Caenorhabditis elegans response to salt

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Zout smaak in *Caenorhabditis elegans*

Thesis

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Born in Lagos, Nigeria

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To my family and friends
biological and non-biological
I am blessed to have you all in my life all this while,
for the love, encouragement, support and care

Contents

Scope of this thesis

This thesis describes my work, where I used genetic methods to identify new genes involved in salt taste in *C. elegans.* In addition, I used calcium imaging to characterize the cellular response of *C. elegans* to salt. The thesis is divided into five sections and each section is summarized below.

In chapter 1, I discuss the importance of salt for our health and subsequently our current knowledge about the different taste modalities and how their sensations are regulated in mammals and *Drosophila.* Next, I discuss several sensory behaviours of *C. elegans* and the signal transduction pathways involved, with an emphasis on salt chemotaxis. Furthermore, I discuss different types of learning and memory observed in *C. elegans* and neurotransmitters involved. Finally, I discuss the importance of *C. elegans* as a model organism and the behavioural and neuronal methods commonly employed to study *C. elegans* response to salt.

Chapter 2 shows that *C. elegans* uses two genetic pathways for its response to salt. One pathway involves GCY-14/22 (receptor type guanylyl cyclases), TAX-2/CNG-3 (cyclic nucleotide gated channel subunits β and α), ODR-3 (Gα protein), OSM-9 (transient receptor potential vanilloid channel subunit) and GCY-35 (soluble guanylyl cyclase). The second pathway involves GCY-22, TAX-4/TAX-2 (cyclic nucleotide gated channel subunits α and β) and TAX-6/CNB-1 (calcineurin - Ca^{2+} activated phosphatase).

Chapter 3 entails the characterization of the neuronal response of *C. elegans* in a simple learning behaviour. In this behaviour (gustatory plasticity), animals learn to avoid NaCl by pre-exposing them to NaCl in the absence of food. Ca^{2+} imaging in specific neurons shows that gustatory plasticity involves desensitization of NaCl-attractive neurons and sensitization of avoidance mediating neurons.

Chapter 4 describes a forward genetic screen to identify additional genes involved in salt chemotaxis in *C. elegans.* We analysed thirteen independent mutants that are defective in NaCl chemotaxis with whole genome sequencing and/or Sanger (dideoxy) sequencing. We identified mutations in genes known to be involved in NaCl chemotaxis in eleven of them. The mutated genes that cause the NaCl chemotaxis defects in the remaining two mutants have not yet been identified.

In chapter 5, our findings on the molecular mechanisms of salt taste and gustatory plasticity in *C. elegans* and possible future experiments that can be done to further

our understanding of these processes are discussed.

1

Introduction

1.1 Importance of salt

NaCl is important in the body. The sodium and chloride ions in salt are electrolytes and therefore can conduct electricity when dissolved in water. This property of salt is very useful for many of the body activities because electrolytes carry electrical impulses to our nerves and muscles. Both sodium and chloride ions of salt perform various roles. Chloride ions help in maintaining the acid-base balance of body fluids in addition to other functions. Sodium is, among others, necessary for the contraction of muscles, including the heart, and transmission of neuronal information. In addition, the presence of salt in food is directly linked to the palatability of many foods and it is the most common taste enhancer used in food. The enhancement of palatability of food by salt could drive excessive salt intake in some individuals, which provides an important risk of disease. High levels of salt in the body can cause hypertension (high blood pressure). Hypertension aggravates the risk of heart attack, renal problems, stroke and increases water retention in the body. Low levels of salt also have their effects. For example, it can cause bradycardia (slow heart rate) and slow and irregular muscle contractions which have adverse effects on the proper functioning of heart and muscles.

1.2 Chemosensation

The sensory system of all organisms detects environmental signals and transmits such signals to generate appropriate behavioural responses. The senses of taste and smell detect chemical components (chemosensation) and the senses of vision, touch and hearing detect physical components of the environment. An organism can be attracted, repelled or be indifferent to a chemical stimulus. The ability to respond will enable it to identify a potential food source, danger or mating partner (*1*). In nature, an organism is always exposed to a mixture of different chemical signals. The senses of taste and smell are the main sensory systems that function in driving feeding and drinking behaviour but the texture and temperature of food and emotional states of an individual also affect feeding (*2*).

Some people suffer from complete or partial loss of taste. This can be caused by lesions in the taste buds or nerve connections from the taste buds to the brain, head injury, oral infections, radiotherapy injury in different parts of the mouth and poor hygiene. Other causes of taste defects are surgical manipulations of the tongue and the oral cavity, nutritional deficiency leading to taste aberration, endocrine disorders like diabetes mellitus (*3*) and genetic disorders like type I familiar dysautonomia (Riley-Day syndrome) that causes a defect in taste bud development (*4-6*). Aging is also an important cause of reduced taste. Aging results in changes in taste receptor

cell membrane compositions like ion channels and taste receptors (*7*). It also leads to a decrease in taste bud population and density as a result of a reduction in the number of new cells replacing dead taste receptor cells (*8*). Reduced taste in conjunction with reduced smell leads to loss of appetite which may lead to weight loss, malnutrition, impaired immunity and health deterioration (*9-11*). For example many elderly people generally require a twofold to threefold higher concentration of salt to taste it in tomato soup (*12*). Tendency towards elevated salt intake in the elderly can worsen their medical conditions. Extensive medical solutions to help affected patients are not yet in place. Therefore studies to unravel the mechanisms of taste perception and transduction will help in understanding the process of salt sensation and the players involved. This will be of great importance to develop better diagnoses and treatments to ameliorate the conditions in elderly people and to develop alternatives to NaCl or taste enhancers that allow reduction of NaCl intake.

1.3 Sense of taste

There are five basic taste modalities. They are sweet, sour, bitter, salty and umami (a generally pleasant taste elicited by certain L-amino acids and nucleotides) (*13*). Sweet, umami and the taste of low salt concentrations are commonly acceptable because they signify a potential source of nutrients and they are generally palatable. All organisms from lower invertebrates to mammals are attracted to low salt concentrations and crave for it after a period of salt deprivation (*14-15*). However they avoid higher salt concentrations. There seem to be distinct mechanisms dedicated for sensing both types of salt concentrations (*16-18*). Bitter and sour tastes are generally avoided. These responses help in quick decision making about the intake of potentially harmful foods because many spoilt food and poisonous substances are bitter or sour (*19*). The preference for a particular taste seems to be innate in many organisms. For instance, naïve mice will readily prefer a sweet solution over water when presented the choice and water over bitter, sour or high saline solutions (*2*). Taste can be influenced by the smell, look and texture of the substance and by emotion, state of health, hunger and satiety.

Receptors of taste in mammals and insects.

In mammals, taste is sensed by sensory organs called taste buds which are concentrated on top of the tongue. They are also located at the back, front and side of the tongue and in the throat. Each taste bud contains 50 -100 taste receptor cells (TRC) bundled together. Taste buds are enclosed in epithelial structures called the papillae. In adult mice, TRCs actively regenerate and have an average lifespan of two weeks (*20*). New TRCs replace the dead cells. Each TRC has taste receptors (TR)

located on its surface. Different TRs sense different taste modalities. Sweet tastes are sensed by heterodimeric combination of TIR2 and T1R3 or homodimers of T1R3. T1Rs are G-protein coupled receptors (GPCRs). T1Rs show a wide sequence divergence among different mammalian species (*21*). T1R1/3 senses umami tastes (*22-24*). There are multiple ligand binding sites on the T1R heterodimer complexes and these may explain the broad range of substances sensed by each receptor complex (*25-27*). Mice lacking T1R3 still show a persistent sensitivity to some sugars and a robust preference for umami tastants (*24, 28-29*). This suggests that there are other unknown receptor(s) for sweet and umami taste sensations.

The sensation of sodium salts by mice requires the epithelial sodium channel, ENaC. The ENaC channel can be blocked by amiloride (*16, 30*). ENaC is specific for sodium ions. Pharmacological and genetic evidence suggests that there is also an ENaC-independent salt sensing pathway. This pathway is amiloride-insensitive and also senses other non-sodium salts (*31-32*). A study has suggested that TRPV1 (transient receptor vanilloid) is involved in the amiloride-insensitive pathway but this is still debated in the field (*18*). Amiloride sensitivity does not play a prominent role in salt taste transduction in humans. The molecular mechanism of salt taste and the salt taste receptor in humans are not known.

Bitter substances are sensed by GPCRs, T2R receptors (*33*). T2Rs are low affinity receptors and it has been suggested that this may help to quickly detect any substance as potentially hazardous for consumption (*33*). T2Rs have large sequence diversity with few conserved regions (*34-35*). Each bitter sensing TRC expresses a wide variety of T2Rs which enables it to sense a number of bitter substances (*33*). There is no selectivity in the type of bitter substance that can be sensed by each bitter TRCs (*33*).

The expression of a type of transient receptor potential channel, PKD2L1 (polycystic kidney disease-like) in a subpopulation of taste cells marks the sour-sensing cells, but PKD2L1 itself is not required for sour sensation (*36-37*). The identity of the sour receptor is not yet known. PKD1L3, HNC1 (hyperpolarization-activated cyclic nucleotide-gated channels) and HNC4 have been proposed to be sour taste receptors. It is suggested that sour taste comes about by protons released from food that activate sour receptor channels, producing inward currents and action potentials (*38-39*).

Carbonation is a process whereby CO_2 dissolves in water. Chandrashekar *et al.,* (*40*)

recently showed that the glycosylphosphatidylinositol (GPI)-anchored carbonic anhydrase, Car4, senses carbonation. They showed through genetic ablation of specific populations of TRCs and gene silencing of the Car4 gene that Car4 senses CO_2 in sour-sensing TRCs.

The sensory organs of *Drosophila melanogaster* are called the gustatory receptor neurons, GRN (*41*). Taste receptors called gustatory receptors, Gr, located on the GRNs sense the different taste modalities. GRNs are found on the mouth part (labella), legs and the wings. This is because flies generally move all over their food source. The presence of Grs on different parts of their body readily signals the presence of food. Gr5a receptors sense trehalose and these are found in distinct GRN subpopulations and do not overlap with receptors for other taste modalities (*41*). Gr64f containing GRNs likely sense a wide variety of sugars (*42-44*). Flies do not have any response to umami-tasting substances (*2*). GRNs-expressing Gr5a respond to low concentrations of salt while Gr66a-expressing GRNs are responsive to aversive salt concentrations but the salt receptors are not known (*45-46*). However Gr66a-expressing GRNs are not sufficient for aversion of higher salt concentrations. DEG (degenerin)/ENaC genes, *pickpocket11* and *pickpocket19* contribute to the taste of low salt concentrations (*47*). They form a functional non-selective ENaC channel that is permeable to almost the same extent to Na^+ and K^+, which is in contrast to what occurs in mammals. The disruption of both *pickpocket11* and *pickpocket19* attenuated neuronal reponses to low NaCl concentrations but the response to high salt concentrations was only mildly affected. It was proposed that the response of *Drosophila* to high salt concentrations involves other pathways. Bitter tastants are sensed by the Gr66a receptor but Gr93a-expressing GRNs also sense caffeine (*35, 48*). Flies sense CO_2 with the non-Gr5a expressing GRNs present on the labial palps (*49*).

Signal transduction of taste

Downstream of GPCRs taste information is transduced by G-proteins. For example, bitter sensing TRCs that express T2R receptors activate α-gustducin and α-transducin (*50*). Taste receptors that express T1R3 activate Gα14 (*51*). Activation of the heterotrimeric G protein complex leads to dissociation of the Gα and G$\beta\gamma$ subunits (Figure 1). The G$\beta\gamma$ subunit can subsequently interact with phospholipase Cβ2, PLCβ2, which then produces inositol triphosphate, IP3, and diacylglycerol, DAG, from phosphatidylinositol 4,5-bisphosphate, PIP2. IP3 opens the IP3 receptor ion channel on the endoplasmic reticulum, ER, resulting in calcium release from the ER into the cytosol. Increased cytosolic calcium opens the taste selective cation channel, TRPM5 (*52*). An increase in the cytosolic cation concentration produces a

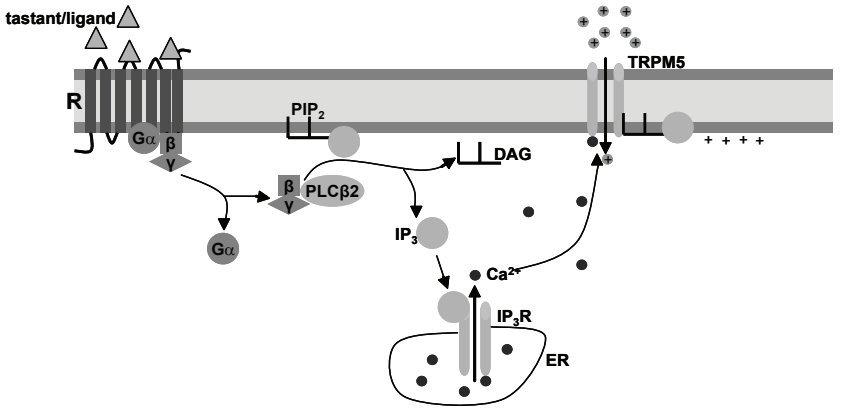

Figure 1. **Model of taste transduction**. Taste ligands bind taste receptor (R) which leads to the dissociation of the heterotrimeric G protein, Gα from Gβγ. Gβγ then activates PLCβ2 which in turn hydrolyzes PIP2 to DAG and IP3. IP3 subsequently activates the IP3R on the ER which releases Ca^{2+} from intracellular stores. Increased intracellular Ca^{2+} opens the non-selective monovalent cation channel TRPM5. Influx of monovalent cations into the cytosol then leads to membrane depolarization.

depolarising membrane potential. Increased cytosolic calcium concentration and a depolarised cytoplasmic membrane lead to the secretion of ATP, the taste bud neurotransmitter, through the ATP-permeable gap junctions hemichannel to the extracelluar space between TRCs (*53*). The hemichannel is made up of pannexin 1 or connexins (*53*). ATP then activates the gustatory afferent nerve fibre (*31*).

1.4 Innate behavioural responses

In mammals, the expression of different taste receptors in distinct TRC populations suggests that each TRC is tuned to a single, non-overlapping taste modality. When TRCs specific to a modality are absent, other taste modalities remain unaffected. The taste preference driven by each TRC is hard-wired to distinct behavioural responses of acceptance or rejection (*2*). These behavioural responses are innate. For example, when the receptor of a tasteless ligand was expressed in the sweet or bitter sensing TRCs, it resulted in the attraction to or avoidance of the normally tasteless ligand respectively (*23*) (*34*). Also when bitter receptors were expressed in sweet TRCs, mice showed very strong attraction to a normally aversive bitter ligand and vice versa. Therefore behavioural responses to tastants are mediated by the identity of the stimulated taste cells and not by the properties of the receptors or the tastants. The mechanism by which the genetic program in the TRCs is hardwired to the appropriate neural circuitry is not known.

The innate response of *Drosophila* to taste can be visualized by the extension and retraction of their proboscis. Exposure to an attractive tastant leads to the extension of the proboscis while the presence of a noxious tastant leads to proboscis retraction. Similar to what is found in the mammalian system, activation of specific gustatory receptor neurons for a taste modality elicits the appropriate innate behavioural response (*45, 54*). The importance of the hard wiring of the system is well illustrated by experiments where expression of a light-activated channel, channelrhodopsin 2, in Gr5a-expressing neurons and stimulation with blue light was sufficient to activate feeding behaviour even in the absence of a tastant (*55*).

Effect of learning and memory on innate behavioural responses

Although the innate responses to different taste modalities are hardwired, the acceptance or rejection of food also depends on previous experience with the food and a change in food preference may occur despite the hardwiring. For example, when coffee is first consumed it is generally not acceptable because of its bitter taste. However, after repeated consumptions, it becomes acceptable. Another example is that when people are presented with food or a novel food, they generally have some expectation about the taste of that food, based on previous exposure to a similar food (*56*). Conditioned taste learning has long been used to study learning and memory in many organisms. Furthermore, there is a phenomenon called conditioned taste aversion (CTA). This is a paradigm that involves the learning of a taste associated with an unpleasant situation (*57*). When sweet food is associated with a bad condition like nausea, a subsequent exposure to the food leads to strong avoidance of the food. However, when the sweet taste is familiar through previous exposure, combined with positive experiences, a subsequent one time experience of the sweet taste with nausea leads to a reduced aversion upon future presentation of the sweet taste. The reduced aversion to food after exposure to the aversive condition shows that the taste was learned and became familiar (*58*). Glutamate and acetylcholine neurotransmission have been shown to be involved in CTA. For example blocking of a glutamate receptor NMDA (*N*-methyl-D-aspartate) eliminated conditioned taste aversion in rats (*59-60*).

1.5 *C. elegans* as a model organism

Our knowledge about the molecular mechanisms of taste as well as the neuronal circuitry involved is very limited. This is mostly caused by the complexity of the nervous systems of mammals. Although *Drosophila* is quite small in size, its nervous system contains many neurons that makes it complex to decipher the complete neuronal circuitry involved in the sensation of taste. *C. elegans* is a good model

organism to study the mechanism and transduction of taste. The relatively low number of neurons in *C. elegans* makes it easier to delineate the neuronal circuit of any behaviour exhibited by the worm. *C. elegans* is a one millimetre, free living soil worm. It is a well studied organism that is widely used in the laboratory to investigate many biological questions because of the conservation of many pathways in evolution. It has other advantages that make it a good model organism. These include its small size, short generation time (3 days), ability to generate and identify mutants within a short time, many techniques to study different processes at the molecular and cellular levels and the low cost of its maintenance in the lab.

Behavioural studies have also benefitted from *C. elegans*. An important advantage of *C. elegans* for these studies is the homogeneity of its genetic background, unlike in mice and *Drosophila* (*61-62*), where genetic differences among strains produce varying behavioural outputs. This genetic heterogeneity often hampers the interpretation of behavioural studies. Also in mice and *Drosophila,* even though the regions of the brain controlling many behaviours are known, it is quite a daunting exercise to map the neuronal circuitries of these behaviours. The low number of neurons in *C. elegans* makes it easier to delineate the neuronal circuit of any behaviour exhibited by the worm.

There are both hermaphrodite and male *C. elegans*. The hermaphrodite has 959 somatic cells of which 302 are neurons and 56 glial and support cells. The male has 381 neurons, 92 glial and support cells and in total 1031 somatic cells (*63-64*). The connectivity of its nervous system with the chemical synapses and gap juctions has been well elucidated through electron microscopy serial sections (*65*). Many genes that regulate neuronal activity and sensation of the environment have been identified and this helps to decipher the signal transduction pathways and neuronal circuitry of a particular sensory behaviour. Most of these genes do not affect reproduction or viability of the worm under laboratory conditions (*66*). This makes the worm easily amenable to experimental manipulations to study detailed mechanisms of neuronal sensory perceptions and regulation of their behavioural outputs (*67*). Many genetic methods have been developed to generate and manipulate *C. elegans* mutants to study different biological processes, many of which are conserved in evolution. Imaging techniques are also well developed to study neuronal activities, circuitry and regulation of different processes in *C. elegans.*

1.6 Sensory perception in *C. elegans*

C. elegans senses a wide range of environmental stimuli. The neurons that sense

these stimuli have been identified through neuronal ablation (*68*). The motor output to a sensory stimulus, indicated by the direction of locomotion, has been mainly used to score sensory responses to tested stimuli (*66*). How the neuronal locomotory circuit functions has therefore been widely studied. *C. elegans* has 95 body wall muscle cells that receive excitatory synaptic input from cholinergic neurons at the neuromuscular junctions (NMJs) and inhibitory synaptic input from GABAergic (gamma aminobutyric acid) neurons at NMJs (*69*). The excitatory DA, DB and inhibitory DD motor neurons are found along the dorsal side of the worm and the excitatory VA, VB and inhibitory VD motor neurons are on the ventral side. They innervate the muscle cells. The neuronal connectivity works such that there are opposing inputs to the muscle cells at the ventral and dorsal sides at the same time. For example, when the ventral side contracts because of excitation by the VA or VB motor neurons, a corresponding inhibition by the DD motor neuron occurs at the dorsal side which then relaxes the muscle cells at the dorsal side. The opposing inputs are necessary for coordinating a smooth sinusoidal movement for *C. elegans* locomotion by preventing simultaneous contraction at the dorsal and ventral body muscles. This was nicely shown with the *unc-49* mutant that is defective in the postsynaptic inhibitory effect of GABA on body wall muscle cells. When *C. elegans* is tapped at the nose or the tail region of its body, it changes direction of movement to the opposite of where it was going before the tap. *unc-49* worms shrank instead of rapidly escaping when mechanically sensitized. The lack of the GABA inhibitory input interfered with the production of an opposing muscle activity necessary for propagating a smooth wave along the worm body length necessary for its movement (*70*).

The modulation of *C. elegans* locomotion direction that helps it to navigate along a stimulus gradient or to its desired location is also being intensely studied. When chemotaxing along an attractive chemical gradient, *C. elegans* senses a change in the attractant concentration along its path (*71*). This showed that it uses a short-term memory system to compare the attractant concentration at its current location to recently experienced concentrations. If the concentration at its current location is higher, it chemotaxes with an increased forward run movement and a low probability of random turn (*71*). If however the concentration at the current location is lower than the concentration at a recent location, it increases its frequency of turns away from the current location. Thus, navigation to its preferred location is modulated by the frequency of turns. This allows the worm to aggregate around the peak of an attractant and away from a repellent. This is similar to the "bias-random walk" strategy in bacteria along a chemical gradient (*72*) and has been termed "the

pirouette model" of chemotaxis in *C. elegans* (*71*) (Figure 2). Another locomotory strategy employed by *C. elegans* along a concentration gradient is called the "weathervane strategy". In this strategy, the worm can slightly bias its navigation to a preferred location by gradually steering its direction during long runs to better align itself with the direction of the gradient (73) (Figure 2). These parallel navigation strategies are effectively used by the worm to chemotax to or away from a gradient peak. The pirouette mechanism has been shown to also regulate navigation in olfactory (74) and thermal (75-76) gradients.

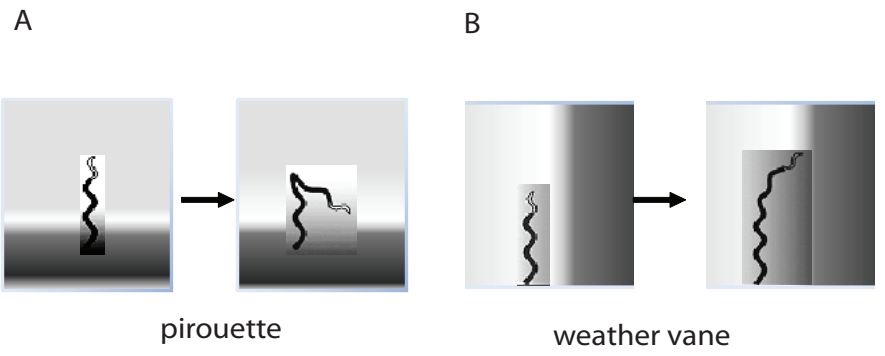

Figure 2. ***C. elegans* movement strategy along a chemical concentration gradient**. A. *C. elegans* can navigate to an attractant by a random bias movement along its path. It modulates its direction by random turns in the direction of the attractant. B. *C. elegans* can also navigate by steering, to better align itself to the direction of the attractant.

C. elegans has sensory mechanisms dedicated for detecting common stimuli in its environment which are also sensed by mammals and Drosophila. Some senses that have been characterized in *C. elegans* and are similar to senses of mammals and/or Drosophila will be discussed below.

1.6.1 Chemosensation

C. elegans senses a wide range of chemical cues through its chemosensory neurons in the head region, called the amphid neurons, and in the tail region, called the phasmid neurons. It has 302 neurons of which 32 are chemosensory neurons. The amphid and phasmid neurons have cilia at the ends of their dendritic processes. The cilia are exposed to the outside of the worm by a special opening in the cuticle formed by two glial cells called the socket and sheath cells (*77*). Accordingly, many mutants with defects in cilia formation are chemosensation deficient (*78-79*). The stimuli sensed by the amphid neurons have been extensively studied.

Chemoattraction to water-soluble compounds

C. elegans senses a wide range of water-soluble chemicals. These include some cations, anions and organic molecules (table 1). The ASE neurons are the main chemosensory neurons for water-soluble chemicals (Figure 3) (*68*). This was shown by laser ablation experiments: animals in which all amphid and phasmid neurons except the ASE neurons were ablated still showed a strong chemotaxis response, and animals in which only the ASE neurons were ablated lost most of their response to water soluble chemicals. However, when only the ASE neurons were ablated, a residual chemosensory response remained. This was contributed by the ADF, ASG, ASI, and ASK neurons (*68, 80*). The ASE neurons are a symmetric pair of neurons with opposing sensitivity to stimuli. The ASE left (ASEL) neuron senses Na^+ while the ASE right (ASER) neuron senses Cl^- and K^+. ASEL is activated by an increase in salt concentration but the ASER neuron is activated by a decrease in salt concentration (*81*). Mutations in two genes that regulate ASE neuron formation, *che-1* and *ceh-36*, greatly reduce chemotaxis to NaCl (*78, 82-83*). Genetic and candidate gene screens have identified some genes necessary for NaCl chemoattraction. These include cyclic nucleotide gated channel subunits *tax-2* and *tax-4 (84-85)*, G protein coupled receptor kinase *grk-2 (15)*, calcineurin (calcium activated phosphatase) subunits A (*tax-6*) (*86*) and B (*cnb-1*) *(15)*, neuronal calcium sensor *ncs-1 (15)*, guanylate cyclases g*cy-14* and *gcy-22 (87)* and *ttx-4* (protein kinase C) (*88*). This shows that cGMP and calcium signal transduction pathways are important in NaCl sensation.

