un total de 1000 cellules (somatiques). Le *C. elegans* répond à une grande variété de stimulus externes et peut modifier son comportement d'après son expérience. Le *C. elegans* sera bientôt le premier organisme multicellulaire dont le génome soit entièrement séquencé. Cet organisme est aussi particulièrement adapté pour l'analyse génétique, il représente donc un système très attractif pour étudier la fonctions de gènes codant pour des protéines comme NCS-1.

Par conséquent mes objectifs (principaux) étaient :

- De caractériser un homologue de NCS-1 dans le nématode *C. elegans*.
- D'étudier sa distribution cellulaire.
- De générer et d'analyser le phénotype de *C. elegans* mutants avec le gène *ncs*-1 inactivé.

Ces résultats ainsi que ma participation à d'autres projets ont conduit aux publications suivantes :

1. Nef S, Fiumelli H, De Castro E, Raes M-B, Nef P (1995). **"Identification of a neuronal calcium sensor (NCS-1) possibly involved in the regulation of receptor phosphorylation."** J. Recept. Res. 15(1-4), 365-378. (1995)

2. De Castro E, Nef S, Fiumelli H, Lenz SE, Kawamura S, Nef P (1995). **"Regulation of rhodopsin phosphorylation by a family of neuronal calcium sensors."** Biochem. Biophys. Res. Commun. 216:133-140. (1995)

3. Nef S., Allaman I., Fiumelli H., De Castro E., Nef P. (1996). **"Olfaction in birds: differential embryonic expression of nine putative odorant receptor genes in the avian olfactory system."** Mechanisms of Development 55:65-77. (1996)

4. Schaad N., De Castro E., Nef S., Hegi S., Hinrichsen R., Martone M., Ellisman M., Sikkink R., Rusnak F., Sygush J., Nef P. **"Direct modulation of calmodulin targets by the neuronal calcium sensor NCS-1."** Proc. Natl. Acad. Sci. USA 93:9253-9258. (1996)

5. De Castro E., Nef P. Characterization and analysis of *Caenorhabditis elegans* Neuronal Calcium Sensor-1. In preparation.

Introduction

Les cellules eucaryotes maintiennent dans leur cytosole une concentration de Ca^{2+} libre très faible (10-100nM). Le Ca^{2+} extracellulaire est lui beaucoup plus élevé (1mM), ce gradient est maintenu grâce à un mécanisme d'homéostasie (principalement via diverses pompes). Lors d'une stimulation par des hormones, des neurotransmetteurs, des photons, des odeurs ou une activité électrique, le taux de Ca^{2+} intracellulaire peut rapidement augmenter (il peut s'élever jusqu'à quelques μM), soit via le relâchement de calcium depuis des réservoirs de Ca^{2+} intracellulaire, soit via l'entrée de Ca^{2+} depuis l'extérieur à travers la membrane plasmique (par l'ouverture de canaux calciques). Le calcium une fois dans le cytosole, fonctionne comme messager second qui peut réguler, par exemple, la contraction musculaire, la sécrétion, le cycle cellulaire, la différentiation, l'apoptose, la plasticité neuronale, la transcription, etc…

Dans la plupart des cas, l'action du Ca^{2+} dépend de son interaction avec certaines protéines liant le Ca^{2+} (« calcium binding protein » ; CBP). Ces protéines médiant l'effet du calcium sont des **senseurs** de calcium (comme la calmoduline, la recoverine) qui peuvent altérer la fonction de protéines cibles en réponse à un changement de la concentration de Ca^{2+} libre, transmettant le message calcique. Il existe aussi des CBP nommées **tampons** qui ne font que tamponner la concentration du Ca^{2+} libre et qui n'interagissent pas avec une cible (de manière calcium dépendante).

Remarquablement, la liaison du Ca^{2+} sur un senseur de calcium induit généralement chez celui-ci, un changement de conformation, qui provoque l'exposition de surfaces hydrophobes, permettant l'interaction avec des cibles (qui sont dans le cas de la calmoduline, par exemple des kinases, phosphatases, des canaux, la synthétase d'oxyde nitrique). Les tampons eux ne montrent habituellement pas de changement de conformation lors de la liaison du Ca^{2+}.

Un large groupe de protéines liant le calcium possède des motifs main-EF (« EF-hand ») comme domaines de liaisons au Ca^{2+} (il faut noter qu'il existe aussi d'autres motifs de liaison au Ca^{2+}, par exemple les motifs annexines et C2) formant la super-famille de protéines liant le calcium à main-EF (« EF-Hand CBP ») . Le motif main-EF est un domaine canonique d'environ 30 acides aminés arrangé en une boucle liant le Ca^{2+}, entourée de deux hélices presque perpendiculaires. Le Ca^{2+} est lié, dans la boucle par 7 ligands oxygène fournis, pour 5 d'entre eux, par des chaînes latérales acides (ou alcool), pour un, par un groupe carbonyle du squelette peptidique, et pour un autre, par une molécule d'eau ; le tout formant une coordination bipyramidale-pentagonale. Les motifs EF se trouvent presque toujours intimement liées en pairs formant un domaine tandem globulaire (où les domaines EF individuels sont plus stable que lorsqu'ils ne sont pas appariés). Actuellement la super famille des « EF-Hand CBP » peut être divisée en 39 sous-familles, et inclut plus de 250 protéines.

