***Caenorhabditis elegans* feeding behaviours**

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Summary

The microscopic free-living nematode worm *Caenorhabditis elegans* was the first metazoan to have its genome sequenced and for many decades has served as a genetically tractable model for the investigation of neural mechanisms of behavioural plasticity. Many of its behaviours involve the detection of its food, bacteria, which are ingested and transported to the intestine by a muscular pharynx. The structure of the pharynx and the circuitry of the pharyngeal nervous system which regulates pharyngeal activity have been described in some detail. This has

provided a platform for understanding how this simple organism finely tunes its feeding behaviour in response to the changing availability and quality of its food, and in the context of its own nutritional status. This resonates with fundamental principles of energy homeostasis that occur throughout the animal kingdom.

Key Words: Nematode; pharynx; behaviour; adaptation; neurotransmission

Introduction

Caenorhabditis elegans is a translucent free-living nematode which is about 1mm long and has a life cycle of 3.5 days when grown on *Escherichia coli* at 20°C (Brenner 1974). The standard wild-type laboratory strain, N2, is derived from a natural isolate from a compost heap in Bristol. The hermaphrodite has 302 neurons and was the first metazoan to have its genome sequenced, with over 19,000 genes (*C. elegans* sequencing consortium, 1998). Newly hatched *C. elegans* larvae are 0.25mm long and develop through four stages, L1-L4, to reach the adult stage after 3 days. The hermaphrodite lays around 300 eggs and can live for up to 20 days. An alternative life cycle is triggered by a stressful environment e.g. starvation, in which larvae enter a metabolically arrested, stress-resistant, non-feeding dauer state. Re-introduction of favourable conditions reinstates progression to adulthood. Male *C. elegans* occur at a frequency of 0.1- 0.2% (Ward and Carrel 1979). Males, which are smaller than females, can be distinguished from hermaphrodites by their specialised tail which contains the copulatory apparatus. Males possess 1031 somatic nuclei compared to 959 in adult hermaphrodites and also have more neurons, 385 (Sulston and Horvitz 1977; Sulston et al. 1980; Sammut et al. 2015).

C. elegans eat bacteria and feed almost continuously through their life cycle, except during moult or diapause stages. To achieve this they detect food in their environment, move towards it and once in the presence of food initiate pharyngeal pumping which enables them to ingest the bacteria. Whilst at first sight this seems a simple repertoire of behaviours, *C. elegans*, just like more complex animals, makes elaborate decisions in the context of feeding (Busch and Olofsson 2012). For example, their feeding behaviour is influenced by their nutritional state (Olofsson 2014) and they can learn to avoid pathogenic bacteria (Zhang et al. 2005). Intriguingly, males unlike hermaphrodites will abandon food to find a mate (Ryan et al. 2014; Lipton et al. 2004).

Probing the neural basis of these food-related decisions has been made tractable by the construction of a 'wiring diagram' or connectome for the *C. elegans* nervous system (Albertson and Thomson 1976; White et al. 1986). Each neuron is identified by a 3 letter nomenclature and this is available in a searchable format www.wormatlas.org and provides a superb resource for neuroscientists interested in the neural basis of feeding behaviour despite the fact that a few functional synapses are not resolved (Bhatla et al. 2015; Trojanowski and Raizen 2015). Therefore, using the connectome and the genetic tools available for *C. elegans* progress has been made towards providing an integrative understanding of how an animal regulates its feeding behaviour, from the suite of food-dependent behaviours that the animal exhibits, through to the cellular and molecular determinants of these behaviours. These have highlighted an arguably surprising complexity and serve to illustrate how even a 'simple' animal has evolved an exquisitely sophisticated neural system to adapt and thrive in its environment. An online resource of movies of *C. elegans* development and behaviour is available at http://labs.bio.unc.edu/Goldstein/movies.html.

Detecting Food- Sensory Neurons

C. elegans must be able to detect food, bacteria, in their environment in order to feed, either at a distance or in their immediate vicinity. There are several ways in which *C. elegans* can detect food, *viz*, smell, taste, mechanical stimulation, oxygen and temperature (Bargmann 2006; Gray et al. 2004; Reddy et al. 2009; Sawin et al. 2000). Detection is through a range of ciliated sensory neurons (60), localized in the head (amphids), the inner labia, and the tail (phasmids, deirids). The primary sensory organ is a collection of amphid neurons whose dendrites extend to the anterior end of the animal and are ADF, ADL, AFD, ASE, ASG, ASH, ASI, ASJ, ASK, AWA, AWB, AWC (Inglis et al. 2007). The inner labial neurons surround the mouth of *C. elegans* are IL1 and IL2. The cephalic or CEP neurons are mechanosensory, as are ASH, IL1, OLL, OLQ, FLP, ADE, and PDE neurons.

Finding food- Locomotion and Nictation

C. elegans explores its environment by crawling on solid surfaces or by swimming in liquid. The chemosensory regulation of locomotion is critical for it to find food (Hilliard et al. 2002; Gray et al. 2005; Milward et al. 2011; Bargmann 2006). Locomotion is often interrupted by backward movements or reversals and turns that allow a change of direction (de Bono and Maricq 2005). Reversals are characterised by the number of head swings followed by reorientation. Short reversals occur when there are only one or two head swings, while three or more head swings are termed long reversals (Gray et al. 2005). The largest reorientation of the worm's direction is called an "omega" turn because the shape taken by the worm resembles the Greek letter Ω. Periods of rapid direction change are called "pirouettes" and the frequency of pirouettes changes in relation to food availability or the detection of an attractant chemical. Pirouettes become more frequent as the environment becomes more unfavourable (Pierce-Shimomura et al. 1999) i.e. as the animal moves away from the food source. Pirouettes are accompanied by long reversals and Ω turns which enables the worm to change its direction of travel. Another strategy, weathervane strategy, can direct *C. elegans* during chemotaxis towards an attractant (Lino and Yoshida 2009). Here the animal gradually curves towards the higher concentration of the chemical cue that signifies the presence of food. For effective chemotaxis both pirouetting and weathervaning need to occur in parallel. Head oscillations also occur during forward locomotion to aid in food search since the sensory neurons are in the tip of the nose. Both ASE sensory neurons and AIZ interneurons are required for pirouette and weathervane mechanisms.

Interestingly, *C. elegans* dauer larvae are also able to initiate a nictation behaviour in which the larvae stand on their tail to hitch a lift on a flying insect as a dispersal and survival strategy to, for example, locate a more remote food source (Lee et al. 2012).

Food Availability and Feeding

C. elegans can modulate its locomotory rate depending on whether or not food is present, moving at a faster rate off the bacterial lawn and slowing down when it reaches a food source (Sawin et al. 2000).

In the presence of food, *C. elegans* spontaneously switches between two locomotory behaviours, dwelling and roaming (Ben Arous et al. 2009; Fujiwara et al. 2002)(Video 1). Sensory perception through amphid neurons is required to extend roaming while internal metabolic

perception of food nutritional value is responsible for dwelling. The time spent in each state is modulated by previous nutritional experience of the animal. Dwelling corresponds to a reduced speed and an increase in turning frequency, allowing the worm to remain on a small area of food. Roaming is characterised by a higher speed but fewer turning events which allows exploration of the food area. These behaviours probably reflect an exploration-exploitation decision concerning the value of the local environment, *viz*, decision-making, as *C. elegans* spends less time dwelling and more time roaming when the quality of the food is low (Ben Arous et al. 2009). Conversely, when worms are on good food roaming is rare (Shtonda and Avery 2006). AIY amphid interneurons are required for effective foraging and also extend the duration of roaming.

In the absence of food, *C. elegans* displays an exploratory behaviour that can be divided into two phases, an early phase termed local area search or area-restricted search (ARS), and a late phase where worms exhibit long range dispersal (Hills et al. 2004; Gray et al. 2005). Following the removal of food, *C. elegans* undergoes ARS behaviour where it spends about 15 minutes undergoing bursts of turning reversals, equivalent to worms searching for food in a restricted area. Both dopamine and glutamate are involved in this behaviour (Hills et al. 2004). ARS behaviour is followed by foraging where worms undertake forward runs, associated with fewer reversals, in which the worm explores new areas for food (Gray et al. 2005).

