Neuronal SKN-1B Modulates Nutritional Signalling Pathways and Mitochondrial Networks to Control Satiety

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Abstract

Correct responses to nutrient type and availability are a crucial mater for all living organisms. In animals, food-related decisions require the communication of the sensory nervous system with internal body cues. Hunger and satiety play an important role here, controlling food intake and maintaining nutrient homeostasis. In *C. elegans*, chemosensory neurons sense food and relay information to the rest of the animal via hormones, neurotransmitters, and neuropeptides. These responses have a direct impact in worms' behaviour and physiology. This study shows that SKN-1B, an ortholog of the mammalian NF-E2 related transcription factors (Nrfs), acts in the two hypothalamus-like ASI chemosensory neurons to sense food, communicate nutritional status to the organism, and control food-related behaviours. SKN-1B modulates IIS and TGF-β pathways to suppress satiety-induced quiescence and promote exploratory behaviour. Finally, SKN-1B influences physiology by promoting a robust mitochondrial network which is required for energy homeostasis. The work presented here, suggests an exciting role for mammalian Nrf proteins in food-sensing and satiety.

Chapter 1 - Introduction

Food-related behaviour in animals

The ever-changing environment demands that animals adapt constantly. Energy acquisition, storage, and mobilization play a fundamental role in survival and reproduction. Across animal taxa, a common observation indicates that food intake is not constant. In some cases, animals find themselves in environment with more than one food source and some of them can be dangerous. Therefore, they need to distinguish which food is beneficial or harmful to them. In some instances, to avoid immediate danger, animals need to suppress feeding, even if they are still hungry.

Appetite control overview

Studies have shown that in mammals the hypothalamus is the executive centre for appetite control and receives input from all over the brain (Alejo Efeyan et al., 2015; Osborne et al., 1918; Young et al., 1932). Feeding is regulated by input from several areas, including the reward circuits. These are comprised by the nucleus accumbens and the limbic system, including amygdala. Unravelling the circuitry which regulates appetite and identifying the neurotransmitters which are involved, is especially important. Food related decisions are essential for organisms and controlled by appetite (Alejo Efeyan et al., 2015; Osborne et al., 1918).

In cases where a selection of food is offered, animals will choose the diet that supports growth best (Alejo Efeyan et al., 2015; Young et al., 1932). The nutritional value of the diet, which is associated with macronutrient composition, strongly influences animal physiology and

lifespan (Shtonda & Avery, 2006). Appetite is formed when missing nutrients from the diet drive the animal to a state which alters its feeding behaviour. Brain and nervous system play an important role in appetite control together with the gut (Alejo Efeyan et al., 2015; Michael W. Schwartz et al., 2000; Shtonda & Avery, 2006)

Experiments in rats where bilateral lesions were introduced in the hypothalamus, altered feeding behaviour and led to an increase in food intake, body weight, and adiposity (Hetherington AW, 1940; Michael W. Schwartz et al., 2000). In contrast, removal of adjacent parts caused the opposite effects, rats instead stopped eating and starved to death (Hetherington AW, 1940; Michael W. Schwartz et al., 2000).This was the first evidence of neuronal control of appetite.

The gut also plays an important role in appetite control (Gibbs et al., 1997; Gibbs et al., 1973; Young-Jai You et al., 2012). Signals from the gut, received by the brain can modify food-related behaviour. In addition, satiety can only be perceived when ingested food is accumulated and passed into the small intestine. In other words, nutrients must reach the gut to be sensed, and subsequently signals are sent to the brain, relaying nutritional satisfaction, and developing the satiety circuit.

Diet and animal behaviour

Diet can influence animal behaviour and appetite through brain-gut communication (Elizalde et al., 1990; Pérez et al., 1999). These food-related behaviours are taste-independent and nutrient content-dependent. Integration of intestinal and neuronal signals allows the perception of food sources and determines food-related behaviours. In addition, diet can influence behaviour and is demonstrated in mammalian paradigms where food serves as a reward (Elizalde et al., 1990; Garcia et al., 1968; Pavlov, 1927). Otherwise, irrelevant stimuli

can be associated with food availability, forming associative animal behaviour/memory with these specific factors. Another example is aversive behaviour, which can be learned by animals when exposed to poisonous food. Previous experience of a noxious food source is memorised, promoting an aversive/avoidance behaviour. The nutritional value of the diet, which supports growth and development is also linked with animal behaviour. However, the exact mechanism remains elusive.

Animals which possess a complex brain render appetite control studies difficult (Efeyan et al., 2015). Humans are thought to have approximately 100 billion neurons, which may form more than 1000 trillion synapses (Efeyan et al., 2015; Michael W. Schwartz et al., 2000). Thus, decoding the appetite and food related behaviour circuits can be a challenging task. Fortunately, these are fundamental for survival functions, and found to be conserved among species. Molecular pathways and genes are highly conserved in simpler organisms. That allows the usage of animal models, including *Caenorhabditis elegans*, to study food-related behaviours (Brenner, 1974; Shtonda & Avery, 2006).

Nutrient sensing mechanisms

Nutrient homeostasis is a crucial matter for all living organisms. The correct sensation of nutrient abundance is an important element of organismal survival. In animals, there are specific mechanisms by which nutrients are sensed. In this section, essential nutrients such as lipids, amino acids, and carbohydrates will be discussed. The nature and role of these nutrients, as well as the mechanisms by which these are sensed in mammals will be analysed. In addition, similarities and differences between humans and *C. elegans* will be presented. Despite the extensive research in the field of nutrient sensing, many questions are yet to be answered.

Macronutrients

Nutrients (or macronutrients) are simple organic compounds, important for biochemical processes which generate energy, or serve as important elements of cellular biomass (Alejo Efeyan et al., 2015; Zečić et al., 2019). Lipids, amino acids, and glucose-related sugars are all essential nutrients for cellular needs. The detection of a specific nutrient may occur by the direct binding of the molecule to its sensor or is mediated by an indirect mechanism dependent on the recognition of a surrogate molecule that indicates nutrient abundance.

Different nutrient-sensing mechanisms exist between unicellular organisms and multicellular eukaryotes (Alejo Efeyan et al., 2015; Zečić et al., 2019). Unicellular organisms are directly exposed to environmental fluctuations of nutrients. They also detect both intracellular and environmental levels of nutrients. In contrast, the majority of cells in multicellular eukaryotes are not directly exposed to changes in environmental nutrients. In order to maintain nutrient homeostasis, circulating nutrient levels are maintained within a narrow range. Nevertheless, circulating nutrient levels fluctuate and thus, intracellular, and extracellular nutrient sensing

mechanisms exist in mammals to monitor this. In addition, multicellular organisms respond to nutrients through the release of hormones, acting as long-range signals with cell non-autonomous effects, mediating the coordination of coherent responses in the whole organism.

Lipids

Lipids are involved in cellular processes such as energy storage and membrane biosynthesis. Lipids are found in different forms, such as fatty acids or cholesterol (Alejo Efeyan et al., 2015). Due to their hydrophobic carbon backbones, lipids are rarely found free in a soluble form in the organism. They are usually packaged into lipoproteins or bound by albumin in the serum (Richieri & Kleinfeld, 1995). The mechanisms of lipid-sensing are not well understood, and many questions remain to be answered.

GPR40 and GPR120

Long-chain unsaturated fatty acids are detected by G-protein-coupled receptors, GPR40 and GPR120. At the plasma membrane of pancreatic β-cells, GPR40 responds to the presence of the free fatty-acid, leading to glucose-stimulated insulin release (Itoh et al., 2003). In addition, GPR120 stimulates insulin response by an indirect mechanism associated with GLP1 (Hirasawa et al., 2005). GLP1 belongs to a group of gastrointestinal hormones, known as incretins, and promotes insulin release in β-cells (Hirasawa et al., 2005). GPR120 promotes the production of GLP1 in the gut and its subsequent release and circulation, where it exerts its effects. Another role of GPR120 together with CD36 receptor is the direct binding and uptake of intestinal lumen fatty acids (Pepino et al., 2014). These receptors, including GPR40, demonstrate fatty-acid detection properties in cells within the oral epithelium and are

involved in gustatory perception (Cartoni et al., 2010; Laugerette, 2005; Martin et al., 2011; Pepino et al., 2012). In addition, accumulation of fatty acid can lead to an immediate response, which increases glucose levels. GPR120 at the plasma membrane of white adipocytes promotes a signal transduction cascade that activates PI3K and AKT, causing cell-autonomous induction of glucose uptake (Oh et al., 2010). Individuals who are obese demonstrate mutations that impair GPR120 function. In mice, GPR120 ablation leads to a diet-induced obesity. Taken together, this signal transduction pathway is important in the systemic regulation of nutrient homeostasis (Ichimura et al., 2012).

Adipokines

Nutrient homeostasis is regulated by hormones known as adipokines. They are secreted by adipocytes, and they have systemic effects. Several biological processes are regulated by adipokines such as appetite control and energy expenditure. Adipokine levels are not a response to the amount of lipids in circulation, however, they are linked to lipid storage (K. Birsoy et al., 2008). Leptin is one of the adipokines which can act as a surrogate indicator of lipid-storage abundance (Wrann et al., 2012). Leptin receptor, LEPR, found to be expressed in both the central nervous system as well as in peripheral tissues, coordinating food intake and organismal metabolism. Leptin operates in hypothalamic neurons that suppress appetite (anorexigenic neurons) and antagonizes the action of appetite-promoting neuropeptides and neurotransmitters. Fasting promotes lipid mobilization through adipocytes, which in turn reduces leptin production. That leads to the stimulation of appetite and nutrient acquisition behaviour. Individuals who have mutations in the *LEPR* gene found to be morbidly obese (Clément et al., 1998). In addition, mice with *Lep*[28] (Yiying Zhang et al., 1994) or *Lepr*[29] (G.-H. Lee et al., 1996) mutations demonstrate eating disorders, which in some cases leads to doubling of body mass compared to normal mice.

Amino acids

In cells, amino acids are the most abundant macromolecule and the essential element for protein synthesis (Alejo Efeyan et al., 2015; Zečić et al., 2019). Due to the energetically costly process of protein synthesis, detection of extracellular and intracellular levels is crucial. During amino acid scarcity, catabolic programs provide amino acids by degrading proteins. Proteasome-mediated degradation and autophagy are key players in this process. Recycled amino acids are used for the synthesis of essential proteins under limited nutrient conditions. In addition, amino acids are catabolized for energy generation in the form of glucose and ketone bodies under extended starvation periods. This response is crucial for organismal survival and provides the necessary resources in organs needed for optimal function. Amino acid detection is a crucial process due to its involvement in processes such as amino acid synthesis, catabolism, and food intake control.

Nonderepressible 2 (GCN2)

Amino acid sensors are still under investigation and many questions haven't been answered yet regarding the precise mechanisms and molecules involved (Alejo Efeyan et al., 2015). Nevertheless, nonderepressible 2 (GCN2) kinase has been found to be an important player in amino acid detection. During protein synthesis, no amino acid compensates for the absence another, and the cell must detect this in order for optimal peptide-chain synthesis to occur (Ibba & Söll, 2000). Amino acid scarcity leads to stalled ribosomes which in turn leads to unfinished peptide chains. In this case, initiation of translation is prevented.

GCN2 can sense any uncharged tRNA, despite of its amino-acid specificity, allowing the recognition of low levels of any amino acid, even when the other 19 amino acids are present in relative abundance. GCN2 demonstrates high affinity to all uncharged tRNAs (Dong et al.,

2000) and acts as an amino acid sensor by detecting a surrogate molecule. During low intracellular amino acid levels, GCN2 binds to a given uncharged tRNA and promotes conformation change. That results in kinase activation and inhibitory phosphorylation, of the early activator of translation initiation: the eukaryotic translation initiator factor 2 α (eIF2α) (Berlanga et al., 1999). The key roles of GCN2 and eIF2a in amino acid sensation and response have been reported in mouse models. In addition, the amino acid sensing pathway acts in the central nervous system, sensing possible amino acid deficits in food, a response which is taste independent (Guo & Cavener, 2007; Hao et al., 2005; Maurin et al., 2005).

Taste receptors of amino acids

Mammals are heterotrophs and they must acquire energy and nutrients from the food sources in the environment. The ability of animals to evaluate the nutritional value of food prior to digestion permits optimal food source selection and expectation of future nutrient satisfaction. Nutrient detection in humans, occurs mostly at the level of the oral taste buds and is considered as a mechanism of extra-organismal detection, which evaluates potential food sources (Alejo Efeyan et al., 2015). In humans, taste is divided into five group: sweet, umami, bitter, sour, and salty. Taste receptors found in the cells of tongue, palatal, and oesophageal epithelium generate signals which eventually form the sensation of taste. These receptors are exposed in the apical membrane with a toward orientation to the environment (Bachmanov & Beauchamp, 2007).

Taste receptors are members of the T1R and T2R families of G-protein-coupled receptors. They demonstrate seven transmembrane domains with an extracellular N-terminus and an intracellular C-terminus (Bachmanov & Beauchamp, 2007). T2R receptor family is important for the recognition of bitter molecules including potentially toxic compounds. T1R1 and T1R3 belong to the T1R receptor family and have been found to be important for the recognition of

amino acids (umami taste). Despite the existence of other receptors important for taste, T1R1-T1R3 heterodimer is essential for the recognition of amino acids (Bachmanov & Beauchamp, 2007; Damak et al., 2003). In particular, receptors which detect amino acids in humans demonstrate high affinity to glutamate, together with other L-amino acids which act as ligands, in contrast to D-amino acids (Nelson et al., 2002). The mechanism of amino-acid sensation begins when amino-acids bind to the taste receptor and initiate signal transduction through the plasma membrane, followed by G-protein activation and neurotransmitter release (Chaudhari & Roper, 2010). This response is combined with other neurotransmission events, at the level of the central nervous system.

Apart from the oral epithelium, taste receptors are found in endocrine cells at specific regions of the gut (S. V. Wu et al., 2002). Similar to the oral epithelium, taste receptors in the intestine operate through G-protein activation. However, after their activation, the mechanisms which elicit responses are different. Instead of promoting neurotransmitter release, activated gut receptors promote the release of incretins into the blood circulation. Incretins act as an anticipatory signal for the upcoming increase in nutrients prior to digestion.

Glucose

Glucose and related sugars are crucial for mammals (Alejo Efeyan et al., 2015; Zečić et al., 2019). There are multiple regulatory mechanisms which maintain glucose levels within a physiological range, controlling glucose intake, storage, mobilization, and breakdown. The detection of glucose can occur at extra-organismal, extracellular, and intracellular levels. A network of hormone signals such as insulin and glucagon control responses according to systemic glucose levels, in distant organs.

Glucokinase (GCK)

An important nutrient-sensing mechanism of intracellular glucose levels is glucokinase. Glucokinase catalyzes the initial step of glucose storage and consumption, synthesis of glycogen and glycolysis, maintaining systemic glucose homeostasis. Other hexokinases exist, however, glucokinase plays a major role in glucose detection and regulation (Printz et al., 1993). Glucokinase found to be activated only during glucose abundance, regulating systemic glucose levels by acting in the liver and pancreas. The liver, through the process of gluconeogenesis can sustain glycaemia. During high abundance of glucose in the circulation, the liver upregulates the storage of glucose in the form of glycogen, and promotes glycogen breakdown when glucose levels are low (Nordlie et al., 1999).

Compared to the other hexokinases in the liver, glucokinase is the most abundant (Alejo Efeyan et al., 2015). Due to its mode of action and under restricted glucose conditions, glucokinase allows unphosphorylated glucose to be exported from the liver to satisfy the energetic needs of brain and muscles. In cases where the levels of glucose in the liver are high, glucokinase promotes the conversion of glucose to G6P, in order to participate into glycolysis or glycogen synthesis, for energy generation or for storage, respectively. Glucokinase found to be expressed in β-cells, as well as in neurons and glial cells in the hypothalamus. Ongoing research aims to answer questions about hypothalamic glucokinase action as glucose sensor in the brain with recent findings demonstrating its involvement in feeding responses and insulin release (Ogunnowo-Bada et al., 2014).

