Gustatory behaviour in *Caenorhabditis elegans*

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Outline of this thesis

It is essential for the survival of an organism that it interacts with its environment. The animal has to be able to distinguish hazardous situations from possible food sources. Salt sensing is important for finding food and also for water-homeostasis. However, relatively little is known about the molecular mechanisms of salt taste.

The nematode *C. elegans* is an ideal model-organism to study the genetics of behaviour (Brenner, 1974). It is capable of sensing salts and we discriminate three different responses: it is attracted to low salt concentrations (Ward, 1973; Dusenbery *et al.*, 1974), it avoids high salt concentrations that give osmotic problems (Culotti & Russell, 1978), and the response to NaCl shows plasticity: normally attractive salt concentrations are avoided after pre-exposure to salt (Saeki *et al.*, 2001; Jansen *et al.*, 2002).

The goal of this study was to unravel the molecular mechanisms and the cellular circuitry behind the different responses of *C. elegans* to NaCl. A candidate gene approach was used in which mutants for 123 genes were tested for their role in salt responses. A complete overview of all genes tested is given in the Chapter 5. We found 22 genes involved in attraction, 57 genes involved in avoidance and 87 genes involved in gustatory plasticity. Several of the most interesting results were followed up and are discussed in this thesis.

In Chapter 1, a summary is given of what is known about the molecular mechanisms of taste, an introduction to *C. elegans* as a model organism, and a brief overview of the *C. elegans* nervous system and neurotransmission. Furthermore, various sensory behaviours of *C. elegans* and their plasticity are discussed.

Chapter 2 focuses on chemotaxis to NaCl and discusses five newly identified genes, which function in two genetic pathways that mediate NaCl chemotaxis. Chapter 3 discusses the role of G protein signalling in gustatory plasticity and identifies multiple neurons as well as a genetic pathway involved in this process. Chapter 4 discusses the roles of different neurotransmitters in the different responses of *C. elegans* to NaCl. An overall discussion and summary are given in the final chapter. Taken together, this study provides new insights into the processes of NaCl chemotaxis and its plasticity in *C. elegans*. We expect that these insights can be extrapolated to mammals.

Chapter 1

Introduction

1

Gustation

1.1.1 Hard-wiring

Organisms need to interact with their environment in order to avoid hazardous situations and to be able to find food. Taste is very important for finding food. Organisms have to distinguish between attractive or good, and toxic or nasty compounds. The gustatory system distinguishes only a few taste categories, unlike for example the olfactory or auditory system. In humans, taste is categorized into five modalities: sweet, bitter, salt, sour, and umami. Umami was first described in 1908 as the taste of glutamate in dried kelp (Yamaguchi & Ninomiya, 2000). In general, sweet, umami, and low salt concentrations are associated with positive behaviours and lead to food acceptance. In contrast, bitter and sour lead to avoidance behaviours. Studies in rats indicate the existence of a sixth taste modality: a taste for dietary fat (Gilbertson *et al.*, 1997; Tsuruta *et al.*, 1999; Kitagawa & Shingai, 2001). Unfortunately, relatively little is known about the exact processes involved in taste. What is known about the gustatory system in humans is mostly concerned with the hard wiring of the system.

In *Drosophila,* taste is mediated by sensory neurons on the proboscis, internal mouthpart organs, legs and wings, and oviposter (Stocker, 1994). The sensory structures for taste are innervated by two to four gustatory neurons and a single mechanosensory neuron (Falk *et al.*, 1976). Unlike the mammalian gustatory system, taste information in *Drosophila* is directly relayed to the brain. The adult *Drosophila* brain contains about 100,000 neurons, with the cell bodies in an outer shell surrounding a fibrous core. The subesophageal ganglion, which is the most ventral region of the brain, receives gustatory projections (Stocker & Schorderet, 1981; Rajashekar & Singh, 1994). This subesophageal ganglion contains a map of the different taste organs. Both position and quality of taste are represented in this map (Thorne *et al.*, 2004; Wang *et al.*, 2004).

In vertebrates, taste begins on the tongue and palate where epithelial-derived taste cells detect chemical cues (Reviewed in Lindemann, 1996). Three morphological structures of taste detecting papillae are topographically arranged on the tongue. Fungiform papillae decorate the anterior two-thirds of the tongue, foliate papillae are on the lateral edges, and circumvillate papillae are found on the posterior two-thirds. These mammalian taste cells are not classic neurons; they do not send axonal projections to the brain. Instead, primary gustatory fibres contact multiple taste cells; these cells can be located within different taste buds. The fibres that carry taste information make their synapses centrally in the medulla, in a

slender line of cells called the nucleus of the solitary tract.

It is not quite clear how sensory taste information is further processed in the brain. Three different models have been proposed to describe how sensory information is processed in vertebrates (Laurent, 1999; Smith & St.John 1999; Scott, 2004): (I) The labelled line model; in this model neurons respond to selective cues in the periphery and this information remains segregated in the brain. (II) The mixed line model; in this model neurons respond to multiple stimuli with different levels of activity. (III) The third model predicts that the precise firing pattern of action potentials conveys information about the nature of the sensory stimuli. Experiments in animal models have shown that cells respond selectively to different taste modalities, demonstrating that taste information is segregated in the periphery. This is most consistent with the labelled-line model of taste coding (Scott, 2005).

1.1.2 Signal transduction

Relatively little is known about the signal transduction mechanisms involved in taste. Genomic database searches identified a family of 68 candidate gustatory receptors in *Drosophila* (Clyne *et al*., 2000; Dunipace *et al*., 2001; Scott *et al*., 2001; Robertson *et al*., 2003). These gustatory receptors do not show sequence homology to members of the mammalian T1R or T2R taste receptor families. Neither do they show homology to *C. elegans* chemoreceptors. The *Drosophila* Gr5a receptor was found to specifically recognize trehalose (Dahanukar et al., 2001; Ueno et al., 2001; Chyb et al., 2003). Cells that contain this trehalose-receptor recognize many sugars besides trehalose. Therefore, these cells are expected to express additional receptors (Dahanukar *et al*., 2001; Ueno *et al*., 2001; Chyb *et al*., 2003; Wang *et al*., 2004). A second population of cells can be defined by the expression of the Gr66a receptor. Multiple different Gr66a expressing cells exist and they can specifically recognize bitter compounds (Thorne *et al*., 2004; Wang *et al*., 2004; Marella *et al*., 2006). The observation that there is a specific population of cells necessary for the detection of bitter and another population of cells to specifically recognize sweet, shows a striking resemblance to the organization of gustatory cells in mammals (Scott, 2005). By expressing an exogenous ligand-gated ion channel in the taste cells and using *in vivo* imaging with G-CaMP, a fluorescent Ca^{2+} probe, it was shown that cellular activity is sufficient to drive taste behaviour (Marella *et al*., 2006).

Thus far, in mammals two taste receptor families have been identified. The first family of taste receptors identified is the T1R family, which consists of three

genes (Hoon *et al.*, 1999; Bachmanov *et al.*, 2001; Kitagawa *et al.*, 2001; Max *et al.*, 2001; Montmayeur *et al.*, 2001; Nelson *et al.*, 2001; Sainz *et al.*, 2001). The T1R receptors belong to the family of type C G protein coupled receptors (GPCRs), mostly related to pheromone receptors and metabotropic glutamate receptors. Receptors from this family function as homo- and heterodimers to detect their ligands (Pin *et al.*, 2005). The heterodimer of T1R2 plus T1R3 seems to detect all natural sugars and artificial sweeteners, and also functions as the umami receptor in humans (Nelson *et al.*, 2001; Li *et al.*, 2002; Damak *et al.*, 2003; Zhao *et al.*, 2003). The T1R2 extracellular amino terminal domain binds most sugars, the T1R3 transmembrane carboxy terminal domain binds some artificial sweeteners, and the T1R2 transmembrane carboxy terminal domain binds G proteins (Xu *et al.*, 2004; Jiang *et al.*, 2004, 2005a, 2005b).

The second family of taste receptors that has been identified in mammals is the T2R family of receptors (Adler *et al.*, 2000; Matsunami *et al.*, 2000). This family consists of 25 genes in humans and 35 genes in mouse and rat (Go *et al.*, 2005). T2Rs have a short extracellular amino terminal domain and are distantly related to mammalian V1R pheromone receptors and opsins. T2Rs function as receptors for different bitter compounds (Chandrashekar *et al.*, 2000; Bufe *et al.*, 2002; Behrens *et al.*, 2004). Expression studies showed that multiple T2Rs could be expressed in a single taste cell, suggesting that bitter cells recognize multiple compounds and no distinction can be made between these different bitter compounds (Adler *et al.*, 2000; Mueller *et al.*, 2005).

No co-expression has been found of T1R1, T1R2, and T2Rs. Therefore no cell has been found that detects sugars, amino acids (umami), as well as bitter compounds (Hoon *et al.*, 1999; Adler *et al.*, 2000; Nelson *et al.*, 2001). The differential expression of different taste receptors argues that there is a topographic map of taste sensitivity on the tongue. However, the tongue is not clearly segregated into different regions that exclusively recognize different tastes (Scott, 2005). There is no clear evidence that any kind of spatial segregation of sensitivities contributes to a neuronal representation of taste quality, although there are some slight differences in sensitivity across the tongue and palate, especially in rodents (Smith & Margolskee, 2001).

The first downstream signalling molecule involved in taste that has been identified is Gustducin, a Gα protein preferentially expressed in the tongue (McLaughlin *et al.*, 1992). Mice lacking Gustducin show reduced responses to sugars, amino acids, and bitter compounds both in behavioural assays and electrophysiological analyses. This argues that Gustducin is a critical transducer of

taste in general (Wong *et al.*, 1996; Glendinning *et al.*, 2005). Activated Gustducin causes changes in cAMP levels. Downstream of the G protein, phospholipase 2B and the TRPM5 cationic channels, which are selectively expressed in taste cells, are required for the detection of sugars, amino acids, and bitter compounds (Rossler *et al.*, 1998; Asano-Miyoshi *et al.*, 2000; Asano-Miyoshi *et al.*, 2001; Perez *et al.*, 2002; Zhang *et al.*, 2003). Overall, the signalling pathway emerging for sweet, umami, and bitter taste is that GPCRs activate G proteins and PLC, leading to activation of transient receptor potential (TRP) channels and depolarisation of the cell.

Recently, also in *Drosophila,* G protein signalling was shown to be involved in sugar reception (Ishimoto *et al.*, 2006). Knockout of the Gγ subunit 1 resulted in lowered responses to sugars, both behaviourally and electrophysiologically. This indicates that indeed the Gγ subunit is involved in tasting sugar, but it also shows that another pathway exists.

All signal transduction molecules discussed thus far play a role in tasting sugars, amino acids, and bitter compounds. Signal transduction of salt and sour taste appears to use other signal transduction pathways that may not be mediated by G protein signalling, since the absence of Gustducin, PLC, or TRP5M does not have any effect on salt or sour perception (Wong *et al.*, 1996; Zhang *et al.*, 2003). In contrast, ion channels have been proposed to mediate the detection of these taste modalities (Reviewed in Lindemann, 1996; Boughter & Smith, 1998; Smith & Margolskee, 2001).

The sense of sour is mediated by acids or protons that permeate through cells and potentially influence ion channel activity (DeSimone *et al.*, 2001). Acid-sensing or proton channels that have been suggested include an H^+-gated Ca^{2+} channel (Miyamoto *et al.*, 1998), the inhibition of a K^+ channel (Kinnamon & Roper, 1988), an amiloride sensitive Na^+ channel (Gilbertson *et al.*, 1993), a Cl^- channel (Miyamoto *et al.*, 1998), an acid-sensing degenerin (Ugawa *et al.*, 1998; Liu & Simon, 2001), and a hyperpolarisation-activated cyclic nucleotide-gated channel (Stevens *et al.*, 2001).

The detection of salts involves two types of taste receptors in mammals (Frank *et al.*, 1983; Stewart *et al.*, 1997): a Na^+ specific receptor and a second receptor that does not discriminate between Na^+, K^+, and NH_4^+. In the anterior tongue of humans the Na^+ sensitive receptor in the fungiform taste receptor cells is the amiloride sensitive epithelial channel (ENaC). Amiloride is a diuretic that specifically blocks ENaCs. The effects of amiloride on gustatory responses were not limited to NaCl. Suppression of responses to KCl, HCl, and saccharine also

occurred. No gustatory responses to amiloride itself were found (Halpern, 1998; Reviewed in Lindemann, 2001). However, a significant part of the taste responses to NaCl is amiloride insensitive. The amiloride insensitive salt taste receptor is a constitutively active non-selective cation channel derived from the VR1 gene (Lyall *et al.*, 2004). VR1 knockout mice demonstrate no functional amiloride insensitive salt taste. The differential distribution of the ENaC and the amiloride insensitive transduction pathways vary widely among species. However, any downstream signalling remains unknown.

1.2 Behavioural plasticity

An organims is continuously exposed to various stimuli among which taste, therefore the nervous system shows plasticiy in order to cope with any changes. Plasticity may occur at many sites in the nervous system, both at the molecular and cellular level, and eventually at the behavioural level. Virtually all animals demonstrate a degree of behavioural plasticity. One form of behavioural plasticity is learning, which may be defined as an adaptive change in behaviour caused by experience.

There are a number of different types of learning, ranging from relatively simple to very complex (Table 1). Simple forms of behavioural plasticity are habituation, sensitisation, and adaptation; a more complex form is conditioning; and even more complex forms of learning are latent learning, observational learning, and imprinting. Ultimately, learning can lead to memory, which involves long-term potentiation (LTP) and long-term depression (LTD).

The simplest form of behavioural plasticity is habituation, which is defined as the decrease in a behavioural response as a result of repeated presentation of a stimulus. Habituation is a universal phenomenon and involves changes in the intensity of a response, not the nature of the response itself. Habituation can be divided into short-term and long-term habituation, in which short-term habituation may involve a decrease in the amount of neurotransmitter released and long-term habituation may involve a decrease in the number of active synapses as well as a decreased output at a given synaps. Long-term habituation is dependent on protein synthesis, whereas short-term habituation is not. Adaptation may have the same effect on a response, albeit that habituation can be reversed by an independent harsh stimulus and adaptation cannot.

In contrast to habituation, sensitisation is defined as the enhancement of a response after exposure to a strong stimulus, different from the stimulus that elicits the response itself. In contrast with habituation, sensitisation increases the chance of neurotransmitter being released, thereby increasing the strength of a synaps. Long-term training can produce long-lasting sensitisation. The effects can be eliminated by desensitisation.

Table 1: **Different types of behavioural plasticity** (Shepherd GM, Neurobiology 3rd ed.)

Types of learning	
Simple	Habituation Sensitisation Adaptation
Associative	Classical or passive Operant or instrumental One-trial or aversion
Complex	Latent Observational Imprinting

Conditioning, which is a more complex form of behavioural plasticity, can de divided into classical conditioning or associative learning and operant conditioning. Associative learning uses a molecular mechanism similar to that of sensitisation. In this form of learning an animal associates a neutral stimulus with a second stimulus that is either punishing or rewarding. The most famous example of associative learning is an experiment performed by Pavlov in the early 1900's. A dog was conditioned by offering food with a simultaneous sound of a bell. After conditioning, the dog started producing saliva in anticipation of food whenever the bell sounded.

In associative learning or classical conditioning, the animal is a passive participant. By contrast, an animal may be asked to learn a task or solve a problem; this is called operant or instrumental conditioning. Since the animal usually makes mistakes before learning the task, it is also referred to as trial-and-error learning. In this form of behavioural plasticity, the strength of the response depends on the strength of the reward or punishment.

A special form of conditioning is aversion learning, which differs in several aspects from normal conditioning. In aversion learning, the taste for food may be lost when it is suspected to be sick making. It is also referred to as one-trial learning. Unfortunately almost nothing is known about the mechanisms that mediate this type of learning.

Very complex forms of behavioural plasticity are latent learning, observational learning, and imprinting. Latent learning helps an animal to perform better in operant tasks and occurs when an animal is introduced to a new environment. In a new environment an animal normally starts exploring without reward or punishment. Observational learning occurs when an animal observes another animal performing a task. After this observation the animal learns the task more rapidly. Finally, imprinting is a process in which a preference is set in the nervous system at juvenile stages. An example of imprinting is the process whereby a young animal forms a behavioural attachment to a parent.

Ultimately, learning can lead to the formation of long-term or associative memory. Memory may be defined as the storage and recall of previous experience. In mammals, the main model for memory involves LTP and LTD. LTP is a long lasting enhancement of synaptic transmission first reported by Bliss and Lomo (Bliss & Lomo, 1973), it can be induced by high frequency stimulation of neurons. In contrast to LTP, LTD is a long lasting reduction in synaptic transmission and can be induced by delivering low frequency stimulations to neurons.

1.3 *Caenorhabditis elegans* as a model organism

The 1 mm large soil nematode *Caenorhabditis elegans* was first used in the 1970's to study the genetics of development and behaviour (Figure 1; Brenner, 1974). Since then studies using this model organism have provided valuable insights into the molecular mechanisms that govern various biological processes. The natural isolate N2 from Bristol is referred to as the standard wild type strain.

C. elegans develops in four larval stages (L1-L4) to become either a self-fertilising hermaphrodite or a male. The hermaphrodites are useful to maintain strains, but the occasional males allow genetic crosses. Each larval stage is terminated by a moult, since the animal is ensheated by a tough impermeable elastic cuticle. The nematode has a short generation time. Hermaphrodites have a lifespan of about two weeks and lay approximately 300 eggs. The development to an adult depends on the temperature and therefore can be manipulated by shifting

temperatures. It takes about three days to become an adult at 20°C. Under harsh conditions L2 larvae can also develop to an alternative third stage, referred to as the dauer stage. These dauer larvae show an increased lifespan and can survive starvation and overcrowding conditions. Dauer larvae can recover from this alternative stage and develop to adults when environmental conditions improve.

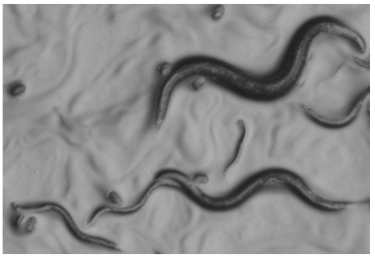

Figure 1: **Wild type *C. elegans*.**

C. elegans is used as a model organism because of its simplicity; genetic screens are fast and cheap and can be done on large scale. Moreover, many biological processes have been conserved from worm to man. In addition, several genetic tools are available. Importantly, the complete genome, which consists of five pairs of autosomes and one pair of sex chromosomes, has been fully sequenced and annotated (The *C. elegans* Sequencing Consortium, 1998). Different strains with single nucleotide polymorphisms (SNPs) compared to wild type Bristol N2, for example the Hawaiian strain CB4856, have been isolated and sequenced (Koch *et al.*, 2000; Wicks *et al.*, 2001). These SNPs can be used to map random mutations in the genome (Wicks *et al.*, 2001). Mutations can be obtained by using transposon or chemical mutagenesis (Zwaal *et al.*, 1993; Jansen *et al.*, 1997; Liu *et al.*, 1999). Genes can also be knocked down using RNAi techniques either by injection or by feeding (Kamath *et al.*, 2003). Transgenic animals can be generated within a few days by germ line transformation (Mello *et al*, 1991). A last advantage of *C. elegans* is its transparency, which allows *in vivo* imaging techniques with all kinds of fluorescent constructs and dyes.

The development of *C. elegans* has been extensively studied and the complete embryonic lineage has been determined (Sulston *et al.*, 1983; Schnabel *et al.*, 1997). Hermaphrodites consist of a fixed number of 959 somatic cells. Together these cells form various tissues like the cuticle, muscles, the pharynx, the gut, the reproductive system, and the nervous system. Most of the neurons in *C. elegans* are located around the pharynx where their processes form a ring called the nerve ring (White *et al.*, 1986). The nerve ring is the central region of processing that contains around 175 nerve endings. Running from the nerve ring is a set of longitudinal bundles that connect the nerve ring to sensory neurons, motor neurons and several

small ganglia in the tail. Contacts are made using gap junctions, chemical synapses, or neuromuscular junctions. Neurons have simple branching structures, which appear to be largely invariant between different animals and can be assigned into 118 neuronal classes based on their morphology and connectivity (White *et al.*, 1986). The invariant positions and connections of all 302 neurons that make up the nervous system have been determined (White *et al.*, 1986). This allows studying the cellular circuits that play a role in specific behaviours. Even the role of a single neuron in behaviour can be studied. This is a main advantage compared to other 'simple' model organisms, which all still have very complex nervous systems; for example *Drosophila* has 100,000 neurons. Despite its simplicity, *C. elegans* shows various rather complex behaviours (reviewed in Hobert, 2003).

1.4 *C. elegans* sensory behaviour

C. elegans shows all sensory behaviours, except for hearing and seeing. It can respond to touch, osmolarity, temperature, oxygen, and a variety of water-soluble and volatile chemicals. The sensory receptor cells of *C. elegans* are arranged in sensilla. Each sensillum contains a number of ciliated nerve endings and two non-neuronal cells: a sheath cell, which is a glia-like cell enveloping the sensory endings and a socket cell, which joins the sensillum to the hypodermis (Ward *et al.*, 1975; Ware *et al.*, 1975). The amphids are two large sensilla, one on the left and one on the right side of the head. They have an opening to the environment formed by a channel of the sheath and socket cells, through the cuticle. Two analogous, but simpler structures are located in the tail. These are called the phasmids. Together, the amphids and phasmids have been shown to be the main chemoreceptive organs (Bargmann *et al.*, 1990; Hilliard *et al.*, 2002).

Three classes of sensory neurons exist in the amphids (White *et al.*, 1986). The first class consists of cells with one or two slender cilia directly exposed to the environment through the amphid pore. These are the eight neurons ADF, ADL, ASE, ASG, ASH, ASI, ASJ, and ASK, which detect water-soluble compounds (Bargmann *et al.*, 1990; Bargmann & Horvitz, 1991; Kaplan & Horvitz, 1993; Troemel *et al.*, 1995). The second class includes three cell types – AWA, AWB, and AWC – with flattened branched cilia near the amphid pore, enclosed by sheath cells. These neurons are involved in the detection of volatile chemicals (Bargmann *et al.*, 1993). The third class contains only one cell type: the ADF neurons. The ADF neurons have complex brush like structures at the sensory ending embedded in the amphid sheath cells for the detection of temperature changes (Mori & Ohshima,

1995). The functions of the amphid sensory neurons are summarised in Table 2. The neurons of the amphids have synaptic outputs that are mainly focussed onto four interneurons: AIA, AIB, AIY, and AIZ (White *et al.*, 1986).

Laser ablation studies have shown that the amphid neurons are not needed for postembryonic viability. However, when all eight types of amphid sensory neurons were killed with a laser, *C. elegans* shows no chemotaxis (Bargmann & Horvitz, 1991). No two types of the amphid sensory neurons have identical functions, except for the left and right cell of one type. Most neurons recognize several different chemical cues. The ASH neurons can even sense stimuli from different modalities, such as chemicals and touch.

For a long time the left and right cells of a neuron pair were thought to be functionally identical. However, the left and right members of the AWC and ASE neuron pairs have been shown to be functionally different and to express different genes. The fate of the left and right AWC neuron is randomly determined. The decision for left or right is made by a signalling pathway using Ca^{2+} and MAP kinases (Troemel *et al.*, 1999; Wang *et al.*, 2001; Sagasti *et al.*, 2001; Tanaka-Hino *et al.*, 2002; Davies *et al.*, 2003; Chuang & Bargmann, 2005).

Table 2: **The roles of the 12 pairs of amphid sensory neurons in *C. elegans*.** Adapted from Bargmann *et al.*, 1990.

	Amphid sensory neurons	
Taste	ASE	Na^+, Cl^-, K^+, cAMP, biotin, lysine, Cu^{2+}, Cd^{2+}, protons
	ADF	Na^+, Cl^-, cAMP, biotin, protons, dauer
	ASG	Na^+, Cl^-, cAMP, biotin, lysine, dauer
	ASI	Na^+, Cl^-, cAMP, biotin, lysine, dauer
	ASJ	dauer
	ASK	lysine, dauer
Nociception	ASH	osmolarity, touch, odorants, heavy metals, protons, bitter compounds
	ADL	octanol, Cu^{2+}, Cd^{2+}
	AWB	2-nonanone
Olfaction	AWA	diacetyl, pyrazine, 2,4,5-trimethylthiazole
	AWC	benzaldehyde, butanone, isoamylalcohol, 2,3-pentanedione, 2,4,5-trimethylthiazole
Thermosensation	AFD	temperature

The fates of the left and right ASE neurons, the main salt sensing cells, are regulated as well. Functionally, the left ASE neuron is mainly sensitive to Na^+, whereas the right ASE neuron is mainly sensitive to Cl^- and K^-. The *lim-6* homeobox gene is required for this functional asymmetry and also for the ability to distinguish Na^+ from Cl^- (Pierce-Shimomura *et al.*, 2001). *lim-6* regulates the

asymmetric expression of several guanylate cyclases (gcy's) (Hobert *et al.*, 1999). It is required to repress expression of the ASER specific guanylate cyclase *gcy-5* in ASEL through homeobox gene *cog-1* and Groucho homologue *unc-37* (Chang *et al.*, 2003). The guanylate cyclases *gcy-6* and *gcy-7* are specifically expressed in ASEL independent of *lim-6*. In addition, left/right asymmetry of ASE neurons is controlled by the microRNA *lsy-6*, which in turn is activated by the zinc-finger transcription factor *die-1* and in turn represses *cog-1* activity in ASEL (Johnston & Hobert, 2003; Chang *et al.*, 2003).

1.4.1 Taste

C. elegans can detect and discriminate between a wide range of water-soluble compounds: cyclic nucleotides – cAMP and cGMP -, biotin, anions – Cl^-, Br^- I^-, SO_4^-, NO_3^- -, cations – Na^+, Li^+, K^+, Mg^{2+}, Ca^{2+} -, and amino acids – lysine, cysteine and histidine (Dusenbery *et al.*, 1974). *C. elegans* can even discriminate compounds that are sensed by the same cell, suggesting discrimination occurs within a single cell (Bargmann *et al.*, 1990). Animals can chemotax by placing their head in a particular relationship to the sensed (gradient of) attractant, rather than maximizing the speed at which it approaches the attractant (Ward, 1973).

Three different assays exist to analyse chemotaxis of *C. elegans* to water-soluble compounds. The first analyses of chemotaxis were done using gradient assays (Ward, 1973). In these assays shallow gradients were formed by radial diffusion from a point-source of an attractant and subsequently the fraction of worms that accumulate in the centre of the gradient was determined (Figure 2A). In these assays it is not quite clear to which concentration the animals respond. A second assay uses a steep instead of a shallow gradient, and gives a choice between two concentrations or two different attractants by the use of quadrant plates (Wicks *et al.*, 2000; Jansen *et al.*, 2002). Different quadrants of these plates can be filled with agar of different constitution (Figure 2B). The dispersal of worms over the different quadrants is a measure for the attraction to a compound. The third assay is called a step response (Miller *et al.*, 2005). Animals are individually placed on a thin porous membrane over a pair of inverted showerheads. Each showerhead emits a solution with a different concentration of the compound tested. Stepwise, temporal changes in concentration are delivered by sliding the showerheads relatively to the animal. Behaviour is scored as the transitions that occurred between four states: forward, reversal, omega turn, and other. These are different states of the locomotory program of *C. elegans*.

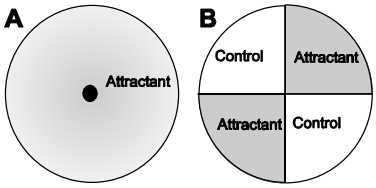

Figure 2: **Schematic overview of soluble compound chemotaxis assay plates.** (A) gradient chemotaxis assay plate. (B) quadrant chemotaxis assay plate.

Laser ablation studies showed that the ASE neurons are both necessary and sufficient for chemotaxis to water-soluble compounds, such as cAMP, biotin, Na^+, and Cl^- (Bargmann & Horvitz, 1991). After killing the ASE neurons, a residual response was left, which was completely abolished after killing the ADF, ASG, and ASI neurons. However, killing the ADF, ASG, ASI neurons, and leaving ASE neurons intact, had no effect on chemotaxis. This suggests that these cells have redundant functions. To abolish chemotaxis to lysine not only the ASE, ASG, and ASI neurons, but also the ASK neurons had to be killed. Taken together, the laser ablation experiments showed that the ASE neurons are most important, and in addition the ASG and ASI neurons sense lysine, cAMP and other compounds. The ADF neurons sense cAMP, biotin, NaCl, but not lysine. The ASK neurons only sense lysine (Bargmann *et al.*, 1990). However, in these behavioural assays the compounds were tested as ammonium or acetate salts. In the shallow gradient assays animals showed no response to NH_4Ac. However, other analyses showed that *C. elegans* is strongly attracted to NH_4Ac (Wicks *et al.*, 2000; Jansen *et al.*, 2002; Chang *et al.*, 2004), therefore it is not exactly clear to what compounds the animals responded in the previous laser ablation experiments.

Several screens were performed to find genes involved in chemotaxis to water-soluble compounds using the gradient assays. Many of the mutations found in these screens affected the structure of the ciliated neurons (Dusenbery *et al.*, 1975; Lewis & Hodgkin, 1977; Perkins *et al*, 1986; Bargmann & Horvitz, 1991). This indicates that the ciliated neurons are required for normal chemotaxis, but it does not yield any information about the signal transduction mechanisms involved. In addition, some of the mutations found in the screens affected the fate of specific amphid sensory neurons, for example, the C_2H_2-type zinc finger transcription factor *che-1*, which is needed for normal function of the ASE neurons (Uchida *et al.*, 2003).

A few genes from the genetic screens that were cloned encode signalling molecules. The *tax-2* and *tax-4* genes encode β and α type subunits of a cGMP gated channel and the *tax-6* gene encodes the A subunit of the calcium activated

phosphatase calcineurin (Coburn & Bargmann, 1996; Komatsu *et al*., 1996; Kuhara *et al.*, 2002). This suggests that the second messengers cGMP and calcium are important for chemotaxis to NaCl in *C. elegans.*

In mammals salts are sensed by amiloride sensitive ENaC channels or via TRPV channels (Heck *et al*., 1984; Canessa *et al*., 1994; Lyall *et al*., 2004). The *C. elegans* genome contains 28 ENaC channels and five TRPV channels (Colbert *et al*., 1997; Tobin *et al*., 2002; Goodman & Schwarz, 2003). However, none of the ENaCs have been implicated in taste in *C. elegans* thus far. Only a few mutant strains exist for the ENaCs and most of those cannot be tested in behavioural assays, since they have uncoordinated phenotypes. Moreover, amiloride did not affect salt chemotaxis (Hukema & Jansen, unpublished results). In addition, also the TRPV channel subunits have not been implicated in salt chemotaxis (Jansen *et al*., 2002).

Finally, RNAi techniques revealed a role for *kvs-1* and *mps*-genes in chemotaxis to biotin and lysine (Bianchi *et al*., 2003). *mps*-genes encode Mink related peptides (MiRPs) that associate with an voltage-gated K^+ channel encoded by *kvs-1*. *kvs-1* and the *mps*-genes are expressed in the ASE and ADF neurons, which both play a role in chemotaxis (Bianchi *et al*., 2003; Park *et al*., 2005; Bargmann & Horvitz, 1991). Analysis of loss-of-function or deletion mutants for *kvs-1* and the *mps*-genes will have to further elucidate the exact role of the genes in chemotaxis to NaCl.

1.4.2 Olfaction

The AWA and AWC amphid sensory neurons are used to detect attractive volatile chemicals, the AWB, ASH, and ADL neurons are used to avoid repellent volatile chemicals (Bargmann *et al*., 1993; Troemel *et al*., 1995; 1997). Olfactory cues are most likely detected by GPCRs, one of which has been identified as ODR-10 (Sengupta *et al*., 1996). Each olfactory neuron expresses a certain repertoire of GPCRs. This repertoire defines the animal's capacity to respond to a certain compound and also defines the nature of its response, either attraction or avoidance (Troemel *et al*., 1997).

Detection of odorants can be tested using chemotaxis assays in which animals can move towards or away from a point source of an odorant on an agar plate (Bargmann *et al*., 1993; Troemel *et al*., 1997). Using these behavioural assays it has been found that the AWA and AWC neurons use different signal transduction machineries.

In the AWA neurons the Gα protein ODR-3 constitutes the main signal for odorant detection (Roayaie *et al.*, 1998). GPA-3 and GOA-1 also mediate stimulatory signals in the AWA neurons, whereas GPA-5 mediates an inhibitory signal (Lans *et al.*, 2004; Matsuki *et al.*, 2006). Downstream of the G proteins the TRP channel subunit OSM-9 is required (Colbert *et al.*, 1997). OSM-9 can form a channel together with the other TRP channel subunit OCR-2 and they promote each other's localization to the cilia (Tobin *et al.*, 2002). Polyunsaturated fatty acids (PUFAs) are thought to be able to modulate TRP channels. Indeed, it appears that PUFAs function as endogenous modulators of TRP channels in olfactory signalling in the AWA neurons (Kahn-Kirby *et al.*, 2004). Moreover, the nPKCε/η TTX-4 and the PKCδ/θ TPA-1 are both required for AWA mediated olfaction. These proteins are both activated by the DAG-analog PMA (Okochi *et al.*, 2005) and are also involved in PUFA signalling. A schematic overview of olfactory signalling in the AWA neurons is given in Figure 3A.

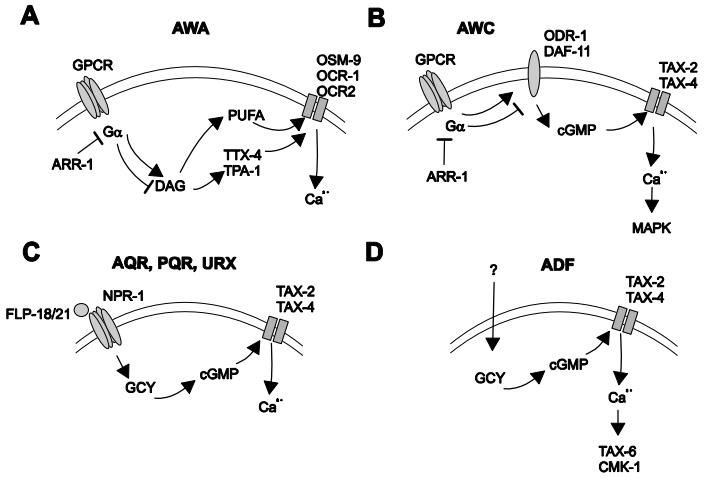

Figure 3: **Different signal transduction pathways involved in sensory behaviours in *C. elegans*.** (A) Olfactory signalling in the AWA neurons. (B) Olfactory signalling in the AWC neurons. (C) Aerotaxis signalling in the AQR, PQR, URX neurons. (D) Thermotaxis signalling in the ADF neurons. For details and references see text.

In the AWC neurons olfaction is mediated by the second messenger cGMP. First, the odorant signal is transduced by Gα proteins. The main signalling route requires ODR-3, but GPA-3 and GPA-13 act redundantly in attraction to AWC-sensed odorants. In addition, GPA-2 has an inhibitory as well as stimulatory function (Roayaie *et al.*, 1998; Lans *et al.*, 2004). Downstream of the G proteins the guanylate cyclases DAF-11 and ODR-1 are required for normal AWC function in

olfaction (Bargmann *et al.*, 1993; Vowels and Thomas, 1994; Birnby *et al.*, 2000; L'Etoile & Bargmann, 2000). In turn, cGMP can activate a cGMP-gated channel formed by the β and α subunits TAX-2 and TAX-4 (Coburn & Bargmann, 1996; Komatsu *et al.*, 1996). TAX-2 and TAX-4 co-localise to the cilia of the AWC neurons, making it likely that they form a hetero-multimeric channel (Komatsu *et al.*, 1996; 1999). Downstream of the cGMP signalling, a MAP kinase pathway may function in the AWC neurons, since LET-60 Ras is required for odorant detection, which is dependent on TAX-2/TAX-4 and the voltage-activated Ca^{2+} channel subunit UNC-2 (Hirotsu *et al.*, 2000). A schematic overview of olfactory signalling in the AWC neurons is given in Figure 3B.