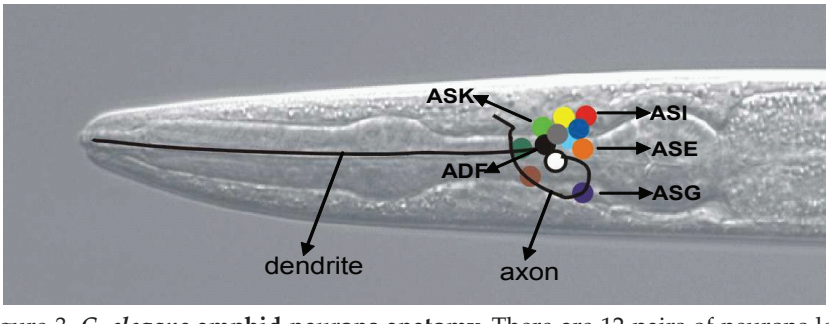

Figure 3. ***C. elegans* amphid neurons anatomy.** There are 12 pairs of neurons located in the amphid region of *C. elegans'* body. The five known salt sensing neurons are highlighted.

In addition, neuronal contributions (other than that of ASE) to NaCl sensation have been highlighted by neuronal calcium imaging. The ADF left and right neurons are activated by an increase in NaCl and the ASH neurons are activated by a decrease in NaCl concentrations (*89*). Two other minor NaCl sensing neurons, ASG an ASI, did not show any neuronal response to either an increase or a decrease in NaCl

concentration.

Chemoattraction to volatile compounds

C. elegans also senses a wide range of volatile compounds using the AWA and AWC neurons (table 1) (*90*). Unlike the water-soluble chemicals, which are sensed at the micro- and millimolar ranges, many of these volatile compounds are sensed at the nanomolar range. AWA senses diacetyl, pyrazine, and 2,4,5-trimethylthiazole and the AWC senses benzaldehyde, butanone, isoamyl alcohol, 2,3-pentanedione, and 2,4,5-trimethylthiazole (*90*). In addition, the AWC neurons are functionally asymmetric.

Neuron	Stimuli and Response		Reference
	Attraction	Avoidance	
ASE	Na^+, K^+, Cl^-, cAMP, cGMP, serotonin, biotin	Cu^{2+}, Cd^+	(*68*)
ADF	Na^+, Cl^-, Biotin, cAMP		(*68*)
ASG	Na^+, Cl^-, Lysine, Biotin, cAMP		(*68*)
ASI	Na^+, Cl^-, Lysine, Biotin, cAMP		(*68*)
ASK	Lysine	SDS, quinine	*(68)*
AWA	Diacetyl, 2,3-pentanedione, pyrazine, thiazole		(*90*) (*97*)
AWB		2-nonanone, 1-octanol (off food)	(*98*) (*99*)
AWC	benzaldehyde, butanone, thiazole, isoamyl alcohol diacetyl, 2,3-pentanedione		(*90*) (*97*)
ASH		High osmolarity, SDS, 1-octanol, Quinine, Cd^{2+}, Cu^{2+}	(*100-103*)
ADL		Cd^{2+}, Cu^{2+} , 1-octanol	(*99-100, 103*)
AQR, PQR and URX	oxygen		(*1, 104-106*)
PHA, PHB		SDS (antagonistic to ASH response)	(*101*)

Table 1. **Chemosensory neurons in *C. elegans*.** The known stimuli sensed by the different chemosensory neurons are shown. They are either attractants or repellents.

Asymmetry can be visualized by looking at the expression of a GPCR, *str-2*, which is randomly expressed in one of the 2 AWC cells (*91*). The neuron expressing the *str-2* gene is termed the AWC^{on} cell and the neuron not expressing it is termed the AWC^{off} cell (*91-92*). The AWC^{on} cell senses butanone and AWC^{off} senses 2,3-pentanedione. The two neurons are both required for the sensation of benzaldehyde. The cilia of the AWA and AWC neurons are buried in the sheath cell (*93*) and odorants may reach their cilia by diffusion or transport across the sheath cell (*1*). The Gα protein

odr-3 among others, is important in mediating olfactory responses in the AWA and AWC neurons (*94*). cGMP signaling is essential for AWC olfactory response. The genes involved in this pathway include the cyclic nucleotide gated channel subunits *tax-2* and *tax-4 (84-85)* and transmembrane guanylyl cyclases *odr-1 and daf-11 (99-100).* Channels formed by the TRPV channel subunits OSM-9, OCR-1 and OCR-2 play important role in AWA olfactory sensation (*95*). TRPV channels are regulated by polyunsaturated fatty acids (PUFA) in AWA (*96*). Thus, the two main olfactory neurons in *C. elegans* use different sensory transduction mechanisms in odor sensation.

1.6.2 Oxygen and carbondioxide sensation

C. elegans can sense different levels of oxygen and carbondioxide in its environment. It prefers an intermediate level of oxygen (8%) and avoids both low (<4%) and high (>12%) levels in a linear oxygen gradient (*106-107*). The AQR, PQR and URX neurons sense oxygen levels in the worm (table 1) (*104-106*). The AQR and PQR neurons have the cilia at the tip of their dendrites exposed in the worm coelomic body fluid, while the URX neuron has its dendrite extended to the tip of the nose (*108*). Oxygen level regulates aggregation behaviour of the worm (*106*). Worms that aggregate do so at 21% oxygen. Aggregated worms disperse when the oxygen level is reduced to the preferred 8% oxygen concentration. Aggregation is also regulated by the ASH and ADL (stimulatory input) and the ASI (inhibitory input) neurons (*108*). Oxygen concentration upshift is sensed by soluble guanylyl cyclases (sGCs), GCY-35 and GCY-36 in URX and downshift by GCY-31 and GCY-33 in the BAG neurons (*109-110*). These sGCYs have a haem-binding domain which preferentially binds oxygen, unlike the haem domains of other soluble guanylyl cyclases in some organisms that bind nitric oxide (*106*). A neuropeptide receptor, NPR-1, together with a globin domain containing protein, GLB-5, regulate the behavioural response of *C. elegans* to changes in oxygen concentration (*109-110*).

C. elegans senses carbondioxide (CO_2) and generally avoids it at concentrations above 5%. It requires the ciliated amphid BAG neurons for CO_2 sensation and avoidance. CO_2 avoidance is mediated by multiple signaling pathways which include the cGMP-gated channels subunits TAX-2 and TAX-4 and a receptor guanylyl cyclase GCY-9. Many signaling molecules and pathways including the neuropeptide Y receptor NPR-1, calcineurin subunits TAX-6 and CNB-1, insulin and transforming growth factor β (TGFb) pathways modulate the CO_2 avoidance response (*111-113*).

1.6.3 Pheromones

The *C. elegans* pheromone, daumone, signals population density and regulates dauer formation. When L2 worms sense overpopulation by the presence of a high amount of the pheromone or if the temperature is high and food supply low, they enter an alternative larval stage called the dauer larva (figure 4) (*114-116*). The dauer is a long lived and developmentally arrested larval stage. When environmental conditions become favourable, the dauer larva re-enters a reproductive cycle. Ablation of the ASI, ASG and the ADF neurons together make the worms enter a constitutive dauer stage (*117*). ASI and ADF have a redundant role in dauer formation with ASG playing a minor role (*117*). ASI expresses a TGFb-like gene, *daf-7*, which signals to prevent the worm from entering the dauer stage (*118-119*). The ASG and ADF neurons express insulin-like ligands for the insulin receptor DAF-2 (*120-121*). Both the insulin and TGFb signaling pathways signal to prevent entry into the dauer stage. *daf-7* expression is transcriptionally regulated by the dauer pheromone in the ASI neurons (*119*). ASJ and ASK have antagonistic functions to the ASI, ASG and ADF neurons in that they promote dauer formation (*119*). Ablation of the ASJ neurons leads to a dauer-defective phenotype (*117*) which indicates that the ASJ neurons are necessary for dauer formation.

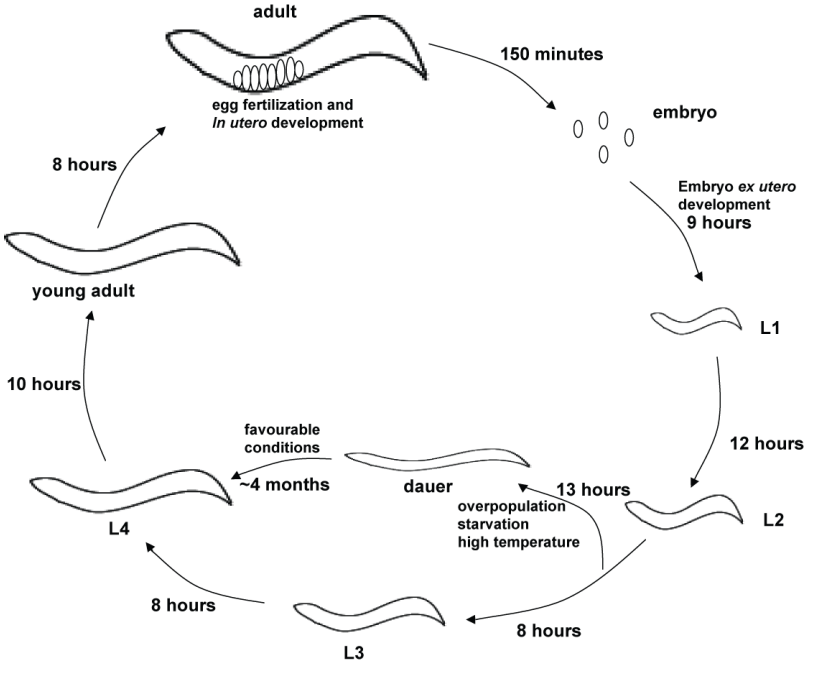

Figure 4. **Life cycle of *C. elegans*.**

Some of the neuroendocrine systems that regulate dauer formation also function in adult *C. elegans* where they regulate aging. However, dauer pheromone does not extend longevity of adult worms (*122*). *daf-2* mutation prolongs lifespan in close association with *daf-16* (FOXO transcription factor) and *age-1* (phosphatidyl inositol 3 kinase) in adult worms (*123-126*). They all act in the same pathway in the regulation of aging. Some chemosensory neurons that influence dauer formation are also involved in the regulation of lifespan extension. This was first speculated when it was observed that some sensory mutants showed extended adult lifespan (*127*). The neurons involved include the ASI, ASG, ASK and ASJ neurons. Ablation of the ASI and/or ASG neurons caused a small but significant increase in lifespan but this was suppressed by the ablation of the ASJ and/or ASK neurons (*122*). This showed that some neurons promote and others antagonize longevity. Two olfactory neurons also affected longevity in *C. elegans.* The absence of AWA extended lifespan while the absence of AWC did not (*122*). However, ablation of both AWA and AWC further increased lifespan. This suggests that there is some kind of redundancy in the antagonistic function of AWA and AWC in longevity. The effect of ASI on lifespan is mediated through the *daf-2* pathway (*122*).

Another neuroendocrine system reacts to the sex hormone. Adult hermaphrodites secrete a sex-specific pheromone to attract males but not other hermaphrodites (*128*). The sex pheromone in *C. elegans* thus serves a mating function. The sex pheromone is a synergistic mixture of different ascarocides comprising ascr#2, ascr#3, ascr#4 and ascr#8 (*129-130*). These ascarocides also make up the dauer pheromone but with different compositions of ascarocides. *C. elegans* pheromone serves as a sex pheromone at lower concentrations, usually at the picomolar range but at higher concentrations, they serve as a dauer pheromone (*130*). Ascarocides expression is influenced developmentally and by environmental conditions (*130*). The male specific neurons CEM are necessary for male attraction to sex pheromone. The olfactory neurons AWA and AWC and ASK neurons are also important for male attraction to sex pheromone (*130-131*).

1.5.4 Nociceptive sensation

C. elegans avoids a large range of chemical and mechanical stimuli. It is repelled by high osmolarity, some organic odors like octanol, bitter alkaloids like quinine, heavy metals like Cu^{2+} and Cd^{2+}, detergents like sodium dodecyl sulphate, SDS, and touch to the tip of the nose (*79, 95, 101-102, 132*). Upon contact with a chemical repellent, it rapidly turns and reverses it direction of locomotion (*79*). It also avoids gentle and hard touches to its body. The ASH neurons are polymodal sensory

neurons that sense both chemical repellents and mechanical stimulations (*101-103, 133-134*). Two Gα proteins, ODR-3 and GPA-3, have distinct functions in the ASH neurons in directing nociception to different repellents. *odr-3* regulates osmotic and mechanical avoidance while *gpa-3* regulates avoidance of copper and quinine (*94, 102, 135*). Osmotic sensation also requires the cytoplasmic protein *osm-10* (*136*). Transient receptor potential (TRP) channels, OSM-9, OCR-1 and OCR-2 mediate most of the ASH responses (*95, 137*). Mutations in *fat-3* (δ6 fatty acid desaturase) that catalyzes the rate-limiting step in the conversion of two essential fatty acids into C20 polyunsaturated fatty acids (PUFAs), also cause strong defects in all ASH-mediated behaviours, probably because PUFAs generated by *fat-3* are essential as modulators of TRPV channels in ASH (*96*).

The ADL neurons contribute to the avoidance of some chemical cues like Cu^{2+} and Cd^{2+} and 1-octanol in the absence of food (*101*). *osm-9* and *ocr-2* are also co-expressed in the ADL neurons (*137*). The AWB neurons are responsible for avoiding some noxious odors like 2-nonanol and, in the absence of food, the avoidance of 1-octanol (*98-99*). Two phasmid (tail) neurons, PHA and PHB, mediate SDS avoidance but their response is antagonistic to ASH (*101*). Starvation alters the chemoavoidance of some organic odors. It downregulates chemosensory input from ASH while simultaneously recruiting ADL and AWB to nociception (*98*). For example, well-fed worms avoid 1-octanol exclusively via ASH, but upon starvation, ASH, ADL and AWB are necessary for the avoidance of 1-octanol (*98*).

1.7 Other sensations in *C. elegans*

1.7.1 Thermosensation

C. elegans can grow and reproduce between 12-27°C. Temperatures above 33°C increase the turn/reversal rate and thus elicit a robust escape from the increased temperature (*138*). When placed on a temperature gradient, *C. elegans* seeks the temperature at which it was most recently cultivated in the presence of food (*139-140*). Upon transfer to a new temperature in the presence of food, it resets its preference within 2-4 hours to the new temperature. A starved worm however, avoids the most recent temperature associated with the absence of food. The AFD neurons are the main temperature sensing neurons of *C. elegans* (*140*). Absence of the AFD neurons makes the worms become cryophilic on a temperature gradient irrespective of their cultivation temperature (*140-142*). The AFD neurons have extensive ciliated endings at the tip of their dendrites that look like a microvilli structure (*143*). Mutations that disrupt this ciliated structure also affect thermosensation (*142, 144*).

The AWC neurons, which have long been known as olfactory neurons, were recently shown to also function as temperature sensing neurons (*145-146*). Calcium imaging experiments showed that the AWC neurons respond to an increase and a decrease in temperature (*145-146*).

1.7.2 Mechanosensation

C. elegans responds to light touch at both the anterior and posterior parts of its body. The AVM and the ALM neurons sense anterior body touch, which elicits backward movement, and the PVM and PLM neurons sense posterior body touch, which elicits forward movement (*69*) (Figure 5). These neurons make chemical and electrical synapses to command interneurons which synapse to motor neurons that coordinate forward or backward movement. The nature of the chemical synapses is not known (*66*). The connections of the sensory neurons, interneurons and motor neurons are such to facilitate a withdrawal response to body touch and to determine the choice between forward or backward movement.

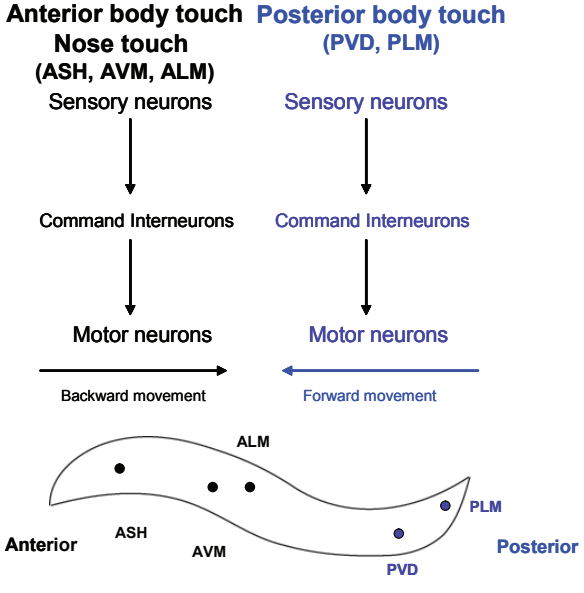

Figure 5. **Neurons mediating *C. elegans* response to body touch.** Coordination of signals from command interneurons to different motor neurons determines the direction of movement of *C. elegans* to touch to the anterior or posterior parts of its body.

The PVD neurons mediate sensation of harsh touch to the midbody (*147-148*) (Figure 5). Animals in which the PVD neurons were ablated did not respond to harsh body touch to the central part of the body but responded well to harsh touch to the head and the tail regions of the body of the worm (*147*). Noxious signals sensed by PVD seem to promote an escape response shown by increased speed and reduced pauses and reversals (*149*).

1.8 Signal transduction molecules in chemosensation

The known molecules involved in *C. elegans* chemosensation signal transduction have mostly been identified by isolating mutants defective in the behavioural response to chemical cues. Reverse genetics has also been used to identify such genes (*150-151*). G-protein coupled receptors (GPCRs) play a major role in chemosensation in *C. elegans.* There are over 1000 predicted GPCR genes encoded in *C. elegans* genome which make up about 7% of *C. elegans* genes (*100*). This is a huge number when compared to the number of GPCRs encoded by the genomes of mammals (~2%) and *Drosophila* (~1%) (*152*). Cyclic nucleotide-gated channels, Ca^{2+} signaling, heterotrimeric G-proteins and TRP channels also play crucial roles in chemosensation in *C. elegans.* Each chemosensory neuron expresses many chemosensory receptors (CRs). This makes the worm chemosensory system similar to the bitter sensing cells in mammals which express many bitter sensing receptors per taste cell (*153*). It has been challenging to identify the specific ligand(s) that binds a receptor. This could be caused by redundancy among the various receptors. Downstream of the GPCRs, G-proteins activate guanylate cyclases which produce cGMP. cGMP then activates cGMP-gated channel subunits, TAX-2 and TAX-4, which are used by many neurons for cation influx into the cells (Figure 6) (*153*). The absence of either TAX-2 or TAX-4 diminishes or abolishes the neuronal response to a stimulus (*84, 154*). Other neurons use the OSM-9 and OCR-2 transient receptor potential channels for signal transduction (*95, 137*) (Figure 6). These may be gated by polyunsaturated fatty acids (PUFAs) (*96*).

The convergence of different signaling pathways to common downstream signaling molecules raises the challenging question: how can the worm discriminate among stimuli sensed by the same neuron? One of the ways the worm achieves this is by segregating responses to different stimuli to the left and right sisters of a neuron pair. For example, the ASEL senses an upstep (increase) in NaCl concentration while the downstep (decrease) in salt concentration is sensed by the ASER (*81*). Another mechanism by which neurons can discriminate different stimuli is by using additional signaling molecules in a stimulus-specific manner, although the core

signal transduction pathway(s) is shared (*153*).

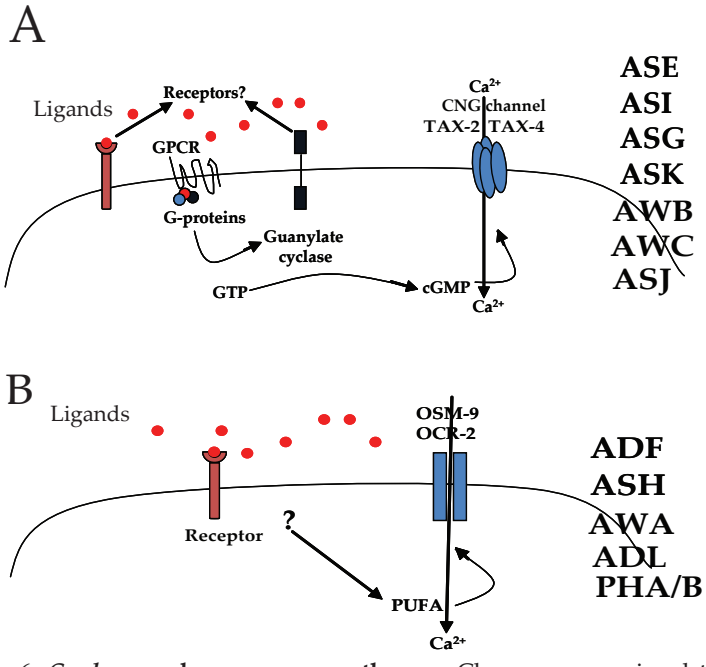

Figure 6. ***C. elegans* chemosensory pathways.** Chemosensory signal transduction usually involves A. cGMP and B. TRPV in *C. elegans.* The chemosensory neurons in which these pathways are believed to occur are listed on the right of the pictures.

1.9 Behavioural plasticity

The behavioural response to a stimulus may vary depending on the context in which the stimulus is presented and also on previous experience with the stimulus, the organism's age and its stage of development. This behavioural change is generally referred to as behavioural plasticity. Behavioural plasticity can involve learning and memory (*155*). Changes in behaviour are elicited by neuronal plasticity. Neuronal plasticity can occur for example at the synapses by changes in the synaptic connections between neurons (*156-157*). However, neuronal plasticity can also occur at the level of intracellular signaling, e.g. by changes in receptor concentration or (in)activation of signaling molecules. *C. elegans* shows different kinds of behavioural plasticity similar to what is observed in higher organisms. These include adaptation to the stimulus after repeated stimulus presentation, learning and memory.

Animals display different kinds of behavioural plasticity. These include adaptation,

habituation, sensitization, associative learning etc. Adaptation is a process that involves the inability of an organism to respond efficiently to a repeated stimulus due to neuronal fatigue. The animal can still sense the stimulus but not as efficiently as the first time the animal was exposed to the stimulus. Habituation involves a decrease in a behavioural response after repetition of a stimulus. It is different from adaptation because it does not involve neuronal fatigue. Sensitization is a type of non-associative learning that involves an increase in a behavioural response of an animal as a result of stimulus repetition. Associative learning involves a change in behaviour after association between two stimuli.

As indicated in the preceding paragraph, distinctions have been made among various behavioural changes in mammals and some other organisms, but these distinctions are not really clear cut in *C. elegans*. I will discuss below some examples of behavioural plasticity seen in *C. elegans.*

1.9.1 Desensitization/sensory adaptation

When *C. elegans* is exposed to a stimulus for a prolonged time, the response of the sensory neuron decreases over time. The worm generally recovers from adaptation after removal of the stimulus (*139, 158-159*). For example, prolonged exposure to an odorant decreases the sensitivity of the worm to that odorant (*159*). This generally leads to adaptation to that odorant but sensitivity to other odorants sensed by the same neuron is usually spared (*159*).

1.9.2 Learning and Memory

Learning is the ability to change a behavioural response to a stimulus as a result of experience and memory is the ability to store and recall the experience-dependent behavioural changes to the stimulus (*157*). There are two main types of learning - associative and non-associative learning. Non-associative learning involves a behavioural change in an organism when exposed to a single stimulus. Associative learning is a type of learning that integrates the presence of a neutral (conditioned) stimulus, with a more significant (unconditioned) stimulus. The animal generally has an innate (reflex) response to the unconditioned stimulus but may not necessarily show any obvious behavioural response to the conditioned stimulus. Upon pairing of the two stimuli, the animal learns to associate the conditioned stimulus with the unconditioned stimulus at its next encounter and shows an appropriate behavioural response based on its past experience. The classic example of this is Pavlov's dog and food (salivation) experiment. A dog normally salivates at the sight of food. Ian Pavlov noticed that the dogs salivated when food was not present. He found out

later that every time the dogs were fed, the person that fed them wore a lab coat and the dogs associated the presence of the lab coat with feeding time. He went further to study the cause of reflex responses. One of the experiments he did was ringing of a bell when food was brought to the dogs. The dogs learnt that the ringing of bell related to their feeding. They started salivating at the mere sound of a bell. This showed that environmental signals that normally have no relation to a reflex action could trigger a reflex response through experience.

1.9.2.1 Non-associative learning in *C. elegans*.

Habituation

Habituation is a decrement in the response of a worm to a stimulus after repeated exposure to the stimulus. An example is the tap withdrawal response in the worm. When the side of a Petri dish containing a worm is tapped, the worm immediately backs off. After repeated tapping, the distance and frequency of the backward movement of the worm diminishes (*160*). Habituation differs from sensory adaptation in that there is a slower habituation response to a stimulus if the stimulus is very intense or if there is a longer interval between stimuli exposures. However sensory adaptation occurs at the same frequency with different stimulus intensities. Another difference is that although the two responses diminish over time after stimulus removal, the rate of recovery from habituation depends on the interstimulus intervals during the repeated exposures. An interstimulus interval is the time between two given stimuli. Shorter interstimulus intervals results in faster habituation as well as quicker recovery from habituation (*161*). Finally, habituation is rapidly reversible when the habituated worm is exposed to a new or a noxious stimulus (*157, 162*). For example, centrifugation of worms rapidly dishabituates the worm that has habituated to diacetyl (*163*). Sensory adaptation however persists to a stimulus in the presence of a new stimulus. The worm only recovers from adaptation after the stimulus is removed and sufficient time elapses before reexposure to the stimulus.

Sensitization

Sensitization is the enhancement of a response to a stimulus by raising the arousal level of the animal to the stimulus (*164*). For example, if *C. elegans* is given a single tap to the side of the plate followed by a rapid succession of taps, then a single tap again, its response to the second single tap is higher than to the first single tap (*160*).

1.9.2.2 Associative learning in *C. elegans*.

Olfactory plasticity

Worms can modulate their response to an odorant based on previous experience. For example, if worms are exposed simultaneously to attractive and aversive volatile compounds, they learn to avoid the attractive compound upon subsequent presentation of the attractive compound alone. This form of olfactory plasticity has been demonstrated by exposing animals to diacetyl in combination with acetic acid, acetic acid being a noxious substance to worms. The worms learned to avoid diacetyl after pre-exposure in the presence of acetic acid (*165*). Moreover, the attraction of the worm to some volatile compounds can be enhanced after preexposure to the compounds in the presence of food. *C. elegans* thus learns to associate the presence of the compound with food (*166-167*).