La famille des senseurs de calcium neuronaux NCS

Récemment, des protéines formant une famille, jusqu'alors inconnue, de senseurs de calcium neuronaux ont été caractérisées. Cette branche émergante dans la superfamille des protéines à main-EF est nommée famille des «neuronal calcium sensors» (NCS) (senseurs de calcium neuronaux) où aussi famille recoverine/vilip (d'après le nom des premières séquences caractérisées).

Les membres de cette famille NCS montrent un haut degré d'homologie entre eux avec 36 à 100% d'identité au niveau des acides aminées. Ce sont tous des protéines d'environ 200 acides aminés (avec un poids moléculaire de 22-24kD), acides (avec un point iso-éléctrique entre 4-6). Elles possèdent tout quatre main-EF potentielles, bien que fréquemment certaines d'entre elles semblent être clairement dégénérées et ne peuvent probablement plus lier le Ca^{2+}. D'après la similarité de leurs séquences ainsi que par des études phylogénétiques on peut grouper ces protéines en différentes sous-familles/sous-groupes (voir figure 7).
Un de ces groupes est formé par les **NCS spécifiques à la rétine** (exprimée dans la rétine des vertébrés) comme la recoverine, la visinine et la S-moduline.
Un autre groupe comprend les protéines de type **NCS-1** (principalement étudié par notre laboratoire) incluant des séquences hautements homologues (voire figure 10) isolées chez le rat, la souris, le poulet (NCS-1 rat/souris/poulet, les 3 totalement identiques), jusqu'à, étonnamment, la levure, en passant par le nématode *Caenorhabditis elegans* (*Ce*-NCS-1, l'objet de cette thèse) et la drosophile (fréquenine). Ces protéines semblent être les différentes versions d'une même protéine dans différentes espèces (des orthologues).
D'autres NCS comme Vilip1,2,3 de rat/poulet, l'hippocalcine de rat/humaine, la neurocalcine bovine ont été groupées formant la **sous-famille neurocalcine/vilip**.
Le membre le plus divergent de cette famille est pour l'instant *Ce*-NCS-2 chez le *Caenorhabditis elegans*, formant à lui tout seul une nouvelle branche dans l'arbre phylogénétique des NCS (et qui représente peut-être une séquence spécifique au nématodes).

Le rôle physiologique précis de la majorité de ces NCS est inconnu, excepté pour les NCS rétinales qui sont impliquées dans la modulation de la sensibilité à la lumière, ainsi que pour la fréquenine, une NCS-1 de *Drosophila* qui est connue pour moduler le relachement de neurotransmetteur à la jonction neuro-musculaire.

Modulation de la sensibilité à la lumière par les NCS rétinales

La visinine (de poulet), la recoverine (boeuf/rat/humain), la S-moduline (de grenouille), ont été les premiers membres de la famille NCS à être caractérisés. Ces protéines (probablement) orthologues sont exprimées dans les photorécepteurs. Des expériences aussi bien *in vitro* que *in vivo* ont montré que la recoverine ainsi que la S-moduline empêchent, à haut calcium, le « quenching » du photorécepteur rhodopsine en inhibant sa phosphorylation. Ces NCS rétinales agissent en inhibant la rhodopsine kinase (l'enzyme phosphorylant la rhodopsine) à haut calcium (voir figure 9). Par conséquent, ces protéines augmentent la sensibilité aux photons dans la pénombre. Dans un tel cas, la stimulation des photorécepteurs est faible, leur concentration en calcium est élevé (dans les photorécepteurs, à l'opposé de pratiquement toutes les autres cellules, le taux de Ca^{2+} est élevé en absence de stimulation, et baisse localement lors d'une stimulation), ce qui permet aux NCS rétinales d'être actives et d'empêcher (de ralentir) la phosphorylation et donc l'inactivation de la rhodopsine photo activée, prolongeant ainsi la durée de vie de la réponse aux photons. Avec une lumière ambiante plus forte, la concentration de Ca^{2+} dans les photorecepteurs est (localement) plus faible et ne permet plus aux NCS rétinales de ralentir l'inactivation de la rhodospine, diminuant ainsi la sensibilité à la lumière. Les NCS rétinales participent donc à l'ajustement, l'adaptation de la sensibilité visuelle en fonction des conditions de lumière.

Régulation du relâchement de neurotransmetteurs par la fréquenine

La fréquenine dans la mouche du vinaigre *Drosophila* est exprimée de manière prépondérante dans le système nerveux et apparaît concentrée dans les régions synaptiques particulièrement à la jonction neuro-musculaire. La fréquenine partage 41% d'identité au niveau des acides aminés avec la recoverine, et 72% avec NCS-1 de rat/souris/poulet (qui semble être son orthologue).

