“The implication of DEL-4, an ENaC/DEG protein family member, in neuronal activity and acid sensation.”

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“Ο ρόλος της πρωτεΐνης DEL-4, μέλος της οικογένειας καναλιών ENaC/DEG, στην φυσιολογική λειτουργία των νευρώνων και την αντίληψη όξινου περιβάλλοντος”

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Abstract

DEL-4, a sodium channel present in *C. elegans* neuronal cells, is a member of a broad and diverse protein family, the DEG/ENaC. The properties of this channel appear to differ from the rest identified members of this family. A constitutively active gate, generating inward sodium current possibly exerts a regulatory role in neuronal transmission since it rapidly closes under low pH. From an in silico analysis, it was found that *del-4* promoter possesses binding sites for DAF-16, SKN-1 and HSF-1 transcriptions factors. These three factors are the primary regulators of starvation, oxidative stress and heat stress responses, respectively. Collectively, we hypothesize that DEL-4 participates in sensory integration, modulating neurotransmission. From aldicarb behavioural assay and conducted experiments of the lab, involving dopaminergic signalling and locomotion, we come to support the notion that DEL-4 indeed alters physiological synaptic release. To get a more comprehensive look of how a normal or disrupted DEL-4 channel affects neuronal function, we used a voltage sensor expressed in dopaminergic neurons. The observation was that in both mutant and a stress induction cases, the cell membrane was becoming hyperpolarized. Notably, we also checked if the malfunctioning channel alters the integrity of the neurons. We noticed increased neurodegeneration events in mutants and intriguingly a more neuroprotective effect of DEL-4 overexpression. Our results provide new insights into how sodium channels participate in the mechanism of neuronal integration.

Περίληψη

Η πρωτεΐνη DEL-4 (degenerin like), αποτελεί ένα κανάλι που άγει ιόντα νατρίου και εντοπίζεται στα νευρικά κύτταρα του νηματώδη σκώληκα *Caenorhabditis elegans*. Ακόμη, αποτελεί μέρος της εκτενούς και ποικιλόμορφης οικογένειας πρωτεϊνών των Epithelial Sodium Channel/ Degenerin (DEG/EnaC). Οι ιδιότητες της DEL-4 φαίνεται να είναι διαφορετικές από αντίστοιχες πρωτεΐνες της ίδιας οικογένειας αποτελώντας ένα μονίμως ανοιχτό κανάλι, το οποίο δημιουργεί ρεύμα άγοντας ιόντα στο εσωτερικό του κυττάρου καθώς κλείνει σε χαμηλές τιμές ph. Επιπλέον, η περιοχή του υποκινητή του γονιδίου της φαίνεται να περιέχει θέσεις πρόσδεσης για τους μεταγραφικούς παράγοντες DAF-16, SKN-1 και HSF-1. Καθένας από τους εν λόγω παράγοντες αποτελεί κεντρικό ρυθμιστή απόκρισης για τα παρακάτω είδη stress. Το stress κατά την έλλειψη τροφής, οξειδωτικό καθώς και λόγω αυξημένης θερμοκρασίας κατά αντιστοιχία με του προαναφερθείς παράγοντες. Από τα παραπάνω, καταλήγουμε στην υπόθεση ότι το κανάλι συμμετέχει στην αντίληψη στρεσογόνων καταστάσεων ρυθμίζοντας την νευροδιαβίβαση. Από πειράματα που διεξήχθησαν στο εργαστήριο καθώς και από δοκιμή με το φάρμακο aldicarb παρατηρήσαμε αλλαγές στην συναπτκή έκλυση και αλλαγές στη φυσιολογική κίνηση του οργανισμού. Για την καλύτερη κατανόηση της δράσης του καναλιού χρησιμοποιήσαμε έναν ανιχνευτή δυναμικού μεμβράνης, ώστε να διαπιστώσουμε τυχόν αλλαγές στον φυσιολογικό και στον τροποποιημένο κανάλι. Μεταλλαγές του καναλιού καθώς και η επίδραση stress, είχαν το ίδιο αποτέλεσμα, την υπερπόλωση της μεμβράνης. Επιπρόσθετα, παρατηρήσαμε ότι το κανάλι της μεταλλαγμένης αλληλουχίας είχε αυξημένα ποσοστά νευροεκφύλισης σε ντοπαμινεργικούς νευρώνες σε σχέση με τα φυσιολογικά. Αξιοσημείωτο είναι το γεγονός, ότι η υπερέκφραση της φυσιολογικής DEL-4 φαίνεται να είχε προστατευτικό ρόλο σε μοντέλα νευροεκφύλισης. Από όλα τα παραπάνω μπορούμε να εξάγουμε το εξής συμπέρασμα, ένα κανάλι νατρίου φαίνεται να έχει πολύ σημαντική δράση στη νευρική λειτουργία και αντίληψη του stress.

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"You have made your way from worm to man, and much in you is still worm."

— Friedrich Nietzsche, Thus Spoke Zarathustra

-Chapter I: Materials and methods-

A: Materials

A1Nematode strains

N2	Bristol strain, wild type
DG1856	goa-1(sa734) I
BR5270	byIs161[Prab-3::F3DK280;Pmyo-2::mCherry]
pacr-2pHluorin::SNB-1	KP3085 nuIs122 [acr-2p:: pHluorin: :snb-1 + myo-2p::dsRed2]
p_{del-4}DEL-4::GFP; p_{del-4}SNB-1::DSRED	*unc-119*(ed3);Ex[p_{del-4}DEL-4::GFP; ; p_{del-4}SNB-1::DSRED *;unc-119*(+)]
p_{del-4}DEL-4_{1-36}::GFP	N2;Ex[p_{del-4} DEL-4_{1-36}::GFP; *rol-6*]
p_{dat-1}GFP (CGC)	BZ555 egIs1[p dat-1 GFP]
TPH-1::GFP (CGC)	GR1366 mgIs42 [tph-1::GFP]
OSM-10::GFP	HA3 osm-10::GFP
p_{acr-2}GFP	CZ631 acr-2p::GFP + lin-15(+)
del-4 mutant (SHIGEN)	Tm717
dat-1(p)::ASAP1pRF4	

B: C.elegans maintenance and genetics

Strains were maintained at 20o under standard conditions (unless otherwise noted).

B1: Preparation of growth media: NGM agar-based medium (Nematode Growth Medium)

NaCl	3g
Agar	17g
Bactopeptone	2.5g
Streptomycin	0.2g

Add distilled water to 700ml and autoclave, then wait till the medium reaches 55oC to add while stirring the following:

1M MgSO4	5 mg/ml cholesterol (in 100% ethanol)
1M CaCl2	10 mg/ml Nystatin (in 70% ethanol)

1 M KPO buffer pH 6.0 (108.3 g KH2PO4,	1ml
35.6 g K2HPO4, H_2O to 1L)	1ml
1ml	25ml
1ml	

Pour the liquid in petri dishes (60mm for maintenance, 35mm for mating and 100mm for larger cultures) using a pump.
Allow the plates to dry at Room Temperature (RT) for a day or two. Then stock at 4 oC if no contamination was detected.

(Wormbook, C.elegans maintenance chapter)

B2: Preparation of bacterial food source:

C. elegans is usually cultured in a bacterial lawn of an E.coli strain, OP50. This particular strain is auxotroph to uracil which presence is limited to NGM plates generating this desirable limited bacterial lawn. OP50 is cultured on L Broth plates and liquid. A single colony of streaking is used to inoculate a 50 ml of LB flask for 5 hours. The following step is the seeding of NGM plates 200µl for each plate are sufficient to create a favourable food source for the worms after an overnight (O/N) incubation

LB broth (Luria-Bertani medium) (1L):

Bacto-tryptone	10g
Yeast extract	5g
NaCl	10g
*Bacto-agar (in case of LB plates)	15g

Brenner, 1974

B3: Culture of C.elegans:

As previously mentioned all strains in our lab are preferably sustained in 20^{o}C. Manipulation of strains is achieved using a dissecting microscope. For the propagation of the strains transfer of l4 staged worms occurs once per 3 days in new plates to avoid their starvation. However, the transferring frequency depends on the strain and its needs. The transfer is facilitated through a pick made up with a platinum wire piece that can be directly sterilized with flame. All procedures are taking place under sterile conditions using flame (bunsen burner).

B4 Solutions

M9 buffer (1L):	
KH2PO4	3g
Na2HPO4	6g
NaCl	5g
1M MgSO4 (add after autoclave)	1m

M13 buffer:

30 mM Tris–HCl, pH 7.0
100 mM NaCl
10 mM KCl
Adjust pH to 7.0, and make up to 100 mL with water.
Filter through a 0.2 µm membrane.

B5: Synchronization and/or decontamination

- Egg-laying synchronizing method

A quick method to synchronize worms starting from eggs is the egg-laying. 25-30 (depending on the strain) gravid adult worms are placed on NGM plates with OP50 for about 5 hours then the adult worms are removed from the plate. Well synchronized eggs remain into the plate.

-Bleaching protocol

Contaminated worm cultures can be disinfected by treating gravid adult worms, bearing eggs with the bleaching solution, which dissolves the tissues of larvae and adult worms but does not affect the eggs (if the exposure is adequately limited).

Bleaching solution

5% solution of sodium hypochlorite	1ml
NaOCl	2ml
5N NaOH	7m
dd H_2O	

The freshly made solution is used every single time.

Procedure:

Egg-bearing adults are collected with sterile water or M9 from contaminated plates and then placed onto Eppendorf tubes.

Centrifuge for 30sec at 5000rpm. Discard the supernatant and resuspend in ddH_2O (repeat twice).

Resuspend the worms in bleaching solution and ddH_2O (3:1 ratio)

Put the tubes in a shaker or vortex them every two minutes till the solution is clear and no worm bodies are observed.

(Do not leave the solution for more than 5 minutes otherwise, the eggs do not hatch.)

Centrifuge for 1 minute at 13000rpm.

Discard the supernatant and resuspend the egg pellet in ddH_2O (repeat twice).

Transfer the eggs to seeded NGM plates using a pipette.

B5 Freezing :

For the long term storage of worms, a freezing procedure is used. For the optimized survival of post freezing recovery, storage can be achieved at -80°C or in liquid nitrogen (-196°C). The method used is the following 5-10 L4 staged animals are placed on each one of 5 NGM plates. When the second generation of eggs is hatched, the plate is full of starved L1 larvae. Starved L1 have the most significant chance to get revived after a freezing.

Freezing buffer (1L):

NaCl	5.85g
KPO4 1M, PH 6.0	50ml
Glycerol	200ml
MgSO4 (Add after autoclave)	3ml

Collect the animals of the plates with M9 solution (~9ml) and place it to 15 ml falcon (on ice).
Add an equal volume of cold freezing buffer (7.5ml).
Mix the two solutions and aliquot 1.5ml in each of 10 cryovials residing on ice.
Transfer the labelled cryotubes to -80°C.

B5. Generating males:

Hermaphrodites (5AA; XX) is the primary sex of C. elegans. Nevertheless, males are also observed in very low incidence (1‰), and they usually occur by rare nondisjunction of the X chromosome during meiosis producing the male sex (5AA; X0).

The adult male can be distinguished from the hermaphrodite by its distinctive tail and its slim body.

In the lab, male worms are used for crosses to create homozygotes, and they are made with the induction of problematic meiosis by heat stress.

4-5 Hermaphrodite L4 animals are placed in each of two NGM plates and get incubated at 37 °C for 55min. The plates are furtherly maintained at 20 °C while their descendants are examined for the presence of males.

B6. Crossing strains

Transfer 1 or two L4 hermaphrodite worms in the presence of 5-10 males in 35mm plates to ensure their encounter. To maximize the chance of having homozygous strains, we autocross L4 hermaphrodite of the F1 generation with males that bear the gene

locus of interest. From their F1 we take single L4 animals and place them into new plates. Furtherly we check their genotype with single worm PCR or with phenotype screening for this particular gene (depends on the strain).

B7. Generation of transgenic animals

Generation of transgenic animals was with microinjection of ASAP-1 construct with rol-6 sequence bearing plasmid. A selection marker. The protocol used is furtherly explained in standard protocols of the lab.