1.4.3 Nociception

C. elegans is capable of avoiding all kinds of repulsive stimuli, such as touch and noxious chemicals, which is generally referred to as nociception. The first sensory modality to be explored in detail was mechanosensation. Both harsh and light touch of the body elicit avoidance responses (Chalfie *et al.*, 1981). Animals reverse and change direction if a mechanical stimulus is applied to the anterior boy. In contrast, when the stimulus is applied to the posterior body, animals accelerate their forward movement. The neural circuitry for touch avoidance has been traced from the sensory neurons to the motor neurons (Chalfie *et al.*, 1985). Gentle touch is transduced via the ALM, AVM, PLM, and PVM sensory neurons; the PVD neurons are responsible for strong mechanical touch responses; and the ASH, FLP, OLQ, and IL1 neurons are involved in nose touch responses (reviewed in Goodman, 2006). Common to all different forms of mechanosensation is that the AVA and AVD interneurons are involved in forward movement and the AVB and PVC interneurons are involved in reversals (Chalfie *et al.*, 1985; Kaplan & Horvitz, 1993; Wicks & Rankin, 1995).

Mechanosensation is transduced by DEG/ENaC channels and TRP channels. The main DEG/ENaC ion channel complex involved in transduction of touch responses comprises four different molecules: the degenerins MEC-4 and MEC-10, the somatostatin-like protein MEC-2, and the paraoxonase like protein MEC-6 (Chelur *et al.*, 2002; Goodman *et al.*, 2002). Light touch to the nose is sensed by the ASH neurons (Kaplan & Horvitz, 1993) and requires the release of glutamate from these ASH neurons to their command interneurons, as well as the TRP channel subunits OSM-9 and OCR-2 (Colbert *et al.*, 1997; Mellem *et al.*, 2002).

The ASH sensory neurons are polymodal and are involved in a range of avoidance responses. They do not only detect mechanical stimuli, but they are involved in the response to a range of chemical repellents as well. Wild type animals avoid sugars and salts in high concentrations (Culotti & Russell, 1978), detergents (Dusenbery, 1974; Hilliard *et al.*, 2005), heavy metals such as Cd^{2+} and Cu^{2+} (Sambongi *et al.*, 1999), pH lower than 4 (Sambongi *et al.*, 2000), and all kinds of bitter compounds such as denatonium and quinine (Tajima *et al.*, 2001; Hilliard *et al.*, 2004). The avoidance of bitter compounds requires the Gα protein GPA-3 and the unknown protein QUI-1, which are not needed for touch avoidance (Hilliard *et al.*, 2004). Besides the ASH neurons, other neurons are also important for avoidance. The ADL neurons contribute to avoidance of high osmolarity, octanol, Cd^{2+}, and Cu^{2+} (Bargmann *et al.*, 1990; Troemel *et al.*, 1997; Sambongi *et al.*, 1999) and the phasmid sensory neurons are involved in SDS avoidance (Hilliard *et al.*, 2002). In addition, the AWB neurons play a role in the detection and avoidance of volatile chemicals (Troemel *et al.*, 1997).

Distinct pathways in the ASH neurons exist for touch response and osmotic avoidance (Hart *et al.*, 1999). o*sm-10* encodes for a novel cytosolic protein and *osm-10* mutant animals show defects in osmotic avoidance, but no effect was found on the response to nose touch or volatile chemicals. Further evidence for the existence of distinct pathways came from the analysis of *grk-2* mutants (Fukuto *et al.*, 2004). The *grk-2* gene encodes a G protein coupled receptor kinase, and loss-of-function of *grk-2* results in a defect in the avoidance of octanol. In contrast, nose touch avoidance was normal in *grk-2* mutants.

The different pathways in the ASH neurons for touch and osmotic avoidance both transduce their signal to postsynaptic cells with the neurotransmitter glutamate. The different levels of glutamate are released in the different responses, resulting in different glutamate receptors being activated on the post-synaptic neurons (Mellem *et al.*, 2002). The signal transduction involved in the release of glutamate requires G proteins and TRP channels. The Gα protein ODR-3 as well as the TRP channel OSM-9 is essential for ASH mediated avoidance responses (Colbert *et al.*, 1997; Roayaie *et al.*, 1998; Tobin *et al.*, 2002). OSM-9 probably forms a polymodal channel together with OCR-2, since mutation of *ocr-2* leads to similar avoidance defects as *osm-9* mutation. Activity of this OSM-9/OCR-2 channel is probably regulated by PUFAs. Mutant animals with defects in PUFA synthesis are indeed defective in avoidance of hyperosmolarity, heavy metals, volatile repellents, and touch (Kahn-Kirby *et al.*, 2004).

1.4.4 Oxygen sensation

Social feeding was first recognized as a variation in feeding behaviour in cultures of different natural isolates of *C. elegans*. Wild type N2 animals are solitary feeders; in contrast, the Hawaiian strain CB4856 shows social feeding. This difference in feeding behaviour is caused by the expression of different NPY-1 isoforms. NPY-1 is a neuropeptide receptor that exists in two different isoforms in the wild. One isoform, NPR-1 215F, is found exclusively in social strains, while the other isoform, NPR-1 215V, is found exclusively in solitary strains (de Bono & Bargmann, 1998). FLP-18 and FLP-21 are ligands for NPR-1 and differentially activate the two different isoforms. Overexpression of *flp-21* can transform social animals into solitary feeders (Rogers *et al.*, 2003). In addition, aversive inputs from the ASH and ADL neurons, using the TRP channel subunits OSM-9 and OCR-2, play a role in social feeding (Coates & de Bono, 2002). Later, it was found that *C. elegans* is also capable of sensing oxygen gradients in its environment (Gray *et al.*, 2004). It turned out that the different social feeding behaviours are the result of different preference of oxygen levels by the different strains.

Oxygen related behaviours can be tested in aerotaxis assays. In these assays animals are placed in a gas-phase oxygen gradient and are allowed to move freely on an agar surface (Gray *et al.*, 2004). Using these assays it was found that *C. elegans* has a preference for 5-12% oxygen, and avoids higher and lower concentrations. Avoidance of higher oxygen levels requires cGMP signalling via guanylate cyclases - GCY-35 and GCY-36 – and the cGMP gated channel TAX-2/TAX-4. The guanylate cyclases are activated by a drop in oxygen levels, which results in depolarisation of AQR, PQR, and URX body cavity neurons. Expression of GCY-35 and GCY-36 in olfactory neurons transforms these neurons into oxygen sensors. This implied that expression of these guanylate cyclases determines the oxygen sensing capacity of the neurons. Previously it was found that NPR-1 functions in the body cavity neurons and that its activity is modulated by TAX-2/TAX-4 (Coates & de Bono, 2002). These results indicate that aerotaxis and social feeding indeed use the same mechanisms. A schematic overview of aerotaxis signalling in the AQR, PQR, URX neurons is given in Figure 3C.

The response to oxygen is plastic. Culturing at a lower percentage of 1-2% oxygen for a few hours resets the preferred oxygen concentration, seeking instead of avoiding 0-5% oxygen. The body cavity neurons are needed for this plasticity of

aerotaxis as well (Gray *et al.*, 2004; Cheung *et al.*, 2005). GCY-35 and TAX-4 mediate oxygen sensation in the same sensory neurons that control social feeding behaviour. Social feeding occurs only when oxygen levels exceed preferred levels and seems to be a strategy for responding to hyperoxygenic environments (Gray *et al.*, 2004).

1.4.5 Thermotaxis

C. elegans can also respond to changes in its surrounding temperature: thermotaxis (Hedgecock & Russell, 1975). Thermotaxis behaviour can be studied by observing the tracks of individual animals in radial thermal gradients. When exposed to a thermal gradient, wild type animals migrate to their cultivation temperature and move isothermally.

The neuronal circuitry for thermotaxis in *C. elegans* has been identified by laser ablation experiments (Mori & Ohshima, 1995). Ablation of the AFD sensory neurons resulted in severe thermotaxis defects. The AFD neurons transmit their signal to the AIY and AIZ interneurons, since ablation of the AIY interneurons resulted in a cryophilic or cold seeking phenotype and ablation of AIZ interneurons results in a thermophilic or heat seeking phenotype. These interneurons further transmit the thermotaxis signal to command interneurons, of which the RIA interneurons may be important for the integration of the thermotaxis signals.

Besides the hard-wiring, several genes that are important for the signal transduction in thermotaxis have been identified. Three guanylate cyclases - *gcy-8*, *gcy-18*, and *gcy-23* – seem to function redundantly in thermotaxis (Inada *et al.*, 2006). These guanylate cyclase, which are expressed exclusively in the AFD neurons, produce cGMP. The second messenger cGMP may in turn activate the cGMP gated channel, formed by the TAX-2 and TAX-4 subunits, which is essential for thermotaxis (Coburn & Bargmann, 1996; Komatsu *et al.*, 1996). Activation of the TAX-2/TAX-4 channels probably raises the Ca^{2+} levels in the cell, which in turn activates downstream signalling molecules. One of the downstream targets may be the Ca^{2+}/calmodulin-dependent kinase CMK-1 that is required for modulation of normal thermotaxis (Satterlee *et al.*, 2004). In contrast, the calcineurin subunit A TAX-6 has been shown to negatively regulate the neuronal activity of the AFD neurons (Kuhara *et al.*, 2002). A schematic summary of thermotaxis signalling in the ADF neurons is given in Figure 3D.

1.4.6 Pheromones

Pheromones are signals used for communication between individuals of the same species. *C. elegans* also produces a pheromone, called dauer pheromone (Jeong *et al.*, 2005), which is continuously secreted. Upon overcrowding very high concentrations of the dauer pheromone cause the animals to enter the dauer stage (Albert *et al.*, 1981), an alternative developmental stage with distinct features and extended lifespan. The pheromone induces dauer larva formation at the second molt and inhibits recovery from the dauer stage in a dose-dependent manner (Golden & Riddle, 1982). Dauer-formation signal transduction involves two endocrine pathways: the insulin and the TGFβ pathway, which are regulated by G protein signalling (Riddle *et al.*, 1981; Vowels & Thomas, 1994; Zwaal *et al.*, 1997; Gerish *et al.*, 2001; Li *et al.*, 2003).

The amphid sensory neurons are also involved in dauer formation (Bargmann *et al.*, 1990). Killing the ADF, ASG, and ASI neurons results in dauer formation regardless of crowding conditions or food-availability, signals that normally induce dauer formation. The ADF and ASI neurons appear to act redundantly in this process. The ASJ neurons are crucial for dauer recovery, which is not affected by the ADF, ASG, and ASI neurons. This suggests that dauer formation and recovery are clearly distinct mechanisms.

1.5 Behavioural plasticity in *C. elegans*

All different forms of sensory behaviour discussed above show plasticity (reviewed in Hobert, 2003), allowing us to dissect the molecular pathways involved in learning behaviours.

1.5.1 Gustatory plasticity

The response of *C. elegans* to salts is plastic. Two different behavioural assays have been developed to study this gustatory plasticity. The first plasticity assay uses gradient plates to test chemotaxis behaviour. In this assay animals are conditioned on NGM-plates for four hours. When animals are starved on plates containing NaCl during the four hour conditioning, they show dramatically reduced chemotaxis to NaCl in the subsequent gradient assay (Saeki *et al.*, 2001). This conditioning requires both the presence of NaCl and the absence of food, indicating that it is not merely adaptation or habituation. While chemotaxis to

volatile chemicals is unaffected by pre-exposure to NaCl in the absence of food, it does affect chemotaxis to other water-soluble compounds. The other way around, conditioning with other water-soluble compounds, such as glucose and sorbitol, did not affect chemotaxis to NaCl. Exogenous serotonin blocked the reduction in chemotaxis after starvation in the absence of food. Thus, serotonin may mediate the food signal in this behavioural assay.

Thus far, one gene has been identified that is involved in this process: *hen-1,* encoding an LDL receptor motif containing protein. *hen-1* mutant animals showed a weaker behavioural change after conditioning than wild types (Ishihara *et al.*, 2002). This protein seems to be necessary specifically for the integration of different signals, since detection of NaCl is normal in *hen-1* mutant animals.

The other assay developed to test gustatory plasticity is based on the quadrant chemotaxis assay (Jansen *et al.*, 2002). In these assays animals are pre-exposed in a buffer containing NaCl and subsequently animals are tested on quadrant plates for their chemotaxis behaviour. Prolonged exposure of wild type animals in a buffer abolishes chemotaxis, and even results in avoidance of the salt in a time and concentrations dependent manner. In addition, this behaviour is reversible and partly salt specific. Optimal pre-exposure conditions for this assay are a pre-exposure of 15 minutes in a buffer containing 100 mM NaCl and subsequently testing chemotaxis behaviour to 25 mM NaCl.

Using the quadrant gustatory plasticity assay, it was found that the Gγ subunit GPC-1 is involved in gustatory plasticity (Jansen *et al.*, 2002). GPC-1 is needed for avoidance after pre-exposure to NaAc, NaCl, and NH_4Cl, whereas chemotaxis to these compounds was normal in naïve *gpc-1* mutant animals. The Gγ subunit is expressed in the ADL, ASI, ASJ, ASH, AFD, AWB, and PHB neurons, of which the ASI and ASH neurons have been shown to play a role in salt responses (Bargmann *et al.*, 1990; Jansen *et al.*, 2002). Thus, it seems likely that GPC-1 functions in these neurons in gustatory plasticity. Two other genes previously implicated in olfactory adaptation, *osm-9* and *adp-1*, are also involved in gustatory plasticity (Colbert & Bargmann, 1995; Colbert *et al.*, 1997; Jansen *et al.*, 2002).

1.5.2 Olfactory adaptation

The responses to olfactory cues are regulated in a dynamic way by behavioural context and the animal's previous experience. Prolonged exposure to an odorant leads to a decreased response to that odorant, called olfactory

adaptation. Olfactory adaptation is odorant selective; animals can adapt independently to different odorants sensed by a single pair of olfactory neurons (Colbert & Bargmann, 1995). Olfactory adaptation, and also recovery from adaptation, increases upon starvation. The effect of starvation is antagonised by exogenous serotonin (Colbert & Bargmann, 1997). In olfactory adaptation, the signal of serotonin is not transmitted through GOA-1 as has been shown for egg-laying (Matsuki *et al.*, 2006). However, a balance of the Gα proteins GOA-1 and EGL-30 was found to regulate olfactory adaptation in the AWC neurons. Elevated levels of diacylglycerol (DAG) result in a defect in olfactory adaptation. DAG levels can be elevated either by exogenous addition of a DAG analogue or by mutating genes from the GOA-1 pathway (Matsuki *et al.*, 2006).

Further evidence for the role of G proteins in olfactory adaptation came from *arr-1* mutants, which show significant defects in olfactory adaptation and recovery from adaptation (Palmitessa *et al.*, 2005). In contrast, overexpression of ARR-1 enhances olfactory adaptation. *arr-1* encodes an arrestin, which is thought to regulate G protein activity and often function together with G protein receptor kinases (GRKs). *C. elegans* has two GRKs, GRK-1, and GRK-2, which are broadly expressed. It seems that GRK-2 is not involved in olfactory adaptation, it remains to be seen whether GRK-1 is involved (Fukuto *et al.*, 2004).

Olfactory adaptation can be inhibited by EGTA, which chelates extracellular Ca^{2+}, suggesting that olfactory adaptation requires calcium signalling (Colbert & Bargmann, 1995). The importance of calcium in olfactory adaptation was further stressed by the finding that the TRP channel OSM-9 as well as the calcineurin A subunit TAX-6 are involved in this process (Coburn & Bargmann, 1996; Kuhara *et al.*, 2002). OSM-9 functions in the AWA neurons in olfaction together with OCR-2, but in neurons that express only *oms-9* and not *ocr-2*, OSM-9 resides in the cell body instead of the cilia. In the cell body OSM-9 seems to function in adaptation rather than in sensation (Tobin *et al.*, 2002). *tax-6* mutant animals showed enhanced adaptation, which is suppressed by mutation of *osm-9*. Thus, *tax-6* might negatively regulate the response to odorants by regulating OSM-9 dependent adaptation.

In addition, using a forward genetic screen an additional olfactory adaptation pathway was found. This pathway uses the unknown protein ADP-1. *adp-1* mutant animals show a defect in adaptation to odorants, but not in detection of odorants, suggesting that sensation and adaptation are distinct processes. Moreover, *adp-1; osm-9* double mutant animals have stronger defects than either

single mutant, suggesting that *osm-9* and *adp-1* function in separate pathways (Colbert & Bargmann, 1995).

Not only calcium, but also the second messenger cGMP plays a role in olfactory adaptation, since overexpression of ODR-1 disrupts adaptation to the odorant butanone (L' Etoile *et al.*, 2002). The guanylate cyclase ODR-1 produces cGMP, which can activate for example the cGMP dependent kinase EGL-4. EGL-4 activity is needed for adaptation only in adult animals and requires a predicted NLS after longer odorant exposure, suggesting that nuclear translocation triggers long-term adaptation (L'Etoile *et al.*, 2002). In the nucleus EGL-4 could transmit its signal to the transcription factor TBX-2. This protein is mostly localized to the cytoplasm, but upon olfactory adaptation it is translocated to the nucleus (Miyahera *et al.*, 2004). In addition, EGL-4 acts presynaptically in cholinergic neurons and acts to regulate serotonin levels indirectly in dauer formation (Daniels *et al.*, 2000). Perhaps EGL-4 can regulate serotonin levels in olfactory adaptation as well, since serotonin antagonizes the enhancing effect of starvation on olfactory adaptation.

Other studies have revealed even more complex forms of plasticity of *C. elegans* olfactory responses: habituation, aversion learning, state dependency, and imprinting.

Adaptation has been defined as a decrement in the behavioural response due to sensory fatigue, which cannot be dishabituated. On the other hand, habituation is a form of associative learning in which the decrease in response can be readily reversed. In *C. elegans,* olfactory habituation can be reversed by harsh treatment, such as centrifugation or vortexing (Bernhard & van der Kooy, 2001; Morrison & van der Kooy, 2001). Adaptation is thought to be the result of pre-exposure to high concentrations of an odorant, whereas habituation is the result of pre-exposure to low concentrations (Bernhard & van der Kooy, 2001). Unfortunately, very little is known about the molecular processes involved in olfactory habituation.

C. elegans can also modify its olfactory preferences after exposure to pathogenic bacteria, resulting in avoidance of odorants from the pathogen and increased attraction to odorants from familiar nonpathogenic bacteria (Zhang & Bargmann, 2005). Exposure to pathogenic bacteria increases serotonin levels in the ADF neurons by transcriptional and post-transcriptional mechanisms. Serotonin functions through the MOD-1 serotonin receptor, which is expressed in sensory interneurons, to promote aversion learning.

If olfactory adaptation is acquired during ethanol administration, the adaptation is subsequently displayed only if the ethanol stimulus is present again. This behaviour was called state-dependency and is a form of classical conditioning. *cat-1* and *cat-2* mutant animals are impaired in this process, indicating a function for dopamine and/or serotonin, since CAT-1 and CAT-2 are involved in the transport and synthesis of these neurotransmitters, respectively (Duerr *et al.*, 1999; Lints & Emmons *et al.*, 1999; Bettinger & McIntire, 2004).

A different form of olfactory plasticity is imprinting (Remy & Hobert, 2005). Olfactory imprinting is a process in which the exposure of animals to olfactory cues during a specific and restricted time window leaves a permanent memory that shapes the animal's behaviour upon encountering the olfactory cue at later stages. The imprint is associated with favourable growth conditions and is generated at early juvenile stages. Olfactory imprinting uses a single interneuron pair that is postsynaptic to olfactory neurons: AIY.

Thus far, very little is known about the molecular mechanisms behind these complex forms of olfactory learning and it is not quite clear whether different mechanisms are involved. However, it is very remarkable that most of them seem to involve serotonin signalling.

1.5.3 Tap withdrawal habituation

Many studies on learning behaviour in *C. elegans* have been done using the tap withdrawal habituation assay. *C. elegans* swims backwards in response to a tap delivered to the petri dish it is cultured on. This tap withdrawal response can be both habituated and sensitised(Rankin *et al.*, 1990). Both habituation and recovery are dependent on the frequency of stimulation and the interstimuli interval (ISI). Short ISI produce greater response decrement than long ISI; short ISI induced effects recovers more rapidly than those induced by long ISI. This suggests long term and short term ISI stimulation initiate different processes (Rankin & Broster, 1992).

C. elegans can retain memory for habituation training for over 24 hours. This long-term retention was blocked by heat shock, resulting in a stop of protein synthesis, between training blocks (Beck & Rankin, 1995). Retention of habituation can be affected by associations and context cues can serve as facilitory associative components for retention of habituation training (Rankin, 2000).

The neural circuit that underlies habituation was determined by laser ablating individual neurons and examining the resulting effect on the tap

withdrawal response (Chalfie *et al.*, 1985; Wicks & Rankin, 1995; 1996). Initial genetic analyses showed that the neurotransmitter glutamate is important in this form of learning and memory in *C. elegans*. Mutants for the glutamate transporter EAT-4 show normal tap withdrawal responses, but showed more rapid and complete habituation than wild type animals (Rankin & Wicks, 2000).

Additionally, group reared animals show a much stronger response to mechanical stimuli than animals raised in isolation. Glutamate is also involved in this process, since the glutamate receptor GLR-1 is needed for this effect (Rose *et al.*, 2005). Serotonin was found to be important in olfactory learning, and in the tap withdrawal habituation glutamate is the neurotransmitter involved. Thus, both serotonin and glutamate are important neurotransmitters in behavioural plasticity in *C. elegans,* similar to what was found for learning in vertebrates.

1.5.4 Thermotaxis associative learning

Thermotaxis shows plasticity, since the temperature preference of well-fed animals can be reset by cultivating these animals only for a few hours at another temperature (Mohri *et al.*, 2005). Besides, animals disperse from their preferred temperature after starvation and overcrowding, suggesting that animals can associate their cultivation temperature with environmental cues, such as food availability. The change in feeding state quickly stimulates a switch between attraction to and avoidance of the memorized temperature. However, the acquisition of this new temperature memory establishes more slowly than normal. In a forward screen several *aho* (abnormal hunger orientation) mutants were isolated that are defective in starvation induced cultivation temperature avoidance. Unfortunately, these genes have not been cloned yet. Exogenous serotonin and octopamine can mimic the well-fed state and the starved state of the animal, respectively (Mohri *et al.*, 2005).

The LDL receptor motif protein HEN-1, which is involved in gustatory plasticity, is also needed in thermotaxis associative learning (Ishihara *et al.*, 2002). The HEN-1 protein can be secreted and works non-cell-autonomously. It seems that this protein has a general function in the integration of different signals. Furthermore, it has been shown that temperature avoidance is dependent on Ca^{2+} and requires a functional calcium-binding sensor NCS-1 (Gomez *et al.*, 2001). The temperature associative learning defect of *ncs-1* mutants could be rescued by expressing the *ncs-1* gene in the AIY interneuron, not in the temperature sensing neuron AFD. Overexpression of *ncs-1* improved memory and resulted in enhanced

association with temperature. Thus, the levels of NCS-1 in the animal determine the association between starvation and temperature.

1.6 Neurotransmission

The discussed behaviours and their plasticity all involve multiple neurons, requiring signalling between these different cells. A neurotransmitter is a substance that is released from the presynaptic nerve terminal and subsequently changes the properties of the postsynaptic cell by binding to specific receptors. Over the years different categories of neurotransmitters have been found to function in the vertebrate nervous system. These categories are acetylcholine, the biogenic amines, the amino acids glutamate and GABA, and neuropeptides.

Although the *C. elegans* nervous system has only 302 neurons and it is relatively simple, the diversity of classical neurotransmitters seems comparable to that in vertebrate nervous systems. Almost all known mammalian neurotransmitters are also found in *C. elegans* except for (nor)epinephrine (Rand *et al.*, 2000). However, it has been suggested that the functions of epinephrine and norepinephrine are taken over by tyramine and octopamine in invertebrates (Roeder *et al.*, 2003).

1.6.1 Acetylcholine

In vertebrates, acetylcholine is the excitatory neurotransmitter at neuromuscular junctions, but it also acts in pain and some chemosensory pathways. Acetylcholine is synthesized in nerve terminals from acetyl coenzyme A and choline, in a reaction that is catalysed by choline acetyltransferase (Figure 4A). Like all neurotransmitters, acetylcholine is packed into vesicles that are released in a Ca^{2+} dependent manner. Postsynaptically the signal of acetylcholine is transduced via either nicotinic or muscarinic receptors. Nicotinic acetylcholine receptors are ionotropic receptors and muscarinic receptors are GPCRs. The postsynaptic actions of acetylcholine are not terminated by reuptake, like with other neurotransmitters, but in contrast acetylcholine is hydrolysed by the enzyme acetylcholinesterase. This enzyme hydrolyses acetylcholine into acetate and choline. Subsequently, choline is taken up by cholinergic nerve terminals.

In *C. elegans,* acetylcholine is the main excitatory neurotransmitter and the only one that is essential for viability (Brenner, 1974; Rand *et al.*, 2000). Approximately 115 neurons in *C. elegans* appear to be cholinergic; almost all of

these are motor neurons. Moreover, animals are paralysed by cholinesterase inhibitors and nicotinic agonists. Taken together, it seems that acetylcholine mediates locomotion.

In *C. elegans* at least 40 genes with similarity to nicotinic subunits have been identified, although not all have been shown to be authentic nicotinic receptors (Squire *et al.*, 1995; Ballivet *et al.*, 1996; Bargmann, 1998). These receptor genes can be categorized into five groups based on sequence similarity. In addition, three muscarinic acetylcholine receptors have been identified in *C. elegans*: GAR-1, -2, and -3, of which GAR-3 is most similar to mammalian muscarinic receptors (Park *et al.*, 2003). Genes involved in acetylcholine metabolism have also been found in *C. elegans*: CHA-1 is an acetyltransferase and UNC-17 is a choline transporter (Rand & Russell, 1984; Rand, 1989; Alfonso *et al.*, 1993; 1994).

1.6.2 Glutamate

Glutamate is generally thought to be the most important transmitter for normal vertebrate brain function. Nearly all excitatory neurons in the central nervous system are glutamatergic. It has been estimated that half of the synapses in the brain release this amino acid. The precursor of glutamate in synaptic terminals is glutamine, which is released by glial cells and metabolised to glutamate by the mitochondrial enzyme glutaminase (Figure 4B). Subsequently, glutamate is packed into vesicles, which are released in a Ca^{2+} dependent manner. Postsynaptically, glutamate can bind either to ionotropic glutamate receptors (iGluRs) or metabotropic glutamate receptors. iGluRs comprise a family of diverse ion channels formed by a tetrameric arrangement of receptor subunits. The 18 subunits described in mammals are divided into different groups: NMDA and non-NMDA receptors. The family of non-NMDA receptors is subdivided into AMPA, kainate, and delta subtypes. Functional ligand-gated receptors are formed by heteromerisation of subunits from a single subtype. Excess glutamate is removed from the synaptic cleft by transporters present in both gial cells and presynaptic terminals. Glial cells contain the enzyme glutamine synthetase, which converts glutamate back into glutamine.

Ten genes that encode putative glutamate receptor subunits have been identified in *C. elegans*. The subunits encoded by these genes belong either to non-NMDA (GLR1-8) or the NMDA (NMR-1 and NMR-2) class (Maricq *et al.*, 1995; Hart *et al.*, 1995; Brockie *et al.*, 2001). The EAT-4 glutamate transporter was found to

affect the amount of glutamate released from glutamatergic neurons. It is hypothesized to influence glutamate release by modulating the activity of glutaminase (Lee *et al.*, 1999).

Most of the phenotypes associated with glutamate in *C. elegans* came from the analysis of *glr-1* AMPA-type glutamate receptor mutant animals and *eat-4* glutamate transporter mutant animals. Glutamate is released by many sensory and interneurons in *C. elegans* and some behavioural circuits involving glutamate have been identified. The response to light mechanical stimuli of the head is mediated by the ASH neurons. These neurons produce glutamate and activate the interneurons through the GLR-1 postsynaptic glutamate receptor (Hart *et al.*, 1995; Maricq *et al.*, 1995; Mellem *et al.*, 2002; Chao *et al.*, 2004). The response to strong mechanical stimuli in the anterior body is mediated by the ALM and AVM sensory neurons through the AVR-15 postsynaptic glutamate gated channel (Lee *et al.*, 1999). Also the effects of M3 motor neurons on pharyngeal muscle is mediated through AVR-15 (Li *et al.*, 1997; Dent *et al.*, 2000). The glutamate transporter EAT-4 is involved in habituation of the tap withdrawal response, since *eat-4* mutants habituate more rapidly than wild types, recover more slowly, and show no dishabituation (Rankin *et al.*, 2000; Rose *et al.*, 2002; 2003). NMDA receptors provide currents that modulate the frequency of movement and reversals during foraging (Brockie *et al.*, 2001). Osmotic avoidance involves NMDA and non-NMDA receptors (Kaplan & Horvitz, 1993; Mellem *et al.*, 2002). Furthermore, glutamate plays a role in control of locomotion (Zheng *et al.*, 1999).

1.6.3 GABA

Most inhibitory neurons in the vertebrate brain use γ-aminobutyric acid (GABA) as a neurotransmitter. GABA has the property to inhibit the ability of mammalian neurons to fire action potentials (Curtis *et al.*, 1995). GABA is present in high concentrations throughout the .nervous system, but also in other tissues. It is most commonly found in local-circuit interneurons, where it is synthesized from glutamate by the enzyme glutamic acid decarboxylase, which requires the cofactor pyridoxal phosphate for activity (Figure 4C). After GABA containing vesicles have been released, GABA can bind two types of receptors: $GABA_A$ receptors are ligand-gated ion channels, and $GABA_B$ receptors are metabotropic receptors. The mechanism of removal of GABA from the synaptic cleft is similar to that of glutamate: both neurons and glial cells have high affinity transporters for GABA. Eventually, GABA is converted to succinate by two different enzymes.

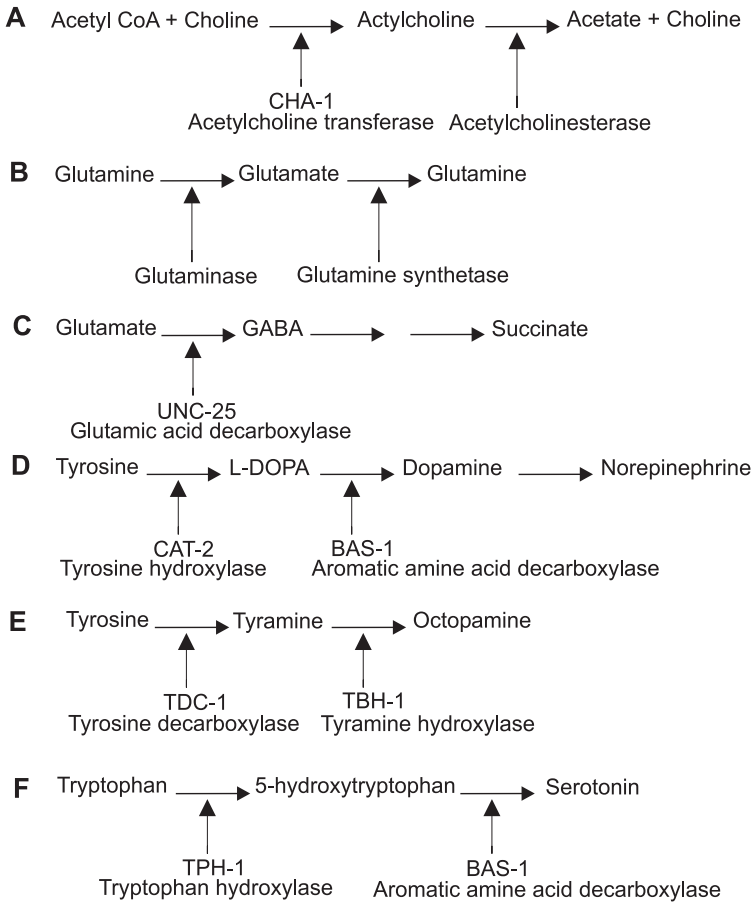

Figure 4: **Overview of the biosynthesis of neurotransmitters with the belonging enzymes.** (A) Synthesis of acetylcholine. (B) Synthesis of glutamate. (C) Synthesis of GABA. (D) Synthesis of dopamine. (E) Synthesis of octopamine. (F) Synthesis of serotonin.

In *C. elegans* multiple genes have been found needed for GABA function. These genes include the *unc-25* glutamic acid decarboxylase (Jin *et al.*, 1999), *unc-47* GABA-transporter (McIntire *et al.*, 1997), *snf-11* GABA transporter (Mullen *et al.*, 2006), *unc-49* inhibitory $GABA_A$ receptor (Bamber *et al.*, 1999), and the *exp-1* excitatory channel receptor (Beg & Jorgensen, 2003). Examination of the *C. elegans* genome reveals several more receptors, including $GABA_B$ receptors (Jorgensen, 2005).

Although GABA is the most abundant neurotransmitter in the vertebrate brain, in *C. elegans* only 26 neurons are GABAergic: 6 DD neurons, 13 VD neurons, 4 RME neurons, RIS, AVL, and AVB (McIntire *et al.*, 1993). Three phenotypes are linked to the GABAergic nervous system in *C. elegans*: (I) the DD and VD neurons have an inhibitory role in locomotion; (II) the RME neurons are associated with

foraging behaviour; (III) the AVL and DVB neurons have an excitatory role in the defecation cycle. No phenotype has been associated with the loss of the RIS interneuron.

Taken together, there are three main differences of the *C. elegans* GABAergic nervous system compared to that in vertebrates. First, GABA acts in neuromuscular junctions in *C. elegans*, whereas it functions in the central nervous system in vertebrates. Second, less than 10% of *C. elegans* neurons are GABAergic, in contrast to 30-40% of the neurons in vertebrates. Third, GABA has an inhibitory role in vertebrates, but can function excitatory as well as inhibitory in *C. elegans*. No GABA-gated cation channel related to EXP-1 has been found in the vertebrate genome.

1.6.4 Biogenic amines

The group of biogenic amines contains the catecholamines – octopamine, dopamine, norepinephrine, and epinephrine – and serotonin. All catecholamines are derived from a common precursor, the amino acid tyrosine (Figure 4D,E). The first rate-limiting step in the synthesis of catecholamines is catalysed by tyrosine hydroxylase and results in the synthesis of dihydrozyphenylalanine (DOPA). Dopamine is produced from DOPA by DOPA-decarboxylase and it is present in several brain regions, but its major role is in the control of body movements (Figure 4D). Next, a third enzyme, dopamine β-hydroxylase, converts dopamine into norepinephrine. This neurotransmitter mainly functions in neurons important in modulating sleep and wakefulness. Epinephrine is present at much lower levels in the brain and is synthesized by phenylethanolamine-*N*-methyltransferase from norepinephrine. Some neurons in the brain are thought to use epinephrine as a neurotransmitter, but its function remains unknown. Not all cells that release catecholamines express all biosynthetic enzymes. The biogenic amine octopamine is also derived from tyrosine, but its synthesis requires other enzymes: tyrosine decarboxylase converts tyrosine into tyramine and tyramine β-hydroxylase converts tyramine into octopamine (Figure 4E).

Serotonin is synthesized from the amino acid tryptophan. Tryptophan is taken up by neurons and hydroxylated in a reaction catalysed by the enzymes tryptophan-5-hydroxylase and 5-hydroxytryptophan decarboxylase or aromatic amine acid decarboxylase (Figure 4F). Interestingly, DOPA decarboxylase and 5-hydroxytryptophan decarboxylase seem to be identical. Thus, the synthesis of both dopamine and serotonin requires the same enzymes.