Les mouches surexprimant cette fréquenine montrent une augmentation de la facilitation (accroissement de la réponse lors d'un stimulus répétitif) du relâchement de neurotransmetteurs à la jonction neuro-musculaire. Dans cette jonction neuro-musculaire, la fréquenine augmente l'excitabilité du terminal présynaptique lors d'un stimulus répété qui pu faire monter durablement la concentration (locale) de Ca^{2+}. Des données électrophysiologiques montrent qu'il y a une modulation de l'échange Na^{+}/Ca^{2+} par la fréquenine qui semble ralentir l'évacuation du Ca^{2+} des sites de relâchements lorsque la concentration en Ca^{2+} atteint un certain niveau (lors d'un stimulus répétitif) augmentant ainsi l'excitabilité du terminal. Dans la jonction neuro-musculaire la fréquenine participe donc à la facilitation du relâchement de neurotransmetteurs, probablement en modulant certains canaux ou pompes impliqués dans l'évacuation du Ca^{2+} terminal. Les cibles exactes de la fréquenine sont pour l'instant inconnues ainsi que sa fonction dans le système nerveux central (SNC). Module-t-elle aussi le relâchement de neurotransmetteur dans le SNC? Quel rôle joue-t-elle dans la post-synapse (où elle peut aussi se trouver)? Les autres NCS-1 ont-elles des rôles similaires?

Ensemble, ces données indiquent que des NCS peuvent être impliquées dans la fine modulation de la sensibilité et de l'excitabilité de certains neurones.

Activitées in vitro et in vivo de NCS-1 (rat/poulet/souris)

Dans le rat, NCS-1 est largement distribuée dans le cerveau et le système nerveux périphérique et est particulièrement concentré dans la rétine, le cervelet et l'hippocampe. Il peut être trouvé, comme la fréquenine, dans des neurones moteurs. Dans les neurones NCS-1 semble être préferentiellement localisé dans les dendrites et les axones. NCS-1 peut donc se trouver à la fois pré- et post- synaptiquement et pourait à la fois moduler l'excitabilité de la pré-synapse (comme la fréquenine) et la sensibilité de la post-synapse (comme la recoverine).

Il à été montré que NCS-1 (ainsi que la plupart des NCS) *in vitro* peut inhiber la phosphorylation de la rhodopsine, comme les NCS rétinales. Par extension, ces résultats peuvent suggérer un rôle potentiel des protéines NCS dans la modulation Ca^{2+} dépendante de la phosphorylation de récepteurs (couplé aux protéines G).

Nous avons aussi montré *in vitro* et *in vivo* que NCS-1 peut se substituer à ou potentialiser la calmoduline dans certain cas (il y a 21% d'identité au niveau des acides aminés entre NCS-1 et la calmoduline).

Ces données indiquent que NCS-1 est probablement un modulateur multifonctionel qui peut partager certaines cibles avec la calmoduline mais possède aussi certainement ses propres cibles. Comme NCS-1 est largement distribué dans le système nerveux, il exerce sans doute différentes fonctions dans différentes régions/cellules. Étant l'orthologue de la fréquenine, NCS-1 doit vraisemblablement avoir des fonctions similaires. Néanmoins, les rôles physiologiques des protéines NCS-1 dans les différentes régions du cerveau restent mal connus.

Le nématode Caenorhabditis elegans

Le *Caenorhabditis elegans* (*C. elegans*) est un petit ver nématode (l'adulte est d'une longueur de 1mm) non parasitaire du sol, se nourrissant de bactéries. Il y a deux sexes: les hermaphrodites qui se reproduisent par auto-fertilisation, et les mâles (apparaissant spontanément à faible fréquence) qui peuvent fertiliser les hermaphrodites (les hermaphrodites ne peuvent se fertiliser entre eux). Les hermaphrodites pondent des oeufs. Le temps de génération (de l'oeuf à l'adulte) du *C. elegans* est de 3 jours (dans des conditions de croissance optimales). La progéniture, après l'éclosion passe par 4 stades appelés larvaires L1 à L4 (bien qu'il n'y ait pas de métamorphose). Un hermaphrodite adulte est fertile durant environ 4 jours et peut après encore vivre 10 à 15 jours. L'espérance de vie totale du C. elegans est donc d'environ 2-3 semaines.

L'hermaphrodite adulte possède 959 noyaux somatiques (+ ~1000 cellule germinales), et le mâle 1031. La taille du génome de *C. elegans* est d'environ $100x10^6$ nucléotides. Il possède 5 chromosomes autosomes (I-V) et un chromosome sexuel (X). Les hermaphrodite sont diploïdes pour les 6 chromosomes (étant donc XX), les mâles eux sont diploïdes pour les 5 autosomes mais n'ont qu'un seul X (haploïde, X0). Les mâles apparaissent spontanément dans les population d'hermaphrodites par non disjonction du chromosome X lors de la méiose(on observe ~1 mâle sur 500 hermaphrodites).

Le *C. elegans* est un excellent organisme expérimental. Ces atouts sont sa simplicité (anatomique, génétique, et comportementale), sont cycle de vie rapide, sa facilité de culture dans le laboratoire, et son adaptation pour l'analyse génétique. De plus, les *C. elegans* peuvent être stockés indéfiniment à l'état congelé.