Glucose transporter 2 (GLUT2)

Another sensor of glucose levels is GLUT2 (Alejo Efeyan et al., 2015). It is part of an important mechanism for the detection of extracellular levels of glucose. GLUT2 permits the transport of glucose across the plasma membrane exclusively during high glycaemia conditions. After feeding, GLUT2 plays an important role in the regulation of glucose. In the liver, GLUT2 mediates glucose import, followed by GCK-dependent phosphorylation for storage and energy generation (Alejo Efeyan et al., 2015). As a response to low glycaemia, hepatic glycogenolysis and gluconeogenesis occurs, increasing the intrahepatic glucose levels. GLUT2 can transport glucose in a bidirectional manner and thus exports glucose into circulation. GLUT2 is considered an important regulator of glucose homeostasis due its role in both importing and exporting glucose during hyperglycaemic states and high levels of intrahepatic glucose respectively. Systemic detection of glucose levels is mediated by the pancreatic β-cells which are important for the synthesis and secretion of insulin. Glucose is being imported in β-cells and phosphorylation occurs by GLUT2 and GCK leading to an increased ATP:ADP ratio.

Taste receptors of glucose and related sugars

Similar to the detection of amino acids in taste buds, the heterodimer of TIR2-TIR3 acts as a taste receptor for glucose (F. Zhang et al., 2008). In addition, the expression of T1R2-T1R3 receptors is found in the intestinal epithelium, with similar sensing capabilities as in the oral epithelium. Nevertheless, the signal transduction in the intestine epithelium does not trigger an afferent signal to the brain but leads to the transient localization of the GLUT2 transporter at the apical membrane. As a result, there is an enhanced glucose absorption from the intestinal lumen after feeding (Dyer et al., 2005; Mace et al., 2007).

Macronutrients in *C. elegans*

Macronutrients are crucial for the biomass composition of cells. *C. elegans* cellular biomass demonstrates similarities with both bacteria and mammalian cells (Zečić et al., 2019). *C. elegans* as a bacterivore animal, relies heavily on bacterial food sources for the acquisition of both macronutrients (lipids, amino acids, carbohydrates) and micronutrients (vitamins, minerals) [Figure 1]. The average *E. coli* cell is rich in nitrogen and its dry weight comprises of approximately 55% protein, 23% nucleic acids (20% RNA and 3% DNA), 7-9% lipids and 6% carbohydrates, whereas vitamins, co-factors, and ions comprise approximately 4% of the dry weight (Bremer & Dennis, 2008; Uzman, 2003). Nevertheless, between bacterial species there is a difference in macro/micronutrient content, especially in terms of carbohydrates (Brooks et al., 2009; MacNeil et al., 2013). Therefore, worms in nature may obtain different amounts of macro/micronutrients compared to animals in the lab, which are fed with the standard laboratory food source *E. coli* OP50 [29, 30]. *C. elegans* dry biomass consists of approximately 60% protein, 20% lipids, 6.5% nucleic acids and 6% carbohydrates (Yilmaz & Walhout, 2016). Similar to worms, the mammalian dry cellular biomass is comprised mostly by proteins, approximately 60%, followed by 13% lipids, 5% nucleic acids and 6% sugars (Feijó Delgado et al., 2013; Uzman, 2003). Overall protein and carbohydrate content is similar between worms, *E. coli*, and mammals. Nevertheless, the relative lipid and nucleic acid content is different between these species.

Lipids in *C. elegans*

C. elegans shows evolutionary conservation with S. cerevisiae, D. melanogaster, and mammals, regarding fats and their existence in lipid droplets (as organelles) (Kühnlein, 2012; Mak, 2012; Murphy, 2012; Radulovic et al., 2013). Worms do not possess adipocytes (Mullaney & Ashrafi, 2009), which is the main component of the adipose tissue and an essential organ in mammals, where adipocytes demonstrate endocrine and immune roles (Kıvanç Birsoy et al., 2013; Coelho et al., 2013). In *C. elegans* the major organ for fat and energy storage is the intestine. Similar to mammals, the worm intestine shows liver and adipose tissue-like roles. In addition, the hypodermis (Mak, 2012; Mullaney & Ashrafi, 2009) and muscle (Liu et al., 2014) of worms demonstrate levels of fat accumulation. Interestingly, an excessive amount of triglycerides and glycogen are observed in dauer larvae, serving as energy source during starvation (Koutarou D. Kimura et al., 1997; O'Riordan & Burnell, 1989).

Amino acids in *C. elegans*

Evolutionary adaptation forced many eukaryotes, including *C. elegans*, to feed on other organisms. That affected amino acid synthesis, with eukaryotes losing the ability to synthesize approximately half of the amino acids compared to plants and fungi, which are able to synthesize them all (Lampitt, 1919; Miflin & Lea, 1977; Payne & Loomis, 2006; Rose, 1937). The dietary essential amino acids for *C. elegans* are arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine, and valine (J.R. Vanfleteren, 1973) [Figure 1]. The same set of amino acids apart from arginine, represent the dietary essential amino acids for rat and human (Albanese AA., 1952; Rose, 1937). In addition, whole-genome sequence analysis showed that the biosynthetic pathways for 10 dietary non-essential amino acids are evolutionary conserved in *C. elegans*, rats, and humans. Nevertheless, the genome

of *C. elegans* does not encode orthologues of enzymes associated with arginine biosynthesis in the human urea cycle (Cynober et al., 1995; Payne & Loomis, 2006).

Glucose in *C. elegans*

Excess glucose is stored as glycogen in both *C. elegans* and humans. In humans, the primary sites of glycogen storage are the liver and skeletal muscle (Bollen et al., 1998). In contrast, *C. elegans* stores glycogen mostly at the intestine but also in some degree at the hypodermis and muscle (Frazier & Roth, 2009; Possik et al., 2015; Seo et al., 2018). Humans possess different isoforms of glycogen synthase in each of the storage tissues (Bollen et al., 1998) where *C. elegans* has only one encoded by *gsy-1* (Frazier & Roth, 2009; Seo et al., 2018). In addition, worms are able to store glucose as the non-reducing disaccharide trehalose, through the activity of trehalose-6-phsophate synthase, whereas humans do not possess such capability, and break down dietary trehalose (Richards AB et al., 2002). In *C. elegans* trehalose-6-phsophate synthase is encoded by the *tps-1* and *tps-2* genes (Hanover et al., 2005; Pellerone et al., 2003). Finally, during the dauer stage in worms, glycogen levels found to be crucial energy sources and important for locomotion (Burnell et al., 2005).

Sterols in *C. elegans*

Sterols are considered essential growth and reproduction factors in *C. elegans* (Dutky SR et al., 1967) [Figure 1]. In humans, sterols (apart from cholesterol), found in human plasma are involved in important functions. Studies using different types of media showed that *C. elegans* needs exogenous ergosterol, β-sitosterol, stigmasterol or cholesterol for growth and development. These are the final products of sterol biosynthesis in plants and mammals (Hieb & Rothstein, 1968; Szewczyk et al., 2003). *C. elegans* lacks important enzymes

which are needed for the final steps of sterol synthesis. The worm genome encodes homologues for mammalian enzymes important for the initial steps of sterol synthesis (up to farnesyl pyrophosphate), however it lacks downstream enzymes such as squalene synthase and squalene cyclase (Chitwood, 1999; Kurzchalia & Ward, 2003). In contrast, mammals possess a complete set of enzymes needed for the de novo synthesis of sterols and acetyl-CoA, under the tight control of SREBP. Mammals do not require exogenous sterol supplementation, in contrast to worms (Espenshade & Hughes, 2007).

In nature, worms obtain dietary sterols by feeding on decaying plant and fungal material, or animal faeces, due to the fact that bacterial sources do not provide them (Kurzchalia & Ward, 2003; Schulenburg & Félix, 2017). In the lab, this requirement for sterols is satisfied by cholesterol supplementation in the media (Lozano et al., 1987; Merris et al., 2003). However, cholesterol is not an essential dietary sterol in worms, despite its use under laboratory conditions. The role of cholesterol in *C. elegans* is not entirely clear. In mammals, cholesterol found to have many important roles; it is a fundamental component of cellular membranes, important for their fluidity and semi-permeability. In addition, cholesterol acts as a precursor for the synthesis of steroid hormones and bile acids. Cholesterol is almost absent from *C. elegans* cellular membranes. That indicates that cholesterol does not hold a crucial structural role in worms. However, studies suggest another important function for cholesterol in worms. It acts in cellular signalling associated with moulting and dauer development (Kurzchalia & Ward, 2003; Matyash et al., 2001; Merris et al., 2003).

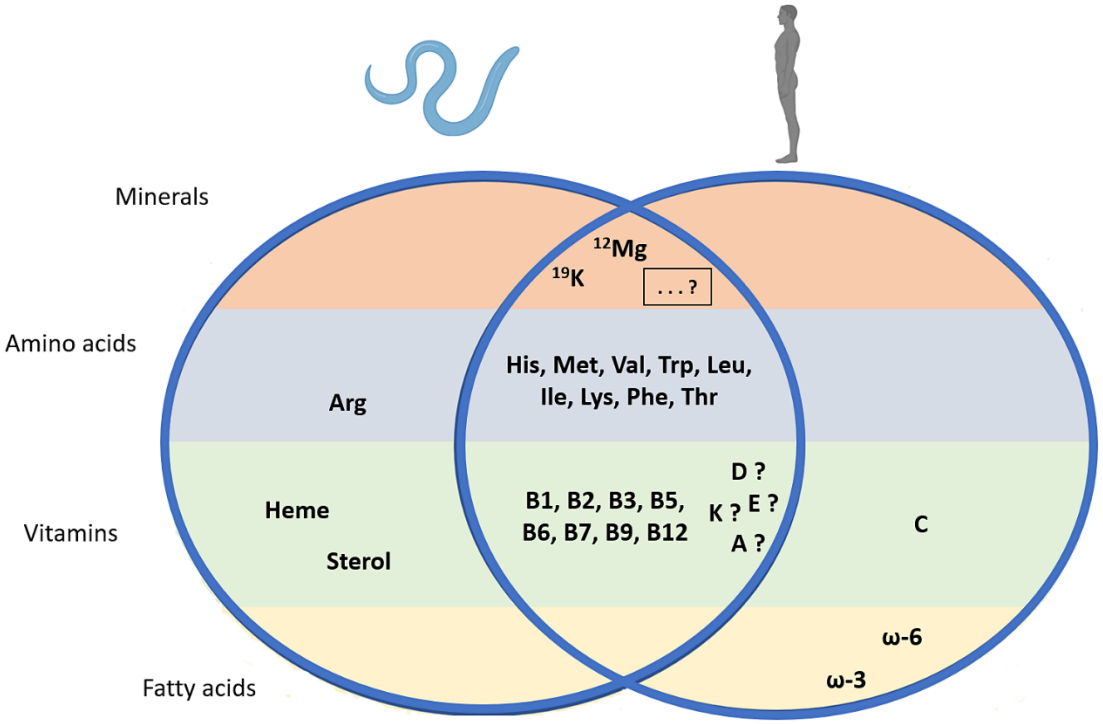


Figure 1: Essential nutrients for *C. elegans* and humans. Essential amino acid requirement is similar between worms and humans, with the exception of arginine, which is an essential amino acid only in worms. Worms are heme and sterol auxotroph organisms, in contrast with humans. Similar requirements for vitamins B1-B12 between worms and humans. Adapted from (Zečić et al., 2019).

Caenorhabditis elegans as a model organism

Caenorhabditis elegans, C. elegans for short, was developed as a model organism by Sydney Brenner to study development and nervous system (Brenner, 1974). Nowadays, *C. elegans* is considered a powerful and widely used genetic tool (Sulston et al., 1983; Sulston et al., 1977; White et al., 1986). Adult worms are approximately 1mm long and are easily viewable with a dissecting microscope. They are self-fertilizing hermaphrodites with a short lifespan compared to other animal models.

C. elegans life cycle

The embryogenesis of *C. elegans* requires approximately 16 hrs at 20C (all the following phases described here, took place at 20C) (Corsi et al., 2015). After fertilization, an eggshell protects the embryo and allows progeny to grow independently of the mother. Usually, the embryo is retained within the parent until the 24-cell stage. Upon hatching, the hermaphrodite embryo has 558 nuclei and becomes a first stage (L1) larva. The worm starts to feed and develop through the four larval stages (L1-L4). The duration of L1 stage is approximately 16 hrs long, whereas the duration of the other stages is 12 hrs long. At the end of each stage, there is a sleep-like period of inactivity known as lethargus (Raizen et al., 2008). At this point a new cuticle is produced. The end of lethargus is accompanied by the moulting of the old cuticle. An approximate period of 12 hrs is required for the animal to reach adulthood after the L4 moult. At this point, the adult hermaphrodite, initiates production of progeny, with a duration of 2-3 days until the exhaustion of its self-produced sperm capability. Mating between sperm-depleted hermaphrodites and males can generate additional progeny. Death by senescence comes several weeks after the reproductive period of the animal.

Egg to adult development takes about 3 days. Each animal lays about 300 eggs which permits large number of animals to be bred cost effectively. Males do appear at low frequency and male enriched populations can be maintained by mating with hermaphrodites. The presence of males means that strains can be easily crossed and resulting mutants can be isolated from a single hermaphrodite. Adult *C. elegans* are post mitotic organisms with 959 somatic cells, including 302 neurons of known embryonic and post embryonic lineages. Furthermore, the haploid genome size of *C. elegans* is only 100 megabase pairs (Mb) in comparison with the 3200 (Mb) of humans (Corsi et al., 2015).

Dauer

C. elegans must adapt and survive under the harsh environmental conditions that exist in the wild (Corsi et al., 2015). A remarkable strategy exists in worms, allowing them to enter a state of diapause during development known as dauer (Ailion & Thomas, 2003; Hu, 2007). During food source depletion, high temperature, or crowded conditions, *C. elegans* activate an alternative life cycle program where growth and development are arrested until environmental conditions improve.

At L2 larval stage, *C. elegans* moult into the alternative L3 larval stage, known as the dauer larva (Hu, 2007). The signal for this transformation is processed by L1 worms, but its effects are expressed at the L2d stage. The cuticle of the dauer larva surrounds the worm, blocks the mouth, and stops the worm from eating, leading to developmental arrest (Cassada & Russell, 1975). Dauers can survive for many months and upon refeeding they shed the mouth plugs, moult, and resume development as slightly different L4 larvae.

There are many morphological differences between the dauer larvae and the normally developed larvae .(Cassada & Russell, 1975; Hu, 2007) Dauer larvae described as radially

constricted and exhibit a thinner diameter compared to L3-stage larvae, as well as the subsequent developmental stages. Under the microscope, the specialised cuticle, as well as their distinctive length and width permits quick identification. Dauer cuticle is resistant to chemicals and protects the animal against environmental stresses and caustic agents.

Apart from the morphological differences, dauers demonstrate distinctive metabolism compared to other larval stages (Hu, 2007). During normal development and between L1 and L2 stages, L1 animals undergo a significant metabolic change (Burnell et al., 2005; Wadsworth & Riddle, 1989). Instead of relying on the activity of the glyoxylate cycle which generates carbohydrates from fat stores, they depend on oxidative metabolism and the TCA cycle. In contrast, dauer larvae continuously rely on and generate lipid storage prior to entrance into dauer. This peculiar metabolic state of dauer larvae provides resistance to metabolic stress, with dauers showing increased SOD activity (Larsen, 1993; Jacques R. Vanfleteren & De Vreese, 1995).

This alternative life cycle program, the dauer stage, acts as survival strategy for *C. elegans*, allowing the animal to survive long enough and reproduce when the environmental conditions improve (Kenyon, 2005). Other organisms use a similar strategy to survive (known collectively as cryptobiosis) however, this remarkable capability is absent in humans. Nevertheless, valuable knowledge can be acquired about the molecular mechanisms which regulate this metabolic adaptation of dauers (Jones & Ashrafi, 2009). Important pathways for development such as the insulin/insulin-like growth factor signalling (IIS) and transforming growth factor β (TGF-β) are disrupted in dauers and that can provide valuable knowledge about their operation (Koutarou D. Kimura et al., 1997; Ren et al., 1996; Wolkow et al., 2000). This is highly important since essential energy metabolism pathways are found to be well conserved between *C. elegans* and humans. Thus, dauers considered as a valuable tool to investigate

how organismal-survival-response acts on physiology and metabolism in order to be successful.

Neuromodulation and foraging behaviour in *C. elegans*

C. elegans is considered an excellent model to study the mechanisms by which neuromodulation regulates foraging behaviour (D. Chase, 2007). Despite its compact nervous system of only 302 neurons, *C. elegans* neuromodulation mechanisms show remarkable similarities with those which operate to control similar processes in mammals. In addition to classical neurotransmitters, *C. elegans* nervous system contains more than 100 neuropeptides and biogenic anime modulators such as dopamine, serotonin, octopamine, and tyramine (D. Chase, 2007). In this section, the role of the biogenic amines in foraging behaviour of worms will be discussed. Understanding the roles of neurotransmitters in the nervous system of *C. elegans* can provide valuable knowledge to understand signalling in the human brain.