After carrying out its function in the synaptic cleft, the catecholamines – dopamine, norepinephrine, and epinephrine – and serotonin are removed from the synaptic cleft by reuptake into the nerve terminals or into surrounding glial cells by an Na^+ dependent transporter. There are two major enzymes involved in the catabolism of catecholamines: mono-amine oxidase and catechol *O*-methyltransferase, which both are targets for numerous psychotic drugs.

In *C. elegans* the genes encoding for the components of dopamine, serotonin, and octopamine synthesis have been identified. The enzymes encoded by *bas-1* and *cat-4* are involved in both serotonin and dopamine synthesis, *cat-2* in needed for dopamine synthesis and *tph-1* is specifically needed for the synthesis of serotonin (Figure 4D, F; Loer & Kenyon, 1993; Lints & Emmons, 1999; Sze *et al.*, 2000). Octopamine biosynthesis requires the enzymes encoded by the genes *tdc-1* and *tbh-1* (Figure 4E; Alkema *et al.*, 2005).

The *C. elegans* genome contains several serotonin and dopamine receptors and transporters. Dopamine and serotonin are transported into the synaps by the synaptic vesicular monoamine transporter CAT-1 (Duerr *et al.*, 1999). *dat-1* and *mod-5* encode for dopamine and serotonin reuptake transporters, respectively (Ranganathan *et al.*, 2001; Jayanthi *et al.*, 1998). In a BLAST search for biogenic amine receptors in the *C. elegans* genome several GPCRs have been found, among which the SER-1 and SER-4 serotonin receptors, the SER-2 tyramine receptor (Rex & Kominiecki, 2002), and the DOP-1, DOP-2, and DOP-3 dopamine receptors (Tsalik *et al.*, 2003; Chase *et al.*, 2004). Furthermore, a serotonin-gated chloride channel MOD-1 was found (Ranganathan *et al.*, 2000).

1.6.4.1 Dopamine

In *C. elegans*, dopamine was found in eight sensory neurons: the CEPV, CEPD, ADE, and PDE neurons (Sulston *et al.*, 1975). Ablation of dopaminergic neurons as well as application of a dopamine receptor agonist eliminates area restricted search behaviour (Hills *et al.*, 2004) and it modulated the plasticity of mechanosensation (Sanyal *et al.*, 2004). In addition, dopamine deficits result in difficulties in sensing and responding to the presence of food (Loer and Kenyon, 1993; Sawin *et al.*, 2000). Exogenous dopamine inhibits locomotion and egg laying (Horvitz, 1982; Dempsey *et al*, 2005). Dopamine acts extrasynaptically to modulate locomotion rate by activating D1 and D2 receptors, which are co-expressed in cholinergic neurons (Chase *et al*., 2004).

1.6.4.2 Serotonin

In *C. elegans* seven neurons from four different classes can be stained with antibodies against serotonin: 2 NSM neurons, 2 ADF neurons, RIH, and 2 AIM neurons (Horvitz, 1982; Desai *et al.*, 1988; Loer and Kenyon, 1993; Duerr *et al.*, 1999). However, only two classes are capable of *de novo* synthesis of serotonin: the ADF and NSM neurons (Sze *et al.*, 2000). Serotonin regulates egg laying, neuronal migration, pharyngeal pumping, male mating, but also the responses to starvation (Loer and Kenyon, 1993; Weinschenker *et al.*, 1995; Schafer *et al.*, 1996; Sawin *et al.*, 2000; Sze *et al.*, 2000; Hardaker *et al.*, 2001; Kindt *et al.*, 2002; Nuttley *et al.*, 2002; Niacaris and Avery, 2003; Shyn *et al.*, 2003; Chao *et al.*, 2004; Dempsey *et al.*, 2005; Mohri *et al.*, 2005). Remarkable is the role that serotonin has in a variety of assays involving behavioural plasticity: in thermotaxis, salt-chemotaxis, and olfaction (Colbert & Bargmann, 1997; Saeki *et al.*, 2001; Bettinger & McIntire, 2004; Mohri *et al.*, 2005; Zhang & Bargmann, 2005).

1.6.4.3 Octopamine and Tyramine

In *C. elegans,* exogenous octopamine inhibits egg laying and stimulates locomotion (Horvitz, 1982 ; Alkema *et al.*, 2005). Moreover, octopamine is involved in the signal transduction of food signals in thermotaxis. Animals disperse from their cultivation temperature after starvation and overcrowding, which can be induced by exogenous octopamine (Mohri *et al.*, 2005).

Tyramine is a precursor of octopamine. Tyramine was found to influence egg laying, modulated reversal behaviours, but also suppresses head oscillations (Alkema *et al.*, 2005). Overall, tyramine influences other behaviours than octopamine, indicating that tyramine can function as a neurotransmitter on its own.

1.6.5 Neuropeptides

A neuropeptide is a small peptide that can act as a neurotransmitter or neuromodulator. Neuropeptides are frequently released as co-transmitters with other classical neurotransmitters, of which they can modulate the impact. They are derived from large precursor or proprotein molecules which can be enzymatically cleaved and post-translationally modified to yield multiple copies of the same peptide or copies of several different bioactive peptides. It is important to note that

the synthesis of neuropeptides requires gene activation, DNA transcription and RNA translation, with the prepropeptide being transported from the ribosomes to the Golgi apparatus, where it is processed into propeptides, which can be transported from the soma to synaptic sites. This in contrast to the classical neurotransmitters, which can be synthesized by cytosolic enzymes at the synaptic sites.

Critical in the cleavage of proproteins into active neuropeptides are proprotein convertases. *C. elegans* has two such proprotein convertases: EGL-3 that regulates mechanosensory behaviours, and EGL-21 that affects locomotion behaviour (Kass *et al.*, 2001; Jacob & Kaplan, 2003). The *C. elegans* genome contains 23 predicted FMRFamide-like (*flp)* peptide genes and 32 predicted neuropeptide-like (*nlp*) genes. Recently, 21 peptides derived from formerly predicted neuropeptide-like genes, 28 predicted FMRFamide-related peptides (FaRPs), and 11 novel peptides were identified, but their receptors remain to be identified (Li *et al.*, 1999; Husson *et al.*, 2005).

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Chapter 2

Two genetic pathways involving TRPV channel-, G protein-, and MAP kinase-signalling mediate chemotaxis to NaCl in *C. elegans*

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2

Manuscript in preparation

Abstract

Not much is known about the molecular mechanisms of NaCl detection. Thus far, two ion channels have been shown to play a role in this process in mammals: an epithelial Na^+ channel (ENaC) and a transient receptor potential channel TRPV. We use *Caenorhabditis elegans* to unravel the molecular mechanisms of NaCl detection. Previous studies have shown that NaCl chemo-attraction in *C. elegans* requires cGMP and Ca^{2+} signalling via the cyclic nucleotide gated channel TAX-2/TAX-4 and the calcineurin A subunit TAX-6. Here we identify five new molecules involved in chemo-attraction to NaCl in *C. elegans* and position them in two different genetic pathways. One pathway involves the genes *tax-4* and *tax-6*, and the newly identified mitogen activated protein (MAP) kinases: *nsy-1* and *sek-1*. The second pathway consists of *tax-2*, as well as the newly identified TRPV channel *osm-9*, the Gα subunit *odr-3*, and the guanylate cyclase *gcy-35*. Our findings show overlap in the molecular mechanisms of NaCl detection between *C. elegans* and mammals, since both use signalling via TRPV channels. In addition, we provide the first evidence for the involvement of G protein- and MAP kinase signalling in NaCl chemotaxis.

Introduction

Salt taste is essential for water homeostasis and can serve as a possible food cue. Very little is known about the molecular mechanisms of how salts are detected (reviewed in Lindemann, 2001). In mammals salts appear to be sensed by two different mechanisms. The first uses the amiloride sensitive epithelial Na^+ channel (ENaC) (Heck *et al.*, 1984; Canessa *et al.*, 1994), the second an amiloride insensitive pathway involving the transient receptor potential (TRP) channel VR1 (Lyall *et al.*, 2004). It has been proposed that Na^+ influx through these channels can activate the cell leading to neurotransmitter release. However, other signalling molecules have not yet been identified.

We use the model organism *C. elegans* to study the molecular mechanisms of salt chemotaxis using the quadrant assay (Wicks *et al.*, 2000; Jansen *et al.*, 2002). In this assay animals, washed with a buffer, are placed on petri dishes divided into four quadrants with plastic spacers. Each quadrant is filled with buffered agar with a certain concentration of a compound. The dispersal of animals over the quadrants with or without the compound serves as a measure of attraction or repulsion of the compound. Using this assay, we can discriminate three responses

of *C. elegans* to NaCl. First, animals are attracted to NaCl concentrations up to 200 mM, and second they avoid higher concentrations. The third response is called gustatory plasticity: avoidance of normally attractive NaCl concentrations after pre-exposure to NaCl.

C. elegans uses only 12 pairs of sensory neurons located in the head, the amphid sensory neurons, to sense its environment (Bargmann *et al.*, 1990). The most important salt sensing cells in *C. elegans* are the ASE neurons (Bargmann & Horvitz, 1991). These neurons are essential for NaCl chemotaxis also in quadrant assays (Hukema *et al.*, 2006). However, three other pairs of neurons have redundant functions in salt detection: the ADF, ASG, and ASI neurons (Bargmann & Horvitz, 1991). In addition, the ASE, ASI, ADF and ASH neurons have a role in gustatory plasticity. Avoidance of high NaCl concentrations is mediated by the nociceptive ASH neurons.

Several genes involved in chemotaxis to NaCl have been found in genetic screens using gradient assays, in which animals are exposed to a shallow gradient of NaCl (Ward, 1973; Dusenbery *et al.*, 1975). These genes include the cGMP gated channel subunit genes *tax-2* and *tax-4*, and the calcineurin subunit A *tax-6* (Coburn & Bargmann, 1996; Komatsu *et al.*, 1996; Kuhara *et al.*, 2002). We confirmed the role of these genes in the quadrant assays and in addition found that also the calcineurin subunit B CNB-1 and the neuronal Ca^{2+} sensor NCS-1 are involved in attraction to NaCl (Hukema *et al.*, 2006). Taken together, this shows that the second messengers cGMP and Ca^{2+} are important for chemotaxis to NaCl in *C. elegans*.

In this study, we identified five new genes that play a role in chemotaxis to NaCl. We show that these genes function in two genetic pathways. The first pathway consists of the *tax-4* and *tax-6* genes, as well as the newly identified MAP kinase genes *nsy-1* and *sek-1.* The second pathway includes *tax-2* as well as genes for the TRP channel subunit *osm-9*, the Gα subunit *odr-3*, and the guanylate cyclase *gcy-35*. Our finding that the TRPV channel subunit OSM-9 is required for chemotaxis to salt in *C. elegans* shows a striking similarity to salt taste in mammals, in which the TRPV channel VR1 has been found to mediate salt responses (Lyall *et al.*, 2004). In addition, we provide the first evidence that G protein and MAP kinase signalling play a role in NaCl chemotaxis.

Results

TAX-2 and TAX-4 function in parallel pathways

Previously it was found that chemotaxis to salts involves the second messenger cGMP (Coburn & Bargmann, 1996; Komatsu *et al.*, 1996). We confirmed the role of the cGMP gated channel (CNG) subunits TAX-2 and TAX-4 also in the quadrant assay (Hukema *et al.*, 2006). In this assay *tax-2* and *tax-4* mutant animals show a very similar defect in chemotaxis to NaCl (Figure 1A): they show no response to 0.1-1 mM NaCl, but there still is a significant, albeit strongly reduced, response to higher NaCl concentrations (10-100 mM). Since TAX-2 and TAX-4 function together in many processes (Coburn & Bargmann, 1996; Komatsu *et al.*, 1996), 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 NaCl (Figure 1A), suggesting that *tax-2* and *tax-4* function, at least partially, in parallel genetic pathways.

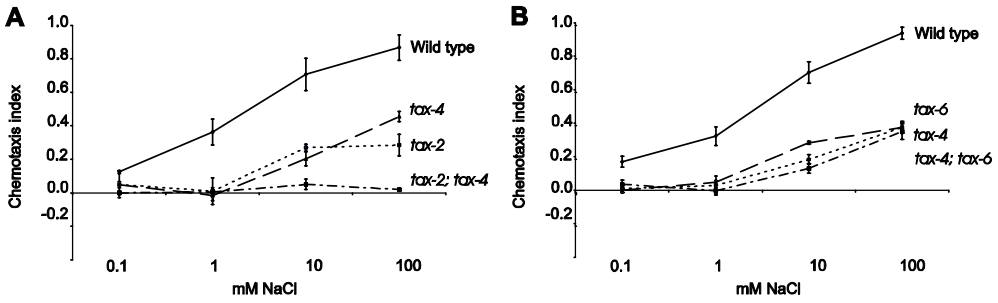

Figure 1: ***tax-2, tax-4,* and *tax-6* function, at least partially, in two genetic pathways in NaCl chemotaxis**. (A) *tax-2* ($p<0.05$) and *tax-4* ($p<0.01$) mutant animals show reduced chemotaxis to NaCl, but do not significantly differ from each other ($p>0.05$). Chemotaxis to NaCl is completely abolished in *tax-2; tax-4* double mutant animals ($p<0.05$ when compared to either of the two single mutant animals), suggesting that *tax-2* and *tax-4* function, at least partially, in parallel genetic pathways. (B) *tax-4 and tax-6* mutant animals show a similar defect in chemotaxis to NaCl ($p<0.05$). *tax-4; tax-6* double mutant animals do not significantly differ from *tax-4* mutant animals ($p>0.05$), suggesting that *tax-4* and *tax-6* function in the same genetic pathway. Indicated are the averages of at least 4 assays ± s.e.m.

Previous studies have shown that the calcineurin A subunit TAX-6 plays a role in salt chemotaxis (Kuhara *et al.*, 2002; Hukema *et al.*, 2006). We found that *tax-6* mutant animals show a very similar response to NaCl as *tax-4* mutant animals

(Figure 1B). To determine whether *tax-6* functions in the same pathway as *tax-4,* we tested *tax-4; tax-6* double mutant animals for their response to NaCl. We found that *tax-4; tax-6* double mutant animals show the same response to NaCl as either of the single mutants, suggesting that they function in the same genetic pathway. The TAX-6 calcineurin subunit A is a Ca^{2+}-activated phosphatase, which might be activated by Ca^{2+} entering the cell via a cGMP-gated channel that includes TAX-4 and perhaps other CNG subunits.

MAP kinase function in chemotaxis

The implication of the phosphatase calcineurin TAX-6/CNB-1 in salt chemotaxis triggered us to test various kinase mutant strains in our quadrant chemotaxis assay (Kuhara *et al.*, 2002; Hukema *et al.*, 2006). The *C. elegans* genome contains 438 predicted kinases (Manning, 2005) several of which have been shown to function in sensory signalling. Of these kinases involved in sensory signalling, we tested 18 different mutants, including mitogen activated protein (MAP) kinases – *jkk-1, jnk-1, pmk-3, nsy-1,* and *sek-1* – (Kawasaki *et al.*, 1999; Berman *et al.*, 2001; Sagasti *et al.*, 2001; Villanueva *et al.*, 2001; Tanaka-Hino *et al.*, 2002), diacylglycerol kinases – *dkg-1, dgk-2,* and *dgk-3* – (Lynch *et al.*, 1995; Hadju-Cronin *et al.*, 1999; Nurrish *et al.*, 1999; Espelt *et al.*, 2005), Ca^{2+}-Calmodulin dependent (CaM) protein kinases – *cmk-1* and *unc-43* – (Eto *et al.*, 1999; Reiner *et al.*,1999), G protein coupled receptor kinases – *grk-1* and *grk-2* – (Fukuto *et al.*, 2004), kinases involved in IP_3 signalling – *age-1, pdk-1*, and *ppk-2* – (Paradis *et al.*, 1999; Wolkow *et al.*, 2002), and others – *egl-4, kin-13,* and *pkc-2* – (Land *et al.*, 1994; Islas-Trejo *et al.*, 1997; L'Etoile *et al.*, 2002). The role of three of these kinases– *dgk-1, egl-4,* and *grk-2* – in the response of *C. elegans* to NaCl has been reported previously (Hukema *et al.*, 2006). Unfortunately, *unc-43* loss- and gain-of-function animals could not be tested in our behavioural assays, because of their severe locomotion defect. The behaviours of the other kinase mutants were tested in the quadrant NaCl chemotaxis assay; most mutants showed the same response as wild type animals (Table 1).

Interestingly, *nsy-1* and *sek-1* mutant animals showed strong defects in chemotaxis to NaCl. We found the defect using two alleles for both genes (Figure 2A, results not shown). Both showed the same behaviour: no response to 0.1-1 mM NaCl, but a clear, albeit significantly reduced, response to 10-100 mM NaCl (Figure 2A). The MAPKKK NSY-1 and the MAPKK SEK-1 function together in a pathway that regulates the asymmetric expression of *str-2* in the AWC neurons, and in a pathway that regulates the innate immune response. In both cases it has been reported that NSY-1 functions upstream of SEK-1 and activates it by

phosphorylation (Sagasti *et al.*, 2001; Kim *et al.*, 2002; Tanaka-Hino *et al.*, 2002). Since the responses of *nsy-1* and *sek-1* mutant animals were very similar, these genes might also function in the same pathway in NaCl chemotaxis. Analysis of *nsy-1; sek-1* double mutant animals indeed showed the same defect in chemotaxis to NaCl as *nsy-1* and *sek-1* single mutants, suggesting they function in the same genetic pathway (Figure 2A).

Table 1: Chemotaxis response of mutant animals for kinases involved in sensory signalling in *C. elegans*.

gene	product	response to 0.1-100 mM NaCl	reference
age-1 (hx546) II	PI_3-kinase	WT	Wolkow *et al.*, 2002
cmk-1 (ok287) IV	CaMK I	WT	Eto *et al.*, 1999
dgk-1 (nu62) (sy428) x	diacylglycerol kinase	WT	Hadju-Cronin *et al.*, 1999 ; Nurrish *et al.*, 1999; Hukema *et al.*, 2006
dgk-2 (gk142) x	diacylglycerol kinase	WT	Espelt *et al.*, 2005
dgk-3 (gk110) III	diacylglycerol kinase	WT	Lynch *et al.*, 1995; Espelt *et al.*, 2005
egl-4 (ky95) (n479) (ok1105) IV	cGMP-dependent protein kinase	WT	L'Etoile *et al.*, 2002 ; Hukema *et al.*, 2006
grk-1 (ok1239) I	G protein coupled receptor kinase	WT	Fukuto *et al.*, 2004
grk-2 (rt97) III	G protein coupled receptor kinase	<<<	Fukuto *et al.*, 2004; Hukema *et al.*, 2006
jkk-1 (km2) x	jnk kinase	WT	Kawasaki *et al.*, 1999; Villanueva *et al.*, 2001
jnk-1 (gk7) IV	Jun N-terminal Kinase	WT	Kawasaki *et al.*, 1999; Villanueva *et al.*, 2001
kin-13 (ok563) V	protein kinase C	WT	Land *et al.*,1994
pdk-1 (mg142) x	IP_3 dependent kinase	WT	Paradis *et al.*, 1999
pkc-2 (ok328) x	protein kinase C	WT	Islas-Trejo *et al.*, 1997
pmk-3 (ok169) IV	MAP kinase (p38)	WT	Berman *et al.*, 2001
ppk-2 (pk1343) III	PIP kinase	WT	D. Weinkove, personal communication
nsy-1 (ag3) (ok593) II	MAPKKK	<<<	Sagasti *et al.*, 2001
sek-1 (ag1) (km4) x	MAPKK	<<<	Tanaka-Hino *et al.*, 2002
unc-43 (e408) (n498gf) IV	CaMK II	ND	Reiner *et al.*, 1999

The response of *nsy-1* mutant animals also resembles the response of *tax-4* mutant animals. Therefore, we analysed the response to NaCl of *nsy-1; tax-4* double mutant animals. No additional defect in chemotaxis was found in *nsy-1;*

tax-4 double mutants, suggesting that *nsy-1* functions in the same genetic pathway as *tax-4* (Figure 2B). We propose that *tax-4*, *tax-6*, *nsy-1* and *sek-1* function in the same genetic pathway that regulates chemotaxis to NaCl.

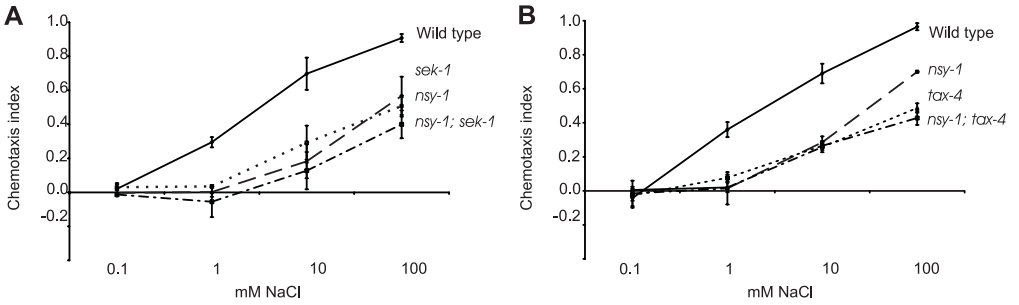

Figure 2: **A new role for MAP kinase signalling in chemotaxis to NaCl.** (A) Both *nsy-1 (ok593)* and *sek-1 (km4)* mutant animals show defects in chemotaxis to 1-100 mM NaCl ($p<0.05$). *nsy-1; sek-1* double mutant animals show a defect in chemotaxis to 0.1-100 mM NaCl ($p<0.05$ when compared to wild type), which is not significantly different from *nsy-1* and *sek-1* single mutant animals ($p>0.05$). (B) *nsy-1; tax-4* double mutant animals show a defect in chemotaxis to 1-100 mM NaCl, which is not significantly different from *tax-4* single mutant animals ($p>0.05$). These results suggest that *nsy*-1, *sek-1,* and *tax-4* function in the same genetic pathway. Indicated are the averages of at least 4 assays ± s.e.m.

The TRPV channel subunit OSM-9 functions in NaCl chemotaxis

In mammals the TRPV channel subunit VR1 plays a role in the response to NaCl (Lyall *et al.*, 2004). We found a role for the TRPV channel subunit OSM-9 in gustatory plasticity (Hukema *et al.*, 2006). Moreover, *osm-9* is expressed in the ASE, ADF, ASI, ASG and ASH neurons that are involved in various responses to NaCl (Bargmann & Horvitz, 1991; Colbert *et al.*, 1997). We wondered if OSM-9 is also involved in attraction to NaCl, although *osm-9* single mutant animals show no defect in chemotaxis to 0.1-100 mM NaCl (Hukema *et al.*, 2006; Figure 3). 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-6* double mutant animals for their response to NaCl. To our surprise, attraction to NaCl in these double mutants was completely abolished (Figure 3). This finding confirms the notion that there are two parallel genetic pathways that mediate chemotaxis to NaCl. In addition, our results show that, like in mammals, TRPV channels are involved in salt taste in *C. elegans*.

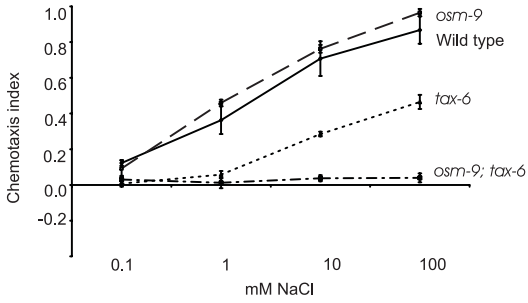

Figure 3: **The TRPV channel subunit OSM-9 functions in chemotaxis to NaCl.** o*sm-9* single mutant animals show wild type responses to 0.1-100 mM NaCl ($p>0.05$). Chemotaxis to 10-100 mM NaCl is completely abolished in *osm-9; tax-6* double mutant animals ($p<0.001$ when compared to both single mutants), suggesting that *tax-6* and *osm-9* function in parallel genetic pathways. Indicated are the averages of at least 4 assays ± s.e.m.

G protein signalling in chemotaxis

The Gα subunit ODR-3 is often found to function in the same pathways as OSM-9. Moreover, we found that ODR-3 plays an important role in gustatory plasticity, where it functions in the ADF neurons (Hukema *et al.*, 2006). The ADF neurons also play a role in attraction to NaCl (Bargmann & Horvitz, 1991). As a consequence we posed that the Gα protein ODR-3 might also function in the ADF neurons in chemotaxis to NaCl.

odr-3 mutant animals show wild type chemotaxis to 0.1-100 mM NaCl (Hukema *et al.*, 2006; Figure 4). However, chemotaxis was completely abolished in *tax-4; odr-3* and *tax-6; odr-3* double mutant animals (Figure 4A,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 4C), suggesting that *tax-2* and *odr-3* function in the same genetic pathway, in parallel to the *tax-4, tax-6* pathway.

We assumed that *odr-3* and *osm-9* would function in the same genetic pathway, since both single mutant animals show wild type chemotaxis and both function in parallel to the *tax-4, tax-6* pathway. This hypothesis was confirmed by the chemotaxis behaviour of *osm-9; odr-3* double mutant animals, since they showed the same, essentially wild type, response to 0.1-100 mM NaCl, similar to either single mutant (results not shown). Taken together, our results suggest that two genetic pathways in chemotaxis to NaCl exist. One pathway includes *tax-4, tax-6, nsy-1,* and *sek-1* and functions in parallel to the second pathway that includes *tax-2, odr-3,* and *osm-9*.

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

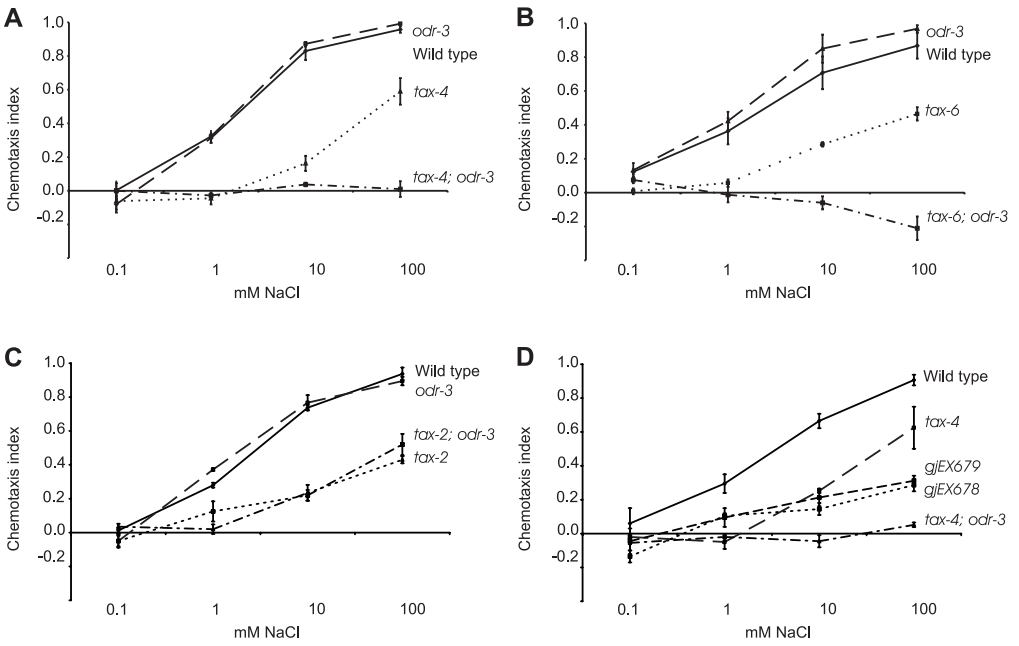

Figure 4: **The Gα protein ODR-3 functions in chemotaxis to NaCl.** *odr-3* single mutant animals show wild type responses to 0.1-100 mM NaCl ($p>0.05$). (A) Chemotaxis to 0.1-100 mM NaCl is completely abolished in *tax-4; odr-3* double mutant animals ($p<0.05$ when compared to *tax-4* single mutant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

process. *odr-3* is expressed in the AWA, AWB, AWC, ADF and ASH neurons (Roayaie *et al*., 1998), of which only the ADF neurons have been implicated in attraction to NaCl (Bargmann & Horvitz, 1991). We have shown that ODR-3 is required in the ADF neurons in gustatory plasticity (Hukema *et al*., 2006). Hence, 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 4D), suggesting that ODR-3 functions in the ADF neurons in chemotaxis to NaCl.

cGMP signalling 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 guanylate cyclase GCY-35 in the body cavity neurons AQR, PQR, and URX (Gray *et al.*, 2004). We found that *gcy-35* also plays a role in the AQR, PQR, and URX neurons in gustatory plasticity (Hukema *et al.*, 2006). 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 (Hukema *et al.*, 2006; Figure 5). In contrast, *gcy-35; tax-4* double mutant animals do not show any response to 0.1-100 mM NaCl (Figure 5A). Thus, the guanylate cyclase GCY-35 is involved in attraction to NaCl, and probably functions in a genetic pathway parallel to *tax-4*. 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, the response to 100 mM NaCl of *tax-2; gcy-35* mutant animals was very similar to the response of *tax-2* mutant animals, although the double mutant animals showed less chemotaxis to 10 mM NaCl as *tax-2* single mutant animals (Figure 5B).

To further confirm that *gcy-35* function in the *tax-2* pathway we analysed the behaviour of *gcy-35; odr-3* and *gcy-35; osm-9* double mutant animals. The responses of these double mutant animals to 0.1-100 mM NaCl were indistinguishable from the wild type response, just like the responses of the different single mutant animals (Figure 5C,D). Taken together, our results suggest that *gcy-35* functions in the same genetic pathway as *tax-2, odr-3* and *osm-9* in parallel to the *tax-4, tax-6, nsy-1, sek-1* pathway.

Discussion

We have identified five new genes that play a role in chemotaxis to NaCl. Our results suggest that these genes function in two different genetic pathways. The first genetic pathway contains the CNG channel subunit *tax-2,* the TRPV channel subunit *osm-9,* the Gα subunit *odr-3*, and the guanylate cyclase *gcy-35;* the second genetic pathway contains the CNG channel subunit *tax-4*, the calcineurin subunit *tax-6*, the MAPKKK *nsy-1* and the MAPKK *sek-1* (Figure 6).

In mammals, salts are thought to be detected via amiloride sensitive ENaCs, where ion influx leads to depolarisation of the cell and neurotransmitter release. In addition, salts are probably detected by the TRPV1 channel, of which

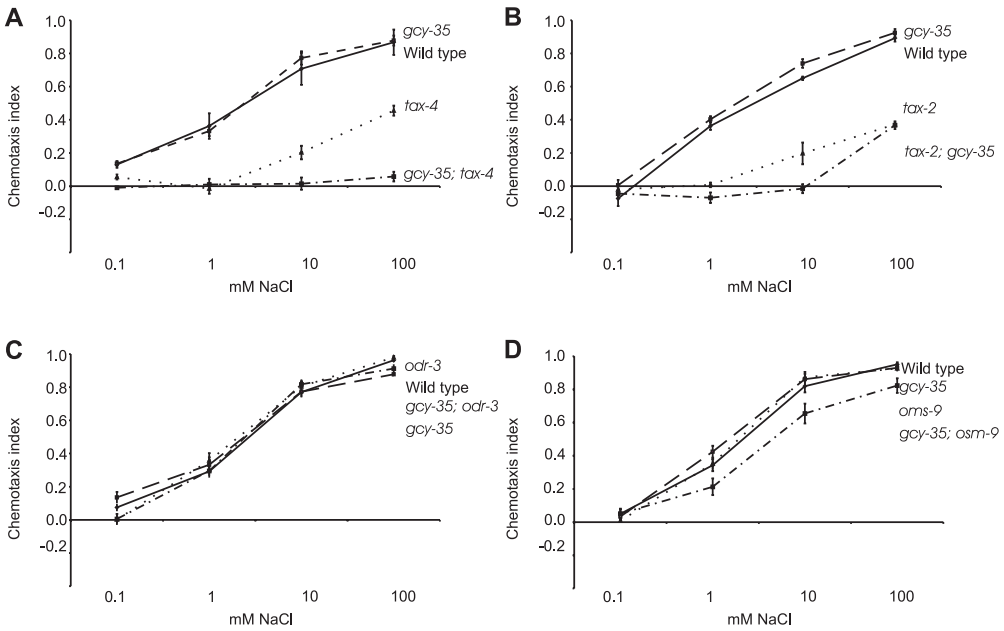

Figure 5: **A role for the guanylate cyclase GCY-35 in chemotaxis to NaCl.** *gcy-35* single mutant animals show wild type responses to 0.1-100 mM NaCl ($p>0.05$). (A) Chemotaxis to 0.1-100 mM NaCl is completely abolished in *gcy-35; tax-4* double mutant animals ($p<0.05$ when compared to *tax-4* single mutant animals for 10-100 mM). (B) Chemotaxis of *tax-2; gcy-35* double mutants to 100 mM NaCl does not significantly differ from *tax-2* single mutants ($p>0.05$). (C) *gcy-35; odr-3* double mutant animals show wild type chemotaxis to 0.1-100 mM NaCl ($p>0.05$ compared to wild type, and *gcy-35* or *odr-3* single mutant animals). (D) *gcy-35; osm-9* double mutant animals show wild type chemotaxis to 0.1-100 mM NaCl ($p>0.05$ compared to wild type, and *gcy-35* or *osm-9* single mutant animals). These results suggest that *gcy-35* functions in the same genetic pathway as *tax-2, odr-3,* and *osm-9*, in parallel to *tax-4*. Indicated are the averages of at least 4 assays ± s.e.m.

the molecular mechanisms remain to be elucidated (Lyall *et al*., 2004). Our finding that the TRPV channel subunit OSM-9 is involved in chemotaxis to NaCl, provides evidence that the molecular mechanism used by *C. elegans* to sense NaCl overlaps with the mammalian salt sensation mechanism. However, at present it is unknown what the molecular function of OSM-9 in NaCl detection is. OSM-9 could function as a salt receptor in the ASE neurons or other sensory neurons involved in chemotaxis to NaCl, in which OSM-9 is expressed. Since loss of function of OSM-9 alone does not affect NaCl chemotaxis, OSM-9 probably functions in parallel to another, unknown NaCl receptor. The *C. elegans* genome contains 28 ENaCs (Goodman & Schwarz, 2003), thus far none of these have been shown to affect chemotaxis to NaCl, however effects could be masked by functional redundancy.

Alternatively, OSM-9 might function as a downstream signalling molecule and participate in a G protein signalling cascade as is used in olfaction and nociception (Colbert *et al.*, 1997; Roayaie *et al.*, 1998).

G proteins have been implicated in several taste responses in mammals but not in salt detection. The Gα subunit gustducin is preferentially expressed in the tongue (McLaughlin *et al.*, 1992), and is involved in the detection of sugar, amino acids, and bitter compounds (Wong *et al.*, 1996; Glendenning *et al.*, 2005). In addition, a recent study showed that in *Drosophila* a Gγ subunit is involved in sugar detection (Ishimoto *et al.*, 2006). 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 in avoidance of high salt concentrations and it is needed in the ADF neurons in gustatory plasticity (Roayaie *et al.*, 1998; Hukema *et al.*, 2006). Here, we have shown that ODR-3 is also needed in the ADF neurons for chemotaxis to NaCl. This suggests that also GPCRs might be involved in the detection of salts just like for sugars, amino acids, and bitter compounds. Alternatively, ODR-3 could function downstream of other receptor types, such as the TRPV channel, or it could play a role in modulating the activity of NaCl detecting channels.

Previously it has been shown that Ras/MAPK signalling is involved in olfaction in *C. elegans* (Hirotsu *et al.*, 2000). In general, neuronal MAPK signalling is mostly involved in the integration of signals, and it has been shown in multiple model systems that MAPK signalling is involved in learning and memory (reviewed in Sharma & Carew, 2004; Sweatt, 2004; Blackwell, 2006). In this study, we have for the first time shown that MAPK signalling is also involved in chemotaxis to NaCl in *C. elegans.* Both the MAPKKK NSY-1 and the MAPKK SEK-1 are required for normal chemotaxis to NaCl. Our results suggest that both MAPK function in the same genetic pathway and therefore it seems likely that NSY-1 phosphorylates and thereby activates SEK-1.