C. elegans constantly assesses the quality of food, sometimes leaving its food patch in search of "better" food. The probability of leaving a food patch is greater from bacteria scored as poor quality food (DA837) compared to those with high quality (HB101), based on their growth promoting potential (Shtonda and Avery 2006). These authors found that large bacteria were a poorer food source compared with small bacteria. When food becomes depleted, the probability of worms leaving the food increases and several sensory neurons have been identified in this food-leaving behaviour (Milward et al. 2011). Neurons expressing the cGMP-gated ion channel subunits TAX-2/TAX-4, which are important for the modulation of chemosensory transduction (Komatsu et al. 1996), promote food leaving since *tax-2* and *tax-4* mutants have a marked reduction in food-leaving probability. Specific rescue of *tax-2* expression in sensory neurons revealed that the CO_2-sensing neurons, ASE and BAG (Bretscher et al. 2008; Bretscher et al. 2011) and one or more of the O_2-sensing neurons, AQR, PQR, URX, increase food leaving events. The detection of CO_2 by ASE, AFD and/or BAG neurons plays a role in food leaving behaviour since worms lacking these neurons display a reduced food-leaving phenotype induced by a rise in CO_2 (Milward et al. 2011). Conversely, OCR-2, a transient receptor potential V-like channel associated subunit, acts in ADF neurons to inhibit food leaving. AFD neurons can either promote or suppress food leaving depending on the context. For example, when ASE and BAG neurons are defective, AFD promotes food leaving but inhibits food leaving if either ASE or BAG neurons are functional.

C. elegans removed from a food environment reduce their feeding rate (Avery and Horvitz 1990; Dalliere et al. 2016; Avery and Horvitz 1989). After a relatively short period off food the feeding rate is very low, less than a fifth of the on food rate (Dalliere et al. 2016). More prolonged food deprivation results in erratic behaviour in which worms exhibit 'fictive' feeding that can intermittently achieve the same on food rate (Avery and Horvitz 1990; Dalliere et al. 2016). It is a puzzle why the worms should try to feed when bacteria are not present but a parsimonious explanation is that they are sampling the immediate environment for the presence of food (Dalliere et al. 2016).

Nutritional Status and Feeding Behaviour

The behavioural response to food depends on the worm's feeding history and resultant nutritional status. Starved animals, subjected to 6 to 12 hours food deprivation, when transferred back onto food show an enhanced pumping rate, proportional to the time spent off food, compared to the rate displayed by well-fed worms raised on food (Avery and Horvitz 1990). This hyperactive feeding is dependent on 5-HT (5-hydroxytryptamine; serotonin) signalling acting through SER-5 receptors (Lemieux et al. 2015). *C. elegans* that have been starved display an increased pumping rate in response to a much lower concentration of food compared to a well-fed worm, indicating that the period of starvation sensitizes *C. elegans* to bacteria (Avery and Horvitz 1990). Evidence indicates that activation of muscarinic receptors upon starvation leads to phosphorylation of MAPK and triggers changes in pharyngeal pumping, allowing an enhanced response when food becomes available (You et al. 2006). In addition, well-fed worms placed in absence of food immediately increase their turning rate and try to explore the near environment, while in contrast, worms starved for several hours when transferred onto a non-seeded plate display almost no reversals and immediately try to escape from the unfavourable environment (Tsalik and Hobert 2003). These behaviours are regulated by a homeostatic system which allows the worm to balance its food intake to match its energy expenditure and recover from a period of fasting through motivation to eat more following depletion of energy stores.

Kynurenic acid has been shown to be a gauge of internal nutrient availability and a key modulator of hyperactive feeding post-fast (Lemieux et al. 2015). In well-fed worms, kynurenic acid, derived from ingested food, levels are high and inhibit the glutamate receptor, NMR-2, on AVA neurons to reduce the release of FLP-18 peptides. In the presence of food cues 5-HT is released from ADF which stimulates pharyngeal pumping, resulting in food ingestion. When *C. elegans* are food deprived kynurenic acid production is suppressed which allows the release of FLP-18 which in turn activates NPR-5 on ADF. However, in the absence of food sensory cues, no 5-HT is released from ADF and pharyngeal pumping is suppressed. When animals are returned to food, sensory cues together with NPR-5 activation by FLP-18 results in the release of 5-HT and hyperactive pharyngeal pumping, a re-bound phenomenon that results in rapid food ingestion. With time and increased food intake, the kynurenic acid pool is rebuilt, leading to inhibition of AVAs and reduction of FLP-18 release, resulting in a reinstatement of normal feeding behaviour.

A state of quiescent behaviour has been described in well fed *C. elegans* where worms become immobile and stop pumping which may be equivalent to satiety in mammals (You et al. 2008). Quiescence is induced when *C. elegans* are fed high quality bacteria but rarely observed when worms are fed low quality bacteria. Quiescence also depends on the previous feeding history, for example, fasting enhances quiescence after refeeding.

Social and Solitary Feeding

A comparison of the feeding behaviours between different strains of *C. elegans* has revealed two distinct feeding behaviours on food. When N2 worms enter food they slow their locomotion rate and disperse across the food lawn which is called solitary feeding. In contrast the Hawaiian CB4856 and German RC301 strains maintain a fast rate and aggregate on the border of the lawn, forming clumps of worms which is called social feeding (de Bono and Bargmann 1998; Rogers et al. 2003). This difference in feeding behaviour is due to a polymorphism in the neuropeptide

Y-like G-protein coupled receptor (NPR) encoding *npr-1* gene. One allele, NPR-1 215F, occurs in social feeders while solitary feeders have the allele, NPR-1 215V. Since *npr-1* mutants are social feeders and expression of the more active form *npr-1* 215V converts social feeders to solitary feeders, the role of NPR-1 is to suppress social feeding (de Bono and Bargmann 1998). Ligands for NPR-1 are FLP-18 and FLP-21 neuropeptides. Disruption of *flp-21* expression enhances social feeding while overexpression suppresses social feeding. *flp-18* is expressed in AVA, AIY, RIM and RIG neurons and in pharyngeal neuron M2 and M3 while *flp-21* is widely expressed including in URA, ADL, ASH and ASE neurons, pharyngeal neurons MC, M2 and M4 (Kubiak et al. 2003; Rogers et al. 2003) and also in ASJ, ASK, FLP, RMG and URX (Macosko et al. 2009). Aggregation and bordering are promoted by the O_2-sensing URX/AQR/PQR neurons under hyperoxic conditions (Gray et al. 2004). These neurons promote hyperoxic avoidance. Due to the high concentration of bacteria in the bacterial lawn border, O_2 levels are reduced compared to the centre or outside the lawn. Thus URX/AQR/PQR may promote aggregation and bordering via their hyperpoxic avoidance role, resulting in worms moving to the border of the bacterial lawn where O_2 is lower (Coates and de Bono 2002; Cheung et al. 2004). *npr-1* mutants also show an increase in food leaving probability and locomotion speed (Bendesky et al. 2011; Milward et al. 2011). NPR-1 is associated with foraging behaviour where social feeders *npr-1* show an increased tendency to disperse from one food patch to another relative to solitary feeders N2 (Gloria-Soria and Azevedo 2008).

The Pharynx

During feeding bacteria are taken up by the mouth and transported to the intestine via the pharynx. Since pharyngeal pumping is essential for feeding in *C. elegans*, the investigation of the pharynx and its control is important in studying feeding behaviour. In adult hermaphrodites the pharynx is a muscular pump, 100µm long with a diameter of 20µm. It consists of a contractile element of 20 muscle cells, 20 neurons which comprise the pharyngeal nervous system, 4 glands cells and 9 marginal cells which are thought to strengthen the integrity of the pharyngeal organ and may also play a role in synchronising its electrical activity (Altun and Hall 2009). This complex of cells is assembled in a triradiate structure which is isolated from the rest of the body by a basement membrane (Albertson and Thomson 1976)(Figure 1). *C. elegans* is a filter-feeder, the radially orientated (a tri-radiate symmetry) muscles of the pharynx, the corpus and anterior isthmus contract to open the lumen. The lumen closes when the radially orientated muscles rapidly relax whilst channels running in between the muscles might allow for expulsion of fluid back through the mouth (Altun and Hall 2009).