There are two important groups of neurotransmitters which operate in *C. elegans*: classical and biogenic amines (D. Chase, 2007). The production of classical neurotransmitters requires acetylcholine (ACh), γ-aminobutyric acid (GABA) and glutamate. From the cell body, classical neurotransmitters are transported and released into the synaptic cleft. The synaptic vesicles carry them and need the protein UNC-13 for synaptic fusion (Richmond et al., 1999; Speese et al., 2007). The synaptic vesicles bind to post-synaptic ionotropic receptors which are important for fast transmission (Smart & Paoletti, 2012). Classical neurotransmitters are important for a range of functions in *C. elegans*, including feeding behaviour, but are most important for motor neuron function. Further discussion on neurotransmitters will focus on biogenic amines, due to their significant role in foraging behaviour, exploration, response to environment changes, and relevance to this study.

Biogenic amines

Studies have shown that the biogenic amines such as dopamine (DO), serotonin (5-HT), octopamine (OA), and tyramine (TA) can be found in *C. elegans* extracts by HPLC analysis, acting as neurotransmitters or neuromodulators (Alkema et al., 2005; Horvitz et al., 1982; Sanyal et al., 2004; Sulston et al., 1975) [Figure 2]. Other signalling amines such as histamine, epinephrine, and norepinephrine are not produced in *C. elegans* (Horvitz et al., 1982; Sanyal et al., 2004). In *C. elegans,* a vesicular monoamine transporter, *cat-1*, is required for all biogenic amines to be loaded into synaptic vesicles. In contrast with the small synaptic vesicles holding classical neurotransmitters, biogenic amines are packaged into large and dense core vesicles (Duerr et al., 1999). Similar to UNC-13, UNC-31 is needed for the amine-containing vesicles to be docked to the plasma membrane. UNC-31 is the homolog of human CAPS (calcium-dependent activator protein for secretion). After docking, the vesicles fuse with the plasma membrane and the biogenic amines and neuropeptides are released (Lin et al., 2010; Speese et al., 2007).

Studies in mammals have identified the roles of dopamine and serotonin in the nervous system. Abnormalities in signalling of these neurotransmitters are associated with many human diseases such as Parkinson's disease, depression, and schizophrenia. Despite several important discoveries so far, many questions have been not fully answered. For instance, in mammals it is not well understood whether octopamine or its biosynthetic precursor tyramine act as neurotransmitters. Research in invertebrates and *C. elegans* showed that octopamine and tyramine can both act as neurotransmitters (Alkema et al., 2005). It has been shown that neuromodulators can drive behaviours in worms, which are associated with different feeding states. The role of each biogenic anime in worms' foraging behaviour will be discussed below.

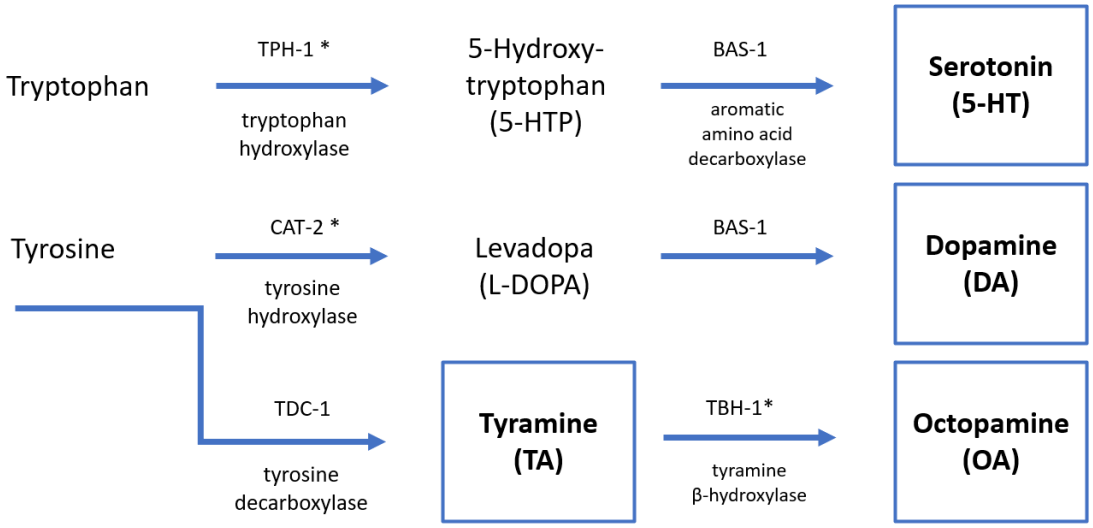


Figure 2: Synthesis of biogenic amines. Serotonin is synthesised from tryptophan using tryptophan hydroxylase (TPH-1), making 5-hydroxy-tryptophan (5-HTP). GTP cyclohydrolase I (CAT-4) * is also needed for this process. 5-HT is synthesised from 5-HTP using BAS-1. **Dopamine** is synthesised from tyrosine by tyrosine hydroxylase (CAT-2) into L-DOPA. In order for this to occur GTP cyclohydrolase I (CAT-4) * is needed. L-DOPA is converted to dopamine likely by BAS-1. **Tyramine** is synthesised from tyrosine using tyrosine decarboxylase (TDC-1). **Octopamine** is synthesised from tyramine by tyramine β-hydroxylase (TBH-1). Adapted from (D. Chase and M. Koelle, 2007).

Dopamine

Synthesis of dopamine

In *C. elegans*, dopamine signalling plays an important role in modulation of locomotion behaviour and learning (Duerr et al., 1999; Hills, T., P.J. Brockie, 2004; Loer & Kenyon, 1993; Sanyal et al., 2004; Sawin et al., 2000).. Inhibition of dopamine release, through neuron ablation or genetic mutation, render the worm unable to respond to environment changes. More about the synthesis and roles of dopamine in *C. elegans* will be discussed here.

Dopamine is synthesised from tyrosine by tyrosine hydroxylase (CAT-2) into L-DOPA [Figure 2] (Lints and Emmons, 1999; Sulston et al., 1975). In order that to occur GTP cyclohydrolase I (CAT-4) is needed (Loer & Kenyon, 1993). L-DOPA is converted to dopamine likely by BAS-1 [Figure 2] (Hare & Loer, 2004). Dopamine transporter DAT-1 is required for dopamine to enter the cells (Lankupalle D. Jayanthi et al., 1998). Dopamine is produced in eight neurons in the hermaphrodite [ADE, PDE, CEPD/V) and in additional six neurons found in the tail of the male [R5A, R7A, R9A), all of them considered to be mechanosensory neurons (14 in total) (Sulston et al., 1975). Known dopamine receptors are: GPCRs DOP-1, 2, 3 (D. L. Chase et al., 2004) and 4 (Sugiura et al., 2005), and ligand-gated Cl^- channel LGC-53 (Ringstad et al., 2009). DOP-5 and DOP-6 are uncharacterised, possible DA GPCRs, with sequence similarity to DOP-3 (Bentley et al., 2016).

Dopamine, locomotion, and foraging behaviour

Foraging behaviour of *C. elegans* is highly associated with locomotion behaviour and its ability to respond to environmental changes. Dopamine governs this response. More specifically, dopamine is required for well-fed WT worms to be able to slow down their locomotion rate, when they encounter a bacterial lawn. This is also known as basal slowing response (Sawin et al., 2000). *cat-2* mutants, or worms in which the dopaminergic neurons have been ablated, are unable to demonstrate basal slowing response. It has been shown that DOP-3 slows locomotion, whilst DOP-1 antagonises DOP-3 in cholinergic neurons (D. L. Chase et al., 2004). Dopamine then stops octopamine from triggering roaming by binding to DOP-2 in SIA neurons and DOP-3 in SIA and RIC neurons (Satoshi Suo et al., 2009).

Dopamine signalling is important for the detection of new food sources in the environment. *C. elegans* uses a food source until it is depleted. Immediately after, worms begin searching the immediate area for additional food before roaming to more distant areas. This localized search for food, is also known as area-restricted searching and is demonstrated in worms through an increased frequency of high-angled turns, soon after the food supply depletion. Dopamine acts on glutamatergic neurons to increase high-angled turns for area-restricted search (Hills et al., 2004).

In case of complete food elimination, worms demonstrate reduced frequency of high-angled turns, which results in roaming-exploratory behaviour to more distant areas. Worms require dopamine signalling for area-restricted searching. This is clearly demonstrated in the case of *cat-2* mutants or worms in which the dopaminergic neurons have been ablated, where the frequency of high-angled turns is diminished, immediately after food supply depletion. Worms spend less time in the immediate area, in which food supply was recently exhausted.

Dopamine signalling has been found to be important for foraging behaviour in *C. elegans*, by modulating at least two distinct behaviours of locomotion. Dopamine is needed to ensure that

worms will remain at the area of the food source. This is particularly important because animals must remain near food sources to survive. Leaving a food source sooner than needed, increases the chances of death. In *C. elegans* dopamine increases the frequency of high-angled turns which stops them from going away from the food source. In case of detecting a previous food source, dopamine signals make the worm slow forward locomotion, increasing the possibility of staying at the food source this time. Nevertheless, upon food source depletion, the elevated frequency of high-angled-turns, makes the worm search the local environment for new food. In case of a complete lack of food, high-angled turns diminish, and the worm starts to explore more distant food sources.

Serotonin

Biogenic amines are important regulators of mood, appetite, and weight (Lam, 2006). Pathologies such as depression, anorexia, and obesity demonstrate symptoms of altered food intake patterns. Dysregulated serotonin signalling is associated with the manifestation of these disorders (Curran & Chalasani, 2012). Serotonin signalling has a great impact on the regulation of feeding behaviour. Treatment of these pathologies includes drugs which increase the amount of 5-HT at the synapses. However, the actual mechanism by which 5-HT levels can influence complex behavioural and metabolic features of these pathologies is not yet fully understood. Serotonin signalling modulates locomotion behaviour and allows the worms to respond to environment changes, directly impacting foraging behaviour.

Synthesis of 5-HT

5-HT is synthesised from tryptophan through tryptophan hydroxylase (TPH-1), making 5-hydroxy-tryptophan (5-HTP) [Figure 2] (Sze et al., 2000). GTP cyclohydrolase I (CAT-4) is also needed for this process (Loer & Kenyon, 1993). 5-HT is synthesised from 5-HTP using BAS-1 [Figure 2] (Hare & Loer, 2004) and is transported into cells using the 5-HT reuptake transporter (SERT) MOD-5 (Ranganathan et al., 2001) and is broken down into 5-hydroxyindole acetic acid (5-HIAA) using monoamine oxidase AMX-2 (Wang et al., 2017). In worms, *tph-1* is expressed in these neurons: ADF, NSM, AIM, RIH, and in adulthood, HSN (in hermaphrodites), CP0-CP06 and B-type ray neurons (R1B, R3B, R9B). The last two types being male-specific (Serrano-Saiz et al., 2017; Sze et al., 2000). Furthermore, under hypoxic conditions (1% O_2), TPH-1 is upregulated in ASG neurons, with increased upregulation increased of NSM and ADF neurons (Pocock & Hobert, 2010).

5-HT, locomotion, and foraging behaviour

Food levels influence locomotion behaviour in *C. elegans* and adaptive responses from fasting to feeding require 5-HT signalling. Behavioural analysis of *tph-1* mutants showed that they have increased fasting quiescence, decreased feeding quiescence, and increased roaming while feeding compared to WT (Churgin et al., 2017; Flavell et al., 2013). This indicates that endogenous 5-HT signalling is needed for both wild-type fasting and feeding behaviours. 5-HT is released in response to food and is required for adapting locomotory behaviours during an appropriate feeding state.

The production of 5-HT occurs primarily in the two head neuron pairs ADF and NSM, as well as one neuron pair close to the vulva, the HSN. Studies which have used fasting conditions, or *daf-2* mutations, showed that there was a reduction in the *tph-1* reporter expression

exclusively in the ADF neurons. This indicates that reduced nutrient signals cause a reduction in 5-HT production in ADF neurons which potentially influence behaviour.

Indeed, the reduced roaming and dwelling behaviour of fasted *tph-1* mutants was restored by expressing wild-type TPH-1 in the ADF neurons (Song et al., 2013). In contrast, wild-type TPH-1 expression in the NSM neurons of fasted *tph-1* mutants caused a further reduction in roaming. Taken together, 5-HT production in ADF neuron is needed for wild-type fasting behaviour, and with respect to roaming, is antagonized by the 5-HT production in the NSM neuron. Treatment with exogenous 5-HT suppresses wild-type quiescence and roaming and promotes dwelling, a well-documented observation that 5-HT is slowing down locomotion (Flavell et al., 2013; Ranganathan et al., 2000). Serotonin is important for food-deprived worms to be able to dramatically slow down their locomotion rate when they detect a bacteria lawn. This "enhanced slowing response" requires serotonin signalling, as *bas-1* and *cat-4* mutants demonstrate impairments in enhanced slowing (Sawin et al., 2000). The deficit of *bas-1; cat-4* double mutants can be rescued after exogenous treatment with serotonin.

Serotonin and dopamine have distinct influence in locomotion behaviour of worms. Serotonin signalling causes a much more dramatic slowing response than dopamine signalling, as food-deprived worms totally stop upon food detection, whereas well-fed worms simply reduce their locomotion rate (see dopamine section). Both serotonin and dopamine play a role in foraging behaviour in worms. Serotonin signalling ensures that food-deprived worms stay on the food source once they have encountered it, whereas dopamine signalling promotes well-fed worms to stay in the proximity of food, without stopping them from exploring other potential food sources.

5-HT receptors and foraging behaviour

5-HT operates through four G-protein-coupled receptors, (SER-1, 4, 5, 7) and two 5-HT-gated chloride channels (MOD-1 and LGC-40) (Ringstad et al., 2009), controlling various processes in worms (Carre-Pierrat et al., 2006; Gürel et al., 2012; Sawin et al., 2000). Exogenous 5-HT treatment was used on single 5-HT receptor mutants, under fasting conditions, to distinguish which of them are important for locomotory-foraging behaviour. *ser-5* and *mod-1* mutants were found to respond more substantially to exogenous 5-HT treatment in comparison with WT animals. 5-HT increased the quiescence of *ser-5* mutants, while roaming was suppressed. This indicates that SER-5 promotes movement in response to 5-HT. On the other hand, exogenous 5-HT suppressed quiescence and dwelling of *mod-1* mutants, while roaming increased. This indicates that 5-HT acts via MOD-1 to suppress movement. It has been reported in many studies that MOD-1 promotes dwelling behaviour in worms (Flavell et al., 2013; Sawin et al., 2000). Taken together, 5-HT operates antagonistically through SER-5 to increase roaming and suppress quiescence, and through MOD-1 to increase dwelling and suppress roaming.

SER-5 is a G-protein-coupled receptor acting in the ASH neurons to mediate behavioural responses to octanol and in muscles to mediate egg laying (Hapiak et al., 2009; Harris et al., 2009). SER-5 is acting in both muscles and neurons to promote movement in response to 5-HT. In addition, MOD-1 is a 5-HT-gated chloride channel that operates in the nervous system to mediate locomotion, learning, and regulation of fat levels (Flavell et al., 2013; Noble et al., 2013; Ranganathan et al., 2000; Yun Zhang et al., 2005b). MOD-1 is acting in the AIY neurons and is important for the sufficient restoration of wild-type responses to 5-HT.

In addition, pharyngeal pumping rate is reduced when mutations are present in receptor genes (except *ser-7*) (K. S. Lee et al., 2017). It has been shown that SER-1 is required for fast pumping, whereas SER-4 is required to sustain the high pumping rate and reduce long pauses,

rendering it essential for a dwelling-feeding state. The neurons which are involved in these behaviours are NSM and HSN (K. S. Lee et al., 2017; Song et al., 2013; Waggoner et al., 1998). In addition to that, 5-HT increases egg-laying while worms are on food. Mutants lacking tryptophan hydroxylase, demonstrate enhanced propensity for developmental arrest. This indicates that the absence of the positive food signal, 5-HT, impairs development into adulthood (Sze et al., 2000). Taken together, all the difference findings regarding of 5-HT operation indicate that, foraging behavioural states can be regulated by serotonin and are dependent on the receptor and nutritional status of the worm.