The MAP kinases NSY-1 and SEK-1 regulate asymmetric cell-fate of the AWC neurons (Sagasti *et al.*, 2001; Tanaka-Hino *et al.*, 2002). It is very well possible that they have a similar function in the asymmetry of the ASE neurons or other NaCl sensing neurons. However, no such effects of NSY-1 and SEK-1 on the ASE neurons are known. Alternatively, the MAP kinases could function in signalling rather than influencing cell fate. It is difficult to speculate where in the cellular circuitry involved in chemotaxis to NaCl the MAP kinases might function. Their

broad expression patterns suggest they could have a function in the sensory neurons, but they could also function more downstream in the integration of signals.

Previously the second messenger cGMP has been implicated in chemotaxis to NaCl (Coburn & Bargmann, 1996; Komatsu *et al.*, 1996; Birnby *et al.*, 2000). Here we identify a role for the guanylate cyclase *gcy-35* in chemotaxis to NaCl. *gcy-35* is not expressed in the sensory neurons that are involved in chemotaxis to NaCl (Bargmann & Horvitz, 1991; Cheung *et al.*, 2004). However, we have previously shown that *gcy-35* is needed in the AQR, PQR, URX neurons for gustatory plasticity (Hukema *et al.*, 2006). Perhaps *gcy-35* also functions in these neurons in chemotaxis to NaCl. However, it is unclear how these neurons contribute to NaCl chemotaxis.

Both *tax-2* and *tax-4* CNG mutant animals show the same defect in chemotaxis to NaCl. Surprisingly, the double mutant showed an additional defect, suggesting these CNG subunits function, at least partially, in different pathways. We conceived two possible explanations for these observations. The first possibility is that *tax-2* and *tax-4* function in two separate pathways (Figure 6, model I): One pathway in which TAX-2 forms a functional channel together with a CNG channel α subunit other than TAX-4. The *C. elegans* genome contains five putative CNG α subunits, including TAX-4, CNG-1, and CNG-3 (Komatsu *et al.*, 1996; Cho *et al.*, 2004; 2005). CNG-1 and CNG-3 are likely candidates to form a channel with TAX-2, since both are expressed in the amphid sensory neurons, CNG-3 is even expressed in the ASE neurons (Cho *et al.*, 2004; 2005). The second pathway would use the α subunit TAX-4, which can form a functional channel on its own, since no alternative β subunit genes have been identified in the *C. elegans* genome. Heterologous expression of TAX-4 in cell culture has shown that this protein can indeed form a functional channel on its own (Komatsu *et al*., 1999).

A second, and to our opinion more likely, explanation would be that TAX-2 and TAX-4 do function in the same pathway and form the main NaCl chemotaxis pathway (Figure 6, model II). Also in other behaviours TAX-2 and TAX-4 function together (Coburn & Bargmann, 1996; Komatsu *et al.*, 1996). We propose that in addition to this pathway a second redundant pathway exists, which signals via *tax-2* together with another CNG α subunit, e.g. CNG-1 or CNG-3, allowing signalling in response to high NaCl concentrations in *tax-4* mutant animals. The response to

NaCl seen in *tax-2* mutant animals can be explained by the fact that TAX-4 can form a functional channel on its own. This model also explains that *osm-9, odr-3,* and *gcy-*35 mutant animals show wild type chemotaxis to NaCl, since mutations in these genes only affect a secondary signalling pathway.

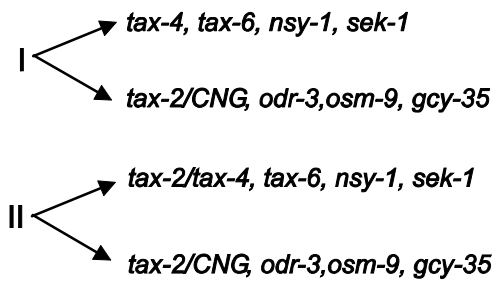

Figure 6: **Two genetic pathways mediate chemotaxis to 0.1-100 mM NaCl in *C. elegans*.** One pathway includes *tax-4, tax-6, nsy-1,* and *sek-1*; the other pathway includes *tax-2, osm-9, odr-3,* and *gcy-35.* We propose two models explaining these pathways. In model I *tax-2* functions in a pathway parallel to *tax-4.* The CNGβ subunit *tax-2* needs another α subunit to form a functional channel; *tax-4* can form a functional channel on its own. In model II TAX-2/TAX-4 together constitute the main salt sensing pathway; in addition, there is a second redundant pathway including *tax-2*, which needs an α subunit other than *tax-4*, to form a functional channel.

Unfortunately, our data do not allow us to order the here identified genes in a putative signalling cascade, although it is tempting to do so based on the molecular function of the encoded proteins. In our favourite working model, TAX-2 and TAX-4 function together in the ASE neurons. Activation of the TAX-2/TAX-4 channel leads to Ca^{2+} influx, which can in turn lead to activation of calcineurin TAX-6 and the MAP kinases NSY-1 and SEK-1, however these latter proteins may also function in other cells. Another TAX-2 channel, together with another CNG α subunit than TAX-4, could also function in the ASE neurons or in other neurons involved in salt chemotaxis, such as the ADF neurons. In our assay, the ASE neurons are essential for NaCl detection, although other assays indicate that also other neurons contribute to NaCl detection. We find that signals from the ADF neurons contribute to NaCl detection. It is unclear if the ADF neurons are directly involved in NaCl detection. It is also possible that they function downstream of the main salt sensing ASE neurons. Perhaps signals from the ASE neurons are required to allow NaCl detection by other neurons, such as the ADF neurons. The Gα subunit ODR-3 functions in the ADF neurons. In the AQR, PQR, URX neurons *gcy-35* might function to activate TAX-4, as it does in aerotaxis (Gray *et al.*, 2004). OSM-

9 might function as salt receptor either in the ASE or the ADF neurons, or it could function downstream of ODR-3 in the ADF neurons.

The overlap we find between salt detection in *C. elegans* and mammals, suggests that molecular genetic studies in *C. elegans* can provide us more information about the molecular mechanisms of mammalian salt taste. Very little is known about the signal transduction involved in salt detection in mammals, but it is intriguing to note that many other sensory signalling pathways in mammals also use TRPV channel subunits, G proteins, and cGMP and Ca^{2+} signalling as we have shown to be involved in salt detection in *C. elegans* in this study.

Materials and Methods

Strains, genetics and germline transformation

Strains used in this work are *tax-2 (p671), gcy-35 (ok769), grk-1 (ok1239), age-1 (hx546), nsy-1 (ag3) (ok593), grk-2 (rt97), dgk-3 (gk110), ppk-2 (pk1343), tax-4 (p678), cmk-1 (ok287), egl-4 (ky95) (n479) (ok584), jnk-1 (gk7), pmk-3 (ok169), osm-9 (ky10), tax-6 (p675), unc-43 (e408) (n498), kin-13 (ok563), odr-3 (n1605), dgk-1 (nu62) (sy428), dgk-2 (gk142), jnk-1 (km2), pdk-1 (mg142), pkc-2 (ok328), sek-1 (ag1) (km4)*. Wild type *C. elegans* used were the strain Bristol N2. Rescue of *odr-3* expression in the ADF neurons was done using a *srh-142::odr-3* construct, as described before, injected at a concentration of 25 ng/µl (Sagasti *et al.*, 1999; Lans *et al.*, 2004). Germline transformation was performed as described (Mello *et al.*, 1991). We used an *elt-2::GFP* construct as co-injection marker (Fukushige *et al.*, 1999).

Behavioural assays

Chemotaxis towards NaCl was assessed as described before (Wicks *et al.*, 2000; Jansen *et al.*, 2002; Hukema *et al.*, 2006). 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 s.e.m.

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Chapter 3

Antagonistic sensory cues generate gustatory plasticity in *Caenorhabditis elegans*

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3

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Abstract

Caenorhabditis elegans shows chemo-attraction to 0.1 – 200 mM NaCl, avoidance of higher NaCl concentrations, and avoidance of otherwise attractive NaCl concentrations after prolonged exposure to NaCl (gustatory plasticity). Previous studies have shown that the ASE and ASH sensory neurons primarily mediate attraction and avoidance of NaCl, respectively. Here we show that balances between at least four sensory cell types, ASE, ASI, ASH, ADF and perhaps ADL, modulate the response to NaCl. Our results suggest that two NaCl-attraction signalling pathways exist, one of which uses Ca^{2+}/cGMP signalling. In addition, we provide evidence that attraction to NaCl is antagonized by G protein signalling in the ASH neurons, which is desensitised by the G protein coupled receptor kinase GRK-2. Finally, the response to NaCl is modulated by G protein signalling in the ASI and ADF neurons, a second G protein pathway in ASH and cGMP signalling in neurons exposed to the body fluid.

Introduction

Salt taste is essential for ion and water homeostasis, however our understanding of the molecular mechanisms of salt perception is relatively limited (Lindemann, 2001). In rodents and *Drosophila*, salts are thought to be primarily sensed by cation influx through degenerin/epithelial Na^+ channels (DEG/ENaC), leading to membrane depolarisation (Lindemann, 2001; Liu *et al.*, 2003). In humans, the contribution of ENaC channels is less pronounced and salts are thought to be detected by a vanilloid receptor-1 (TRPV1) variant, a member of the transient receptor potential (TRP) cation channel family (Lyall *et al.*, 2004). We use the nematode *Caenorhabditis elegans* to unravel the molecular mechanisms that mediate the response to NaCl.

C. elegans shows chemo-attraction to low NaCl and avoidance of high NaCl concentrations (Dusenbery, 1974; Ward, 1973; Culotti & Russell, 1978). Chemo-attraction to salts is mediated by four pairs of amphid sensory neurons (ADF, ASE, ASG and ASI), of which the ASE cells are most important (Bargmann & Horvitz, 1991). Several genes involved in salt detection have been identified. These include the guanylate cyclase DAF-11, the cGMP gated cation channel subunits TAX-2 and TAX-4 and the calcineurin A subunit TAX-6, suggesting that *C. elegans* uses cGMP and Ca^{2+} signalling in salt detection (Birnby *et al.*, 2000; Coburn & Bargmann, 1996; Komatsu *et al.*, 1996; Kuhara *et al.*, 2002). *C. elegans* avoids NaCl concentrations above 200 mM (RKH & GJ, unpublished results). This response is thought to be

due to a general avoidance of high osmotic strength. The ASH sensory neurons play a pivotal role in this process (Bargmann *et al.*, 1990). Osmotic avoidance requires the Gα protein ODR-3, the TRP channel subunits OSM-9 and OCR-2, the cytoplasmic protein OSM-10, the G protein-coupled receptor kinase GRK-2 and glutamatergic neurotransmission, involving the glutamate receptors GLR-1 and NMR-1, the glutamate transporter EAT-4 and the proprotein convertase EGL-3 (Berger *et al.*, 1998; Colbert *et al.*, 1997; Fukuto *et al.*, 2004; Hart *et al.*, 1999; Mellem *et al.*, 2002; Roayaie *et al.*, 1998; Tobin *et al.*, 2002).

The behavioural response to salts is plastic and depends on various cues, including exposure time and salt concentration. We use a new chemotaxis assay to analyse both salt detection and its plasticity (Figure 1A; Jansen *et al.*, 2002; Wicks *et al.*, 2000). In the plasticity assay, animals are washed in a buffer containing 100 mM NaCl and subsequently analysed for chemotaxis. These pre-exposed animals show avoidance of otherwise attractive NaCl concentrations. This behaviour is time and concentration dependent, reversible and partially salt specific. Thus far, two proteins have been shown to contribute to this behaviour, the TRP channel subunit OSM-9 and the Gγ protein GPC-1 (Jansen *et al.*, 2002). The finding that prolonged exposure to NaCl results in avoidance suggests a mechanism that involves more than adaptation or desensitisation. We propose a model in which the behavioural response to salt involves a balance between attraction and avoidance, as has been described for the regulation of the response to benzaldehyde, aggregation behaviour and the integration of sensory signals (de Bono *et al.*, 2002; Ishihara *et al.*, 2002; Nuttley *et al.*, 2001). We call this behaviour gustatory plasticity.

In this paper, we show that four pairs of sensory neurons and neurons exposed to the body fluid are involved in gustatory plasticity. Our results suggest that two signalling pathways mediate attraction to NaCl, likely in the ASE neurons. One of these pathways involves cGMP and Ca^{2+} signalling. We show that attraction to NaCl is antagonized by a G protein-signalling pathway in the ASH neurons, which is desensitised by GRK-2. Furthermore, the response to NaCl is modulated by G protein signalling in the ASI and ADF neurons, an additional G protein pathway in the ASH neurons and cGMP signalling in neurons exposed to the body fluid.

Results

Antagonistic inputs from three pairs of sensory neurons modulate gustatory behaviour

Previously, it was shown that the ASE neurons act as primary sensory neurons for NaCl detection (Bargmann & Horvitz, 1991). To confirm this role of the ASE cells, we tested the behaviour of *che-1* and *ceh-36* mutant animals. *che-1* mutants lack functional ASE neurons due to mutation of a zinc finger protein similar to the *Drosophila* GLASS transcription factor (Uchida *et al.*, 2003). *che-1* is predominantly expressed in the ASE neurons; only occasionally expression was observed in other neurons (Uchida *et al.*, 2003). *ceh-36* encodes an *Otx*-related homeodomain protein expressed in the ASE and the AWC neurons, which specifies the identities of these neurons (Lanjuin *et al.*, 2003). Both *che-1* and *ceh-36* mutant animals showed no significant chemotaxis to NaCl (0.1 – 100 mM, Figure 1B). In contrast, these animals showed wild type or even increased avoidance of 1 M NaCl (Figure 1C). Surprisingly, *che-1* and *ceh-36* animals showed no response to NaCl after pre-exposure to 100 mM NaCl (Figure 1D). The chemotaxis defects observed in *che-1* animals could be rescued by introduction of the wild-type *che-1* gene as a transgene (Figure 1B, D). Our results confirm that the ASE neurons are essential for chemotaxis to NaCl, also in our assay, and that these cells are not required for avoidance of 1 M NaCl (Bargmann & Horvitz, 1991; Uchida *et al.*, 2003). Importantly, our results indicate that signalling via the ASE neurons is essential for gustatory plasticity.

To identify additional cells involved in gustatory plasticity we used the Gγ subunit, GPC-1. *gpc-1* mutants showed reduced avoidance of, or even attraction to NaCl after pre-exposure (Jansen *et al.*, 2002). *gpc-1* is not expressed in the ASE neurons, but it is expressed in three other pairs of neurons involved in salt perception (Jansen *et al.*, 2002): the ASI cells, which have a minor function in attraction to salts (Bargmann & Horvitz, 1991), and the ADL and ASH nociceptive neurons (Bargmann *et al.*, 1990). Introduction of the wild type *gpc-1* gene as a transgene in *gpc-1* animals could restore the response to NaCl after pre-exposure (Figure 2A; Jansen *et al.*, 2002). However, we never obtained full rescue of the defect. Since overexpression of *gpc-1* induced a gustatory plasticity defect (Jansen *et al.*, 2002), we surmise that probably the levels of GPC-1 are crucial for the behavioural response. Specific expression of GPC-1 in the ASI or ASH

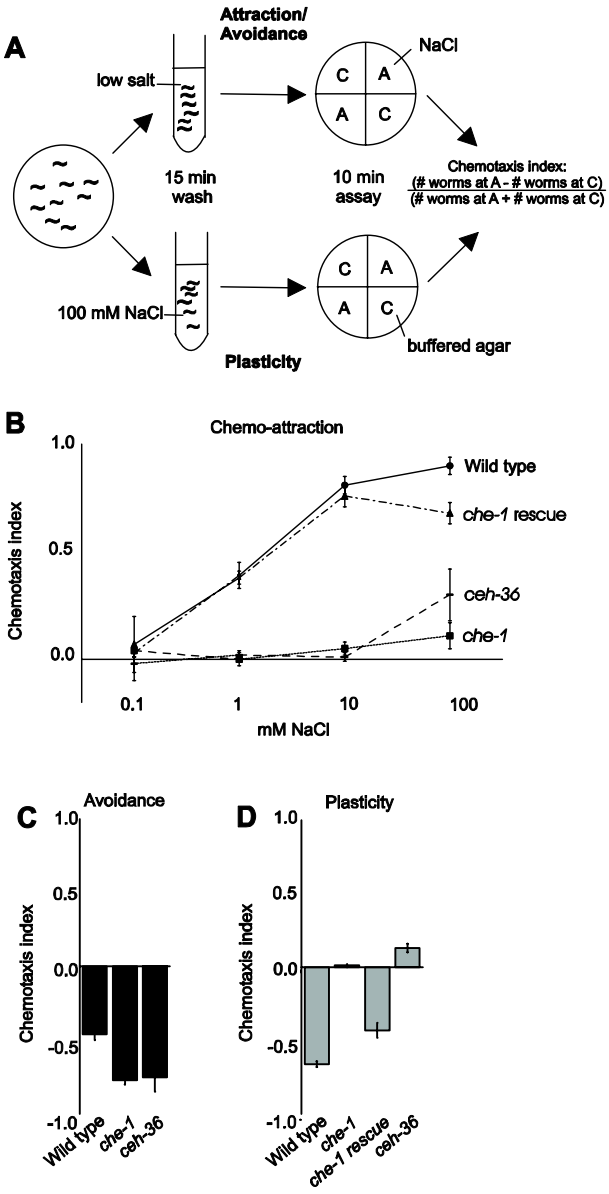

Figure 1: **Both chemo-attraction to NaCl and gustatory plasticity require the ASE neurons.** (A) Worms are washed in a low salt buffer or a buffer containing 100 mM NaCl and tested for chemotaxis. The assay plates contain two quadrants with only buffered agar (C) and two quadrants with buffered agar and NaCl (A). We use 0.1, 1, 10 or 100 mM NaCl to test for chemo-attraction, 1 M NaCl to test for avoidance and 25 mM NaCl in the gustatory plasticity assay. The distribution of the animals over the quadrants is determined after 10 minutes, and the chemotaxis index is calculated. (B) ASE function is required for the detection of NaCl, since chemotaxis to NaCl was abolished in *che-1* and *ceh-36* mutants ($p<0.001$). Chemotaxis could be restored by introduction of a *che-1* transgene (*che-1 rescue*, $p<0.001$). (C) *che-1* and *ceh-36* animals strongly avoided 1 M NaCl. (D) *che-1* and *ceh-36* animals showed no significant response to NaCl after pre-exposure to 100 mM NaCl ($p<0.001$ compared to wild type animals). The *che-1* defect could be rescued by introduction of a *che-1* transgene (*che-1 rescue*).
In all figures: panels show mean $\pm$ s.e.m.; $n \geq 4$ for all assays. Open bars indicate chemotaxis to 25 mM NaCl; gray bars indicate the response to 25 mM NaCl after pre-exposure to 100 mM NaCl; black bars show the response to 1 M NaCl.

neurons partially restored avoidance after pre-exposure in *gpc-1* mutants (Figure 2A). Expression of GPC-1 both in the ASI and ASH neurons did not yield better rescue (results not shown). These results suggest that ASI and ASH do not have additive functions, however since *gpc-1* expression levels seem a crucial

determinant in this behaviour these experiments are not conclusive. Expression of GPC-1 in the ADL and ASH nociceptive neurons resulted in better rescue than expression in the ASH neurons alone (Figure 2A), suggesting that GPC-1 probably also functions in ADL. Expression of *gpc-1* in the ASE neurons did not restore gustatory plasticity (Figure 2A), suggesting that GPC-1 does not function non-cell-autonomously. Our findings indicate that GPC-1 acts both in the ASI and ASH neurons, and probably also in the ADL neurons, to modulate the response to NaCl. Avoidance of 1 M NaCl is not affected in *gpc-1* animals, suggesting that separate pathways exist in the nociceptive neurons for osmotic avoidance and the modulation of the response to attractive NaCl concentrations.

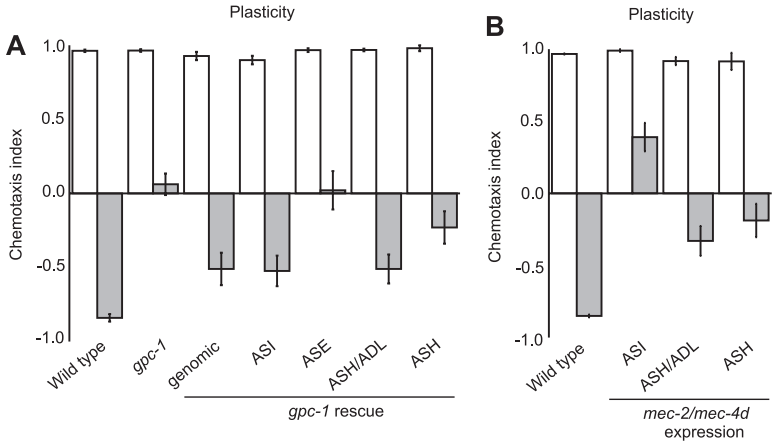

Figure 2: **Identification of sensory neurons involved in gustatory plasticity**. (A) Avoidance after pre-exposure could be restored by introducing the wild type *gpc*-1 gene in *gpc-1* animals (genomic), or by expressing *gpc-1* in the ASI, ASH or ASH and ADL neurons ($p<0.01$). Expression in ASE did not rescue the plasticity defect ($p>0.05$). (B) Expression of *mec-4d*/*mec-2* constructs in the ASI neurons, in the ASH/ADL nociceptive neurons, or the ASH neurons significantly impaired gustatory plasticity ($p<0.01$).

To validate the importance of the ASI, ADL and ASH neurons in gustatory plasticity, we expressed a dominant mutant DEG/ENaC channel MEC-4d in specific neurons to inactivate these cells (Harbinder *et al.*, 1997). To increase the efficacy we co-expressed *mec-2* constructs, since MEC-2 has been shown to increase MEC-4d activity (Goodman *et al.*, 2002). Expression of the *mec-4d*/*mec-2* constructs in the ASI neurons strongly affected gustatory plasticity, while expression in the ASH or ADL cells only mildly interfered with plasticity (Figure 2B). Expression of the *mec-4d*/*mec-2* constructs in the ASI, ASH or ADL neurons did not affect salt detection (Figure 2B, results not shown). The *mec-4d*/*mec-2* data confirm that the ASI, ASH and probably ADL neurons are involved in gustatory plasticity.

Our results indicate that gustatory plasticity is the result of integration of input from three to four pairs of sensory neurons. Signalling via the ASE cells is essential for both attraction to salt and avoidance after pre-exposure. However, the ASE neurons are not involved in avoidance of 1 M NaCl. This response is mediated by the ASH neurons (Bargmann *et al.*, 1990). Avoidance after pre-exposure involves cues from the ASI and ASH cells, and probably the ADL neurons.

G protein signalling in the ADF neurons modulates gustatory plasticity

Our finding that the Gγ subunit GPC-1 plays a role in gustatory plasticity prompted us to test mutants of the Gα subunits expressed in the gustatory neurons in our behavioural assays. None of the sensory Gα mutants showed a defect in chemotaxis to NaCl (Table I). However, two Gα mutants showed impaired gustatory plasticity: GPA-1 and ODR-3 (Figure 3A, B).

Thus far, no function has been described for GPA-1 (Jansen *et al.*, 1999). We found that *gpa-1* animals have a defect in gustatory plasticity, whereas they showed wild type avoidance of 1 M NaCl (results not shown). The behaviour of *gpa-1* animals was very similar to that of *gpc-1* animals, and GPA-1 is expressed in the ASI neurons and the nociceptive neurons (Jansen *et al.*, 1999), suggesting that GPA-1 might function together with GPC-1 in these neurons. To test this hypothesis we generated a *gpa-1 gpc-1* double mutant. The behaviour of the double mutant was not significantly different from the behaviour of the two single mutants (Figure 3A), indicating that *gpa-1* and *gpc-1* function in the same genetic pathway that modulates gustatory plasticity.

ODR-3 is essential for olfaction and chemo-avoidance and is expressed in the AWA, AWB and AWC olfactory neurons and in the ADF and ASH neurons (Roayaie *et al.*, 1998). *odr*-3 animals showed a very strong gustatory plasticity defect (Figure 3B). To test if this is caused by the osmotic avoidance defect of *odr-3* animals we tested if expression of the *odr-3* gene in the ASH neurons could restore gustatory plasticity. Expression of ODR-3 in the ASH neurons of *odr-3* animals fully restored avoidance of 1 M NaCl (Figure 3C), confirming that ODR-3 in the ASH neurons is essential for this response (Roayaie *et al.*, 1998). However, avoidance after pre-exposure could not be restored by expressing *odr-3* in the ASH neurons (Figure 3B), indicating that ODR-3 mediated signalling in the ASH neurons is not sufficient for gustatory plasticity. To test if the ADF neurons are involved in gustatory plasticity, we expressed the *odr-3* gene in these neurons.

Table 1: **Overview of strains tested for salt perception**.

gene	reference	function	taste	plasticity
arr-1 (ok401)	Fukuto *et al.*, 2004	arrestin	WT	-0.12 ± 0.17***
ceh-36(ks86)	Lanjuin *et al.*, 2003	transcription factor	m***	0.13 ± 0.03***
che-1 (p679)	Uchida *et al.*, 2000	transcription factor	m	0.01 ± 0.02***
cnb-1 (jh103)	Bandyopadhyay *et al.*, 2002	calcineurin B	m*	0.74 ± 0.04**
daf-11 (m47)	Birnby *et al.*, 2000	gyanylate cyclase	WT	WT
dgk-1 (sy428)	Hadju-Cronin *et al.*, 1999; Nurrish *et al.*, 1999	diacylglycerol kinase	WT	0.72 ± 0.05***
(nu62)			WT	0.27 ± 0.10***
egl-4 (ky95)	Daniels *et al..*, 2000	PKG	WT	0.06 ± 0.06 ***
(ok1105)			WT	-0.06 ± 0.08 ***
fat-1 (wa9)	Watts & Browse, 2002	ω-3 fatty acyl desaturase	WT	-0.02 ± 0.16**
fat-3 (wa22)	Watts & Browse, 2002	δ-6 fatty acid desaturase	WT	0.20 ± 0.06***
fat-4 (wa14)	Watts & Browse, 2002	δ-5 fatty acid desaturase	WT	0.31 ± 0.09***
(ok958)			WT	0.10 ± 0.03***
gcy-32 (ok995)	Coates & de Boneo, 2002	guanylate cyclase	WT	WT
gcy-35 (ok769)	Cheung *et al.*, 2004	gyanylate cyclase	WT	-0.10 ± 0.10*
gpa-1 (pk15)	Jansen *et al.*, 1999	Gα subunit	WT	0.17 ± 0.06**
gpa-2 (pk16)	Zwaal *et al.*, 1997	Gα subunit	WT	WT
gpa-3 (pk35)	Zwaal *et al.*, 1997	Gα subunit	WT	WT
gpa-4 (pk381)	Jansen *et al.*, 1999	Gα subunit	WT	WT
gpa-10 (pk362)	Jansen *et al.*, 1999	Gα subunit	WT	WT
gpa-11 (pk349)	Jansen *et al.*, 1999	Gα subunit	WT	WT
gpa-13 (pk1270)	Jansen *et al.*, 1999	Gα subunit	WT	WT
gpa-14 (pk347)	Jansen *et al.*, 1999	Gα subunit	WT	WT
gpa-15 (pk477)	Jansen *et al.*, 1999	Gα subunit	WT	WT
grk-2 (rt97)	Fukuto *et al.*, 2004	GRK	m***	-0.61 ± 0.06***#
itr-1 (sa73)	Baylis *et al.*, 1999; Dal Santo *et al.*, 1999	IP_3 receptor	WT	0.82 ± 0.06***
ncs-1 (qa406)	Gomez *et al.*, 2001	Ca^{2+} sensor	m**	-0.13 ± 0.14**
ocr-1 (ok132)	Tobin *et al.*, 2002	TRPV channel	WT	-0.20 ± 0.08***
(ak46)			WT	-0.25 ± 0.14***
ocr-2 (ak47)	Tobin *et al.*, 2002	TRPV channel	WT	-0.18 ± 0.12***
odr-3 (n1605)	Roayaie *et al.*, 1998	Gα subunit	WT	0.85 ± 0.04***
osm-9 (ky10)	Colbert *et al.*, 1997	TRPV channel	WT	0.06 ± 0.05**
pdl-1 (gk157)	Smith & Pilgrim, personal communication	phosphodiesterase	WT	0.13 ± 0.09 **
tax-2 (p671)	Coburn & Bargmann, 1996	cGMP-gated channel	m***	0.83 ± 0.04***
tax-4 (p678)	Komatsu *et al.*, 1996	cGMP-gated channel	m***	0.83 ± 0.03***
tax-6 (p675)	Kuhara *et al.*, 2002	calcineurin A	m***	0.64 ± 0.05***

32 Mutant strains were tested for chemotaxis to 0.1, 1, 10 and 100 mM NaCl (taste) or chemotaxis to 25 mM NaCl after pre-exposure to 100 mM NaCl (plasticity) and their responses were compared to wild type animals: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; unc = locomotion defect; these strains did not perform well in the assay; # pre-exposure for only 5 minutes, response stronger than wild types.

ADF specific expression of *odr-3* restored avoidance after pre-exposure (Figure 3B), but did not restore avoidance of 1 M NaCl (Figure 3C). Expression of *odr-3* in both ASH and ADF did not yield better rescue, but reproduced the ASH specific rescue of avoidance of 1 M NaCl and ADF specific rescue of gustatory plasticity (results not shown). Hence, ODR-3 functions in two separate processes. It mediates avoidance of 1 M NaCl in the ASH neurons and it mediates a signal in the ADF neurons that modulates gustatory plasticity.

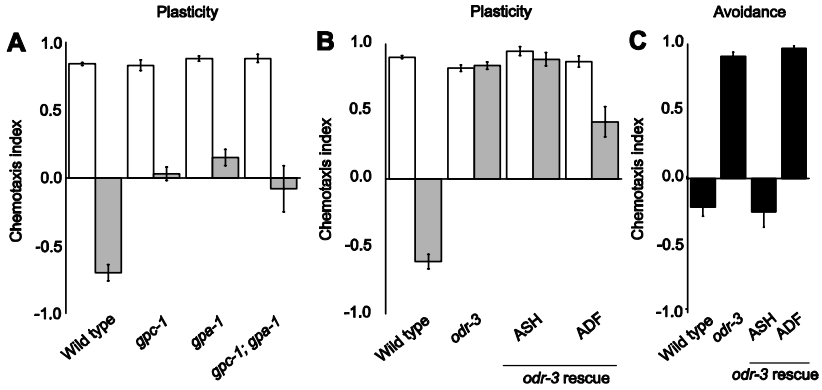

Figure 3: **Gα subunits GPA-1 and ODR-3 modulate gustatory plasticity**. (A) GPA-1 is involved in gustatory plasticity ($p<0.0001$) and functions in the same genetic pathway as GPC-1, since *gpa-1 gpc-1* animals showed the same response as the single mutants ($p>0.05$) (B) Also ODR-3 is involved in gustatory plasticity ($p<0.0001$). Avoidance after pre-exposure could be restored by expressing ODR-3 in the ADF neurons ($p<0.0001$), but not by expressing it in the ASH neurons ($p>0.05$). (C) ODR-3 signalling mediates avoidance of 1 M NaCl ($p<0.0001$). Avoidance could be restored by expressing ODR-3 in the ASH neurons ($p<0.0001$), but not by expressing it in the ADF neurons ($p>0.05$).

Excessive avoidance in *grk-2* animals counterbalances chemo-attraction to NaCl

G protein signal transduction can be modulated by G protein coupled receptor kinases (GRKs) and arrestins. Studies in mammals and *Drosophila* have shown that GRKs inhibit G protein signalling by phosphorylating activated G Protein Coupled Receptors (GPCRs) followed by arrestin binding and receptor internalisation (Ferguson, 2001). In addition to this classical function, recent studies indicate a role for GRKs and arrestins as signal transduction molecules (Perry & Lefkowitz, 2002; Kim *et al.*, 2005; Ren *et al.*, 2005). The *C. elegans* genome contains two GRKs, *grk-1* and *grk-2*, and one arrestin gene, *arr-1* (Fukuto *et al.*, 2004). Loss-of-function of *grk-2* impairs the response to attractive odorants and several repellents, including odorants, quinine and 1-4 M glycerol, but seems to have no or

little involvement in olfactory adaptation. These results suggest that GRK-2 functions as a signal transduction molecule in the nociceptive neurons and olfactory neurons (Fukuto *et al.*, 2004).

Loss-of-function of *grk-2* severely impaired chemotaxis to NaCl (Figure 4A). However, *grk-2* animals did show avoidance after pre-exposure (Figure 4B), indicating that these animals can detect NaCl. When these animals were only briefly pre-exposed to NaCl they even showed enhanced avoidance after pre-exposure (Figure 4C). Since the ASE cells are essential for both salt taste and gustatory plasticity, we surmised that GRK-2 does not function in the ASE neurons, but in the nociceptive neurons instead. We first tested the response of *grk-2* animals to 1 M NaCl. Surprisingly, *grk-2* animals showed strong avoidance of 1 M NaCl (Figure 4D), in contrast to the previously reported defect in avoidance of 1-4 M glycerol (Fukuto *et al.*, 2004). These results suggest that different signalling routes exist for avoidance of high concentrations of glycerol and NaCl.

We next tested if specific expression of *grk-2* in the nociceptive neurons ADL and ASH could restore chemotaxis of the *grk-*2 mutants. Indeed, a *srb-6::grk-2* construct restored the response to 1 - 100 mM NaCl partially (*gjEx601* and 2 other strains) or fully (*gjEx602*) (Figure 4A). Probably, *gjEx601* animals did not express sufficient GRK-2 in the nociceptive neurons for full rescue, since avoidance of 1M NaCl was not restored (Figure 4C). In contrast, *gjEx602* animals probably over-expressed GRK-2, diminishing the avoidance responses (Figure 4A and D), and restoring chemo-attraction. To further confirm GRK-2 function in ASH, we also used an *osm-10::grk-2* construct to express *grk-2* in the ASH and ASI neurons. Also this construct could restore the NaCl chemo-attraction defect of *grk-2* animals (*gjEx639* in Figure 4A, B and D). In addition, specific expression of *grk-2* in the ASH neurons could restore avoidance of octanol and 1 M glycerol (results not shown). In contrast, expression of GRK-2 in interneurons, but not the sensory neurons, using a *glr-1::grk-2* construct did not restore chemotaxis to NaCl (results not shown). Our results suggest that GRK-2 modulates the strength of the NaCl avoidance signal mediated by the nociceptive neurons. Such a function fits with a classical function for GRK-2 in desensitisation of the NaCl avoidance signalling pathway (Figure 7), suggesting that GRK-2 has two different functions in separate signalling pathways in the ASH cells. Moreover, these results show that signals derived from the nociceptive neurons counterbalance chemo-attraction to NaCl mediated by the ASE neurons.