Pharyngeal Muscle

The pharynx is made up of 20 muscle cells, some syncytial, which form eight muscle layers, pm1-8. These muscle cells form the three compartments of the pharynx, *viz*, the corpus (divided into pro- and meta-), isthmus, and the terminal bulb which is most posterior. The grinder, a cuticular specialisation of the muscles in the terminal bulb, facilitates the breaking up of the bacteria before they pass to the intestine. Pharyngeal behaviour is made up of two main components, the pharyngeal pumps and isthmus peristalsis (Avery and You 2012). Pharyngeal pumping can be considered in three stages. Initially food enters the corpus which contracts, together with the anterior isthmus, initiating the start of the pump leading to the opening of the

lumen, allowing fluid and bacteria to enter the mouth. The corpus then relaxes, closing the lumen, allowing expulsion of the fluids while the bacteria remain trapped and are transported posteriorly. This filtering system is aided by the anterior tip of the corpus relaxing a few milliseconds before the rest of the organ (Fang-Yen et al. 2009). Simultaneously with the contraction of the corpus, the terminal bulb contracts, this leads to the rotation of the three cuticular plates of the grinder which smash the bacteria and push the debris into the intestine. During pumping, the posterior isthmus remains closed and isolates the corpus from the terminal bulb. Bacteria are transported in the terminal bulb only during isthmus peristalsis, an event which takes place approximately every four pumps (Avery and Horvitz 1987, 1989). This allows bacteria to be concentrated in the isthmus before they reach the terminal bulb (Fang-Yen et al. 2009; Avery and Shtonda 2003). When food is scarce, for example, when food is diluted, the corpus still pumps rapidly, allowing the maximum amount to be ingested, but the isthmus and terminal bulb contraction rates are reduced since less food is ingested (Chiang et al. 2006). Reducing food density has a similar effect to ablating pharyngeal motor neuron M4, suggesting that M4 may be important for mediating the food density response of the isthmus/ terminal bulb contractions.

The pharyngeal muscles are electrically coupled by gap junctions, which play an important role in synchronizing contractions (Starich et al. 1996). The *C. elegans* genome contains a family of gap junction-forming proteins, innexins (Phelan et al. 1998; Phelan 2005). In the innexin *eat-5(ad464)* mutant, the corpus and terminal bulb contractions are desynchronized (Starich et al. 1996). This network is thought to play an important role in coordinating the muscle response which, despite an intrinsic myogenicity, is largely coordinated by the pharyngeal nervous system (Li et al. 2003; Starich et al. 2003).

The Pharyngeal Nervous System

The 20 neurons of the pharyngeal nervous system consist of 14 anatomical types with six bilaterally paired and eight single neurons (Albertson and Thomson 1976) which are linked to the extra-pharyngeal nervous system by a pair of RIP (ring/pharyngeal) interneurons (White et al. 1986). RIP neurons interact with the pharyngeal nervous system via a gap junction with a pair of pharyngeal neurons, I1. No chemical postsynaptic synapse has been identified between RIP neurons and the pharyngeal nervous system. However, I1 neurons interact with a number of pharyngeal neurons both through chemical synapses and gap junctions (www.wormatlas.org)(White et al. 1986). It is interesting that the absence of RIP neurons, which provide the sole route for neural connectivity between the pharyngeal and extrapharyngeal nervous systems, has no obvious effect on pumping rate either in the presence or absence of food (Avery and Thomas 1997; Dalliere et al. 2016). This indicates that neurohormonal volume transmission and, or, signalling intrinsic to the pharynx must be important in maintaining feeding. The pharyngeal neurons release three classical fast transmitters, *viz*, acetylcholine (ACh), glutamate, and 5-HT. However, pharyngeal neurons can also express receptors for transmitters which they cannot synthesise, such as, dopamine (Sugiura et al. 2005). This suggests that dopamine and possibly other transmitters can act as neurohormones i.e. are released from neurons in the extrapharyngeal nervous system to regulate feeding behaviour. Indeed, there is evidence that extrapharyngeal 5-HT can influence pharyngeal pumping (Cunningham et al. 2012). Conversely, it has been shown that 5-HT released from a pharyngeal neuron, NSM, can act in neurohormonal fashion to regulate locomotion (Flavell et al. 2013). Thus, as in more

complex animals, there is bidirectional signalling between the 'gut' and 'brain' to coordinate feeding behaviour.

Recording Activity of the Pharyngeal Neuromuscular System

As the worm is translucent, the activity of the pharynx can be visually scored in the intact freely moving worm in different environmental contexts by counting the number of pharyngeal pumps with one pump defined as one contraction relaxation cycle of the terminal bulb grinder (Video 2). This provides an excellent experimental approach to investigate the plasticity of feeding behaviour but it lacks detail in terms of the pharyngeal sub-behaviours that underpin feeding. These sub-behaviours can be resolved by video analysis (Avery and Thomas 1997). The activity of the neuromuscular network that underpins the pharyngeal behaviours may be further probed by electrophysiological recordings (Raizen and Avery 1994; Cook et al. 2006).

Electrophysiological recordings have provided fine resolution of the activity of the pharyngeal neuromuscular system. In a widely used approach an extracellular recording can be made by drawing the mouth of the worm into a suction electrode, termed an electropharyngeogram (EPG). EPGs can be recorded either from intact worms or from cut heads. The first EPGs were recorded by Raizen and Avery (1994) and the technique used to study pharyngeal excitation in *C. elegans* (Raizen et al. 1995). A typical EPG is about 200msec in duration and begins with a small excitatory postsynaptic potential (epsp), E1, immediately followed by a large depolarizing spike, E2. The EPG is terminated by a repolarizing spike, R1, representing the repolarization of the corpus which is followed by a much smaller repolarizing spike, R2, representing repolarization of the terminal bulb. Between E2 and R1 are usually a series of inhibitory postsynaptic potentials (ipsps). However, EPG amplitude is very dependent on the position of the recording electrode and the seal between the electrode and the worm (Cook et al. 2006). Recently a microfluidic chip with the capability to resolve the fine detail of the EPG waveform has been introduced with potential to improve reproducibility and throughput of recordings (Hu et al. 2013).

Alternately, a more technically challenging approach is to make intracellular recordings from individual muscle cells from the corpus, isthmus or terminal bulb (Raizen and Avery 1994; Cook et al. 2006; Franks et al. 2009). These have mainly provided information about the intrinsic excitable properties of the pharyngeal muscle. Its resting membrane potential is around -75mV and is mainly due to the potassium gradient together with a contribution from an ouabain-sensitive electrogenic pump (Franks et al. 2002). The amplitude and duration of the muscle action potential (AP) is dependent on the extracellular calcium concentration though sodium ions may play a role (Vinogradova et al. 2006). However, there are no obvious candidate genes for a voltage-gated sodium channel in the *C. elegans* genome (Bargmann 1998). Information regarding the currents underlying the muscle AP have been obtained using whole cell recording methods (Shtonda and Avery 2005; Steger et al. 2005).

Electrophysiological approaches may also be combined with optogenetics and optical imaging to investigate the cellular mechanisms regulating pharyngeal function (Schafer 2006; Franks et al. 2009; Trojanowski et al. 2016; Schüler et al. 2015).

Pharyngeal Neurons and Pharyngeal Pumping

Pharyngeal recordings combined with laser ablation of specific neurons and analysis of mutants has resolved the contribution of specific neurotransmitter signalling pathways in coordinated and rapid pharyngeal pumping that is observed in the presence of food.

Three pharyngeal motoneuron types play a key role in the regulation of pharyngeal pumping, *viz*, MCs, M3s and M4. MCs and M4 are cholinergic while M3s are glutamatergic. M4 which synapses with the posterior part of pm5 (isthmus) was considered essential for growth and viability of *C. elegans* (Avery and Horvitz 1989). However, there is evidence that when fed on small bacteria, worms are viable and fertile (Avery 2010). M4 ablation stops growth when worms are fed on normal sized bacteria as they cannot ingest food since the posterior part of the isthmus remains relaxed and the lumen closed (Avery and Horvitz 1989). M3 motoneurons which synapse with pm4 (metacorpus) control pumping duration by triggering the end of a pump by initiating pharyngeal relaxation (Avery et al. 1993). Following M3 ablation, the pump duration increases and so reduces the pumping rate (Raizen et al. 1995). Activation of M3 releases glutamate which activates the glutamate-gated chloride channel AVR15, *avr-15* being expressed on pm4 and pm5 (Dent et al. 1997). MCs also synapse with pm4 and are responsible for the fast pharyngeal pumping rate. Following MC ablation the pumping rate falls from around 250 to around 50 pumps per minute which reduces food intake and growth (Avery and Horvitz 1989). MCs may also act as mechanosensory neurons since their free subcuticular endings between pm3 and pm4 could detect the physical presence of bacteria in the pharynx (White et al. 1986). Following ablation of all pharyngeal nervous system neurons, the pharynx still continues to pump slowly, indicating an underlying myogenic activity (Avery and Horvitz 1989). The role of the pharyngeal nervous system in pharyngeal pumping has been further investigated by Trojanowski and colleagues (Trojanowski et al. 2014; Trojanowski et al. 2016). Optogenetic hyperpolarization of pharyngeal motoneurons, *viz*, MCs, M2s, M4 and I2s, failed to completely stop pumping. These authors found that MCs, M2s and M4 can directly stimulate pumping while I1s stimulate pumping through MCs and M2s. It is also possible that subthreshold myogenic oscillations occur in pharyngeal muscle and excitatory input from MC allows the oscillations to develop into muscle contractions. Overall, there is evidence to support an important pacemaker role for MC and a rate-permitting role for M3.