C: Behavioural analysis

Acute aldicarb response

We used aldicarb (Sigma Aldrich Aldicarb PESTANAL®, analytical standard), as proposed from Mahoney et al. We differentiated the protocol by adding the worms in drop with 10mM concentration of the drug. (Instead of using NGM plates with aldicarb) We diluted the drug in M9 and used a drop of 15µl and monitored when worms stopped moving. 6 worms per condition in each experiment were used, with a total number of 6 replicates.

Monitoring Heat stress resistance

For: heat stress (2hrs at 37 ^{o}C and O/N recovery) D1 animals and then recovery one day Starvation for imaging (O/N on empty plates)D1 worms imaged at D2.

D: C.elegans Imaging- Microscopy

Dissecting Microscopes strain maintenance and genetics are performed at 10-50X magnification with a dissecting microscope. Plates must be illuminated from underneath the stage.

Mounting animals for imaging: with 30mM Levamisole, a nicotinic acetylcholine receptor agonist that causes continued stimulation of worm muscles, leading to paralysis. (Sigma-Aldrich (−)-Tetramisole hydrochloride)

Zeiss Axio Imager Z2

EVOS™ M7000 Imaging System ThermoFisher

LSM710 Zeiss confocal microscope, Axio-observer Z1.

E: Molecular Methods and cloning

E1. Single worm DNA isolation and PCR

Worm Lysis Buffer (WLB):
50 mM KCl
10mM Tris, pH 8.5
2.5mM MgCl2
NP-40 0.45%
Tween-20 0.45%
Proteinase K 10 mg/ml (ratio 1/10 add before use)
In 5µl of WLB place the progenitor of the plate or 3 smaller worms.
Freeze the tubes at -80oC for at least 1 hour or O/N.
Lyse the worms and elicit the genomic DNA by heating to 65 oC for 60 min.
Inactivate the PK by heating to 95 oC for 15 min.
Perform PCR.

E2. Genomic DNA isolation

For the Genomic DNA isolation expansion of the plate in two larger 60mm plates is necessary to achieve increased efficiency of isolation. For this method, the following kit is used. From MACHEREY-NAGEL® NucleoSpin Tissue, Mini kit for DNA from cells and tissue.

The protocol used corresponds to protocol 5 of the handbook.

Reagents used	Final concentration	PCR parameters
DNA template		Initialization: 95°C for 3-5 min Denaturation: 95°C for 30 sec Annealing: 55-65°C for 45 sec (based on Tm of the primers) Elongation: 72°C ~1 min/1 kb Repetition of steps for 30-35 cycles Final elongation: -72°C for 10 mim Hold: 4°C
Forward primer (100 pmol/µl)	25pmol/ µl	
Reverse primer (100 pmol/µl)	25pmol/ µl	
dNTPs (2mM)	0.2mM	
Enzyme Buffer 10x	1x	
Enzyme (10U/µl)	~0.2U/µl	

E3. PCR DNA amplification

PCR Purification and gel extraction DNA isolation were performed with QIAquick PCR Purification Kit and QIAquick Gel Extraction Kit respectively (QIAGEN®)

E5. Molecular Cloning:

5i. Restriction Endonuclease Reaction

All Enzymes were provided for Qenzyme (Minotech) and New England Biolabs (NEB).

5ii. Ligation and Topo Ligation

Ligation T4 enzyme was provided by Minotech and Topo Vector for Topo Ligation from Invitrogen (PCRIITOPO Cloning) and were applied according to the instructions of the manufacture.

5iii. Transformation

Electro-competent XL-1 blue cells were used for the bacterial transformation.

Competent cells are acquired via a method to create electro-competent cells which takes place in the lab and are stored at -80oC.

A proportion of Ligation mix ~2μl is added in the competent cells after the 5 min of their incubation on ice.

The bacteria are transferred with the DNA in a cuvette with caution not to form bubbles.

Then Electroporation is taking place in the Electro Square Porator with the following parameters

MODE: HV
Polarity: unipolar
Internal: 500ms
p.lens:0.100MS
Voltage: 2000V

Add 1ml of LB in the cells to recover from the electro-shock and incubate for 1hour at 37oC
Centrifuge for 1min, Discard 800μl and plate the rest in warm LB plates plus Ampicillin.
O/N at 37oC.

5iv. Mini culture preparation and isolation

This is a small-scale plasmid isolation from bacterial cultures. This method is based on alkaline lysis method, and the buffers are provided from QIAGEN® named P1 Resuspension solution plus RNAse, P2 Lysis solution and P3 the Neutralization Buffer.

P1 and P3 are used chilled while P2 must not be incubated longer than 5 min.

The extended protocol is used for the isolation is in the manufacture handbook.

5v. Plasmid DNA isolation

For larger scale, plasmid isolation for alliance in microinjection or Bombardment Additional reagents are needed for the plasmid purification.

Additionally, columns are used Qtip 20 and Q tip 100.

Additional buffers are used except for P1, P2 and P3 the following:

QBT (Equilibration Buffer), QC (washing) and QF (elution). These Buffers are used to extract the DNA after its binding in the column.

All buffers and Columns are provided from QIAGEN®

F: Data processing and Statistical analysis

Analysis of Images with Image J 1.48V and BioLens

Statistical analysis was conducted with GraphPad Prism 6.01 (GraphPad Software, USA)

-Chapter II: Introduction-

A: *C. elegans* as model organism

Caenorhabditis elegans was introduced into the scientific community by Sydney Brenner in 1963, as an ideal model, studying developmental biology and neurobiological functions. This lilliputian nematode, (roundworm) is found in both terrestrial and aquatic ecosystems, and its size varies from 0.25mm as a newly hatched larvae to 1mm as an adult worm[1,2].

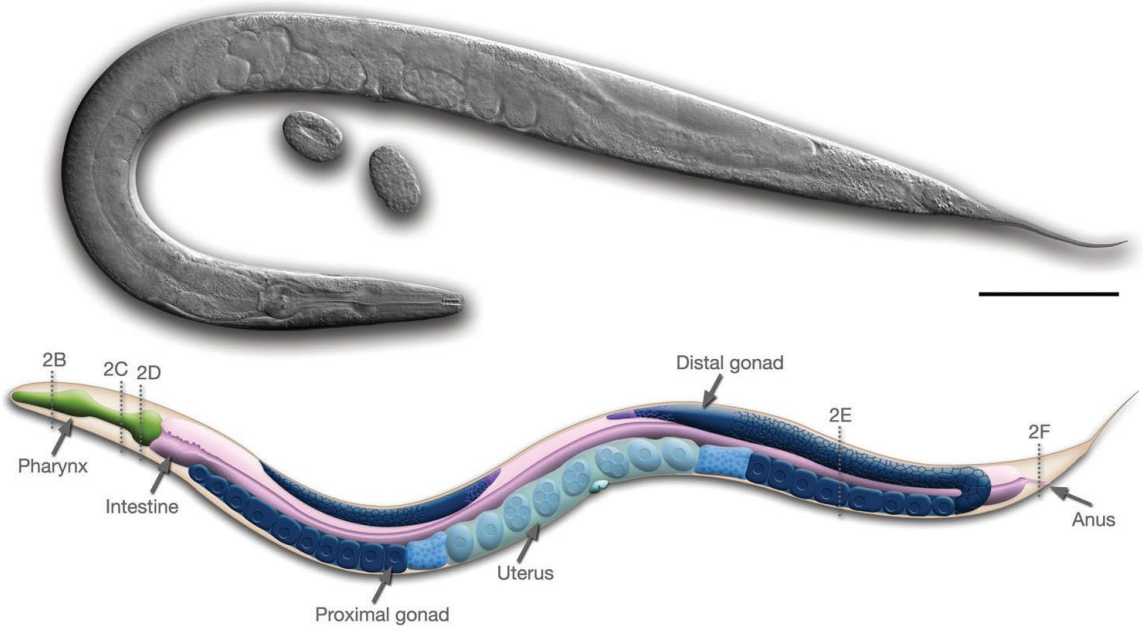


Figure2. 1: Depiction of an adult Hermaphrodite C. elegans, actual visualization (the former image) and model illustrating anatomical features of a hermaphrodite. Scale bar, 100µm. Image adapted from Meneely et al., 2019 [2].

C. elegans is predominantly observed as a self-fertilizing hermaphrodite, albeit males also exist, in a minor population of 0.2%, naturally occurring. Hermaphrodites produce haploid sperm, which remains stored in their spermatheca at the L4 larval stage. During

adulthood, oocyte production initiates. In the event of male existence in the population, cross-fertilization takes place, whereas, in the absence of males, the stored sperm is capable of fertilizing the entire population of progeny (~300). Self-fertilization simplifies maintaining stocks, as a single animal can give rise to an entire population.

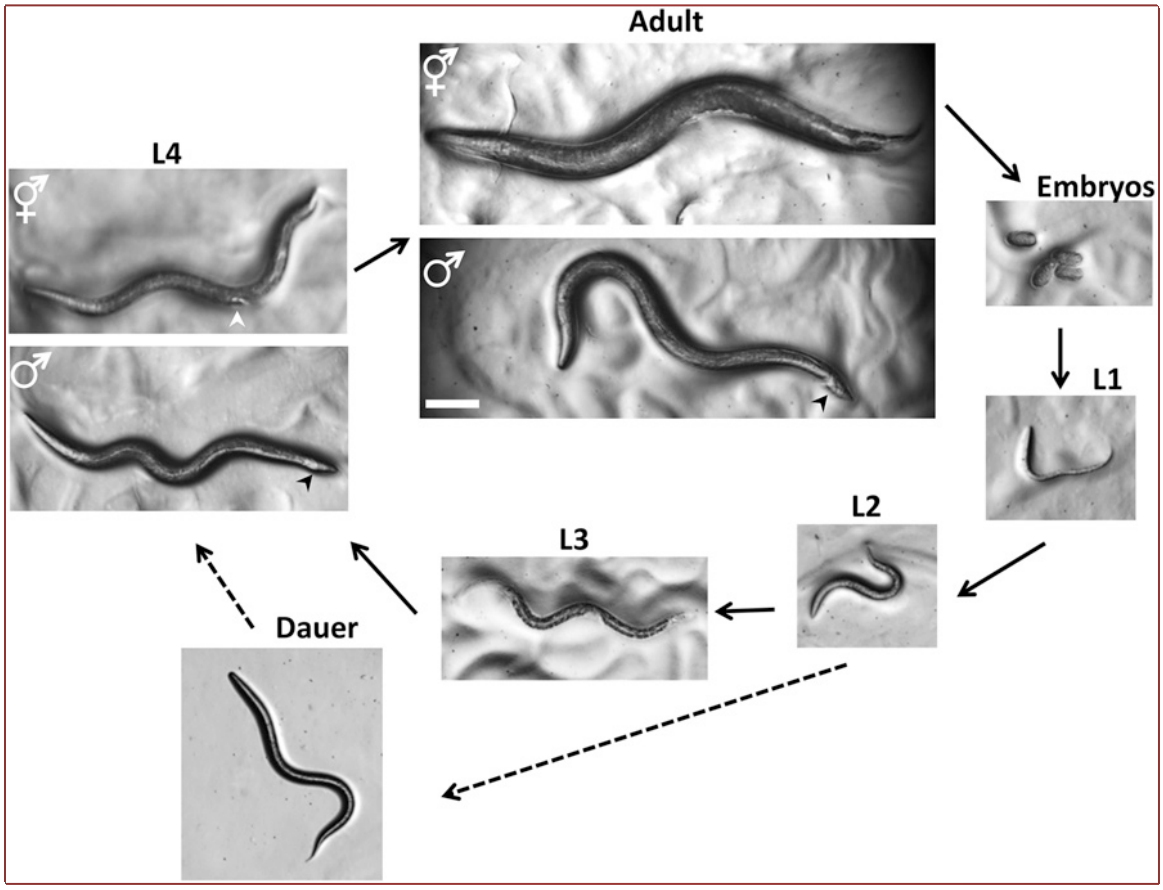


Figure2. 2 ***The Developmental life cycle of C.elegans:*** *Newly hatched L1 larvae pass through L2, L3 and L4 larval state to reach the adult reproductive state. The alternative dauer state is also depicted. White arrows point the distinguishable valva of L4 animals, whereas the black one, points out the differentiated tail of males. Images are taken from petri dishes seeded with bacteria. Scale bar 100µm.Image adapted from Corsi et al., 2015*[1].