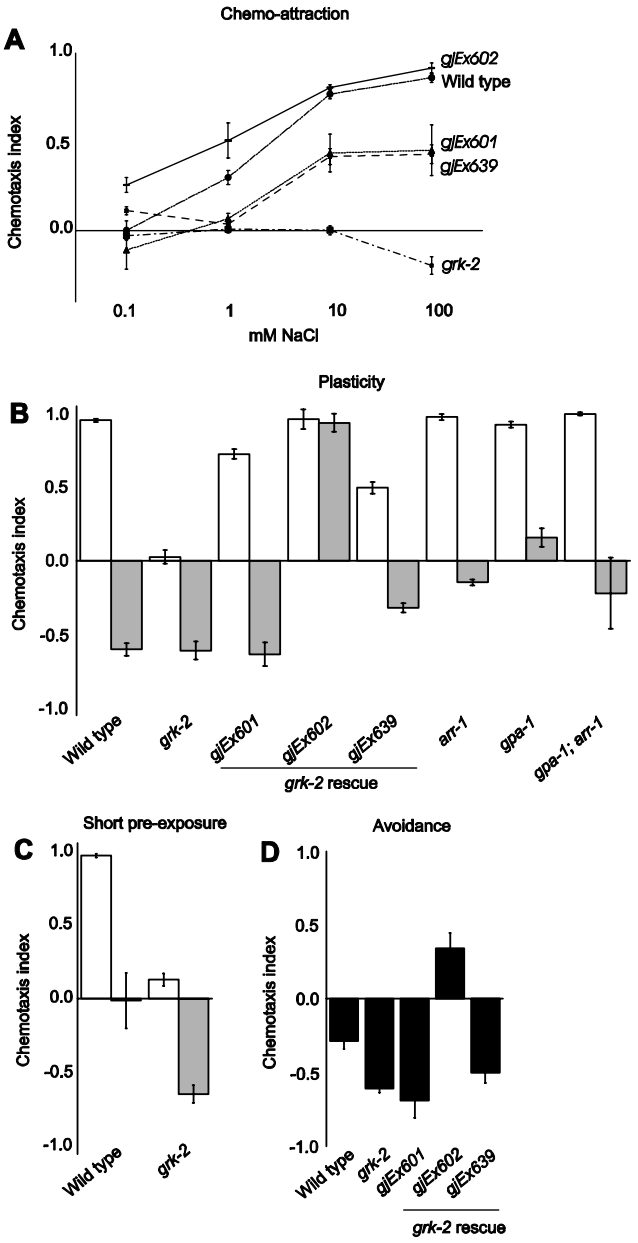

Figure 4: **Excessive avoidance in *grk-2* animals counterbalances chemo-attraction to NaCl** (A) Chemotaxis to 0.1 –100 mM NaCl was impaired in *grk-2* animals ($p<0.0001$). The *grk-2* defect could be restored by expressing GRK-2 in the nociceptive neurons in strains *grk-2 gjEx601* ($p<0.05$), *gjEx602* ($p<0.001$), and *gjEx639* ($p<0.001$). (A) *grk-2* mutants showed no chemo-attraction to 25 mM NaCl, but strong avoidance after pre-exposure to 100 mM NaCl. Expression of GRK-2 in the nociceptive neurons restored chemo-attraction in *grk-2 gjEx601* animals ($p<0.001$) and *grk-2 gjEx639* animals (($p<0.0001$). *grk-2 gjEx602* animals showed even better rescue of chemo-attraction ($p<0.0001$), but impaired avoidance after pre-exposure ($p<0.0001$). Mutation of *arr-1* significantly reduced avoidance after pre-exposure ($p<0.001$), to a similar level as loss-of *gpa-1*. The behaviour of the *gpa-1 arr-1* double mutant suggests that both genes function in the same pathway ($p>0.05$). (C) *grk-2* animals showed enhanced avoidance after pre-exposure to NaCl for only 5 minutes ($p<0.001$). (D) *grk-2* animals showed strong avoidance of 1 M NaCl, which was not affected in *grk-2 gjEx601* and *gjEx639* animals ($p>0.05$), but impaired in *grk-2 gjEx602* animals ($p<0.001$).

The *C. elegans* arrestin, *arr-1*, is required for adaptation and recovery from adaptation to various odorants (Palmitessa *et al.*, 2005). In our assays, mutation of *arr-1* reduced avoidance after pre-exposure, while chemo-attraction or avoidance

responses were not affected (Figure 4B, results not shown). These results are not in agreement with a function of ARR-1 in receptor or channel internalisation in the ASE or nociceptive neurons. Rather, *arr-1* animals displayed behavioural deficits comparable to those observed in *gpc-1* and *gpa-1* animals. Indeed, *gpa-1 arr-1* double mutant animals showed the same response after pre-exposure as the two single mutants (Figure 4B), indicating that these genes function in the same genetic pathway. However, *arr-1* is broadly expressed in the nervous system of *C. elegans* (Fukuto *et al.*, 2004; Palmitessa *et al.*, 2005). Therefore, it is unclear in which cells ARR-1 functions in gustatory plasticity.

Gustatory plasticity requires cGMP signalling in the body cavity neurons

A frequently used second messenger in G protein signal transduction is cGMP. cGMP can be generated by guanylate cyclases and it can activate other signalling molecules such as cGMP-gated channels or protein kinases (PKG). Previous studies have shown that cGMP signalling plays an important role in chemotaxis to NaCl. Mutation of the guanylate cyclase DAF-11 and the cGMP-gated channel subunits TAX-2 and TAX-4 abolish chemotaxis to NaCl (Birnby *et al.*., 2000; Coburn & Bargmann, 1996; Komatsu *et al.*., 1996). Surprisingly, mutation of *daf-11* did not affect chemotaxis to NaCl in our assay (Figure 5A), whereas mutation of *tax-2* and *tax-4* strongly affected chemotaxis to 0.1-10 mM NaCl. However, these latter mutants still showed strong chemo-attraction to 100 mM NaCl (Figure 5A). These results confirm that cGMP signalling is involved in chemotaxis to NaCl, although it is unclear which proteins activate the TAX-2/TAX-4 channel. In addition, our results suggest that another pathway exists for the detection of NaCl, which functions in parallel to the cGMP pathway.

Mutations in *tax-2* and *tax-4* also abolish gustatory plasticity (results not shown). However, since these proteins play important roles in chemotaxis to NaCl, we cannot conclude whether TAX-2 and TAX-4 function in gustatory plasticity. Analysis of other cGMP signalling molecules did indicate a role of cGMP signalling in gustatory plasticity. Mutations in the cGMP dependent kinase EGL-4 and the phosphodiesterase delta-like protein PDL-1 affected gustatory plasticity (Figure 5C). EGL-4 is expressed in a large number of neurons (Fujiwara *et al.*, 2002; Hirose et al., 2003) and PDL-1, responsible for cGMP breakdown, shows pan-neuronal expression (J. Smith & D. Pilgrim, personal communication). Hence, further cell-specific rescue and epistasis analysis are needed to determine the specific functions of these proteins.

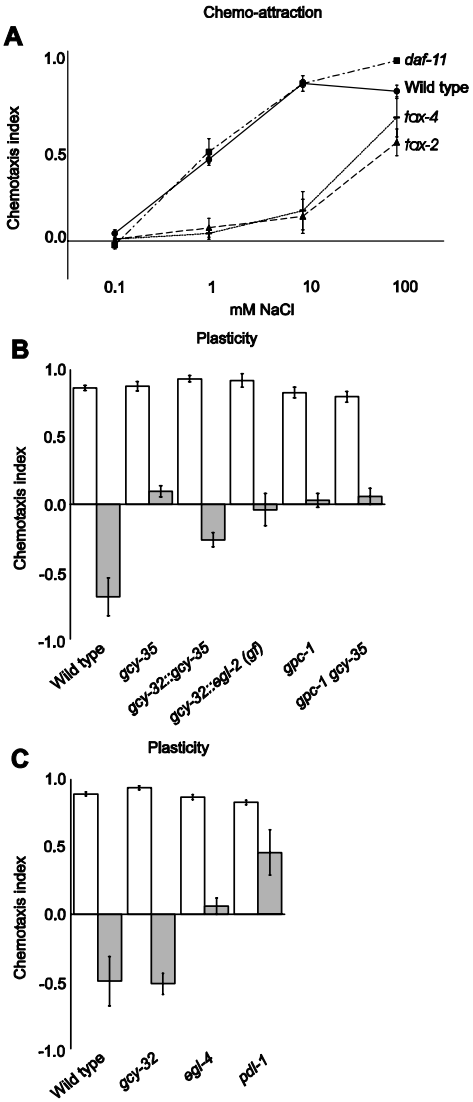

Figure 5: **Gustatory plasticity requires cGMP** (A) Salt detection was not affected in *daf*-11 animals ($p>0.05$), but significantly impaired in *tax-2* and *tax-4* animals ($p<0.01$ at 1 and 10 mM NaCl). These mutants still responded to 100 mM NaCl. (B) Mutation of *gcy-35* affected gustatory plasticity ($p<0.001$). This defect could be rescued by expression of *gcy*-35 in the AQR, PQR, URX neurons ($p<0.0001$). Expression of a *gcy-32::egl-2(gf)* construct to inhibit the activity of AQR, PQR and URX neurons reduced avoidance after pre-exposure ($p<0.05$).Analysis of a *gpc-1 gcy-35* double mutant strain indicated that these genes function in the same genetic pathway in gustatory plasticity ($p>0.05$ compared to the single mutants). (C) Mutation of the cGMP signalling molecules *egl-4* ($p<0.001$) and *pdl-1* ($p<0.01$) affected gustatory plasticity, whereas mutation of gcy-35 did not ($p>0.05$).

We also identified a guanylate cyclase that plays a role in gustatory plasticity. Mutations in the soluble guanylate cyclase *gcy-35* affected this behaviour (Figure 5B), but did not affect chemo-attraction or avoidance of high NaCl concentrations. *gcy-35* is expressed, among others, in the body cavity neurons AQR, PQR and URX (Cheung *et al.*, 2004; Gray *et al.*, 2004). These neurons are directly exposed to the pseudocoelomic body fluid (White *et al.*, 1986) and regulate social feeding or aggregation behaviour (Coates & de Bono, 2002). We tested whether GCY-35 functions in these body cavity neurons during plasticity by specific expression of *gcy-35* in these neurons. Indeed, avoidance after pre-

exposure was restored in *gcy-35* animals expressing a *gcy-32::gcy-35* construct (Figure 5B). We did not obtain full rescue, suggesting that *gcy-35* might also function in other cells in gustatory plasticity.

To confirm that the AQR, PQR and URX cells are involved in gustatory plasticity we expressed a gain-of-function mutant form of the *eag*-type potassium channel EGL-2 in these neurons, using a *gcy-32::egl-2(gf)* construct. Expression of this construct has been shown to inhibit neuronal activity (Coates & de Bono, 2002). We found that expression of EGL-2(gf) in these neurons significantly reduced avoidance after pre-exposure to 100 mM NaCl, without affecting chemotaxis (Figure 5B, results not shown). These results show that the AQR, PQR and URX body cavity neurons mediate a signal that modulates the response to NaCl.

The phenotype of the *gcy-35* animals was very comparable to that of the *arr-1*, *gpa-1* and *gpc-1* animals. To test if also *gcy-35* functions in the same genetic pathway, we tested the behaviour of *gpc-1 gcy-35* double mutant animals. The double mutant strain behaved as the two single mutants (Figure 5B). Therefore, we hypothesize that cGMP signalling via *gcy-35* in the AQR, PQR and/or URX neurons functions in the same genetic pathway as *gpc-1*, *gpa-1* and *arr-1* to modulate the response to NaCl after prolonged exposure.

Gustatory plasticity requires Ca^{2+} signalling

Another frequently used second messenger is Ca^{2+}. Ca^{2+} levels in the cell can be increased by influx via transient receptor potential (TRP) channels or cGMP-gated channels, and by depletion of intracellular stores after IP_3-receptor activation. Increased Ca^{2+} levels can subsequently activate various downstream effector molecules, including calcineurin and the neuronal calcium sensor NCS-1. Previous studies have shown that Ca^{2+} signalling is important for chemotaxis to NaCl, since mutation of the cGMP-gated channel TAX-2/TAX-4, which upon activation leads to Ca^{2+} influx, affects chemotaxis to NaCl (Coburn & Bargmann, 1996; Komatsu *et al.*, 1996; Figure 5A). In addition, it was shown that mutation of the calcineurin A subunit TAX-6 affects chemotaxis to NaCl (Kuhara *et al.*, 2002). Calcineurin is a phosphatase consisting of two subunits A and B, encoded by *tax-6* and *cnb-1*, respectively (Bandyopadhyay *et al.*, 2002; Kuhara *et al.*, 2002). We tested *tax-6* and *cnb-1* mutants in our assays, as well as *ncs-1* mutants, which lack a functional neuronal calcium sensor 1 (Gomez *et al.*, 2001). All three mutants showed chemotaxis defects comparable to those of the *tax-2* and *tax-4* mutants

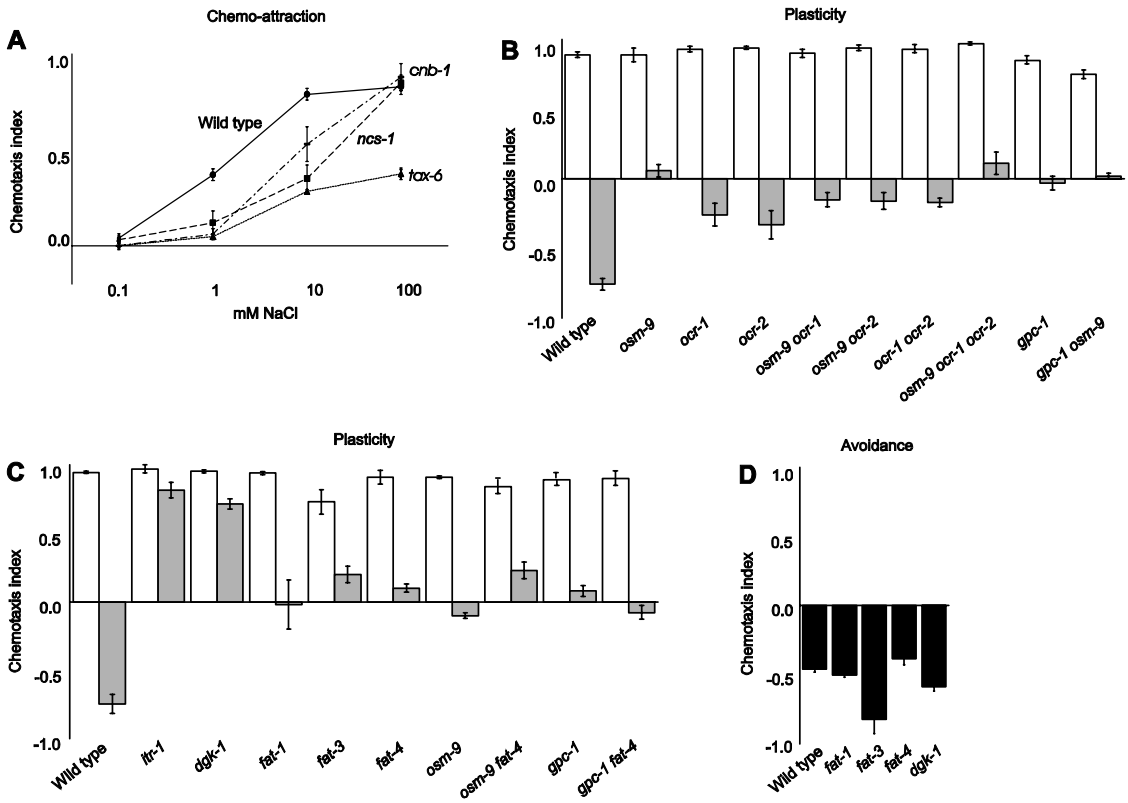

Figure 6: **Gutatory plasticity requires Ca^{2+} signalling.** (A) Salt detection was significantly impaired in *tax-6, cnb-1* and *ncs-1* animals ($p<0.01$ at 1 and 10 mM NaCl). These mutants still responded to 100 mM NaCl. The behaviour of *tax-4 tax-6* double mutants was not significantly different from the behaviour of the two single mutants ($p>0.05$). (B) TRP channel mutants *osm-9, ocr-1* and *ocr-2* showed defects in plasticity ($p<0.001$). TRP-channel double mutants showed similar defects to the single mutants ($p>0.05$). The TRP-channel triple mutant showed a very small additive effect of inactivation of all three subunits ($p\leq0.05$, compared to *ocr-1* and *ocr-2* animals). The TRP channels function in the same genetic pathway as *gpc-1*, since the response of the *gpc-1 osm-9* double did not differ significantly from the single mutants ($p>0.05$). (C) *itr-1, dgk-1, fat-1, fat-3* and *fat-4* showed defects in plasticity ($p<0.001$). FAT-4 functions in the same pathway as OSM-9 and GPC-1, since no significant difference could be detected between double and single mutants ($p>0.05$). (D) Avoidance of 1 M NaCl was not reduced in *fat-1, fat-3, fat-4,* or *dgk-1* animals.

(Figure 5A, 6A): strongly reduced chemotaxis to low NaCl concentrations (0.1-10 mM), but stronegr or even wild type responses to 100 mM NaCl. Also these mutants showed defects in gustatory plasticity, however, because of their function in chemotaxis, their role in gustatory plasticity remains unclear.

The similarities of the NaCl chemotaxis phenotypes of the *tax-2*, *tax-4*, *tax-6*, *cnb-1* and *ncs-1* animals suggested that these genes might function in the same

pathway. In agreement with this hypothesis, we observed no significant difference between the responses of the tax-4 tax-6 double mutant animals and the two single mutants (Figure 6A). The expression patterns of these genes overlap only in the ASE neurons (Bandyopadhyay *et al.*, 2002; Coburn & Bargmann, 1996; Gomez *et al.*, 2001; Komatsu *et al.*, 1996; Kuhara *et al.*, 2002). Since the ASE neurons are essential for NaCl detection, it is likely that the cGMP and Ca^{2+} signalling molecules for the detection of NaCl act in the ASE neurons. However, at present we cannot rule out a function for these proteins in other sensory neurons or interneurons. Moreover, it is unclear which signals activate the cGMP/Ca^{2+} NaCl detection pathway.

Besides cGMP-gated channels also TRP channels mediate Ca^{2+} influx. We have previously shown a function for the TRPV channel subunit OSM-9 in gustatory plasticity (Jansen *et al.*, 2002). Here we show that also the OCR-1 and OCR-2 TRPV channel subunits are involved (Figure 6B). The responses of the three TRPV channel mutants did not differ significantly from each other, although the defect seems a little more severe in the *osm-9* animals (Figure 6B). In addition, the behaviour of doubles between these three mutants did not differ significantly from the single mutants (Figure 6B), suggesting that these three subunits function in the same genetic pathway. In the *ocr-1, ocr-2, osm-9* triple mutant we observed a very small additive effect of inactivation of all three TRP channel subunits compared to the *ocr-1* and *ocr-2* single mutants (Figure 6B).

The plasticity defects of the TRPV channel mutants were very similar to the defects of the *gpa-1*, *gpc-1*, *arr-1* and *gcy-35* mutants. The behaviour of *gpc-1 osm-9* double mutant animals was not significantly different from the single mutants (Figure 6B), suggesting that also the TRPV channel subunits function in this genetic gustatory plasticity pathway. The expression patterns of these TRPV genes are restricted to subsets of sensory neurons, overlapping with neurons that play a role in gustatory plasticity: ASH and ADL (Colbert *et al.*, 1997; Tobin *et al.*, 2002). TRP channels can be activated in various ways, including by phospholipase C (PLC) dependent mechanisms (Montell *et al.*, 2002). Activation of PLC by G proteins leads to the production of inositol (1,4,5) trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 can activate IP_3 receptors resulting in Ca^{2+} release from intracellular stores, which can activate TRP channels. TRP channels can also be activated by DAG, or its derivatives, polyunsaturated fatty acids (PUFAs; Kahn-Kirby *et al.*, 2004). We tested if these signalling molecules are involved in gustatory plasticity.

Mutation of both the IP_3 receptor ITR-1 (Baylis *et al.*, 1999; Dal Santo *et al.*, 1999) and the diacylglycerol kinase DGK-1, essential for reduction of DAG levels

(Hajdu-Cronin *et al.*, 1999; Nurrish *et al.*, 1999), strongly reduced avoidance after pre-exposure (Figure 6C). However, these mutations did not affect chemo-attraction to 0.1 – 100 mM NaCl or avoidance of 1 M NaCl (Figure 6C, D, results not shown), indicating that IP_3 and DAG signalling are only involved in the plasticity response. It is possible that these second messengers activate TRP channels, but they could also affect other signalling routes such as the $G_o\alpha/G_q\alpha$ network, which is involved in gustatory plasticity as well (RKH and GJ, unpublished results).

To test if PUFAs are involved in gustatory plasticity, we tested three lipid desaturase mutants that affect PUFA synthesis, *fat-1, fat-3* and *fat-4* (Watts & Browse, 2002). Recently, strong avoidance defects were described for *fat-3* mutant animals, including a defect in avoidance of 1 M glycerol; mild or no defects were observed for *fat-1* or *fat-4* animals (Kahn-Kirby *et al.*, 2004). In our assays, the PUFA synthesis mutants showed no defect in avoidance of 1 M NaCl (Figure 6D), again indicating that separate pathways exist for the detection of 1 M glycerol and 1 M NaCl. However, all three mutants showed aberrant behaviour in our gustatory plasticity assay (Figure 6C), indicating that PUFAs play a role in this process.

Since the PUFA synthesis mutants showed a very comparable behaviour to *gpa-1*, *gpc-1*, *arr-1, osm-9* and *gcy-35* animals, we analysed the behaviour of *gpc-1 fat-4* and *osm-9 fat-4* double mutants. The behaviour of these double mutants did not differ significantly from the behaviour of the single mutants, indicating that these genes all function in the same genetic pathway (Figure 6C).

Discussion

Model for gustatory plasticity in *C. elegans*

We can discriminate three responses of *C. elegans* to NaCl: first, chemotaxis to NaCl concentrations between 0.1 – 200 mM, second, avoidance of NaCl above 200 mM, and third, avoidance of otherwise attractive NaCl concentrations (25 mM) after pre-exposure to 100 mM NaCl. Data presented here and by others indicate that these responses require input from at least 4 chemosensory neurons and the neurons exposed to the body fluid. We propose a model to explain the different gustatory responses (Figure 7).

The ASE neurons are essential for chemotaxis to low NaCl concentrations (Figure 7). Chemo-attraction is antagonized by avoidance, mediated by the ASH neurons (Figure 7). The ASH neurons are not activated at low NaCl concentrations, but become activated at high NaCl concentrations (Hilliard *et al.*, 2004). It is unclear

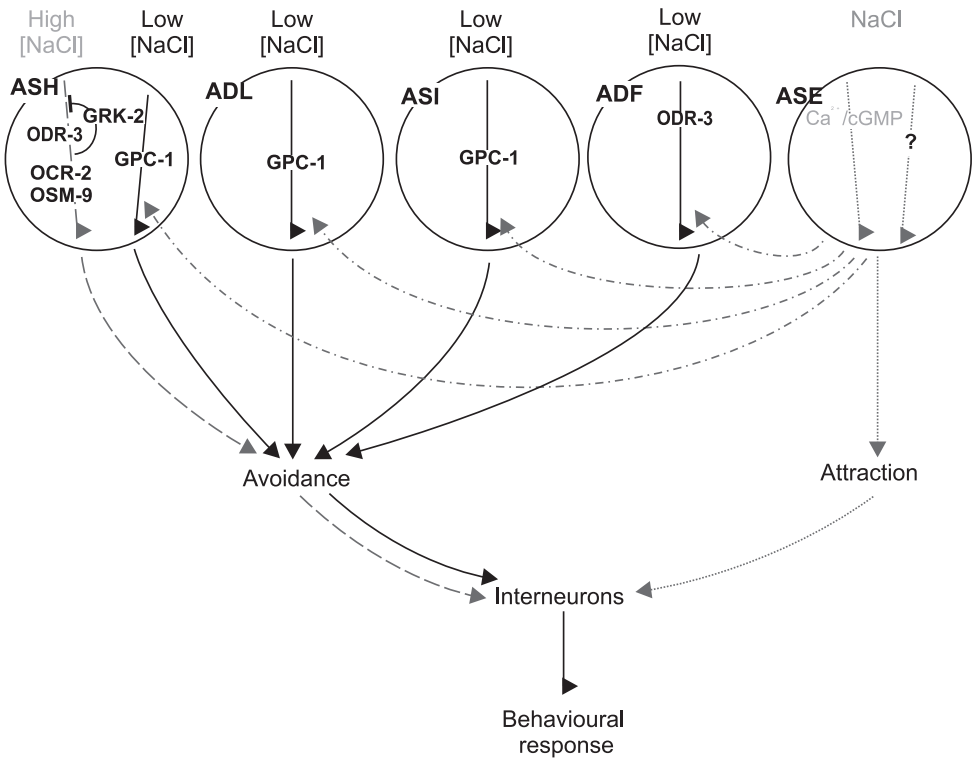

Figure 7: **Model for NaCl detection and its plasticity in *C. elegans***. NaCl is detected by the ASE sensory neurons, probably using cGMP/Ca^{2+} signalling and an unknown signalling pathway. Attraction is antagonized by avoidance mediated by the ASH, using ODR-3, OSM-9, OCR2 and the inhibitor GRK-2. We propose that upon prolonged exposure to attractive NaCl concentrations an ASE derived signal sensitises the ASH, ADL, ASI and ADF neurons, resulting in avoidance of normally attractive NaCl concentrations. We have shown that the ADF neurons use ODR-3, and the ASI neurons, a second pathway in the ASH neurons and probably the ADL neurons use GPC-1. Integration of these signals ultimately leads to the behavioural response of the animal.

where the ASE and ASH derived signals are integrated and where the choice between attraction and avoidance is made. It is also not known why avoidance is preferred over attraction at NaCl concentrations above 200 mM. Perhaps ASE signalling is blocked at high salt concentrations, or alternatively, preference for avoidance might be the default state, e.g. determined by the wiring of the nervous system. Our model suggests that also the ADF, ADL and ASI neurons are not or only weakly activated at low NaCl concentrations. We propose that upon prolonged exposure to 100 mM NaCl the ASE neurons produce a signal (blue arrows in Figure 7) that sensitises the ADF, ADL, ASI and ASH neurons, resulting in avoidance of otherwise attractive NaCl concentrations (black arrows in Figure

7). We suggest that the ASE neurons signal via the body cavity neurons, AQR, PQR and URX, but it is also possible that the body cavity neurons function downstream of the ADF, ADL, ASI and ASH neurons.

Based on the cellular circuit described above, expression patterns and our cell specific rescue experiments, we can place several molecules in our model (Figure 7). We propose that stimulation of the ASE neurons by low NaCl concentrations activates a cGMP and Ca^{2+} signalling pathway and another unknown pathway. Avoidance of 1 M NaCl, mediated by the ASH neurons, requires the Gα subunit ODR-3 and the TRP channel subunits OSM-9 and OCR-2, and is inhibited by GRK-2. ODR-3 also functions in the ADF neurons in gustatory plasticity, where it might either transduce the avoidance signal, or alternatively transduce the ASE derived sensitising signal.

In addition, we have identified a genetic pathway that mediates gustatory plasticity. This pathway involves the Gγ GPC-1, the Gα GPA-1, the arrestin ARR-1, the TRPV channel subunits OSM-9, OCR-1 and OCR-2, PUFA signalling (FAT-4) and the guanylate cyclase GCY-35. It is unclear if these proteins function in the same cells, but our and previously published data are consistent with a function for GPC-1 in the ASI, ASH and perhaps ADL neurons (based on rescue experiments), GPA-1 and OSM-9 in these same neurons (based on expression patterns, Colbert *et al.*, 1997; Jansen *et al.*, 1999), OCR-1 in ADL and OCR-2 in ADL, ASH and ADF (Tobin *et al.*, 2002) and GCY-35 in the AQR, PQR and URX body cavity neurons (based on rescue experiments). We cannot discriminate if these proteins transduce the avoidance response or function in a pathway that mediates the ASE derived sensitising signal. As indicated, part of this model is based on previously described expression patterns. Most of these expression patterns have been derived using GFP reporter constructs. These constructs may not fully represent the gene's expression pattern. Therefore, additional cell-specific rescue experiments will be needed to confirm our model.

Materials and methods

Strains, genetics and germline transformation

Strains used in this work are listed in Table I. Wild type *C. elegans* were strain Bristol N2. Germline transformation and transgene integration were performed as described (Mello *et al.*, 1991). We used an *elt-2::GFP* construct (30 ng/μl) as co-injection marker (Fukushige *et al.*, 1999). Rescue of *che-1*, *gpc-1*, *odr-3* and *grk-2* and cell inactivation using *mec-4d* and *mec-2* or *egl-2(gf)* was tested using

5 or more transgenic strains for each clone injected at various concentrations (1 – 100 ng/µl). Although cell specific expression of *mec-2*/*mec-4d* or *egl-2gf* did affect gustatory plasticity we did not observe neuronal degeneration.

Molecular biology

Details of plasmid construction are available on request. Promoters used for cell-specific rescue or cell inactivation were: *flp-5* (ASE; Li *et al.*, 1999), *gpa-4* (ASI; Jansen *et al.*, 1999), *gpa-11* (ADL, ASH; Jansen *et al.*, 1999), *gpa-13* (ADF, ASH, AWC; Jansen *et al.*, 1999), *sra-6* (ASH, ASI faint; Troemel *et al.*, 1995), *srb-6* (ADL, ASH; Troemel *et al.*, 1995), *glr-1* (17 classes of neurons; Hart *et al.*, 1995; Maricq *et al.*, 1995), *srh-142* (ADF; Sagasti *et al.*, 1999). Expression patterns were confirmed using GFP-fusion constructs driven by the same promoter.

Behavioural assays

Chemotaxis towards NaCl and gustatory plasticity were assessed as described before (Wicks *et al.*, 2000; Jansen *et al.*, 2002), using 0.1, 1, 10 or 100 mM or 1 M NaCl; after pre-exposure to 100 mM NaCl animals were tested for chemotaxis to 25 mM NaCl. A chemotaxis-index was calculated: (A-C)/(A+C); where A is the number of worms at the quadrants with NaCl, C is the number of worms at the quadrants without attractant. Statistical significance was determined using the two-tailed *t*-test. Error bars represent the s.e.m.

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Chapter 4

Gustatory plasticity in *C. elegans* involves the neurotransmitters glutamate, serotonin, dopamine, and octopamine

Renate K Hukema, Suzanne Rademakers, Gert Jansen

4

Manuscript in preparation

Abstract

Caenorhabditis elegans shows chemo-attraction to 0.1-200 mM NaCl, avoidance of higher NaCl concentrations and avoidance of otherwise attractive NaCl concentrations after prolonged exposure to NaCl, which is called gustatory plasticity. Here we show that *C. elegans* only shows avoidance of relatively low salt concentrations after pre-exposure to salts in the absence of food, suggesting that in gustatory plasticity *C. elegans* associates low NaCl concentrations to this cue. In addition, starvation enhances gustatory plasticity. In a previous study we identified several sensory neurons and many genes involved in gustatory plasticity. Here we identify several neurotransmitter systems used in gustatory plasticity. We show that serotonin, dopamine, and octopamine are involved in gustatory plasticity. Both serotonin and dopamine are acutely needed during the gustatory plasticity response. In addition, serotonin is required during development. We found a role for dopamine in GABAergic neurons and the DA and DB motor neurons. Moreover, we found that glutamate is necessary for chemotaxis to NaCl and also for gustatory plasticity. Surprisingly, of all mutants that affect neurotransmission tested only loss-of-function of the glutamate gated Cl^- channel *avr-15*, the propotein convertase *egl-3*, and the dopamine reuptake transporter *dat-1* affect starvation enhanced gustatory plasticity.

Introduction

Mammals are attracted to low concentrations and repelled by high concentrations of NaCl. Also the nematode *C. elegans* shows attraction to relatively low NaCl concentrations and avoids higher NaCl concentrations (Ward, 1973; Culotti & Russell, 1978). In addition, *C. elegans* avoids otherwise attractive NaCl concentrations after pre-exposure to NaCl (Jansen *et al.*, 2002). We call this latter response gustatory plasticity. We try to decipher the molecular mechanisms and cellular circuitry that mediate gustatory plasticity.

C. elegans senses chemical from its environment using only eleven pairs of sensory neurons: the amphid sensory neurons (Bargmann & Horvitz, 1991). Of the amphid sensory neurons the ASE neurons are essential for chemotaxis to NaCl (Bargmann & Horvitz, 1991). The ADF, ASG, and ASI neurons have a redundant role in chemotaxis to NaCl (Bargmann & Horvitz, 1991). Avoidance of high salt concentrations is mediated by the ASH nociceptive neurons (Bargmann & Horvitz, 1991). Gustatory plasticity involves signals from at least five pairs of sensory

neurons (Hukema *et al.*, 2006). The ASE neurons are essential, but also signals from the ADF, ASI, ASH, and probably ADL neurons are involved.

Relatively little is known about the molecular mechanisms of NaCl perception. Thus far, the cGMP gated channel TAX-2/TAX-4, the calcineurin TAX-6/CNB-1, and the neuronal calcium sensor NCS-1 have been shown to be involved (Coburn & Bargmann, 1996; Komatsu *et al.*, 1996; Kuhara *et al.*, 2002; Hukema *et al.*, 2006). It is likely that these molecules function in the ASE neurons, but this has not been proven yet. Avoidance of high NaCl concentrations is generally thought to be due to avoidance of high osmotic strength. Osmotic avoidance requires the Gα subunit ODR-3, the TRP channel subunits OSM-9 and OCR-2, the cytoplasmic protein OSM-10, the G protein coupled receptor kinase GRK-2, and glutamatergic neurotransmission involving the glutamate receptors GLR-1 and NMR-1, the glutamate transporter EAT-4, and the proprotein convertase EGL-3 (Colbert *et al.*, 1997; Berger *et al.*, 1998; Roayaie *et al.*, 1998; Hart *et al.*, 1999; Mellem *et al.*, 2002; Tobin *et al.*, 2002; Fukuto *et al.*, 2004). Many genes have been found to function in gustatory plasticity (Hukema *et al.*, 2006). Some of these genes have been arranged in a genetic pathway, including the arrestin *arr-1,* G protein subunits *gpa-1* and *gpc-1,* the guanylate cyclase *gcy-35,* the TRPV channel subunit *osm-9,* and *fat-4* that is needed for the synthesis of polyunsaturated fatty acids. Of these genes, *gpc-1* is required in the ASI, ASH, and probably ADL neurons. *gcy-35* functions in the AQR, PQR, URX neurons. In addition, G protein signalling via ODR-3 is required in the ADF neurons, and GRK-2 functions in the ASH neurons.

Gustatory plasticity depends on the time of pre-exposure and the concentration of NaCl during pre-exposure (Jansen *et al.*, 2002). However, since pre-exposure not only reduces attraction to NaCl, but even results in avoidance, we surmised that this behaviour involves more than adaptation or desensitisation and that perhaps additional cues are required to induce gustatory plasticity. Previously, NaCl chemotaxis was reported to be affected by starvation (Saeki *et al.*, 2001). Serotonin can block the starvation effect on chemotaxis in these gradient assays. The plasticity of several other behaviours that have been described in *C. elegans* is also modulated by food signals: olfactory adaptation, basal slowing responses, and thermotaxis (Colbert & Bargmann, 1997; Sawin *et al.*, 2000; Mohri *et al.*, 2005). Starvation enhances basal slowing responses and olfactory adaptation; it also causes animals to avoid their cultivation temperature. In plasticity of thermotaxis exogenous serotonin and octopamine can mimic the well-fed state and the starved state of the animal, respectively (Mohri *et al.*, 2005). Also the effect of starvation on olfactory adaptation is antagonised by serotonin (Colbert &

Bargmann, 1997). Moreover, *C. elegans* locomotory rate is modulated by serotonin as well as dopamine (Sawin *et al.*, 2000).

In mammals, the biogenic amines dopamine and serotonin are important neurotransmitters involved in many behaviours and have been implicated in a variety of brain disorders. Serotonin and dopamine are synthesized from tyrosine and tryptophan respectively by a series of enzymatic reactions and subsequently transported in synaptic vesicles. After exocytosis, the neurotransmitters exert their effects on postsynaptic neurons by binding their receptors; excess neurotransmitter is taken up into postsynaptic neurons by reuptake transporters.