Likewise, worms display a remarkable sense of food discrimination. They learn to avoid pathogenic bacteria and prefer non-pathogenic bacteria after preexposure to different bacterial strains (*168*). This learning can be achieved in about 4 hours. When presented with a choice of pathogenic or non pathogenic bacteria on either side of a plate, they aggregate eventually at the non-pathogenic bacteria side of the plate. Serotonin is required for both olfactory and food discrimination learning (*168*). Besides pathogenicity, worms can also learn a preference for different food choices based on the quality of the bacteria. Worms prefer small and non-sticky bacteria (high quality food) over sticky and difficult-to-eat bacteria (low quality food) when presented the choice after pre-exposure to either one of the bacteria (*169*).

Gustatory plasticity

C. elegans shows a remarkable ability to learn and remember different environmental cues associated with food and aversive stimuli. It can learn to associate the presence of NaCl with the presence or absence of food (*15, 170-171*). In general, *C. elegans* is attracted to NaCl up to 200 mM and it avoids higher concentrations. This response is mediated by the ASE neurons, the main salt sensing neurons, with minor contributions from the ASI, ADF, ASG and ASK neurons. The ASH neurons are the main salt avoidance neurons. When an attractive NaCl concentration is paired with lack of food, subsequent re-exposure to NaCl leads to avoidance of NaCl. If *C. elegans* is pre-exposed to NaCl in the presence of bacteria (food), it remains attracted to NaCl upon re-exposure. This learning behaviour, called gustatory plasticity, occurs in as little as 10 minutes exposure to NaCl without food. A model has been proposed in which prolonged exposure to NaCl leads to the desensitization of the

ASE neurons (the main NaCl sensing neuron), thereby decreasing attraction to NaCl. In addition, the ASE neurons send a signal that sensitizes other sensory neurons, including the ASH neurons, allowing them to detect low NaCl concentrations thus making the worm to avoid normally attractive NaCl concentration (*15*). Hukema *et al* also showed that learned avoidance of NaCl can also occur if pre-exposure to NaCl occurs in the presence of a repellent (*15*). Therefore worms learn to associate an unconditioned cue (NaCl) with a repellent during pre-exposure and subsequently avoid NaCl upon re-exposure.

Several genes involved in gustatory plasticity have been identified including the Gα proteins, ODR-3 (functions in the ADF neurons) and GPA-1, the Gγ protein, GPC-1 (functions in the ASI and ASH neurons) and the TRPV channel proteins, OSM-9, OCR-1 and OCR-2 (*15, 170*). The precise roles of these proteins in the gustatory plasticity signal transduction pathway is not known. However, it has been suggested that they could be transducing the avoidance signal after pre-exposure to NaCl or they may be transducing the sensitizing signal from the ASE neurons (*15*). Serotonergic, dopaminergic and glutamatergic signaling are also involved in gustatory plasticity (*172*).

Prolonged starvation results in stronger avoidance. We call this starvation enhanced gustatory plasticity, but this seems very similar to another salt avoidance learning paradigm, called salt chemotaxis learning (*173*). In salt chemotaxis learning, the worms are incubated on a NaCl-containing plate without food for one to four hours. The worms are then tested for attraction to NaCl. Tomioka *et al* showed that worms strongly avoid NaCl afterwards and that the insulin pathway is involved in this behaviour (*173*). They found that the ASER neuron is necessary for salt chemotaxis learning. However calcium imaging of the ASER neuron tested after pre-exposure to NaCl in the absence of food for 20 minutes, showed very similar responses in insulin mutants and wild type animals. This suggests that possibly additional neuron(s) are involved in starvation enhanced gustatory plasticity.

Aerotaxis plasticity

Worms prefer an oxygen concentration of 5-12% under standard laboratory conditions. However, after pre-exposure for 4-6 hours to 1% oxygen in the presence of food, they preferentially migrate to 0-7% oxygen in a gradient of 0-21% (*104*). The worms learn to associate 1% oxygen with the presence of food because worms pre-exposed to 1% oxygen in the absence of food did not show a preference for 0-7%.

The soluble guanylyl cyclases GCY-32 and GCY-34 are required for this oxygen-food learning. These genes are expressed in the three oxygen sensing neurons of *C. elegans*, AQR, PQR and URX (*104, 106, 174*).

1.10 Neurotransmitters

Changes in animal behaviours require communication among different neurons in a circuitry. This communication is largely mediated by neurotransmitters. (*157*). They can act humorally as found in systemic neurohormones or act on a specific cell in a circuit via synaptic connections. There are seven known neurotransmitters in *C. elegans* as well as neuropeptides (*175*). They are acetylcholine, γ-amino butyric acid (GABA), serotonin, dopamine, tyramine, octopamine, glutamate and different types of neuropeptides. I discuss below biogenic amines, glutamate and neuropeptides, which are involved in gustatory plasticity.

1.10.1 Aminergic neurotransmitters

Biogenic amines are biologically active organic poly-ionic amines derived from aromatic or cationic amino acids (*176*). They have diverse physiological roles in mammals ranging from regulation of homeostasis to control of cognition. They can be made endogenously or obtained exogenously, mostly from food. There are four known biogenic amines in *C. elegans* which are dopamine, serotonin, tyramine and octopamine (*177-180*). They modulate behaviour of worms in response to changes in environmental cues (*181*). They act on neurons and muscles to affect locomotion, egg laying, learning and pharyngeal pumping.

Dopamine

Dopamine is produced in eight neurons in the hermaphrodite and in six additional neurons in the male (*177*). Ablation of these neurons causes defects in the ability of the worm to respond to changes in its environment, egg-laying, defecation and basal motor activity (*64, 177, 182-189*). Dopamine is made from tyrosine (Figure 7). CAT-2, tyrosine hydroxylase, converts tyrosine to 1-dihydroxyphenyl-l-alaninelevadopa (1-DOPA) (*183*). 1-DOPA is then converted by BAS-1 (aromatic amino acid decarboxylase) to dopamine (*185*) (Figure 4). CAT-2 requires a cofactor BH_4 (tetrahydrobiopterin) for its activity, whose synthesis depends on the GTP cyclohydrolase enzyme, CAT-4 (*190-191*). Cytosolic dopamine is packaged into synaptic vesicles by CAT-1 (vesicular monoamine transporter) (*187*) where it is stored until it is secreted upon neuronal depolarization. Synaptic dopamine is taken up by DAT-1 (dopamine transporter) (*192*). Dopamine reuptake primarily terminates dopamine signaling. Four dopamine receptors have been identified in *C. elegans*. As

in mammals two types of receptors can be discriminated: D1- (increases cAMP level after stimulation by dopamine) and D2-like (decreases cAMP level after stimulation by dopamine) receptors (*193-194*). DOP-1 and DOP-4 are D1-like receptors and DOP-2 and 3 are D-2 like receptors (*178, 195-197*).

Dopamine modulates the locomotory behaviour of *C. elegans* in response to changes in the environment. For example, in well fed wild type worms, dopamine signaling makes the worms slow down their locomotion rate when they encounter food. This phenomenon is termed "the basal slowing response". This mechanism is thought to increase the dwelling time of the worm on food, thus increasing its chance of survival by preventing starvation. However, *cat-2* (tyrosine hydroxylase) mutant animals do not exhibit the basal slowing response (*189*). In addition, *dop-3* animals do not slow down locomotion upon contact with food but this is rescued by a mutation in another dopamine receptor, *dop-1*. This shows that both DOP-3 and DOP-1 antagonize each other to control the rate of locomotion in response to changes in environment (*181*).

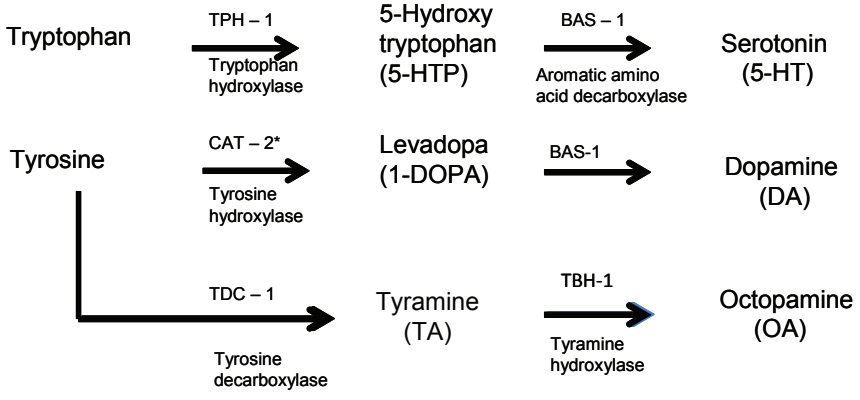

Figure 7: ***C. elegans* monoamine biosynthetic pathways**. * TPH-1, CAT-2 and TBH-1 require BH4 (tetrahydrobiopterin) as a cofactor.

Dopamine is also involved in learning in *C. elegans*. For example, *C. elegans* habituates to tapping. However, *cat-2* and *dop-1* animals habituate faster than the wild type. Exposure of *cat-2* animals to exogenous dopamine restores the habituation response to the wild type level (*178*). This shows that dopamine regulates how fast an animal can habituate.

Serotonin

Serotonin is synthesized in eight neurons of *C. elegans* including the NSM, HSN, ADF and RIH neurons (*179, 185, 198-199*). Serotonin is made from tryptophan in two

steps (*200*) (Figure 4): tryptophan is converted by tryptophan hydroxylase (TPH-1) to 5-hydroxyltryptophan (5-HTP). 5-HTP is converted by BAS-1 (same enzyme involved in dopamine biosynthesis) to serotonin (5-HT) (Figure 7) (*201*). Serotonin is transported into and stored in vesicles by the vesicular monoamine transporter, CAT-1 (*187*). It is released from the presynaptic nerve terminal into the synapse by exocytosis. Serotonin can thereafter bind its cognate receptors on the postsynaptic membrane. Serotonin signaling is primarily terminated by its reuptake into cells by the serotonin reuptake transporter (MOD-5) (*202*). There are four known serotonin receptors. SER-1, SER-4 and SER-7 are metabotropic G-protein coupled receptors and MOD-1 is a serotonin-gated chloride channel (*203-206*). Gαo and *dgk-1* (diacylglycerol kinase) mediate serotonin signaling downstream of the receptors (*207-209*).

Serotonin signals the presence of food in *C. elegans* (*175, 209-210*). Serotonin mediates an "enhanced slowing response" in starved worms when they encounter food (*189*). This response is different from the basal slowing response of well-fed animals. The enhanced slowing response is a stop in movement by starved worms while the basal slowing response is only reduced movement upon encounter with food. Serotonin therefore signals to prevent starved animals from leaving a food source. *bas-1* and *cat-4* mutant animals have a defect in the enhanced slowing response (*189*). MOD-1 is required for the enhanced slowing response (*189, 205*). The cells involved in this response are not known. SER-1, SER-4 and SER-7 are expressed in the pharyngeal muscles and neurons and they may play a role in the regulation of pharyngeal activities (*211-214*).

Serotonin signaling underlies many food related behavioural changes in *C. elegans (215)*. For example serotonin is involved in gustatory plasticity (*172, 216*), in increased attraction to benzaldehyde after worms are pre-exposed to it in the presence of food (*167*) and learned food (bacteria) preference (*168*). Serotonin also stimulates the aversive response of ASH neurons to octanol *(217).*

Tyramine

Tyramine is synthesized from tyrosine by TDC-1 (tyrosine decarboxylase enzyme) (*180*) (Figure 7). TDC-1 is expressed in the RIC, RIM, UV1 neurons and in the gonadal sheath cells (*180*). It is converted to octopamine by TBH-1 (tyramine β-hydroxylase) (*180*). TBH-1 is expressed in the RIC neuron and the gonadal sheath cells. Tyramine plays a role in egg laying, consistent with its expression in the gonadal sheath cells, as *tdc-1* mutants are weakly hyperactive in egg laying (*180*). Exogenous tyramine also inhibits egg laying. This suggests that tyramine inhibits egg laying *in vivo*. Two

cells, the RIM motorneuron and the UV1 neuroendocrine cell, that express TDC-1 but not TBH-1, may release tyramine as a neurotransmitter. *tdc-1* mutants exhibit some behavioural defects that are not shared with the *tbh-1* mutants. These include the inhibition of head oscillation to the anterior part of the body as the worm backs off in response to a light body touch, inhibition of egg laying and modulation of spontaneous reversals during locomotion *(180, 218-219)*. Two tyramine receptors, SER-2 and TYRA-2, have been identified and they bind tyramine with high affinity when expressed in cell culture (*218*).

Octopamine

Octopamine is synthesized from tyramine by TBH-1 (*180*) (Figure 7). *tbh-1* is expressed in the RIC neuron and the gonadal sheath cells (*180*). *tbh-1* mutant animals share many behavioural defects with *tdc-1* mutants, which are defective in tyramine and octopamine synthesis. Exposure of animals to exogenous octopamine inhibits egg laying and pharyngeal pumping (*180*). SER-3 is the only characterized octopamine receptor in *C. elegans (220)* and it is present on the cholinergic SIA neurons. Octopamine binds to SER-3 on the SIA neurons and this leads to CREB activation in these neurons. This stimulates foraging at more distant locations instead of searching locally (*220*). This effect of octopamine is inhibited by dopamine released in the presence of food (*221*).

Neuropeptides

Neuropeptides are peptides that can serve as neurotransmitters or hormones (*157, 222*). There are three families of neuropeptides in *C. elegans*: the insulin-like peptides (INS) e.g. DAF-28 which promotes reproductive growth, the FMRFamide (Phe-Met-Arg-Phe-NH2)-related peptides (FLP) e.g. FLP-1 which promotes locomotion and pharyngeal pumping, and the neuropeptide-like proteins (NLP) e.g. NLP-1 which modulates AWC behaviours (*120, 223-225*).

Neuropeptides are usually derived from precursor proteins which undergo posttranslational processing and modifications to yield the mature neuropeptides. A single precursor protein can be cleaved by endoproteolytic enzymes (proprotein convertases) to yield a single neuropeptide, multiple neuropeptides or multiple copies of neuropeptide(s) (*226*). There are four proprotein convertases in *C. elegans*. These are *kpc-1*, *egl-3*/*kpc-2*, *aex-5*/*kpc-3*, and *bli-4*/*kpc-4* (*227*). Loss of *egl-3*/*kpc-2* leads to egg-laying, mechanosensation and locomotion defects (*228-229*) suggesting that neuropeptides derived from precursor proteins that could not be cleaved due to the lack of *egl-3*/*kpc-2* endoprotease are involved in diverse biological processes in

C. elegans. Mutations in *kpc-1* and *aex-5/kpc-3* cause defects that range from mild locomotory defects to slow growth and defecation defects (*230-231*). BLI-4/KPC-4 is mostly involved in converting procollagen to collagen which helps in maintaining cuticle integrity in the worm. A null mutation in *bli-4/kpc-4* causes lethality at late embryogenesis (*232*).

Neuropeptides are localized in dense core vesicles derived from the trans-Golgi network (TGN). They are first cleaved at a dibasic residue at the C-terminus of the peptide followed by removal of the basic residue from the peptide and finally modified at the N and C-termini to prevent degradation. The processing of neuropeptides begins at the endoplasmic reticulum (ER) and continues in the TGN and the dense core vesicles themselves. Dense core vesicles are not localised to synaptic zones where small clear vesicles that contain classic neurotransmitters localize (*226*). Dense core vesicles can be found more diffusely within the nerve terminals. After release from the neuron into the synaptic cleft, neuropeptides are degraded by proteolytic enzymes and thus cannot be recycled back to the neuron but must be made *de novo* in the neuron cell body (*233*).

As in mammals, neuropeptides released from neurons and non-neuronal cells can also act as hormones in *C. elegans (234)*. Neuropeptides act in diverse biological pathways in *C. elegans.* The insulin signaling (INS) pathway is involved in dauer development. Insulin does this in parallel to the transforming growth factor ß pathway (*235*). Loss of either pathway results in a constitutive dauer phenotype (*236*).

Members of the insulin pathway have also been shown to be involved in learning and memory in *C. elegans*. An *ins-1* mutant did not avoid the cultivation temperature on a temperature gradient after starvation (*140*). The phenotype was partially suppressed by mutations in *daf-2* (insulin-like receptor) and *age-1* (PI 3 kinase) mutants. Several insulin signaling mutants are also defective in starvation-induced salt learning (*173*). They include *ins-1* (insulin), *daf-2*, *pdk-1* (phosphoinositide-dependent kinase), *akt-1* (protein kinase B) and *age-1*. *age-1* and *daf-2* were shown to function in the ASER neuron and not in the ASEL neuron.

It has been difficult to characterize the functions of the FLP neuropeptide family because of redundancy in their activities. FLP-1 peptides are necessary for downregulating egg laying in the absence of food (*237*). No *nlp* mutant has been examined for neuronal function yet. However several *nlp* genes may have antimicrobial activities in the worm (*238*). Attempts have been made to identify the

receptors for several neuropeptides but this has been complicated because of the promiscuous binding of many neuropeptides and their cognate receptors. An example is the neuropeptide Y receptor-related protein, NPR-1, which when mutated causes an aggregation phenotype at the edges of the bacterial lawn (*239*). *C. elegans* does not have a clear homologue of mammalian neuropeptide Y. Rogers and colleagues (*240*) undertook a *Xenopus* oocyte assay in which a NPR-1 construct was injected and various FLP peptides were tested for the receptor activation. They found that FLP-21 and some peptides encoded by the *flp-18* gene activated the receptor (*240*). This is a nice example of identifying a receptor and its ligands. Another example is the pigment dispersing factor (PDF) receptor, *pdfr-1,* which encodes three alternatively spliced variants of the same gene. They are G-protein coupled receptors for PDF peptides which regulates locomotion and egg-laying in *C. elegans* (*241-242*).

Glutamate

In *C. elegans*, glutamate is involved in rapid synaptic transmission (*243*). It is stored in vesicles in pre-synaptic cells. Presynaptic sensory neurons respond to sensory stimuli by releasing glutamate. Glutamate transmission is mediated by postsynaptic glutamate receptors (GluRs) which can be ionotropic or metabotropic (G-protein coupled) GluRs (*244*). Ionotropic GluRs mediate rapid excitatory synaptic transmission and they consist of NMDA (*N*-methyl-D-aspartate) or non-NMDA (AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and Kainate) subtypes (*244*). Activation of GluRs present on the post-synaptic interneurons leads to a motor response (*245*). Excess glutamate is dangerous to the post-synaptic cells and usually causes excitotoxicity, i.e. excessive activation of GluRs and the subsequent calcium influx resulting in neuronal damage (*246*). Excess intracellular calcium is highly injurious to mitochondria (*247*). Glutamate is therefore usually quickly removed from synapses by the action of glutamate transporters which are mainly localised in the membrane of glial cells around the synapse. Some transporters are also located on presynaptic cells (*247*). In the glial cells, glutamate is converted to glutamine which is released from the glial cells and taken up by pre-synaptic cells and converted to glutamate by the enzyme glutaminase. EAT-4 (glutamate transporter) loads synthesized glutamate into synaptic vesicles for release (*248*). Glutamate can also have an inhibitory effect by gating of hyperpolarizing chloride channels (*249-250*).

Glutamate signaling is implicated in learning and memory (*251-252*). Two predicted NMDA-type GluR, *nmr-1* and *nmr-2* are necessary for memory retention in *C. elegans (253).* They also invoke *in vivo* NMDA-gated currents. They both function in the RIM

interneurons to mediate memory retention. Glutamate is also involved in habituation of the tap-withdrawal response. *eat-4* worms habituate faster but dishabituate slower than wildtype worms (*254*). Long-term memory of habituation (24 hour) is also modulated by glutamate. *glr-1*, an AMPA-type GluR, or *eat-4* animals were not capable of retaining long-term memory of habituation (*255-256*). It was suggested that the mechanism of long-term memory retention in habituation is mediated by downregulation of glutamate receptors.

Behavioural study of *C. elegans* response to NaCl.

C. elegans is a well established model organism for studying many sensory and learning paradigms. Different methods are used to study the response of *C. elegans* to NaCl. One is the quadrant plate assay (*170, 257*). In this assay, an X-Petri dish divided into four quadrants by plastic spacers is filled with buffered agar containing the desired concentration of NaCl. Opposite quadrants are filled with the same NaCl concentration. A thin layer of buffered agar without NaCl is put on top of the quadrant ridges just before the assay begins. The worms are placed at the centre of the plate and after 10 minutes incubation time, they are counted and the chemotaxis index is scored with the formula shown in figure 8.

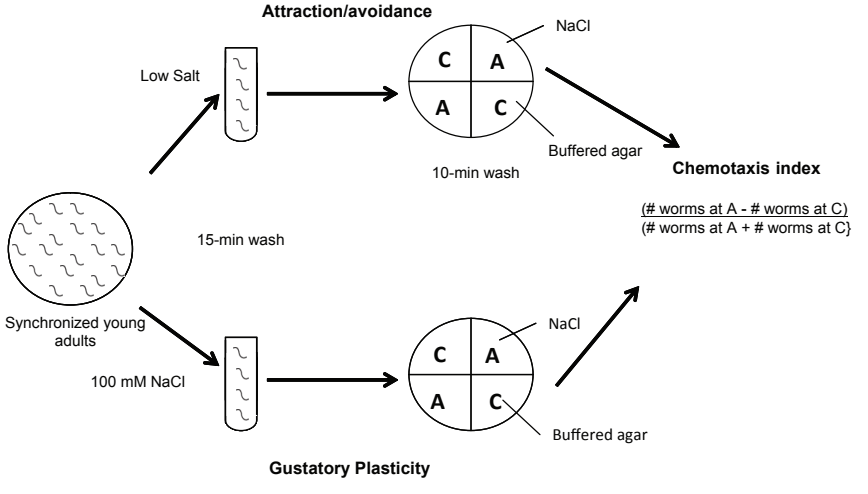

Figure 8. **Quadrant assay method used to study *C. elegans* behavioural responses to NaCl.**

The other chemotaxis assay employed to study *C. elegans'* behavioural response to NaCl is the gradient method (*68, 158*). This measures the migration pattern of the worm on a NaCl concentration gradient. Here, a buffered agar plate is made and a 6 mm agar plug is bored out at one end of the plate. This plug is replaced with a similar plug which has been soaked in a desired NaCl solution. NaCl diffuses

from the agar plug and forms a gradient in the agar plate for about 12-24 hours. At the time of the assay, the agar plug is removed and this position is regarded as the point with the peak concentration of NaCl. A similar size agar plug is bored out of the agar plate at the diametrically opposite position in the plate. This serves as the control point. The worms to be tested for chemotaxis are placed in the middle of the plate and allowed to migrate for a desired amount of time. The main difference between the two methods is that the quadrant plate exposes the animals to a steep concentration gradient while the gradient method exposes the animals to a shallow concentration gradient. A drawback of the shallow gradient assay method is that the exact concentration at the point at which the assayed worms are at the time of scoring is not precisely known. However, recently a method has been developed to estimate the NaCl concentrations along the gradient (*173*).

Cellular study of *C. elegans* response to NaCl.

To understand the molecular processes underlying *C. elegans* behavioural responses to different environmental cues, cellular responses are usually studied. The transparency of *C. elegans* makes it very convenient to study the neuronal response to any environmental cue. Many years of behavioural studies have identified particular neurons involved in sensing specific compounds or environmental cues. These were done by neuronal ablation, genetically induced neuronal degeneration and studies with mutants with defective or non-functional neurons. Cellular imaging is employed to visualize neuron(s) that are activated by a stimulus and are thus involved in the neuronal circuitry for the behavioural response. The main ion that generates action potential in the *C. elegans* is calcium. Thus, genetically encoded neuronal calcium sensors are ideal to study neuronal responses in *C. elegans.* This has aided the delineation of the neuronal circuitry of *C. elegans* responses to many stimuli.

Two genetically encoded calcium sensors have been mainly used in *C. elegans.* These are yellow cameleon and GCaMP (*81, 258*). Cameleon action is based on the principle of Forster (fluorescence) resonance energy transfer (FRET) from a donor to an acceptor fluorophore. The cameleon protein is a fusion protein containing two green fluorescent protein (GFP)-variants, CFP (cyan) and YFP (yellow) (Figure 69). Calmodulin (CaM) and the CaM–binding peptide from myosin light chain kinase (M13) are fused between the CFP and YFP proteins. When CaM binds calcium its conformation changes and condenses around the M13 peptide. The condensation brings CFP and YFP close together, which allows energy transfer from CFP to YFP, leading to an increase in YFP emission and a concomitant decrease in CFP

emission (*259*). The cameleon protein was first used in *C. elegans* by Schafer and colleagues to study neuronal and pharyngeal muscle activation (*260*). Cameleon has subsequently been widely used to study neuronal activation to different stimuli in *C. elegans*. An advantage is that it can visualize both increases and decreases in calcium concentration.