Both sexes are diploid for the five autosomal chromosomes named linkage group (LG) I, II, III, IV, and V. Therefore, X chromosome distinguishes males from hermaphrodites. Males are haploid for the X chromosome, which arises from nondisjunction during

meiosis. The X determines sex to autosome (X: A) ratio[3]. Distinct phenotypical features of males are the slimmer girth and a fan-shaped tail (Figure2.2). The entire *C. elegans* genome is 100 Mb (*C. elegans* Sequencing Consortium 1998) and has approximately 20,000 protein-coding genes. It has been the first multicellular eukaryotic system to have its whole genome sequenced.

C. elegans life cycle is relatively rapid; it takes three days at 20^{o}C for a fertilized egg to develop to a ground adult, producing its fertilizing eggs. *C. elegans* embryogenesis lasts approximately 16hr at 20oC. Embryos are kept within the worm until the stage of 24-cell, and then they are laid. When the embryo is ready to hatch, it proceeds to the L1 larval stage. L1 will grow and molt (replacing its cuticle) to become an L2 larva (during 16hr) and so forth to L3 and L4 (12hr each transition). The final molt is from an L4 larva to an adult hermaphrodite, which then begins laying its eggs 12 hr later as a ground adult. As the reproductive period starts, they begin to produce progeny for ~6 days. Animals can live several more weeks (~3-4) before they die. In cases of food depletion, overcrowding or experiencing elevated temperatures, L2 larvae activate an alternative developmental pathway where they transcend into an L3 larval stage called the dauer larval state. The dauer cuticle surrounds the animal and plugs its mouth, preventing it from eating, leading to developmental arrestment. This larval form is more resistant and manages to survive under environmental stresses until conditions become favourable again. Dauer larvae can survive for many months, and they are the most dispersal form inhabiting nature. 3 (WormBook)

Its main features, including small size, rapid life cycle and a fully annotated genome, lay *C. elegans* as an import organism to address numerous biological processes. Among these features making *C. elegans* an appealing model to work with, there are also the eases that this model offers in maintaining and culturing *C. elegans* in the lab. With barely minimal nutrition and growth requirements, it produces a large number of descendants with a rapid life cycle. Its transparency, the simple anatomy and the relatively short lifespan render *C.elegans* as a critical research organism. In fact, many

novel techniques and ideas are initially tested in C. elegans because of its versatility and ease to screen and test a plethora of drugs and conditions.

B: The nervous system of *C.elegans*

C.elegans nervous system appears to be the most complex organ of this "simple" organism. In hermaphrodites consist of 302 neurons and 56 glial counting for the one-third of whole worms cells (959)[4]. Respectively, 381neurons and 92 glial and supporting cells form the nervous system in males, a more extended one due to the additional neurons lying in their differentiated tail[5].

Such a great expense deciphered in one, and only organ could be due to their need to encounter with the ever-changing environment. Responding in changes of temperature, osmolality and chemical environment. They do sense mechanical and chemical stimuli and respond to them with various mechanisms to cope with the changes. Locomotion, development, egg-laying and feeding are some features that can be altered due to external stimuli. Various intriguing behaviours addressing the neuronal function of *C.elegans* are under investigation, particularly chemotaxis, thermotaxis, responses to touch social feeding and associative along with non-associative learning are some of these behaviours[6]. Physiological stresses, including heat, starvation, hypoxia, and oxidative stress have been found to result in alterations in organismal homeostasis, behaviour and survival. The nervous system is the main sensor of environmental changes. Thus, to compensate these changes, altered activity, plasticity or signal transduction are applied[7,8].

A great majority of neuronal cell bodies are placed in a few ganglia in the head, the ventral cord and the tail[1]. Figure 2.3 depicts the neurons and ganglia formed in the whole body. Taking into consideration their topology, these 302 neurons are assigned to 118 classes. Albeit, each cell possesses a unique combination of properties, such as

morphology, connectivity, function and position. The classical classification of neurons into sensory, interneurons, and motor is partially applicable in *C.elegans* since each neuron combine two or more functions per se. In general, neuronal cells have simple morphologies, being monopolar with one or two neurites (or processes) branching from the cell body. Although some mechanosensory neurons, such as FLP and PVD, have elaborated branched neurites.

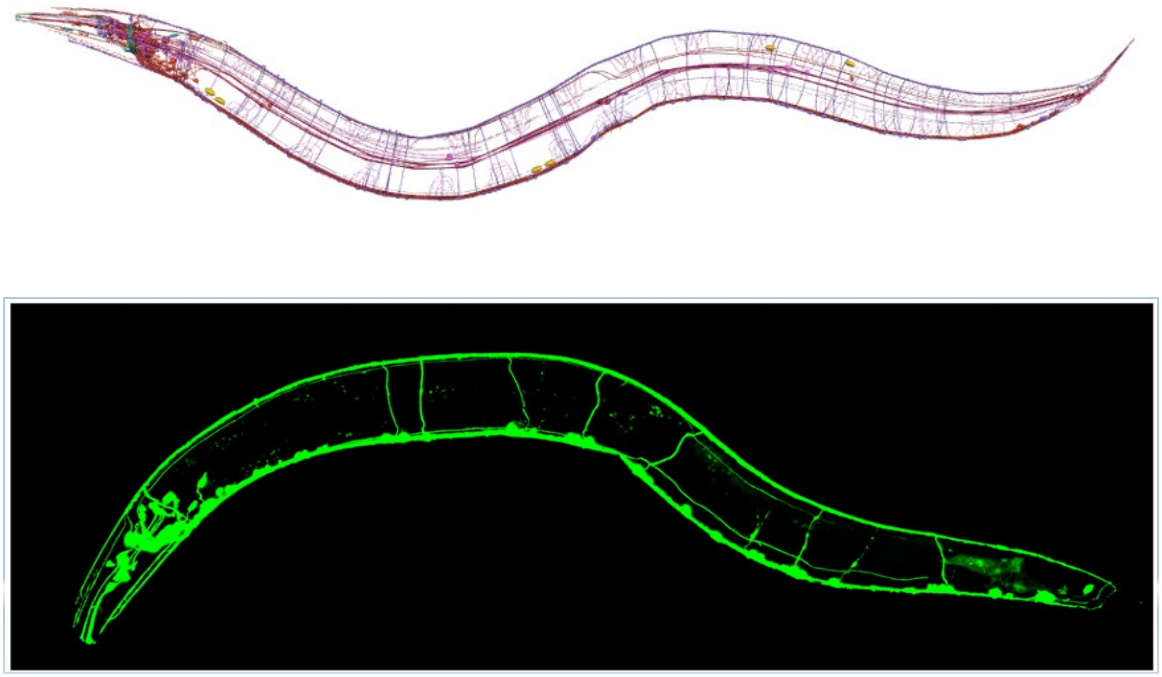


Figure2. 3 ***Nervous system****: The former is a model of all neurons of C.elegans. The later image, the fluorescent image, is showing the nervous system labelled with a GFP reporter (sto-6::gfp). Images adapted from Open Worm and Corsi et al., 2015*

C: Synapse formation

The complete wiring diagram of *C.elegans* neurons and their synapses has been annotated utilizing serial dissection and electron microscopy. *C. elegans* neurons make more than 7000 chemical synapses and gap junction connections[5]. A comprehensive

description of the anatomy of a single worm was reported to form this complete connectome (The mind of the worm). Most of the connections are made en passant (alongside the neurites). The types of synapses that occur in *C.elegans* are relatively similar to that of the known organisms. Nevertheless, connections are present also between classes that do not usually synapse to each other. Chemical synapses occur alongside adjacent neurites. Each cell could form 1 to 19 synapses with the most common to be 5. There is a visible specialization of the pre-synaptic cells, possessing vesicle-filled varicosity and a region adjoining to the connecting post-synaptic cell. Gap junctions are formed in-between neurons, and in-between muscle cells, characterized by electron microscopy as a flatten region of the parallel membranes of the proximate neurons. In addition to the connections as mentioned above, neuromuscular junctions (NMJ), the chemical synapses between neurons with muscle cells as recipient post-synaptic cells are numerous and vital for *C. elegans* locomotion. Contrary to other organisms, in *C.elegans*, motor neurons do not send processes that synapse onto the muscle; instead, muscle cells send cell projections to motor neurons to get innervated.

D: Neurotransmission and Vesicle Release

C. elegans uses many of the most common neurotransmitters, including acetylcholine (Ach), glutamate, g-aminobutyric acid (GABA), biogenic amines (dopamine, serotonin and octapamine). Present are as well their receptors for their detection[9]. Signal transduction is also modulated by numerous neuropeptides (in the synapse), which also act as neuroendocrine signals (long-range signalling molecules)[10]. In *C. elegans* have been identified 113 neuropeptide genes encoding for over 250 neuropeptides. These are separated into two prominent families of insulin-like peptides and FLPs [FMRFamide (Phe-Met-Arg-Phe-NH2)-related peptides]. This number in *C.elegans* is enough to counteract the analogues molecules in mammals as they act with redundancy and bind to multiple receptors and vice versa. Dopamine is produced in eight neurons of the hermaphrodite and other six neurons are located in the tail of the male; all of them being mechanosensory. Correspondingly, serotonin is produced in eight types of

neurons in *C. elegans*[11]. Octapamine synthesis is limited to the RIC interneurons and the gonadal sheath cells[12]. At excitatory NMJs, secreted ACh leads to muscle contraction, whereas inhibitory NMJs release g-aminobutyric acid (GABA) causing muscle relaxation[13].

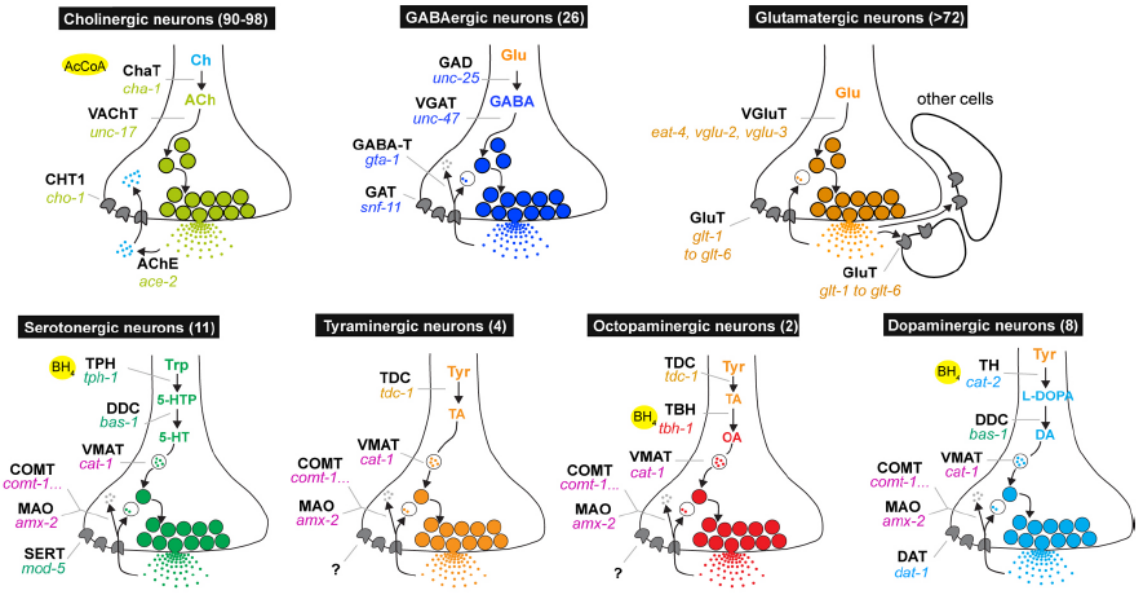


Figure2. 4 Neurotransmitters pathways: The pathway of each neurotransmitter in the C.elegans is depicted along with the genes needed for its synthesis, transportation, secretion or reabsorption. In addition, the number of neurons which are implicated in each of these pathways is referred in the top of each subgraph[9]. Graphical Image adapted from Oliver Hobert 2013 for Workbook.