All results obtained thus far suggest that serotonin and dopamine synthesis and signalling are conserved in *C. elegans.* Serotonin is present in seven neurons (Horvitz *et al.*, 1982), but it is synthesized only in the ADF and NSM neurons (Sze *et al.*, 2000). Serotonin synthesis requires the tryptophan hydroxylase *tph-1,* the aromatic amino acid decarboxylase *bas-1,* and the tyrosine hydroxylase *cat-2* (Lints & Emmons, 1999; Sze *et al.*, 2000; Hare & Loer, 2004). Dopamine was found in eight sensory neurons (Sulston *et al.*, 1975) and its synthesis requires the aromatic amino acid decarboxylase *bas-1* and the GTP cyclohydroxylase *cat-4* (Sawin *et al.*, 2000; Hare & Loer, 2004). After synthesis both serotonin and dopamine are transported by the synaptic vesicular transporter CAT-1 (Duerr *et al.*, 1999). Subsequently, the neurotransmitters can bind several receptors (Ranganathan *et al.*, 2000; Tsalik *et al.*, 2003; Chase *et al.*, 2004), and they are removed from the synaptic cleft by MOD-5 and DAT-1 reuptake transporters (Jayanthi *et al.*, 1998; Ranganathan *et al.*, 2001). *C. elegans* synthesizes an additional biogenic amine, octopamine, which requires the tyrosin hydroxylase *tdc-1* and the tyramine hydroxylase *tbh-1* (Alkema *et al.*, 2005).

Here, we show that gustatory plasticity requires absence of food. This suggests that in this assay *C. elegans* can associate low salt concentrations to the absence of food. Prolonged starvation enhances gustatory plasticity. We show that the neurotransmitters serotonin, dopamine, octopamine, and glutamate are required for gustatory plasticity. Glutamate is also important for attraction to NaCl. Surprisingly, all neurotransmitter mutants showed normal starvation enhanced gustatory plasticity except for *avr-15* and *dat-1* mutant animals, suggesting that glutamate and excess dopamine signalling can block this process.

Results

Food signals modulate gustatory plasticity

Gustatory plasticity depends on the salt concentrations and exposure time used during pre-exposure (Jansen *et al.*, 2002). We wondered if this response involves additional cues. Starvation and a food signal have been implicated in behavioural plasticity of *C. elegans*. To test if food is also important in gustatory plasticity, we pre-exposed animals to NaCl in absence or in the presence of food. To do this, we pre-exposed animals on agar-plates instead of in a buffer. If animals were pre-exposed to 100 mM NaCl in the presence of bacteria, chemotaxis to 25 mM NaCl was unaffected and the animals showed the same response as naïve animals (Figure 1A). However, if we pre-exposed animals to 100 mM NaCl on plates in the absence of bacteria, they avoided 25 mM NaCl (Figure 1A). Thus, it seems that absence of food is essential for gustatory plasticity, suggesting the *C. elegans* associates low NaCl concentrations with the absence of food.

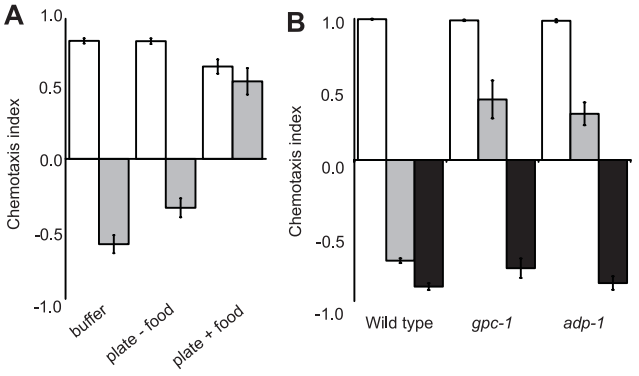

Figure 1**: Starvation is required for gustatory plasticity**. (A) Wild type animals avoid NaCl after pre-exposure on plates in the absence of food, but not in the presence of food. (B) Starvation enhances avoidance after pre-exposure in wild type animals ($p<0.05$). *gpc-1* and *adp-1* mutant animals showed a defect in gustatory plasticity ($p<0.0001$), but they showed wild type levels of starvation enhanced gustatory plasticity ($p>0.05$). Indicated are the averages of at least 4 assays ± s.e.m. Open bars indicate chemotaxis to 25 mM NaCl; grey bars indicate the response to 25 mM NaCl after pre-exposure to 100 mM NaCl; black bars indicates the response to 25 mM NaCl after pre-exposure to 100 mM NaCl after starvation.

Next we wondered if prolonged starvation of animals could modulate gustatory plasticity. Therefore, we first starved animals for four hours on agar plates before testing them in the plasticity assay. Although pre-exposed animals show strong avoidance, four hours of starvation resulted in even stronger avoidance after pre-exposure (Figure 1B). We call this latter behaviour starvation

enhanced gustatory plasticity. Chemotaxis to NaCl was not affected when animals were starved in the absence of NaCl (results not shown).

Subsequently, we tested the known gustatory plasticity mutant animals *gpc-1* and *adp-1* (Jansen *et al.*, 2002). Both *gpc-1* and *adp-1* mutant animals showed a defect a gustatory plasticity when they were pre-exposed to 50 mM NaCl, but starvation before the assay resulted in wild type levels of avoidance after pre-exposure (Figure 1B). Again this shows that starvation leads to enhanced gustatory plasticity. However, this also shows that neither *gpc-1* nor *adp-1* mediate the starvation signal. Finally, these results show that neither GPC-1 nor ADP-1 are required for strong avoidance of low salt concentrations per se, suggesting that these proteins function in the process that integrates absence of food with presence of 100 mM NaCl.

Serotonin is involved in gustatory plasticity

Various plastic behaviours of *C. elegans* are modulated by starvation (Colbert & Bargmann, 1997; Sawin *et al.*, 2000; Mohri *et al.*, 2005). In many of these behaviours the food signal is mediated by serotonin (Colbert & Bargmann, 1997; Mohri *et al.*, 2005). Therefore, we tested if serotonin also acts as the food signal in gustatory plasticity. First, we analysed the behaviour of mutant animals that have defects in the synthesis of serotonin: *bas-1* and *cat-4* mutant animals, which have reduced serotonin as well as dopamine levels, and *tph-1* mutant animals, which have reduced serotonin levels (Sawin *et al.*, 2000; Sze *et al.*, 2000). Unfortunately, *tph-1* mutant animals did not perform well in our behavioural assays due to locomotion defects.

Gustatory plasticity was strongly affected in *bas-1* and *cat-4* mutant animals (Figure 2A). In addition, chemotaxis to NaCl was slightly, but significantly, stronger in *bas-1* and *cat-4* mutant animals (Figure 2A, B). These results suggest that serotonin and/or dopamine are involved in the response of *C. elegans* to NaCl .

To further confirm the role of serotonin in gustatory plasticity, we tested if exogenous serotonin could restore avoidance after pre-exposure in *bas-1* mutant animals. We performed a time series, in which *bas-1* mutant animals were cultured on plates containing 2 mM of serotonin. Indeed, exogenous application of serotonin restored avoidance after pre-exposure in *bas-1* mutant animals (Figure 2C). Incubations to 96 hours, i.e. including the parental generation, were needed, which implies that serotonin plays a role in development. However, exposure of

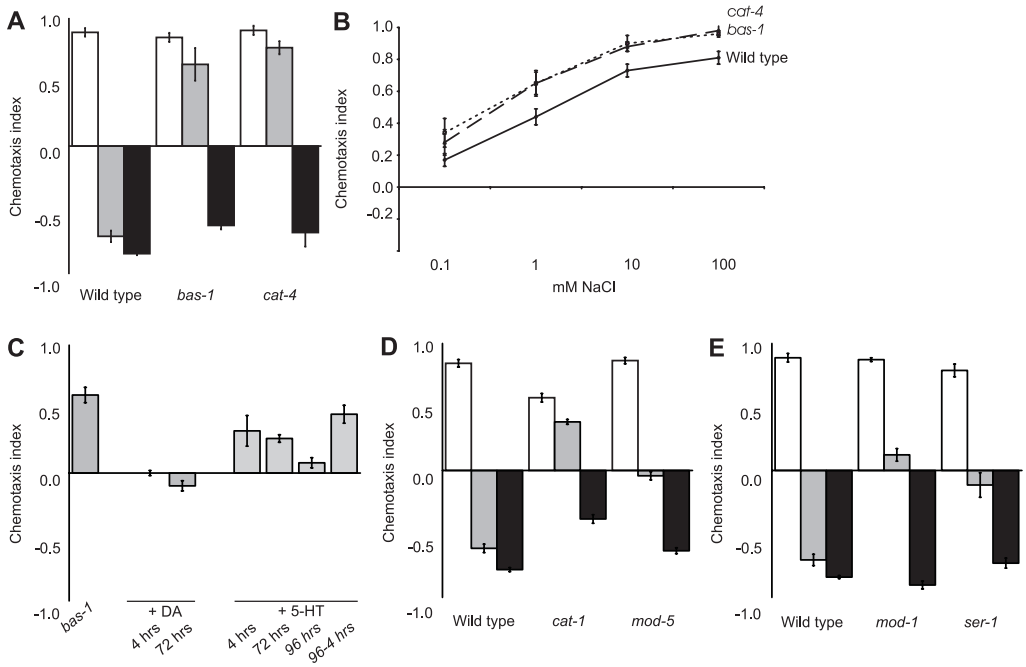

Figure 2: **Serotonin is required for gustatory plasticity**. (A) Mutants with defects in serotonin synthesis, *bas-1* and *cat-4*, showed defects in gustatory plasticity ($p<0.01$), but showed strong avoidance after pre-exposure when they were starved. (B) Chemotaxis to 1-100 mM NaCl is enhanced in *bas-1* and *cat-4* mutant animals when compared to wild type animals ($p<0.05$). (C) Plasticity could partially be restored in *bas-1* mutant animals by exogenously applying 2 mM dopamine for four hours prior to the assay ($p<0.01$). Gustatory plasticity of *bas-1* mutant animals could only be restored by supplying 2 mM serotonin for 72 hours or longer ($p<0.01$). (D) *cat-1* and *mod-5* mutant animals both showed defects in gustatory plasticity ($p<0.001$), they did show gustatory plasticity after starvation. (E) *mod-1* and *ser-1* serotonin receptor mutant animals both showed defects in gustatory plasticity ($p<0.01$), but showed normal gustatory plasticity after starvation. Indicated are the averages of at least 4 assays ± s.e.m. Open bars indicate chemotaxis to 25 mM NaCl; grey bars indicate the response to 25 mM NaCl after pre-exposure to 100 mM NaCl; black bars indicates the response to 25 mM NaCl after pre-exposure to 100 mM NaCl after starvation.

bas-1 mutant animals to serotonin for 96 hours, but not for the last four hours before the assays did not restore gustatory plasticity. Taken together, these results

indicate that serotonin is acutely needed in the process of gustatory plasticity and that it is also required during development.

Serotonin transporters and receptors are involved in gustatory plasticity

To further study the role of serotonin in gustatory plasticity, we analysed the behaviour of other serotonin signalling mutants . Serotonin levels are not only regulated by *de novo* synthesis, but also by its transport in the nerve terminal and by reuptake from the synaptic cleft. Therefore, we tested animals with mutations in the synaptic vesicular monoamine transporter CAT-1 (Duerr *et al.*, 1999) and in the re-uptake transporter MOD-5 (Ranganathan *et al.*, 2001). Behavioural phenotypes of *cat*-1 mutant animals reflect deficiency in biogenic amines (Duerr *et al.*, 1999), whereas *mod-5* mutant animals are hypersensitive to exogenous serotonin (Ranganathan *et al.*, 2001). Both *cat-1* and *mod-5* mutant animals showed defects in gustatory plasticity (Figure 2D). This suggests that not only reduced levels of serotonin, but also too high levels of serotonin affect gustatory plasticity.

Serotonin can have systemic effects on the nervous system regardless of where it is synthesized. Only two pairs of neurons synthesize serotonin *de novo*, but several other neurons have been shown to contain the neurotransmitter (Horvitz *et al.*, 1982; Sze *et al.*, 2000). Therefore, we next tested several serotonin receptor mutants: the serotonin gated Cl^- channel MOD-1 (Ranganathan *et al.*, 2000), and the serpentine receptors SER-1 and SER-4 (Tsalik *et al.*, 2003). We observed defects in *mod-1* and *ser-1* mutant animals in avoidance after pre-exposure (Figure 2E). *ser-4* mutant animals showed wild type responses (results not shown). Unfortunately, the neuronal expression patterns of the serotonin receptors have not been extensively studied.

Serotonin is not required for starvation enhanced gustatory plasticity

We next addressed the question if serotonin plays a role in starvation enhanced gustatory plasticity. Therefore, we starved the serotonin synthesis and serotonin signalling mutant animals before testing gustatory plasticity behaviour. Remarkably, *bas-1, cat-4, cat-1, mod-5, mod-1,* and *ser-1* mutant animals were all capable of strong avoidance after pre-exposure when these animals were starved for four hours (Figure 2A, D, E). These results show that altered serotonin signalling, either increased or decreased, does not affect starvation enhanced avoidance after pre-exposure, suggesting that serotonin does not mediate the starvation signal in this assay, and that serotonin is not required for avoidance of low NaCl concentrations per se.

Dopamine is involved in gustatory plasticity

In *C. elegans* dopamine is often found to act antagonistically to serotonin. In addition, *bas-1* and *cat-4* mutant animals do not only lack serotonin, but also have reduced dopamine levels. Therefore, we wondered if also dopamine might be involved in gustatory plasticity. First, we tested *cat-2* mutant animals, which have reduced dopamine levels but normal serotonin levels (Lints & Emmons, 1999). *cat-2* mutant animals showed normal chemotaxis to NaCl, however gustatory plasticity was strongly affected (Figure 3A). The defect in *cat-2* animals was less strong than the defect of *bas-1* and *cat-4* mutant animals. Taken together, these results suggest that besides serotonin also dopamine is needed for gustatory plasticity.

To confirm the role of dopamine we determined if exogenous dopamine could restore plasticity in *bas-1* mutant animals. Culturing *bas-1* mutant animals for only four hours on plates containing 2 mM dopamine restored gustatory plasticity (Figure 2C), indicating an acute need for dopamine in this process. Longer incubations times did not have additional effects on gustatory plasticity.

Dopamine transporter and receptors involved in gustatory plasticity

To further confirm the importance of dopamine in gustatory plasticity we analysed *dat-1* mutant animals. *dat*-1 mutant animals are thought to have potentiated dopamine activity, since *dat-1* encodes a dopamine re-uptake transporter (Jayanthi *et al.*, 1998). Mutation of *dat-1* resulted in a small but significant defect in chemotaxis to NaCl (Figure 3A,B). These results suggest that dopamine modulates the response of *C. elegans* to NaCl. However, it is also possible that this defect in chemotaxis is caused by a general effect of excessive dopamine signalling on the responsiveness of the animals.

dat-1 mutant animals also showed a defect in gustatory plasticity (Figure 3A). This defect of *dat-1* mutant animals suggests that not only reduced levels of dopamine, but also enhanced activity of dopamine disrupt avoidance after pre-exposure. However, there is only a small defect in gustatory plasticity that could also be caused by the defect in chemotaxis to NaCl. Therefore these results are not conclusive.

To analyse where dopamine might function in the nervous system, we also analysed the behaviour of dopamine receptor mutants. We tested the response of *dop-1* and *dop-2* D_2-like dopamine receptor mutant animals, and *dop-3* D_1-like dopamine receptor mutant animals (Chase *et al.*, 2004). All three mutants showed a

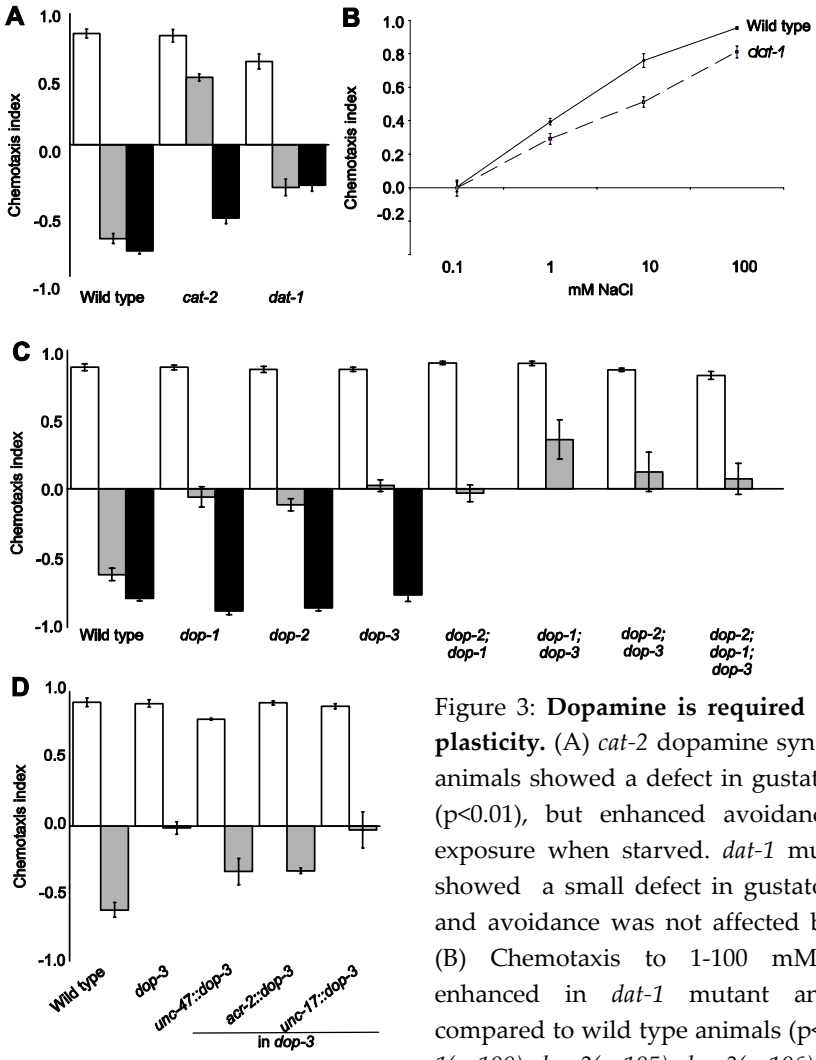

Figure 3: **Dopamine is required for gustatory plasticity.** (A) *cat-2* dopamine synthesis mutant animals showed a defect in gustatory plasticity ($p<0.01$), but enhanced avoidance after pre-exposure when starved. *dat-1* mutant animals showed a small defect in gustatory plasticity, and avoidance was not affected by starvation. (B) Chemotaxis to 1-100 mM NaCl was enhanced in *dat-1* mutant animals when compared to wild type animals ($p<0.05$). C) *dop-1(vs100), dop-2(vs105), dop-3(vs106),* as well as all possible double and triple mutants(showed reduced gustatory plasticity ($p<0.01$). The behaviour of double and triple mutants did not differ significantly from the single mutants ($p>0.05$). *dop-1, dop-2* and *dop-3* mutant animals did show plasticity when starved. (D) Gustatory plasticity could be restored when expressing *dop-3* under control of the *unc-47* or the *acr-2* promoter in *dop-3 (vs106)* mutant animals ($p<0.05$). Expression of *dop-3* driven by the *unc-17* promoter did not affect gustatory plasticity of *dop-3 (vs106)* mutant animals ($p>0.05$). Open bars indicate chemotaxis to 25 mM NaCl; grey bars indicate the response to 25 mM NaCl after pre-exposure to 100 mM NaCl; black bars indicates the response to 25 mM NaCl after pre-exposure to 100 mM NaCl after starvation.

defect in gustatory plasticity (Figure 3C). No additive defects were found in *dop-2; dop-1*, *dop-1; dop-3*, *dop-2; dop-3* double mutant animals, or in *dop-2; dop-1; dop-3* triple mutant animals (Figure 3C). Taken together, this suggests that both D_1-like

and D_2-like dopamine receptors are involved in gustatory plasticity and that they function in the same genetic pathway.

DOP-3 function in DA, DB, and GABAergic neurons

To study in which neurons the D_1-like dopamine receptor DOP-3 functions, we rescued expression of *dop-3* in the GABA-ergic or the cholinergic neurons of *dop-3* mutant animals (Figure 3D). Previously it has been shown that DOP-3 receptors function in these neurons to regulate movement (Chase *et al.*, 2004). Expression of *dop-3* in the GABAergic neurons using the *unc-47* promoter partially rescued the plasticity defect of *dop-3* mutant animals (Figure 3D). Also expression of *dop-3* under control of the *acr-2* promoter resulted in a stronger avoidance after pre-exposure in *dop-3* mutant animals (Figure 3D). However, expression of *dop-3* under control of the *unc-17* promoter did not result in rescue of the plasticity defect of *dop-3* mutant animals. The *unc*-47 promoter drives expression in GABAergic neurons, the *acr*-2 promoter drives expression in the cholinergic DA and DB motor neurons, and the *unc-17* promoter drives expression in all cholinergic neurons (Alfonso *et al.*, 1993; McIntire *et al.*, 1997; Hallam *et al.*, 2000). Taken together, these results suggest that DOP-3 functions in the GABAergic neurons, and in the DA and DB neurons, but seems not to function in the other cholinergic neurons. Since the *unc-17* promoter also drives expression in the DA and DB neurons, we expected that gustatory plasticity would be rescued by expressing *dop-3* using this promoter. Perhaps the expression levels of *dop-3* in the DA and DB neurons were too low in these animals, or other cholinergic neurons could have antagonistic roles in gustatory plasticity.

Dopamine is not required for starvation enhanced gustatory plasticity

Next we tested gustatory plasticity behaviour of *cat-2* mutant animals after starvation. Four hours starvation before the assays resulted in very strong avoidance after pre-exposure in *cat-2* mutant animals (Figure 3A). Also *dop-1, dop-2*, and *dop-3* mutant animals showed avoidance after pre-exposure after starvation, suggesting that also dopamine is not required for starvation enhanced gustatory plasticity and that dopamine is not required for avoidance of 25 mM NaCl per se (Figure 3C). In contrast, starvation of *dat-1* mutant animals did not result in stronger avoidance after pre-exposure (Figure 3A). However, mutation of *dat-1* also affected chemotaxis to NaCl, therefore these results are not conclusive.

Octopamine is involved in gustatory plasticity

Next we tested the role of octopamine in gustatory plasticity, since this neurotransmitter has been found to mediate the starvation signal in thermotaxis behaviour (Mohri *et al.*, 2005). We analysed *tbh-1* and *tdc-1* mutant animals, which have a defect in the synthesis of octopamine (Alkema *et al.*, 2005) and *ser-2* mutant animals, which lack a tyramine receptor (Rex & Kominiecki, 2002). These mutant animals all showed wild type chemotaxis to NaCl, but defective gustatory plasticity (Figure 4). Thus, it seems that octopamine and/or tyramine function in gustatory plasticity. However, octopamine or tyramine are not required for starvation enhanced gustatory plasticity, since *tbh-1*, *tdc-1*, and *ser-2* mutant animals all showed very strong avoidance after pre-exposure when they were starved (Figure 4).

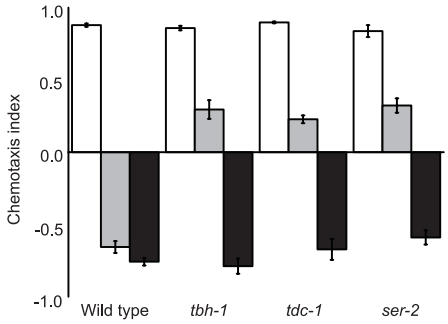

Figure 4: **The role of octopamine in gustatory plasticity.** *tbh-1(ok1196)* and *tdc-1(ok914)* octopamine synthesis mutants , as well as *ser-2* tyramine receptor mutants have defects in gustatory plasticity, but showed stronger avoidance after pre-exposure after starvation ($p<0.001$ compared to non-starved animals). This suggests that octopamine, and perhaps tyramine, are needed for gustatory plasticity, but they do not transduce the starvation signal. Indicated are the averages of at least 4 assays ± s.e.m. Open bars indicate chemotaxis to 25 mM NaCl; grey bars indicate the response to 25 mM NaCl after pre-exposure to 100 mM NaCl; black bars indicates the response to 25 mM NaCl after pre-exposure to 100 mM NaCl after starvation.

Neuropeptides are involved in the response to NaCl

Next, we analysed the role of other neurotransmission systems in gustatory plasticity. Neuropeptides are small peptides cleaved from larger precursors, the proproteins. Neuropeptides are thought to be co-released with classic neurotransmitters and to modulate their function (reviewed in Kupfermann *et al.*, 1991). Neuropeptides are involved in plasticity of the response of *C. elegans* to oxygen (Rogers *et al.*, 2003). Several neuropeptides have been identified in *C. elegans* (Li *et al.*, 1999; Nathoo *et al.*, 2001; Husson *et al.*, 2005). However, only very few loss-of-function alleles of these neuropeptides exist. Therefore, we studied the role of neuropeptides in gustatory plasticity using *egl-3* mutant animals. *egl-3* encodes a proprotein convertase that is involved in the processing of neuropeptides (Kass *et al.*, 2001). *egl-3* mutant animals showed wild type or even

enhanced chemo-attraction to 0.1-100 mM NaCl (Figure 5A). In addition, *egl-3* mutant animals showed a severe defect in gustatory plasticity; they were still very strongly attracted to NaCl after pre-exposure (Figure 5B). On the other hand, starved *egl-3* animals did show avoidance after pre-exposure, although not as strong as in wild type animals (Figure 5B). This suggests that neuropeptides transduce part of the starvation signal in gustatory plasticity. Alternatively, this defect in starvation enhanced gustatory plasticity could also indicate an important role for neuropeptides in avoidance of low salt concentrations.

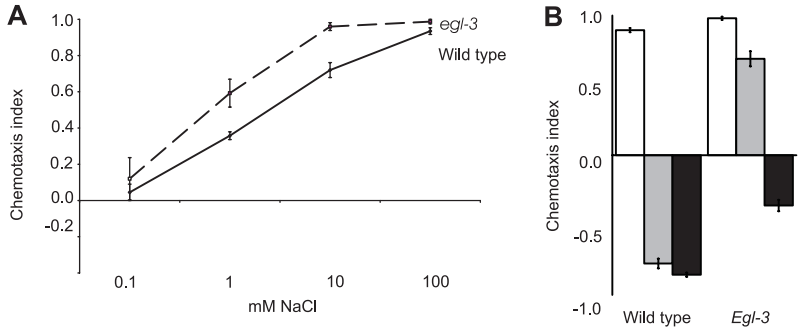

Figure 5: ***egl-3* is needed for gustatory plasticity and normal chemotaxis**. (A) Chemotaxis to 0.1-100 mM NaCl was enhanced in *egl-3(n150)* mutant animals when compared to wild type animals ($p<0.01$). (B) *egl-3(n150)* mutant animals were attracted to NaCl after pre-exposure, but showed avoidance after starvation. Indicated are the averages of at least 4 assays ± s.e.m. Open bars indicate chemotaxis to 25 mM NaCl; grey bars indicate the response to 25 mM NaCl after pre-exposure to 100 mM NaCl; black bars indicates the response to 25 mM NaCl after pre-exposure to 100 mM NaCl after starvation.

Glutamate is involved in chemotaxis to NaCl

Next, we tested whether glutamate is involved in chemotaxis or gustatory plasticity, since glutamate is involved in various behavioural responses in several species. We tested several mutant strains with defects in glutamatergic signalling. First, we tested the role of *eat-4*, which encodes a putative transporter associated with synaptic vesicles and the regulation of transmitter release (Lee *et al.*, 1999). Mutations in the *eat-4* gene, which is expressed in at least 38 neurons, are known to affect behaviours mediated by glutamatergic signalling and seem not to affect behaviours mediated by other neurotransmitters (Lee *et al.*, 1999). We found that mutation of *eat-4* strongly affected chemo-attraction to NaCl (Figure 6A). This suggests that the neurotransmitter glutamate is involved in the response of *C. elegans* to NaCl. In addition, *eat-4* mutant animals did not show avoidance after pre-exposure, although they were capable of avoiding 1 M NaCl (results not

shown). This does not necessarily mean that *eat-4* is involved in gustatory plasticity, since these animals have a defect in chemotaxis to NaCl.

To confirm the role of glutamate in the response of *C. elegans* to NaCl, we tested mutants for the glutamate-gated chloride channels AVR-14 and AVR-15 (Dent *et al.*, 1997; 2000). *avr-14* mutant animals showed a defect in chemotaxis to NaCl, although not as severe as *eat-4* mutant animals; *avr-14* mutant animals still showed a small, but significantly reduced response to 10-100 mM NaCl (Figure 6B). These results are comparable to those found earlier for *tax-2, tax-4, tax-6,* and *ncs-1* mutant animals (Hukema *et al.*, 2006). In contrast, *avr-15* mutant animals showed wild type or even stronger chemotaxis to NaCl (Figure 6B). Mutations in both genes also affect avoidance of 1 M NaCl; *avr-15* mutant animals showed reduced avoidance, whereas *avr-14* mutant animals showed stronger avoidance than wild type animals (results not shown). In addition, mutations in both genes seem to affect gustatory plasticity, since both *avr-14* and *avr-15* mutant animals showed attraction to NaCl after pre-exposure, instead of avoidance (Figure 6C). For *avr-14* mutant animals these results are not conclusive, since these animals also showed defects in chemotaxis to NaCl. On the other hand, the defect in gustatory plasticity of the *avr-15* mutant animals indicates that glutamate is also involved in gustatory plasticity.

Since the AVR-14 and AVR-15 channel subunits seemed to have opposite functions in the response of *C. elegans* to NaCl, we analysed *avr-14; avr-15* double mutant animals for their responses to NaCl. Surprisingly, *avr-14; avr-15* double mutant animals showed wild type attraction to 0.1-100 mM NaCl and wild type gustatory plasticity (Figure 6B, C). The only response of the double mutant animals different from wild type was avoidance of 1 M NaCl, which was stronger than in wild type animals (results not shown). Taken together, the two subunits AVR-14 and AVR-15 seem to have antagonistic effects on salt perception in *C. elegans*. Unfortunately, the expression patterns of the two genes have not been extensively studied.

Glutamate is involved in gustatory plasticity

To further analyse the role of glutamate in gustatory plasticity, we also tested several mutants for ionotropic glutamate receptors. Ten genes that encode putative glutamate receptor subunits have been identified in *C. elegans*: eight non-NMDA (*glr-1-8*) and two NMDA (*nmr-1* and *nmr-2*) class receptors (Maricq *et al.*, 1995; Hart *et al.*, 1995; Brockie *et al.*, 2001). Mutation of *glr-1, glr-2,* or *nmr-1* did not affect chemo-attraction to NaCl (Figure 6D). However, we did find reduced

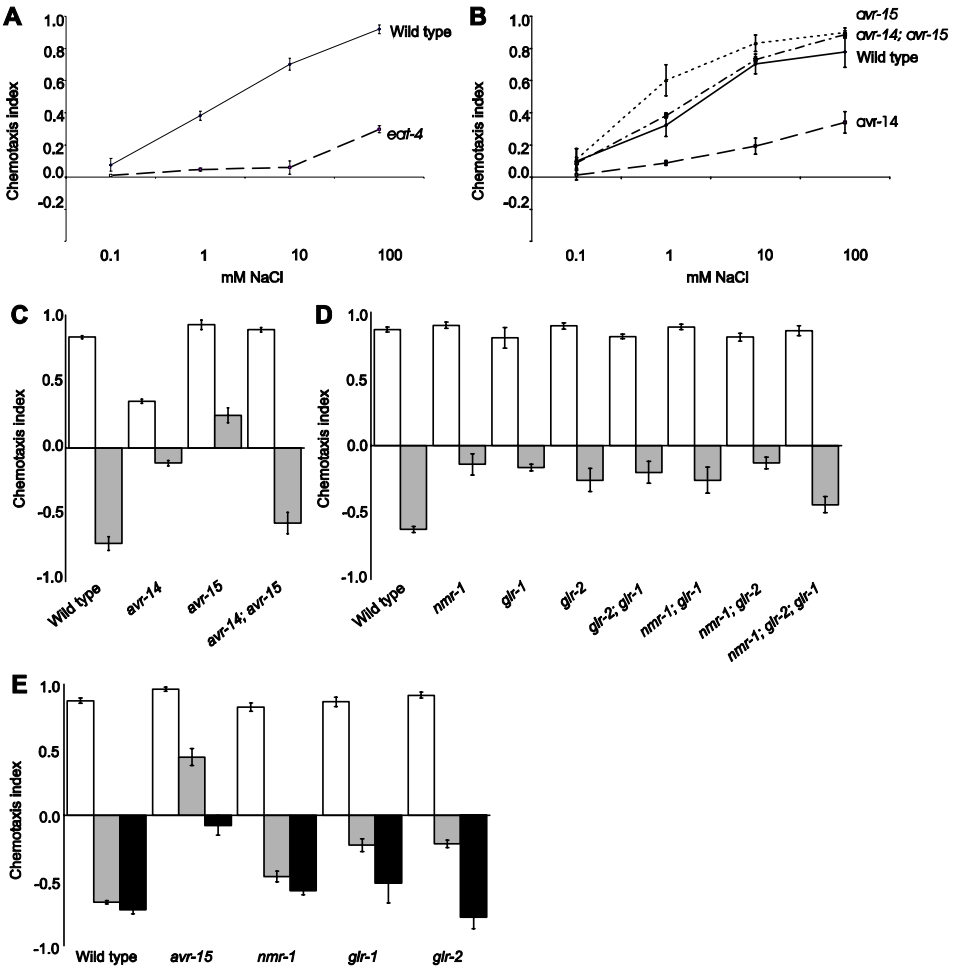

Figure 6: **The role of glutamate in NaCl perception**. (A) *eat-4 (ky5)* mutants showed a strong defect in chemotaxis to 1-100 mM NaCl ($p<0.001$). (B) *avr-14* mutants showed a decreased attraction to 1-100 mM NaCl ($p<0.05$), while *avr-15* mutantsshowed enhanced attraction to 10-100 mM NaCl ($p<0.001$) when compared to wild types. However, *avr-14; avr-15* double mutants showed wild type responses to 0.1-100 mM NaCl ($p>0.05$). (C) *avr-14* mutants showed a defect in attraction to 25 mM NaCl ($p<0.0001$); avr-15 mutants showed wild stronger attraction to 25 mM NaCl when compared to wild type ($p<0.05$). Both *avr-14* and *avr-15* showed defects in gustatory plasticity ($p<0.0001$), but *avr-14; avr-15* double mutants showed wild type avoidance after pre-exposure ($p>0.05$). (D) *glr-1, glr-2, and nmr-1* glutamate receptor mutants showed defects in gustatory plasticity ($p<0.001$). *glr-1; glr-2, glr-1; nmr-1, glr-2; nmr-1* double mutants also showed defects in plasticity when compared to wild types ($p<0.001$), but these defects were not significantly different from the single mutants ($p>0.05$). The defect of *glr-1; glr-2; nmr-1* triple mutants was not significantly different from the *glr-2* mutants ($p>0.05$). Attraction to 25 mM NaCl was not affected in these mutants ($p>0.05$). (E) Glutamate receptor mutants – *avr-15, glr-1, glr-2, nmr-1* – showed stronger avoidance after pre-exposure after starvation ($p<0.$ when compared to non-starved animals). Indicated are the averages of at least 4 assays ± s.e.m. Open bars indicate chemotaxis to 25 mM NaCl; grey bars indicate the response to 25 mM NaCl after pre-exposure to 100 mM NaCl; black bars indicates the response to 25 mM NaCl after pre-exposure to 100 mM NaCl after starvation.

gustatory plasticity in the NMDA type glutamate receptor mutant *nmr-1* and in the non-NMDA type glutamate receptor mutants *glr-1* and *glr-2* (Figure 6D). All three mutant strains show the same response to NaCl after pre-exposure. Next, we tested *glr-2; glr-1, nmr-1; grl-1* and *nmr-1; glr-2* double mutant animals and *nmr-1; glr-1, glr-2* triple mutant animals to see whether possible stronger defects were masked by functional redundancy. However, we did not find increased defects in *glr-1; glr-2, nmr-1; glr-1,* or *nmr-1; glr-2* double mutant animals (Figure 6D). Also the defect of *nmr-1; glr-2; glr-1* triple mutant animals was not significantly different from that of the *glr-2* single mutant animals (Figure 6D). Thus, it seems likely that these glutamate receptor subunits are part of the same genetic pathway involved in gustatory plasticity.