Neuronal Regulation of Feeding Behaviours

Evidence for the vital role of the nervous system in sustaining pharyngeal pumping and feeding behaviour is explicitly provided by observations on the mutant *unc-13*. This gene encodes a synaptic protein that regulates transmitter release by altering the conformation of syntaxin (Kohn et al. 2000). UNC-13 proteins are required for normal pharyngeal pumping and feeding behaviour (Richmond et al. 2001). This indicates that neurotransmitters play a key role in regulating pharyngeal pumping and so their roles will now be discussed. An overview of the genes related to specific neurotransmitter pathways that are expressed in the pharyngeal nervous system is provided in Table 1.

5-HT and Pharyngeal Pumping

5-HT is present in two types of pharyngeal neurons, NSMs and I5 (Chase and Koelle 2007). Application of 5-HT in the presence of food causes a small increase in pumping rate (Avery and Horvitz 1990; Niacaris and Avery 2003) while 5-HT application results in a large stimulation of pumping rate in the absence of food to a level similar to that found in the presence of food (Sze

et al. 2000). *tph-1* mutants which are deficient in 5-HT synthesis show a reduction in pumping rate in the presence of food (Sze et al. 2000). However, in the prolonged absence of food, these mutants have increased pumping compared to N2, suggesting 5-HT has an inhibitory effect in this context (Dalliere et al. 2016). In the presence of food, 5-HT stimulates pumping via activation of pharyngeal neurons MCs and M3s (Niacaris and Avery 2003). 5-HT had two actions on pharyngeal pumping, it decreased the pump duration through stimulation of MC and enhanced the activity of M3, mimicking the effect of the presence of food. These effects can increase the pumping rate to 250 pumps per minute and reduce pump duration to <240msec. This suggests that 5-HT modifies the electrophysiological properties of the pharynx to allow rapid contraction/relaxation cycles. 5-HT also modulates the activity of the cholinergic M4 motoneuron through SER-7 receptors to activate isthmus peristalsis (Song and Avery 2012). The action of 5-HT on MC is through the Gsα signalling pathway while that on M4 is through the $G_{12}\alpha$ signalling pathway. The physiological source of 5-HT was initially suggested to be NSM, however, it has been proposed that ADF, a chemosensory head neuron, may primarily contribute to the physiological increase in pumping in response to food through activation of SER-5 receptors on AVJ interneurons (Cunningham et al. 2012). These authors propose that AVJ is essential for both basal and 5-HT-mediated increase in pumping with 5-HT modulating feeding behaviour through inhibition of AMP-activated kinase (AMPK) in neurons that are the site of action of HLH-34, such as AVJ neurons. 5-HT also activates MC, M3 and M4 through SER-7 serotonin receptors present on these neurons (Hobson et al. 2003; Hobson et al. 2006). Other 5-HT receptors implicated in the regulation of the pharynx are SER-1, found on pharyngeal muscles and neurons, and SER-4 which is only neuronal (Tsalik et al. 2003). Although 5-HT-induced feeding is completely abolished in *ser-7* mutants (Gomez-Amaro et al. 2015) showing SER-7 is essential for 5-HT stimulation of pumping these mutants can still pump in the presence of food (Hobson et al. 2006) as can *tph-1* (Sze et al. 2000; Dalliere et al. 2016) albeit at a reduced rate. It is clear that the stimulation of feeding behaviour has a complex neuronal basis (Dalliere et al. 2016; Srinivasan et al. 2008).

5-HT and Food-Related Locomotion Behaviours

5-HT also plays a key role in the control of *C. elegans* locomotion in relation to feeding. *tph-1* mutants, which lack the ability to synthesise 5-HT, display a dwelling-like behaviour in the absence of food with an increase in short reversals relative to N2 (wild type) during both local area search and following dispersion (Gray et al. 2005). Ablation of ADF neurons, which synthesise 5-HT, results in a reduction of forward movement duration off food, which correlates with an increase in rate of reversals (Wakabayashi et al. 2004). On food, there is evidence that 5-HT released from ADF preferentially regulates pumping while 5-HT released from NSM regulates locomotory behaviour (Cunningham et al. 2012). Therefore, 5-HT released from ADF can modulate both pumping and locomotory behaviours. In the presence of food NSMs promote dwelling behaviour and these cells may detect the presence of food in the pharynx and release 5-HT in response to food to modulate locomotion to stop worms leaving the food (Flavell et al. 2013). Thus 5-HT promotes feeding by enhancing pumping rate and reducing locomotion. 5-HT release from NSM is also responsible for enhanced slowing response seen with food-deprived worms reintroduced to food (Sawin et al. 2000; Iwanir et al. 2016). This shows that 5-HT plays a modulatory role in the behavioural response to a new encounter with food. The 5-HT receptor, SER-4 (Komuniecki et al. 2004) controls the 5-HT dependent reduction of locomotion on food as does the 5-HT gated chloride channel, MOD-1, (Ranganathan et al. 2000). In addition, the

latter also promotes dwelling locomotion on food thus coordinating the feeding behavioural repertoire (Flavell et al. 2013).

Glutamate and Pharyngeal Pumping

5-HT released in response to food triggers the activity of the glutamatergic pharyngeal neurons, M3 (Niacaris and Avery 2003) and this glutamate signalling contributes to the high pharyngeal pumping rate. Mutants for *eat-4*, encoding a glutamate vesicular transporter (Lee et al. 1999), exhibit a reduced pumping rate in the presence of food (Dalliere et al. 2016; Greer et al. 2008; Lee et al. 2008) similar to that seen for *tph-1* (Dalliere et al. 2016). The fast inhibitory glutamatergic transmission is mediated by a glutamate-gated chloride channel encoded by *avr-15* expressed on the pharynx muscles pm4 and pm5 (Dent et al. 1997). The action of glutamate via AVR-15 leads to chloride-dependent relaxation that speeds up the termination of each pump (Avery 1993). Thus the release of glutamate generates fast inhibitory postsynaptic potentials (ipsps) in the pharyngeal muscle, shortening pump duration which leads to an increase in pumping frequency. There is evidence that the glutamatergic neuron M3 is activated in response to pharyngeal muscle contractions through proprioceptive feedback (Raizen and Avery 1994; Trojanowski et al. 2016) and that as a result, mutants deficient for glutamate signalling show a reduced pumping rate (Greer et al. 2008; Lee et al. 2008; Li et al. 2012).

Interestingly the role of glutamate signalling in regulating feeding is context dependent i.e. it depends on whether or not food is present. Whilst it has an excitatory/facilitatory role in the presence of food, in the absence of food its role is inhibitory. Thus, in the absence of food, *eat-4* mutants show enhanced pumping, indicating that glutamate inhibits pumping when food is absent (Dalliere et al. 2016). In this context it is noteworthy that mechanosensory inputs slow down pumping, for example, tapping the tail of adult worms and that this response is also *eat-4* dependent through AVR-14 and AVR-15 (Keane and Avery 2003) and it is possible that the absence of food and mechanical stimulation engage a similar circuit to suppress pumping.

There is also evidence that the metabotropic glutamate receptor, MGL-1, plays a role in feeding behaviour following acute removal of food in *C. elegans* (Dillon et al. 2015). Following acute removal of food, *mgl-1* mutants pump at a raised rate compared to N2, indicating that activation of MGL-1 (which is widely expressed in the extrapharyngeal and pharyngeal nervous system including NSM) leads to inhibition of pharyngeal pumping.