The cycle of biogenesis, trafficking and secretion of Synaptic Vesicles (SV) and Dense Core Vesicles (DCV) affects the whole process of signal transduction. The types as mentioned above of secretory vesicles differ in many aspects. The most profound among them is their cargo; SVs are in charge of the release neurotransmitters while DCVs enclose mainly bioactive neuropeptides. SV exocytosis entails the steps of docking, priming and fusion, followed by the endocytosis in which vesicle proteins and membrane regions are retrieved to restore synaptic homeostasis[13]. Vesicles with integrated neurotransmitters transporters load the vesicle. The newly formed synaptic vesicle have to pass through the docking process, in which SVs are gathered near to the

release regions of synaptic terminals, adjacent to the plasma membrane, albeit without being fused. These docked SVs create a readily releasable pool of SV forming an, as called, active zone in the pre-synaptic terminus[13]. The next step, as it is also illustrated in Figure 2.6, is the selectively priming of the already docked SVs. During priming vesicles undergo a change to become fusion competent. The final signal to trigger SV release is the fluctuations in spatially Ca^{++} concentration[13]. For DCV release the process is quite different.

As previously noted, DCVs transfer bioactive neuropeptides, thus the formation of these vesicles starts at the endoplasmic reticulum where modifications of precursor molecules occur and continue to trans-Golgi from where the vesicles emerge and translocate to the axon terminals[14]. In contrary with SVs, DCVs are docked throughout the pre-synaptic compartment, primed and ready to get fused with the membrane[14,15]. Notably, in order to evoke a synaptic release of DCVs, higher levels of stimulus are needed in contrary with SVs. The last feature which distinguishes these two types of synaptic release relies on the fact that SVs after their release, follow endocytosis, continuing a vicious cycle of exocytosis and endocytosis. By contrast, DCVs are resupplied from trans-Golgi with the formation of new neuropeptides[15].

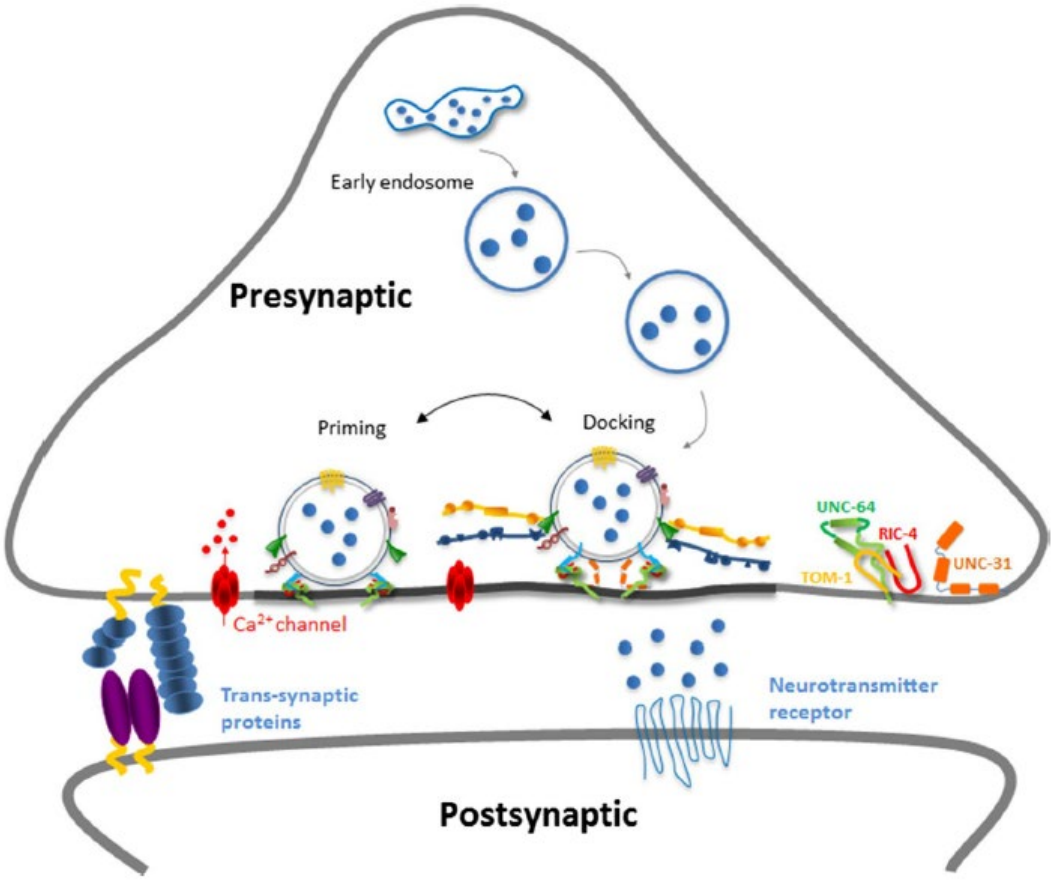


Figure2. ***5 The cycle of biogenesis*** *and exocytosis of synaptic vesicles: Factors necessary for the proper docking and priming of the vesicles are illustrated in the graph*[16]*. Image adapted from Calahorro et al ., 2018.*

E: Activation potentials

The voltage conduction in *C.elegans* appears to be differentiated from the corresponding conduction in vertebrates. No classical voltage-dependent sodium channels and sodium-dependent action potentials have been identified in the genome of *C.elegans.* In addition, there is increased neuronal resistance. Hence in most cases, nerve conduction in *C.elegans* is exhibited passively, without standard active potentials being evoked[17,18]. This may be the case for *C. elegans* exerting the final effect on synaptic releases; as reported in a study by Mellem et al, for C.elegans, there is "something else"[19]. It was also presented that a neuron was shifting between two resting membrane potentials, an event which may be the case for many *C.elegans* neurons[20]. Four main types of regenerative neuronal activity are

proposed, the action potentials, graded potentials, intrinsic oscillations, and plateau potentials[19]. Active potentials are the well-characterized threshold potentials which trigger to excitation (all-or-non). For the rest three little is known, graded potentials are proportionally sensitive to the intensity of the signal (wavelength and amplitude) in contrast with action potentials. Plateau potentials are exhibited as prolonged all-or-none depolarizations that can be evoked from sudden positive current or stopped by a spontaneous negative pulse. Intrinsic oscillations are rhythmic or repetitive patterns of the neural activity causing spontaneous alterations in the membrane probably caused by delicate balances of antagonistic voltage-dependent currents. In RMD neurons plateau potentials was suggested to be dependent on a current carried mainly by Na^+ and $Ca2^+$.[20]

In *C. elegans*, it was found that action potentials were abolished in the absence of extracellular Na^+ in patch-clamp recordings[21]. Despite the lack of candidate genes as voltage-dependent sodium channels, many clues support that Na^+ current contributes to neurons firing. Blockage of Na^+ channels with procaine and quinidine resulted in inhibition of action potentials, while administration of a factor which causes activation of voltage Na^+ channels, veratridine, led to increased frequency of activation potentials. A paper published by Franks et al. suggested the presence of a sodium-dependent pacemaker potential, up-regulated by 5-HT. Furtherly, this kind of pacemaker could be present in both neuron and muscle, providing excitation signals for the pharyngeal pumping[21].

F: Epithelium Sodium Channels/ Degenerins (DEG/ENaC)

The DEG/ENaC protein family encompasses a plethora of proteins expresses in almost all metazoans, in various tissues, exerting multiple roles and various features. The story of this superfamily starts at the early 90s when, Chalfie and Wolinsky, in a screen for touch-sensitive mutants, identified a gene, which they named *deg-1*[22]. This gene was involved in the sensory touch transduction and, when mutated, could lead to the

specific, late onset of neuronal loss. Consequently, *mec-4,* a proposed additional member of this family, was reported to exert the same degenerative phenotype in *C. elegans*[23]. Cannessa and colleagues in 1993 identified the first amiloride-sensitive, epithelium sodium channel subunit, arENaC in vertebrates[24]. Intriguingly, this channel exhibited a remarkable similarity with the recently newly identified degenerins in *C. elegans*, albeit their expression was specialized in lung and kidneys functioning for Na^+ reabsorption. Although the tissue expression was different, these proteins seem to share great sequence and functional similarities, being expressed in very different organisms[24]. Later, members of the DEG/ENaC superfamily have been found in nematodes, flies, snails, and vertebrates. Currently, from this family, 7 different branches can emerge from phylogenetic analysis depending on the organism or the tissue they are expressed.

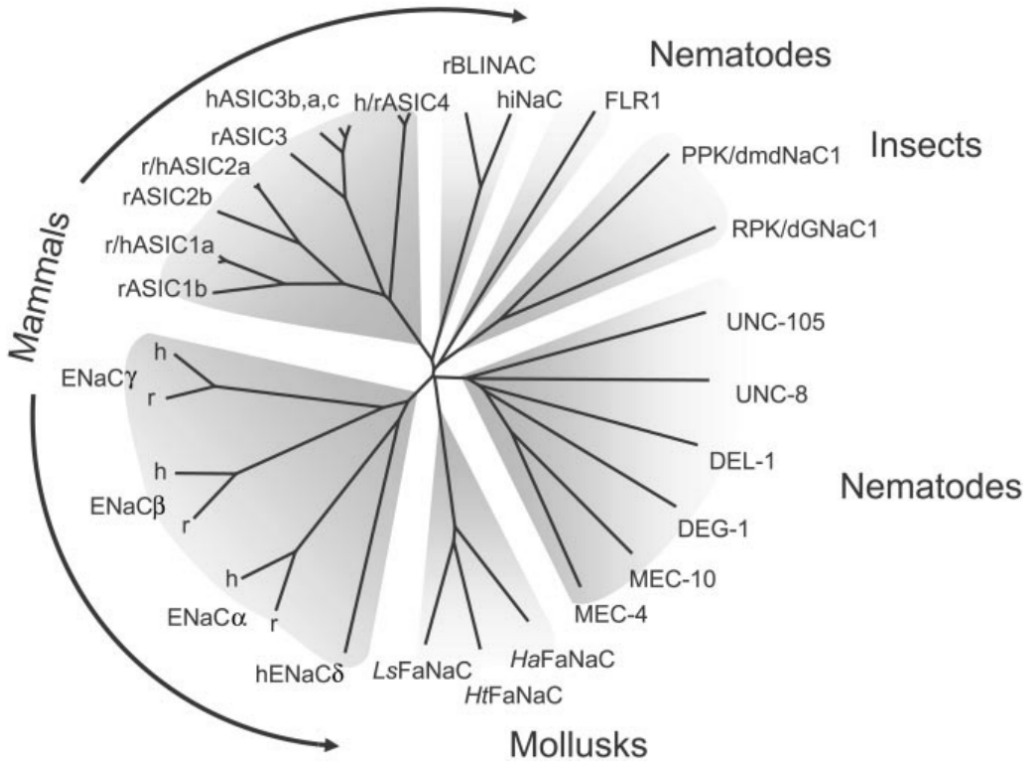


Figure2.6 ***Phylogenetic tree of the epithelial sodium channel (DEG/ENaC) family*** *showing the organization into subfamilies of related sequences differing between the species*[25]*. Graph adapted from Kellenberger et al 2002.*

In Figure 2.6, phylogenetic analysis is depicted, depended on the organismal expression. The three main subfamilies encompass the *C.elegans* degenerins (UNC, MEC, DEG, and DELs), the ASICS and the human SCNN1 genes, which encode the ENaC subunits. Additional subfamilies are also reported in different organisms including the RPK/dGNaC1 and PPK/ dmdNaC1 expressed in *Drosophila*, the peptide-gated Na channel FaNaC of molluscs. The following groups are distinct from their relatives in *C.elegans* and mammals. FLR-1 in *C. elegans* that are clearly distinguishable from the degenerins and the mammalian, BLINaC (brain- liver-intestine amiloride-sensitive Na channel) and hINaC (human intestine Na channel)[26].