Mutation of *avr-15* reduces starvation enhanced gustatory plasticity

We subsequently asked if glutamate mediates the signal that is required for enhanced gustatory plasticity after starvation. *glr-1, glr-2,* and *nmr-1* mutant animals showed wild type plasticity responses when they were starved (Figure 6E). However, *avr-15* mutant animals showed enhanced avoidance after pre-exposure when compared to non-starved *avr-15* mutant animals, but this avoidance did not reach wild type levels (Figure 6E). These results suggest that either glutamate signalling via AVR-15 mediates the starvation signal, or that *avr-15* and glutamate play a very important role in avoidance of 25 mM NaCl.

Acetylcholine and GABA

Finally, we tested if cholinergic and GABA-ergic neurotransmission is involved in gustatory plasticity or other responses to NaCl in *C. elegans*. However, we could not test the different mutant strains for cholinergic or GABA-ergic neurotransmission, since these mutant animals have uncoordinated phenotypes, as a result of which they did not perform well in our behavioural assays. Therefore we cannot confirm or rule out a role for these neurotransmitters in gustatory plasticity.

Discussion

C. elegans can show two responses to low salt concentrations: naïve animals are attracted to 0.1-200 mM NaCl, and animals pre-exposed to 100 mM NaCl avoid low salt concentrations (gustatory plasticity). Here we show that this

latter process requires the absence of food, suggesting that *C. elegans* can associate low salt concentrations with this cue. We also show that prolonged starvation can enhance avoidance after pre-exposure. Previously it was reported that plasticity of chemotaxis requires both the presence of NaCl and the absence of food (zaeki *et al.*, 2001). In addition we identify several neurotransmitters involved in chemotaxis to NaCl, gustatory plasticity, as well as starvation enhanced gustatory plasticity.

Neurotransmission in chemotaxis to NaCl involves glutamate

In this study we identify several mutants that showed defects in chemotaxis to NaCl. First, we find that glutamate levels play a critical role in the response of *C. elegans* to NaCl. In *eat-4* animals, which probably have strongly reduced glutamate neurotransmission, chemotaxis to NaCl was strongly reduced. However, the phenotypes of *avr-14* and *avr-15* glutamate gated Cl channel subunit mutants suggest that glutamate can both stimulate and inhibit chemotaxis to NaCl.

Second, chemotaxis to NaCl is modulated by neuropeptides since changes in neuropeptide levels in *egl-3* mutants, which lack the proprotein convertase required to generate neuropeptides (Kass *et al.*, 2001) resulted in stronger attraction to NaCl than in wild type animals.

Third, chemotaxis to NaCl is probably modulated by dopamine. *bas-1* and *cat-4* mutant animals, which have reduced serotonin and dopamine levels both show stronger attraction to NaCl than wild type animals. We propose that this is due to diminished dopamine signalling, since excessive dopamine signalling, in *dat-1* mutant animals, reduces chemotaxis to NaCl.

Neurotransmission in gustatory plasticity

In this study, we show that gustatory plasticity requires neurotransmission using serotonin, dopamine, octopamine, and glutamate. Serotonin and dopamine are often suggested to have antagonistic functions. However, we found that lack of either of these neurotransmitters reduces gustatory plasticity. We have shown that the gustatory plasticity defect of serotonin and/or dopamine synthesis mutants can be rescued by exogenously applying these compounds. Both serotonin and dopamine are acutely needed in the process of gustatory plasticity; serotonin also has a role in development.

Serotonin is produced by two cells and dopamine is found in eight cells (Sulston *et al.*, 1975; Sze *et al.*, 2000). It is not exactly clear which of these neurons play a role in gustatory plasticity. However, we have previously shown that the ADF neurons, which produce serotonin, are involved in gustatory plasiticty

(Hukema *et al.*, 2006). We expect that serotonin released from the ADF neurons is required for gustatory plasticity.

A next step to unravel the mechanisms of neurotransmission in gustatory plasticity is to identify the receptors. This would allow the identification of the cells involved in the process. Both dopamine and serotonin can modulate neurons that are not necessarily located postsynaptically to the neurons that produce the compounds and thus they can function as systemic neuromodulators. The expression patterns of the receptors that transduce their signals might therefore tell us more about the specific site of action. Unfortunately, the expression patterns of the serotonin receptors we found to play a role in gustatory plasticity have not been described precisely. The dopamine receptors *dop-1, dop-2*, and *dop-3* are expressed in a subset of interneurons (Chase *et al.*, 2004). Our results show that *dop-3* functions in the GABAergic neurons as well as the DA and DB motor neurons.

Although we could not test whether GABA and acetylcholine are involved in gustatory plasticity, our results suggest that both GABAergic neurons and the cholinergic DA and DB motor neurons are involved in gustatory plasticity. Taken together, we propose a model in which, upon prolonged exposure in the absence of food, serotonin is produced by the ADF neurons and dopamine by unidentified neurons. These signals play a role in the integration of food and salt signals, leading to avoidance of low salt concentrations. It is not known on which cells serotonin acts. Our results suggest that dopamine acts more downstream in the cellular circuitry on GABAergic command interneurons and the DA and DB motor neurons, ultimately leading to gustatory plasticity. However, other signals can bypass the requirement of serotonin and dopamine resulting in strong avoidance of low salt concentrations after starvation.

Changes in glutamate levels also affect gustatory plasticity. The glutamate signal is transduced via both NMDA (NMR-1) and non-NMDA like (GLR-1 and GLR-2) ionotropic glutamate receptors. Our results suggest that these receptors function in the same genetic pathway, since double and triple mutant animals for these receptors show the same defects as the single mutant animals. It could be that they even function in the same complex, since they are expressed in the same cells: the AVA, AVD, AVE, AVG, and PVC neurons.

The glutamate gated Cl^- channel subunits AVR-14 and AVR-15 are involved in gustatory plasticity, in which they seem to have opposite functions. The results for the *avr-14* mutant animals are not conclusive, since they also show a defect in chemotaxis to NaCl. Gustatory plasticity is comparable to wild type

animals in *avr-14; avr-15* double mutant animals and defective in both single mutant animals. It seems likely that the opposite effects of both channel subunits depend on their expression patterns. Unfortunately, the expression patterns of these genes have not been deduced to the exact cells.

Changes in neuropeptide levels also disrupt gustatory plasticity, since *egl-3* mutant animals do not show avoidance after pre-exposure. Neuropeptides can be co-released with classical neurotransmitters and thereby influence their activity. It seems possible that neuropeptides affect glutamate activity in gustatory plasticity as has been proposed for touch and osmotic stimuli (Mellem *et al.*, 2002).

Enhanced gustatory plasticity after starvation

Starvation for four hours before the assays strongly enhanced avoidance after pre-exposure. This process of starvation enhanced gustatory plasticity does not require serotonin, dopamine, or octopamine. Mutant animals with decreased signalling using these neurotransmitters all showed wild type levels of starvation enhanced gustatory plasticity, despite their defects in gustatory plasticity. This suggests that in these animals the mechanism that mediates avoidance of low salt concentrations is still intact, and that serotonin, dopamine, and octopamine play a role in the mechanism whereby *C. elegans* associates low food with salt. We propose that prolonged starvation bypasses or sensitises the mechanism that associates the presence of salt with absence of food, resulting in strong avoidance even in the absence of important neurotransmitters such as serotonin, dopamine and octopamine.

We found three mutants that affect starvation enhanced gustatory plasticity. First, starvation did not enhance gustatory plasticity in *dat-1* mutant animals. In *dat-1* mutant animals dopamine signalling is prolonged because dopamine is not properly cleared from the synaptic cleft (Jayanthi *et al.*, 1998). A possible explanation for the defect in starvation enhanced gustatory plasticity is that the excess dopamine blocks the starvation signal. Alternatively, the excess dopamine could result in less responsiveness of the animals in general, which could also explain the chemotaxis defect of *dat-1* mutants.

Second, mutation of the glutamate gated Cl channel subunits *avr-15* affected starvation enhanced gustatory plasticity. However, since mutation of *avr-15* also affects gustatory plasticity, it is not clear if AVR-15 mediates the starvation signal or functions in the process that mediates avoidance of low salt concentrations per se.

Third, *egl-3* mutant animals showed the same response as *avr-15* animals: no avoidance after pre-exposure in well-fed animals and reduced starvation enhanced gustatory plasticity. It is therefore unclear where neuropeptides function in this process.

Materials and Methods

Strains used in this work are *avr-14(ad1302)*, *mod-5(n822)*, *tdc-1(n3247)*, *tdc-1(ok1197)*, *cat-2(e1112)*, *tph-1(mg280)*, *nmr-1(ky4)*, *adp-1(ky20)* *glr-1(n2461)*, *glr-2(tm669)*, *eat-4(ad806)(n2474)(ky5)*, *bas-1(ad446)*, *dat-1(tm903)*, *ser-4(ok512)*, *avr-15(ad1051)*, *egl-3(n150)(n588)*, *cat-4(e1141)*, *dop-2(vs105)(ok1038)*, *mod-1(ok103)*, *cat-1(e1111)*, *dop-1(vs100)(ok398)*, *dop-3(vs106)(ok295)*, *ser-1(ok345)*, *tbh-1(n3247)*, *tbh-1(ok914)*, *ser-2(pk1357)*, and *gpc-1(pk298)*. Wild type *C. elegans* used were the strain Bristol N2. Germline transformation was performed as described (Mello *et al.*, 1991). We used an *elt-2::GFP* construct as co-injection marker (Fukushige *et al.*, 1999). Promoters used for rescue of *dop-3* mutant animals were: *acr-2* (DA and DB motor neurons; Hallam *et al.*, 2000), *unc-17* (cholinergic neurons; Alfonso *et al.*, 1993), and *unc-47* (GABAergic neurons; McIntire *et al.*, 1997). Plasmids were as described before (Chase *et al.*, 2004).

Behavioural assays

Chemotaxis towards NaCl was assessed as described before (Wicks *et al.*, 2000; Jansen *et al.*, 2002). To test if starvation affected gustatory plasticity animals were starved on CTX-plates for four hours before the assay. 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 s.e.m.

Supplementation studies

Rescue of serotonin and dopamine deficiencies were performed by culturing animals on NGM-plates containing either 2 mM of dopamine hydrochloride or 2 mM of serotonin creatinine sulfate complex dissolved in 0.1 N HCl. Animals were cultured on the plates for 4 or 72 hours before the assays were performed. In case of serotonin supplementation animals were also cultured for 96 hours before the assay. In the latter case also the parents of the animals tested were cultured on plates containing serotonin. The concentration of 2 mM for both

substances did not influence behaviour of wild type animals. Higher concentrations affected locomotion, resulting is poor performance in the behavioural assays, whereas lower concentrations were less effective.

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Chapter 5

Candidate gene approach identifies genes involved in the responses of *Caenorhabditis elegans* to NaCl

Renate K Hukema, Gert Jansen

5

Introduction

Salt taste is very important for the detection of food and it is essential for water homeostasis. In general, animals are attracted to low concentrations of salt and they avoid high concentrations, probably due to osmotic avoidance. These responses are plastic and can change depending on past experiences (reviewed in Scott *et al.*, 2005). In mammals, salt is detected using two different receptors: amiloride sensitive epithelial Na^+ channels (ENaCs) (Heck *et al.*, 1984; Canessa *et al.*, 1994), and transient receptor potential (TRP) channel subunits (Lyall *et al.*, 2004).

We use the nematode *C. elegans* to study the different salt taste responses. Several assays exist to analyse chemotaxis behaviour of *C. elegans* (Ward *et al.*, 1973; Wicks *et al.*, 2000; Miller *et al.*, 2005). We use the quadrant assays (Wicks *et al.*, 2000), in which animals are given a choice between quadrants filled with buffered agar containing a concentration of a certain compound and quadrants filled with only buffered agar. Using these assays we can discriminate three responses to NaCl (Jansen *et al.*, 2002; Hukema *et al.*, 2006). First, *C. elegans* is attracted to low salt concentrations up to 200 mM; second, they avoid NaCl concentrations higher than 200 mM. Third, *C. elegans* shows gustatory plasticity: avoidance of normally attractive NaCl concentrations after prolonged pre-exposure to attractive salt concentrations.

Chemotaxis to NaCl involves four different amphid sensory neurons. The ASE neurons are the main salt sensing cells, but the ADF, ASG, and ASI neurons have a redundant role in chemotaxis to NaCl (Bargmann & Horvitz, 1991). In *C. elegans* no receptor has been identified, but several downstream molecules have been identified, including the cGMP gated channel subunits TAX-2, and TAX-4, the calcineurin A subunit TAX-6, and the guanylate cyclase DAF-11 (Coburn & Bargmann, 1996; Komatsu *et al.*, 1996; Birnby *et al.*, 2000; Kuhara *et al.*, 2002).

Osmotic avoidance via the polymodal nociceptive ASH neurons (Bargmann & Horvitz, 1991) requires the Gα protein ODR-3, the TRP channel subunits OSM-9 and OCR-2, the cytoplasmic protein OSM-10, the G protein coupled receptor kinase GRK-2, and glutamatergic neurotransmission using glutamate receptors GLR-1 and NMR-1, glutamate transporter EAT-4, and proprotein convertase EGL-3 (Colbert *et al.*, 1997; Berger *et al.*, 1998; Roayaie *et al.*, 1998; Hart *et al.*, 1999; Mellem *et al.*, 2002; Tobin *et al.*, 2002; Fukuto *et al.*, 2004).

Gustatory plasticity requires the Gγ protein GPC-1, the TRP channel subunit OSM-9 and the unknown protein ADP-1 (Jansen *et al.*, 2002). GPC-1 is not

expressed in the ASE neurons, suggesting other neurons are involved in gustatory plasticity (Jansen *et al.*, 2002).

We used a candidate gene approach to identify genes involved in the three responses of *C. elegans* to NaCl. We focussed our efforts on the identification of genes involved in gustatory plasticity. We tested many mutants, which we selected based on our knowledge of salt attraction and avoidance behaviours, and behavioural plasticity in both *C. elegans* and mammals. Taken together, this lead us to test genes involved in G protein signalling (Table 1-3), cGMP signalling (Table 4), Ca^{2+} signalling (Table 5), PUFA synthesis (Table 6), IP_3 signalling (Table 7), MAP kinase signalling (Table 8), and some other genes involved in general signal transduction (Table 9). To find out which cells function in gustatory plasticity we tested mutant animals that specifically lack the function of certain cells (Table 10) and to unravel which neurotransmitters are involved we tested mutants in general neurotransmission (Table 11), GABAergic and cholinergic neurotransmission (Table 11), glutamatergic neurotransmission (Table 12), dopaminergic and serotonergic neurotransmission (Table 13), and finally octopaminergic neurotransmission (Table 14).

In total we tested mutant animals for 123 genes. Seven genes we could not test in our behavioural assays, because they did not perform due to their uncoordinated phenotypes (marked ND in Table1-14). We found 22 genes involved in attraction to NaCl, 57 genes involved in avoidance, and 87 genes involved in gustatory plasticity. Most of the mutants that have a defect in attraction to NaCl are also defective in gustatory plasticity. However, these results are not conclusive since we cannot rule out that a defect in chemotaxis could be the cause of the plasticity defect. Even though there are also mutants that have a defect in chemotaxis, but not in plasticity.

The most interesting results were studied further and are discussed in Chapter 2-4. The other results are summarised in Table 1-14. Further experiments will have to elucidate the precise role of these genes in the responses of *C. elegans* to NaCl.

Table 1: **G protein subunits expressed in amphid sensory neurons**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
gpa-1 (pk15) V	Gα subunit	ADL ASH ASI ASJ PHA PHB	Jansen *et al.*, 1999	WT	<<	>>>
gpa-2 (pk16) V	Gα subunit	AIA AWC I5 IL1L IL2L OLL/URB M1 M5 PHA PHB PVT	Zwaal *et al.*, 1997	WT	WT	WT
gpa-3 (pk35) V	Gα subunit	ADF ADL AIZ ASE ASG ASH ASI ASJ ASK AWA AWC PHA PHB	Zwaal *et al.*, 1997	WT	WT	>>>
gpa-4 (pk381) IV	Gα subunit	ASI	Jansen *et al.*, 1999	WT	WT	>>>
gpa-10 (pk362) V	Gα subunit	ADF ASI ASJ	Jansen *et al.*, 1999	WT	WT	>>>
gpa-11 (pk349) II	Gα subunit	ADL ASH	Jansen *et al.*, 1999	WT	WT	WT
gpa-13 (pk1270) V	Gα subunit	ADF ASH AWC PHA PHB	Jansen *et al.*, 1999	WT	WT	<<<
gpa-14 (pk347) I	Gα subunit	ADE ALA ASH ASI ASJ ASK AVA CAN DVA PHA PHB PVQ RIA	Jansen *et al.*, 1999	WT	WT	WT
gpa-15 (pk477) I	Gα subunit	ADL ASH ASK PHA PHB	Jansen *et al.*, 1999	WT	WT	>
odr-3 (n1605) V	Gα subunit	ADF ASH AWA AWB AWC	Roayaie *et al.*, 1998	WT	<<<	<<<
gpc-1 (pk298) x	Gγ subunit	ADL ASH AJ AFD ASI PHB	Jansen *et al.*, 2002	WT	<<<	>>>

Table 2: **Genes from the $G_o\alpha/G_q\alpha$ network**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
goa-1 (n1134) (n363) I	$G_o\alpha$ subunit	all neurons	Mendel *et al.*, 1995; Segalat *et al.*, 1995	WT	<<<	WT
egl-30 (n686) (ad806) I	$G_q\alpha$ subunit	all neurons	Brundage *et al.*, 1995; Lackner *et al.*, 1999	ND	>>	>>>
gpb-2 (sa603) (pk751) I	Gβ subunit	all neurons	Chase *et al.*, 2001; van der Linden *et al.*, 2001; Robatzek *et al.*, 2001	WT	<<<	WT
eat-16 (sa609) I	RGS protein	all neurons	Hadju-Cronin *et al.*, 1999; Robatzek *et al.*, 2000	WT	<<<	WT
egl-10 (n692) (md176) V	RGS protein	all neurons	Koelle *et al.*, 1996	WT	>	>>>
egl-8 (ok934) (n488) V	phospholipase Cβ	all neurons	Lackner *et al.*, 1999; Miller *et al.*, 1999	ND	<<<	>>>
dgk-1 (nu62) (sy428) x	diacylglycerol kinase	all neurons	Hadju-Cronin *et al.*, 1999 ; Nurrish *et al.*, 1999	WT	<<<	WT

Table 3: **RGS proteins and other modulators of G protein activity**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
rgs-1 (nr2017) III	RGS protein	all neurons	Dong *et al.*, 2000	WT	WT	>>>
rgs-2 (vs17) x	RGS protein	ventral cord; head and tail neurons	Dong *et al.*, 2000	WT	<<	>>>
rgs-3 (vs19) II	RGS protein	ADL ASH ASJ ASK AWB AWC PHA PHB	Dong *et al.*, 2000	WT	<	?
rgs-4 (vs93) II	RGS protein		Dong *et al.*, 2000	WT	<<	?
rgs-6 (vs62) x	RGS protein	all neurons	Dong *et al.*, 2000	WT	WT	?
rgs-8 (vs64) x	RGS protein		Dong *et al.*, 2000	ND	ND	ND
rgs-9 (vs94) x	RGS protein		Dong *et al.*, 2000	WT	<<<	?
rgs-10/11 (vs109) x	RGS protein	all neurons	Dong *et al.*, 2000	WT	<<<	?
grk-1 (ok1239) I	G protein coupled receptor kinase		Fukuto *et al.*, 2004	WT	<<	WT
grk-2 (rt97) III	G protein coupled receptor kinase		Fukuto *et al.*, 2004	<<<	>>>	>>>
arr-1 (ok401) x	arrestin		Palmitessa *et al.*, 2005	WT	<<<	>>>

Table 4: **Genes involved in cGMP signalling**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
daf-11 (m47) V	guanylate cyclase	ASI ASJ ASK AWB AWC	Birnby *et al.*, 2000	WT	WT	>>>
odr-1 (n1936) x	guanylate cyclase	ASI ASJ ASK AWB AWC	L'Etoile & Bargmann, 2000	WT	WT	>>>
gcy-5 (ok921) (ok930) II	guanylate cyclase	ASER	Yu *et al.*, 1997	WT	<<<	WT
gcy-7 (tm901) V	guanylate cyclase	ASEL	Yu *et al.*, 1997	WT	<<<	WT
gcy-31 (ok296) x	guanylate cyclase	BAG	Yu *et al.*, 1997	>>	<<	WT
gcy-32 (ok995) V	guanylate cyclase	AQR PQR URX	Yu *et al.*, 1997	WT	WT	WT
gcy-33 (ok232) V	guanylate cyclase	BAG	Yu *et al.*, 1997	WT	<<<	>>>
gcy-35 (ok769) I	guanylate cyclase	ALN AQR AVM BDU PLM PLN PQR SDQ URX	Yu *et al.*, 1997 ; Cheung *et al.*, 2004	WT	<<<	>>>
gcy-36 (db42) (db66) x	guanylate cyclase	AQR PQR URX	Yu *et al.*, 1997	WT	<<	WT
gcy-37 (ok384) IV	guanylate cyclase	AQR PQR URX	Yu *et al.*, 1997	>	<<	WT
tax-2 (p671) (p694) (ks31) I	CNG-channel β subunit	AFD ASE ASG ASJ ASI ASK AQR AWB AWC BAG PQR	Coburn & Bargmann, 1996	<<<	<<<	>>>
tax-4 (p678)(ks11) (ks28) III	CNG-channel α subuni	ASE ASG ASI ASJ ASK BAG AWC URX	Komatsu *et al.*, 1996	<<<	<<<	>>>
cng-3 (jh113) IV	CNG-channel α subuni	AFD ASI ASE AWB AWC	Cho *et al.*, 2004	WT	WT	>>
egl-4 (ky95) (n479) (ok1105) IV	cGMP-dependent protein kinase	ASE ASH ASK AVJ AWC DVB DVC PQR RIB RMDD RMDV SMDD SMDV	Stansberry *et al.*, 2001; Hirose *et al.*, 2003	WT	<<<	>>>
eat-7 (ad450) IV	gain-of-function allele of *eat-4*		Raizen, personal communication	WT	<<<	WT
pdl-1 (gk157) II	phosphodiesterase		Smith & McKay, personal communication; McKay *et al.*, 2003	WT	<<	>>>

Table 5: **Genes involved in Ca^{2+} signalling**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
ocr-1 (ak46) (ok132) V	TRPV channel subunit	ADL AWA	Tobin *et al.*, 2002	WT	<<<	<<
ocr-2 (ak47) IV	TRPV channel subunit	ADF ADL ASH AWA PHA PHB	Tobin *et al.*, 2002	WT	<<<	<<<
osm-9 (ky10) IV	TRPV channel subunit	ADF ADL ASE ASG ASH ASI ASJ ASK AWA AWC OLQ PHA PHB	Colbert *et al.*, 1997	WT	<<	<<<
trp-1 (ok323) III	TRPC channel subunit	BAG, motor neurons, interneurons	Colbert *et al.*, 1997	WT	<<	>>>
itr-1 (sa73) IV	IP_3 receptor	LUA PDA nerve ring, ventral nerve cord	Bayliss *et al.*, 1999; Dal Santo *et al.*, 1999; Gower *et al.*, 2001	WT	<<<	>>>
tax-6 (p675) (jh107) IV	calcineurin A subunit	ADF ADL AFD AIM ASE ASH ASI ASK AUA AVA AVE AVK AWA AWC PHA PHB RMDV	Kuhara *et al.*, 2002	<<<	<<<	>>>
cnb-1 (jh103) V	calcineurin B subunit	ADF ADL AFD AIM ASE ASH ASI ASK AUA AVA AVE AVK AWA AWC PHA PHB RMDV	Bandhyopadhyay *et al.*, 2002	<	<<	>>>
ncs-1 (qa406) x	neuronal calcium sensor	ADF AFD AIY ASE ASG AVK AWA AWB AWC BAG PHB RMG	Gomez *et al.*, 2001	<<	<<	>>>

Table 6: **Genes involved in the synthesis of polyunsaturated fatty acids**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
elo-1 (gk48) (wa7) IV	PUFA elongase		Watts & Browse, 2002	WT	<<<	WT
fat-1 (wa9) IV	ω-3 fatty acyl desaturase		Watts & Browse, 2002	WT	<<<	WT
fat-3 (wa22) IV	δ-6 fatty acid desaturase	several head and tail neurons	Watts & Browse, 2002; Watts *et al.*, 2003	WT	<<<	WT
fat-4 (ok958) (wa14) IV	δ-5 fatty acid desaturase		Watts & Browse, 2002	WT	<<<	>>>
dgk-1 (nu62) (sy428) x	diacylglycerol kinase	all neurons	Hadju-Cronin *et al.*, 1999 ; Nurrish *et al.*, 1999	WT	<<<	WT
dgk-2 (gk142) x	diacylglycerol kinase		Espelt *et al.*, 2005	WT	WT	WT
dgk-3 (gk110) III	diacylglycerol kinase	4 nuclei of nerve ring; 8 nuclei of head ganglia	Lynch *et al.*, 1995; Espelt *et al.*, 2005	WT	WT	WT
egl-8 (ok934) (n488) V	phospholipase Cβ	all neurons	Lackner *et al.*, 1999; Miller *et al.*, 1999	unc	<<<	>>>

Table 7: **Genes involved in IP_3 signalling**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
itr-1 (sa73) IV	IP_3 receptor	LUA PDA nerve ring, ventral nerve cord	Bayliss *et al.*, 1999; Dal Santo *et al.*, 1999; Gower *et al.*, 2001	WT	<<<	>>>
age-1 (hx546) II	PI_3-kinase		Wolkow *et al.*, 2002; McKay *et al.*, 2003	WT	<	?
aap-1 (ok282) I	PI_3-kinase adaptor subunit	neurons	Wolkow *et al.*, 2002	WT	<	>>
daf-2 (e1370) III			McKay *et al.*, 2003	WT	<	?
ppk-2 (pk1343) III	PIP kinase		Weinkove, personal communicaiton; McKay *et al.*, 2003	WT	WT	?
pdk-1 (mg142) x	IP_3 dependent kinase	majority of neurons in head and tail	Paradis *et al.*, 1999	WT	WT	>

Table 8: **MAP kinases**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
jnk-1 (gk7) IV	Jun N-terminal Kinase	ALM AVM BDU CAN HSN PDE PVD SDQ	Kawasaki *et al.*, 1999; Villanueva *et al.*, 2001	WT	<<	?
jkk-1 (km2) x	jnk kinase	ALM AVM BDU CAN HSN PDE PVD SDQ	Kawasaki *et al.*, 1999; Villanueva *et al.*, 2001	WT	<<<	?
pmk-3 (ok169) IV	MAP kinase (p38)		Berman *et al.*, 2003	WT	WT	?
nsy-1 (ag3) (ok593) II	MAPKKK	several neurons	Sagasti *et al.*, 2001	<<<	WT	?
sek-1 (ag1) (km4) x	MAPKK	several neurons	Tanaka-Hino *et al.*, 2002	<<<	WT	?

Table 9: **Genes involved in general signal transduction and behavioural plasticity**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
che-6 (e1126) IV			Lewis & Hodgkin, 1977	WT	WT	WT
crh-1 (tz2) IV	CREB		Kimura *et al.*, 2002	WT	<<	>>>
cmk-1 (ok287) IV	CaMK I		Eto *et al.*, 1999	WT	WT	>>>
egl-2 (n693) (n2656) V	voltage-gated K^+ channel	AFD ALN AQR ASE AWC BAG IL2 PLN PQR URX	Weinschenker *et al.*, 1995	WT	<<<	?
hab-1 (cn308) I			Xu *et al.*, 2002	<	<<<	?
hen-1 (tm501) x	LDL receptor like protein	AIY ASE	Ishihara *et al.*, 2002	WT	<<<	>>>
kin-13 (ok563) V	protein kinase C	ALM BDU PDE PHA PHB PLM PVD PVM SDQ	Land *et al.*, 1994	WT	<<	
lin-17 (n3091) (e1456) I	frizzled		Sawa *et al.*, 1996	WT	<<	>>
lin-18 (e620) x	receptor tyrosine kinase		Inoue *et al.*¸2004	<<	<<<	>>>
osr-1 (rm1) (ok959) I			Solomon *et al.*, 2004	WT	WT	WT
pkc-2 (ok328) x	protein kinase C		Islas-Trejo *et al.*, 1997	WT	WT	>>>
unc-43 (e408) (e382) III	CaMK II	dorsal nerve cord, ventral nerve cord, nerve ring	Reiner *et al.*, 1999	ND	ND	ND

Table 10: **Genes required for the function of certain cells**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
che-1 (p679) I	transcription factor	ASE	Uchida *et al.*, 2003	<<<	<<<	>>>
ceh-36 (ks86) x	transcription factor	ASE AWC	Lanjuin *et al.*, 2003	<<<	<<<	>>>
cog-1 (sy275) II	transcription factor	ADL ASE ASJ	Palmer *et al.*, 2002	WT	<	?
fkh-2 (ok683) x	transcription factor	ADF AWB	Molin *et al.*, 2000	WT	<<<	WT
lim-4 (yz12) (ky403)x	transcription factor	AWB RMED RMEV RMDD SAID SIAV SMDV SMDD	Sagasti *et al.*, 1999	<<	<<<	>>
lim-6 (nr2073) x	transcription factor	ASEL ASG AVL AWA DVB RIS RME	Hobert *et al.*, 1999	WT	<<<	WT
osm-6 (p811) V			Collet *et al.*, 1998	ND	ND	ND
osm-10 (n1602) III		ASH ASI PHA PHB	Hart *et al.*, 1999	<<	<	<<<
ttx-3 (ot22) (mg158) x	transcription factor	AIY	Hobert *et al.*, 1997 ; Altun-Gultekin *et al.*, 2001	WT	<<<	>>
unc-42 (e419) V	transcription factor	AIN ASH AVA AVD AVE AVH AVJ AVK RIV RMD SMB SAA SIB	Baran *et al.*, 1999	ND	ND	ND

Table 11: **Genes encoding proteins involved in general neurotransmission, peptidergic neurotransmission, GABAergic neurotransmission, and cholinergic neurotransmission**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
syd-1 (ju82) II	PDZ domain protein	all neurons	Hallam *et al.*, 2002	WT	WT	>>>
sad-1 (ky289) x	protein kinase	all neurons	Crump *et al.*, 2001	WT	<<<	>>>
egl-3 (n150) (n588) (n589) V	proprotein convertase	ALM ASH AVM AVB AVD HSN PVC RIG SDQ	Kass *et al.*, 2001	WT	<<	>>>
flp-21 (ok889) V	FMRFamide-related neuropeptide (FaRP)	ADL ASE ASI ASH FLP MC M4 M2 URA	Rogers *et al.*, 2003; Kim & Li, 2004	WT	<	WT
nlp-1 (ok1469) (ok1470) x	neuropeptide-like protein	ASI AWC BDU PHB	Li *et al.*, 1999; Nathoo *et al.*, 2001	WT	WT	WT
unc-25 (n2379) (e156) III	glutamic acid decarboxylase	AVL DVB DD RME RIS VD	McIntire *et al.*, 1993; Eastman *et al.*, 1999; Jin *et al.*, 1999	ND	ND	ND
unc-49 (e407) (e382) III	$GABA_A$ receptor	head ganglia	Bamber *et al.*, 1999	ND	ND	ND
cha-1 (n2411) (p1151) IV	choline acetyltransferase	IL2 URA URB SAA SAB SIA SIB SMB SMD RMD RIM VA VB VC DA DB AS SDQ HSN ALN PLN	Rand *et al.*, 1989	ND	ND	ND

Table 12: **Genes involved in glutamatergic neurotransmission**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
avr-14 (ad1302) I	glutamate-gated chloride channel	head neurons	Dent *et al.*, 2000	<<	<<<	>>>
avr-15 (ad1051) V	glutamate-gated chloride channel	head neurons	Dent *et al.*, 1997; 2000	WT	<<<	<<<
glr-1 (n2461) III	AMPA receptor	AIB AVA AVB AVD AVE AVJ AVG DVC PVC PVQ RIG RIM RIS RMD RME SMD URY	Maricg *et al.*, 1995; Hart *et al.*, 1995; Zheng *et al.*,1999; Brockie *et al.*,2001a	WT	<<	<<<
glr-2 (tm669) III	AMPA receptor	AIA AIB AVA AVD AVE AVG PVC RIA RIG RIR RMDD RMDV	Brockie *et al.*, 2001a	WT	<	WT
nmr-1 (ak4) II	NMDA receptor	AVA AVD AVE AVG PVC RIM	Brockie *et al.*, 2001b	WT	<<<	>>>
mgl-2 (tm355) I	metobotropic glutamate receptor	AIB AVE RIB RMDV RMDD SMDV SMDD tail neurons	Katsura, personal communication	WT	<<	?
eat-4 (ad819) (n2474) (ky5) III	vesicular glutamate transporter	ADA AIN ALM ASH ASK AUA AVJ AVM FLP IL1 LUA OLL OLQ PVD PLM PVR	Lee *et al.*, 1999	<<<	<<<	WT

Table 13: **Genes involved in dopaminergic and serotonergic neurotransmission**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
bas-1 (ad446) III	aromatic amino acid decarboxylase		Hare & Loer, 2004	WT	<<<	>>>
cat-2 (e1112) II	tyrosine hydroxylase	ADE CEPV CEPD PDE	Lints & Emmons, 1999	WT	<<	WT
cat-4 (e1141) V	GTP cyclohydroxylase I		Sawin *et al.*, 2000	WT	<<<	WT
tph-1 (mg280) II	tryptophan hydroxylase	ADF AIM HSN NSM RIH	Sze *et al.*, 2000	unc	<	?
dop-1 (vs100) (ok398) x	D_2 dopamine receptor	AVM ALM ALN PLN PVQ RIS	Chase *et al.*, 2004	WT	<<<	WT
dop-2 (vs105) (ok1038) V	D_2 dopamine receptor	PDA RIA SIA SIB	Chase *et al.*, 2004	WT	<	WT
dop-3 (vs106) (ok295) x	D_1 dopamine receptor		Chase *et al.*, 2004	WT	<<<	<<
mod-1 (ok103) V	serotonin receptor		Ranganathan *et al.*, 2000	WT	<<	WT
ser-1 (ok345) x	serotonin receptor		Tsalik *et al.*, 2003	WT	<	<
ser-4 (ok512) III	serotonin receptor	DVA DVC PVT RIB RIS	Tsalik *et al.*, 2003	WT	WT	?
mod-5 (n822) I	serotonin reuptake tranporter		Ranganathan *et al.*, 2001	WT	<<	>>>
dat-1 (tm903) III	dopamine reuptake transporter	ADE CEPV CEPD PDE	Jayanthi *et al.*, 1998	<	<<	WT
cat-1 (ok411) (e1111) x	synaptic vesicular monoamine transporter	ADE ADF AIM AIY CAN CEP HSN NSM PDE RIH VC4 VC5	Duerr *et al.*, 1999	WT	<<<	WT

Table 14: **Genes involved in octopaminergic and tyraminergic neurotransmission**

gene	product	neuronal expression pattern	reference	attraction	plasticity	avoidance
tbh-1 (n3247) (ok1196) x	tyramine hydroxylase	RIC	Alkema *et al.*, 2005	WT	<<<	?
tdc-1 (n3419) (ok914) II	tyrosin decarboxylase	RIC RIM	Alkema *et al.*, 2005	WT	<<<	?
ser-2 (pk1357) x	tyramine receptor	AIY AIZ ALN AVH AUA CAN DA9 LUA OLL PVC PVD RIA RIC RID SABC SABD SDQ	Tsalik *et al.*, 2003	WT	<<<	?