Glutamate and Food-Related Locomotion Behaviours

Glutamate is important for foraging behaviour. In the presence of food, N2 worms reduce their foraging by decreasing their speed and body bend frequency while *eat-4* mutants show a hyperactive foraging behaviour with an increase in body bend frequency (Lee et al. 2008). Glutamate signalling is required for the high turn rate observed in wild-type animals immediately (up to 12 minutes) after food withdrawal as *eat-4* mutants display a reduced low reversal and turn rate(Hills et al. 2004)(Chalasani et al. 2007). Glutamate signalling from AWC olfactory neurons is directly involved in this although evidence suggests other neurons also play a role (Chalasani et al. 2007). The glutamatergic gustatory ASK neurons also promote reversals and reduce forward locomotion in the absence of food (Gray et al. 2004; Wakabayashi et al. 2004). AWC and ASK neurons act through the AIB interneuron to modulate local area search (Gray et al. 2005). The ionotropic glutamate receptors GLR-1 and GLR-2 and the glutamate-gated

chloride channel GLC-3 are required for the high-angle turn and omega turn frequency in response to food removal (Hills et al. 2004; Chalasani et al. 2007). AWCs release glutamate onto AIY, AIA and AIB interneurons which have opposing effects depending on the receptor activated, *viz,* glutamate activates AIB through GLR-1 while inhibiting AIY and AIA through GLC-3.

γ-Aminobutyric acid (GABA) and Pharyngeal Pumping

There is no evidence for either the release of GABA or its synthesis in the pharyngeal system (Jorgensen 2005). However, *unc-25* mutants which have mutations in the biosynthetic enzyme for GABA, glutamic acid decarboxylase, have reduced pumping both in the presence and absence of food, suggesting it has a stimulatory role (Dalliere et al. 2016) consistent with key roles for extrapharyngeal mechanisms in regulating feeding behaviour.

GABA and and Food-Related Locomotion Behaviours

GABA plays an important role in modulation locomotion (Schuske et al. 2004) and mediates foraging behaviour (Schuske et al. 2004; Jorgensen 2005). While exploring its environment, the tip of the worm oscillates from side to side within a narrow range, possibly to enhance its chances of finding environmental cues. When the four GABA-containing RME neurons are ablated these head movements become exaggerated, indicating these neurons limit the range of head swings during foraging and weather-vaning behaviour.

Acetylcholine and Pharyngeal Pumping

ACh is the major neurotransmitter at excitatory body wall neuromuscular junctions in *C. elegans*. It is essential for development and null mutants for the synaptic vesicle ACh transporter (VAChT), *unc-17*, are lethal (Alfonso et al. 1993), hence the necessity to use hypomorphic mutations to investigate adult behaviours. In the presence or absence of food *unc-17* mutants have a severely reduced pumping rate, indicating the important stimulatory role for ACh for pharyngeal pumping (Dalliere et al. 2016). The pharyngeal nervous system has at least six types of cholinergic neuron, *viz*, M1, M2s, M4, M5, I6 and MCs (Franks et al. 2006). However, it is MC, as discussed above, which has been identified as the key player in the cholinergic stimulation of the pharynx (Avery and Horvitz 1989). Following 5-HT stimulation in response to food, MCs release ACh onto nicotinic receptors at the pharyngeal muscle neuromuscular junctions (Raizen et al. 1995; Niacaris and Avery 2003). ACh released from MCs acts on pm4 via EAT-2, a nicotinic cholinergic receptor (McKay et al. 2004). *eat-2* mutants, like MC ablated worms have a reduced pumping rate in the presence of food (McKay et al. 2004; Raizen et al. 1995). Pharyngeal muscle also expresses a muscarinic receptor, GAR-3, which contributes to the control of muscle membrane potential and the excitation-coupling of pharyngeal muscle through two different mechanisms (Steger and Avery 2004). These involve changes in intracellular calcium signalling which adjusts the kinetics of pharyngeal muscle function to allow optimal feeding. Activation of the GAR-3 pathway increases both the duration of the muscle action potential and the strength of contraction. Starvation in *C. elegans* activates MAPK, Mitogen-Activated Protein Kinase, in pharyngeal muscles through a muscarinic ACh receptor as part of a hunger signal in the pharynx (You et al. 2006). These authors propose that during starvation, the muscarinic pathway to MAPK is active which changes pharyngeal muscle physiology to increase

ingestion of food when it is available. Overall these results show that both nicotinic and muscarinic receptors modulate feeding behaviour in *C. elegans.*

Acetylcholine and Food-Related Locomotion Behaviours

Interestingly, *eat-2* mutants show an enhanced probability of leaving a food patch relative to N2 (Shtonda and Avery 2006; Olofsson 2014). Due to the pumping defect of *eat-2* mutants, it is possible the higher leaving probability on low-quality food is due to the lower feeding rate of the mutant as less nutrient is ingested from the environmentIn this context it is interesting to note *C. elegans* mutants that are chronically undernourished due to alimentary tract defects will also leave a food patch more readily and even show avoidance of bacteria that are otherwise attractive to wild-type animals (Olofsson 2014).This makes a case for coupling between feeding or nutritional intake and the locomotory circuits and has provided an interesting route to investigating 'decision-making' behaviour of the worm in the relation to its food environment (Milward et al. 2011). (Olofsson 2014)

Dopamine and Pharyngeal Pumping

There is no evidence that exogenous application of dopamine modulates the pumping rate on food (Barros et al. 2014). However, in *cat-2* mutants defective in the synthesis of catecholamines, such as, dopamine, , there is a small but significant reduction in pumping during the early phase of food deprivation which is consistent with an excitatory effect during the early phase of the behaviour (Dalliere et al. 2016).

Dopamine and Food-Related Locomotion Behaviours

Dopamine is required for the slowing response on food, as *cat-2* mutants do not show reduced locomotion in the presence of food (Sawin et al. 2000). However, dopamine is not involved in the enhanced slowing to food as *cat-2* mutants deprived of food for 30 minutes show a similar response to food as N2 worms. Ablation of individual dopamine-containing neurons only shows a slight reduction in slowing to food but ablation of all of them abolishes this behaviour (Sawin et al. 2000). Dopamine also plays a role in local area search behaviour (Hills et al. 2004). Thus dopamine regulates two distinct locomotory behaviours ensuring worms remain on a restricted area either to keep feeding or to explore in a priority local area when seeking food. Dopamine can also act to signal the presence of food in *C. elegans* (Ezcurra et al. 2011). Responses to repellents are enhanced in the presence of food due to direct food sensing by dopamine neurons which is partly mediated by the dopamine receptor DOP-4 present on ASH nociceptors, increasing sensory responses. This work has been extended to show that neuropeptide release is also involved in this dopamine effect (Ezcurra et al. 2016). Neuropeptides inhibit escape responses to noxious stimuli in the absence of food but not in its presence. Two receptors, NPR-1 and NPR-2, present in ASH neurons inhibit avoidance to repellents by increasing adaptation to these stimuli when food is scarce. Dopamine, in contrast, enhances ASH responses and decreases adaptation in the presence of food. These dopamine effects are dependent on NPR-1 and mediated through activation of DOP-1 receptors on AUA interneurons which release one or more neuropeptides to activate ASH nociceptors. Thus feeding state can modulate nociception via the interaction of dopamine and neuropeptide signalling. Interestingly, neither FLP-18 nor FLP-21, established ligands for NPR-1 (Rogers et al. 2003), increase ASH adaptation suggesting other neuropeptide ligands mediate this effect.

Octopamine/Tyramine and Pharyngeal Pumping

Neither octopamine nor tyramine is synthesised by neurons in the pharyngeal nervous system but both, when applied exogenously either to whole worms or cut head preparations, reduce pumping rate (Horvitz et al. 1982; Li et al. 2012; Packham et al. 2010; Rex et al. 2004; Rogers et al. 2001). Therefore, if these amines have a physiological action on pharyngeal pumping then they must be acting as neurohormones. Tyrosine decarboxylase 1 (TDC-1) which is responsible for the synthesis of tyramine occurs in only three cell types in *C. elegans*, *viz*, RIM, RIC and the uv1 neuroendocrine cells (Alkema et al. 2005). Since tyramine-β-hydroxylase (TBH-1), the enzyme which converts tyramine to octopamine, occurs in RIC then it is likely that tyramine is released only from RIM and uv1 cells while octopamine is released from RIC (Suo et al. 2006). Octopamine has the opposite effect to 5-HT, increasing the pharynx action potential duration by suppressing M3 activity (Niacaris and Avery 2003; Rogers et al. 2001) thereby reducing pumping rate. *tbh-1* and *tdc-1* mutants show no aberrant behaviour in the presence or absence of food (Dalliere et al. 2016; Greer et al. 2008; Li et al. 2012). Tyramine acts through SER-2 receptors to inhibit pumping (Rex et al. 2004; Li et al. 2012). This receptor is found on NSM neurons and pm1 and pm6 muscles (Tsalik et al. 2003; Rex et al. 2004). When the transcriptional regulator DAF-3 is inhibited in RIM and RIC neurons, this prevents release of octopamine and tyramine and activation of SER-2 receptors, allowing pharyngeal pumping rate to increase (Greer et al. 2008). A second tyramine receptor, TYRA-2 is expressed in MC and NSM neurons and may play a role in *C. elegans*' feeding behaviours (Rex et al. 2005). It has been suggested that both tyramine and octopamine are released in response to repellent odours in order to reduce feeding (Li et al. 2012). The octopamine receptor, SER-3, is found on pharyngeal muscle (Carre-Pierrat et al. 2006) and *ser-3* mutants have a slightly lower pumping rate compared to N2 in the absence of food. SER-3 occurs on the cholinergic SIA neurons and is required for SIA activation upon food deprivation triggered by octopamine release from RIC neurons (Suo et al. 2006; Yoshida et al. 2014). However, the role of SIA neurons on feeding behaviour is not known.