Des mutants peuvent être facilement générés par des agents mutagènes chimiques ou par irradiation, permettant d'effectuer des criblages génétiques classiques et aussi de faire de la génétique inverse en sélectionnant (par PCR) des vers avec des délétions dans un gène particulier. Les allèles récessifs peuvent être exposés du fait de la reproduction par self-fertilisation (chez l'hermaphrodite). Des vers transgéniques sont facilement obtenus en injectant des constructions (ADN) dans les gonades des hermaphrodites.

D'autre part, il y a une profusion d'informations descriptives disponibles sur le *C. elegans* comme le lignage cellulaire complet (les relations filiales de toutes les cellules durant le développement), l'anatomie complète à la résolution du microscope électronique, la carte physique entière du génome, et bientôt la séquence complète de son génome (le *C. elegans* sera bientôt le premier organisme multicellulaire dont le génome soit entièrement séquencé). De plus le *C. elegans* montre une assez grande similarité génétique avec les mammifères.

Le système nerveux du *C. elegans* (hermaphrodite) est composé de 302 neurones (sur un total de 959 noyaux somatiques). Le « câblage » complet de son système nerveux est connu.

Le *C. elegans* peut se déplacer (en avant et en arrière, par des mouvements ondulatoires), faire des mouvements exploratoires avec sa tête, manger, déféquer, pondre des oeufs et s'accoupler. Grâce à son système sensoriel, le *C. elegans* répond à une grande variété de stimuli externes (toucher, température, lumière, composé chimiques, pression osmotique) et peut modifier son comportement d'après de son expérience.

Le *C. elegans* représente donc un système très attractif pour (entre autres) des études génétiques et comportementales en neurobiologie.

Résultats et discussion

Clonage du cDNA de *Ce*-NCS-1 (*Caenorhabditis elegans* Neuronal Calcium Sensor-1)

Afin de déterminer si des séquences de type Neuronal Calcium Sensor-1 existent dans le *C. elegans*, j'ai criblé une librairie de cADN de *C. elegans* en utilisant le cADN de NCS-1 de poulet comme sonde. J'ai pu isoler différents phages positifs contenant des insertions encodant une protéine hautement similaire au poulet/rat NCS-1. Nous avons nommé cette protéine *Caenorhabditis elegans* Neuronal Calcium Sensor-1 (*Ce*-NCS-1). La séquence déduite en acide aminés de *Ce*-NCS-1 possède 75% d'identité avec NCS-1 de rat/poulet et 65% avec NCS-1 de la levure *S. pombe*. Ensemble, ces résultats suggèrent que ces protéines soient des homologues d'espèces (des orthologues). NCS-1 est la seule protéine NCS qui a un homologue dans la levure et est donc la séquence la plus conservée de la famille NCS.
Afin d'estimé le degrer de diversité génétique des NCS dans le *C. elegans*, j'ai criblé a nouveau la librairie de cADN de *C. elegans*, cette fois à très basse stringeance, en utilisant le cADN de *Ce*-NCS-1 comme sonde. J'ai isolé plusieurs phages positifs encodant ou pour *Ce*-NCS-1 ou pour une unique nouvelle protéine appartenant à la famille NCS. Cette nouvelle séquence a été nommée *Ce*-NCS-2. Cette protéine montre seulement 45% d'identité au niveau des acides aminés avec *Ce*-NCS-1 et 44 % avec NCS-1 de poulet/rat. Par ce criblage aucune autre NCS n'a été trouvée dans le *C. elegans*.

Les protéines *Ce*-NCS-1 et *Ce*-NCS-2

Les deux protéines sont apparentées à la famille NCS. *Ce*-NCS-1 est hautement similaire à NCS-1 de rat/poulet (avec 75% d'identité au niveau des acides aminés). *Ce*-NCS-2 est pour l'instant la protéine la plus divergente de la famille NCS, partageant seulement de 37 à 49 % d'identité (au niveau des acides aminés) avec les autres membres de la famille NCS. *Ce*-NCS-2 ne possède pas d'équivalents connus d'en d'autres espèces et est donc probablement une NCS spécifique aux nématodes. Certaines caractéristiques des protéines *Ce*-NCS-1 et *Ce*-NCS-2 sont montrées dans la table I.

Analyse	**protéine *Ce*-NCS-1**	protéine *Ce*-NCS-2
Poids moléculaire	22023.50	21986.80
Longueur	191	190
1 microgramme =	45.406 pMoles	45.482 pMoles
Coefficient d'extinction molaire	19180 ±5%	16860 ±5%
1 unité d'absorbance à 280nm {1 A(280)}=	1.15 mg/ml	1.30 mg/ml
Point Isoélectrique	5.02	5.00
Charge au pH 7	-6.59	-6.82

Table I. Caractéristiques (théorique, calculée) de *Ce*-NCS-1 et *Ce*-NCS-2.

"Northern blots"

Afin de vérifier si ces 2 gènes NCS sont réellement exprimé dans le *C. elegans* type sauvage ainsi que pour comparer l'abondance relative des deux messagers et connaître leur taille, j'ai effectué des analyses 'Northern blot' sur de l'ARN total de *C. elegans* en utilisant les cDNA de mes deux NCS de *C. elegans* comme sondes.