1

GCaMP is a circularly permuted fluorescent protein based on split GFP (Figure 9). The native GFP structure is made up of a ß-barrel formed by eleven β-sheets which form a cylindrically shaped structure termed the "β-can". An α-helix threads along the axis of the cylinder and contains the fluorescent chromophore which is very close to the centre of the ß-can. The chromophore is made up of a tripeptide, serine, tyrosine and glycine, which spontaneously autocatalyses a reaction with oxygen to form a fluorescent imidazolidone ring that forms multiple hydrogen bonds with

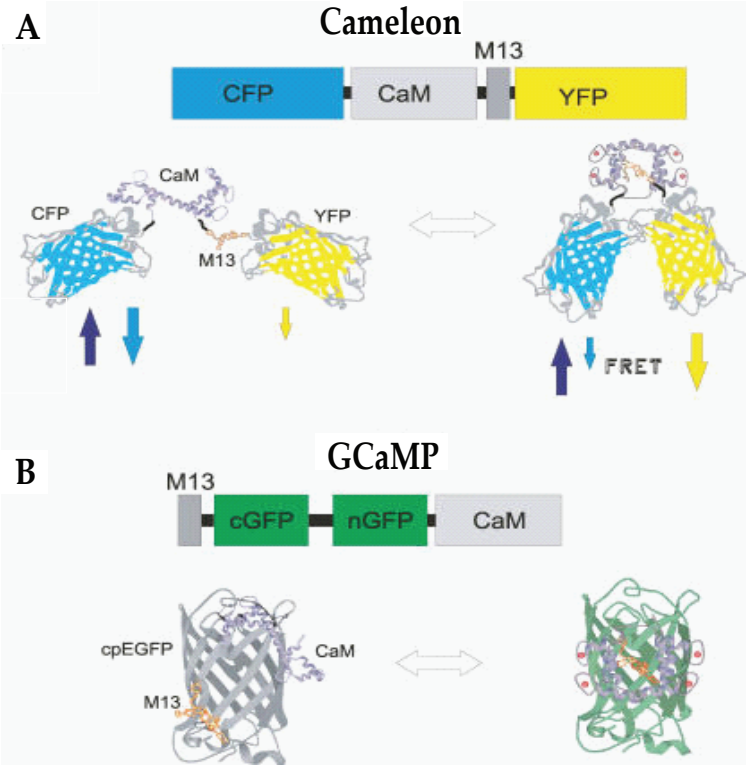

Figure 9. **Genetically encoded calcium indicators (GECI).** A. Yellow cameleon protein composition is depicted with the two fluorescent proteins and calmodulin and its binding peptide, M13. Below the conformational change that occurs when there is an increase in calcium concentration is shown. Circles are calcium molecules, up arrows depict excitation and down arrows depict emission. B. GCaMP protein composition is shown with the circularly

permuted GFP fragments (n and cGFP), calmodulin (CaM) and its binding partner (M13). Below the conformational change that occurs in the GCaMP molecule upon calcium binding is shown (Adapted with permission from (263)).

basic residues in the surrounding β-sheets (*261*). Roger Tsien and colleagues (*262*) discovered that GFP can tolerate insertion of relatively large peptides at various positions in the ß-sheets and the β-sheets linker regions. With this, they developed a circularly permuted GFP variant. They split the GFP protein sequence into two halves which are joined together by linker peptide sequences. Calmodulin and its protein binding domain, M13, were inserted into the rearranged GFP molecule such that calmodulin and M13 protein are on either end of the split and inverted GFP (Figure 9). In the absence or low level of calcium, calmodulin is not activated and does not interact with M13. Binding of calcium to CaM results in a conformation change of CaM and binding to M13. This leads to a rearrangement in the whole fusion protein and the closing of the β-barrel around the chromophore (*263*), which leads to an increase in fluorescence of GCaMP.

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2

Two genetic pathways mediate chemotaxis to NaCl in *C. elegans*

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Abstract

NaCl is essential for maintaining salt and water homeostasis in the body. However, our understanding of the molecular mechanisms of NaCl detection is still quite limited. In mice, the epithelial sodium channel (ENaC) has been shown to be involved in NaCl detection. However, an ENaC independent salt taste mechanism also exists. We use the nematode *C. elegans* to identify genes involved in NaCl taste. Several genes involved in NaCl chemoattraction in *C. elegans* have been identified. These include the guanylyl cyclases *gcy-14* and *gcy-22*, *tax-2* and *tax-4* cyclic nucleotide gated (CNG) channel subunits and *tax-6* and *cnb-1* calcineurin subunits. In our assays, in which we exposed the worms to a very steep NaCl gradient, these mutants showed reduced chemotaxis to NaCl, but they were still significantly attracted to higher NaCl concentrations. By analyzing the behaviour of double mutants and testing additional mutants in a candidate gene approach, we found that chemotaxis to NaCl involves two genetic pathways. The first pathway involves five genes that have been previously characterized, *gcy-22*, *tax-2*, *tax-4*, *tax-6* and *cnb-1*. The second pathway involves *gcy-14*, *gcy-22*, *tax-2*, the CNG channel α subunit *cng-3*, the Gα protein *odr-3*, the TRPV channel subunit, *osm-9*, and the guanylyl cyclase, *gcy-35*. By using cell specific rescue and calcium imaging we show that *tax-2* likely functions in the ASE neurons and *tax-4* in amphid neurons other than ASE. Together, we found that TAX-2/CNG-3 and TAX-2/TAX-4 may form functional channels for NaCl chemotaxis *in vivo*. We also found that *odr-3* functions in the ADF neurons.

Introduction

Salt taste is essential for water and salt homeostasis and can serve as a food cue. Unfortunately, very little is known about the molecular mechanism of salt detection (reviewed in (*20*)). In mammals salt seems to be sensed by two different mechanisms. The first uses the amiloride sensitive epithelial Na^+ channel (ENaC) (*16, 30, 261*) and the second mechanism which is amiloride insensitive, has been suggested to involve the non-selective cation-channel TRPV1, transient receptor potential (*18*). It seems likely that Na^+ influx through these channels leads to activation of the cell and neurotransmitter release.

We use the model organism *C. elegans* to study the molecular mechanisms of NaCl chemotaxis. *C. elegans* is a simple organism with only 302 neurons, of which the complete connectivity has been established (*262*). Despite its simplicity *C. elegans* shows complex behaviours such as chemotaxis to volatile and water-soluble compounds. Sensation of cues in its environment is mainly mediated by twelve pairs of neurons located in the head, the amphid sensory neurons (*263*).

We can discriminate three responses of *C. elegans* to NaCl using the quadrant assay method (*15, 167, 254*). In this assay, animals are given a choice between two quadrants filled with buffered agar containing a given concentration of NaCl and two control quadrants filled with buffered agar without NaCl (*254*). These assays show that naive animals are attracted to concentrations up to 200 mM and avoid higher concentrations. In a third response, called gustatory plasticity, the animals avoid normally attractive NaCl concentrations after 15 minutes pre-exposure to NaCl in the absence of food (*167*).

The main salt sensing cells of *C. elegans* are one pair of neurons, the ASE neurons (*68*). These neurons are essential for NaCl chemotaxis also in the quadrant assays (*15*). However, three other pairs of neurons have redundant functions in salt detection: the ADF, ASG, and ASI neurons (*68*). Structurally, the ASE neurons seem bilaterally symmetrical, but they respond asymmetrically to some stimuli. ASE left (ASEL) preferentially senses Na^+, Mg^{2+} and Li^+ while ASER right (ASER) senses K^+, Cl^-, Br^- and I^- ions (*87, 264*). ASEL and ASER also differ in their response to changes in NaCl concentrations. ASEL responds to an increase in NaCl concentration, while ASER responds to a decrease in NaCl concentration (*81*). Imaging of neuronal responses to NaCl in other neurons involved in NaCl sensation showed that the two ADF neurons respond to an increase in NaCl concentration (*89*). The ASH neurons are nociceptive neurons and sense high NaCl concentrations (*263*), thus mediating avoidance of high

NaCl concentrations. In addition, the ASH neurons respond to a decrease in NaCl concentration(*89*). The responses of ADF and ASH depend on input from other cells. No response to a change in NaCl concentrations was observed in the ASI and ASG neurons (*89*).

Several genes involved in chemotaxis to NaCl have been identified in genetic screens using the gradient assay method. In this method, animals are exposed to a shallow gradient of NaCl (*155, 265*). Genes identified include the cGMP gated channel subunit genes *tax-2* (β subunit) and *tax-4* (*α* subunit), and the calcineurin subunit A, *tax-6 (84-86).* We confirmed the roles of these genes in our quadrant assays and in addition found that the calcineurin subunit B, CNB-1, and the neuronal Ca^{2+} sensor, NCS-1, are involved in attraction to NaCl (*15*). Finally, a reverse genetic approach has shown that two receptor type guanylyl cyclases GCY-14 and GCY-22 play roles in chemotaxis to Na^+ and Cl^- respectively (*87*). Taken together, these results show that the second messengers cGMP and Ca^{2+} play important roles in chemotaxis to NaCl in *C. elegans*.

In this study, we identify four new genes that play a role in chemotaxis to NaCl. Cell specific rescue confirmed their involvement in NaCl sensation. We show that these genes function in two genetic pathways. The first pathway consists of the *gcy-22*, *tax-2*, *tax-4*, *tax-6* and *cnb-1* genes. The second pathway includes *gcy-14*, *gcy-22*, *tax-2* as well as another CNG channel α subunit, *cng-3*, the TRPV1 channel subunit *osm-9*, the Gα subunit *odr-3*, and the guanylyl cyclase *gcy-35*.

Results

TAX-2 and TAX-4 function in parallel pathways

Previously it was found that chemotaxis to salts involves the second messenger cGMP (*84-85*). We confirmed the role of the cGMP gated channel (CNG) *α* and β subunits, TAX-4 and TAX-2, in the quadrant assay (*15*). In this assay *tax-2(p671)* and *tax-4(p678)* mutant animals showed a very similar defect in chemotaxis to NaCl (Figure 1). They showed no response to 0.1-1 mM NaCl, but a significant residual response to higher NaCl concentrations (10-100 mM). A possible explanation of the partial NaCl chemotaxis phenotype of these mutants is that the TAX proteins in these mutants still have residual functions, although both alleles are considered to be null alleles. The *tax-2(p671)* allele contains a substitution of a highly conserved arginine for a cysteine in the first membrane-spanning domain and the *tax-4(p678)* allele contains a stopcodon near the N-terminus (*84-85*). We tested two other *tax-2* alleles:

p691, which carries a serine to a proline missense mutation in the pore region and *p694*, which abolishes expression of *tax-2* in the ASE, AFD, AQR and BAG neurons due to the deletion of the first exon and 1.8 kb of upstream sequence (*84*). Both alleles showed very similar effects on NaCl chemotaxis in our assays (results not shown). Also other *tax-4* alleles, *ks11*, a proline to leucine mutation in the pore region and *ks28,* an aspartic acid to valine mutation in the second transmembrane domain H2 (*85*), still showed significant chemotaxis to higher NaCl concentrations (results not shown). These results made it unlikely that the partial NaCl chemotaxis defect of the *tax-2* and *tax-4* mutants is caused by residual activity of the TAX proteins.

Since TAX-2 and TAX-4 are expressed and function together in many processes (*84-85*), we wondered if *tax-2* and *tax-4* function in the same genetic pathway in NaCl chemotaxis. Therefore, we tested *tax-2; tax-4* double mutant animals for their response to NaCl. To our surprise, loss of both *tax-2* and *tax-4* completely abolished chemotaxis to 0.1-100 mM NaCl (Figure 1), suggesting that *tax-2* and *tax-4* function, at least partially, in parallel genetic pathways.

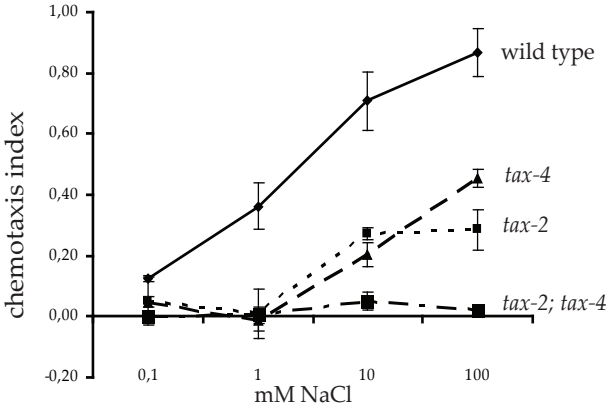

Figure 1: ***tax-2* and *tax-4* function, at least partially, in two genetic pathways in NaCl chemotaxis.** t*ax-2(p671)* ($p<0.05$) and *tax-4(p678)* mutant animals showed reduced chemotaxis to NaCl compared to wild type animals ($p<0.01$), but did not significantly differ from each other ($p>0.05$). Chemotaxis to NaCl was completely abolished in *tax-2; tax-4* double mutant animals ($p<0.05$ when compared to either of the two single mutant animals). Indicated are the averages of at least 4 assays ± s.e.m.

CNG-3 and TAX-4 function in parallel pathways

Although CNG channels are thought to be composed of α and β subunits, α

subunits, including TAX-4, have the capability of forming functional channels when expressed in heterologous systems (*84, 266*). β subunits, including TAX-2, could not. Also *in vivo* in *C. elegans* TAX-4 can form a functional channel without TAX-2, since overexpression of *tax-4* in the *tax-2* mutant could rescue the chemotaxis defect of *tax-2* animals (*84*). Based on these data we propose that the partial chemotaxis defect of *tax-2(p671)* animals can be explained by the activity of the TAX-4 homomeric channel.

The complete loss of chemotaxis to NaCl of the *tax-2; tax-4* mutant suggests that in the *tax-4* mutant, another CNG α subunit forms a functional heteromeric channel with TAX-2 which could account for the residual chemotaxis seen in *tax-4* animals. *cng-1* and *cng-3* are two other *C. elegans* CNG α subunits. *cng-1* is expressed in unidentified amphid neurons (*267*) and *cng-3* is expressed in the AFD, ASE, ASI, AWB and AWC amphid neurons (*268*). *cng-1(jh111)* (results not shown) and *cng-3(jh113)* single mutants showed no salt chemotaxis defects (Fgure 2). To test if *cng-1* or *cng-3* function in either of the two NaCl chemotaxis pathways, we generated double mutants between these CNG channel mutants and *tax-2* and *tax-4*.

Doubles between *cng-1* and *tax-2* or *tax-4* did not reveal a function of *cng-1* in NaCl chemotaxis (results not shown). However, the analysis of double mutants did reveal a function of *cng-3* in NaCl chemotaxis: *cng-3; tax-2* animals showed the same behaviour as *tax-2* single mutants, whereas *cng-3; tax-4* had lost all chemotaxis to NaCl (Figure 2A and B). This suggests that *cng-3* acts in the *tax-2* pathway and in parallel to the *tax-4* pathway. To confirm that the NaCl chemotaxis phenotype of *cng-3; tax-4* animals was caused by mutation of *cng-3*, we re-introduced the wild type *cng-3* gene as a transgene in *cng-3; tax-4* animals. Indeed, this transgene rescued the phenotype of the *cng-3; tax-4* animals (Figure 2C). Based on our results, we propose that NaCl chemotaxis in *C. elegans* uses two CNG channels, the TAX-2/TAX-4 channel and the TAX-2/CNG-3 channel.

TAX-2 probably functions in the ASE neurons in NaCl chemosensation

Thus far, our results suggest that NaCl chemotaxis in *C. elegans* uses two CNG channels, TAX-2/TAX-4 and TAX-2/CNG-3. *tax-2* gene expression has been reported in the AFD, ASE, ASG, ASI, ASJ, ASK, AWB, AWC, AQR, PQR and BAG neurons (*84, 108*). From these cells the AFD, ASE, AQR and BAG neurons seem most important, because NaCl chemotaxis was also affected in the *tax-2(p694)* animals, which have lost *tax-2* expression only in these cells (*84*). It is most likely that *tax-2* functions in the ASE neurons since they are the main NaCl sensing cells. Indeed, *tax-2(p671)*

animals showed no Ca^{2+} response in either the ASEL neuron after an increase in NaCl concentration from 40 to 80 mM or in the ASER neuron after a decrease from

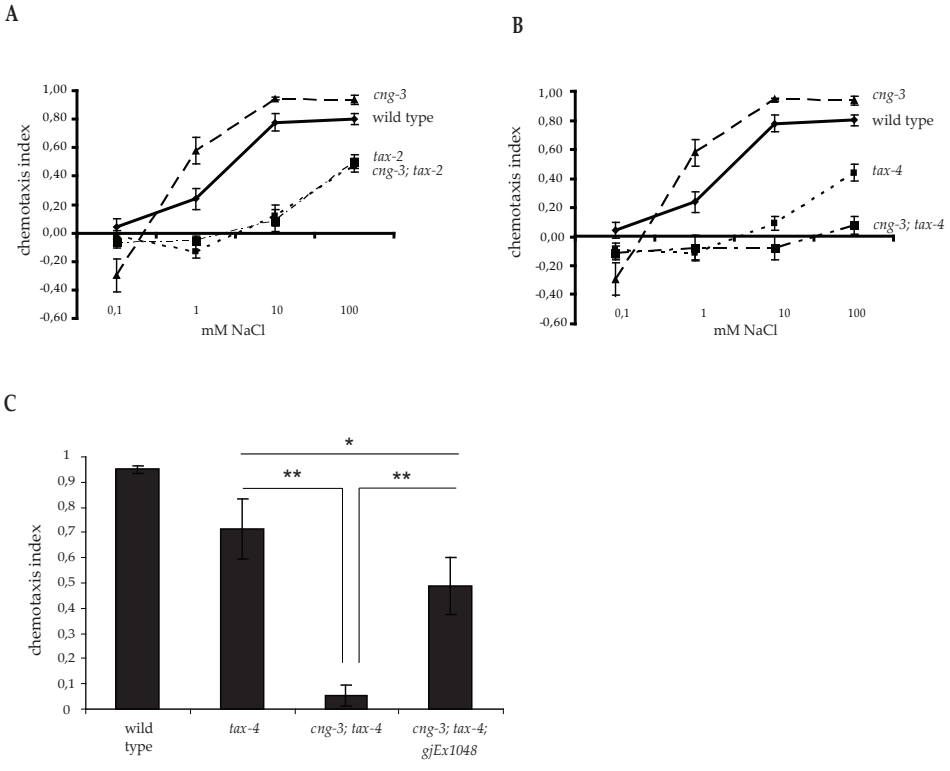

Figure 2: ***cng-3* acts in NaCl chemotaxis in the same pathway as *tax-2* and in parallel to *tax-4*.** (A) *cng-3* animals showed wild type chemotaxis to NaCl ($p>0.05$ compared to wild type animals); *cng-3; tax-2* animals showed the same response to NaCl as *tax-2* animals ($p>0.05$). (B) *cng-3; tax-4* animals showed significantly reduced chemotaxis to NaCl, compared to *tax-4* mutant animals ($p<0.001$). (C) Chemotaxis to 100 mM NaCl of *cng-3; tax-4* animals could be restored by introduction of the wild type *cng-3* gene as a transgene (*gjEx1048*) in *cng-3; tax-4* animals (* - $P>0.05$, ** - $P<0.0001$). Indicated are the averages of at least 4 assays ± s.e.m.

40 to 0 mM NaCl (*81*). We have confirmed these results using a sensitive neuronal Ca^{2+} reporter, GCaMP3 Ca^{2+} (*259, 269-270*) imaging and found no responses in the ASEL neurons of *tax-2(p671)* animals when exposed to 100 mM NaCl from a baseline of 0 mM NaCl (results not shown). These results show that *tax-2* is required for the calcium response in the ASE neurons. However, these data do not exclude that *tax-2* might also function in the AFD, AQR or BAG neurons in NaCl chemotaxis.

TAX-4 functions in neuron(s) other than the ASE neurons in NaCl chemosensation

The *tax-4* gene is expressed in the AFD, ASE, ASG, ASI, ASJ, ASK, AWB, AWC, BAG, AQR, PQR and URX neurons (*85*, *108*). Previously, it has been shown that *tax-4(p678)* animals showed no Ca^{2+} response in the ASEL neuron after an increase in NaCl concentration from 40 to 80 mM or in the ASER neuron after a decrease from 40 to 0 mM NaCl (*81*), indicating that *tax-4* is required for the response of these cells to NaCl. To confirm these results we performed Ca^{2+} imaging in the ASE neurons of *tax-4(p678)* animals using GCaMP3. Interestingly, *tax-4(p678)* animals did show calcium responses in the ASEL neurons when exposed to an increase in NaCl concentrations (Figure 3A). In general, these responses were quite late, more than one second after exposure to NaCl, whereas wild type animals showed very rapid responses upon exposure to a stimulus. These experiments show that mutation of *tax-4* affects Ca^{2+} responses in ASEL neurons, but is not absolutely required for it.

To identify in which neurons *tax-4* functions in NaCl chemotaxis, we performed cell specific rescue experiments. First, we expressed the *tax-4* gene in the ASE neurons of *tax-4* animals using the p_{flp-5}*::tax-4* construct. Surprisingly, 8 independent transgenic strains showed no rescue of the NaCl chemotaxis defect at 10 mM NaCl (Figure 3B). To confirm that *tax-4* functions in cells other than the ASE neurons, we tried rescue using the p_{odr-4}*::tax-4* construct. *odr-4* is expressed in all amphid and phasmid sensory neurons, except the ASE gustatory and AFD thermosensory neurons (*271*). We confirmed that these animals indeed lack expression of *tax-4* in the ASE neurons by visualizing GFP expressed from an artificial operon in the same construct (*108*). In agreement with our ASE-rescue experiments, 6 out of 7 p_{odr-4}*::tax-4* strains showed significant rescue of NaCl chemotaxis (Figure 3B). Expression of *tax-4* in all amphid chemosensory neurons using both p_{flp-5}*::tax-4* and p_{odr-4}*::tax-4* did not further improve chemotaxis, suggesting that expression of *tax-4* in the ASE neurons is not required for NaCl chemotaxis (Figure 3B).

tax-4 is also expressed in the AQR, PQR and URX neurons. We have previously shown that these cells play a role in gustatory plasticity (*15*). To test if *tax-4* functions in these neurons in NaCl chemotaxis, we restored *tax-4* expression in these neurons using the p_{gcy-32}*::tax-4* construct. However, none of the 4 p_{gcy-32}*::tax-4* strains showed rescue of NaCl chemotaxis (Figure 3B). Combining p_{gcy-32}*::tax-4* with p_{odr-4}*::tax-4* did not improve rescue with p_{odr-4}*::tax-4* alone, suggesting that *tax-4* does not function in the AQR, PQR and URX neurons in NaCl chemotaxis (Figure 3B). Also expression of *tax-4* in the ASI neurons did not rescue the chemotaxis defect (Figure 3B). Together

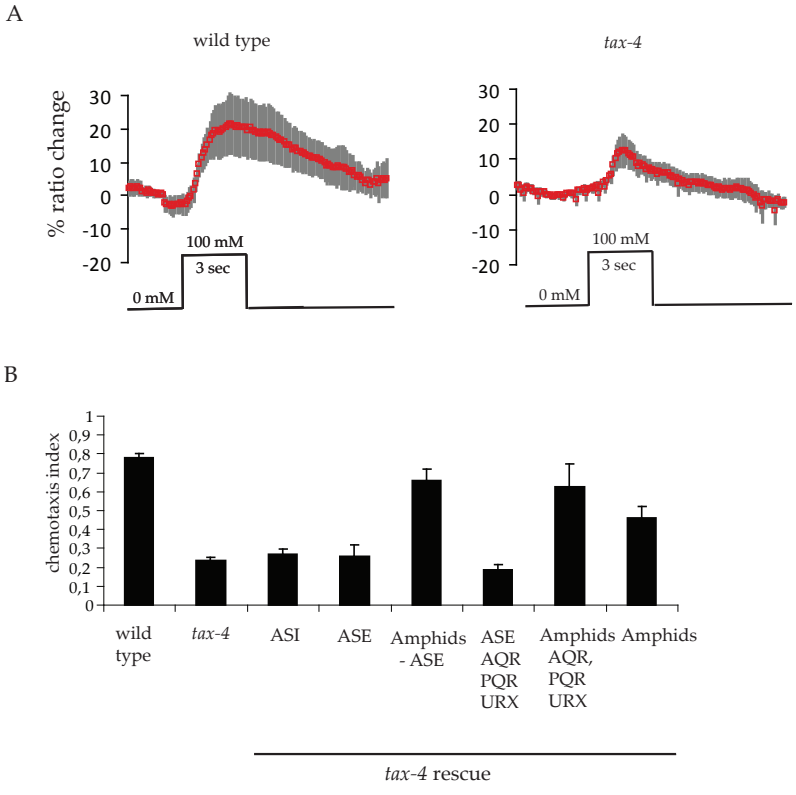

Figure 3. ***tax-4* is not required in the ASE neurons for NaCl chemotaxis.** (A) *tax-4* mutant animals showed Ca^{2+} fluxes in ASEL neurons after exposure to 100 mM NaCl (wild type n = 9; *tax-4* n = 6). Gray traces along data represent standard error of the mean (SEM). (B) *tax-4* expression was rescued in ASE and in various other neurons. No rescue was observed when *tax-4* was expressed only in the ASE, ASI or in AQR, PQR and URX neurons ($p>0.05$). Rescue was observed when *tax-4* was expressed in all amphids neurons and in all amphids neurons except the ASE neurons ($p<0.0001$). Assays were preformed with 10 mM NaCl. Indicated are the averages of at least 4 assays ± s.e.m.

our data suggest that TAX-4 acts in amphid chemosensory neurons other than the ASE neurons in chemotaxis to NaCl.