Octopamine/Tyramine and Food-Related Locomotion Behaviours

Head oscillations are a component of foraging behaviour and *tdc-1* mutants but not *tbh-1* mutants show a head oscillation phenotype: They do not suppress head oscillations during touch-induced backward movements in the absence of food (Alkema et al. 2005). Reversal behaviour is also modulated by tyramine as *tdc-1*, but not *tbh-1*, shows an increase in spontaneous reversals. Ablation of the tyraminergic neuron RIM phenocopies this effect again implicating tyramine signalling in the food dependent behaviour. An interesting study using quantitative trait loci analysis (QTL) has implicated the tyramine receptor, TYRA-3, in food-leaving behaviour suggesting tyraminergic signalling has a role in the decision-making behaviour of the worm in the context of its food environment (Bendesky et al. 2011).

Neuropeptides and Pharyngeal Pumping

A large number of neuropeptides occur in *C. elegans* including FMRFamide-like peptides, FLPs, neuropeptide-like peptides, NLPs, insulin-like peptides, INSs (Kim and Li 2004; Lau and Chalasani 2014; Li 2005; Li and Kim 2014; Nathoo et al. 2001) and many play a key role in pharyngeal pumping, particularly in the absence of food (Dalliere et al. 2016). In both *egl-3* mutants that lack proprotein convertase and *egl-21* mutants that lack carboxypeptidase and are thus deficient in neuropeptides (Kass et al. 2001; Jacob and Kaplan 2003; Husson et al. 2006;

Husson et al. 2007), pumping rate is reduced when *C. elegans* are either in the presence of food or when food is absent. The reduction of pumping is particularly striking in the absence of food and suggests that neuropeptide signalling may be important for maintaining a low level of pumping while the worm searches for food. UNC-31 is required for dense core vesicle-mediated exocytosis (Speese et al. 2007) i.e. neuropeptide release, and while *unc-31* mutants exhibit normal pumping rates on food, in the absence of food they show constitutive pumping with a rate of over 100 pumps per minute (Avery et al. 1993; Dalliere et al. 2016). This is in sharp contrast to the situation for *egl-3* and *egl-21* and may be explained by the fact that *egl-3* mutants are deficient in FLPs and NLPs while *unc-31* mutants may be deficient in additional neuropeptides. Importantly this observation shows that in the absence of food an *unc-31* dependent pathway is required to impose active suppression of feeding. When the double mutant, *unc-31;egl-3*, was examined it pumped at the same rate as *egl-3* both on and off food. This fits with a model in which an inhibitory neuropeptide signal from UNC-31 acts upstream of an excitatory neuropeptide signal from EGL-3 to stimulate pharyngeal pumping. Since both *unc-31* and *eat-4* mutants pump constitutively off food, the pumping rate of the double mutant, *unc-31;eat-4* was tested (Dalliere et al. 2016). This double mutant pumped at a lower rate than N2 in the presence of food but in the absence of food it pumped at such a high rate as if the worm had not recognized the absence of food. In contrast the double mutant, *eat-4;egl-3*, pumped in a similar manner to N2 off food. Taken together this analysis provides evidence for two distinct pathways regulating pharyngeal pumping which have *unc-31*/*egl-3* and *eat-4* dependence.

Another approach has been to study the action of exogenous neuropeptides on pharyngeal pumping rate in cut heads (Papaioannou et al. 2005; Papaioannou et al. 2008a). Nine FLPs were excitatory of which FLP-17A, FLP-17B and FLP-8 were the most potent. FLP-8 is the same sequence as the nematode peptide first isolated from the parasitic nematode *Ascaris suum* i.e. AF1 (KNEFIRFamide) (Cowden et al. 1989) FLP-17s are expressed in the pharyngeal motoneuron M5 but FLP-8 is absent from the pharyngeal nervous system (Kim and Li 2004). Thus, FLP-17 peptides are endogenous to the pharyngeal nervous system and potential candidates to regulate pumping in the presence or absence of food (Dalliere et al. 2016). The other excitatory FLPs which are expressed in pharyngeal neurons are FLP-2 (I5, MC, M4), FLP-4 (I5, I6, NSM), FLP-5 (I4, M4 and possibly I2), and FLP-6 (I1, I4). Twelve FLPs inhibited pump activity in the presence of 5-HT, of which FLP-11A and FLP-13A were the most potent. While FLP-11 is not expressed in the pharyngeal nervous system FLP-13 is expressed in the pharyngeal neurons I5, M3 and M5 (Kim and Li 2004). Other inhibitory FLPs which are expressed in pharyngeal neurons are FLP-1 (M5), FLP-15 (I2), FLP-18 (M2, M3), and FLP-21 (MC, M4, M2). Five NLPs have been tested on pharyngeal pumping, of which NLP-8, present in I2, was weakly inhibitory while NLP-1, NLP-2 and NLP-3 were weakly excitatory and NLP-10 more strongly excitatory (Papaioannou et al. 2008a). NLP-10 is expressed in two unidentified anterior pharyngeal neurons while NLP-3 is expressed in nine types of pharyngeal neurons, *viz*, I1-I4, I6, M1-M3, and NSM (Nathoo et al. 2001). Thus a number of neuropeptides could influence pharyngeal pumping through their release from pharyngeal neurons.

An opioid-like system, involving the NLP- 24 peptides, has been shown to stimulate pumping in the absence of MC function (Cheong et al. 2015) and these peptides are also required for roaming. NLP-24 acts on a receptor NPR-17 on ASI sensory neurons to increase pumping and exploration and it has been suggested this helps worms to survive starvation. The link with an opioid system is that the opiate morphine stimulates while the opiate antagonist naloxone inhibits

feeding in starved worms and these effects require NPR-17. Opioid agonists, together with one NLP-24 peptide, activated NPR-17 expressed in HEK-293 cells while a kappa opioid agonist stimulated pumping in starved N2 but not *npr-17* mutant *C. elegans*.

In addition to the neuropeptide receptor NPR-1, which has a key role in regulating feeding behaviours, other NPRs have also been implicated. NPR-4 and NPR-5, activated by FLP-18 peptides released from AIY interneurons, modulate responses to odour and play a role in local area search behaviour (Cohen et al. 2009). For example, *npr-4* mutants fail to switch from local area search to dispersal. Both receptors are widely expressed and also play a role in the regulation of fat accumulation. Overexpression of FLP-18 triggers a marked reduction in locomotion rate by acting on both NPR-4 and NPR-5. NPR-11 and its ligand NLP-1 regulate olfactory adaptation and off food search behaviour via a feedback signal to AWC olfactory neurons (Chalasani et al. 2010). *npr-11* mutants also have defects in local area search behaviour. *nlp-1* mutants have a higher turning rate than N2 during local area search, indicating that NLP-1 limits AWC-induced turning behaviour.

Insulin-like peptides have also been shown to have a role in feeding behaviour (Chalasani et al. 2010). Insulin-related peptide, INS-1, inhibits turning since *ins-1* mutants show increased turning in AWC-dependent local search behaviour. The insulin/IGF receptor, DAF-2, has been implicated in the control of feeding behaviours, for example, *daf-2* mutants are defective in the gradual slow increase of pumping rate normally seen in the absence of food (Dwyer and Aamodt 2013). These authors concluded that high levels of insulin/IGF-1 signalling are required to maintain high levels of pumping during starvation. *daf-2* mutants can be divided into class I and class II depending on their developmental and behavioural defects with Class II *daf-2* mutants showing reduced mobility and a rapid decline in pharyngeal pumping rate with advancing age (Gems et al. 1998). Both on and off food some *daf-2* mutants move more slowly than N2 worms and fail to initiate dispersal following prolonged food deprivation, suggesting a role for insulin in this adaptive behaviour (Dillon et al. 2016). *daf-2* mutants also have a reduced pumping rate both on and off food. In contrast to *daf-2* mutants, *daf-18* mutants show an increase in pumping rate off food relative to N2 which is similar to the constitutive pumping off food observed with *eat-4* and *unc-31* mutants (Dalliere et al. 2016). This constitutive pumping off food indicates there are signals which actively inhibit pumping rate. Using an isolated head preparation, the pumping rate of some *daf-2* mutants show reduced excitation to 5-HT than N2 worms while the excitatory response to a neuropeptide previously shown to potently excite the pharynx, FLP-17A (Papaioannou et al. 2008b)is unaffected (Dillon et al. 2016).