Les résultats de ces expériences (voir figure 17) montrent que les deux gènes sont exprimés dans le *C. elegans* type sauvage, que le messager pour *Ce*-NCS-2 semble plus abondant (que *Ce*-NCS-1). La taille du messager RNA pour *Ce*-NCS-1 est d'environ 1 kilo-bases (kb), celui de *Ce*-NCS-2 environ 1.4kb (ces tailles approximatives correspondant à la taille des cADN clonés).

Production et purification de *Ce*-NCS-1 et *Ce*-NCS-2

J'ai surexprimmé dans la bactérie *E. coli* les protéines *Ce*-NCS-1 et *Ce*-NCS-2 en utilisant la partie codante de leur cADN insérée dans un vecteur d'expression inductible. J'ai purifié (voir figure 18) les protéines surexprimées grâce à une colonne hydrophobe. Une caractéristique commune des protéines modulatrices liant le calcium (comme la calmoduline ou nos NCS-1) est que le Ca^{2+} induit un changement de conformation conduisant à l'exposition de surfaces fortement hydrophobes (leur permettant d'interagir avec des protéines cibles). Ces protéines peuvent donc être retenues sur une colonne hydrophobe (qui joue ici le rôle d'une colonne d'affinité) en présence de Ca^{2+}, eô éluées en supprimant le Ca^{2+} libre (avec de l'EDTA).

Tests *in vitro* (avec les protéines recombinantes purifiées)

Phosphorylation de la rhodopsine

Il à été montré que *Ce*-NCS-1 ainsi que *Ce*-NCS-2 et la NCS vilip1 peuvent, *in vitro*, inhiber la phosphorylation de la rhodopsine (la protéine photoreceptrice des vertébrés) d'une facon calcium dépendante. Par extension, ces résultats suggèrent un role potentiel des NCS dans la modulation (calcium dépendante) de la phosphorylation, et par la même de l'inactivation, de certains recepteurs.

Activation de la 3' :5' – phosphodiestérase de nucléotides cycliques

Nous avons observé, *in vitro*, que *Ce*-NCS-1 peut directement activer l'enzyme 3' :5' – phosphodiestérase de nucléotides cycliques (PDE), une cible bien connue de la calmoduline, donnant une stimulation de l'activité de 4.6x ±1.5 (n=5, 1µM *Ce*-NCS-1, 100µM Ca^{2+}) avec un EC_{50} à environ 200nM (la calmoduline provoque une stimulation de 6 à 7 fois avec un EC_{50} à 1nM). Le même effect à été montré avec NCS-1 de poulet/rat. Il semble donc que *in vitro* *Ce*-NCS-1 peut remplacer la calmoduline dans l'activation d'au moins une de ses cibles.
Ensemble, ces résultats *in vitro* suggèrent que *Ce*-NCS-1 (ainsi que ses orthologues) possède différentes cibles, et est donc un modulateur multifonctionel. Néanmoins les véritables cibles (*in vivo*) des proteines NCS-1 ainsi que leurs fonctions exactes restent mal connues.

« Overlay » au ^{45}Ca

Afin de prouver que *Ce*-NCS-1 (et *Ce*-NCS-2) sont bien de vraies protéines pouvant lier le calcium (« Calcium Binding Protein », CBP), j'ai effectué avec celles-ci un « assay » direct de liaison au calcium. Il à été montré que la plupart des protéines liant le calcium après un gel SDS-PAGE et un transfert sur une membrane de nitrocellulose, peuvent garder leur capacité à lier le calcium. En incubant la membrane avec du calcium radioactif (isotope 45) il est possible de mettre en évidence les protéines liant le calcium par autoradiographie. Cette technique est appelée : « overlay » au ^{45}Ca.
J'ai effectué cet « overlay » sur *Ce*-NCS-1, *Ce*-NCS-2. L'autoradiographie montre un signal à la position de *Ce*-NCS-1 et *Ce*-NCS-2 démontrant que ces protéines peuvent lier le calcium (voir figure 19). *Ce*-NCS-1 ainsi que *Ce*-NCS-2 sont des authentiques protéines de *C. elegans* liant le calcium.

Production d'anticorps polyclonaux

J'ai immunisé des lapins avec les protéines recombinantes *Ce*-NCS-1 et *Ce*-NCS-2 afin d'obtenir des anticorps (polyclonaux) contre ces deux protéines. J'ai immuno-purifié les sérums de lapin contenant les anticorps spécifiques par chromatographie contre les antigènes purs (les protéines recombinantes elles-mêmes) puis testés les anticorps ainsi purifiés par ELISA (« enzyme-linked-immuno-assay ») et par « western blot » (voir figure 20)
Avec les tests ELISA, on observe que le titre des anticorps purifiés est élevé (dilution donnant la moitié de la réponse maximale : pour *Ce*-NCS-1 1:100000, pour *Ce*-NCS-2 1 :20000), qu'ils sont sélectifs (l'anticorps anti-*Ce*-NCS-1 sur la protéine *Ce*-NCS-2 « réagit » légèrement, seulement à une dilution de 1:100, il n'y à pas de « réaction » de l'anticorps anti-*Ce*-NCS-2 sur *Ce*-NCS-1). Sur les « western blots » on n'observe pas de réaction croisée des anticorps avec les antigènes (l'anticorps anti-*Ce*-NCS-1 ne reconnaît pas *Ce*-NCS-2 à la dilution utilisé, et vice versa) même avec les quantités élevées de protéines recombinantes présente (1µgr). Les deux anticorps reconnaissent une bande de la bonne taille (~20kD) sur un extrait total de protéines de *C. elegans*. Dans cet extrait total, *Ce*-NCS-1 semble être moins abondant que *Ce*-NCS-2, ceci correspondant avec les résultats obtenus dans les « northern blots » où le messager pour *Ce*-NCS-1 apparaissait moins abondant que celui pour *Ce*-NCS-2.