Receptor-type guanylyl cyclases function in two NaCl chemosensory pathways

GCY-14 and GCY-22 are receptor-type guanylyl cyclases expressed in ASEL and ASER respectively (*171, 272*). Ortiz *et al.* showed that *gcy-14* animals are slightly

defective in Na^+ sensation, while *gcy-22* animals displayed significant defects in chemotaxis to Cl^-, Br^- and I^- salts but no defect in Na^+ sensation (*87*). In our assay, *gcy-14* mutant animals showed wild type chemotaxis to NaCl (Figure 4A). *gcy-22* mutant animals showed a defect in NaCl chemotaxis very similar to that of *tax-2* and *tax-4* animals. *gcy-14; tax-2* animals showed the same response as *tax-2*, suggesting that *gcy-14* might function in the *tax-2* pathway. Interestingly, *gcy-14; tax-4* animals showed a stronger NaCl chemotaxis defect than *tax-4* animals, confirming the role of *gcy-14* in NaCl chemotaxis and suggesting that it functions in a genetic pathway in parallel to *tax-4*. Double mutants between *gcy-22* and *tax-2* or *tax-4* showed the same behaviours as the single mutants, suggesting that *gcy-22* functions in both the

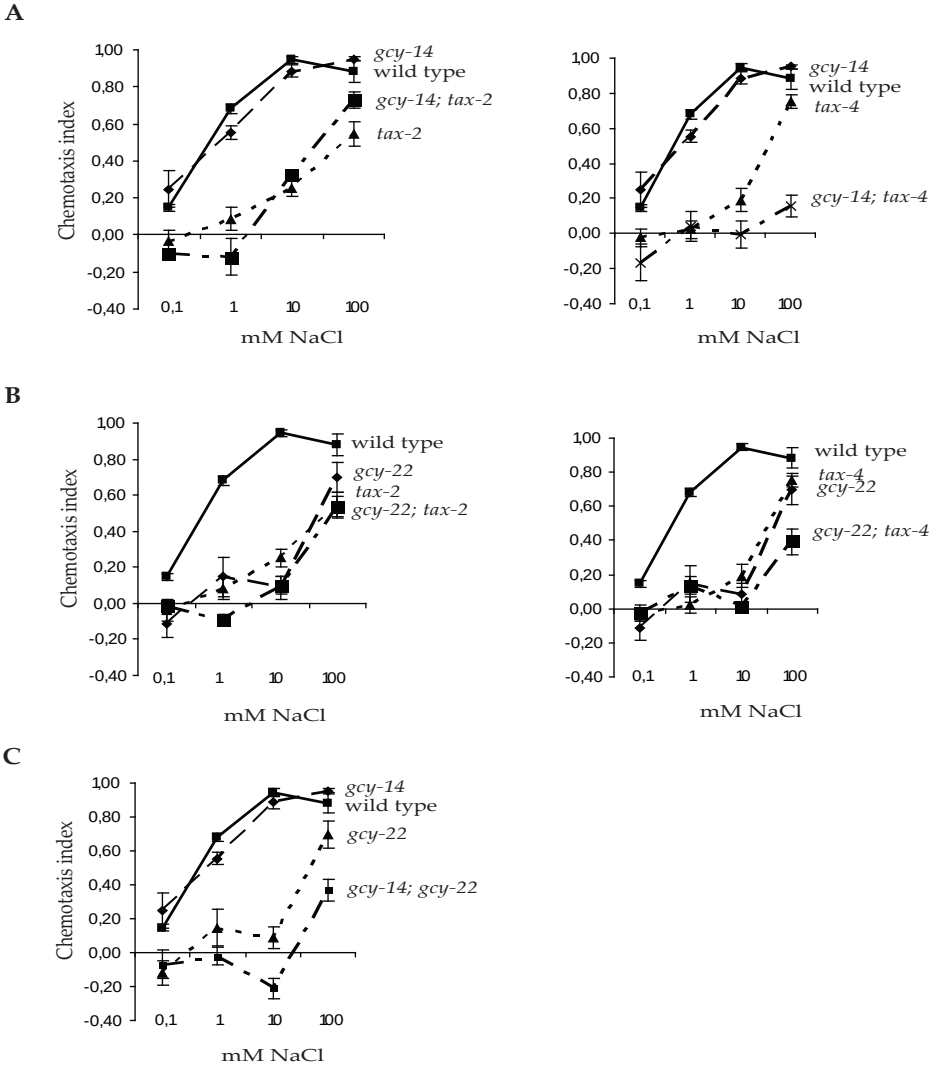

Figure 4. ***gcy-14* and *gcy-22* are involved in NaCl chemotaxis in *C. elegans*.** (A) *gcy-14* animals behaved like wild type animals in the quadrant NaCl assay. *gcy-14; tax-2* animals showed the same behaviour as *tax-2* animals, p>0.05. *gcy-14; tax-4* double mutant animals were defective in NaCl chemotaxis, p<0.0001 compared to *gcy-14, tax-4* and wild type single mutant animals. (B) *gcy-22; tax-2* double mutant animals showed the same NaCl chemotaxis as *gcy-22* and *tax-2* single mutant animals, p>0.05. Similarly, *gcy-22; tax-4* double mutant animals showed the same response as *tax-4* and *gcy-22* single mutant animals, p>0.05. (C) *gcy-14; gcy-22* animals showed less chemotaxis to NaCl compared to *gcy-14* and *gcy-22* single mutant animals, although this difference was not significant, p>0.05 among all. Indicated are the averages of at least 4 assays ± s.e.m.

tax-2 and *tax-4* pathways. Consistent with these results, we found that *gcy-14; gcy-22* double mutant animals have a stronger chemotaxis defect than the *gcy-22* single mutants, although this difference was not significant (Figure 4C). Together, these results suggest that *gcy-14* and *gcy-22* both function in NaCl chemotaxis, probably partially in parallel pathways. *gcy-14* functions in the *tax-2* pathway and *gcy-22* functions in both the *tax-2* and *tax-4* pathways.

TAX-6 acts in the TAX-4 pathway

Previous studies have shown that calcineurin plays a role in NaCl chemotaxis in *C. elegans* (*15, 86*). Calcineurin is a Ca^{2+} activated phosphatase that consists of two subunits, the catalytic A subunit TAX-6 and the regulatory B subunit CNB-1 (*273-275*). *tax-6* mutant animals showed a very similar response to NaCl as *tax-4* or *tax-2* mutant animals, but *cnb-1* mutant animals showed a wild type response (Figure 5A

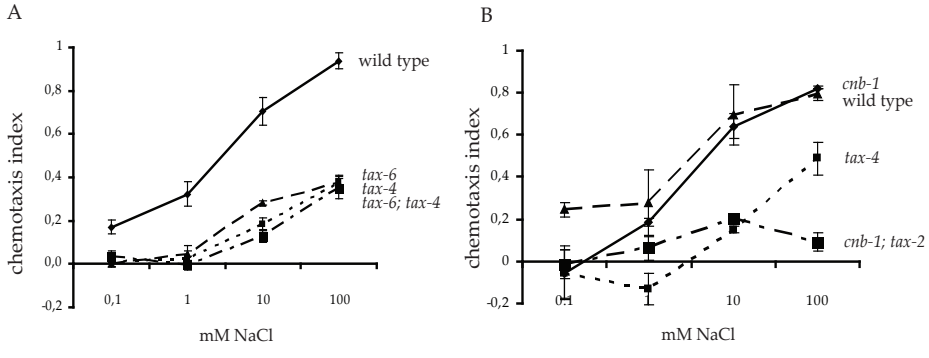

Figure 5: ***tax-6* and *cnb-1* act in the same pathway as *tax-4*.** (A) *tax-6* and *tax-4* mutant animals showed a similar defect in chemotaxis to NaCl (p>0.05). The responses of *tax-4; tax-6* double mutant animals did not significantly differ from either single mutant (p>0.05). (B) *cnb-1;*

tax-2 double mutant animals showed strongly reduced chemotaxis to NaCl ($p<0.001$), suggesting that *cnb-1* acts in parallel to *tax-2*. These results suggest that *tax-4, tax-6* and *cnb-1* function in the same genetic pathway. Indicated are the averages of at least 4 assays ± s.e.m.

and B). To determine in which pathway calcineurin functions, we generated double mutants between *tax-6* or *cnb-1* and *tax-2* or *tax-4*. Unfortunately, we could not make *tax-6; tax-2* or *cnb-1; tax-4* animals. *tax-6; tax-4* double mutant animals showed the same response to NaCl as either of the single mutants (figure 5A). In contrast, *cnb-1; tax-2* double mutant animals showed an almost complete loss of chemotaxis to NaCl (figure 5B). Taken together, these results suggest that both *tax-6* and *cnb-1* function in the same genetic pathway as *tax-4* in parallel to *tax-2*. Since calcineurin is a Ca^{2+}-activated phosphatase, it might act downstream of the TAX-2/TAX-4 CNG channel.

The TRPV channel subunit OSM-9 functions in NaCl chemotaxis

It has been suggested that the TRPV channel subunit VR1 plays a role in NaCl taste in mammals (*18*). In *C. elegans*, the TRPV channel subunit OSM-9 has been implicated in a behavioural response to NaCl, gustatory plasticity (*15*). Moreover, *osm-9* is expressed in the ASE, ADF, ASI, ASG and ASH neurons and these neurons are involved in different responses to NaCl (*68, 95*). We wondered if OSM-9 is also involved in attraction to NaCl, although *osm-9* single mutant animals showed no defect in chemotaxis to 0.1-100 mM NaCl (*15*) (Figure 6). We speculated that a function of *osm-9* in NaCl chemotaxis could be masked by the fact that there are two parallel pathways that mediate chemotaxis to NaCl. Therefore, we tested *osm-9; tax-4* animals and *osm-9; tax-6* animals for their response to NaCl, and found that attraction to NaCl in these double mutants was significantly diminished or completely abolished (Figure 6A and 6B). Unfortunately, we could not generate *osm-9; tax-2* double mutant animals. These findings suggest that *osm-9* functions in the *gcy-14*/*gcy-22*/*tax-2*/*cng-3* pathway, in parallel to the *gcy-22*/*tax-4*/*tax-6*/*cnb-1* pathway. In addition, our results show that, as suggested for mammals, TRPV channels are involved in salt taste in *C. elegans*.

G protein signaling in chemotaxis

The Gα subunit ODR-3 is often found to function in the same pathways as OSM-9 (*94, 102*). Moreover, we found that ODR-3 plays an important role in gustatory plasticity, where it functions in the ADF neurons (*15*). The ADF neurons also play a role in attraction to NaCl (*68*). Therefore we tested whether ODR-3 might also function in

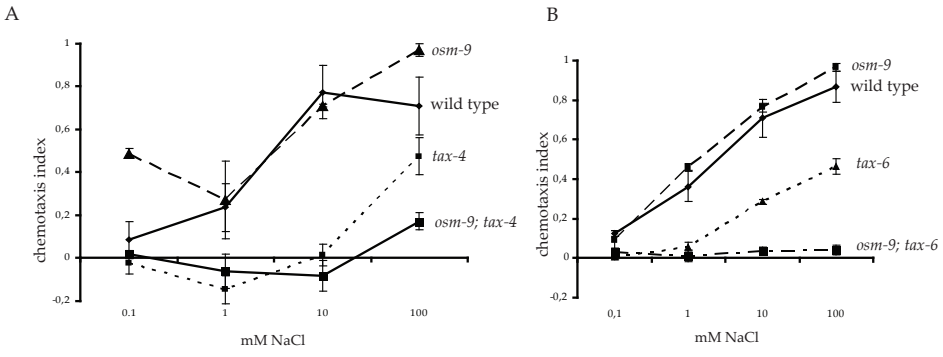

Figure 6: **The TRPV channel subunit OSM-9 functions in chemotaxis to NaCl.** *osm-9* single mutant animals showed wild type responses to 0.1-100 mM NaCl ($p>0.05$). (A) Chemotaxis is reduced in *osm-9; tax-4* double mutant animals ($p<0.018$ compared to *tax-4* at 100 mM) (B) Chemotaxis to 10-100 mM NaCl is completely abolished in *osm-9; tax-6* double mutant animals ($p<0.001$ when compared to both single mutants). Indicated are the averages of at least 4 assays ± s.e.m.

the ADF neurons in chemotaxis to NaCl. *odr-3* mutant animals showed wild type chemotaxis to 0.1-100 mM NaCl (*15*) (Figure 7). However, chemotaxis was completely abolished in *tax-4; odr-3* and *tax-6; odr-3* double mutant animals (Figure 7A and B), suggesting that *odr-3* functions in a genetic pathway parallel to the *tax-4*/*tax-6* pathway. In contrast, *tax-2; odr-3* double mutant animals showed the same response to NaCl as *tax-2* single mutant animals (Figure 7C), suggesting that *tax-2* and *odr-3* function in the same genetic pathway, in parallel to the *gcy-22*/*tax-4*/*tax-6*/*cnb-1* pathway.

Thus far, no role for G protein signal transduction in attraction to NaCl had been described. Here we show that the Gα protein ODR-3 is involved in this process. *odr-3* is expressed in the AWA, AWB, AWC, ADF and ASH neurons (*94*) of which only the ADF neurons have been implicated in attraction to NaCl (*68*). As we have previously shown that ODR-3 is required in the ADF neurons in gustatory plasticity (*15*), we expressed *odr-3* specifically in the ADF neurons of *tax-4; odr-3* double mutant animals. Expression of *odr-3* in the ADF neurons partially restored NaCl chemotaxis in *tax-4; odr-3* mutant animals (Figure 7D), suggesting that ODR-3 functions in the ADF neurons in chemotaxis to NaCl.

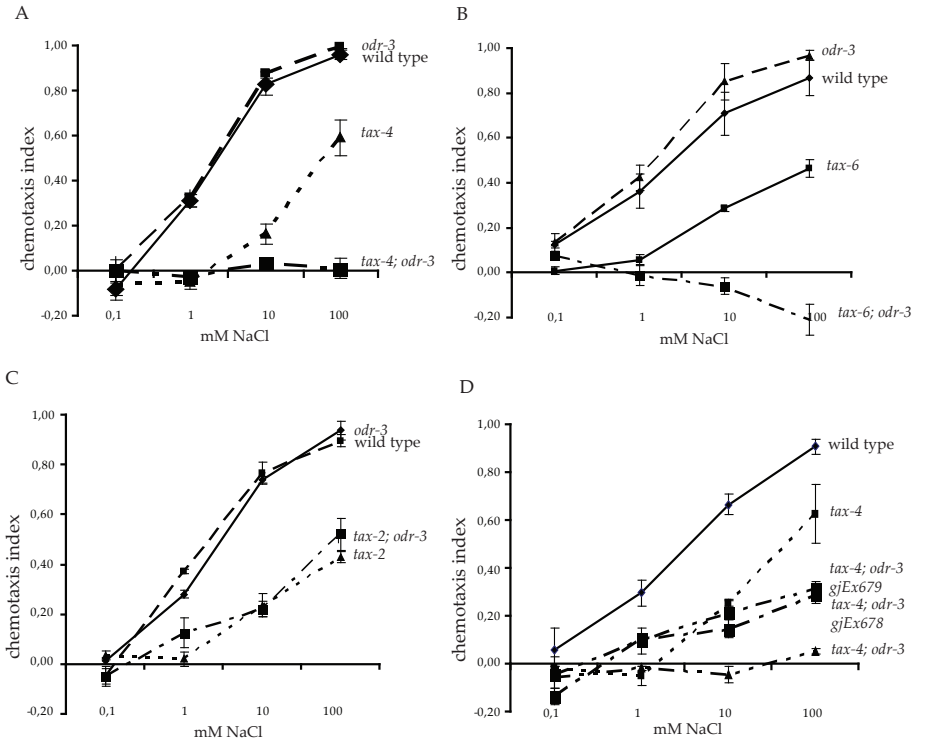

Figure 7: **The Gα protein ODR-3 functions in chemotaxis to NaCl.** *odr-3* single mutant animals showed wild type responses to 0.1-100 mM NaCl ($p>0.05$). (A) Chemotaxis to 0.1-100 mM NaCl was completely abolished in *tax-4; odr-3* double mutant animals ($p<0.05$ when compared to *tax-4* single mutant animals for 10-100 mM). (B) Chemotaxis to 0.1-100 mM NaCl was completely abolished in *tax-6; odr-3* double mutant animals ($p<0.0001$ when compared to *tax-6* single mutant animals for 10-100 mM). (C) *tax-2; odr-3* double mutants showed a defect in chemotaxis ($p<0.01$ when compared to wild type), which is the same as that of *tax-2* single mutants ($p>0.05$). (D) Expression of *odr-3* in the ADF neurons of *tax-4; odr-3* double mutants partially rescued chemotaxis to NaCl ($p<0.05$). Shown are the responses of two strains, which showed very similar responses. Indicated are the averages of at least 4 assays ± s.e.m.

GMP signaling in chemotaxis

Previous studies have shown that TAX-4 also plays a role in aerotaxis, the response of *C. elegans* to oxygen. TAX-4 mediates aerotaxis together with the guanylyl cyclase GCY-35 in the body cavity neurons AQR, PQR, and URX (*106*). We found that *gcy-35* also plays a role in the AQR, PQR, and URX neurons in gustatory plasticity (*15*). Since *gcy-35* is involved in salt responses, we proposed that, like *tax-4*, it might also be of importance in attraction. *gcy-35* mutant animals showed wild type attraction

to 0.1-100 mM NaCl (*15*) (Figure 8). In contrast, *tax-4; gcy-35* double mutant animals did not show any response to 0.1-100 mM NaCl (Figure 8A). Next, we asked whether *gcy-35* functions in the same genetic pathway as *tax-2* by analysing the behaviour of *tax-2; gcy-35* double mutant animals. As expected, *tax-2; gcy-35* mutant animals showed very similar responses to NaCl as *tax-2* mutant animals (Figure 8B). Thus, the guanylyl cyclase GCY-35 is involved in attraction to NaCl, and probably functions in the *tax-2* pathway in parallel to the *tax-4* pathway.

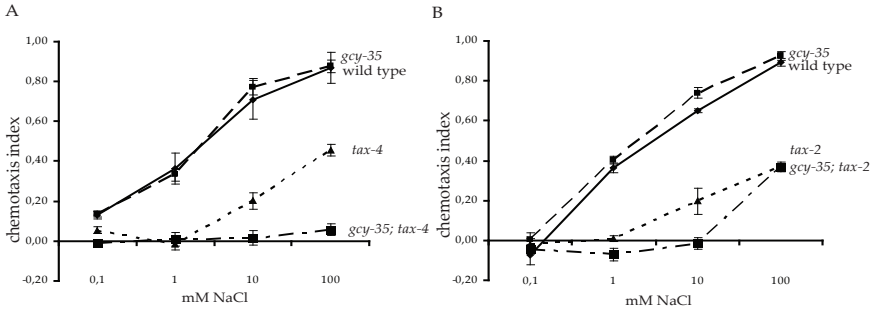

Figure 8: **A role for the guanylyl cyclase GCY-35 in chemotaxis to NaCl**. *gcy-35* single mutant animals showed wild type responses to 0.1-100 mM NaCl ($p>0.05$). (A) Chemotaxis to 0.1-100 mM NaCl was completely abolished in *gcy-35; tax-4* double mutant animals ($p<0.05$ when compared to *tax-4* single mutant animals for 10-100 mM). (B) Chemotaxis of *tax-2; gcy-35* double mutants to 100 mM NaCl was not significantly different from *tax-2* single mutants ($p>0.05$). Indicated are the averages of at least 4 assays ± s.e.m.

Taken together, our results suggest that *gcy-35* functions in the same genetic pathway as *gcy-14*, *gcy-22*, *tax-2*, *cng-3*, *odr-3* and *osm-9* but in parallel to the *gcy-22*, *tax-4*, *tax-6* and *cnb-1* pathway.

Discussion

We have identified four new genes that play a role in chemotaxis to NaCl in the nematode *Caenorhaditis elegans*. Our results suggest that these genes function in two different genetic pathways. The first genetic pathway contains the guanylyl cyclases *gcy-14*, *gcy-22* and *gcy-35*, the CNG channel subunits *tax-2* and *cng-3*, the TRPV channel subunit *osm-9* and the Gα subunit *odr-3*. The second genetic pathway contains the guanylyl cyclase *gcy-22*, the CNG channel subunits *tax-2* and *tax-4*, and the calcineurin subunits *tax-6* and *cnb-1* (Figure 9).

What does this mean molecularly? In mammals, NaCl is detected via the amiloride-sensitive channel, ENaC, where ion influx leads to depolarisation of cells and

neurotransmitter release (*30*). *C. elegans* NaCl sensation is not affected by amiloride (Hukema and Jansen unpublished results) and thus far no ENaC/DEG channel has been shown to function in NaCl chemotaxis in *C. elegans.* In addition, a putative amiloride-insensitive nonspecific salt sensation mechanism exists. It has been suggested that this pathway uses the TRPV1 channel for taste transduction (*18*). However, two recent publications showed that TRPV1 knockout mice still showed a robust preference for NaCl over water in the presence of amiloride (*276-277*).

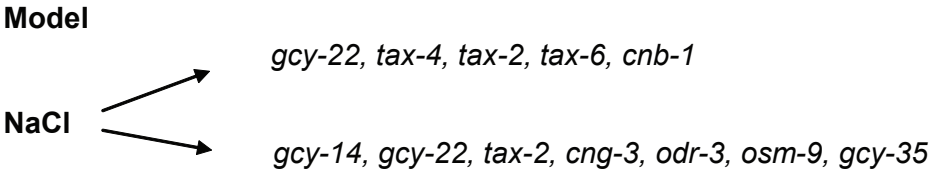

Figure 9: **Two genetic pathways mediate chemotaxis to 0.1-100 mM NaCl in *C. elegans.*** One pathway includes *gcy-22, tax-4, tax-2, tax-6* and *cnb-1* and the other pathway includes *gcy-14, gcy-22, tax-2, cng-3, osm-9, odr-3,* and *gcy-35.*

It has therefore been proposed that in addition to the TRPV1 pathway other amiloride-insensitive NaCl transduction mechanisms exist in mice. We found that the TRPV channel subunit OSM-9 is involved in chemotaxis to NaCl in *C. elegans,* suggesting that the molecular mechanism used by *C. elegans* to sense NaCl overlaps with the mammalian salt sensation mechanism. However, we do not know the molecular function of OSM-9 in NaCl detection. OSM-9 could function as an additional salt taste transduction channel in the ASE neurons or in other sensory neurons involved in chemotaxis to NaCl like ADF, ASI or ASG, but it could also function as a downstream signal transduction protein.

The most likely NaCl receptors identified thus far are the transmembrane receptor-type guanylyl cyclase (GCY) proteins, GCY-14 and GCY-22 (*87*). However whether they function as receptors has not been proven. Furthermore, *gcy-14; gcy-22* double mutant animals still show significant chemotaxis to NaCl suggesting that additional signaling molecules function in parallel to these GCYs. It is not clear if similar proteins function in NaCl taste in other animals. Guanylyl cyclase activity has been shown cytologically in rabbit foliate taste buds. This activity was localized to the atypical portion (microvilli and neck) of taste buds which is the likely site of interaction of

the taste stimuli and the taste cells (*278*). This suggests that guanylyl cyclases are involved in taste transduction in mammals, but whether they are involved in salt taste is unclear.

Since *gcy-14* and *gcy-22* function in ASEL and ASER respectively (*87*) and produce cGMP, it seems likely that they function upstream of the CNG channels: TAX-2/TAX-4 and TAX-2/CNG-3. Of these channels TAX-2/TAX-4 seems most important for chemotaxis to NaCl since mutations in these genes give the strongest phenotypes. Mutations in either *tax-2* or *tax-4* affect calcium responses in the ASE neurons, suggesting that this channel functions in these neurons. However, we could not rescue the *tax-4* defect by expressing *tax-4* in the ASE neurons, suggesting that *tax-4* at least also function in other sensory neurons in NaCl chemotaxis. Which neurons they are is not yet known.

Although the order of the different genes that act in the two NaCl chemotaxis pathways is not known, it seems likely that calcineurin TAX-6/CNB-1 functions downstream of the TAX-4/TAX-2 channel and responds to a calcium influx via this CNG channel. Alternatively, calcineurin may play a regulatory role in the signal transduction pathway downstream of the calcium influx via TAX-2/TAX-4. A similar regulatory function of calcineurin has been observed in *C. elegans* quiescence (*279*), in the modulation of CO_2 avoidance (*111*) and in the regulation of responses of some sensory neurons (*86*). In rats and mice, it has also been shown that calcineurin desensitizes TRPV1 by dephosphorylating it (*280*). Since TPRV1 has been shown to play a role in salt taste, calcineurin may regulate salt taste in mammals.

We identified a Gα subunit *odr-3* and an additional GCY *gcy-35* that function in the *gcy-14*/*gcy-22*/*tax-2*/*cng-3*/*osm-9* pathway. G proteins have been implicated in several taste responses in mammals but not in salt detection. For example, the Gα subunit gustducin is involved in the detection of sugar, amino acids, and bitter compounds (*281-282*). We have for the first time identified a G protein that is involved in chemotaxis to NaCl. The Gα protein subunit ODR-3 is involved in all three responses to NaCl that we distinguish. ODR-3 is needed in the ASH neurons for avoidance of high salt concentrations and it is needed in the ADF neurons in gustatory plasticity (*15, 94*). Here we have shown that ODR-3 is also needed in the ADF neurons for chemotaxis to NaCl. At present it is unclear in which signaling pathway ODR-3 functions in NaCl chemotaxis but it is tempting to speculate it functions together with OSM-9. OSM-9 and OCR-2 are expressed in the ADF neurons together with ODR-3. Since ODR-3 acts in ADF in NaCl sensation, we propose that OSM-9 and perhaps OCR-2

may function in conjunction with G-protein signaling in NaCl sensation in the ADF neurons. Alternatively, ODR-3 could function downstream of the OSM-9 TRPV channel, or it could play a role in modulating the activity of NaCl detecting channels in ADF.

Our analyses have identified four new genes involved in NaCl taste in *C. elegans*. Since very little is known about the signal transduction of salt sensation in mammals, it would be worthwhile to investigate the involvement of homologues of these genes in NaCl sensation in mammals.

Materials and Methods

Strains, genetics and germline transformation

Strains used in this work are *tax-2(p671)*, *tax-2(p691)*, *tax-2(p694)*, *tax-2(ks10)*, *tax-2(ks15)*, *gcy-35(ok769)*, *tax-4(p678)*, *tax-4(ks11)*, *tax-4(ks28)*, *osm-9(ky10)*, *tax-6(p675)*, *cnb-1(jh103)*, *odr-3(n1605)*, *gcy-14(pe1102)*, *gcy-22(tm2364)* and *cng-3 (jh114)*. Wild type *C. elegans* used was the Bristol N2 strain. Rescue of *odr-3* expression in the ADF neurons was done using a *srh-142::odr-3* construct injected at a concentration of 25 ng/µl (*283-284*). Rescue of *tax-4* was achieved using constructs in which the *tax-4* cDNA is placed in an artificial operon with *gfp (108, 285)* and injected at a concentration of 5 - 25 ng/µl. Neuronal specific rescue was carried out using the following constructs: ASE - *flp-5::tax-4*, ASI – *gpa-4::tax-4*, ASE and ASI – *gpa-4::tax-4* and *flp-5::tax-4*, AQR, PQR and URX – *gcy-32::tax-4*, ASE, AQR, PQR and URX – *flp-5::tax-4* and *gcy-32::tax-4*, in all amphids neurons – *flp-5::tax-4* and *odr-4::tax-4* and in all the amphids neurons except ASE – *odr-4::tax-4*. Germline transformation was performed as described (*286*). We used an *elt-2::GFP* construct as co-injection marker (*287*).

Behavioural assays

Chemotaxis towards NaCl was assessed as described before (*15, 167, 254*). A chemotaxis index was calculated: (A-C)/(A+C), where A is the number of animals at the quadrants with NaCl, and C is the number of animals at the quadrants without attractant. Statistical significance was determined using the two-tailed t-test, error bars represent standard error of means S.E.M. One-way ANOVA and Bonferroni tests were used to compare datasets with more than two samples.