In the presence of food the TGF-β signalling mutants *daf-7* (Ren et al. 1996)and *daf-1* (Georgi et al. 1990) pump at a reduced rate compared with N2 while in food deprived *C. elegans* the pumping rate is reduced to 50% for *daf-7* and *daf-1* mutants and for N2 (Greer et al. 2008). These authors investigated the role of DAF-7 in the regulation of food intake and fat content and found evidence that the two events were coordinated by independent outputs of the nervous system. Food activates ASI sensory neurons to release DAF-7 which activates its receptors, DAF-1 and DAF-4, to inhibit DAF-3 (a co-SMAD protein) formation in RIM and RIC neurons. This prevents the release of tyramine and octopamine from RIM and RIC neurons, respectively, and their activation of SER-2 receptors on pharyngeal neurons, so stimulating feeding rate. It has been proposed that satiety-induced quiescence is regulated by insulin and TGF-β in *C. elegans* (You et al. 2008). *egl-3*, *egl-21* and *unc-31* mutants do not exhibit quiescence while quiescence

is reduced in *daf-2* and *daf-7* mutants. Quiescence can be restored in *daf-7* mutants when *daf-7* is expressed in ASI neurons. DAF-7/TGF-β signalling also acts to promote roaming behaviours in the presence of food (Ben Arous et al. 2009), food leaving through ASI (Milward et al. 2011) and is involved in the control of social feeding behaviour, acting in a parallel pathway to NPR-1 (de Bono et al. 2002).

SEB-3 is a CRF-like (corticotropin-releasing factor) receptor in *C. elegans* and *seb-3* mutants have suppressed dwelling behaviour (Jee et al. 2013) while NPR-9, a galanin-like receptor, also affects roaming and dwelling (Bendena et al. 2008). PDFR-1, a pigment dispersing factor receptor, influences roaming states in response to food (Flavell et al. 2013).

Summary of Excitatory and Inhibitory Neural Signals Regulating Feeding Behaviour

Bacteria provide a complex environmental cue that triggers olfactory, gustatory and mechanosensory modalities and downstream neural signalling pathways involving both extrapharyngeal and intrapharyngeal systems (Dalliere et al. 2016; Bhatla and Horvitz 2015). The presence of food facilitates pumping through several transmitters that act at the level of the pharynx, *viz*, ACh, glutamate, 5-HT, neuropeptides or via neurohormonally mediated signalling, *viz*, GABA, 5-HT and neuropeptides. The removal of food results in activation of both excitatory and inhibitory pathways that modulate the level of pharyngeal activity to around 20% of the on food rate. In the early phase of food deprivation both glutamate and neuropeptides inhibit pharyngeal pumping while ACh, GABA, dopamine and neuropeptides increase pumping. In the later phase, glutamate, 5-HT and neuropeptides inhibit while ACh, GABA and neuropeptides increase pumping. Glutamate signalling is important in this context-dependent regulation as it is excitatoryin the presence of food but inhibitory in its absence. Independent of glutamate inhibition is an UNC-31-dependent circuit that imposes an inhibitory tone through inhibition of net neuropeptide excitation.

Circuits Associated with Feeding

The circuit mediating the behavioural response to the removal of food has been investigated by a painstaking analysis which observed the locomotory behaviour of worms in which single classes of neuron had been laser ablated (Gray et al. 2005). This circuit uses sensory cues to modulate turning rates associated with local area search and long-range dispersal after prolonged absence of food. Information passes from amphid sensory neurons to two layers of interneurons and command interneurons to forward and backward motoneurons. Local area search behaviour is triggered by olfactory AWC neurons, gustatory ASK neurons and AIB interneurons. AWC and ASK activate AIB which in turn activates RIB interneurons and these then activate RIV and SMD head motoneurons to induce omega turns. Interneurons and motoneurons downstream of AIB and AIY encode specific aspects of reversals and turns. The motoneurons involved in these manoeuvres are SMD, RIV and SMB. AIB can also activate command interneurons AVA to induce reversals. AWC can inhibit AIY but can be inhibited by ASI gustatory neurons. AIY can activate RIM motoneurons which can inhibit AVA interneurons and reduce reversals. Thus ASI and AIY decrease reversals and omega turns and are important for the dispersal state. The first order interneurons identified in this circuit are AIY, AIZ, AIB and AIA while RIA, RIB and SAA are second order interneurons and AVA, AVB and PVC are command interneurons. 50% of the synaptic output from the amphid neurons is onto these first order interneurons and most of their synaptic output is to RIM and SMB motoneurons.

The AWC chemosensory neurons respond rapidly to the removal of food (Chalasani et al. 2007) and in turn activate AIB interneurons through a glutamate receptor, GLR-1. AIB activation lasts several minutes which may provide a neural correlate of the prolonged behavioural responses to the food deprivation. At the same time AWCs inhibit AIY interneurons through a glutamate-gated chloride channel, GLC-3. When *C. elegans* is exposed to a food odour, this inhibition is blocked and AIYs are rapidly activated. These opposite effects of AWCs on AIY and AIB may underpin a coordinated locomotor behaviour response in the absence of food. The AIY interneurons are also major postsynaptic targets of the ASE gustatory neurons and the thermosensory AFD neurons (Mori and Ohshima 1995; Tsalik and Hobert 2003). In a later paper it was shown that activation of AIY by AWC, AFD and ASE, releases FLP-18 peptides when *C. elegans* encounters sensory input from food (Cohen et al. 2009). These peptides act on NPR-4 receptors, located on RIV and AVA interneurons, to regulate olfactory responses and foraging behaviour. As AIYs do not directly synapse onto RIV and AVA, FLP-18s act as neurohormones.

The circuit controlling feeding behaviour in *C. elegans* associated with either attractive or repellent stimuli has been investigated (Li et al. 2012). The central integrating circuit receives input from different sensory modalities. Repellents are sensed by ASH neurons which activate RIM/RIC neurons to release tyramine/octopamine to suppress feeding while attractant odours act via AWA and AWC neurons to activate NSM neurons to release 5-HT which facilitates feeding. AWA and AWC neurons release glutamate which activates *glr-7* expressed in pharyngeal neurons, including I1, I2, I3, I6, MI and M3, in addition to NSM neurons. There is reciprocal inhibition between the two circuits with NSM neurons inhibiting RIM/RIC neurons activity via MOD-1 receptors and RIM/RIC neurons inhibiting NSM via SER-2 receptors. The authors describe this system as a 'flip-flop' circuit for feeding regulation, activation of one or the other circuit depending on the concentration of repellent or attractant stimuli applied.

The neurons involved in the control of the persistent behaviours, dwelling and roaming, have been investigated in *C. elegans* (Flavell et al. 2013). AIY, RIF and ASI interneurons promote roaming. These interneurons express MOD-1 5-HT-gated chloride channels which when activated by 5-HT released from NSM and HSN motoneurons inhibit roaming and promote dwelling.. In contrast Pigment Dispersing Factor, PDF, released from PVP interneurons promotes roaming through activation of PDFR-1 receptors, located primarily on AIY, RIA and RIM interneurons. Both 5-HT and PDF act as neurohormones in this circuit while AIY has synaptic links with RIA and RIM while RIF has synaptic links with RIM, AVB and PVP.

Using noxious light as a stimulus three circuits have been described which alter pumping in *C. elegans* (Bhatla and Horvitz 2015). In the first circuit light activation of I2 neurons induced direct synaptic inputs onto pm3 corpus muscles to inhibit pumping through the release of glutamate which acts on AVR-15 glutamate-gated chloride channels. In the second circuit polysynaptic activation of RIP neurons by noxious light inhibited I1 neurons, preventing activation of MC neurons and hence the excitatory effect of MC neurons on pm4 corpus muscles. This resulted in inhibition of pharyngeal pumping. The third circuit involved light activation of M1 neurons which stimulate pm3 muscles to contract expelling fluid from the mouth, termed spitting by the authors (Trojanowski and Raizen 2015).