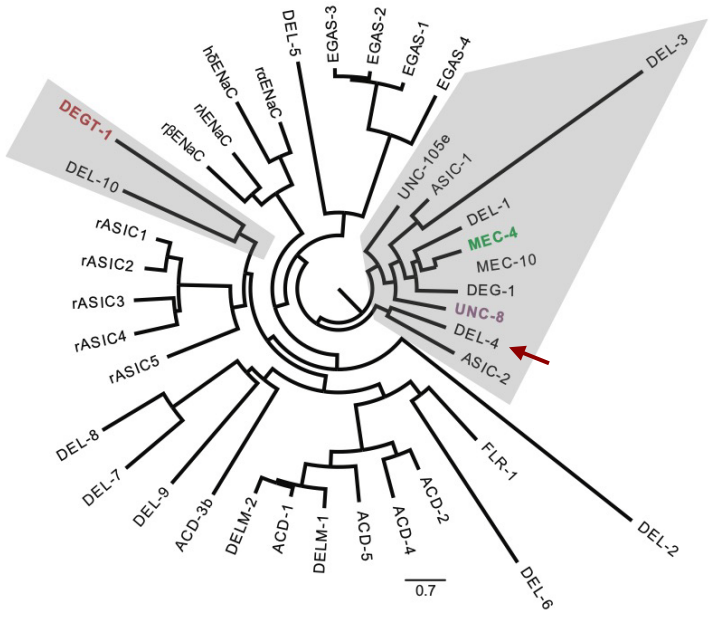


Figure2. 6 A second Phylogenetic tree of the 30 DEG/ENaC/ASIC subunits of C.elegans in association with the respective mammalian ENaC and ASIC subunits7. Arrow pointing, the protein of interest in this study. Illustration adapted from Fechner et al 2020

Beyond this diversity, highly conserved subunit structure and sequence similarities are profound. The overall secondary structure subunits encoded by these genes are highly conserved. Each subunit, range from 500 to 1000 amino acids and share sequence similarities. The existing unique protein common structural topology as following described. The channel possesses two transmembrane domains, N and C terminus facing in the cytoplasm and the two Cysteine Rich Domains (CRDs) and the rest of the

protein forms a loop facing the extracellular matrix[27–29]. DEG/ENaC channels have been implicated a broad spectrum of cellular functions, including mechanosensation, proprioception, pain sensation, gametogenesis, epithelial Na gating, heat sensitivity, taste and learning[28,30–32].

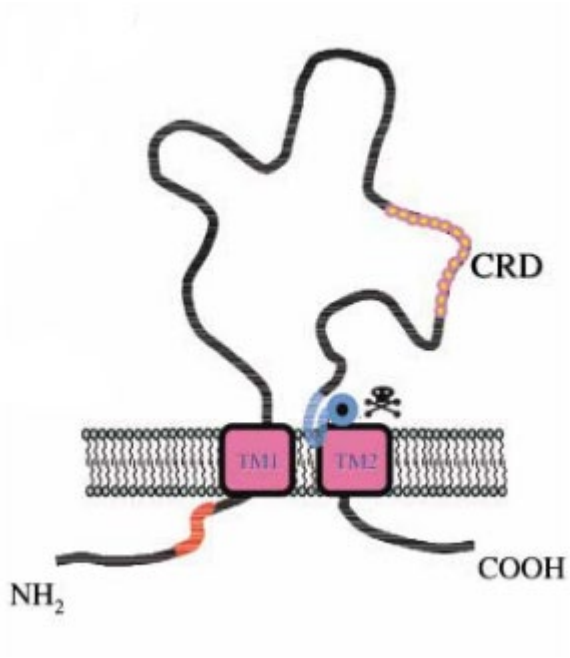


Figure2. 7 Common structural model of DEG/ENaC subunits as described in the text[28]. Image adapted from Bianchi and Driscoll 2002.

In mammals, ENaCs are expressed in diverse tissues like kidney, lungs, gastrointestinal tract, skin, sweat glands, pancreas, even the cochlea and the retina possible, mediating sodium gating and homeostasis. ASICs are abundantly expressed in the central and peripheral nervous system and are implicated in nociception and pain perception and associative learning, while they differ from ENaCs in preferably gating protons[26].

Degenerins, the *C. elegans* subfamily which occupies a large part of the family, is mainly expressed in the nervous system. In *C. elegans* some degenerins are implicated in shear stress and mechanosensation. It has been shown that some of them are activated by mechanical stimuli and are essential in touch sensation and proprioception[31,33].ASICs are

involved in pathologies of the Central and Peripheral Nervous System. Increasing evidence implicates them in several neurodegenerative diseases. Degenerins, in *C.elegans*, as their name implies are also associated with neurodegenerative events when their function is perturbed. Degenerative models in *C.elegans* have been developed over the past few years, enabling the research of complex disease in a simplified model such as *C.elegans*[34].

G: C.elegans as a model for degenerative disorders

At both cellular and molecular level, most of the neurobiology questions can be studied in the worm. In *C. elegans*, some individual neurons perform functions that would recapitulate the function of multiple neurons in mammals. It is estimated that 42% of neuronal genes have orthologues in the genome of *C. elegans*. Nowadays, neurodegenerative diseases are exhibiting an accelerated augmented prevalence into the elderly. Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), amyotrophic lateral sclerosis (ALS) and others, affect millions of people around the globe. Their common feature includes the progressive loss of neurons, due to aggregated proteins accumulating in the neurons or extracellularly[35]. Abundant models have been generated in *C.elegans* both genetic and toxicant, to delineate the mechanisms of these diseases and for the screening of plausible drugs[35,36]. Some of the former, are described below in Figure 2.9. Thereinafter furtherly described a model of PD and tauopathy.

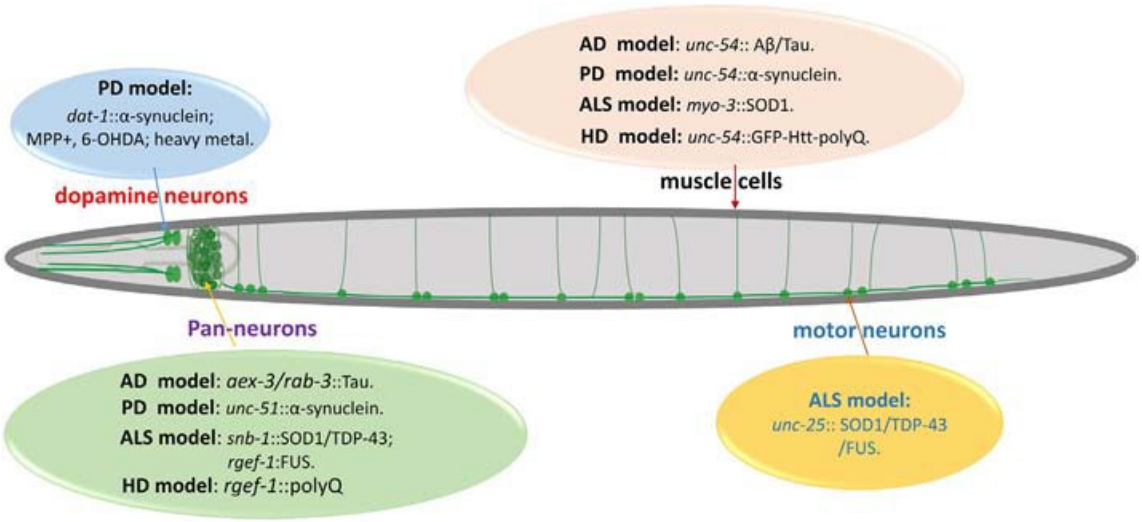


Figure2. 8 A representation of the most used models of neurodegenerative disease in C.elegans[35]. Image adapted from Li and Le 2013.

PD is a progressive, mainly, late-onset neurodegenerative disease, manifesting impaired movement and reduced cognitive function. A profound characteristic of this disease is the aggregation of the a-synuclein protein into the Lewy bodies intracellularly[37]. Subsequently, there is a progressive loss of dopaminergic neurons in the substantia nigra of the brain. Strains in *C.elegans* expressing the human a-synuclein, since there is no orthologue expressed in the worm, have been generated. The expression can be targeted to specific tissues and even neurons with the use of transcriptional reporters for specific neurons, such as the widely used dopamine transporter dat-1. The aggregation, of this protein in DA neurons of the worm, causes neuronal loss after adulthood[38,39].

Ultimately, aggregation of tau in a non or hyper-phosphorylated state is a common cause of a group of diseases, as called, Tauopathies. A model was introduced by Fatouros et al. A mutation in the human sequence of tau led to increased nucleation when expressed in *C.elegans*, leading to severe proteotoxicity and neuronal defects[40].

Study Objectives

In this study we seek to delineate the plausible role of DEL-4 sodium channel, member of the DEG/EnaC family, in neuronal function and neuro transduction.

We have characterized the channel and taking into consideration its properties we carried out the following:

- ∇ We tested synaptic release in the context of normal DEL-4 channel, and mutated.

- ∇ We performed an analysis, with the use of voltage indicators, of resting membrane potential of dopaminergic neurons in normal and under DEL-4 malfunction.

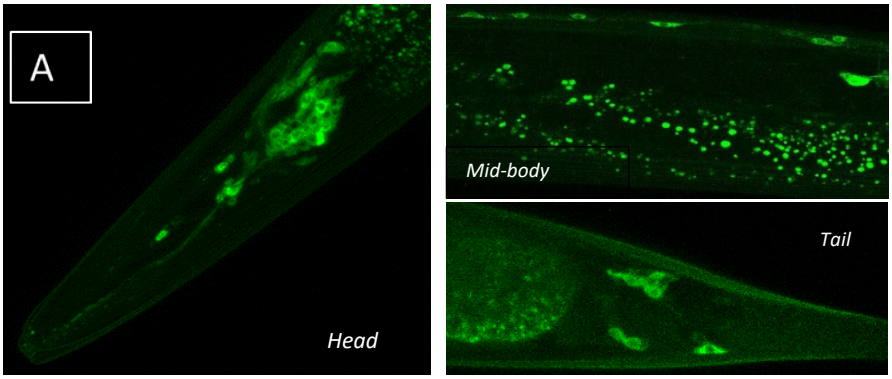
- ∇ We evaluated changes due to physiological stresses in neuronal and organismal function and the role of DEL-4 in these responses.

- ∇ On a final note, we assessed the implication of the DEL-4 mutated channel in the progression of neurodegenerative diseases, in neurodegenerative models.

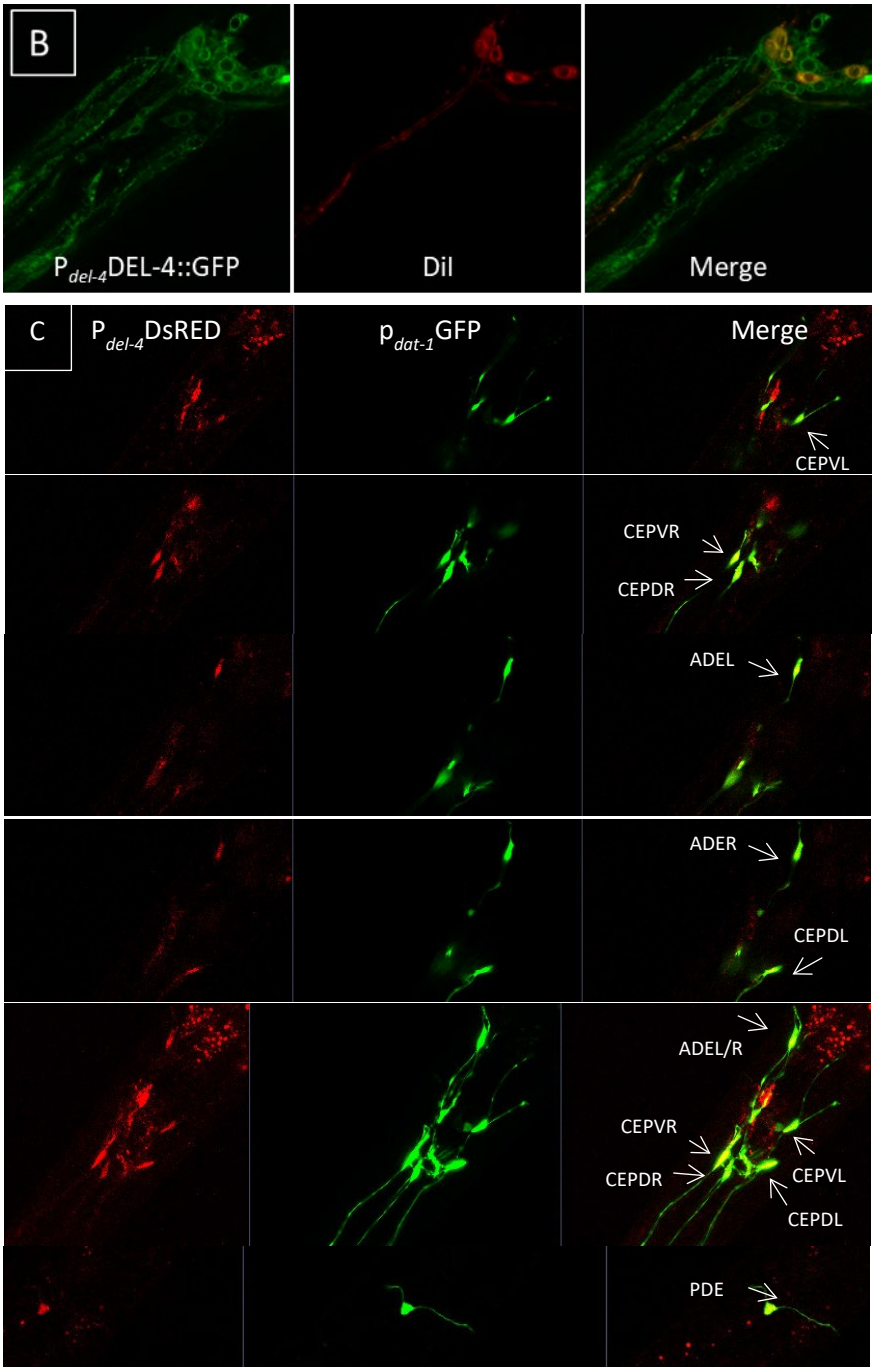
-Chapter III: Experimental Results-

A: DEL-4 expression pattern

As confocal images dictate, DEL-4 is expressed in multiple neurons of different types, lengthwise the entire worm (Figure 1.A). Data (not shown) also suggest a relatively steady expression of DEL-4 during development.