Octopamine

Octopamine is structurally similar to the fight-or-flight hormones adrenaline and noradrenaline (Yong Li et al., 2017). It is found primarily in invertebrates; however, it exists in trace amounts in mammals. Octopamine is involved in starvation-related behaviours. An example of this is found in *Drosophila melanogaster*, where octopamine signalling is needed for the hyperactivity of starved animals. It has been proposed that hyperactivity demonstrates the drive of starved animals to detect food sources. In worms, octopamine inhibits behaviours such as egg laying and feeding, and modulates response rate to aversive stimuli (Alkema et al., 2005; Mills et al., 2012; Wragg et al., 2007). Studies in both flies and worms showed that octopamine induces hyperactivity upon fasting, as well as a wake-promoting state. Studying the role of these in signals in invertebrates may help us understand the molecular basis of more complex behaviours in mammals, such as aggression and anxiety.

Synthesis of octopamine

Octopamine is synthesised from tyramine by tyramine β-hydroxylase (TBH-1) [figure 2] (Alkema et al., 2005; Yong Li et al., 2017). Examination of TBH-1 expression revealed that the production of octopamine is restricted to the RIC interneurons and the gonadal sheath cells (Alkema et al., 2005). Neurons that produce octopamine can also use/and release tyramine as neurotransmitter [Figure 2]. Also, tyramine needs to be synthesized and/or transported into these neurons to make octopamine. Octopamine acts through 5 receptors, OCTR-1, SER-3 (which is shared with tyramine), SER-6, SRX-43, and SRX-44. Octopamine and tyramine can both bind to OCTR-1 with high affinity. In addition, OCTR-1 is expressed in the ASI, ASH, AIY, ADE and CEP neurons, as well as in a non-neuronal manner in the spermatheca and uterine toroid cell (Wragg et al., 2007).

Octopamine, locomotion, and foraging behaviour

Studying the function of octopamine can be challenging due to the fact that cells that produce octopamine can also release tyramine as neurotransmitter, or tyramine as the precursor in octopamine synthesis. *tbh-1* mutants demonstrate behavioural impairments similar to *tdc-1* mutants which are unable to synthesize tyramine or octopamine, making the sole study of octopamine a challenging task. In this section, the action of octopamine with regards to locomotion and foraging behaviour of the worms will be discussed.

Under fasting conditions, *tdc-1* mutants demonstrate enhanced quiescence, dwelling, and reduced roaming behaviour in comparison with WT animals. *tdc-1* mutants are unable to produce both tyramine and octopamine. Addition of exogenous octopamine suppressed the increased fasting quiescence in *tdc-1* mutants, as well as in wild type. Nevertheless, exogenous tyramine caused a slight increase in fasting quiescence, but not in *tdc-1* mutants. Taken

together, these results indicate that octopamine is needed for wild-type levels of fasting quiescence, and also indicate that octopamine can suppress quiescence.

Dwelling and roaming is being affected by tyramine and octopamine with them having opposite roles in locomotion. More specifically, treatment with tyramine caused increased dwelling and reduced roaming in *tdc-1* and wild-type animals. In contrast, treatment with octopamine caused slightly reduced dwelling and increased roaming. Taken together, these results indicate that octopamine and tyramine influence locomotion in a different manner. Tyramine promotes reduced locomotion, characteristic of fed worms, while octopamine promotes increased locomotion, characteristic of fasted worms.

Hyperactivity of fasted animals in both worms and flies is controlled by octopamine (Yang et al., 2015). *tbh-1* mutants which are unable to synthesise octopamine, demonstrate enhanced quiescence, dwelling, and reduced roaming under fasted conditions compared to wild-type animals. Exogenous treatment with octopamine was able to reverse the quiescence and roaming defects, but not dwelling. Experiments in worms also showed that octopamine acts to promote movement under both fed and fasted conditions.

Octopamine receptors and foraging behaviour

In worms, SER-3 and SER-6 are Gq-coupled GPCRs on cholinergic SIA neurons and are required for effects of octopamine on locomotion. Exogenous treatment with octopamine significantly increases roaming and decreases quiescence in wild-type and *octr-1* animals. In contrast, *ser-3* and *ser-6* mutants do not respond to exogenous octopamine. These results indicate that octopamine requires SER-3 and SER-6 receptors to influence locomotion. It has been reported that SER-3 and SER-6 operate in the SIA neurons to mediate the transcription of the starvation-

response transcription factor, cAMP response element-binding protein (CREB) (Yoshida et al., 2014).

Rescue experiments of SER-3 an SER-6 in the SIA neurons were able to restore wild-type locomotory response to octopamine. More specifically, CREB transcription factor in the SIA neurons is activated in response to fasting or treatment with exogenous octopamine (S. Suo et al., 2006; Yoshida et al., 2014). In order for this to occur, functional SER-3 and SER-6 are required. These receptors act in the SIA neurons and mediate the effects of octopamine on locomotory behaviour under starved conditions, either through dimerization or threshold effect.

C. elegans roaming, dwelling, quiescence, and other food-related behaviours

C. elegans is considered as a powerful animal model to investigate behavioural related questions. Several studies have described worms' behaviour in detail, providing valuable knowledge and direction for future studies. The bacteria-eating roundworm changes its behaviour in the presence or absence of food. More specifically, *C. elegans* alternates between active and inactive phases under standard conditions (Bargmann, 1993; De Bono & Maricq, 2005; Schafer, 2005). Worms on OP50 found to spend 80% of their time in an inactive state known as dwelling and 20% in an active state known as roaming (Fujiwara et al., 2002; Boris Borisovich Shtonda & Avery, 2006a). It has been shown that during dwelling, animals maintain a low speed and alternate frequently between backward and forward movement and being restricted in a small area of the bacteria lawn. During roaming, animals demonstrate straight displacement of sustained forward movement with no alterations in direction. A third behavioural state exists, known as quiescence and is characterized by complete absence of movement or pharyngeal pumping (Trojanowski & Raizen, 2016). Usually under well fed

conditions quiescence periods are limited and thus, mostly dwelling and roaming exist. However, under fasted and re-fed conditions, quiescence periods found to be increased. Roaming, dwelling, and quiescence together, comprise the overall exploratory behaviour in worms (Ben Arous et al., 2009). *C. elegans* exploratory behaviour provides a valuable tool to study the impact of food in animal behaviour and homeostasis.

C. elegans detects food through two pairs of neurons, AWA and AWC, which act to recognize attractive odorants (Pereira & van der Kooy, 2012; Troemel et al., 1997). On the other hand, worms can identify and avoid harmful chemicals and pathogens through the neuronal pair, AWB (Pradel et al., 2007). Avoidance of pathogenic bacteria can be achieved either by odour or after ingestion (Meisel et al., 2014; Schulenburg & Ewbank, 2007; Zhang et al., 2005). It has been shown that *C. elegans* does not actively avoid laboratory food sources such as *E. coli* OP50 or HT115 however, these bacterial diets can be pathogenic over time (Couillault & Ewbank, 2002). *C. elegans* chooses a less pathogenic diet such as *B. subtilis* against OP50 if given dietary choice (Clark & Hodgkin, 2014; Gusarov et al., 2013). In contrast, pathogenic bacteria such as *Pseudomonas aeruginosa* PA14, are initially attractive to the worm but within a short time period the worm begins to avoid them (Beale et al., 2006). Bacterial avoidance also found when non-pathogenic bacteria lawns are specifically engineered to cause the knockdown of crucial *C. elegans* genes (Melo & Ruvkun, 2012). Studies indicate that this *C. elegans* behaviour derives from the ingestion of bacteria which synthesize compounds that disable crucial cellular pathways like protein synthesis and mitochondria operation (Breen et al., 1986; Huss et al., 2002; Tercero et al., 1996).

C. elegans can detect the nutritional quality of the food source and alter its behaviour and physiology accordingly (Leon Avery & Boris B. Shtonda, 2003). As an example, when worms found themselves in a less desirable diet, they increase pharyngeal pumping, pharyngeal autophagy, and demonstrate extended roaming periods. Thus, worms will change their

behaviour to locate optimal food sources, to achieve maximum energy acquisition, and sustain homeostasis (Avery et al., 1993; Chiang et al., 2006; Kang & Avery, 2009). It has been shown that bacterial food acts as a sensory stimulus for *C. elegans* however, limited work has been done on how food acts as reinforcement, providing feedback in the worm upon ingestion (Boris Borisovich Shtonda & Avery, 2006a).

C. elegans as a genetic tool

Conserved behaviours and mechanisms, as well as the simplicity of its nervous system renders this model ideal to study neuronal circuits (Corsi et al., 2015). Neuron ablation using a laser can be used to target individual neurons and study their function. *C. elegans* is the only organism who's entire neuronal network is mapped by electron microscopy reconstruction, as well as, by other methods, such as the multicolour *C. elegans* transgene called "NeuroPAL" (a Neuronal Polychromatic Atlas of Landmarks) (Yemini et al., 2021). The proteome of *C. elegans* has been reported to share 83% homology to humans and can be easily manipulated by a process known as RNA interference (RNAi) and by other methods, such as usage of genetic mutants and genome editing/CRISPR-Cas9. RNAi method simply involves the incorporation of dsDNA-expressing bacteria into their food source, to silence specific protein coding mRNA (The *C. elegans* Sequencing Consortium, 1998; Corsi et al., 2015).

Working with *C. elegans* is a quick and easy process which allows large number of progeny (millions or billions) to grow in a small space within a week. This advantage is of crucial importance in genetics, making possible large scale genetic screens to cover the entire genome. Discoveries and breakthroughs such as the study of insulin signalling in ageing found to be widely conserved, including in mice and potentially in humans (Friedman & Johnson, 1988; Soerensen et al., 2015) . Studies in worms revealed lifespan regulating genes involved in different longevity processes, including the Target of Rapamycin pathway, Insulin signalling

pathway and many others (Vellai et al., 2003). Apart from this, *C. elegans* genetics, provide valuable insights about mitochondria function, which will be discussed in more detail later (Van Raamsdonk et al., 2010). Finally, the small size of the worms makes them tractable and reliable for the measurement of long scale behaviours, see below (Owen-Smith et al., 2010).

C. elegans and longevity

Recently, much attention focused on single gene mutations which lead to lifespan extension. The most robust effect on lifespan extension and health span derived from gene mutations which either influence the quantity of food ingested or interfere with animals' perception of the amount of food ingested. The effects of these mutations such as lifespan extension, health span, and stress resistance, indicate the existence of a complex molecular and genetic network which integrates diet acquisition, utilization, and organismal physiology (Gottlieb & Ruvkun, 1994; Harder et al., 2004; Züchner et al., 2004). *C. elegans* can utilize a wide range of bacterial diets found in the wild as well as, is able to adapt to the food source provided (Boris Borisovich Shtonda & Avery, 2006a). These two abilities of the bacterivore worm led to the elucidation of molecular mechanisms which control dietary adaptation. Signe gene mutations of animals', which fed with the two most common bacterial diets in the laboratory (*E. coli* B-OP50 and *E. coli* K12-HT115), revealed the existence of diet-gene pares or genes crucial for a specific diet type but nonessential on others (Khanna et al., 2014; Maier et al., 2010; Pang et al., 2014; Xiao et al., 2015; Pang et al., 2014). Even though both diets are *E. coli* based, it is evident that they are nutritionally distinct, and ingestion of these diets shown to influence organismal metabolism differently.

C. elegans signalling pathways overview

Insulin and IGF1-like signalling (IIS) pathway

In animals, nutrient status, growth, and metabolism are coupled through the insulin and IGF1 like signalling (IIS), which is a highly conserved process. IIS pathway is involved in animals' behavioural responses especially associated with food. The degree of evolutionary conservation in the general IIS pathway is evident and found in both simple organisms such as *C. elegans* and in more complex such as humans.

One significant difference between worms and humans is the number of IIS receptors. In mammals there are three IIS receptors, the insulin receptor (INSR), IGF1R, and insulin receptor-related receptor (IRR) (Boucher et al., 2010; Deyev et al., 2011; Hirayama et al., 1999; Mathi et al., 1995). In contrast, worms found to have only one insulin receptor, encoded by the *daf-2* gene. DAF-2 receptor is analogous in identity to IGF1R and INSR in mammals.

Nevertheless, there are some essential differences among organisms (Papatheodorou et al., 2014). In *C. elegans*, insulin signalling acts upon the interaction between insulin-like peptides (ILP), which are released as a cell-non autonomous signal from distant cells, and their receptor DAF-2 (Murphy et al., 2013; Kimura et al., 1997; Li et al., 2003). ILPs levels fluctuate according to the sensory input and nutrient availability. In worms, there are approximately 40 genes encoding putative ILPs, including both agonist and antagonist of the DAF-2 receptor (Fernandes de Abreu et al., 2014; W. Li et al., 2003; Pierce et al., 2001).

The mammalian insulin-like superfamily contains 10 ILPs. However, only insulin and insulin-growth factors (IGF-1 and IGF-2) demonstrate IIS-related tyrosine kinase binding (specifically involving the IGF1R and INSR). IGF-1 and IGF-2 levels are controlled by growth hormone signalling and IGF-binding proteins (Werner et al., 2008). DAF-2 receptor in worms, demonstrates approximately 30% sequence homology with the human INSR receptor. More

specifically, the two heterodimers with an extracellular α domain composition of IIS receptors found to be conserved across species (Koutarou D. Kimura et al., 1997).

Upon DAF-2 and ILPs binding, there is an activation of the *C. elegans* orthologue of the mammalian class 1 phosphatidylinositol 3-kinase (PI3K) group of kinases, AGE-1. In turn, AGE-1 generates PIP3 from PIP2 in the plasma membrane, initiating a signalling cascade via PDK-1 which leads in the activation of AKT1/2 and SGK1 phosphorylation [Figure 3] (Hertweck et al., 2004; Morris et al., 1996; Paradis & Ruvkun, 1998; Paradis et al., 1999). Then, AKT-1 phosphorylates the transcription factors DAF-16 and SKN-1 which enable a transcriptional program related with longevity and restricted growth (Murphy et al., 2013; Ogg et al., 1997; Tullet, 2015).

Reduced insulin signalling through genetic manipulation results in lifespan extension up to tenfold in case of *age-1* mutants compared to wild type (Kenyon et al., 1993; Arantes-Oliveira et al., 2003). Research in mice and *D. melanogaster* has revealed that pro-longevity effects of downregulated insulin signalling are well-conserved (Blüher et al., 2003; Clancy et al., 2001; Kappeler et al., 2008; Maria E Giannakou et al., 2004; Selman et al., 2008). In addition, it has been suggested that specific alleles of FOXO3A (*daf-16* in *C. elegans*) found overrepresented in long lived humans (Li et al., 2009).

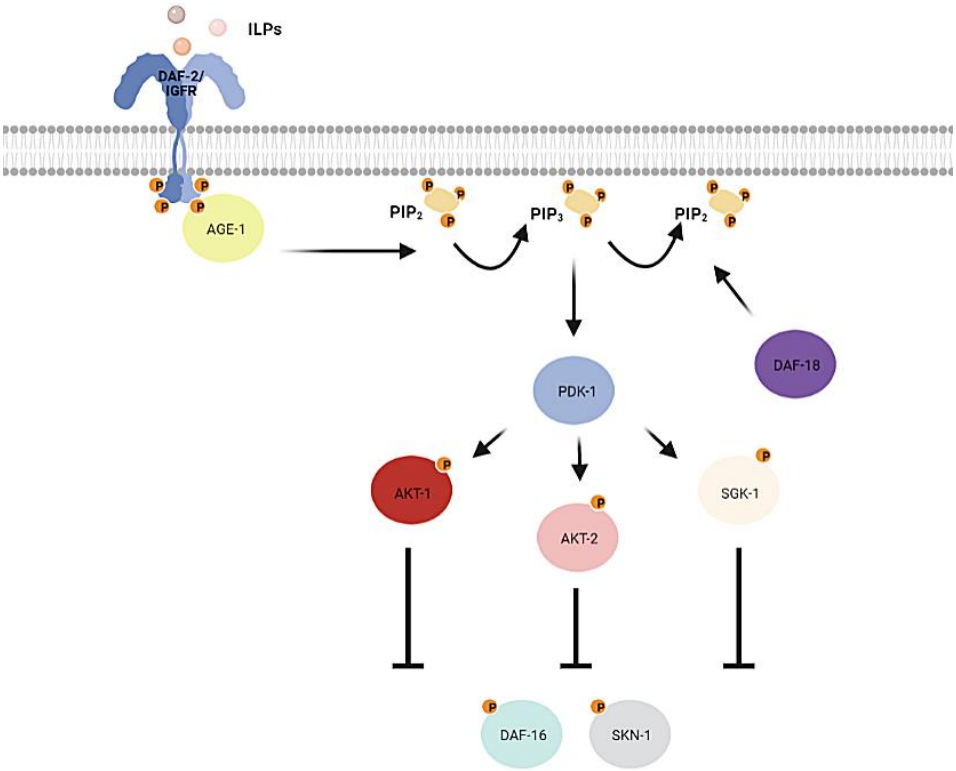


Figure 3: Insulin IGF-like signalling in *C. elegans*. DAF-2 is activated (or repressed by antagonistic ILPs) by its ligands, ILPs. This results in the activation of AGE-1 which drives the conversion of PIP2 to PIP3 and ultimately leads to the inhibitory phosphorylation of transcription factors SKN-1 and DAF-16. Active DAF-16/FoxO and SKN-1/Nrf, in the absence of IIS signalling, regulate genes involved in (oxidative) stress responses, development, UPR, mitochondrial function and therefore, longevity. Orange circles represent phosphorylation. Adapted from (Coleen T. Murphy, 2013).