Conclusion

Unpicking the neural basis of feeding and food-dependent behavioural plasticity in *C. elegans* has revealed a fundamentally evolutionarily conserved framework for feeding behaviour. At the core of this are mechanisms that enable the worm to balance its food intake against its nutritional requirements (Figure 2) but layered onto this are circuits which permit decisions about the quality and safety of the food source, the relative toxicity of the environment and even the drive for reproduction. As in more complex animals, these decisions require bidirectional signalling between the gut and the brain involving point to point communication through defined, context-dependent circuits and neurohormonal volume transmission.

Acknowledgements: Nicolas Dallière was funded by the Gerald Kerkut Charitable Trust.

Table 1: The Expression of Neurotransmitter Related Genes in the Pharyngeal Nervous System. The genes listed encode proteins required for neurotransmitter synthesis or storage or encode neurotransmitter receptors. This characterization is a 'work in progress' and the listing should not be regarded as comprehensive.

neuron	genes
M1	***nlp-3; unc-17***
M2	***flp-18; flp-21; nlp-3; unc-17***
M3	***eat-4; flp-13; flp-18; glr-3; nlp-3; nlp-7; ser-7***
M4	***flp-2; flp-5; flp-21;ser-7; unc-17***
M5	***flp-1; flp-13; flp-17; unc-17***
I1	***flp-6; glr-7; nlp-3***
I2	***flp-5?; flp-15; glr-7; nlp-3; nlp-8***
I3	***glr-7; nlp-3***
I4	***flp-5; flp-6; nlp-3***
I5	***eat-4; flp-2; flp-4; flp-13***
I6	***flp-4; glr-7; nlp-3; unc-17***
NSM	***eat-4; flp-4; glr-7; mgl-1; nlp-3; ser-2; tph-1; tyra-3***
MC	***flp-2; flp-21; ser-7; tyra-3 unc-17***
MI	***glr-7; nlp-3; unc-17;***

Figure 1. The Organisation of the Pharyngeal System. The pharynx has a dumbbell shape, about 100 microns long in the adult, and is a syncytium of radial muscle cells (pm) organised into functional regions as indicated. The pharyngeal interneurons and motor neurons that regulate the contraction-relaxation cycle of the pharyngeal muscle, and some of which also perform a sensory role (not shown)(Bhatla and Horvitz 2015), are encapsulated within the pharyngeal basement membrane. A subset of the 20 pharyngeal neurons is depicted along with their major neurotransmitter phenotype. The pharyngeal nervous system is connected to the extrapharyngeal system by a gap junction linking RIP to I1. In addition, neurotransmitters released from neurons either within the pharyngeal system, or by the extrapharyngeal system, may act in a neurohormonal fashion to exert effects that do not require neural connectivity (Flavell et al. 2013). For a more comprehensive description of the pharyngeal circuitry see (Franks et al. 2006).

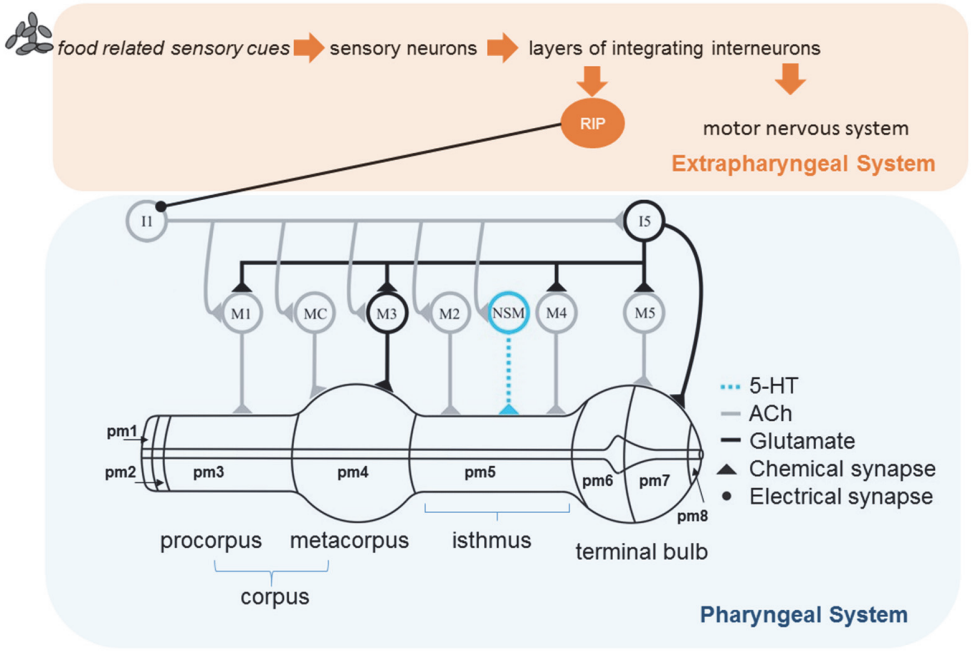
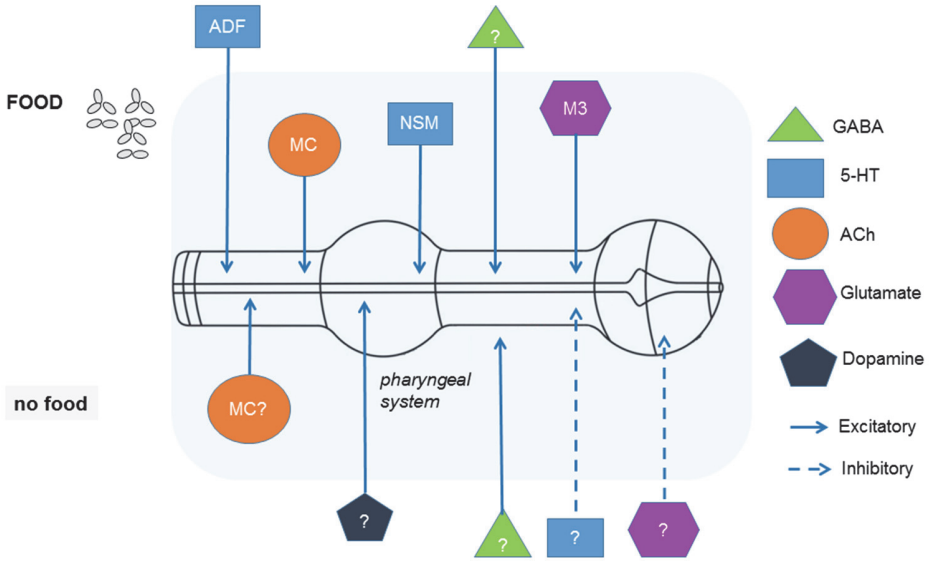


Figure 2. Neural Signals Contributing to the Coordination of Pharyngeal Behaviour in *C. elegans*. A simplified view of the major neurotransmitter signals that have been shown to regulate pharyngeal pumping in the presence and absence of food. In the presence of food excitatory signals are provided by 5-HT, ACh, GABA and glutamate neurons, both from within and outside the pharyngeal circuit as indicated. In the absence of food there is also a cholinergic excitatory drive, which may also be provided from MC, in coordination with dopamine and GABA. Intriguingly in the absence of food 5-HT and glutamate appear to inhibit pharyngeal activity (Dalliere et al. 2016). Neuropeptides are also important regulators of feeding both on and off food. The organisation of these pathways is still to be resolved.



Video 1. The Locomotory Behaviour of *C. elegans* in the Context of a Bacterial Lawn. Seven adult hermaphrodites were placed on a bacterial lawn (50 µl of *E. coli* OP50 o.d. 0.8) and video was captured for 2 hours. The border of the bacterial lawn is indicated with a white arrow. The video was recorded at 27.5 frames/minute and the playback speed is 10 frames/second: It provides examples of the range of behaviours observed in the context of food encompassing dwelling, roaming, bordering and food leaving, indicated in the captions.

Video 2. Visualising Feeding Behaviour in Freely Moving Worms. *C. elegans* are translucent and in adults the activity of the pharyngeal muscle can easily be seen under a dissecting microscope with ~60 x magnification as the worm feeds. At the beginning of the video the location of the terminal bulb in the anterior of two of the worms is indicated with white arrows. As the worm moves the contraction and relaxation of the terminal bulb can be observed. This provides a means to score the rate of feeding in the intact animal as it explores its environment. The video is shown in slow motion and is shot on a depleted bacterial lawn. Typically on abundant food the terminal bulb pumps at a rate of 4 Hz.

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