Imaging

To express GCaMP (*269*) in the ASE neurons, we injected a *flp-6::*GCaMP3 construct

into wild type, *tax-2(p671)* and *tax-4(p678)* animals. Animals were glued onto dried 2% agarose pads using Nexaband veterinary glue (World Precision Instruments, Sarasota Florida). The glued animals were then incubated in CTX buffer (5 mM KPO_4, pH 6.6, 1 mM $MgSO_4$, 1 mM $CaCl_2$). The osmolarity of the buffer was kept constant at 325 mosmol with glycerol. The test stimuli were made by adding the required quantity of NaCl to the buffer. The animals were exposed to the stimuli through a capillary that moved close to the nose of the animals. Images were acquired with a Zeiss Axiovert 200M microscope, fitted with a Harvard apparatus MC-27 flow chamber. We used a custom automation in Improvision Openlab to control the movement of the capillary and to acquire images. Neuronal calcium responses were determined by measuring changes in fluorescence of GCaMP upon stimulation with NaCl and comparing to the baseline GCaMP fluorescent levels before stimulation with NaCl.

Acknowledgements
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3

Gustatory plasticity in *Caenorhabditis elegans* involves opposing changes in the sensitivities of gustatory and nociceptive neurons

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Abstract

Neuronal plasticity allows animals to alter their behavioural responses based on previous experiences. Despite increasing knowledge about the molecular mechanisms underlying neuronal plasticity, it remains a major challenge to directly correlate these processes to the behaviour of living animals. We study the plasticity of *C. elegans'* behavioural response to NaCl. *C. elegans* is attracted to low and avoids high concentrations of NaCl. However, after prolonged exposure to attractive NaCl concentrations in the absence of food, *C. elegans* avoids previously attractive NaCl concentrations. This behavioural change is called gustatory plasticity. By using single cell Ca^{2+} imaging in awake animals, we show that pre-exposure to NaCl affects the sensitivity of both gustatory and nociceptive neurons. The ASEL gustatory neuron that normally responds to NaCl increase is desensitized, while the ASER gustatory neuron that responds to a decrease in NaCl concentrations and the ASH nociceptive neurons are sensitized after pre-exposure. Desensitization of ASEL involves serotonin indicating that this process is at least partially non-cell autonomous. The sensitization of the ASH neurons requires signals from the ASE neurons and is strongly affected in serotonin, dopamine, glutamate and neuropeptide signaling mutants. We propose that gustatory plasticity in *C. elegans* is driven by opposing changes in the sensitivities of gustatory and nociceptive neurons.

Introduction

Neuronal plasticity allows animals to alter their behavioural responses as a result of previous experiences and underlies the processes of associative and non-associative learning. Results obtained in the last decades have taught us much about the molecular mechanisms of neuronal plasticity. However, learning processes require information processing in neural circuits involving various cells and many synaptic connections (*288*). One of the major challenges in neuroscience is to directly correlate neuronal plasticity throughout a neural circuit to the behaviour of a living animal.

C. elegans senses many chemical and physical cues in its environment and its response to these cues can be reversibly regulated by prior experience (*136-137*). We study a behavioural switch in the response of *C. elegans* to NaCl. Naïve *C. elegans* is attracted to NaCl concentrations up to 200 mM but avoids higher concentrations (*15, 68, 79, 155, 167, 263*). However, after 15 minutes pre-exposure to 100 mM NaCl in the absence of food, it strongly avoids all NaCl concentrations. This response is called gustatory plasticity. Longer pre-exposure to NaCl in the absence of food induces a starvation response and causes even stronger avoidance of NaCl called starvation-enhanced gustatory plasticity or salt chemotaxis learning (*169-170*). Previous studies have identified a number of proteins that play a role in gustatory plasticity and have shown that the ASE gustatory neurons, the ASH nociceptive neurons and the ASI and ADF neurons are involved (*15*). Communication between these and perhaps other neurons involves serotonin, dopamine, glutamate and neuropeptide neurotransmission (*169*).

Attraction to NaCl is primarily mediated by the bilaterally asymmetric ASE neurons (4, 12). *che-1* mutant animals, which lack functional ASE neurons (*82*), are not attracted to low NaCl concentrations, but avoid high NaCl concentrations just like wild type animals, confirming that the ASE neurons are essential for attraction, but not required for avoidance (*15*). Avoidance of high NaCl concentrations (osmotic avoidance) is mediated by the ASH neurons (*130, 263*). *odr-3* mutant animals, which lack the Gα protein ODR-3 essential for ASH activity, showed strong attraction to even lethal concentrations of NaCl (*15*). These data suggest that the naïve response to NaCl is a balance between attraction mediated primarily by the ASE neurons and avoidance mediated by ASH. We hypothesized that prolonged exposure to NaCl in the absence of food modulates these pathways making the animals avoid previously attractive NaCl concentrations. Here we used cell specific Ca^{2+} imaging in awake animals to visualize the responses of the ASE gustatory and ASH nociceptive neurons, both in naïve animals and in animals pre-exposed to NaCl.

Results

We determined the dynamic range of the naïve responses to NaCl of the ASE and ASH neurons, using the Ca^{2+} reporter Yellow Cameleon (*257*, *289*). Suzuki *et al (81)* have shown that the left ASE neuron, ASEL, produces Ca^{2+} transients in response to an increase in NaCl concentration, whereas ASER responds to a decrease in NaCl. The ASH neurons yield Ca^{2+} transients in response to various nociceptive compounds, including osmotic stimuli (*101*). We were able to reproduce these responses in the ASE and ASH neurons. In agreement with our behavioural NaCl chemotaxis data, ASEL neurons showed Ca^{2+} fluxes in response to a 3 seconds exposure to both low and high NaCl concentrations (Figure 1A). In contrast, the ASER neurons did not respond to a 3 seconds exposure to NaCl neither at low nor at high concentrations (Supplementary Figure 1). In addition, we found that the ASH neurons showed a gradual increase in Ca^{2+} transients in response to increasing concentrations of NaCl starting at 200 mM NaCl (Figure 1B). Together, our imaging and previous behavio-

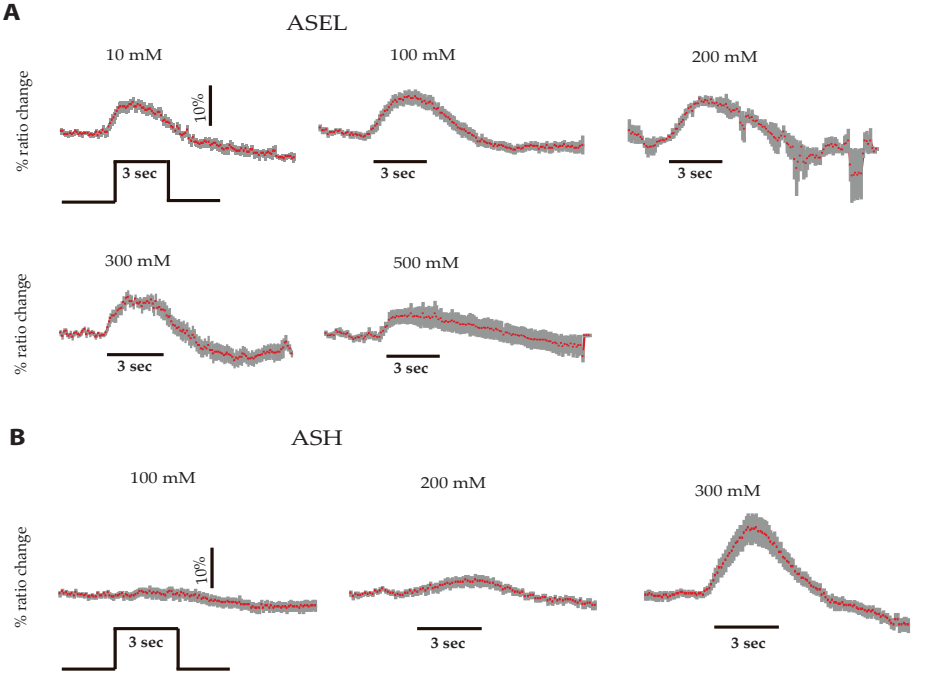

Figure 1. **Naïve responses of ASEL and ASH to different concentrations of NaCl.** Animals were stimulated for 3 seconds with different concentrations of NaCl from a baseline of 0 mM. In these and other experiments average traces of neuronal Ca^{2+} transients in each neuron are shown and traces indicate average percentage change in R/R_o where R is the fluorescence emission ratio and R_o the baseline fluorescence emission ratio before stimulation with NaCl. Gray traces along data represent standard error of the mean (SEM). (A) ASEL responded to

10-500 mM NaCl. 10 mM (n = 10), 100 mM (n = 38), 200 mM (n = 9), 300 mM (n = 15), 500 mM (n = 4). (B) ASH showed gradually increasing Ca^{2+} fluxes to increasing NaCl concentrations. 100 mM (n = 18), 200 mM (n = 18), 300 mM (n = 22).

ural data in naïve animals fit a two-factor model in which *C. elegans* is attracted to all NaCl concentrations, mediated primarily by the ASE neurons, but that this attraction can be overruled by osmotic avoidance, mediated by the ASH neurons, resulting in avoidance of NaCl above 200 mM.

What neuronal events occur after prolonged exposure to NaCl? The observed avoidance of *C. elegans* after pre-exposure to NaCl could be the result of desensitization of attraction and/or sensitization of avoidance, or an alternative factor that overrules the naïve behaviour. To determine if the Ca^{2+} transients of the ASE and/or ASH neurons change after pre-exposure, we exposed animals to 100 mM NaCl for a period ranging from 30 seconds to 10 minutes and after a brief wash (30 - 60 seconds) tested their response to 100 mM NaCl. Ca^{2+} responses in ASEL neurons were strongly reduced or even abolished after 5 or 10 minutes pre-exposure to 100 mM NaCl, but unaffected or only slightly reduced after 1 or 2 minutes pre-exposure (Figure 2A and results not shown). This is in line with behavioural data which showed gradually

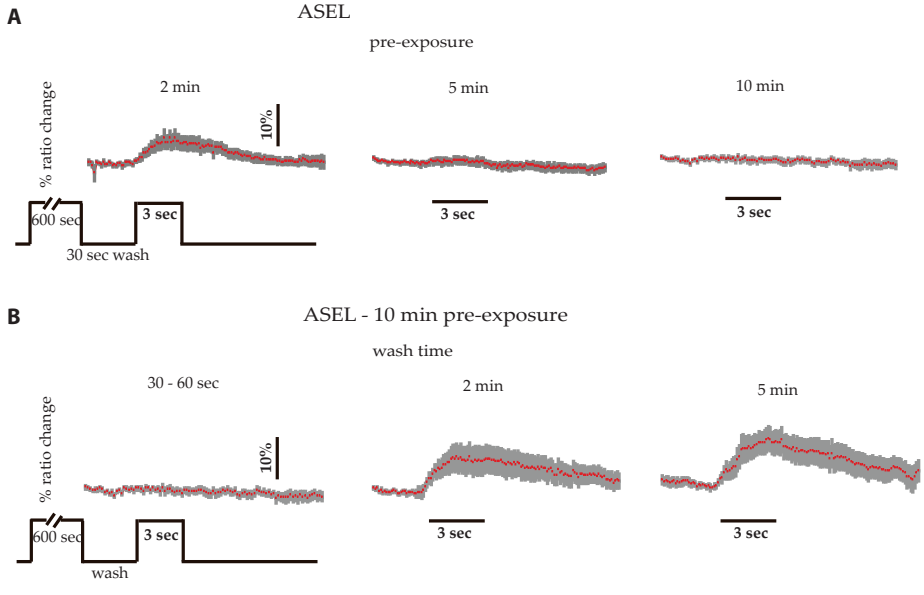

Figure 2: **Pre-exposure desensitizes ASEL.** (A) Animals were pre-exposed to 100 mM for different lengths of time before re-exposure to 100 mM. The Ca^{2+} response of ASEL neurons

upon 3 seconds exposure to 100 mM NaCl was lost after 5 or 10 minute pre-exposure to 100 mM NaCl but not after 2 minutes pre-exposure. Pre-exposure 2 min (n = 7), 5 min (n = 7), 10 minutes (n = 17). 30 sec – wash (30 – 60 seconds wash between pre-exposure and re-exposure to NaCl). (B) Animals were pre-exposed to 100 mM NaCl for 10 minutes, washed for 1, 2 or 5 minutes with a NaCl-free buffer and re-exposed to 100 mM NaCl. 2 minutes or longer wash with a NaCl-free buffer restores the Ca^{2+} response of ASEL to 100 mM NaCl. Wash time 1 minute (n = 17), 2 minutes (n = 9), 5 minutes (n = 8).

reduced attraction to NaAc with increasing pre-exposure times (*167*). Desensitization of ASEL is however short-lived. When animals were first pre-exposed to 100 mM for 10 minutes and then exposed to a NaCl-free buffer for two minutes or longer before re-exposure to NaCl, we did observe Ca^{2+} transients in ASEL neurons (Figure 2B). Also these responses are in agreement with behavioural data where we have shown that attraction to NaCl after pre-exposure is restored by a 5 minutes wash (8).

Suzuki *et al.* (*81*) have shown that the ASER neurons respond to a decrease in NaCl concentrations. We could not detect a Ca^{2+} flux in the ASER neurons in response to a decrease in NaCl concentration from a baseline of 100 mM to 0 mM after 30 seconds exposure to NaCl (Figure 3A). However, longer exposure to NaCl did yield Ca^{2+} fluxes in ASER, which increased with longer exposure time, indicating that the ASER neurons became sensitized by prolonged exposure to NaCl (Figure 3A).

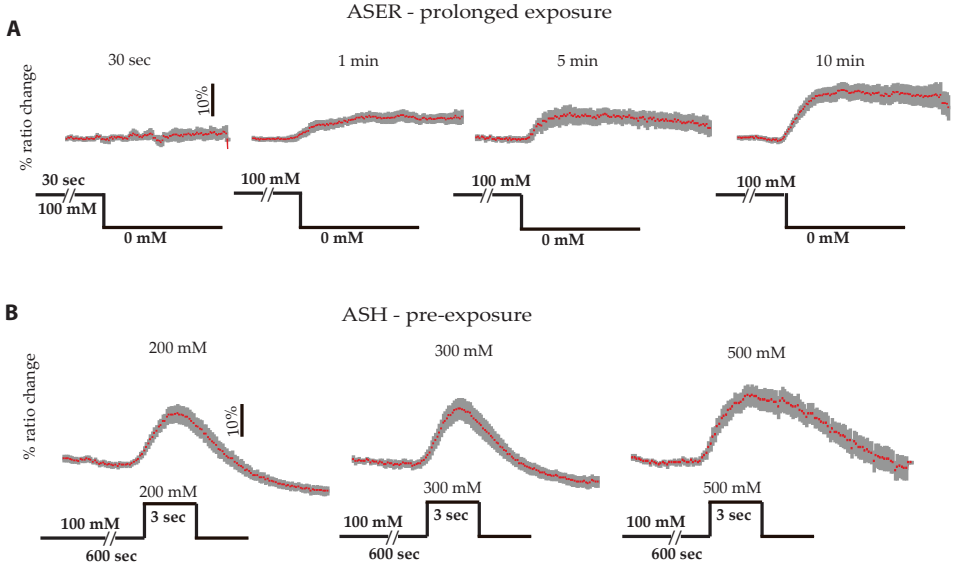

Figure 3: **Pre-exposure sensitizes ASER and ASH.** (A) 30 seconds exposure to 100 mM NaCl did not result in a response in ASER upon removal of NaCl. Increasing pre-exposure times

gave increasing responses in ASER. 30 seconds (n = 8), 1 minute (n = 12), 5 minutes (n = 6), 10 minutes (n = 15). (B) The response of ASH to 200 mM NaCl was increased after pre-exposure to 100 mM NaCl, while the responses to 300 and 500 mM NaCl were unchanged (compare to Figure 1C). 200 mM (n = 17), 300 mM (n = 11), 500 mM (n = 24).

We observed significant Ca^{2+} transients in the ASH neurons after exposure to NaCl concentrations above 200 mM but not to lower concentrations (Figure 3B). To test if the response of the ASH neurons is affected after pre-exposure to 100 mM NaCl, we exposed animals to 100 mM NaCl for 10 minutes and subsequently exposed them to 200 mM NaCl. This procedure resulted in significant responses to 200 mM NaCl, indicating that the ASH neurons are also sensitized during pre-exposure to NaCl (Figure 3B). Pre-exposure did not affect Ca^{2+} transients in ASH neurons in response to higher NaCl concentrations (Figure 3B). Taken together, our results show that gustatory plasticity involves desensitization of ASEL and sensitization of ASER and ASH.

As we have shown previously that the ASE neurons are required for naïve NaCl chemotaxis and for gustatory plasticity (*15*), we next tested if the ASE neurons are required for sensitization of ASH. We tested the Ca^{2+} response of the ASH neurons of *che-1* animals, which lack functional ASE neurons (*82*). The naive response of *che-1* animals to 200 mM NaCl was very similar to the response of wild type animals (Figure 4A). Interestingly, *che-1* animals pre-exposed to 100 mM NaCl for 10 minutes did not or only very weakly respond when exposed to 200 mM NaCl (Figure 4B).

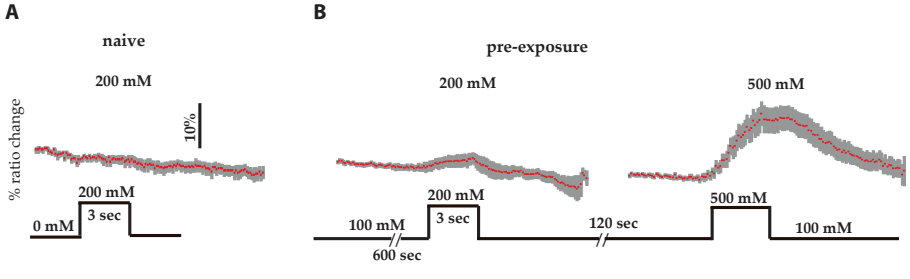

Figure 4: **ASH neurons of *che-1* animals are not sensitized after pre-exposure.** (A) Naïve *che-1* animals did not respond to 200 mM NaCl (n = 6). (B) After 10 minutes pre-exposure to 100 mM NaCl, no response to 200 mM NaCl was observed in *che-1* animals (n = 11, 3 worms showed a slight response). The same *che-1* animals did respond to 500 mM NaCl after exposure to 200 mM (n = 11).

These results suggest that input from the ASE neurons is necessary to sensitize the

ASH neurons. To control for the responsiveness of the ASH neurons of the tested animals, we confirmed that these same animals did respond to 500 mM NaCl (Figure 4B).

Our results indicate that gustatory plasticity involves communication between the ASE and the ASH neurons. To identify neurotransmitters involved in this process, we visualized the Ca^{2+} responses of the ASE and ASH neurons of several synaptic transmission mutants. We tested *unc-13(e51)* mutants which have a defect in synaptic vesicle release, *eat-4(ad819)* mutants which have a defect in synaptic glutamate transport and *unc-31(e928)* and *egl-3(ok979)* mutants which have neuropeptide signaling defects. Mutations in these genes did not affect ASEL desensitization or ASER sensitization (data not shown). However, all four mutations did affect sensitization of the ASH neurons (Figure 5 and supplementary figure 2). These results confirm that sensitization of ASH is non-cell autonomous and requires glutamate and neuropeptide neurotransmission which is in agreement with behavioural analyses (*169*).

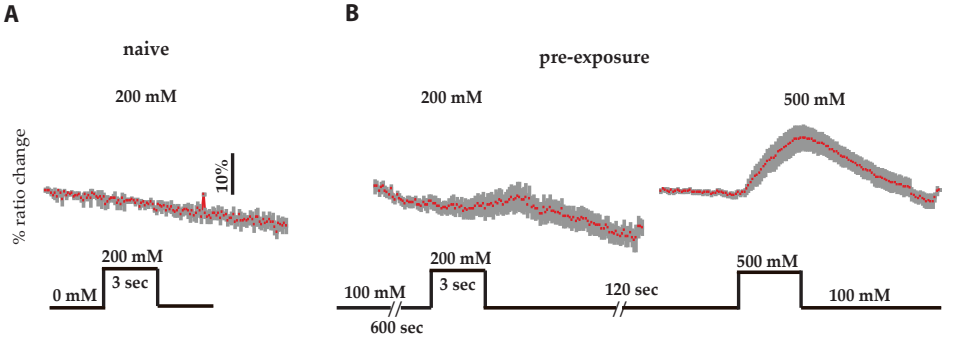

Figure 5: **ASH neurons of *unc-13* mutants are not sensitized after pre-exposure.** (A) ASH neurons of naïve *unc-13* animals did not respond to 200 mM (n = 3). (B) Pre-exposure to 100 mM NaCl did not sensitize the ASH neurons (n = 8). The same ASH neurons however did respond to 500 mM NaCl (n = 8).

Next, we tested serotonin (*tph-1)* and dopamine (*cat-2)* synthesis mutants, since these neuromodulators are involved in gustatory plasticity (*169*). The ASER neurons of *tph-1* animals showed wild type sensitization (data not shown). Interestingly, *tph-1* mutant animals showed less desensitization of ASEL; 5 out of 9 animals tested showed a response to NaCl after pre-exposure (Figure 6A). To confirm that the defects in ASEL responses of *tph-1* animals were caused by lack of serotonin, we cultured *tph-1*

animals for 72 hours on plates containing 2 mM serotonin and subsequently tested the Ca^{2+} responses in the ASEL neurons of these animals. 7 out of 9 *tph-1* animals cultured on serotonin showed desensitization of ASEL (Figure 6B), confirming that this neurotransmitter plays a role in desensitization of this cell. These results also suggest that desensitization of ASEL after pre-exposure is not cell autonomous and requires input from one or more other cells. Also sensitization of the ASH neurons was affected in *tph-1* mutant animals (Figure 6C). ASH sensitization could be rescued by culturing *tph-1* animals on serotonin (Figure 6D).

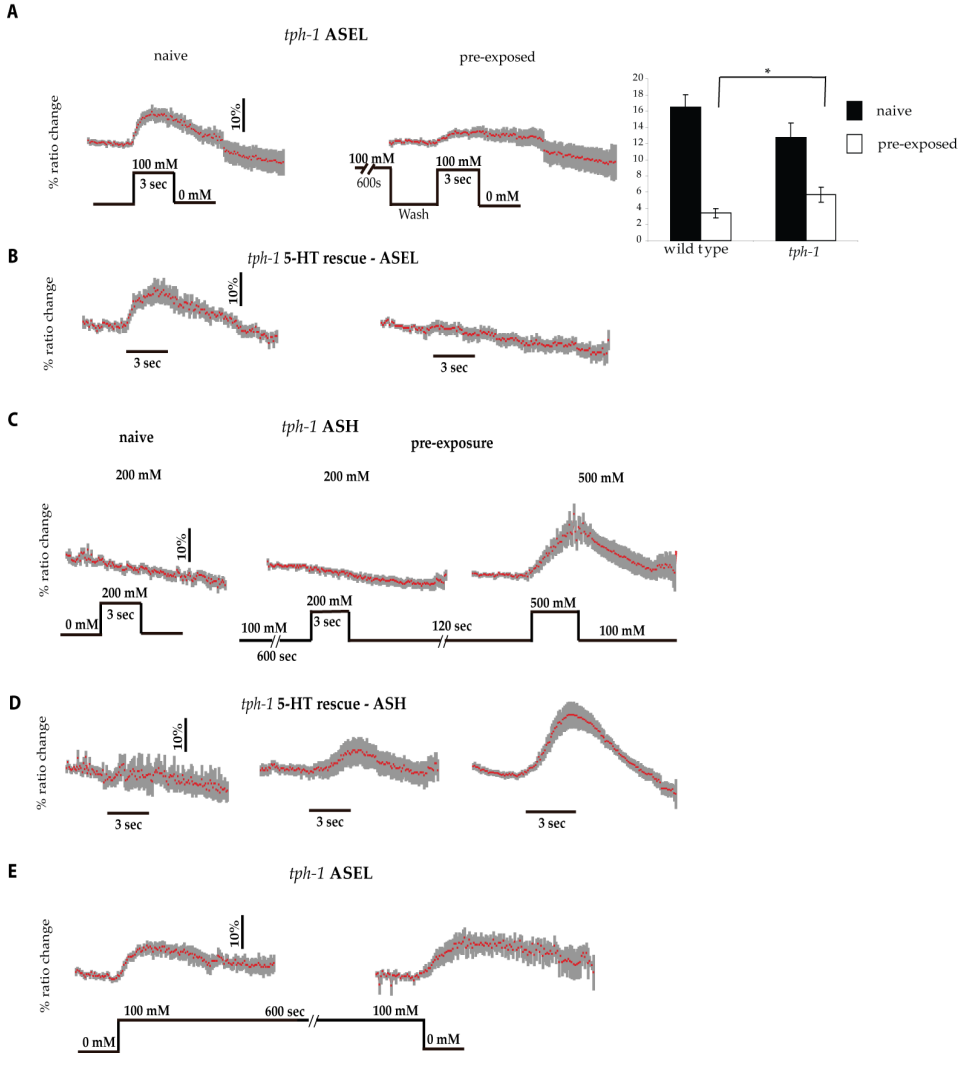

Figure 6: **Serotonin signaling is involved in gustatory plasticity.** (A) Ca^{2+} imaging in ASEL

neurons of *tph-1* animals showed wild type responses to 100 mM. After pre-exposure 5 out of 9 animals still showed responses to 100 mM NaCl. Naïve (n = 9), pre-exposed (n = 9). When compared to wild type animals, the response seen in the ASEL neurons of *tph-1* animals was significantly different from that seen in the wild type animals after pre-exposure. * - $p<0.036$. (B) 5-HT rescued the response in the ASEL neurons of *tph-1* animals leading to desensitization after pre-exposure . Naïve (n = 9), pre-exposed (n = 9, 2 worms gave a 4 and 4.5% ratio change). (C) The ASH neurons of *tph-1* animals did not show any Ca^{2+} transients upon exposure to 200 mM NaCl after pre-exposure to 100 mM NaCl. 200 mM naïve (n = 5), 200 mM pre-exposed (n = 8), 500 mM control (n = 8). (D) 5-HT rescued the response in the ASH neurons of *tph-1* animals leading to sensitization after pre-exposure. 200 mM naïve (n = 10), 200 mM pre-exposed (n = 10), 500 mM control (n = 10). (E) The ASEL neurons of 6 *tph-1* worms responded to both an increase and a decrease of 100 mM NaCl. (n = 6 in both cases).