Transforming Growth Factor-β (TGF-β) signalling pathway

The TGF-β superfamily of intercellular signalling molecules is a fundamental means of cell-to cell communication in eukaryotic animals (Robertis, 2008). TGF-β signalling is essential during development and homeostasis for several vital processes (M. Y. Wu & Hill, 2009). That includes patterning the embryonic body plan and later specifying and sustaining cell identities. Dysregulated TGF-β signalling leads to birth defects, disorders, and diseases in humans (Padua & Massagué, 2009; M. Y. Wu & Hill, 2009). In humans there are more than 30 TGF-β members (Patterson & Padgett, 2000). In contrast, *C. elegans* has only five TGF-β ligands with non-redundant, non-lethal functions (Patterson & Padgett, 2000). Between *C. elegans* and higher

organisms there is high conservation of TGF-β signalling pathways at both molecular and functional levels.

The *daf-7* pathway was described initially by its control of the dauer/continuous developmental switch. Under favourable growth conditions, in early larval stages, the DAF-7 TGF-β ligand is secreted by the ASI chemosensory neurons [Figure 4]. DAF-7 binds to the widely expressed DAF-1 and DAF-4 type I and type II TGF-β receptors to promote reproductive growth (Estevez et al., 1993; Pierce et al., 2001; Schackwitz et al., 1996). Under adverse conditions, such as increased population density, low food availability, and high concentrations of secreted pheromone, *daf-7* expression is reduced. This results in the downregulation of TGF-β and insulin signalling, and promotes entry into the alternative third larval dauer stage (Gottlieb & Ruvkun, 1994). In addition, *daf-7* TGF-β pathway regulates the expression of all chemoreceptors known to be expressed in the ASI chemosensory neurons, as well as chemoreceptors expressed in the ASH sensory neurons (Nolan et al., 2002).

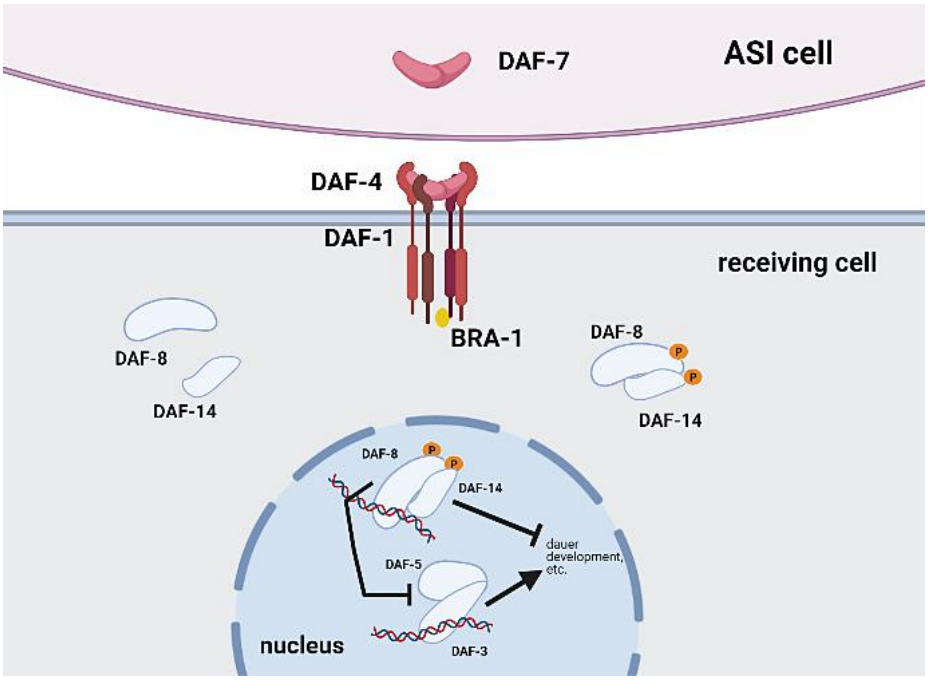


Figure 4: Illustration of the TGF-β pathway (dauer specific). DAF-7 promotes continuous, non-dauer development. A heterotetrameric receptor composed of two DAF-1 type I receptor and two DAF-4 type II receptor subunits receives the DAF-7 signal. BRA-1/BMP receptor associated protein (BRAM) is a negative intracellular regulator of DAF-1. The DAF-7 signal is transduced by DAF-8 and DAF-14 Smads. These components, when activated, inhibit the functions of DAF-3/Co-Smad and DAF-5/Sno/Ski, which promote dauer development. Adapted from (Gumienny, 2013).

cGMP signalling pathway

As in other organisms, G protein-coupled receptors (GPCRs) play a fundamental role in worms' development and behavioural responses (Bargmann et al., 2006; Dryer, 1999; Tsunozaki et al., 2008). Examples of these can be found when worms alter their motility behaviour in response to food, or when chemo-attractants and repellents modify chemotaxis, and avoidance behaviour, respectively. Furthermore, the cGMP pathway promotes entry into, and exit from, the alternative dauer developmental stage in *C. elegans* (Dryer, 1999; Tsunozaki et al., 2008).

The behaviours which respond to chemosensory cues are regulated primarily by the amphid chemosensory organs, which contain eleven pairs of chemosensory neurons (Bargmann et al.,

2006). A specific set of candidate receptor genes found to be expressed in each amphid sensory neuron detect a distinctive set of attractants, repellents, or pheromones. There are about 500 to 1000 different GPCRs expressed in chemosensory neurons and these may be supplemented by alternative sensory pathways as well (Dryer et al., 1999; Tsunozaki et al., 2008). Downstream of GPCRs there are two signal transduction systems which are prominent in chemosensation. The first one, uses cGMP as a second messenger to open cGMP-gated channels and the second relies upon TRPV channels [Figure 5]. Kinases and phosphatases modulate and fine-tune these sensory pathways. *C. elegans* detects chemicals through chemosensory neurons that penetrate the cuticle and expose their sensory cilia to the environment: the amphid, phasmid, and inner labial neurons. In general, chemosensory neurons belong to bilaterally symmetric pairs in which the left and right members of each class are structurally identical. In *C. elegans*, olfactory and pheromone-sensing neurons use DAF-11 and ODR-1 as ligand-independent receptor-like guanylate cyclases (RGCs) downstream of G protein signalling (Bargmann et al., 2006; Dryer et al., 1999; Tsunozaki et al., 2008).

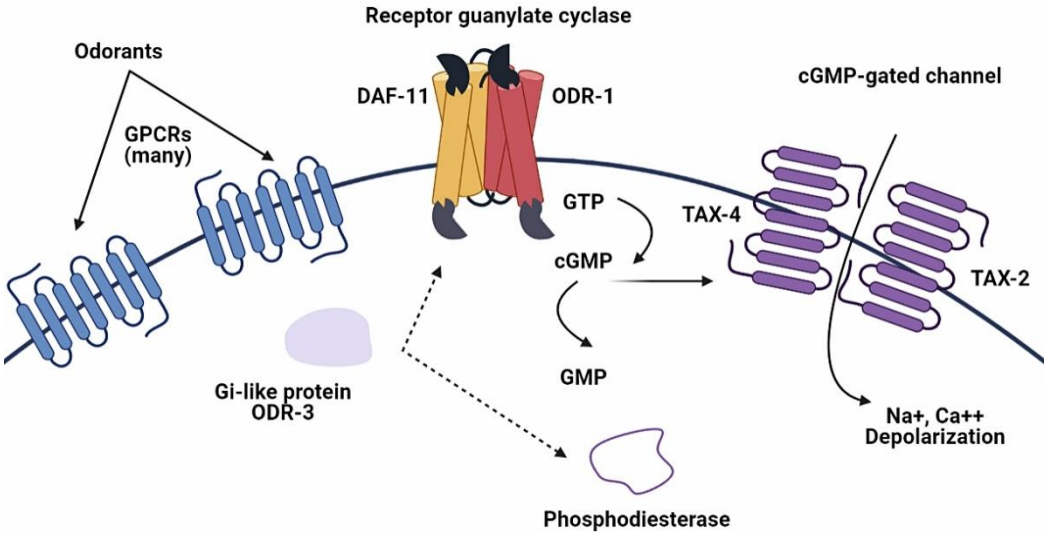


Figure 5: Potential signal transduction pathway for odour detection in AWC cilia. Studies have shown that G protein-coupled receptors (GPCRs), GI-like proteins, receptor guanylate cyclases, and the

cGMP-gated channel have crucial roles in odour detection through AWC neurons. Suggested model indicates that odours are sensed by GPCRs that activate the GI-like protein ODR-3. ODR-3 regulates either cGMP production by receptor-like guanylate cyclases (ODR-1, DAF-11), or cGMP consumption by phosphodiesterases. cGMP opens the cGMP-gated channels encoded by TAX-2 and TAX-4 to depolarize the cell. Adapted from (Bargmann Cornelia I, 2006).

Prevention of dauer development requires *daf-11*, which is expressed in the ASI and ASJ neurons that control dauer formation and recovery. In addition, *daf-11* is expressed in AWC, AWB, and ASK neurons and is needed for AWC and AWB olfactory behaviours (Bargmann et al., 2006). Furthermore, *daf-11* plays an important role in ASE water-soluble chemotaxis however, it is not expressed in ASE neurons, indicating that its function in these neurons may be non cell-autonomous. *daf-11* encodes a transmembrane guanylyl cyclase (TM-GC) and provides the first example of a TM-GC that functions in taste and smell transduction. *daf-11* mutants demonstrate chemosensory impairments to pheromone and to volatile and non-volatile attractants (Bargmann et al., 2006). In addition, DAF-11 operation provides the first genetic model for this class of proteins. The cyclic GMP analogue 8-bromo-cGMP suppresses the pheromone response defect in *daf-11* mutants, showing that DAF-11 has a role in guanylyl cyclase operation. *daf-11::gfp* expression is observed in five specific pairs of sensory neurons (ASJ, AWC, ASI, ASK, and AWB) in a pattern consistent with most *daf-11* mutant phenotypes (Bargmann et al., 2006). In addition, *daf-11* mutants demonstrate avoidance defects of the volatile repellent 2-nonanone.

The Mitochondrion: An organelle important for energy balance

Mitochondria, the powerhouses of the cell, are important organelles for energy production. Despite well-studied roles of mitochondria in energy generation, the involvement of mitochondrial dynamics in animal behaviour demands more research. In this section, mitochondrial origin, structure, and dynamics will be analysed. In addition, mitochondria-related pathologies will be discussed.

Origin of mitochondria

The recognition of intracellular structures which correspond to mitochondria dates back to the 19th century (Ernster et al., 1981). In 1890, Altmann observed a cytoplasmic structure which resembled a bacterium and lived as an independent organism within the cell (Altmann, 1890). The endosymbiotic theory was proposed in 1926 by Ivan Willin and continued by Lynn Margulis in 1967, hypothesizing the origin of mitochondria (Sagan, 1967; Wallin, 1926). The theory states that around 1.5-2 million years ago an early ancestor of eukaryotic cells (host) absorbed α-proteobacteria (endosymbiont) (Gray, 2012; Gray et al., 1999). This endosymbiotic relationship, under the pressure of evolution caused the exchange, or the loss, of genes from the bacterium to the host genome and led to the evolution of the mitochondrion as we know it. The unique characteristics of mitochondria such as the existence of a double membrane, the presence of distinctive circular mitochondrial genome, the ability of host-independent division, and the homology between mitochondrial and bacterial genes support this theory.

Structure of mitochondria

Mitochondria structure is comprised by two membranes, an outer membrane (OM) and an inner membrane (IM) (Schiavi & Ventura, 2014; Tsang & Lemire, 2003). The region between OM and IM is known as inter membrane space (IMS). The area which IM surrounds is known as the matrix. IM and OM show morphological and structural differences. IM is described as a convoluted membrane with protrusions that reach into the matrix known as cristae. In contrast, OM is described as a smooth membrane, responsible for the characteristic, elongated cylinder shape of mitochondria. The diameter of mitochondrial structures is around 0.5-1 μm. Mitochondrial membranes do not only differ morphologically but also structurally. More specifically, OM includes transport proteins which allow the formation of channels across the membrane allowing the movement of particles with a size smaller than 5000 Daltons into the IMS but not the movement through the IM. One reason for that is the enrichment of the IM with cardiolipin, a phospholipid that inhibits the movement of ions into the matrix. Similarly, IM contains a variety of transport proteins which selectively permit the passage of small molecules essential for mitochondrial operation within the matrix. The IM of mitochondria contains five-electron transport chain (ETC) complexes, with four of them mediating redox-reactions in the oxidative phosphorylation (OXPHOS) pathway, oxidizing sugar, fats, and proteins, finally leading to the production of adenosine triphosphate (ATP) in the fifth complex, the ATPase. In addition, mitochondria have distinctive circular genome, mitochondria DNA (mtDNA), which is found in the matrix. *C. elegans* mtDNA is slightly smaller in comparison with the human mtDNA. More specifically, *C. elegans* mtDNA is made up of 13,794 nucleotides and encodes 36 genes: 2 ribosomal RNAs (12S rRNA and 16 rRNA), 22 transfer RNAs, and 12 ETC subunits, in contrast with the human mtDNA, which is made up of 16,569 nucleotides and encodes 37 genes (Maglioni & Ventura, 2016). Also, *C. elegans* mtDNA

is lacking ATP8 gene which encodes a subunit of complex V encoded in all mammalian mtDNA (Tsang & Lemire, 2003).

Mitochondrial dynamics

Mitochondria are dynamic organelles and often adapt their morphology in response to cellular needs (Okimoto et al., 1992). Cells control mitochondria morphology in conditions where energy is required, or when nutrient availability is altered. They do this by joining together (fusion) or dividing (fission). Thus, changes in the mitochondrial network must occur dynamically, always in coordination with cell requirements [Figure 6]. Research in mitochondrial dynamics revealed a fascinating relationship between energy demand, supply balance, and mitochondrial morphology. Changes in mitochondrial morphology are highly-controlled processes within cells and dependent on the activity of evolutionary conserved GTPases (Liesa & Shirihai, 2013).

Mitochondria network can be found in a tubular (fused/elongated) or fragmented (separated) state (Okimoto et al., 1992). The morphological appearance of mitochondria is controlled by fusion and fission events. Mitochondrial fusion requires mitofusins for the fusion of the outer membrane (MFN1 and MFN2 in mammals, Fzo1 in budding yeast, and FZO-1 in *C. elegans*), and for the inner membrane fusion (OPA1 in mammals, Mgm1 in budding yeast, and EAT-3 in *C. elegans*) (Kanazawa et al., 2008; Liesa & Shirihai, 2013). Mitofusin proteins establish dimeric associations across the outer mitochondria membranes which tether neighbouring mitochondria and induce fusion via GTP hydrolysis. OPA1 is a dynamin-like GTPase important for the fusion of the inner mitochondrial membrane and for the maintenance of the internal cristae network.

The conserved regulation of mitochondrial fission is demonstrated in yeast, invertebrates, and mammals, and requires the dynamin-related protein DNML1 (Dnm1 in budding yeast and DRP-1 in *C. elegans*). Mitochondrial fission is mediated by the dynamin-related protein 1 (Drp1) and its association with the binding partners Fis1 and/or mitochondrial fission factor (Mff) (Rolland et al., 2009; Y. Yoon et al., 2003). The efficiency of mitochondrial fission depends upon a wide range of interactions such as phosphorylation, sumoylation, and ubiquitination with Drp1 (Cribbs & Strack, 2007; Otera et al., 2010). Phosphorylation of Drp1 at S616 by Cdk1/cyclin B leads to enhanced Drp1 fission efficiency (Cribbs & Strack, 2007). In contrast, phosphorylation of Drp1 at S637 by PKA reduces fission by maintaining Drp1 in the cytosol, whereas dephosphorylation of S637 by calcineurin leads to the translocation of Drp1 to the mitochondria promoting mitochondrial fission (Cribbs & Strack, 2007; Harder et al., 2004; Otera et al., 2010).