Immunolocalisation de *Ce*-NCS-1

Afin de mettre en évidence l'expression de la protéine *Ce*-NCS-1 dans le *C. elegans* (*in situ* localisation de la protéine) j'ai utilisé l'anticorps anti-*Ce*-NCS-1 pour immunolocaliser *Ce*-NCS-1 en effectuant des expériences d'immunofluorescence (IF). Des *C. elegans* fixés et perméabilisés ont été « marqués » avec l'anticorps anti-*Ce*-NCS-1.
Par ces études (voir figure 21), *Ce*-NCS-1 apparaît être localisé (chez l'hermaphrodite adulte) dans des extensions neuronales caractéristiques de neurones sensoriels dans la tête et dans la queue ainsi que dans des extensions dans l'anneau nerveux (entre les deux bulbes du pharynx) et dans la corde (nerveuse) ventrale. *Ce*-NCS-1 est aussi détecté dans certains muscles : dans la cellule musculaire pharynguale pm1, dans des muscles de la vulve, dans le muscle dépresseur anal. L'expression de *Ce*-NCS-1 est aussi détectée à tous les stades larvaires. L'expression dans les embryons (oeufs) et dans les mâles n'a pas été examinée.

Dans les neurones, la protéine est presque complètement absente des corps cellulaires et semble donc être localisée préférentiellement dans les neurites (axones et dendrites). Des contrôles avec un mutant n'exprimant plus *Ce*-NCS-1 ('knock-out' mutant, voir après) ne montrent aucun signal en immunofluorescence indiquant que les marquages obtenus sont bien spécifiques pour *Ce*-NCS-1. Comme les corps cellulaires (dans les neurones) ne sont pas marqués il est difficile de déterminer précisément les cellules positives. Des vers transgéniques portant une construction avec un gène reporter pour ncs-1, seront utilisés pour identifier précisément les cellules exprimants *Ce*-NCS-1 (voir après).

Clonage des gènes *ncs*-1 et *ncs*-2

J'ai isolé les gènes *ncs*-1 et *ncs*-2 en criblant (à haute stringeance) une librairies génomique de *C. elegans* en utilisant les cDNA pour *Ce*-NCS-1 et *Ce*-NCS-2 comme sondes. J'ai isolé plusieurs phages positifs avec de larges inserts contenant les gènes *ncs*-1 et *ncs*-2 (voir figures 22, 23, 24).

Localisation chromosomique des gènes *ncs*-1 and *ncs*-2

	***Ce*-NCS-1**	***Ce*-NCS-2**
YAC positifs	Y55B3	Y64B2, Y62E9
Cosmides positifs	F53H8, C44C1	C09D5, K09B7, AC2
Localisation chromosomique	X: -18.85 (entre les gènes aex-3, unc-1)	I: 4.46 (entre les gènes tba-1, lin-11)

Table II. Information sur la localisation chromosomique des gènes *ncs-1*, *ncs-2*.

GFP comme marqueur de l'expression du gene *ncs*-1

Afin de localiser précisément les cellules où le gène *ncs*-1 s'exprime, j'ai utilisé le promoteur de ncs-1 (un fragment de 3kb en amont de la séquence codante, supposé promoteur) pour diriger l'expression d'un gène reporteur GFP (« green fluorescent protein » ; protéine fluoresceant en vert) dans des vers transgéniques (qui portent donc une construction ncs-1 « promoteur » : : GFP). En alignant la fluorescence GFP avec les images d'interférence différentielle Nomarski (mettant en évidence les noyaux), l'expression peut être assignée aux cellules individuelles (car l'anatomie complète du *C. elegans* est connue au niveau cellulaire).
Dans les adultes (hermaphrodite) l'expression de GFP (et par là-même l'expression de *ncs*-1) a été observée dans 20 (10 paires bilatéralement symétriques [Gauche, Droite]) neurones sensoriels (il y a 60 neurones sensoriels dans le *C. elegans*); comprenant des neurones sensoriels de la tête (neurones « amphid » + BAG) : paires **AWC, ASE, AWB, BAG** (expression forte), AWA, AFD, ADF, ASF (expression + faible, + rare) ; des neurones sensoriels de la queue (neurones « phasmid ») : **PHB** (expression forte), PHA (expression + faible, + rare); des inter-neurones : **AVK** (expression forte) et AIY (expression + faible, + rare) et les neurones moteurs : RMG (expression faible). Il y aussi expression dans la cellule musculaire pharynguale **pm1**. Avec le GFP on n'observe pas de marquage dans le muscle dépresseur anal (contrairement aux résultats obtenus en immunofluorescence) ni dans les muscles de la vulve (bien qu'un marquage très faible soit observé dans certains vers, voir figure 24j). L'expression de GFP est observée aussi à tout les stades larvaires. L'expression dans les mâles ainsi que dans les embryons n'a pas été examinée.