Previous studies have shown that serotonin signals the presence of food in *C. elegans (290)*. It is unclear how serotonin functions in gustatory plasticity where the presence of NaCl is associated with the absence of food. Interestingly, the ASEL neurons of 6 *tph-1* animals showed Ca^{2+} responses to both an increase and a decrease in NaCl concentration (Figure 6E) suggesting that these animals have lost ASEL/R asymmetry. This phenotype could indicate a requirement of serotonin for the proper development of the ASE neurons.

To determine whether dopamine plays a role in sensitization or desensitization of the ASE and/or ASH neurons, we tested *cat-2* animals, which do not synthesize dopamine. These animals showed wild type levels of desensitization of their ASEL neurons. However, the ASH neurons of *cat-2* animals were not sensitized after pre-exposure (Figure 7A and B). This supports the behavioural data that dopamine is also required for gustatory plasticity (*169*).

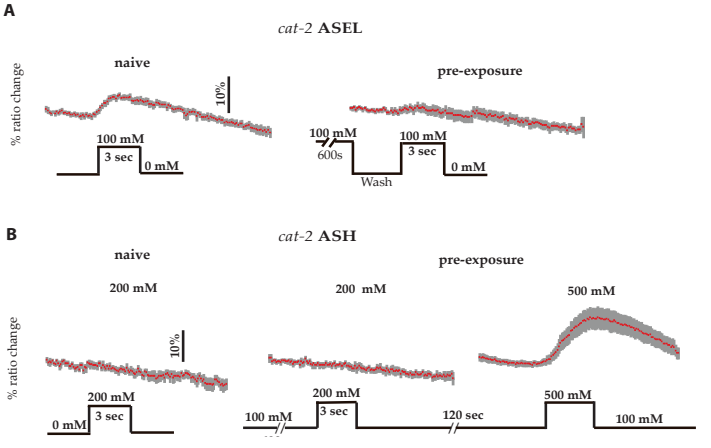

Figure 7: **Dopamine signaling is involved in gustatory plasticity.** (A) The ASEL neurons of *cat-2* animals are desensitized by pre-exposure to 100 mM NaCl (n = 9 in both cases). (B) The ASH neurons of *cat-2* are not sensitized after pre-exposure to 100 mM NaCl. 200 mM naïve (n = 8), 200 mM pre-exposed (n = 9), 500 mM control (n = 9). Gray traces along data represent standard error of the mean (SEM).

Taken together, our results confirm that serotonin and dopamine play important roles in gustatory plasticity (*169*). Serotonin plays a role in the proper development of the ASE neurons and in desensitization of ASEL. Furthermore, both serotonin and dopamine are required for sensitization of ASH, although this defect in *tph-1* animals might be secondary to the asymmetry defect seen in the ASEL neuron of *tph-1* animals. The identities of the neurons that release serotonin and dopamine, as well as where in the circuit the two neurotransmitters function are not yet known. However, since the serotonergic ADF neurons are common contacts between ASE and ASH neurons, and they have been shown to play a role in gustatory plasticity, at least part of the serotonin signal could originate from these cells.

Based on our results we propose that the response of *C. elegans* to NaCl is regulated at different levels. Naïve chemotaxis to NaCl is mediated by a core NaCl chemosensation machinery, comprised of the ASE neurons that mediate NaCl attraction and the ASH neurons that mediate avoidance of osmotic stresses resulting in a balance between attraction and avoidance (Figure 8). Pre-exposure to 100 mM NaCl in the absence of food results in changed dynamic ranges of both the ASE and ASH neurons. The ASEL neuron becomes desensitized while ASER and ASH become sensitized. Desensitization of the ASEL neuron involves serotonin and thus, signals from one or more other neurons. The identity of these neurons is not known, but these might be the ADF neurons since they are serotonergic and/or the ASI neurons since they are involved in sensation of food cues (*291*). Thus far, we have not identified a neurotransmitter that plays a role in ASER neuron sensitization after prolonged exposure to NaCl, suggesting that ASER sensitization might be cell autonomous. Sensitization of ASH requires signals from the ASE neurons, glutamatergic, serotonergic, dopaminergic and neuropeptide signaling, illustrating the complexity of this seemingly simple behavioural paradigm. This relatively simple experimental system provides a direct correlation of neuronal plasticity to behaviour, which will allow a further detailed study of the molecular and cellular mechanisms of behavioural plasticity.

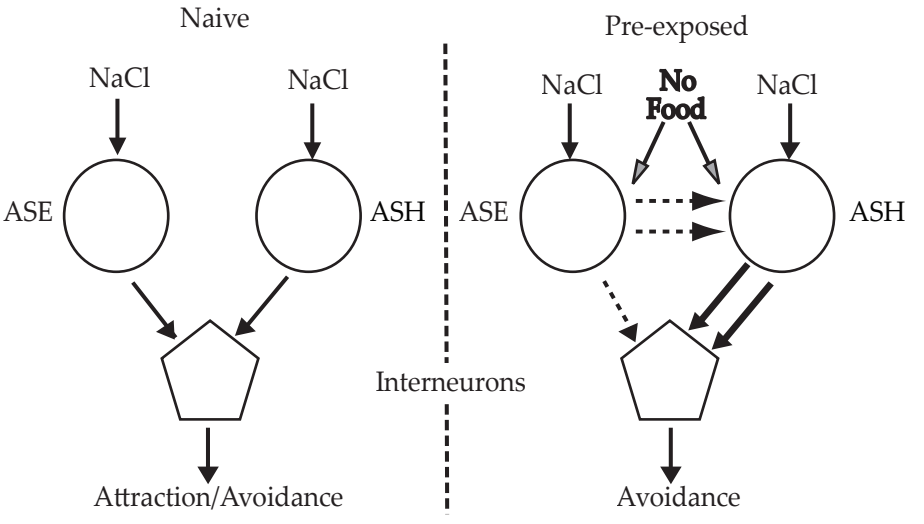

Figure 8. **Model of NaCl chemosensation of naïve animals or animals pre-exposed to 100 mM NaCl.**

Experimental Procedures

Strains and germline transformation

Wild-type *C. elegans* strain used was Bristol N2. The following mutant alleles were used: *che-1(p679), tph-1(mg280), cat-2(tm2261), eat-4(ad819), unc-13(e51), unc-31(e928)* and *egl-3(ok979).* Germline transformation was performed as described (*286*), using an *elt-2::GFP* construct as co-injection marker (*287*). Promoters used for expressing the Yellow Cameleon (YC3.60) construct (*289*) were *sra-6* for ASH (*100*) and *flp-6* for ASE (*292*).

Cameleon imaging

Images were acquired with a Zeiss Axiovert 200M microscope, fitted with a Harvard apparatus MC-27 flow chamber. The naïve wash buffer contained 5 mM K_2HPO_4/ KH_2PO_4, pH 6.6, 1 mM $MgSO_4$, 1 mM $CaCl_2$, the pre-exposure buffer contained an additional 100 mM NaCl. The osmolarity of both buffers was set to 325 mosmol, using glycerol. The stimuli for the range tests were made by adding the required quantity of NaCl to either of the buffers. Animals were glued onto 2% agarose pads using Nexaband® veterinary glue (World precision Instruments, Sarasota, Florida). Stimuli were applied by moving a capillary into the buffer close to the nose of the worm. We used a custom automation in Improvision Openlab to control the movement of

the capillary and to acquire the images. The acquired image was split into a CFP and YFP part with an Optical Insights Dualview beamsplitter (dichroic mirror 505 nm, 465/30 nm and 535/30 nm emission filters), and the intensities of the CFP and YFP fluorescent areas were recorded, normalized to the 2 seconds prior to the stimulus. The fluorescent ratio was determined by (YFP intensity)/(CFP intensity)-0.6, where the 0.6 factor corrects the bleedthrough of CFP in the YFP channel (*293*). Each datapoint represents the average of at least four independent recordings, all in different neurons.

Statistical analysis

All results are given as a mean +/- standard error of the mean.

Acknowledgements

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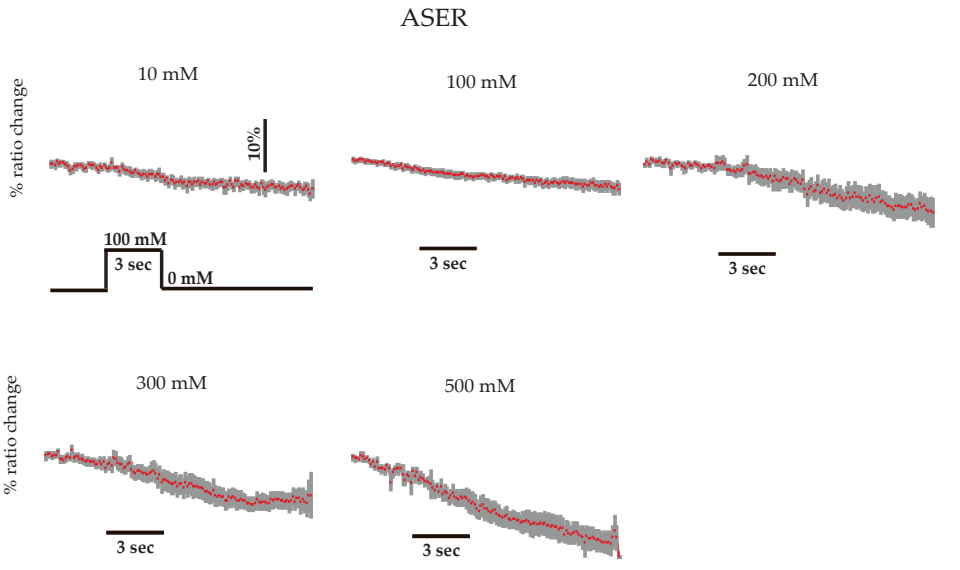
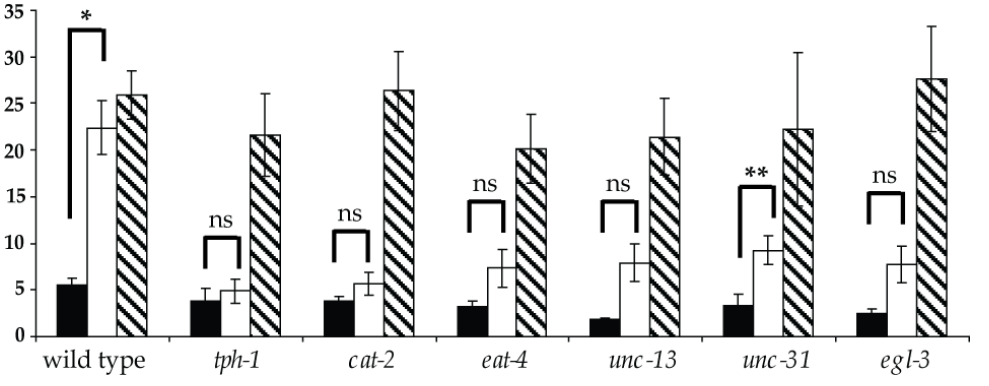
Supplementary Data.



Supplementary figure 1: The ASER neurons did not respond to a 3 seconds exposure to different concentrations of NaCl from a baseline of 0 mM. 10 mM (n = 8), 100 mM (n = 30), 200 mM (n = 5), 300 mM (n = 9), 500 mM (n = 4)



Supplementary figure 2: Ca^{2+} fluxes in the ASH neurons of naïve wild type, *tph-1*, *cat-2*, *eat-4*, *unc-13*, *unc-31* and *egl-3* animals exposed to 200 Mm NaCl from a baseline of 0 mM NaCl, (naïve – black bars), exposure to 200 mM NaCl after 10 minutes exposure to 100 mM NaCl (pre-exposed – white bars) and same animals exposed to 500 mM NaCl (controls – stripped

bars). Naïve animals did not respond to 200 mM NaCl, but after pre-exposure to 100 mM NaCl, the wild type animals ASH neurons were sensitized, * - $p<0.001$. The ASH neurons of all the mutants except *unc-31* were not sensitized after pre-exposure to 100 mM NaCl, **- $p<0.05$, ns - not significant. To test for the responsiveness of the ASH neurons of the animals, we confirmed that the same animals did respond to 500 mM NaCl.

4

Identification of genes involved in *C. elegans* chemotaxis to NaCl

Oluwatoroti Umuerri, Wilfred van Ijcken, Gert Jansen

Work in progress

Abstract

C. elegans is attracted to low concentrations of NaCl up to 200 mM and repelled by concentrations above 200 mM NaCl. However, little is known about the signal transduction pathway(s) involved in *C. elegans* salt chemotaxis. We carried out a forward genetic screen to identify additional genes that function in NaCl chemotaxis. We found 27 mutants that showed little or reduced chemotaxis to 100 mM NaCl. Using single nucleotide polymorphism (SNP) mapping and whole genome sequencing we identified likely causal mutations in 11 mutants. In all these mutants, mutations were found in genes already known to play a role in salt chemotaxis. Whole genome sequencing of 2 mutants did not reveal mutations in known NaCl chemotaxis genes. These mutants likely contain mutations in new NaCl chemoaxis genes.

Introduction

Little is known about the molecular mechanism of salt taste. Since many genes have been conserved during evolution, *C. elegans* is a good model to characterize the molecular mechanism of salt taste. *C. elegans* is robustly attracted to low concentrations of NaCl but it avoids concentrations above 200 mM. Some genes involved in NaCl chemotaxis in *C. elegans* have been identified. These include *tax-2* and *tax-4* (cyclic nucleotide-gated channel subunits) (*68*), *tax-6* and *cnb-1* (calcineurin subunits) and *gcy-14* and *gcy-22* (receptor-type guanylyl cyclases) (chapter 2) (*87*). GCY-14 and GCY-22 have been suggested to function as Na^+ and Cl^- receptors, respectively, but this remains to be proven. In addition, some genes involved in avoidance of high concentrations of NaCl have been identified. These include *odr-3* (Gα protein) (*15*) and *osm-9* (TRPV channel subunit) (*101*). In mice, ENaC (epithelial sodium channel) (*30*) is required for NaCl sensation, but there are no indications that ENaC channel plays a role in NaCl taste in *C. elegans*. To better understand how NaCl is detected by *C. elegans*, more genes involved in salt sensation have to be identified and their functions delineated.

C. elegans behavioral response to NaCl is studied using two main methods. One method uses a diffuse NaCl gradient assay method in which the worms navigate on an agar plate containing a diffuse NaCl gradient emanating from a point source (*294*). The other method uses a steep NaCl gradient in which two different NaCl concentrations are clearly demarcated by 4 quadrants of an assay plate (*167, 254*). Opposite quadrants have different NaCl concentrations. In the diffuse assay method, *tax-2* and *tax-4* single mutant worms showed no significant chemotaxis to NaCl (*68*). However, in the gradient plate assay method, they did show significant chemotaxis to NaCl, although not to the level of wild type animals (*15*). Using the latter assay method, we have shown that *C. elegans* uses two parallel genetic pathways for NaCl chemotaxis. One pathway involves *gcy-14, gcy-22, tax-2, cng-3* (cyclic nucleotide-gated channel subunit 3), *odr-3, osm-9* and *gcy-35* (soluble guanylate cyclase) and the other pathway involves *gcy-22, tax-2, tax-4, tax-6* and *cnb-1* (chapter 2).

To identify additional genes that function in NaCl chemotaxis in *C. elegans*, we performed a forward genetic screen. To increase our chances of finding mutants we used *odr-3* loss-of-function animals in our screen. *odr-3* animals show wild type levels of NaCl chemotaxis, but double mutants of *odr-3* with *tax-4* or *tax-6* have completely lost chemotaxis to NaCl. *tax-2; odr-3* animals behaved just like *tax-2* animals. We isolated 27 mutants from EMS mutagenic screen of *odr-3* animals. Whole genome sequencing of 13 independent mutants identified mutations in already known NaCl

chemotaxis genes in 11 mutants. The remaining 2 mutants do not have mutations in any known NaCl chemotaxis genes. We are currently mapping the mutated genes in these 2 mutants.

Results

To optimize the screen conditions, we first tested the behaviors of wild type, *tax-4*, *odr-3* and *tax-4; odr*-3 animals in NaCl chemotaxis assays using 1, 10, 25, 50 and 100 mM NaCl (Figure 1). The assays in which we gave the animals a choice between 100 mM and 0 mM NaCl discriminated best between *tax-4; odr*-3 and the 3 other strains tested.

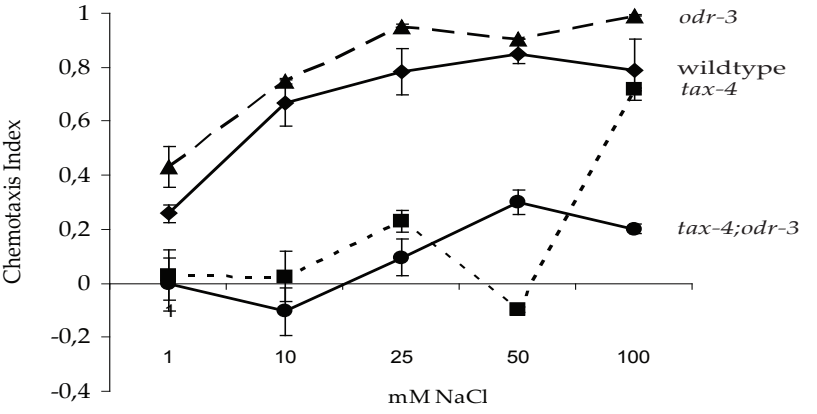


Figure 1: **Chemotaxis responses at different NaCl concentrations.** wildtype, *tax-4*, *odr-3* and *tax-4; odr-3* animals were tested for their responses to 1, 10, 25, 50 and 100 mM NaCl. *tax-4; odr-3* animals showed a highly reduced response at 100 mM NaCl, which is distinct from the responses of wild type, *tax-4* and *odr-3* animals. Shown are averages of at least 4 assays ± s.e.m.

Five L4 animals, mutagenized with 50 mM EMS (P0s) were transferred onto a plate (20 plates in total per EMS screen) and allowed to lay eggs for 24 hours. Subsequently, the P0 animals were removed and the F1 animals were allowed to grow to adulthood. There were approximately 110 F1 animals per plate. The F1 animals were collected and bleached and the eggs (F2) were transferred to new plates. Adult F2 animals were assayed with a modified quadrant assay method. In these assays, one half of the quadrant plate was filled with buffered agar containing 100 mM NaCl (test half) and the other half was filled with only buffered agar (control half). The animals that migrated to the control halves were washed off after 10 minutes and plated on a new

agar plate with food. To enrich for real mutants, we repeated the assays on the same day and again kept the animals that migrated to the control halves. The recovered animals were cultured on an agar plate with food and assayed for a third time on the next day. Finally, we picked the worms that went to the control halves individually onto new plates. The progeny of these 3 times enriched, putative mutants were staged by bleaching and tested for chemotaxis to 100 mM NaCl. Cultures that showed a NaCl chemotaxis defect were saved for further characterization.

In total, we screened approximately 22,000 genomes for NaCl chemotaxis defective mutants in five screens. The 3-fold enrichment resulted in assays of over 350 individual putative mutants of which 28 showed a chemotaxis defect (Figure 2). 1 mutant showed a dye filling defect which suggests a ciliary defect and was discarded. Of the remaining 27 mutants the following mutants were also isolated from the same cultures: *gj1015*, *gj1016*, *gj1024* and *gj1044*; *gj1020* and *gj1021*; *gj1017*, *gj1022*, *gj1023* and *gj1043*; *gj1039*, *gj1041* and *gj1042*; and *gj1026* and *gj1034*. The phenotypes of some mutants were quite weak and they were not further characterized. These include *gj1013*, *gj1016*, *gj1018*, *gj1030* and *gj1035*. This leaves 13 independent mutants and these were further analyzed.

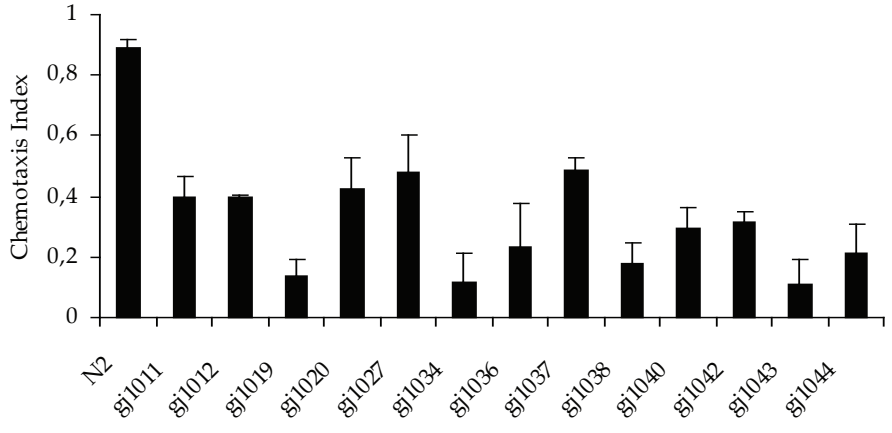


Figure 2: **13 mutants isolated in four EMS screens.** Chemotaxis to 100 mM NaCl was defective in 13 independent mutants isolated from 4 different EMS screens. Indicated are averages of at least 4 assays ± s.e.m.

Mutants *gj1042*, *gj1043*, and *gj1044* were selected for sequencing of the *che-1* and the *ceh-36* genes by Sanger sequencing because they showed the strongest NaCl

chemotaxis defects from two different plates. Of these, only *gj1044* contains a mutation in the *che-1* gene: a G → A transition at the splice donor site of intron 4 of *gj1044* animals (Figure 3A). To confirm that the chemotaxis defect of *gj1044* was caused by mutation in *che-1* gene, we introduced wild type *che-1* gene in *gj1044* animals (Figure 3B). As a control, a similar rescue experiment was performed on *che-1(p679)* mutant animals. Chemotaxis to NaCl was restored by the transgene in both *gj1044* and *che-1(p679)* animals (Figure 3B). *gj1044* was not further characterized.

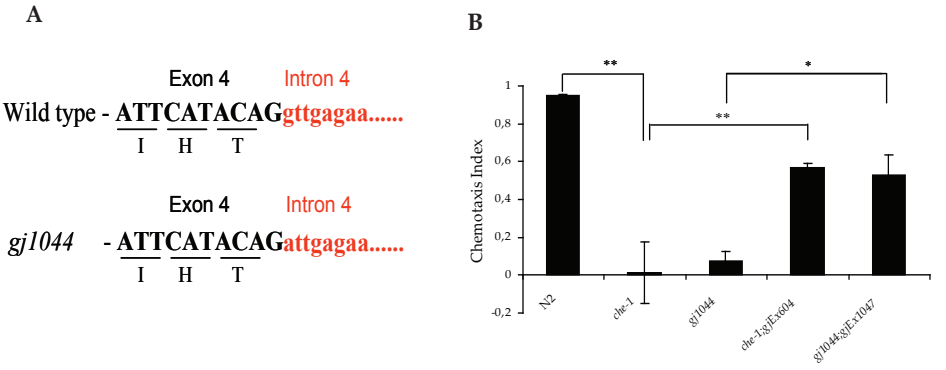


Figure 3. ***gj1044* is a *che-1* allele.** (A) *gj1044* contains a G → A transition which affects the splice donor site in intron 4 of the *che-1* gene. (B) Chemotaxis to 100 mM NaCl of wild type, *che-1*, gj1044 and *che-1* and *gj1044* strains rescued with the wild type *che-1* transgene (*gjEx604* and *gjEx601047* respectively). * - p<0.05, ** - p< 0.0001. Shown are averages of at least 4 assays ± s.e.m.

To identify the genes mutated in *gj1042* and *gj1043*, we mapped the mutant loci using single nucleotide polymorphism (SNP) mapping (*295-298*). The most common SNP analysis used in *C. elegans* is based on SNPs in the genomes of two wild type *C. elegans* strains; CB4856 isolated in Hawaii, USA and N2 strain isolated in Bristol, UK. On average, the genomes of these two *C. elegans* strains contain one SNP at every 3000 bp. Some SNPs affect restriction enzyme sites in either the N2 or CB4856 genome, which can be used to visualize the presence of N2 or CB4856 SNPs. We crossed *gj1042* and *gj1043*, which were generated in the N2 background, with CB4856 males and tested the chemotaxis response of F3 cultures derived from individual F2 cross progeny. Using this approach we selected over 30 cultures from *gj1042* crossed with CB4856 that showed a mutant NaCl chemotaxis phenotype and over 50 cultures that showed a wild type phenotype. We obtained similar numbers of backcrossed strains for *gj1043*/CB4856 cross showing either a wild type or mutant phenotype. DNA was isolated from pooled animals from all *gj1042*/CB4856 mutant strains, all *gj1042*/

CB4856 wild type strains, *gj1043*/CB4856 mutant strains and *gj1043*/CB4856 wild type strains. Subsequently, for each of *C. elegans* 6 chromosomes, SNPs covering the whole chromosome were amplified from each of the 4 DNA pools, N2 and CB4856 control DNA samples. PCR fragments were digested with the appropriate restriction enzymes and fragments separated on an agarose gel.

This analysis showed more N2 chromosome I DNA in *gj1043*/CB4856 mutant animals than in *gj1043*/CB4856 wild type animals (Figure 4) while the frequency of N2/CB4856 DNA did not differ for the other chromosomes (Figure 4). The frequency of N2 DNA was highest at SNPs 1.4 and 1.5, indicating that *gj1043* mapped to the right arm of chromosome I. *gj1042* mapped to the middle of chromosome III. Subsequently, the genome sequence of these mutants was determined using the Illumina HiSeq 2000® sequencer yielding on average 13 times coverage. The genome sequences were analyzed using MaqGene software (*299*) which compares the sequencing data to the N2 genome sequence and reports mutations found and uncovered regions in the sequencing data. The uncovered regions may be deletions, sequencing errors or problems with the analysis. MaqGene sorts the output based on the genomic positions of the mutations, the frequency at which a mutation is seen and the type of mutation. *gj1042* turned out to be a pre-mature stop mutation in the *tax-4* gene (table 1). The mutation is a G → A transition in exon 4 and it converts a tryptophan to a stop codon. g*j1043* contains a missense mutation in exon 8 of the *tax-2* gene (table 1). The mutation converts a proline to a serine at amino acid position 428 of TAX-2. The mutation is the same as the mutation found in the *tax-2(p691)* mutant allele that affects the pore-forming region of TAX-2. *gj1042* and *gj1043* were not further analyzed since they are already known NaCl chemotaxis genes.