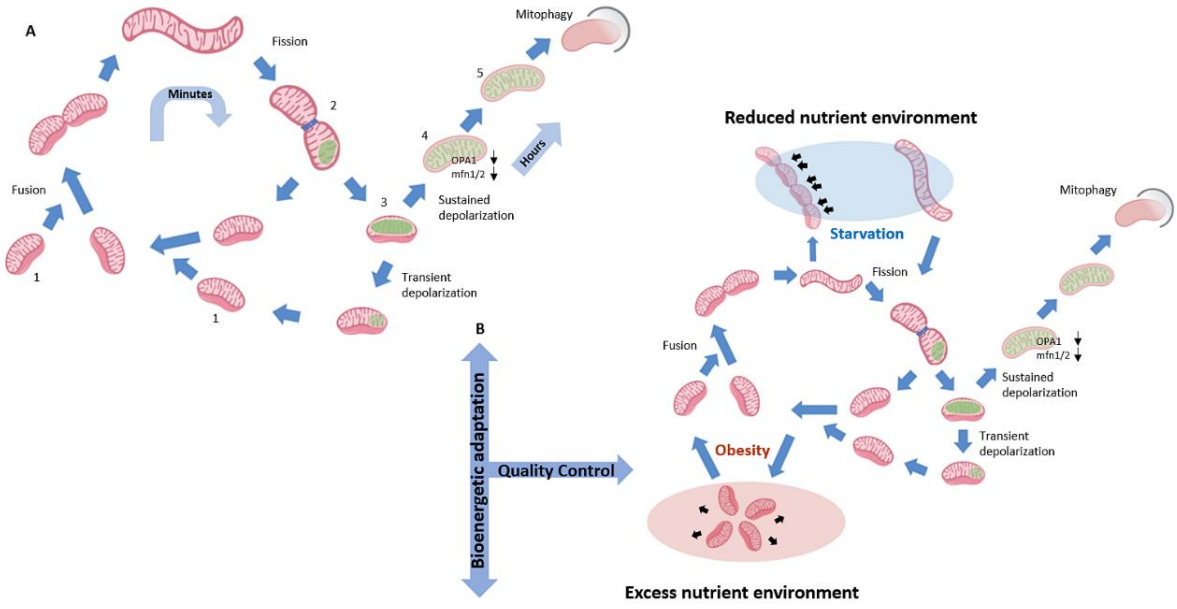


Figure 6: Mitochondrial dynamics and response to nutrient availability. A) The mitochondria life cycle is comprised of repetitive mitochondria fusion and fission events. Mitochondrial fusion is controlled by mitofusin (Mfn) 1 and Mfn 2 (both essential for the fusion of the mitochondrial outer membrane) and optic atrophy 1 (Opa1) (essential for the fusion of the inner mitochondrial membrane).

Fusion leads to the generation of a network where two mitochondria mix and reorganize their components (1). Mitochondrial fission is controlled by dynamin-related protein 1 (Drp1), mitochondrial fission factor (Mff), and fission 1 (Fis1). Minutes after, fission leads to the division of fused mitochondria into two daughter mitochondria with distinct membrane potentials (2). The daughter mitochondrion with the higher membrane potential returns immediately to the cycle of fusion and fission, in contrast to the daughter mitochondrion with the depolarized membrane potential (green colour) which remains in solidarity until the recovery of membrane potential occurs (3). The persistent depolarized membrane potential (green colour) of the daughter mitochondrion inhibits its ability to fuse and promotes its recruitment to the pre-autophagic pool which eventually leads to autophagy with a delay of 1-3 hr. (4,5). There is an accumulation of the tensin homolog-induced putative kinase protein 1 (PINK1) at the surface of the dysfunctional mitochondria which leads to the recruitment of Parkin. Then, Parkin-induced ubiquitylation of the outer membrane promotes the recruitment of autophagosomes leading to degradation upon fusion with a lysosome. **B)** Energy demand and nutrient availability influence the life cycle of mitochondria by extending post fusion (elongation) or post fission (fragmentation) states. Enhanced fusion or restricted fission activity leads to mitochondria elongation. Mitochondrial elongation occurs in starvation, acute stress, and senescence where increased energy efficiency is required. Enhanced fission or restricted fusion activity leads to shorter mitochondria. This occurs in states where reduced bioenergetic efficiency is required (with increased uncoupled respiration). High energy supply demands bioenergetic adaptation. This is mediated by the arrest of the mitochondria life cycle due to prolonged exposure to excess nutrient conditions, compromising the quality control of mitochondria and leading to reduced longevity. (Adapted from (Liesa & Shirihai, 2013), generated using BioRender)

Mitochondrial fusion

In conditions where nutrient supply is limited fusion events occur to promote cell survival (Gao et al., 2014; Hailey et al., 2010). Responding to starvation mitochondrial fusion occurs, preventing them from autophagic degradation (Gomes et al., 2011). Fusion is a key factor for autophagosome biogenesis during starvation (Hailey et al., 2010). It has been shown that an extended rate of mitochondrial fusion can be achieved by an additive nutrient starvation effect which acts as a protection response for mitochondria against sequestration and subsequent autophagosomal degradation (Rambold et al., 2011). Mitochondrial fusion not only protects from mitophagy but also serves as mitochondria quality control, due to the inability of dysfunctional mitochondria to fuse together (Chen et al., 2005; Gomes et al., 2011; Rambold et al., 2011; Twig et al., 2008). Eventually, defective mitochondrial are recycled. Cytoplasmic components are the initial degradation material during the first period of

starvation, in contrast with the mitochondria which become a substrate much later, indicating fusion as a protection mechanism (Kristensen et al., 2008).

Mitochondrial fusion acts as a defensive mechanism through mitochondrial DNA exchange, re-organization of mitochondrial cristae, and by delaying apoptosis (Chen et al., 2010; Lee et al., 2004; Tondera et al., 2009). It is also needed to provide high ATP production to meet the demands of G1/S phase of the cell cycle (Mitra et al., 2009). Biogenic processes require increased ATP synthesis rates during G1/S phase in order cell duplication to occur. Increased mitochondrial ATP synthesis during mitochondria fusion is also involved in cell senescence (Lee et al., 2007; Yoon et al., 2006). Senescence reduces the capacity of processes such as proliferation, homeostatic imbalance, and mitochondrial biogenesis. Adaptation response to decreased mitochondrial biogenesis occurs by the fusion of mitochondria which promotes ATP synthesis capacity and/or bioenergetic efficiency (Liesa & Shirihai, 2013). By complementation, fusion maintains functional mitochondria with elevated copies of mutated mitochondrial DNA (mtDNA) per senescent cell (Liesa & Shirihai, 2013).

Mitochondria fusion is depended on Mfn1 and Opa1, which require two Drp1 post translational modifications, after experiencing nutrient limitation (Rambold et al., 2011). In contrast to nutrient excess conditions, starvation promotes an acute inhibition of mitochondrial fission (Gomes et al., 2011; Rambold et al., 2011). More specifically, mitochondrial Drp1 function is inhibited, followed by unopposed fusion events which lead to elongated mitochondria. In conclusion, under starvation periods, mitochondria fuse together to increase their capacity of ATP synthesis to answer ATP demands (Liesa & Shirihai, 2013; Otera et al., 2010; Rolland et al., 2009). The reason why elongated mitochondria cannot be targeted for autophagy is still under investigation.

Mitochondrial fission

Mitochondrial fission occurs as a response to reductive stress and elevated ROS production (Liesa & Shirihai, 2013). Upon nutrient excess, mitochondrial fission improves the negative impact of reductive stress and ROS production, by decreasing mitochondrial membrane potential through cristae reconstruction and OPA1 regulation. Fission can be induced by mechanisms or physiological processes for the depolarization of mitochondria. This amplifies or enhances the capacity of these processes, reducing the mitochondrial membrane potential.

Mitochondrial fission acts as a quality control mechanism by producing two bioenergetically distinct mitochondria. One with elevated membrane potential and one with reduced membrane potential (Gomes et al., 2011; Liesa & Shirihai, 2013). From that point, the single daughter mitochondrion with the reduced membrane potential, either recovers its membrane potential and restores its ability of reconnecting with the network or remains in that depolarized state. If mitochondrion remains in that state for a prolonged period, OPA1 is degraded, inhibiting the capacity of the mitochondrion to reconnect with the network, leading to mitophagy. In some respects, fission is acting as a mechanism which isolates potentially damaged organelles and selective fusion determines the course of the mitochondria to be autophagocytosed. Prolonged inhibition of fission impacts mitochondria bioenergetics by the build-up of permanent dysfunctional mitochondria that are not able to segregate.

Prolonged fission inhibits mitochondrial fusion and leads to a disrupted cycle of fusion, fission, and autophagy (Chen et al., 2010; Kristensen et al., 2008). That results to an inhibition of the mitochondrial complementation and therefore, enhanced subcellular mitochondrial heterogeneity (Tondera et al., 2009). Disruption of selective mitochondrial removal and the existence of depolarized mitochondria has a negative impact even to the mitochondrial biogenesis. Nutrient excess can also disrupt autophagic flux, by restricting lysosomes which are needed for autophagic degradation (Chen et al., 2010). This leads to the accumulation of

damaged mitochondria, which are negatively affect mitochondria produced *de novo* (by unselective fusion and/or enhanced ROS generation). In conclusion, mitochondrial fission is important for mitophagy, mitotic segregation of mitochondria to daughter cell, and distribution of mitochondria to subcellular locations like neuronal axons (Lee et al., 2004; Liesa & Shirihai, 2013). Mitochondrial fission is crucial for progression of apoptosis and M phase, and for the degradation of defective mitochondria.

Mitochondrial changes in ageing and disease

Ongoing research reveals the involvement of mitochondria in a plethora of conditions. That includes metabolic disorders such as diabetes and obesity, neuropathies such as Parkinson's and Charcot-Marie-Tooth disease (Cereghetti et al., 2008; Las et al., 2011; S. Lee et al., 2007; Liesa & Shirihai, 2013; Mitra et al., 2009; Mollica et al., 2006; Twig et al., 2008; Wikstrom et al., 2007; Yoon et al., 2006). Regarding insulin resistance, obesity, and type-2 diabetes mellitus (T2DM), a term known as metabolic inflexibility is often used to describe the disrupted capacity of alternating between energy substrates, with mitochondria dysfunction being placed at the centre of these metabolic disorders.

Ageing has a negative effect in mitochondria function, maintenance of energy balance, and production of ROS in both invertebrates and vertebrates. Even though ageing is associated with inadequate control of mitochondrial quality and turnover mechanisms like autophagy, type-2 diabetes and obesity involve responses against excess nutrient conditions. Both ageing and metabolic diseases are affected by periods of exposure to excess nutrition conditions, raising questions about opposing actions of them to deal with excess nutrients and sustain quality control. Failure of maintaining homeostasis, mitochondria health and function is compromised, with consequences in larger processes within an organism, such neuronal circuitry function.

Disrupted mitochondrial dynamics are associated with neuronal and skeletal muscle symptoms. More than 100 mutations within the gene encoding mitofusin 2 (MFN2) are associated to subtype 2A of Charcot-Marie-Tooth disease, which is considered as the most common heritable axonal neuropathy. The initial discovery of OPA1 came from the examination of the autosomal dominant optic atrophy, the most frequent condition of hereditary optic neuropathy, and more than 250 pathogenic mutations have been identified. DRP1 mutation can also lead to a severe type of infantile neurodegenerative disease. In addition, over-activation of DRP1-mediated fission is involved to several diseases such as Parkinson's and Huntington's disease.

Interventions such as frequent exercise and reduced food intake proven to be beneficial against these disorders however, increasing metabolic pathologies demand measures to be taken (Andreux et al., 2013; Chandhok et al., 2018; Gomes & Scorrano, 2013). Pharmacological targeting of mitochondrial metabolism suggested to be an effective intervention against the rapid spreading of metabolic disorders (Olichon et al., 2006). Thus, elucidation of how dysfunctional mitochondrial dynamics influence the overall health of an organism is crucial and research is needed to delineate the biological role of fusion and fission dynamics in disease.

Mitochondria are involved in several cellular processes such as production of energy (ATP) through nutrient breakdown, autophagy/mitophagy, and apoptosis. Despite the number of evidence which indicate mitochondrial response to conditions of altered nutrient amount, such as fasting/caloric restriction (CR) or high-fat diet, the actual mechanism and the elements which are associated with, remain elusive (Chan, 2006; Olichon et al., 2006). In mice, knockout of either MFN1/2, OPA1 or DRP1 is embryonically lethal. In contrast*, C. elegans* tolerates individual knockouts of these mitochondria associated proteins, with animals remain viable, allowing the delineation of each individual mitochondrial fission and fusion proteins.

The Nrf transcription factor SKN-1

In *C. elegans* the *skn-1* gene encodes a transcription factor which is the functional orthologue of mammalian Nrf bZip transcription factors (also known as NF-E2 related factors) (Walker et al., 2000). In mammals, Nrf proteins must dimerise to bind the DNA through their ZIP motif. In *C. elegans*, the ZIP domain is absent, and the dimerization is substituted by a more efficient DNA binding motif (Kophengnavong et al., 1999). There is a significant degree of divergence between mammalian NRFs and SKN-1, in terms of both sequence and structure. There are four possible isoforms of *skn-1 (a-d)* with three of these *(a-c)* have been detected in vivo [Figure 7] (Blackwell et al., 2015). Among *skn-1* isoforms there is divergence, in terms of structure, expression patterns, cellular localisation, and function. Some isoforms are more closely resembling individual NRF proteins. Studies using mutant alleles which knock out all *skn-1* isoforms have shown interesting results however, it is difficult to attribute functions to individual isoforms and it is generally accepted that the combined functions of SKN-1 isoforms reflect the combined functions of Nrfs (Blackwell et al., 2015).

The isoforms are differentially regulated at the transcriptional level. SKN-1A is expressed from an operon promotor upstream, whereas SKN-1C and SKN-1B are predicted to be expressed from their own unique promotor [Figure 7, 8] (An et al., 2005; An & Blackwell, 2003; Bishop & Guarente, 2007). The SKN-1 DNA binding domain found to be conserved between all four isoforms and includes a Cap N Collar domain (CnC), a basic region (BR), and a flexible NH2 terminal arm important for the interaction with the DNAs minor groove [Figure 8] (Kophengnavong et al., 1999). Dimerization is absent thus SKN-1 binding site diverge from the mammalian antioxidant response element (ARE) and SKN-1 will bind to a single sequence of WWTRTCAT, with the NH2 arm binding to the WWT via the minor groove (An & Blackwell, 2003).

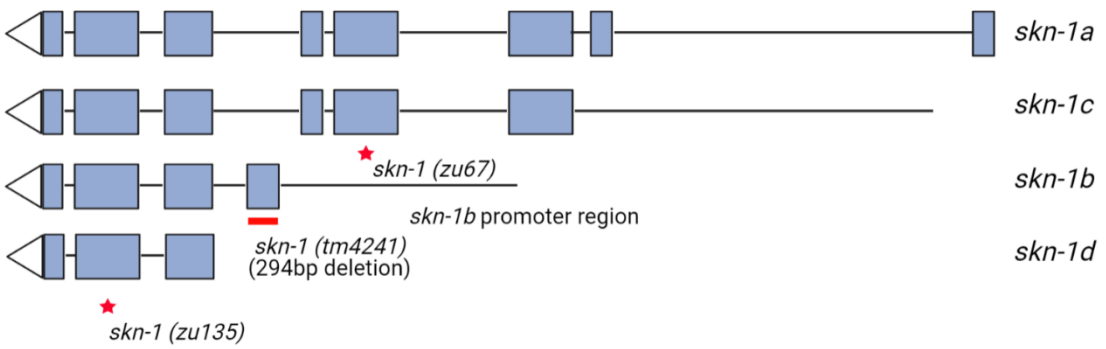


Figure 7: Genomic structure and classical mutant alleles of *skn-1.* Commonly used mutations in *skn-1*, which allow to study the function of this gene. The *zu135* (Bowerman, Eaton and Priess, 1992) mutation introduce premature stop codon which abolish all *skn-1* expression, while *zu67* mutation abolish *skn-1a/c* expression. *skn-1(tm4241)* mutation causes the deletion of a *skn-1b* specific exon. Adapted from (Blackwell et al., 2015).