To take a closer look at the protein's topology, we dyed the membranes of the neurons with a dye, DiI, which stains the membranes in Red. The concomitant expression of DEL-4 translational reporter with GFP tagging showed a wide colocalization in the cell membrane. By these means, we demonstrated the anticipated expression of DEL-4 in the plasma membrane of its expressing neurons (Figure 1.B)[41,42].


B
P del-4 DEL-4::GFP
DiI
Merge
C
P del-4 DsRED
p dat-1 GFP
Merge
CEPVL
CEPVR
CEPDR
ADEL
ADER
CEPDL
ADEL/R
CEPVR
CEPDR
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PDE

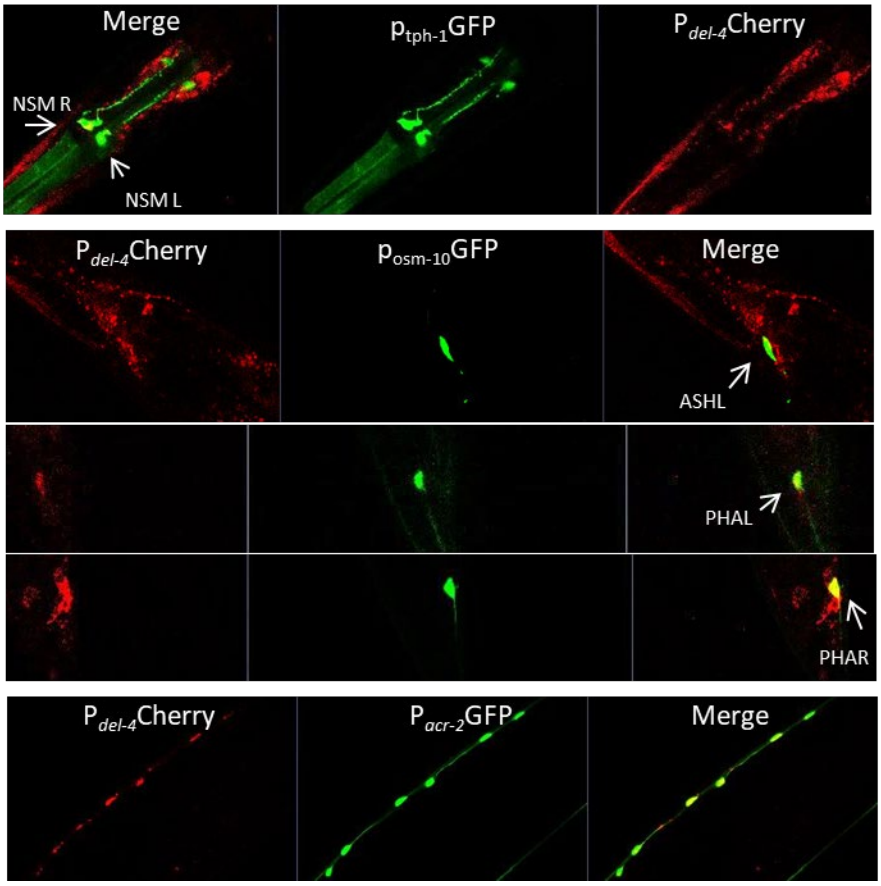


Figure 1: DEL-4 expression pattern. A. Expressed through the body. B.DEL-4 localizes at the cytoplasmic membrane. C .It is expressed in all dopaminergic neurons, in serotonergic, chemosensory and motor neurons. Specifically, DEL-4 is found in ASH, PHA, CEPDL/R, CEPVL/R, ADEL/R, PDE, motor neurons (VA/B, DA/B). Confocal images (z-stacks unless otherwise indicated) of strains that came up by crossing del-4 transcriptional reporter (seen in red) with the respective neuronal reporters (seen in green).

Using strains co-expressing both transcriptional reporters of specific neuronal types and DEL-4 protein transcriptional reporters, we can assess the expression pattern of DEL-4 (Figure 1.C). Here, there are briefly reported markers used to identify and characterize neuronal types in which del-4 is expressed[43,44]. Regarding the imaging, DEL-4 is expressed in dopaminergic (CEPDL/R, CEPVL/R, ADEL/R, PDE), identified with dat-1 expression, the dopamine transporter expressing gene. It is also expressed in chemosensory neurons (ASH, PHA/B/C, and ASEL/R) and cholinergic motor neurons (VA/B, DA/B), reported with the acr-2 promoter. The furtherly expression is identified in

polymodal neuron IL1 (mechanosensory, motor neuron, and interneuron) and mechanosensory PVM.

Another point that we wanted to address is the possibility of DEL-4 localizing precisely to the synapses. To test this hypothesis, we used confocal fluorescent microscopy again, with a magnitude 63x. Transgenic worms expressing translations reporter (GFP) of del-4, along with the translational reporter of synaptobrevin under the promoter of the del-4 gene, are depicted below (Figure 2.C). Synaptobrevin gene encodes for the SNB-1 protein, a neuronally expressed C.elegans orthologue of the Synaptic Vesicle associated membrane protein (VAMP2)[45]. This co-expression enabled us to conclude that although DEL-4 colocalize with SNB-1 to a great extent, DEL-4 seems to have a broader expression pattern being expressed in both synapses and the rest of the neuron. Conclusively, DEL-4 appears to be implicated in a more general regulation of sodium homeostasis.

B: Physiological characterization of DEL-4

For the characterization of DEL-4 protein, electrophysiological studies were performed. Wild type protein DEL-4 or product from del-4(tm717) mutant was ectopically expressed on *Xenopus laevis* oocytes, and Two-Electrode Voltage Clamp (TEVC) was executed. Inward Na^+ currents were recorded with homomeric DEL-4 expression. In the presence of 500 μM of the DEG/ENaC channel blocker amiloride, the currents were terminated, suggesting that the channel is constitutively open and closes in the presence of a blocker (Figure 2.A). The product of the mutant del-4(tm717) did not elicit Na+ currents, either. Regarding ion selectively, DEL-4 homomeric channel is permeable to monovalent cations preferentially Na+ during perfusion of oocytes with NaCl, LiCl, or KCl solution (Na+ > Li+ > K+) as is shown in Figure 2.B.

The most notable part of the electrophysiological study is the perfusion of DEL-4 expressing *Xenopus* oocytes with solutions of gradually reduced pH (Figure 2.C). It is clear that the DEL-4 channel is blocked when pH drops from 6 to 5. These results indicate that the DEL-4 channel is continuously open and closes it in the presence of

amiloride and low pH. This feature sets it apart from other acid-sensing sodium channels (ASICs), which are blocked in low pH in contrary to the DEL-4 channel.

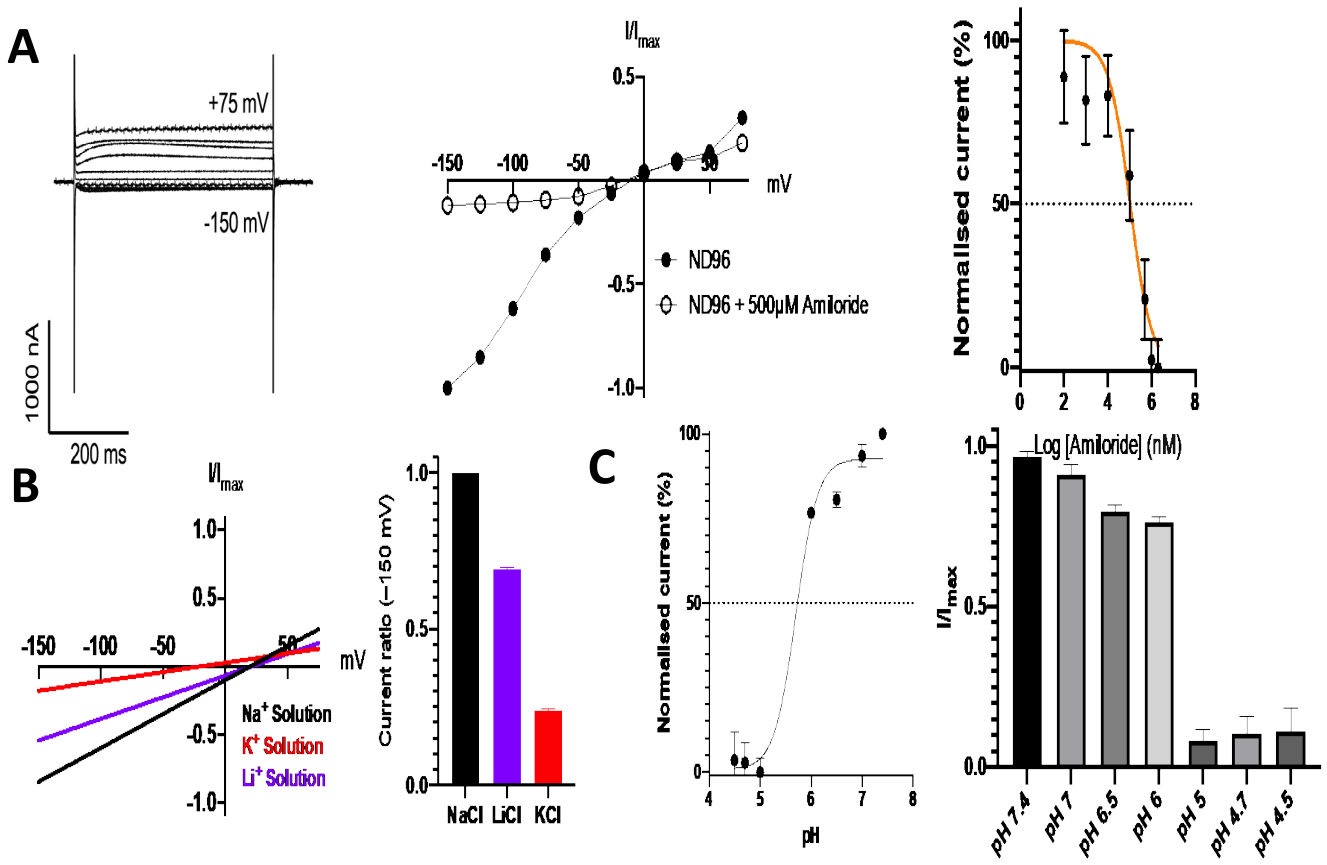


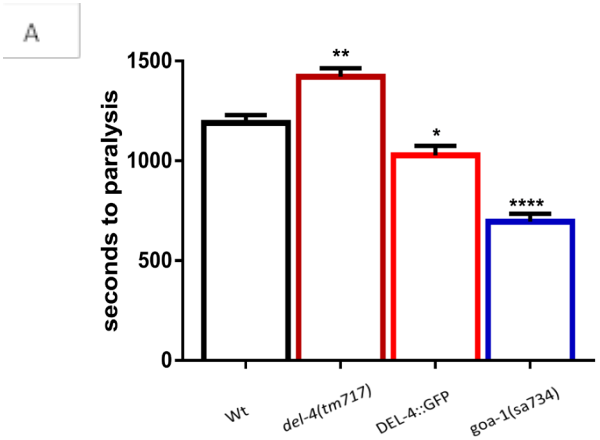
Figure 2 Electrophysiological characterization of DEL-4:. (A) Current–voltage (IV) relationships for Xenopus oocytes injected with del-4 cRNA when the oocytes are perfused with a physiological NaCl solution (ND96) (black circles), and in presence of 500 µM of the DEG/ENaC channel blocker amiloride (open circles) (N=10). Normalised current of amiloride dose–response curves for the DEL-4 homomer showing a half maximal inhibitory concentration (IC_{50}) of 105 µM ($LogIC_{50}$ = 5.022 nM) (N = 4) indicated by the dashed line. B. Preferable permeability to cations C.DEL-4 subunits can form a constitutively open channel at neutral pH that is blocked by low extracellular pH. Currents were normalized to maximal currents and best fitted with the Hill's equation. Error bars represent Mean and SEM.