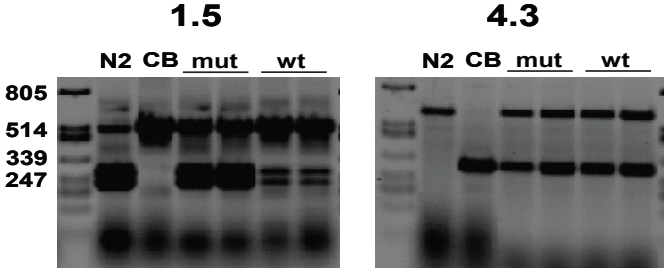


Figure 4: **SNP analysis gel maps** *gj1043* **to chromosome I.** SNP analysis was performed on *gj1043* genomic DNA. Primer sets covering SNP 1.5 located on chromosome I and SNP 4.3 located on chromosome IV were used. PCRs were preformed on Bristol N2 (wild type), CB4856, *gj1043*/CB4856 mutant strains (mut), and *gj1043*/CB4856 wild type strain (wt), digested with

EcoRI (SNP 1.5) or HindII (4.3) and separated on a 2% agarose gel. The SNP 1.5 PCR fragment of N2 animals yielded 2 fragments of 241 and 285 bp while CB4856 remains uncut. SNP 4.3 PCR fragments of N2 animals remained uncut while CB4856 DNA yields 2 fragments of 303 and 317 bp.

Next we sequenced the regions mutated in *gj1042, gj1043* and *gj1044* in the mutants that were isolated from the same cultures as *gj1042, gj1043* and *gj1044* using Sanger sequencing. *gj1017* and *gj1022* were isolated from the same culture as *gj1043* and also contain the same mutation in *tax-2. gj1039* and *gj1041* were isolated from the same culture as *gj1042* and also contain the mutation found in *tax-4. gj1015* and *gj1016* were isolated from the same cultures as *gj1044*, and *gj1015* contained the same mutation as in *che-1* as *gj1044*, but *gj1016* did not. *gj1016* also showed a weaker NaCl chemotaxis defect than *gj1044*, confirming that it is an independent mutant.

Mutant	Sequence depth	Gene	Mutation	Position	Effect on protein	Sequence output
gj1011	54	*tax-4*	Arg→stop	Exon 4	truncated protein	46x mut, 2x wt
gj1012	60	*gcy-22*	Trp→stop	Exon 9	truncated protein	65x mut, 0x wt
gj1019	46	*ift-20*	Met→Lys	Exon 1	Non-start	52x mut, 0x wt
gj1020	48	*tax-4*	Arg→stop	Exon 7	truncated protein	47x mut, 0x wt
gj1027	52	unknown				
gj1034	52	*grk-2*	Gly→Glu	Exon 5	Affects KD	43x mut, 0x wt
gj1036	47	*dyf-3*	Glu→Lys	Exon 5	Unknown	36x mut, 0x wt
gj1037	49	*che-1*	Gln→stop	Exon 2	truncated protein	51x mut, 0x wt
gj1038	61	unknown				
gj1040	27	*osm-5*	Gln→stop	Exon 10	truncated protein	24x mut, 0x wt
gj1042	27	*tax-4*	Trp→stop	Exon 4	truncated protein	20x mut, 0x wt
gj1043	29	*tax-2*	Pro→Ser	Exon 8	Affects PD	13x mut, 0x wt
gj1044	-	*che-1*	Gly→Val	Exon 4	Affects SS	-

KD – kinase domain, PD – pore domain, SS – splice site

Table 1. **Summary of whole genome sequencing analysis of 13 mutants.** The mutated gene, codon change, effect on protein and the frequency of the mutation in the mutant are shown. mut – mutant, wt – wild type. *gj1044* was sequenced by Sanger sequencing method.

We next performed whole genome sequencing of 10 more independent NaCl chemotaxis mutants. Eight mutants turned out to carry mutations in genes already

known to play a role in NaCl chemotaxis. *gj1011* contains a premature stop in exon 4 of *tax-4. gj1012* contains a mutation in *gcy-22* (receptor-type guanylyl cyclase) changing a tryptophan to a stop codon in exon 9. *gj1019* contains a non-start mutation in the *ift-20* gene, where the start codon was converted to a lysine. Further analysis of this mutant showed that it had a dye-filling defect and thus a cilia defect. This observation was missed at the beginning of this screen, when all the isolated mutants were first checked for cilia defects. *gj1020* contains a pre-mature stop codon in exon 7 of the *tax-*4 gene. *gj1034* contains a glycine to a glutamate mutation in exon 5 of *grk-2* (G protein coupled receptor kinase), which affects the kinase domain of GRK-2. *gj1036* contains a glutamine to a lysine mutation in the *dyf-3* gene. Although it did not affect any known domain of the DYF-3 protein, it suggests that these animals have a cilia defect because DYF-3 is required for normal cilia assembly (*300*). *gj1037* contains a pre-mature stop mutation in exon 2 of *che-1. gj1040* contains a pre-mature stop codon in exon 10 of the *osm-5* gene. It affects the tetratricopeptide repeat (TPR) motif region of OSM-5 (*301*). The TPR is a structural motif that plays an important role in protein-protein interactions (*302*). OSM-5 is an intraflagellar transport protein, suggesting that *gj1040* affects the cilia. Indeed *gj1040* animals are dye filling defective.

The mutated genes causing the NaCl chemotaxis defect in *gj1027* and *gj1038* are not yet known. Whole genome sequencing of *gj1027* and *gj1038* revealed over 100 mutated genes in their genomes but none of these mutations is known to affect NaCl chemotaxis. SNP mapping is currently being performed to delimit the chromosomal regions of the mutation(s) that cause the NaCl chemotaxis defect and the causal mutations for the NaCl chemotaxis defects will be determined.

Materials and methods

Strains

C. elegans strains used are Bristol N2, CB4856, *odr-3(n1605), tax-4(p678)* and *che-1(p679). odr-3*(*n1605)* was used for all the EMS mutagenesis.

EMS mutagenesis

Young L4 animals were treated with 50 mM EMS in M9 buffer at room temperature for 4 hours. Afterwards, the animals were washed three times with M9 and allowed to recover on an agar plate containing food. After 2 hours, L4 animals were picked onto 20 agar plates (5 per plate) and allowed to lay eggs for 24 hours. The mothers were subsequently removed from the plates to avoid production of too much progeny. The worms were allowed to reach adulthood and then their eggs were isolated by

bleaching the animals. The progeny that hatched from these eggs (the F2 progeny of the mutagenized animals) were assayed for NaCl chemotaxis. EMS screens were carried out with an average of 2200 F1 animals per EMS screen.

Assay method

In the screen, young adult animals were assayed using a modified quadrant assay method (*167, 254*). Instead of opposite quadrants being filled with the same NaCl concentration, two neighboring quadrants were filled with the same concentration of NaCl. A thin layer of buffered agar was placed on top of the plastic ridges of the quadrants before the assay. The animals were washed three times with CTX buffer (1 mM $MgSO_4$, 1 mM $CaCl_2$ and 5 mM KPO_4) for 15 minutes and then plated on a straight line along the border of the 2 halves of the assay plates where they were given a choice between agar with 100 mM NaCl and agar without NaCl. Worms that went to the half without salt were washed off with CTX buffer and transferred to new culture plates. They were re-assayed for two more times, once on the same day and once on the second day. This is to enrich for mutants. At the end of the second re-assay, individual worms were picked onto separate plates. After the plates were full, the animals were bleached and assayed to determine their responses to NaCl. For other assays, animals were tested as decribed in (*15, 167, 254*).

Dye-filling

Isolated mutant animals were dye-filled as described in (*303*) using the fluorescent dye DiI (Molecular Probes, Eugene, OR).

SNP mapping

SNP mapping on mutants was done by crossing mutant hermaphrodites with CB4856 males. All the mutants were generated in the Bristol N2 background. After crossing the EMS mutants to CB4856 males, F1 cross progeny was identified by PCR using SNP primer set 1.4 (1.4F - GCACCTCGTCTTTGAATCTG and 1.4R – AGAACTTCTTGCGTCAGCTC). Digestion of the 691 bp fragment with HaeIII cut the CB4856 DNA into fragments of 468 and 213 bp but left the N2 DNA uncut. The F2 progeny from F1 that were cross progeny were picked and grown individually. After the plates were full, animals from each plate were assayed for NaCl chemotaxis. For each EMS mutant, cultures showing wild type and mutant phenotypes were saved while the cultures showing heterozygous phenotypes were discarded. Animals from all cultures showing a mutant phenotype were pooled and their genomic DNA was extracted. The same was done for animals from cultures showing wild type behaviour. The mutant and the wild type genomes were genotyped by PCR using

known SNP primer sets. Finally, the amplified DNA was digested with restriction endonucleases that differentially digest N2 and CB4856. The region that contains mostly N2 DNA in the mutant animals and mostly CB4856 DNA in the wild type behaving animals contains the mutated gene responsible for the phenotype.

Whole genome sequencing and analysis

Genome DNA of the mutant strain was purified and then sequenced using the ILLUMINA HiSeq 2000® sequencer (single read – single plex of 36 bp). The raw sequence data output file was uploaded onto the MaqGene website (*299*) for analysis. Various output files were generated from MaqGene which contain information about the percentage of genome coverage, a detailed list of mutations found per sequenced genome, uncovered regions and more.

Sanger sequencing

The *che-1* and *ceh-36* genes of *gj1042*, *gj1043* and *gj1044* mutant animals were sequenced with different primer sets covering all the exons and splice junctions of these genes. *che-1* and *ceh-36* genes were amplified from purified genomic DNA of the mutant animals. All DNA fragments were sequenced using the Sanger sequencing method as described in (*304-305*).

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5

General Discussion and Future Perspectives

***C. elegans:* A model for NaCl sensation**

Despite the enormous importance of NaCl to our health, how we sense NaCl is still poorly understood. One protein has been identified to play an important role in NaCl sensation in mice; the epithelial sodium channel (ENaC) (*30*). In addition, the transient receptor potential vanilloid (TRPV) channel seems to play a role in NaCl sensation (*18*), although this is not yet well defined. The amiloride-sensitive pathway is the prevalent NaCl sensation mechanism in some organisms like rodents (3), but it plays a less important role in human NaCl sensation (*306*). It seems plausible that two different pathways dedicated to NaCl sensation exist in mice, since it allows the animals to recognize and accept healthy NaCl concentrations and avoid those that are hazardous.

C. elegans is a good model organism to study the molecular mechanism of NaCl taste. *C. elegans* shows three different responses to NaCl. It is generally attracted to NaCl concentrations up to 200 mM and avoids concentrations above 200 mM. The third type of response observed in *C. elegans* is the avoidance of NaCl after prolonged exposure to attractive NaCl concentrations in the absence of food (this is called gustatory plasticity). Different assay methods are employed to study *C. elegans'* response to NaCl. One assay tests the response to NaCl in a shallow gradient (*155*). This method has been used to discover NaCl taste mutants and has identified some genes necessary for NaCl behavioural plasticity (*68, 307*). The quadrant method is another way of studying the response of *C. elegans* to NaCl (*167, 254*). It is a steep gradient setup where worms are given a choice between two known NaCl concentrations e.g. 0 mM and 10 mM NaCl. This method has also been used to identify some genes involved in NaCl sensation and behavioural plasticity (*15, 167*). These two methods mostly display similar wild type and mutant phenotypes in the worms tested. However in some cases, mutants still show significant attraction in the quadrant method while they show no attraction in the gradient assay method ((*15*) and Umuerri, Hukema and Jansen manuscript in preparation). It will be interesting to see if *C. elegans* can differentiate small changes in NaCl concentrations for example as little as 0.1 or 1 mM difference using the quadrant assay method. This could be useful to test the sensitivity of *C. elegans* to minute changes in NaCl concentration.

Two genetic pathways mediate *C. elegans* response to NaCl

We found that *C. elegans* uses two different genetic pathways to mediate its response to NaCl. *tax-2* and *tax-4*, CNG channel subunit mutants, showed no chemotaxis to NaCl using the gradient assay method (*68*). However, when these mutants were tested in the quadrant assays, they showed significant attraction to NaCl, although

to a lesser extent than wild type animals. This finding inspired us to test *tax-2; tax-4* double mutant animals and subsequently other double mutants. Eventually these analyses identified two genetic pathways that mediate NaCl chemotaxis. One pathway involves *tax-2*, *cng-3* (cyclic nucleotide gated channel subunits), *gcy-14*, *gcy-22* (receptor type guanylyl cyclase), *odr-3* (Gα protein), *osm-9* (transient receptor potential vanilloid protein) and *gcy-35* (soluble guanylyl cyclase) and the second pathway involves *tax-4* and probably *tax-2* (cyclic nucleotide gated channel subunits), *gcy-22 (*receptor type guanylyl cyclase), *tax-6* and *cnb-1* (calcineurin).

The ASE, ADF, ASI, ASG and ASK neurons are NaCl sensing neurons. The ASE neurons are the main NaCl sensing neurons, with minor contributions from the other four neurons. The two NaCl sensing pathways may function in the same neurons or in different neurons. It is also possible that the two pathways crosstalk. The order of the signal transduction pathways is not completely known yet. We speculate that in the *tax-2*/*cng-3* pathway, GCY-14 and GCY-22 may be receptors that sense NaCl and produce cGMP upon activation, which in turn activates the TAX-2/CNG-3 channel. Another possible mechanism is that the Gα protein ODR-3, may act upstream of the TAX-2/CNG-3 channel, as shown for other well known signaling systems (*308*). However, *odr-3* functions in the ADF neurons in NaCl chemotaxis (chapter 2). The receptor that senses NaCl in the ADF neurons is not yet known. *gcy-35* likely also acts upstream of the CNG channels in the AQR, PQR and URX neurons. GCY-22 may be the receptor for NaCl sensation in the *tax-2*/*tax-4* pathway. In this pathway, activation of the CNG channel may in turn activate calcineurin TAX-6/CNB-1. It will be interesting to see if human homologues of these genes are also involved in NaCl sensation as in *C. elegans.*

Another important study that still needs to be done is to determine the neurons where the identified genes function. We have shown that although the ASE neurons are important for NaCl chemotaxis, *tax-4* does not function in the ASE neurons in NaCl chemotaxis. When *tax-4* was expressed in all amphid neurons except the ASE neurons, it rescued the *tax-4* NaCl chemotaxis defect, suggesting that *tax-4* functions in other neuron(s) than ASE. Moreover, we observed Ca^{2+} responses in ASEL neurons when exposed to NaCl. Other neurons where *tax-4* might function in NaCl chemotaxis are ASI, ASG, ASJ and ASK. Expression of *tax-4* in ASI did not rescue the chemotaxis defect. In addition, identification of the neurons where the other NaCl chemotaxis genes function is also essential to delineate a more comprehensive NaCl chemotaxis neuronal circuitry.

Opposing changes in the sensitivities of gustatory and nociceptive neurons mediate gustatory plasticity

C. elegans avoids normally attractive NaCl concentrations after prolonged exposure to NaCl in the absence of food. The mechanism underlying this behavioral plasticity is not well known. Here we show that *C. elegans* ASE gustatory neurons sense all concentrations of NaCl, whereas the ASH nociceptive neurons are only sensitive to NaCl at concentrations above 200 mM. This suggests that the naïve response to attractive or noxious NaCl concentrations results from a balance of inputs from the gustatory and nociceptive neurons. We therefore hypothesized that during prolonged exposure, the gustatory neurons are desensitized while the nociceptive neurons are sensitized making the worms avoid previously attractive NaCl concentrations. We used a FRET based Ca^{2+} imaging technique, using cell specific expression of Cameleon constructs (*81, 309*), to explore the neuronal responses of the neurons involved in NaCl sensation. Indeed we found that in wild type animals, ASEL is desensitized and ASER and ASH neurons were sensitized after 10 minutes exposure to NaCl. We found that sensitization of the ASH neurons involves signals from the ASE cells, since *che-1* mutants, which do not have functional ASE neurons, showed no sensitization of ASH.

To find out which neurotransmission systems mediate sensitization and/or desensitization, we analyzed ASE and ASH Ca^{2+} response in six mutants. These experiments showed that glutamate and neuropeptide transmission are involved in sensitization of ASH. In addition, we found that serotonin is involved in desensitization of ASEL and sensitization of ASH. Finally, dopamine was shown to be involved in sensitization of ASH. Behavioral studies have earlier shown the involvement of serotonin and dopamine in gustatory plasticity (*169*), but where in the circuitry they act was not yet known. The precise molecular mechanisms of serotonin and dopamine regulation of gustatory plasticity remains to be understood. Serotonin is involved in different learning behaviors in *C. elegans (164, 169)* and it could be modulating the signals emanating from ASE to the ASH neurons during pre-exposure. A detailed study of, for example double mutants of *tph-1* and mutants with defective ASEL and ASER, could shed some light on our understanding of how serotonin is involved in gustatory plasticity and how it modulates the responses of these neurons. The same holds true for the involvement of dopamine in gustatory plasticity.

As gustatory plasticity is a form of learning, we expect that understanding the mechanism of gustatory plasticity will contribute to our knowledge of learning and

memory in humans. It will be necessary to determine if other neurons important for NaCl attraction and avoidance also play a role in gustatory plasticity. The ADF, ASI and ASK neurons have a minor contribution to NaCl chemoattraction (*68*) and ADF and ASI also function in gustatory plasticity (*15*). The ADF neurons are very interesting since the produce serotonin. The link between ADF and serotonin in gustatory plasticity should therefore be further investigated. The neuronal contributions of other neurotransmitters that function in gustatory plasticity need also to be investigated.

Searching for new genes mediating NaCl sensation

After finding that there are at least two genetic pathways involved in NaCl taste in *C. elegans*, we reasoned that there may be other uncharacterized genes involved. In a screen for new NaCl taste mutants we uncovered at least 13 independent mutants that showed varying NaCl chemotaxis phenotypes. We tried to identify the responsible mutations in 13 mutants and found mutations in genes already known to be involved in NaCl sensation in 11 mutants. All but one of the mutations are new alleles of known genes. We found two mutants that do not carry mutations in genes known to be involved in *C. elegans* NaCl chemotaxis. SNP mapping is ongoing to identify the affected genes. The next thing that will be done after the identification of the mutant genes is to test the phenotypes of double mutants of these genes with the *tax-2* and *tax-4* mutants. This will enable the delineation of the genetic pathway they belong to.

Although we tested about 20,000 mutagenized haploid genomes, the screen may not yet be saturated. The screen was reliable because we did isolate many mutants that affect known NaCl taste genes. A bigger screen may be necessary to identify additional new genes. This could be daunting though, as mutations in some of the genes involved in NaCl taste in *C. elegans* only show defects when in a double mutant background. This will therefore call for screens to be carried out in different mutant backgrounds. An assay method or modified assay that would allow easy mutant identification would make the screen less laborious. One possibility is doing a screen in worms with a genetic background that is always attracted to and/or always avoid all NaCl concentrations. Mutants could be isolated from worms that migrate to the quadrants not expected for worms of the original genotype. Overall, it will be worth the effort to identify additional genes involved in NaCl taste, since homologues or orthologues of such genes in mammals may be involved in NaCl taste as well.

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6

Addendum

Summary
Samenvatting
Curriculum vitae
PhD portfolio
Acknowledgements

Summary

Taste is one of the five senses present in human. The taste of salt is the least understood molecularly among all the taste modalities known. Salt is essential for maintaining water and salt homeostasis in the body and it is generally obtained from food. The epithelial sodium channel (ENaC) is proposed to be the salt receptor in rodents. However, this channel is less important for salt taste in humans. Other receptors for NaCl sensation are not yet known.

Since many genetic pathways, genes and proteins are conserved between human and *C. elegans, C. elegans* is a good model organism to study the molecular mechanism of salt taste. We identified three new genes involved in salt sensation in *C. elegans.* In addition, we studied how *C. elegans* response to salt is modulated based on experience. The results described in this thesis are summarized below.

In chapter 1, I give a general introduction to what is known about different taste modalities in mammals and *Drosophila.* Next, I discuss different sensory behaviors observed in *C. elegans* and the signal transduction pathways that mediate these behaviors. These behaviors include different types of learning and memory observed in *C. elegans*. Subsequently, I discuss the neurotransmitters involved in regulating learning and memory in *C. elegans.* Finally, I discuss the importance of *C. elegans* as a model organism and the behavioral and neuronal methods commonly employed to study *C. elegans* response to salt.

In chapter 2, I describe two genetic pathways that function in NaCl chemotaxis in *C. elegans.* We found that one pathway involves GCY-14, GCY-22 and GCY-35 (guanylyl cyclases), TAX-2/CNG-3 (cyclic nucleotide gated channel subunits β and α), ODR-3 (Gα protein) and OSM-9 (TRPV1 protein). The second pathway involves GCY-22 (guanylyl cyclase), TAX-2/TAX-4 (cyclic nucleotide gated channel subunits) and TAX-6/CNB-1 calcineurin (Ca^{2+} activated phosphatase).

Chapter 3 entails the characterization of the neuronal responses that play a role in gustatory plasticity. In this behavior, *C. elegans* learns to avoid normally attractive NaCl concentrations after prolonged exposure to NaCl in the absence of food. The naïve response to NaCl involves antagonistic contributions from the gustatory and nociceptive neurons. We found that in gustatory plasticity the nociceptive neurons were sensitized while the gustatory neurons were desensitized. The sensitization of the nociceptive neurons requires input from the gustatory neurons and serotonin,

dopamine, glutamate and neuropeptide signaling, indicating that this response is non-cell autonomous.

In chapter 4, I describe a forward genetic screen carried out to identify genes involved in NaCl chemotaxis in *C. elegans.* We analyzed 13 independent mutants by whole genome sequencing and/or Sanger sequencing. 11 of the 13 mutants contained mutations in genes already known to affect chemotaxis to NaCl in *C. elegans.* The identities of the mutated gene(s) that cause the NaCl chemotaxis defect in the remaining 2 mutants are not yet known.

In chapter 5, I discuss the results presented in chapters 2, 3 and 4 and suggest possible future experiments that can be done to further unravel the molecular mechanisms of NaCl taste and gustatory plasticity.

Samenvatting

Smaak is een van de vijf klassieke zintuigen van de mens. Van de primaire smaken (zuur, zout, zoet, bitter en umami) is er over het moleculaire mechanisme van het proeven van zout het minst bekend. Zout is essentieel voor het behoud van water en zout homeostase in het lichaam en wordt over het algemeen opgenomen uit voedsel. Bij knaagdieren is het aangetoond dat het natrium kanaal ENaC functioneert als receptor voor zout. Echter, dit kanaal is minder belangrijk voor het proeven van zout bij mensen. Andere receptoren voor het proeven NaCl zijn op dit moment nog niet bekend.

Omdat veel genetische paden, genen en eiwitten geconserveerd zijn tussen mens en *C. elegans*, is *C. elegans* een goed modelorganisme om het moleculaire mechanisme van het proeven van zout te bestuderen. We identificeerden drie nieuwe genen die betrokken zijn bij proeven van zout in *C. elegans*. Daarnaast hebben we onderzocht in hoeverre de reactie van *C. elegans* op zout gebaseerd is op ervaring. De resultaten beschreven in dit proefschrift zijn hieronder samengevat.

In hoofdstuk 1 geef ik een algemene inleiding over wat er bekend is over smaak in zoogdieren en *Drosophila*. Vervolgens bespreek ik de verschillende vormen van zintuiglijk gedrag zoals waargenomen in *C. elegans* en de signaaltransductie cascades die dit gedrag reguleren. Dit gedrag omvat de verschillende vormen van leren en geheugen die worden waargenomen in *C. elegans*. Vervolgens bespreek ik de neurotransmitters die betrokken zijn bij het reguleren van leren en geheugen in *C. elegans*. Tot slot bespreek ik het belang van *C. elegans* als modelorganisme en de neuronale en gedragsmethoden die vaak gebruikt worden om in *C. elegans* de reactie op zout te bestuderen.

In hoofdstuk 2 beschrijf ik twee genetische paden die betrokken zijn bij NaCl chemotaxis in *C. elegans*. We ontdekten dat één genetisch pad de guanylyl cyclases GCY-14, GCY-22 en GCY-35, een cyclisch nucleotide kanaal dat gevormd wordt door TAX-2 en CNG-3, het Gα eiwit ODR-3 en het TRPV kanaal OSM-9 bevat. Het tweede genetische pad bevat de guanylyl cyclase GCY-22, een cyclisch nucleotide kanaal dat gevormd wordt door TAX-2 en TAX-4 en de fosfatase TAX-6/CNB-1.

Hoofdstuk 3 omvat de karakterisering van de neuronale responsen die een rol spelen in de plasticiteit van smaak. Tijdens dit gedrag leert *C. elegans* om NaCl concentraties die normaal als aangenaam worden beschouwd te vermijden, wanneer ze eerder

langdurig zijn blootgesteld aan NaCl in de afwezigheid van voedsel. De naïeve reactie op NaCl wordt gevormd door antagonistische bijdragen van smaak- en nociceptieve neuronen. We vonden dat in smaakplasticiteit van de nociceptieve neuronen gevoelig waren, terwijl de smaakneuronen ongevoelig waren. De gevoeligheid van de nociceptieve neuronen vereist de bijdragen van de smaak neuronen en ook van serotonine, dopamine, glutamaat en neuropeptide signalen, wat aangeeft dat deze reactie niet cel-autonoom is.

In hoofdstuk 4 beschrijf ik de kandidaat gen benadering die we gebruikt hebben om nieuwe genen die betrokken zijn in NaCl chemotaxis in *C. elegans* te identificeren. We hebben 13 onafhankelijke mutanten geanalyseerd met behulp van whole genome sequencing en/of Sanger sequencing. 11 van de 13 mutanten bevatten een mutatie in een gen waarvan het al bekend was dat het in *C. elegans* NaCl chemotaxis kan beïnvloeden. De identiteit van de gemuteerde gen(en) in de twee overgebleven mutanten zijn nog onbekend.
In hoofdstuk 5 bespreek ik de resultaten zoals beschreven in de hoofdstukken 2, 3 en 4 en de mogelijke toekomstige experimenten die gedaan kunnen worden om de moleculaire mechanismen van het proeven van zout en plasticiteit van smaak verder te ontrafelen.