SKN-1 is required for several key, physiological processes

Development: SKN-1 is crucial for early development. It has been shown that SKN-1 is unevenly expressed at the 4-cell stage, where it is important for the initiation of the endodermal and mesodermal tissue development (Blackwell et al., 2015). Strains carrying mutations which knock out either *a* and *c* isoforms or all *skn-1* isoforms simultaneously are embryonic lethal (Bowerman et al., 1992). Balancers are used to maintain strains carrying embryonic lethal *skn-1* mutations allows the study of these isoforms.

Normal lifespan: In *C. elegans*, SKN-1 is highly involved in the ageing process. More specifically, mutation of *skn-1 c/a* causes lifespan reduction, whereas overexpression of *skn-1* extends lifespan (Blackwell et al., 2015; Tullet et al., 2008).

Lifespan extension by modulating key signalling pathways: More specifically, SKN-1 is essential for lifespan extension from at least three pro-longevity interventions in worms. *skn-1c* which is placed downstream of insulin signalling, is essential for the full longevity effect

mediated from the *daf-2* mutation (Ewald et al., 2015; Tullet, Jennifer M A, Hertweck, Maren, An, Jae Hyung Baker et al., 2008). *skn-1* is essential for the longevity mediated by inhibiting the TOR pathway (Robida-Stubbs et al., 2012).

Lifespan extension caused by dietary restriction: *skn-1b* which is expressed exclusively in the ASI neurons, is essential for the longevity mediated from at least one dietary restriction method (Bishop & Guarente, 2007).

Stress resistance: The absence of *skn-1* renders mutant animals sensitive to oxidative stress mediated by specific stressors, such as sodium arsenite and TBOOH. In addition, *gst-4* (Glutathione S-transferase 4) one of the *skn-1* target genes, found to be highly upregulated by *skn-1* (Oliveira et al., 2009). *gts-4* gene has an important role in reduction of oxidative stressors by glutathione. The significant *gst-4* regulation by *skn-1* used as proxy for *skn-1* activity (Oliveira et al., 2009). More research on *skn-1* targets using microarray method showed that *skn-1* controls a wide range of collagen genes, to the degree that collagens and extracellular matrix involved genes found to be the major target of *skn-1* whereas, genes associated in the oxidative stress response being the immediate next most highly enriched group. This observation comes in agreement with other studies on *skn-1* target genes (An & Blackwell, 2003; Ewald et al., 2015). It has been showed that collagen remodelling downstream of *skn-1*, under reduced insulin-singling and dietary restriction, is essential and a crucial pro-longevity output of *skn-1* (Ewald et al., 2015). In conclusion, the *skn-1* isoforms demonstrate a different regulation, localisation, structural and functional operation.

Proteosome regulation: The largest protein encoded at the *skn-1* genomic locus is SKN-1A [Figure 8]. SKN-1A described as activator of *skn-1* target processes responding to proteasome dysfunction. *C. elegans* responds to proteasome dysfunction by upregulating genes encoding proteasome subunits, detoxification processes, immune response which alter its behaviour to avoid pathogenic bacterial food (Lehrbach & Ruvkun, 2016). With regards to the mammalian

NRFs, SKN-1A described to be the functional orthologue of the extensively studied NRF1. The mechanism by which NRF1 is activated, is conserved in *C. elegans*. In both humans and worms, NRF1/SKN-1A found to be bound to the membranes, including ER and mitochondrial membranes, via a membrane localisation domain and being constitutively targeted for degradation by the proteasome via the ERAD pathway (ER associated degradation) (Lehrbach & Ruvkun, 2016; Radhakrishnan et al., 2014; Zhe Sha et al., 2014). Upon proteasome dysfunction, SKN-1A is being accumulated on these membranes and eventually cleaved by the protease DDI-1, releasing the protein into the cytoplasm where enters the nucleus and enact the *skn-1* associated program (Radhakrishnan et al., 2014). This allows *skn-1* to detect proteasome dysfunction, allowing the cell to respond. In general, *skn-1a* found to be expressed in intestinal tissues however, it has been reported that *skn-1a* is found in some neurons during larval development (Bishop & Guarente, 2007; Tullet et al., 2008).

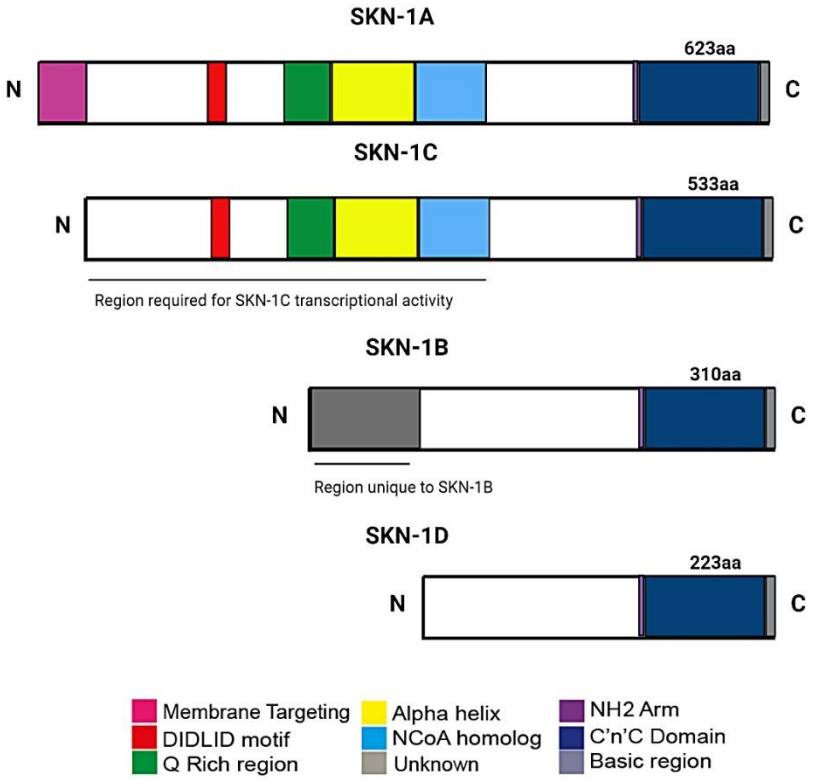


Figure 8: The four protein isoforms of SKN-1. Displayed in descending order of size. SKN-1A and SKN-1C are localised to the *C. elegans* intestine and are activated by independent mechanisms resembling Nrf-1 and Nrf-2 respectively. SKN-1B is confined to the ASI neurons of *C. elegans*. Point mutations in the DIDLID motif radically decrease SKN-1 function (Walker et al., 2000) and this motif is conserved to mammalian NRF proteins. The alpha helix and Glu-rich regions form a second transactivation domain, and the region of NCoA homology is required for interaction with p300 and forms a third transactivation domain (Walker et al., 2000). The DIDLID motif, glu-rich region, alpha helix andregion of NCoA homology, however, are not present in SKN-1B or (should it exist *in vivo*) SKN-1D.

SKN-1 isoforms and expression

Initially, SKN-1 found to be expressed in the ASI neurons and the intestine. More specifically, SKN-1 in the ASI neurons observed to be constitutively nuclear, whereas SKN-1 in the intestine could become nuclear in response to activating processes (An & Blackwell, 2003). One of the few studies which examine *skn-1b*, Bishop and Guarente 2007, showed tissue specificity of the *skn-1b* and *skn-1c* expression. Strain carrying a SKN-1B/C::GFP transgene showed neuronal but not intestinal GFP when *skn-1c* was knocked down and intestinal but not neuronal GFP when *skn-1b* was silenced (Bishop & Guarente, 2007). These results indicate that *skn-1b* is expressed principally in ASI neurons, whereas *skn-1c* is express in the intestine. In terms of structure, *skn-1b* contains a unique exon which is absent in any other *skn-1* isoforms, with unknown roles [Figure 7]. In addition, SKN-1B protein lacks several known domains essential for the activation of SKN-1A and SKN-1C such as the DIDLID motif and regions of the protein needed for the interaction with WDR-23 (Lanlan Tang, 2015), p300 (Walker et al., 2000), and several phosphorylation sites [Figure 8] (Blackwell et al., 2015).

The most extensively studied SKN-1 isoform is SKN-1C, due to its role as the primary effector of most of the effects related with the *skn-1b* gene. SKN-1C is found mostly in the intestine, where it is sequestered in the cytoplasm under normal conditions. In addition, RNAi against

skn-1c inhibits the expression of a SKN-1B/C::GFP transgene specifically in the intestine but not in the ASI neurons (Blackwell et al., 2015). There is a variety of post translational modifications which can regulate the nuclear entry of SKN-1C such as phosphorylation by AKT1/2, SGK, GSK3, PMK-1, (An & Blackwell, 2003; Blackwell et al., 2015; Tullet, Jennifer M A, Hertweck, Maren, An, Jae Hyung Baker et al., 2008) O-GlcNAcylation (Li et al., 2017) and indirectly through WDR-23, which marks SKN-1 for proteasomal degradation (Choe et al., 2009) with the ability to be down or up-regulated controlling *skn-1* activity. Experiments in vitro have shown that the activity of SKN-1C is highly depended on the DIDLID motif and point mutations specifically in this region dramatically impair its activity [Figure 8] (Walker et al., 2000). In addition, this motif found to be conserved all the way to mammalian NRFs. The intestinal activation of *skn-1a/c* is needed for the canonical *skn-1b* functions of stress resistance and normal lifespan. Furthermore, both *skn-1(zu67)* and *skn-1(zu135)* mutants demonstrate sensitivity to a wide range of stresses and exhibiting reduced lifespans [Figure 7] (An et al., 2005). SKN-1C operates in the intestine as a crucial effector of insulin signalling, where it is directly phosphorylated by AKT1/2 and SGK downstream of activated insulin signalling and thus is being restricted from entering intestinal nuclei. SKN-1C constitutively accumulates in the intestinal nuclei upon reduction of the insulin signalling pathway like in *daf-2* mutants (Ewald et al., 2015; Tullet, Jennifer M A, Hertweck, Maren, An, Jae Hyung Baker et al., 2008). Furthermore, activation of *skn-1a/c* in the intestine is essential for rIIS longevity as demonstrated in *daf-2 (e1368)* mutants and *skn-1 (zu67)* mutation can abolish this longevity and that derives from *sgk-1* RNAi as well as abolishing stress resistance derived from rIIS (Tullet, Jennifer M A, Hertweck, Maren, An, Jae Hyung Baker et al., 2008). Intestinal overexpression of *skn-1b/c* promotes lifespan extension. In conclusion, *skn-1c* in the intestine contributes to lifespan downstream of rIIS.

The fourth isoform of *skn-1* has been predicted in vitro, based upon the presence of an SL1 site at the end of this possible transcript (Wormbase version WS259) [Figure 7, 8]. However,

there are no evidence about in vivo presence of *skn-1d*. Expression of *skn-1b* in vivo may confuse the results obtained using previous *skn-1* transgenes due to the risk that some functions of *skn-1d* have been attributed to other SKN-1 isoforms (Choe et al., 2009).

skn-1 isoforms demonstrate differences in aspects such as localisation, regulation, structure, and function. *skn-1* is being involved in the ageing process, as well as in the perception of the environment however, much work remains to identify which isoform specifically is important for each process.

This Thesis

SKN-1B expression in the chemosensory ASI neurons, as well as its involvement in lifespan extension through dietary restriction, raises questions about a potential foraging (environment perception) function. In this study, I will investigate food-related behaviours of animals lacking *skn-1b* and I will test their ability to respond to environmental cues, through different experimental settings.

Chapter 2 – Materials and Methods

C. elegans Strains

Strain name	**Genotype**
CGCM	N2 wildtype
GA1058	*skn-1b(tm4241)*
EU1	*skn-1*(zu67)
EU31	*skn-1(zu135)*
GA1017	*N2 wuEx217[Pskn-1b::skn-1b::GFP; rol-6]*
COP1836	*knu733[wrmScarlet::skn-1b]*
JMT31	*daf-2(e1370)*
JMT32	*daf-2(e1370); skn-1b(tm4241)*
DR1572	*daf-2(e1368)*
GA1060	*daf-2(e1368); skn-1b(tm4241)*
DR47	*daf-11(m47ts)*
JMT70	*daf-11(m47); skn-1b(tm4241)*
CB1372	*daf-7(e1372)*
JMT68	*daf-7(e1372); skn-1b(tm4241)*
PR678	*tax-4(p678)*

MT1072	*egl-4(n477)*
SJ4103	*zcIs14 [myo-3::GFP(mit)]*
JMT90	*skn-1b(tm4241); zcIs14 [myo-3::GFP(mit)]*
WBM671	*wbmEx289[myo-3p::tomm20(aa1-49)::GFP::unc54 3'UTR]*
JMT76	*skn-1b(tm4241); wbmEx289[myo-3p::tomm20(aa1-49)::GFP::unc54 3'UTR]*
JMT50	*drcSI7[unc-119; Pdaf-7::Venus]*
JMT75	*skn-1b(tm4241); drcSi7[Pdaf-7::Venus]*
GA1064	*muEx227[ges-1p::GFP::daf-16a]*
JMT82	*skn-1b(tm4241); muEx227[ges-1p::GFP::daf-16a]*
JMT66	*skn-1b(tm4241), ukcEx15 [Pskn-1b::skn-1b::GFP; myo-3::mcherry]*
JMT67	*skn-1b(tm4241); ukcEx16 [Pskn-1b::skn-1b::GFP; myo-3::mcherry]*

Nematode Growth Media (NGM)

0.3% NaCl w/v [Fisher Bioreagents], 1.7% granulated agar w/v [Melford], 0.25% Bacto^{tm}peptone w/v [Difco] in dH_2O autoclaved and cooled before the addition of sterile 25mM KH_2PO_4 pH6.0, 1mM $MgSO_4$, 1mM $CaCl_2$, and 5µg/ml cholesterol in ethanol.

M9 buffer

0.3% KH_2PO_4 w/v, 0.6% Na_2HPO_4 w/v, 0.5% NaCl w/v in dH_2O autoclaved and cooled before the addition of sterile 1mM $MgSO_4$.

Luria Broth (LB)

LB agar was made by adding 2.5% LB broth w/v and 1.5% granulated agar w/v in dH_2O and sterilised by autoclave. LB liquid media was made by adding 2.5% LB broth w/v in dH_2O and sterilised by autoclave.

Bacterial cultures

Bacterial cultures were stored in 50% glycerol in dH_2O at -80°C. Frozen bacterial cultures were streaked on LB agar plates and incubated overnight at 37°C. Single colonies obtained from LB agar plates were used to inoculate LB liquid medium and grown overnight at 37°C with agitation. Appropriate antibiotics and accessory components were added to the media for selection or gene induction.

C. elegans husbandry

C. elegans strains were grown on standard NGM plates seeded with OP50 *E. coli* (uracil auxotroph B strain) at 20°C unless otherwise stated. OP50 was cultured in lysogeny broth (LB) [Miller, Fisher Bioreagents] overnight at 37°C and 200µl of the resulting culture used to seed 60mm NGM plates. Bacteria allowed to grow for at least 2 days at room temperature (RT) before placing animals. Leica [model] dissecting microscope used to observe and manipulate *C. elegans* with the usage of a platinum wire melted into a glass pipette. The flat end of platinum wire used to collect and transfer animals to other NGM plates. A Bunsen burner next to the microscope used for sterilization of the platinum wire each time contacting animals or bacteria and provided an upward airflow protecting from contamination by floating particles.

Animal population maintained by transferring 5-8 hermaphrodites onto fresh OP50 seeded NGM plates. Prior conducting any experiments animals allowed to feed ad libitum for at least 3 generations.

Timed-egg laying

Timed-egg laying method performed to generate a synchronous population of *C. elegans*. 3 well-developed adult worms from uncrowded plate were transferred onto a seeded NGM plate. Animals allowed to lay eggs for 4 hrs at 25°C before being removed from the plate. Synchronised L4 stage animals used for experiments or transferred onto seeded NGM plates for further synchronisation.