Inward Na+ currents were recorded when homomeric DEL-4 was expressed on Xenopus oocytes perfused with a sodium-rich solution by stepping membrane voltage from -150 mV to +75 mV, a holding potential of -60 mV .

C: DEL-4 regulating synaptic release

Aldicarb resistance assay

Acetylcholine (Ach) regulates locomotion in C.elegans. Cholinergic motor neurons release Ach pre-synaptically, activating Ach receptors of the muscle cells to contract. Acetylcholinesterase is an enzyme also released in the synaptic cleft, which breaks down acetylcholine, leading to neurotransmission termination. Aldicarb, a pesticide, acts as an acetylcholinesterase inhibitor. In the presence of aldicarb, acetylcholine continues to accumulate, causing constituent muscle contraction and eventually paralysis. Animals with defected Ach release seem to be more resistant to aldicarb induced paralysis, as less Ach is collected in the synapse. Inversely, animals with increased synaptic transmission levels and subsequently increased Ach levels are more sensitive to aldicarb paralyzing effect than control animals[45–48]. We conducted the suggested assays a bit altered by not using NGM plates with aldicarb but instead having a drop with 10mM concentration of the drug. In the graph of Figure 3.A it is depicted the seconds needed for each worm to paralyze and the model from Mahoney et al. The *del-4(tm717*) exhibited resistance to aldicarb induced paralysis. In contrast, DEL-4 overexpressing animals were more susceptible to paralysis. Animals bearing goa-1 mutation gene expression were used as a positive control since they do exert increased sensitivity to aldicarb, as reported in the literature[47].



In addition to the aldicarb assay, a levamisole assay was previously performed in the lab. Levamisole is an Ach agonist that results in hyperactivation of Ach receptors of post-synaptic cells. The persistent contraction of the muscle leads eventually to paralysis. Mutants, as well as overexpressing animals for DEL-4, were not sensitive to levamisole (previous data). Both of these assays point out that DEL-4 is implicated in the pre-synaptic transmission, and its physiological function is impairing the synaptic release.

The synaptic release of cholinergic motor neurons is diminished in a del-(tm717) background.

To estimate the levels of released dopamine, we utilized a pH-sensitive version of EGFP, Super ecliptic phluorin tagged with synaptobrevin expressed in dopaminergic neurons (pasic-1SNB-1::SEpHl)[49]. The fluorescence of SEpHl quenched inside synaptic vesicles where pH is low. Synaptic vesicle release exposes SEpHl to the higher pH of the synaptic cleft, and it exhibits fluorescence increase in transgenic animals expressing super ecliptic phluorin tagged with SNB-1 under acr-2 promoter in the cholinergic motor neurons were crossed with del-4(tm717) mutants. Mutant animals exhibited reduced levels of synaptic release compared to wild type at the level of cholinergic NMJ Figure 3.B.

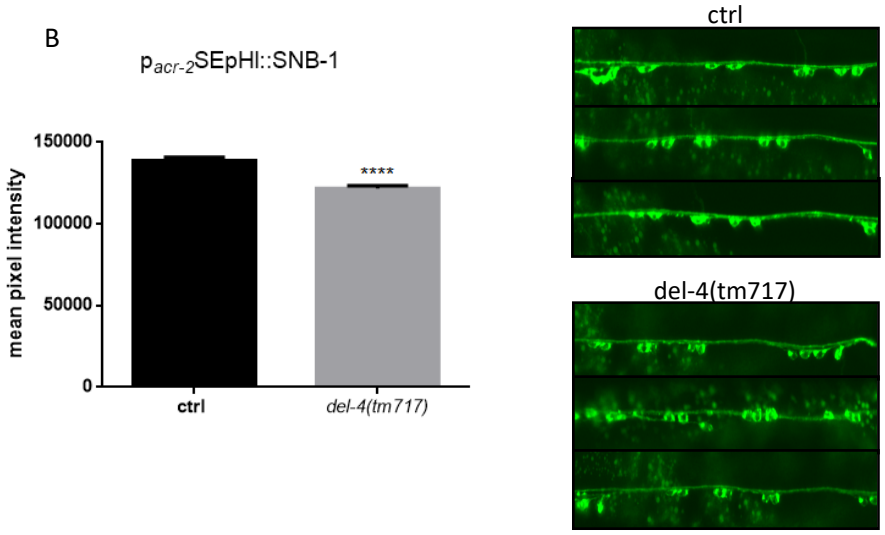


*Figure 3: A. Aldicarb sensitivity assay to 10mM aldicarb. B. Fluorescence measurement of super ecliptic in cholinergic motor neurons at the midbody. ****P < 0.0001 ,n>30 worms , 3 replicates analysed with Unpaired t test.*

D: Dopaminergic neurons hyperpolarize upon DEL-4 downregulation

DEL-4 regulates the resting membrane potential of dopaminergic neurons

In line with the above, we estimate that DEL-4 affects the synaptic transmission, although it is not explicitly expressed in the synapses (Figure1). This leads to the notion that DEL-4 may contribute to synaptic release by altering neurons excitation pattern. According to what is extensively mentioned in the introduction, in C.elegans, there are four types of potentials, which could activate neurons. In addition, Na^+ current was also reported to be essential for the initiation of signal transduction. Closing a Na^+ channel blocks the inward Na^+ current. From all the above, we made the hypothesis of a proton inactivated channel, which leads to hyperpolarization of the cell since the inward current is blocked, and the extracellular charge is getting more positive. To test this hypothesis, we generated voltage indicators expressed under neuron-specific promoters. ASAP1 is based on a voltage-sensitive phosphatase, with the phosphatase region being replaced with a circularly permuted GFP. Membrane potential alterations cause conformational changes of ASAP1 that lead to fluorescence increase upon hyperpolarization and decrease in the case of depolarization [50,51].

We generated four constructs expressing the ASAP-1 voltage indicator under dat-1, tph-1, che-1, and acr-2 promoter. Each promoter is specific for a subgroup of neurons, dat-1 for the dopaminergic, tph-1 for the serotonergic, che-1 for chemosensory and finally acr-2 for cholinergic, as previously reported. We proceeded with the pdat-1ASAP1 plasmid injection in N2 worms along with the *rol-6(e187)* gene sequence to allow us the worm selection. We generated a worm line expressing ASAP-1 in dopaminergic neurons. The next step was to create the crossing with *del-4(tm717)* mutant animals. Finally, we did epifluorescence imaging (20 x magnifications in EVOS). Epifluorescence imaging revealed that *del-4(tm717)* mutant animals had increased fluorescence levels of ASAP1 compared to control animals, meaning a more polarized membrane since ASAP-1 is quince under depolarization and fluorescent in a hyperpolarized environment. Mean expression levels from neuronal somas of all dopaminergic neurons in the head were

counted. Hence, inactive DEL-4 channel hyperpolarizes dopaminergic neurons (Figure 4.B).

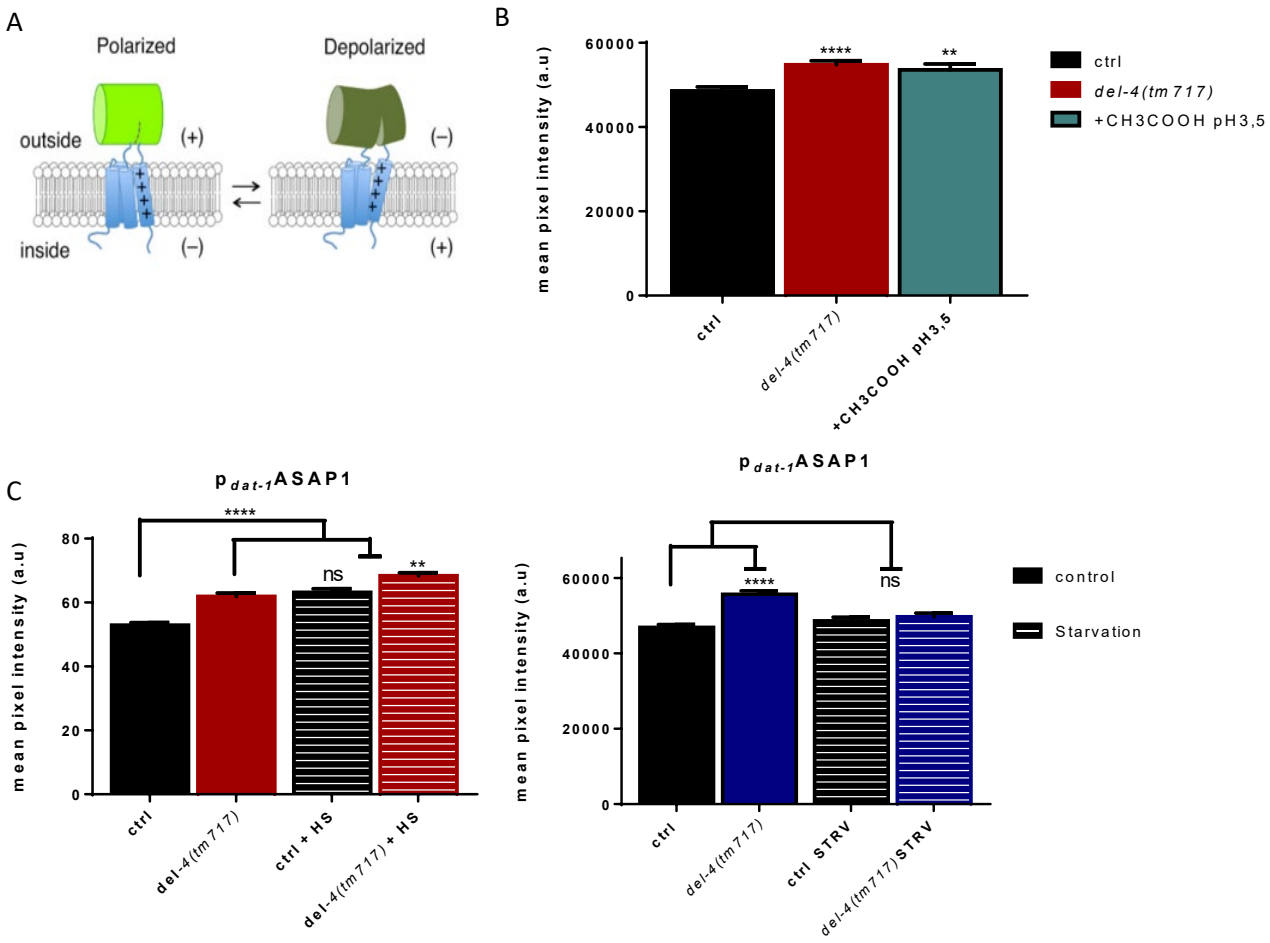


Figure 4: The implication of DEL-4 in neuronal function, using a voltage indicator. A. The voltage indicator ASAP-1. Hyperpolarization under acidic environment and mutated background. C. Stress induced hyperpolarization. Tukey's multiple comparisons test (one-way ANOVA) 3 replicates, n>60 neurons, ***P < 0.001.