Materials and Methods

Strains used in this study are:

LG I: *aap-1(ok282), avr-14(ad1302), che-1(p679), eat-16(sa609), egl-30(n686)(ad806), gcy-35(ok769), goa-1(n1134)(n363), gpa-14(pk347), gpa-15(pk477), gpb-2(sa603)(pk751), grk-1(ok1239), hab-1(cn308), lin-17(n3091)(e1456), mgl-2(tm355), mod-5(n822), osr-1(rm1)(ok959), tax-2(p671)(p694)(ks31), tdc-1(n3419)(ok914)*

LG II: *age-1(hx546), cat-2(e1112), cog-1(sy275), gcy-5(ok921)(ok930), gpa-11(pk349), nmr-1(ak4), nsy-1(ag3)(ok593), pdl-1(gk157), rgs-3(vs19), rgs-4(vs93), syd-1(ju82), tph-1(mg280)*

LG III: *bas-1(ad446), daf-2(e1370), dat-1(tm903), dgk-3(gk110), eat-4(ad819)(n2474)(ky5),glr-1(n2461), glr-2(tm669), grk-2(rt97), osm-10(n1602), ppk-2(pk1343), rgs-1(nr2017), ser-4(ok512), tax-4(p678)(ks11)(ks28), trp-1(ok323), unc-25(n2379)(e156), unc-49(e407)(e382)*

LG IV: *cha-1(n2411)(p1151), che-6(e1126), cmk-1(ok287), cng-3(jh113), crh-1(tz2), eat-7(ad450), egl-4(ky95)(n479)(ok1105), egl-21(n611)(n476), elo-1(gk48)(wa7), fat-1(wa9), fat-3(wa22), fat-4(ok958)(wa14), gcy-37(ok384), gpa-4(pk381), itr-1(sa73), jnk-1(gk7), ocr-2(ak47), osm-9(ky10), pmk-3(ok169), tax-6(p675)(jh107),unc-43(e408)(n498gf)*

LG V: *avr-15(ad1051), cat-4(e1141), cnb-1(jh103), daf-11(m47), dop-2(vs105)(ok1038), egl-2(n693)(n2656), egl-3(n150)(n588)(n589), egl-8(ok934)(n488), egl-10(n692)(md176), flp-21(ok889), gcy-7(tm901), gcy-32(ok995), gcy-33(ok232), gpa-1(pk15), gpa-2(pk16), gpa-3(pk35), gpa-10(pk362), gpa-13(pk1270), kin-13(ok563), mod-1(ok103), ocr-1(ak46)(ok132), odr-3(n1605), osm-6(p811), unc-42(e419)*

LG x: *arr-1(ok401), cat-1(e1111), ceh-36(ks86), dgk-1(nu62)(sy428), dgk-2(gk142), dop-1(vs100)(ok398), dop-3(vs106)(ok295), fkh-2(ok683), gcy-31(ok296),*

gcy-36(db42)(db66), gpc-1(pk298), hen-1(tm501), jkk-1(km2), lim-4(yz12)(ky403), lim-6(nr2073), lin-18(e620), ncs-1(qa406), nlp-1(ok1469)(ok1470), odr-1(n1936), pdk-1(mg142), pkc-2(ok328), rgs-2(vs17), rgs-6(vs62), rgs-8(vs64), rgs-9(vs94), rgs-10/11(vs109), sad-1(ky289),sek-1(ag1)(km4), ser-1(ok345), ser-2(pk1357), tbh-1(n3247)(ok1196), ttx-3(ot22)(mg158)
Wild type *C. elegans* used were the strain Bristol N2.

Behavioural assays

Chemotaxis towards NaCl was assessed as described before (Wicks *et al.*, 2000; Jansen *et al.*, 2002). All strains were tested for chemotaxis to 0.1, 1, 10, 25, and 100 mM NaCl, as well as 1 M NaCl and they were tested for chemotaxis to 25 mM NaCl after pre-exposure to 100 mM NaCl. 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 s.e.m.

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Chapter 6

Discussion & Future directions

6

6.1 Behavioural assays

We distinguish three responses of *C. elegans* to NaCl: attraction to low concentrations up to 200 mM, avoidance of higher NaCl concentrations, and gustatory plasticity. In this latter response, animals avoid normally attractive NaCl concentrations after pre-exposure in the absence of food. Different assays exist to test the response of *C. elegans* to NaCl: gradient assays, quadrant assays, and step response assays (Ward, 1973; Wicks *et al.*, 2000; Jansen *et al.*, 2002; Miller *et al.*, 2005). The step response assays have only been used to test wild type behaviour and to study the role of the ASE neurons in this behaviour (Miller *et al.*, 2005). Unfortunately, no mutant animals have been tested in these assays yet. Forward genetic screens have been done using the gradient assays, in which a shallow gradient is presented to the animals (Ward, 1973; Dusenbery *et al.*, 1975). Unfortunately, it is not quite clear to which concentration of attractant the animals respond in these assays.

We have used the quadrant assays to analyse *C. elegans* chemotaxis behaviour. In these assays animals are given a choice between an attractant and no attractant, a steep gradient is established in these assays. Clearly, there is a difference between the gradient and quadrant assays, and they seem to test different behaviours, since different results can be obtained with the different assays. Many mutants show similar defects in both gradient and quadrant assays, i.e. *tax-2, tax-4, tax-6* (Chapter 2, 3, 5; Coburn & Bargmann, 1996; Komatsu *et al.*, 1996; Kuhara *et al.*, 2002; Hukema *et al.*, 2006). However, *daf-11* mutant animals did show a defect in chemotaxis in the gradient assays, but showed wild type responses in our quadrant assays (Birnby *et al.*, 2000; Hukema *et al.*, 2006). Apparently, DAF-11 is necessary for navigation in a gradient of NaCl, but not for detection of NaCl per se. At present we do not know what the differences between the two behaviours are. This will probably become apparent when more mutant animals are found that show a defect in only one of the two assays.

Gustatory plasticity can be tested using two different assays. The first assay tests chemotaxis behaviour, using gradient assays after pre-exposure on plates for several hours (Saeki *et al.*, 2001). We use quadrant assays to test chemotaxis behaviour after pre-exposure in a buffer (Jansen *et al.*, 2002). In both assays gustatory plasticity requires the presence of NaCl and the absence of food. Unfortunately, relatively few mutant animals have been tested using the 'Saeki'-assays. Several mutants have been found in a forward screen using these assays, but only one cloned gene was found to be involved in gustatory plasticity when using these assays: *hen-1* (Ishihara *et al.*, 2002). We confirmed the role of *hen-1* in

gustatory plasticity using the quadrant assays. Thus far, no differences have been found in signalling involved in the two gustatory plasticity assays.

6.2 Chemotaxis to NaCl (Chapter 2)

We have shown that chemotaxis to NaCl involves two genetic pathways. One pathway includes *tax-4*, *tax-6*, *nsy-1*, and *sek-1*; the other pathway includes *tax-2*, *odr-3*, *osm-9*, and *gcy-35*. The existence of these genetic pathways has been shown by analysing the chemotaxis behaviour of several double mutant animals. It would be interesting to test additional double mutant animals to further confirm the two genetic pathways. Our finding that the *tax*-2 and *tax*-4 CNG subunits functions, at least partially, in two genetic pathways, suggests that perhaps additional CNGs are involved in chemotaxis to naCl. Therefore, it would be interesting to test double mutant animals with CNG α-subunits other than TAX-4, to find out with which α subunit TAX-2 might function.

Furthermore, it would be very interesting to study in which cells the eight genes from the two genetic pathways function. We have shown that the Gα subunit ODR-3 functions in the ADF neurons in chemotaxis to NaCl, but it remains unclear in which cells the other genes function. It seems most likely that they play a role in the amphid sensory neurons that are important in chemotaxis to NaCl: the ASE, ASI, ASG, and ADF neurons (Bargmann & Horvitz, 1990). Cell specific rescue experiments will have to confirm this. However, the guanylate cyclase GCY-35 is not expressed in these amphid sensory neurons. It is expressed in the AQR, PQR, URX neurons, in which it functions in gustatory plasticity, but it is also expressed in other cells (Cheung, *et al.*, 2004; Hukema *et al.*, 2006). Unfortunately, NSY-1 and SEK-1 are very broadly expressed, making it difficult to predict where these proteins might function.

Finally, it will be important to determine how these signalling proteins function together molecularly. Further epistasis analysis together with molecular imaging tools, forward screens, as well as further candidate gene approaches will have to unravel the molecular mechanisms involved.

6.3 G protein signalling in gustatory plasticity (Chapter 3)

We have shown that gustatory plasticity requires G protein signalling in at least four different sensory neurons. Using cell specific rescue experiments and by genetically inactivating cells, we found a role for the ASE, ASI, ASH, and ADF neurons, but also the AQR, PQR, and URX neurons, and perhaps the ADL neurons. A remaining question is what the exact roles of the different cells in

gustatory plasticity are and which signals activate these cells. We proposed a model in which the ASE neurons detect low salt concentrations and sensitise the ADF, ADL, ASI, and ASH neurons, enabling these cells to detect low salt concentrations and mediate avoidance. The role of the AQR, PQR, URX body cavity neurons remains unclear, but they might mediate the sensitisation signal. Alternatively, the ASI and ADF neurons and perhaps the body cavity neurons could be involved in detecting the absence of food. Molecular imaging experiments might reveal the exact roles of the different cells in gustatory plasticity; these experiments might show whether a certain cell has an excitatory or inhibitory role in the process.

We found that *gpa-1, gpc-1, arr-1, osm-9, fat-4,* and *gcy-35* are all part of the same genetic pathway invovled in gustatory plasticity. *odr-3* mutant animals show a more severe defect in plasticity, and *grk-2* mutant animals show the opposite, they show enhanced gustatory plasticity. A very interesting question is how all these different genes interact. It would therefore be very interesting to analyse the behaviour of double mutants with *grk-2*. Preliminary results with *grk-2; odr-3* double mutant animals show intermediate effects on chemotaxis, avoidance, and gustatory plasticity. Further analysis of double mutant animals with other G proteins and other gustatory plasticity mutants will have to elucidate the effects of GRK-2 on the salt responses of *C. elegans*.

6.4 Neurotransmission in the responses of *C. elegans* to NaCl (Chapter 4)

We found several neurotransmitters involved in gustatory plasticity in *C. elegans*, some of which are also involved in chemotaxis to NaCl. Glutamate, serotonin, dopamine, and octopamine are all needed for gustatory plasticity. Glutamate is also required for chemotaxis to NaCl. It is interesting that these neurotransmitters have been implicated in behavioural plasticity and learning behaviours in several other species.

One of the remaining questions is in which cells the neurotransmitters function. Serotonin can be taken up and used as a neurotransmitter by more cells than it is *de novo* synthesised in. Therefore it is useful to study in which cells the receptors are needed. We have already shown that the dopamine receptor DOP-3 is needed in GABAergic as well as cholinergic neurons for gustatory plasticity, but simliar analyses need to be done for the other receptors involved in gustatory plasticity as well.

Another remaining question is how the starvation signal that causes enhanced gustatory plasticity is mediated. Dopamine, serotonin, and octopamine

are not required, but there seems to be a role for glutamate. However, other molecules need to play a role as well. We propose dauer signalling via TGFβ and insulin might be required.

6.5 Candidate gene approach (Chapter 5)

We have identified many genes involved in chemotaxis to and avoidance of NaCl, as well as gustatory plasticity using a candidate gene approach. In total we tested 123 different genes in our assays. We found 22 genes to affect chemotaxis to NaCl, 57 genes affected avoidance of 1 M NaCl, and 87 genes affected gustatory plasticity. We further studied the functions of several genes involved in G protein signalling and neurotransmission, but most genes remain to studied further.

Although this candidate gene approach yielded much information about the responses of *C. elegans* to NaCl, more surprising and unexpected results might be obtained using a forward genetic screen. We tried to use such an approach to identify new genes involved in gustatory plasticity and identified several mutants. To identify the mutations that caused the behavioural defects we used SNP mapping with the Hawaiian strain CB4856. Unfortunately, the CB4856 strain shows a defect in gustatory plasticity, making it very difficult to distinguish mutant animals from the screen from CB4856 animals. We also tried to identify the mutation that causes the gustatory plasticity defect in CB4856 animals, but the our preliminary mapping data suggested that several loci are involved.

Previously, forward genetic screens have been performed to identify genes involved in chemotaxis to NaCl (Dusenbery *et al.*, 1975). Unfortunately, many mutant animals picked up in these screens showed structural defects in the amphid sensory neurons (Perkins *et al.*, 1986). Another approach would perhaps allow us to find genes encoding signalling molecules. Perhaps a slightly adapted quadrant assay can be used for such a screen. Until now all experiments have been done using NaCl, but we have found that responses to NH_4Ac are different from those to NaCl. For example *che-1* mutant animals do not respond to NaCl, but show wild type responses to NH_4Ac. Using the quadrant assays we can give animals a choice between the two salts. In such an assay *C. elegans* prefers NH_4Ac over NaCl. If we use such an assay in a forward genetic screen we might find animals that do show chemotaxis to salt, but have an altered preference. Perhaps using such a screen we can identify novel signalling molecules.

Most, but not all, the mutant animals that show a defect in attraction to NaCl, also show a defect in gustatory plasticity. However, these results are not conclusive. The defect in chemotaxis of these mutant animals could explain the

defect in gustatory plasticity; if animals do not sense NaCl properly, pre-exposure might not result in avoidance. However, both *nsy-1* and *sek-1* mutant animals show defects in chemotaxis to NaCl, but show wild type gustatory plasticity.

tax-6 mutant animals do not show a change in chemotaxis behaviour after pre-exposure. These mutant animals also have a defect in chemotaxis to NaCl. However, a *tax-6* gain-of-function allele showed normal chemotaxis to NaCl, but showed a defect in gustatory plasticity. This suggests that *tax-6* is not only involved in chemotaxis to NaCl, but also in gustatory plasticity.

Remarkably, we found many genes involved in avoidance of 1M NaCl. Only 10 mutants showed less avoidance than wild type animals; 47 different strains showed enhanced avoidance of 1 M NaCl when compared to wild type animals. Most of these 47 strains also showed a defect in gustatory plasticity, they showed less avoidance after pre-exposure. This means that the defect in gustatory plasticity cannot be explained by diminished avoidance behaviour per se. A remaining question is why so many mutants show enhanced avoidance of 1 M NaCl. Our data suggest that the response of *C. elegans* to salt is determined by a balance between attraction and avoidance. It seems that this balance is easily disrupted, resulting in stronger avoidance. However, this is not in agreement with the fact that many of these mutants also show a defect in gustatory plasticity, which means they show less avoidance after pre-exposure. This suggests that avoidance of high NaCl concentrations and avoidance of normally attractive NaCl concentrations after pre-exposure use different mechanisms.

6.6 Cellular model

Gustatory plasticity seems to involve a balance between attraction to and avoidance of NaCl, which is shifted upon pre-exposure. Normal responses to NaCl could be explained by combining attraction and avoidance responses. *C. elegans* is attracted to low NaCl concentrations and it avoids concentrations higher than 200 mM. We propose the low salt concentrations can only activate the ASE neurons, which mediate attraction. It seems likely that at a certain NaCl concentration, of opproximately 100 mM, also the ASH neurons can be activated, and mediate avoidance. At 200 mM NaCl in half of the animals the ASH neurons are not activated, these animals are attracted. In the other half, the ASH neurons are activated and seems to overrule the ASE response leading to avoidance. At higher concentrations this balance shifts towards avoidance. We propose that after pre-exposure this balance between attraction and avoidance is shifted.

It would be very interesting to construct an overall cellular model that explains the different responses of *C. elegans* to NaCl. In Chapter 3 we already proposed a model for gustatory plasticity, in which the ASE neurons detect NaCl, which is antagonised by avoidance mediated by the ASH neurons, depending on the concentration. We proposed that a signal from the ASE neurons sensitises the ASH, ADL, ASI, and ADF neurons, resulting in avoidance of normally attractive NaCl concentrations. The ADF neurons are not only involved in gustatory plasticity, but they also play a role in chemotaxis to NaCl. We suggest that the ASE neurons are essential for chemotaxis to NaCl and that an ASE-derived signal sensitises the ADF neurons and thereby activates a redundant pathway in these neurons. This could work in a similar fashion as in gustatory plasticity; in chemotaxis as well as in gustatory plasticity the ASE neurons are essential and seem to sensitise the ADF neurons. However, in chemotaxis to NaCl this results in attraction to NaCl and in gustatory plasticity it results in avoidance of the same concentrations of NaCl. Both processes require the Gα subunit ODR-3 in the ADF neurons. This suggests that the decision for a different response to NaCl after activation of the ADF neurons seems to be made downstream of ODR-3.

In our cell specific rescue experiments we have shown that in gustatory plasticity the Gγ protein subunit GPC-1 functions in the ASI, ADL, and ASH neurons; the Gα protein subunit functions ODR-3 in the ADF neurons; the G protein coupled receptor kinase GRK-2 functions in the ASH neurons; and the guanylate cyclase GCY-35 functions in the AQR, PQR, URX neurons (Chapter 3). ODR-3 also functions in the ADF neurons in chemotaxis to NaCl (Chapter 2). The TRPV channel subunit OSM-9 as well as ODR-3 has been shown to function in the ASH neurons in osmotic avoidance (Colbert *et al.*, 1997; Roayaie *et al.*, 1998). Thus, these genes can be placed in a cellular model (Figure 1).

It is very attractive to also place other genes in the cellular model. Of course rescue experiments will need to be done to confirm where the different genes function, but we will suggest where they function using their expression patterns. First, the genes involved in chemotaxis to NaCl - *tax-2, tax-4, tax-6, cnb-1,* and *ncs-1* – are all expressed in the ASE neurons (Coburn & Bargmann, 1996; Komatsu *et al.*, 1996; Gomez *et al.*, 2001; Bandyopadhyay *et al.*, 20002; Kuhara *et al.*, 2002). Since the ASE neurons are essential for chemotaxis to NaCl, it seems probable that these genes function in the ASE neurons. These genes are also expressed in the ASI neurons, in which they could also function. The ASI neurons are at least involved in gustatory plasticity and also function in

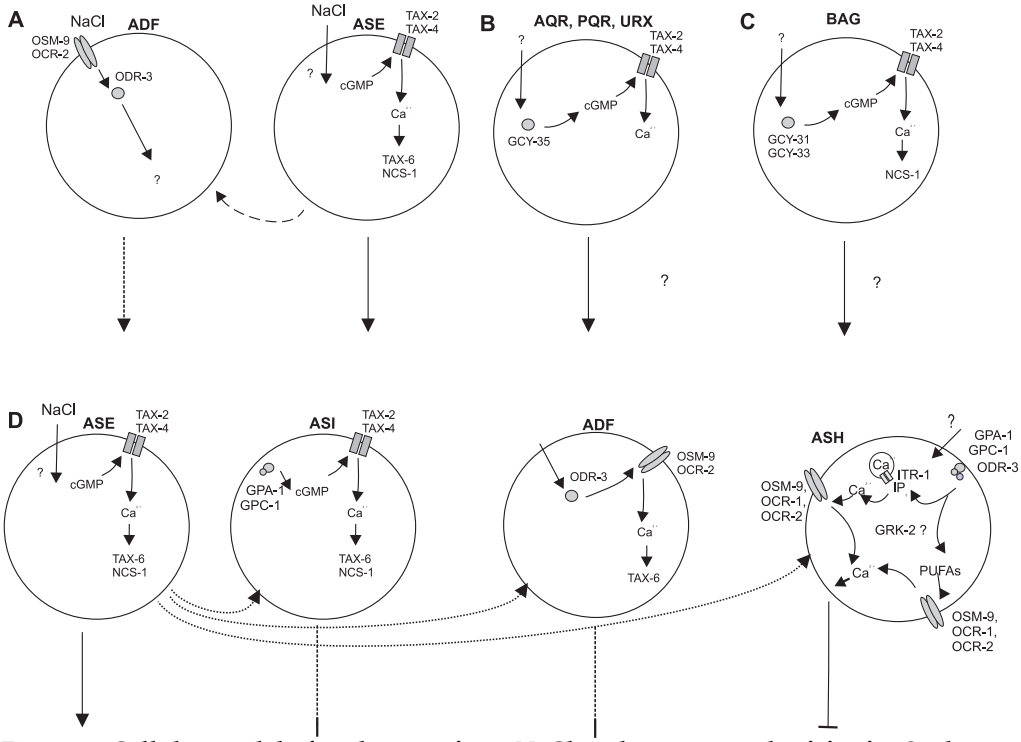

Figure 1: **Cellular models for chemotaxis to NaCl and gustatory plasticity in *C. elegans*.** (A) The ASE neurons are essential for chemotaxis to NaCl and sensitise the ADF neurons. (B) The exact role of the AQR, PQR, URX neurons in gustatory plasticity remains unknown, likely signal transduction molecules that function in these cells are GCY-35, TAX-2, TAX-4. (C) The role of the BAG neurons in the responses of *C. elegans* to NaCl remains unknown, but it seems likely that it uses GCY-31, GCY-33, TAX-2, TAX-4, and NCS-1. (D) The ASE neurons are essential for gustatory plasticity and probably sensitise the ASI, ADF, and ASH neurons, leading to avoidance of otherwise attractive NaCl concentrations after pre-exposure. See text for details. It remains unknown if ODR-3 function upstream or downstream of OSM-9 in the ADF neurons. Both possibilities are given in (A) and (D) and either of them could be relevant in both cases.

chemotaxis to NaCl in the gradient assay (Bargmann & Horvitz, 1991; Hukema *et al.*, 2006). The guanylate cyclases *gcy-5* and *gcy-7* are also expressed in the ASE neurons, in the right and left ASE neuron, respectively (Yu *et al.*, 1997). Both *gcy-5* and *gcy-7* mutant animals show a defect in gustatory plasticity, but not in chemotaxis to NaCl. Transcription factors determining the difference between the left and right ASE neuron are also needed for gustatory plasticity: *lim-6* and *cog-1* (Hobert *et al.*, 1999; Palmer *et al.*, 2002). Unfortunately, we have not yet been able to

determine the role of the left and right ASE neuron in the responses to NaCl in our assay. However, it seems likely that asymmetry is needed for gustatory plasticity.

The TRPV channel subunits OSM-9, OCR-1, and OCR-2 probably function in the ASH neurons in avoidance of NaCl as has been described for other sensory behaviours. However, OSM-9 and OCR-2 are also expressed in the ADF neurons, where they might function in chemotaxis as well as in plasticity (Colbert *et al.*, 1997; Tobin *et al.*, 2000). The TRPV channels can be activated by PUFA signalling or by Ca^{2+}. This suggests that genes involved in the synthesis of PUFAs, such as *dgk-1*, and *egl-8*, might function in the same cells. However, since PUFAs can easily pass the cell membrane, it is also possible they are synthesized in another cell. Ca^{2+} levels in the cell can be raised by release from intracellular stores by ITR-1. We hypothesize that *itr-1* and other IP_3 signalling molecules (*age-1, aap-1)* function in the same cells as the TRPV channels in gustatory plasticity, and can activate the TRPV channels.

The AQR, PQR, URX neurons are also involved in gustatory plasticity (Chapter 3). We have shown that *gcy-35* functions in these cells in gustatory plasticity, but other genes involved in cGMP signalling are expressed in these neurons as well: the cGMP gated channel subunits *tax-2* and *tax-4*, the cGMP dependent kinase *egl-4*, and the guanylate cyclases *gcy-36* and *gcy-37* (Fifure 1B). These genes all display a defect in gustatory plasticity.

In addition, there might be a role for the BAG neurons in chemotaxis to NaCl and its plasticity. The guanylate cyclases *gcy-31* and *gcy-33* are both expressed only in these neurons (Chapter 5; Yu *et al.*, 1997). *gcy-31* and *gcy-33* mutant animals both have a defect in gustatory plasticity and *gcy-31* mutant animals also show enhanced attraction to NaCl. Other genes involved in these responses to NaCl are also expressed in these neurons: the cGMP gated channel subunits *tax-2* and *tax-4*, the TRPC channel subunit *trp-1*, the neuronal calcium sensor *ncs-1*, and the voltage gated K^+ channel *egl-2* (Figure 1C; Weinschenker *et al.*, 1995; Coburn & Bargmann, 1996; Colbert *et al.*, 1997; Gomez *et al.*, 2001).

Further downstream in the cellular circuitry integration of different signals from the sensory neurons will have to take place. Likely candidates for the first step of integration are the AIA and AIB interneurons (White *et al.*, 1986). The neurotransmitters glutamate, dopamine, serotonin, octopamine, as well as neuropeptides are required for the integration. Furthermore, it is difficult ot determine where in the cellular circuitry these neurotransmitters exactly function. We have shown that the dopamine receptor DOP-3 functions in GABAergic neurons – the AVL, DVB, DD, RME, RIS, and VD neurons (McIntire *et al.*, 1997) -

and DA and DB motor neurons (Chapter 4). However, further roles of interneurons and motor neurons will have to be elucidated by cell specific rescue experiments, genetic inactivation of the neurons, or molecular imaging tools.

6.7 Comparison to mammalian behaviour

Our results show two striking similarities between *C. elegans* and mammals. First, we found a role for a TRPV channel subunit in chemotaxis to NaCl in *C. elegans*. In mammals such a TRPV channel subunit has also been found to play a role in salt taste.

Second, many molecules involved in gustatory plasticity in *C. elegans,* also play a role in behavioural plasticity in mammals. Different forms of learning in mammals require the neurotransmitters serotonin and glutamate. AMPA as well as NMDA receptors play a role in LTP, which also requires signalling via cAMP, protein kinase A, and CaM kinases. Postsynaptic changes require IP_3 receptor activation via G protein mediated signalling through PLC. Long-lasting changes during learning involves transcription of CRE-linked genes via CREB, which is opposed by the phosphatase calcineurin. All these different molecules are also involved in gustatory plasticity. Thus far many cGMP signalling molecules are involved in plasticity in *C. elegans*, a role for cAMP remains to be tested.

These similarities between the molecular mechanisms of salt taste and its plasticity of *C. elegans* and mammals suggests that also our other results may be extrapolated to mammals. Here, we show that G protein and MAPK signalling play important roles in salt taste in *C. elegans*. Similar molecules are involved in other taste responses in mammals. We speculate that there might be more overlap of salt and bitter or sweet taste in mammals than anticipated.

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Summary

It is essential for the survival of an organism to interact with its environment. Taste can help an organism to distinguish between attractive and hazardous situations. Unfortunately, relatively little is known about the molecular mechanisms of salt taste. In the first chapter an introduction is given about what is known about taste in mammals and *Drosophila*, behavioural plasticity, *C. elegans* as a model organism, *C. elegans* sensory behaviour and its plasticity, and finally neurotransmission.

C. elegans can sense water-soluble compounds, volatile compounds, touch, oxygen levels, temperature, and pheromones. It mainly uses the amphid sensory neurons for detection of its environment, of which the ASE neurons are the main salt sensing cells. Salt chemotaxis can be tested in three different assays: we use the quadrant assays in which animals are given a choice between an attractant and no attractant. Salt chemotaxis shows plasticity, which we can also test using quadrant assays. In this assay, chemotaxis behaviour is tested after pre-exposure of the animals in a buffer containing 100 mM salt, resulting in avoidance of normally attractive salt concentrations. This behaviour is called gustatory plasticity.

In Chapter 2, we identify five new genes involved in NaCl chemotaxis and position them in two genetic pathways. Chemotaxis to NaCl requires the cGMP gated channel TAX-2/TAX-4 and calcineurin TAX-6. We identified a role for the MAPKKK NSY-1, the MAPKK SEK-1, the TRPV channel subunit OSM-9, the Gα protein subunit ODR-3, and the guanylate cyclase GCY-35. A first genetic pathway consists of *tax-4, tax-6, nsy-1,* and *sek-1*; the second genetic pathway contains *tax-2, osm-9, odr-3,* and *gcy-35*. Our findings show overlap of *C. elegans* NaCl detection with mammalian systems, since both use a TRPV channel subunit. In addition, to our knowledge we provide the first evidence for the involvement of G proteins and MAP kinases in NaCl detection.

In Chapter 3 the role of G protein signalling in gustatory plasticity is discussed. We show that the ASE neurons are essential for gustatory plasticity as well as chemotaxis to NaCl. Gustatory plasticity involves a balance between the ASE, ASI, ASH, ADF, and perhaps ADL neurons. Our results suggest that attraction mediated by the ASE neurons is antagonized by G protein signalling in the ASH nociceptive neurons, which is desensitised by the G protein coupled receptor kinase GRK-2. The response to NaCl is modulated by G protein signalling in the ASI and ADF neurons, a second G protein pathway in the ASH neurons, and cGMP signalling in the AQR, PQR, URX neurons. Finally, we identify a genetic pathway involved in gustatory plasticity including the G proteins *gpa-1* and *gpc-1*,

the arrestin *arr-1*, the TRPV channel subunit *osm-9*, the guanylate cyclase *gcy-35*, and *fat-4*, which is needed for the synthesis of polyunsaturated fatty acids.

In Chapter 4 the roles of different neurotransmitters in the responses of *C. elegans* to NaCl are discussed. We found that *C. elegans* only shows gustatory plasticity when they are pre-exposed to NaCl in the absence of food. Prolonged starvation enhances gustatory plasticity. Glutamate is essential for chemotaxis to NaCl as well as gustatory plasticity, and seems to play a role in starvation enhanced gustatory plasticity, since *avr-15* mutant animals do not show wild type levels of avoidance after pre-exposure when they are starved. In addition, we show that serotonin, dopamine, and octopamine are involved in gustatory plasticity, but they are not required for starvation enhanced gustatory plasticity.

Chapter 5 gives a short overview of the candidate gene approach we used to identify genes involved in the responses to NaCl. In total, we tested mutant animals for 123 different genes. We found 22 genes involved in attraction to NaCl, 57 genes involved in avoidance of 1 M NaCl, and 87 genes involved in gustatory plasticity. These genes are involved in signal transduction via G proteins, Ca^{2+}, cGMP, IP_3, MAP kinases, and neurotransmission via neuropeptides, glutamate, serotonin, dopamine, and octopamine.

Finally, Chapter 6 gives an overall discussion and future directions. This study contributes to the understanding of the different responses of *C. elegans* to NaCl. It shows genetic pathways involved in chemotaxis to NaCl as well as gustatory plasticity. We find overlap with mammalian systems and we expect that this study provides new insights in salt taste and behavioural plasticity in general.

Samenvatting

Het is van essentieel belang voor een organisme om interactie met de omgeving aan te gaan. Smaak helpt een organisme onderscheid te maken tussen aantrekkelijke en gevaarlijke situaties. Helaas is er erg weinig bekend over de moleculaire mechanismen van smaak. In het eerste hoofdstuk van dit proefschrift wordt een inleiding gegeven over smaak in zoogdieren en *Drosophila*, plasticiteit van gedrag, *C. elegans* als een modelorganisme, het gedrag van *C. elegans* en de plasticiteit daarvan en als laatste neurotransmissie.

C. elegans neemt wateroplosbare stoffen, vluchtige stoffen, tast, zuurstof niveaus, temperatuur en feromonen waar. Daarvoor gebruikt het de zogenaamde amphid sensorische neuronen, waarvan de ASE neuronen het belangrijkst zijn voor het detecteren van zout. Zout chemotaxis van *C. elegans* kan getest worden met behulp van drie verschillende assays; wij gebruiken kwadrant assays waarin de dieren een keus krijgen tussen een bepaalde wateroplosbare stof of niets. Zout chemotaxis vertoont plasticiteit, wat ook getest kan worden met behulp van kwadrant assays. In deze assay worden de dieren eerst gedurende 15 minuten blootgesteld aan 100 mM zout in een buffer en vervolgens wordt de reactie van de dieren 25 mM zout getest. Dit resulteert in aversie van normaal aantrekkelijke concentraties zout. Dit gedrag noemen we plasticiteit van smaak.

In hoofdstuk 2 identificeren we vijf nieuwe genen betrokken bij zout chemotaxis en we plaatsen deze in twee genetische paden. Een ion kanaal dat wordt gereguleerd door cGMP gevormd door TAX-2 en TAX-4, en calcineurin TAX-6 zijn benodigd voor chemotaxis van NaCl. Bovendien hebben we een rol gevonden voor de MAPKKK NSY-1, de MAPKK SEK-1, het TRPV kanaal OSM-9, het Gα eiwit ODR-3 en de guanylaat cyclase GCY-35. Het ene genetische pad bevat *tax-4*, *tax-6*, *nsy-1* en *sek-1*; het andere genetische pad bevat *tax-2*, *osm-9*, *odr-3* en *gcy-35*. Onze bevindingen tonen een overeenkomst tussen de detectie van NaCl door *C. elegans* en zoogdieren, aangezien beiden hiervoor TRPV kanalen gebruiken. Bovendien tonen we voor het eerst aan dat MAP kinases en G eiwitten betrokken zijn bij de detectie van NaCl.

In hoofdstuk 3 wordt de rol van G eiwit signaal transductie in de plasticiteit van smaal behandeld. We laten zien dat de ASE neuronen essentieel zijn voor detectie van NaCl, en voor de plasticiteit van smaak. Een balans in singalering tussen de ASE, ASI, ASH, ADF en misschien ook ADL neuronen bepaald de plasticiteit van smaak. Onze resultaten suggereren dat attractie gemedieerd wordt door de ASE neuronen en tegengewerkt wordt door G eiwit signaal transductie in de ASH neuronen, wat gedesensitiseerd wordt door de G eiwit gekoppelde receptor kinase GRK-2. De NaCl respons wordt gemoduleerd

door G eiwit signaal transductie in de ASI en ADF neuronen, een tweede G eiwit gemedieerd pad in de ASH neuronen en cGMP signaal transductie in de AQR, PQR, URX neuronen. Ten slotte, hebben we een genetisch pad geïdentificeerd waarvan de G eiwitten *gpa-1* and *gpc-1*, arrestin *arr-1*, het TRPV kanaal *osm-9*, het guanylaat cyclase *gcy-35* en *fat-4*, dat nodig is voor de synthese van meervoudig onverzadigde vetzuren.

In hoofdstuk 4 wordt de rol van verschillende neurotransmitters in de respons van *C. elegans* op zout besproken. We hebben aangetoond dat er plasticiteit van smaak optreedt als *C. elegans* wordt blootgesteld aan zout in afwezigheid van voedsel. Bovendien wordt de plasticiteit versterkt door de afwezigheid van voedsel voor een paar uur, oftewel verhongering. Glutamaat is essentieel voor chemotaxis van NaCl zowel als de plasticiteit daarvan. Bovendien speelt het een rol in door verhongering versterkte plasticiteit van smaak. *avr-15* mutanten laten verminderde aversie van zout zien na eerdere blootstelling, ook als ze verhongerd zijn. Bovendien laten we zien dat serotonine, dopamine en octopamine betrokken zijn bij de plasticiteit van smaak, maar deze neurotransmitters zijn niet benodigd voor plasticiteit die versterkt wordt door verhongering.

Hoofdstuk 5 geeft een overzicht van een kandidaat gen benadering die we hebben gebruikt om genen te identificeren, die betrokken zijn bij de verschillende responsen van *C. elegans* op zout. In totaal hebben we 123 verschillende genen getest. Daarvan waren 22 genen betrokken bij attractie naar NaCl, 57 genen waren betrokken bij aversie van hoge concentraties NaCl en 87 genen waren betrokken bij de plasticiteit van smaak. Deze genen zijn betrokken bij signaal transductie met behulp van G eiwitten, Ca^{2+}, cGMP, IP_3, MAP kinases en neurotransmissie via neuropeptiden, glutamaat, serotonine, dopamine en octopamine.

Als laatste wordt in hoofdstuk 6 alles bediscussieerd en worden handreikingen voor toekomstige proeven gegeven. Deze studie draagt bij aan het begrijpen van de verschillende responsen van *C. elegans* op NaCl. Het laat genetische paden zien die betrokken zijn bij smaak en de plasticiteit daarvan. Bovendien vinden we overeenkomsten met het systeem in zoogdieren en we verwachten dat onze resultaten worden geextrapoleerd naar hogere organismen zoals de mens en muis. Zo zullen onze resultaten nieuwe inzichten geven in de mechanismen van zout smaak en plasticiteit van gedrag in het algemeen.

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CV

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