Double marquage : GFP + IF

Afin de déterminer si il y a chevauchement des signaux obtenus avec le GFP et avec l'anticorps (immunofluorescence ; IF) j'ai effectué des IF avec l'anticorps anti-*Ce*-NCS-1 sur les vers transgéniques portant la construction GFP. Ces vers ont été marqués en utilisant un anticorps secondaire couplé à la rhodamine donnant un signal rouge. La superposition des signaux GFP en vert et IF en rouge montre qu'il y à chevauchement complet (donnant un marquage jaune/orange) dans tout les extensions neuronales et dans la cellule pharynguale pm1 (voir figure 27). Le marquage par l'anticorps est pratiquement complètement absent des corps cellulaires des neurones positifs, confirmant que *Ce*-NCS-1 est localisé de manière prépondérante dans les axones et les dendrites (neurites) mais exclue des corps cellulaires. Cette localisation préférentielle dans les axones et les dendrites indique que *Ce*-NCS-1 peut-être trouvé à la fois pré et post- synaptiquement et qu'il est probablement associé à la membrane plasmatique. Le signal dans les muscles vulvaux et dans le muscle dépresseur anal n'est observé qu'avec l'anticorps.
La région promotrice choisie dirige l'expression de GFP dans les bonnes cellules (excepté pour les muscles anaux et vulvaux), donc *Ce*-NCS-1 est exprimé dans les cellules positives au GFP susmentionnées.

Fonctions connue des cellules exprimant Ce-NCS-1

Certains neurones où *Ce*-NCS-1 est exprimé sont connus pour être impliquées dans la chimiotaxie (attraction) vers des composés solubles (goût) comme l'AMPc, la biotine, Cl^-, Na^+ pour les neurones **ASE**, ADF, ASG ; dans la chimiotaxie vers des attractants volatiles (odorat) comme le benzaldéhyde, l'isoamyl alcohol, le butanone pour le neurone AWC; dans la répulsion envers des composés volatiles comme l'octanone, nonanone, le benzaldéhyde (à forte concentration) pour les neurones AWB; dans la détection d'une phéromone induisant la formation de la larve « dauer » (forme alternative de développement, très résistante, pour la survivance à long terme et la dispersion) comme les neurones **ADF**, ASG ; la thermotaxie (attraction vers la température à laquelle les vers ont été cultivés) pour les neurones AFD. Le neurone ASE semble aussi moduler la fréquence de la ponte des oeufs en fonction de l'abondance de la nourriture (bactéries). De plus *Ce*-NCS-1 est aussi exprimé dans des muscles de la vulve, nécessaire à l'expulsion (ponte) des oeufs ainsi que dans le muscle dépresseur anal nécessaire à la défécation.

Isolation d'un mutant « knock-out » pour *ncs*-1

Afin de déterminé les processus physiologiques dans lesquelles *Ce*-NCS-1 est impliqué, j'ai isolé une lignée de *C. elegans* mutante avec le gène *ncs*-1 inactivé. Partant d'une souche riche en transposons Tc1, une lignée avec un Tc1 dans le gène *ncs*-1 a pu être isolée ('criblage' par PCR), puis, partant de cette lignée, j'ai isolé (toujours par 'criblage' PCR) une lignée dérivée, possèdant une délétion dans le gène *ncs*-1 résultant d'une réparation impropre après un « saut » du transposon. La délétion enlève 4/5 du gène (l'ATG initiateur compris). Il n'y a plus d'expression de *Ce*-NCS-1 dans ce mutant (homozygote) qui est donc un vrai « knock-out » .
Le mutant est viable, fertile et à l'air en bonne santé. Il répond au toucher et peut se déplacer.

Analyse du phénotype du mutant *ncs*-1 « knock-out »

Sachant que certaines des cellules exprimant *Ce*-NCS-1 sont impliquées (voir avant) dans la chimiosensation, la thermotaxie, le contrôle de la formation de la larve « dauer », la ponte des oeufs et la défécation ; le mutant est actuellement testé pour des défauts dans ces comportements.
Il a été montré que la fréquenine (l'orthologue de NCS-1 dans la mouche), dans la jonction neuro-musculaire de la *Drosophila* facilite le relâchement de neurotransmetteur en réponse à une stimulation répétitive, augmentant l'excitabilité du neurone (probablement en modulant la perméabilité de certains canaux/pompes). La recoverine, une autre NCS, est connue pour moduler la sensibilité des photorécepteurs (dans les vertébrés), l'ajustant aux conditions de lumière ambiante. Par extension, ces données suggèrent que *Ce*-NCS-1 pourrait jouer un rôle similaire dans le *C. elegans*. Notre hypothèse de travail est : dans certain neurones et muscles (cellules excitables) *Ce*-NCS-1 est impliqué dans la modulation de l'excitabilité (comme la fréquenine) et la sensibilité (comme la recoverine) en fonction de la fréquence/ de l'intensité du stimulus. *Ce*-NCS-1 pourrait donc aussi joué un rôle dans la plasticité comportementale (adaptation, apprentissage/mémoire).
Le mutant est actuellement analysé pour détecter des défauts dans la sensibilité/ la plasticité sensorielle ainsi que dans la contraction musculaire.