The second part of this experiment was to check whether the channel's electrophysiological characteristics have the same effect as the mutated one. The question arose; could a more acidic environment lead to inactivation of the channel and furtherly to hyperpolarize the neuron as the mutated did? To determine if this hypothesis stands true, we measured ASAP1 levels of control animals after treatment with an M13 solution of pH 3.5 (adjusted with acetic acid). Animals were incubated in a 15 µl drop of acidic solution or neutral M13 as a control for 30 min before imaging. ASAP1 levels increased in low pH conditions, likewise the mutated. (Additionally, we

checked DEL-4 expression levels with a translational reporter after pH stress that expression levels remained unaffected.) Figure 4.B

E: DEL-4 function is regulated by physiological stress

We know from in silico analysis that *del-4* promoter possesses binding sites for DAF-16, SKN-1 and HSF-1 transcriptions factors. These three factors are the primary regulators of starvation, oxidative stress and heat stress responses, respectively. Furthermore, in not presented data, stresses downregulated DEL-4 expression levels. We already knew from imaging analysis of two heat shock response factors, hsp-16.2 and hsf-1 translational reporters, that when d*el-4* expression is downregulated with RNAi, they exhibit elevated expression. These findings lead to the notion that del-4 mutant, resemble stress like state. Taking into consideration all the above, we hypothesized that the absence of del-4, as observed in the del-4 mt, would have an impact on stress-induced responses, putting the worms in a pre-conditioned state.

Heat stress resistance assay

To begin with, we examined if heat stress affected the lifespan of mutants and DEL-4 overexpressing worms. In heat stress resistance assay, day 1 of adulthood worms incubated for 2 hours in 37^{o}C were recovered for one day. Survival was estimated by counting every day the dead worms. Worms with a mutated background of *del-4* were more resistant to heat shock, while DEL-4 overexpressing animals were more susceptible to stress (Figure 5).

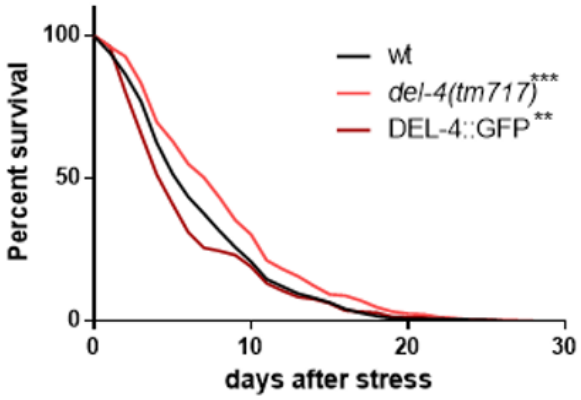


Figure 5: Heatstress resistance curve: Log-rank (Mantel-Cox) test

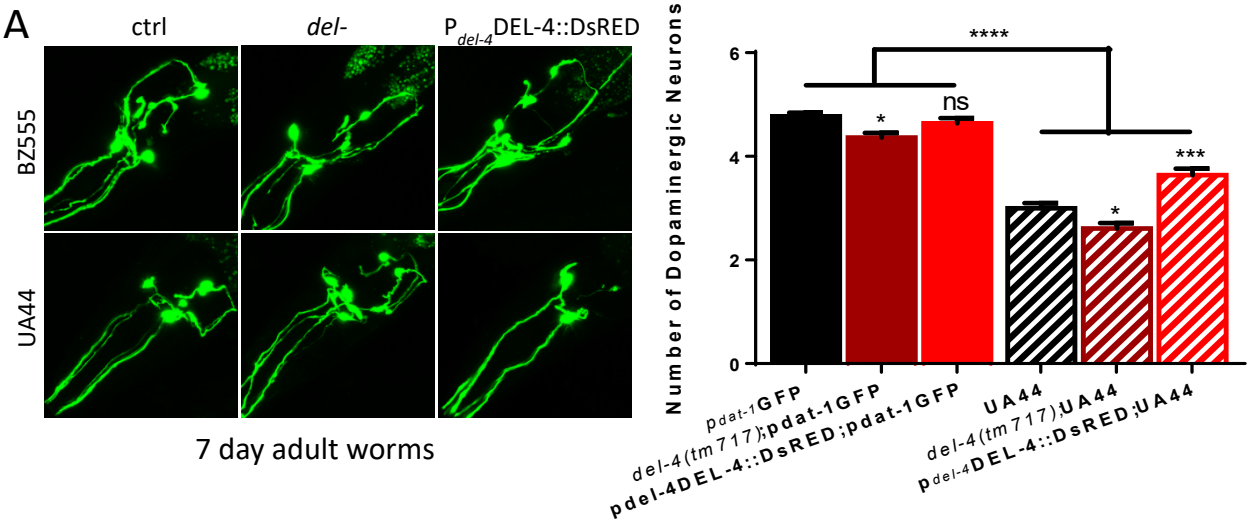
Alteration of membrane potential due to physiological stresses

From not shown experiments, starvation and heat stress limit the expression levels of DEL-4, and DEL-4 reduction causes hyperpolarization of dopaminergic neurons. So, we hypothesized that these two stresses at least should increase ASAP1 levels, too. Starvation on NGM plates without food and heat stress at 37°C for 1hr severely hyperpolarized dopaminergic neurons compared to control. The hyperpolarization level was the same as that of *del-4(tm717)* mutant animals with or without the stress. (Figure 4.C)

F: DEL-4 mutation leads to the progression of degenerative diseases

Neurodegenerative models

We have indicated that DEL-4 perturbed expression leads to altering neuronal function in the expressed cells. As further reported in the introduction, DEG/ENaCs have already been implicated with various neurodegenerative diseases. As referred to the literature, additively to some lab experiments suggesting that DEL-4 inactivation interferes with neurotransmission. Hence, using two models of degenerative diseases, we came to the following results. Mutants of del-4(tm717) and overexpressing strain DEL-4 (pdel-4DEL-4::GFP) were crossed to Parkinson's and Alzheimer's disease models of C. elegans. Parkinson's disease model used the UA44 strain, which expresses human a-synuclein in the dopaminergic neurons, labelled with GFP (pdat-1GFP;pdat-1a-synuclein).



We used the BR5270 strain as a tauopathy disease model, which expresses pan-neuronally the pro-aggregate F3ΔK280 tau fragment[40] BR5270 strain was likewise crossed with the pdat-1GFP reporter to enable the visualization of degenerative dopaminergic neurons. Following animals in D7 and D5 days post adulthood, respectively, for each model, we captured the dopaminergic neurons of the head and by measuring the number of remaining DA neurons (Figure 6.A and B). We observed that overexpressing DEL-4 resulted in reduced degeneration, while DEL-4 downregulation had the opposite effect.

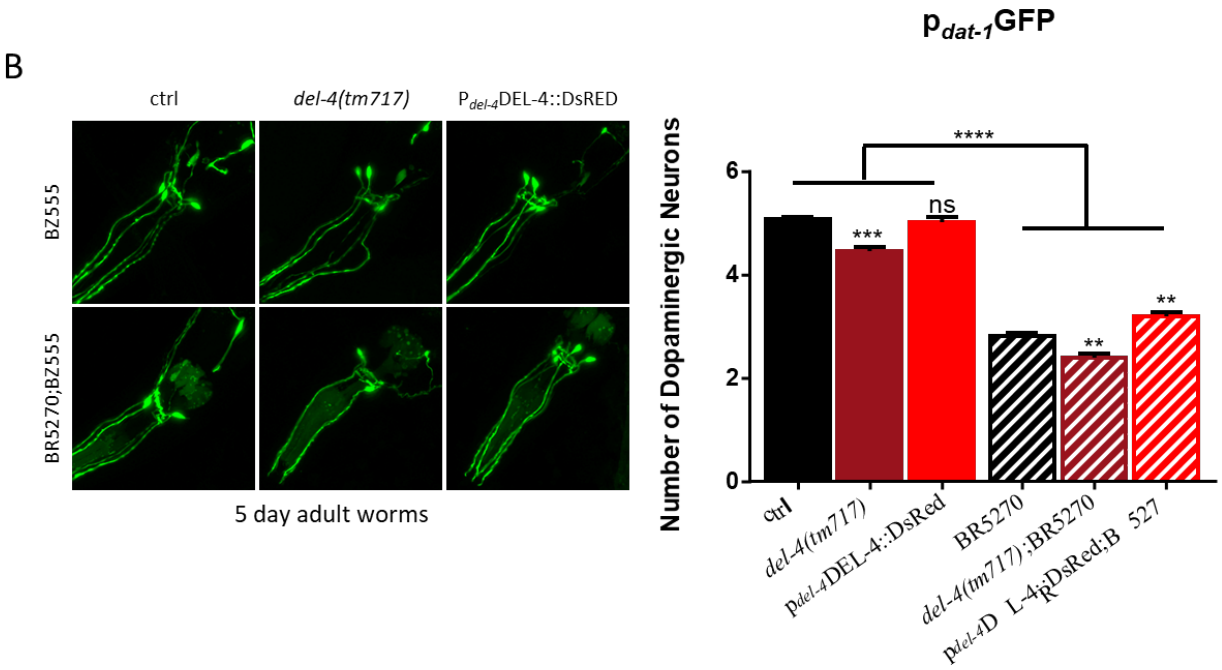


*Figure 6: Neurodegenerative models disease. A model of a-synuclein aggregation. Images taken from day 7 animals. B. Model of taupathy, images taken at day 5 of adulthood. One-way ANOVA for multiple comparisons was used for the estimation of statistical significance 4 replicates (n>40; ***P < 0.001, **P<0.01). Error bars, s.e.m. Images were acquired using Zstacks with confocal microscopy X40 objective lens.*

-Chapter IV: Discussion-

The electrophysiological characterization of DEL-4 provided us with some exciting information about the function of the channel. In mammals homologue channels, ASICs are closed at physiological pH with a small drop in extracellular pH rapidly activating them[52]. Inflammation, ischemic conditions, infections or cancer are some cases that reduce extracellular pH and increase the open probability of ASICs. This feature is antithetical with our protein, which closes in low pH. To our knowledge, the only identified channel which is blocked upon acidosis in *C.elegans* is ACD-1; a proton-gated channel, member of degenerins family. It functions in glial cells to regulate acid avoidance behaviour together with deg-1[53]. Although DEL-4 is expressed in neural cells, it seems to exert a more complicated role in neuronal homeostasis upon stress. We are in the process of conducting the low pH drop test assay, proposed by Wang et al. in addition to a pHtaxis assay. Both these two experiments will provide us with some evidence of if pH sensing characteristic of channel involves the immediate sense of environmental pH or the indirect changes in neuronal region pH-induced as a secondary response to stress. In both cases, DEL-4 seems to exhibit a critical role in controlling neuronal signalling under environmental changes.

Physiological stress is perceived as environmental alterations. *C.elegans* senses these changes via its sensory organ, neuronal cells. Promptly, after the sensation of a stimulus, an event trajectory starts to activate organismal responses to encounter change. Organisms have evolved targeted and specific stress responses dedicated to repair damage and maintain homeostasis. These mechanisms include the unfolded protein response of the endoplasmic reticulum (UPR ER), the unfolded protein response of the mitochondria (UPR MT), the heat shock response (HSR), and the oxidative stress response (OxSR). Organismal responses have been evolved to maintain the homeostasis and repair occurred damage. Heat shock response (HSR) and oxidative stress response

(OxSR) are two of the mechanisms used by *C.elegans* to compensate with physiological stress[8]. Nevertheless, the link between stress perception and these furtherly characterized responses remains to be elucidated. Our data suggest an alleged involvement of DEL-4 in the transmission of a danger signal, likewise increased temperature. Heat resistance assay showed that *del-4* mutants were more resistant to stress. Concomitantly, augmented expression of heat shock response proteins hsp-16.2 and hsf-1 master regulator, is noticed. Another point to mention is that the promoter region of DEL-4 possesses binding sites for critical transcriptional factors of stress responses, for HSF-1, DAF-16 and SKN-1. In addition, as dictated from the voltage sensor experiment, heat stress and possibly starvation leads to the same phenotype with the mutated channel, the membrane hyperpolarization. There are concluding remarks that DEL-4 is transcriptionally regulated from physiological stress responses in addition to mechanistically. The rest of the stresses remain to be investigated to attain a more comprehensive picture of what is happening.

Future experiments are already programmed to investigate, the neuronal activity of stressed worms in wild type and del-4(tm717) background. To accomplish this, we are going to use strains that we already have expressing calcium indicator of channelrhodopsin for optogenetics analysis[54,55]. We also seek to delineate the role of DEL-4 in other neurons except for dopaminergic. We are in the process of generating the transgenic animals with injecting the ASAP-1 voltage sensor under the rest neuronal type promoters.

From all the above, we can extract the following information. DEL-4 channel is expressed in multiple neurons in *C.elegans* and different properties of the rest degenerins as being acid-inactivated. It mediates a fine-tuning in neuronal vesicle transmission, acting as a break when a danger signal appears.

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