Résultats:

Le mutant réagit aux odorants alcool isoamylique et benzaldéhyde, les deux, « sentis » par la paire de neurones AWC, ceci indiquant que ces neurones AWC sont probablement toujours présents et fonctionnels. On peut en déduire que *Ce*-NCS-1 n'est vraisemblablement pas nécessaire au bon développement des neurones l'exprimant (AWC, en tout cas) ni pour les capacités sensorielles de base. La sensibilité « limite » ainsi que l'adaptation à ces odorants est actuellement testée.

Le mutant montre une thermotaxie (vers sa température de culture) anormale. Des vers « sauvages » placés quelques heures à une température donnée (entre 16°c –25°c) en présence de nourriture vont s'adapter à cette température. Placé ensuite dans un gradient thermique (en absence de nourriture), ils vont se souvenir de cette température, s'y diriger et y rester, faisant des parcours « isothermaux ». **Nous avons observé que le mutant ncs-1 « knock-out » ne se « souviens » plus de sa température de culture (ou ne sent plus la température). Ce résultat pourrait indiquer que *Ce*-NCS-1 est impliqué dans certain processus mémoriels.**

Les étude présentées peuvent nous aider à déterminer les processus physiologiques dans lesquels *Ce*-NCS-1 est impliqué et par la même, à comprendre la (les) fonction(s) de cette protéine dans le système nerveux et musculaire du *C. elegans*. Par extension, cela pourrait aussi nous aider à comprendre la fonction de la sous-classe, si conservée dans l'évolution, des protéines NCS-1.

Conclusion

- ***Ce*-NCS-1 est une protéine de *C. elegans*, liant le calcium, hautement similaire aux autres protéines NCS-1**
- *Ce*-NCS-1 semble être la seule séquence NCS-1 de *C. elegans*.
- *Ce*-NCS-2 est la seule autre séquence de NCS trouvée dans le *C. elegans*.
- *In vitro*, *Ce*-NCS-1 peut inhiber la phosphorylation de la rhodopsine d'une manière calcium dépendante
- *In vitro*, *Ce*-NCS-1 peut directement activer la 3':5' PDE de nucléotides cycliques.
- **Dans le *C. elegans* (hermaphrodite), *Ce*-NCS-1 est exprimé à tous les stades larvaires et dans l'adulte** (l'expression dans les embryons ainsi que dans les mâles n'a pas été examinée).
- ***Ce*-NCS-1 est exprimé dans certaines cellules excitables, comprenant:**

Différents types de neurones :

- **10 paires de neurones sensoriels**
  impliqués dans la chimiotaxie vers des odorants volatiles, la chimiotaxie vers des composés solubles, la répulsion envers certains composées chimiques, la thermotaxie, la modulation de la fréquence de ponte, le contrôle de la formation de la larve « dauer ».
- **2 paires d'inter-neurones**
- **1 paire de neurones moteurs**

Des muscles :

- des muscles de la vulve
- le muscle dépresseur anal
- la cellule musculaire pharyngeal pm1

- **Dans les neurones, *Ce*-NCS-1 est trouvé à la fois dans les axones (pre-synaptiquement) et dans les dendrites (post-synaptiquement) mais est significativement exclu des corps cellulaires.**
- *Ce*-NCS-1 est probablement associé à la membrane plasmatique (ou au cytosquelette).
- **Le mutant « knock-out » pour *ncs*-1 est viable, fertile, peut se mouvoir et à l'air en bonne « santé »**
- ***Ce*-NCS-1 n'est pas nécessaire pour les capacités sensorielles ou motrices de base ni pour le bon développement des cellules l'exprimant,** mais doit être probablement impliqué dans la modulation et la plasticité de ces capacités.
- ***Ce*-NCS-1 est vraisemblablement impliqué dans la modulation de l'excitabilité et de la sensibilité cellulaire** (modulant probablement, de manière calcium dépendante, des canaux/pompes et/ou la phosphorylation de certains composants dans la transduction de signaux).
- Le phénotype du mutant « knock-out » est actuellement analysé. Nous nous attendons à détecter des défauts dans la sensibilité, la **plasticité sensorielle** ainsi que dans l'excitabilité de certains muscles.
- **Le mutant « knock-out » montre une thermotaxie anormale. Le mutant ne se « souviens » plus de sa température de culture (ou ne sent plus la température). Ce résultat pourrait indiquer que *Ce*-NCS-1 est impliqué dans certain processus mémoriels.**

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