Freezing and thawing of *C. elegans* stocks

C. elegans can be stored indefinitely at -80ºC. Prior freezing, animals allowed to starve and produce large number of offspring, arrested at the freeze thawing resistant L1 stage. Starved L1 larvae from 5 6cm NGM plates were pooled by washing off animals in M9 and a 1:1 dilution with 2x freezing media (30% glycerol, 5.5mg/ml NaCl, 6.8mg/ml autoclave then add 0.3% 0.1M MgSO4) in the required number of cryo-tubes and placed in a freezing container in a -80°C freezer. Thawing of frozen stocks performed by placing the cryo-tubes on ice and then pipetting onto seeded NGM plates the content using 500µl per plate. Animals allowed to recover at the permissive temperature appropriate for each strain. Then, animals were transferred onto new NGM plates as soon as possible.

Bleach drop cleaning of stocks

Contamination in *C. elegans* stocks either by bacteria or fungi resolved mostly by the bleach drop technique. A 7:8 mixture of thin bleach and 4M NaOH is used to eliminate contamination while allowing *C. elegans* embryos to survive. 5-10µl of this bleach mixture placed onto the edge of a seeded NGM plate and 8-10 gravid adult animals from the contaminated plate were transferred into the bleach drop. Plates were left upright at the permissive temperature appropriate for each strain until visible offspring appear. Then, animals were transferred onto new NGM plates as soon as possible.

Generation of male stock and genetic crosses

Males for genetic crosses were generated by heat shocking L4 stage hermaphrodites at 30^{o}C for 5 hours. Then, animals were placed at permissive temperature and allowed to produce progeny. Males were identified by their district morphological characteristics (tail) and transferred onto a spot of bacteria on an otherwise empty NGM plate together with 2-4 L4 stage hermaphrodites to generate a male stock. Genetic crosses performed by placing L4 stage worms onto a spot of OP50 on an otherwise empty NGM plate at a ratio of 1:7 hermaphrodites-to-males to ensure a high probability of male fertilisation. Then, the clonal F2 generation of worms was genotyped.

RNAi

RNAi treatment of animals performed using the feeding method (Timmons and Fire, 1998). *E. coli* HT115(D3) strain contains an IPTG inducible T7 RNA polymerase which drives the expression of chosen dsRNAi in the bacterial culture. After being ingested and processed by

the endogenous *C. elegans* RNAi machinery, specific degradation of the targeted RNA and finally silencing the corresponding gene occurs. HT115 *E. coli* was grown on LB agar plates containing 50µg/ml ampicillin and 12.5µg/ml tetracycline for 16hrs at 37°C. A single colony used to inoculate LB liquid media which contained 50µg/ml ampicillin and 12.5µg/ml tetracycline and incubated for 16hrs at 37°C. NGM plates containing 50µg/ml ampicillin and 1mM IPTG seeded with the HT115 *E. coli* either expressing empty PL4440 or PL4440 plasmid which carries the relevant RNAi insert. Plates were left for at least 24 hrs allowing induction of dsDNA to occur before transferring animals for feeding.

Genotyping *C. elegans* using PCR

Lysates of single or multiple animals obtained and used as material for PCR. Multiple worm lysis contained genetic material from 8-10 animals in a homogenous population while single worm lysis required for strain validation contained genetic material usually from the F2 parent during a cross.

Animals were lysed in 5µl worm lysis buffer (50mM KCL, 10mM Tris (pH 8.3), 2.5mM MgCl2, 0.45% NP40, 0.45% Tween20, 0.01% Gelatin, 0.1mg/ml proteinase K) in a thermocycler using our lysis protocol (70°C: 60min → 95°C: 15min → 12°C: ∞).

Pre-made PCR mixes, mostly 2x PCRBIO Taq Mix Red (Ingredients listed in the manual as: PCRBIO Taq DNA Polymerase, 6mM MgCl2, 2mM dNTPs, enhancers, stabilizers, and a red dye) were used. PCR reactions were prepared with 10µl Taq Mix, 3µl of 10mM primer mix (all relevant primers at 10mM in MilliQ water) for the specific reaction in question and 2µl of milli-Q H2O. This was added to either 5µl of worm lysate or a DNA solution of the same volume. The reaction mix was placed into a thermocycler and subjected to the chosen PCR protocol (94°C: 60s → 94°C: 30s → X°C (differs between primer sets):30s → 72°C: 60s → Repeat

previous 3 steps 28-32x →72°C: 7min →12°C: ∞). PCR products were then analyzed on 1-2% agarose gels using Promega 1Kb ladder. Visualised using a SynGene GBox.

Exploration assay

Exploration assays were performed as described (Steven W. Flavell et al., 2013). Single OP50 *E. coli* colonies were inoculated in 100 mL of LB and incubated in a shaker-incubator at 37 °C overnight for 16 hrs. 35 mm NGM plates were uniformly seeded with 200 µl of a saturated culture of OP50 and allowed to dry overnight at room temperature. Worms were grown in uncrowded conditions to the L4 stage at 25 °C. Individual L4 animals were placed in the centre of the plates and transferred to 25 °C for 16 hrs. After 16 hrs, the animals were tested to see if they were alive by gently touching them, followed by the plates being immediately photographed. Plates were superimposed on a grid of 3.5 mm squares and the number of squares entered by worm tracks was counted manually. Tracks could enter a maximum of 109 squares. At least 15 (one day adult) animals per genotype were tested on three separate days using different offspring generation. The number of squares entered can vary from day to day, therefore all genetic manipulations are compared to controls tested in parallel.

Food avoidance assay

Food avoidance assays were performed as described (McMullan et al., 2012). Single bacteria colonies were inoculated in 100 ml of LB and incubated in a shaker-incubator at 37 °C overnight for 16 hrs. 55 mm NGM plates were seeded with 100 µl of a saturated bacteria culture as a small spot in the centre of the plate and allowed to dry overnight at room temperature. Only plates with an evenly and defined circular bacteria lawn were used for the assays. 3 well-developed adult worms previously grown in uncrowded conditions at 20 °C

were transferred to each plate. Animals allowed to lay eggs for 4 hours at 25 °C and then they were being removed from the plates. The egg-containing plates transferred at 25 °C for 48 hrs. When animals have reached L4/ one day adult stage their position scored relatively to the bacteria lawn. Animals scored as OFF for a position outside of the bacteria lawn and as ON for a position inside the bacteria lawn. Each genotype tested at least in three trials and each trial contained plates in triplicate. Different offspring generation used between each trial. After the 48 hrs incubation the plates were immediately photographed with the lid on. The percentage of animals being on the bacteria lawn calculated per different genotype/bacteria type.

Bordering assay

To measure bordering behaviour, data from previous food/pathogen avoidance assays were used for further analysis. A modified protocol from (Reddy et al., 2011) used to measure the number of animals on the thicker part of the lawn. More specifically, plates were superimposed on a circle that covers the surface of the bacterial lawn except the thick (outer) part. The distance between the thick (outer) part and the inner (thin) part of the bacterial lawn is approximately 0.5 cm. Animals scored as OUT for a position outside of the circle perimeter (thick bacterial lawn) and as IN for a position inside of the circle perimeter (thin bacterial lawn). The percentage of animals being on the thick (outer) part of the bacterial lawn was calculated.

Thrashing assays

C. elegans motility analysed using the wMicroTracker (NemaMetrix). The system measures the thrashing ability of animals in liquid. The standard protocol followed with some modifications. Sterile 96-well flat-bottom microplates (Sigma) used and 100µL of M9 buffer placed inside each well. 20 synchronized L4 stage animals transferred onto each well. The 96-well microplates were sealed using a PCR tape to prevent evaporation. Worm activity per 96-well microplate recorded for 30 minutes. Worm activity recorded in triplicate for each condition.

Fasting protocol

For quiescence, food intake, and pharyngeal pumping assays, fasting of animals performed as described (Gallagher et al., 2013; Raizen, 2012; You et al., 2008) with some modifications. Animals grew on HB101 (quiescence assay) or OP50 (food intake assay) bacteria at 20^{o}C in non-crowed, non-starved conditions. L4 stage animals were transferred onto plates seeded with the appropriate bacteria for 9-12 hrs until they have reached young adulthood. Then, the animals were transferred with a platinum pick to 60mm NGM plates without food for 16 hrs. Fasted animals should appear pale, thinner in size (compared to fed control animals) and bloated with embryos. After 16 hrs of fasting, animals were transferred onto plates seeded with the appropriate bacteria for re-feeding. For the rest of assays required fasting, L4 stage animals were immediately transferred with a platinum pick to 60mm NGM plates without food for 16 hrs.

Measuring satiety induced quiescence

Satiety induced quiescence assay was performed as described (Gallagher et al., 2013; Raizen, 2012; You et al., 2008) with some modifications. Animals were maintained on NGM plates seeded with HB101 bacteria at 20^{o}C in non-crowed, non-starved conditions. Then, fasting of animals performed as described.

After 16hrs of fasting, individual animals were transferred onto 35mm plates seeded with 50µL of saturated HB101 bacterial culture. The small bacterial lawn allows fast tracking of animals and minimizes disturbance, which can cause exit from quiescence state. Assay plates were placed next to the microscope with enough space between them (not stacking) to avoid vibration. Fasted animals allowed to re-feed for 3 or 6 hrs. During this time vibration and noise around the testing area was minimized. After 3hrs of refeeding, the state of animals examined. The animals should appear quiescent, neither moving nor pumping. Quiescence period is defined as the interval between the beginning of the observation of the quiescent animal to the time at which it resumes feeding and locomotion.

Usually animals exit quiescence by moving backwards, followed by forward movement and resumption of pharyngeal pumping. Occasionally, quiescent animals make a sub-second body jerk or pump once-twice but then immediately return to quiescence state. The end of quiescence period considered when animals sustain movement and pumping for more than 3 seconds. After the measurement of quiescence for the first 3hrs of refeeding, plates placed back next to the microscope until 6hrs of refeeding comes for another measurement. Quiescence behaviour characterized as fragile and sensitive to environmental disturbance. Light, heat, movement-vibration of the plates are all factors that may affect quiescence.

Food intake

A food intake assay performed as described (Gallagher et al., 2013; Raizen, 2012; You et al., 2008) with some modifications. 35mm NGM plates were seeded with an overnight culture of *E. coli* OP50 expressing mCherry. Plates were stored at room temperature for two days. Animals were maintained on NGM plates seeded with OP50 bacteria at 20^{o}C in non-crowed, non-starved conditions. L4 stage animals were transferred onto plates seeded with OP50 bacteria for 9-12hrs until they have reached young adulthood. Then, fasting of animals performed as described and fed control animals were transferred onto OP50 seeded plate for 16 hours. Fed and fasted worms were then placed on the fluorescent OP50 for 5 minutes and allowed to feed. Worms were imaged and the fluorescence intensity within the gut quantified.

Pharyngeal pumping

Animals were maintained on NGM plates seeded with HB101 bacteria at 20^{o}C in non-crowed, non-starved conditions. Fasting of animals performed as described and fed control animals were transferred onto NGM plates seeded with HB101 bacteria for 16 hrs. Animals in both fed and fasted condition were transferred onto NGM plates seeded with HB101 bacteria and allowed to feed/explore for 5 minutes. Then, a video of 30 seconds recorded for each animal and analysed. The number of pumps of each animal multiplied by 2, data were pooled and presented as pumping per minute.

daf-16 nuclear localisation

DAF-16::GFP WT and *skn-1b* animals fed ad libitum on OP50-1 bacteria. L4 stage animals fasted as described and fed control animals transferred onto OP50-1 plates for 16 hrs. After 16 hrs, fasted animals allowed to refeed for 0, 3, and 6 hrs before imaging. Images of whole worms obtained using 300msec exposure time for both fed and fasted condition. Then a grading method applied for quantify the nuclear localisation of DAF-16.

Microscopy

Quantitative fluorescence microscopy

2% of molten agarose in water used to make glass slides. Animals were immobilized on agarose pads in a drop of 10µL 0.06% tetramisole hydrochloride in M9 with a glass cover slip on the top. To acquire images, a Leica DMR microscope with a CoolLED light source and a Leica DFC9000 GT camera used. Images were processed using the FIJI distro of ImageJ. For quantitative fluorescence, the tissues of interest were manually selected as an ROI and mean grey value and integrated densities were recorded. ASIs were individually scored. Animals were synchronized mostly at L4 stage or day 1 of adulthood. ASI neurons were visualized at 40x magnification and whole-body measurements, or intestinal food intake measurements were visualized at 10X magnification.

Calculating mitochondrial coverage

Coverage of the mitochondria area of WT and *skn-1b* animals was examined with age or under fed/fasted conditions. For fed/fasted experiment, animals at L4 stage transferred onto plates seeded with OP50 or onto plates containing no bacteria for 16 hours and kept at 20^{o}C. Then,

animals in both fed and fasted conditions used for imaging the same day. The largest regions of clear body wall muscle were identified between the pharynx and vulva or between the vulva and tail. No significant difference was observed between these two segments and so measurements were pooled. Adjacent regions to the tail, pharynx, and vulva were excluded due to the disrupted mitochondria morphology which naturally occurs there. Images of the mitochondria obtained using 400msec exposure. Measurements were taken by first isolating the muscle fibre using the polygon selection tool [Figure 9] (1, 2). Then, the image type is changed to 16-bit format in order the mosaic plugin to operate properly. In mosaic utility, background subtractor is set to 15 and "clear outside" option is chosen in order the muscle fibre to be isolated and the background to be excluded (3). Threshold applied to create a binary image, where a percentage of the whole fibre covered by fluorescence could be calculated using Fiji (ImageJ) (4). In some cases, further adjustments needed to obtain the best "threshold to visible structures" ratio.

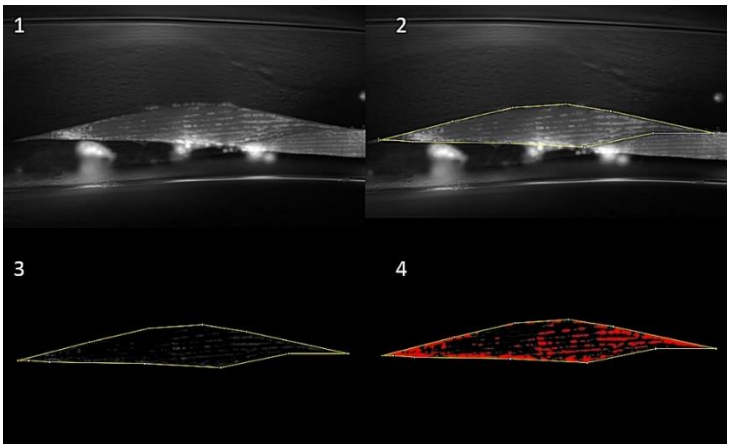


Figure 9: Demonstration of mitochondria area coverage measurement. Threshold applied to create a binary image, where a percentage of the whole fibre covered by fluorescence could be calculated using Fiji (ImageJ).

Characterisation of mitochondrial morphology

Qualitative analysis of the mitochondrial morphology in each muscle section was performed using the same dataset obtained from the mitochondrial coverage experiment. More specifically, images from fed/fasted mitochondria experiment scored as very organised (1), some organisation (2), disorganised (3), and very disorganised (4) [Figure 10]. Briefly, an image scored as (1) when mitochondria structures appear in an oriented tubular-array-formation with no breaks between them, covering the total length of the muscle fibre. An image scored as (2), when mitochondria structures maintain orientation and tubular formation within the muscle fibre but with the appearance of breaking points between them. An image scored as (3), when mitochondria structures appear disoriented and spherical within the muscle fibre. An image scored as (4), when mitochondria structures appear completely disoriented with large spherical structures and breaking points between them. Using the scoring system described the mitochondria morphology was characterized.

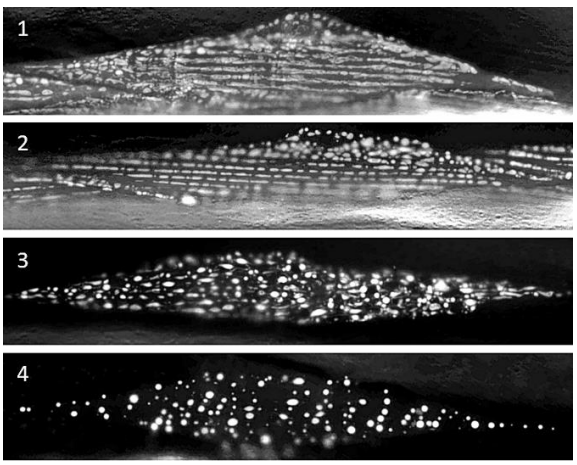


Figure 10: Grading system of mitochondria morphology. 1) Very organised, **2)** Some organisation, **3)** Disorganised, **4)** Very disorganised.

Electron microscopy

100 L4 worms were picked into M9 buffer. M9 was then aspirated off and replaced by ~2mL 2.5% glutaraldehyde fixative in 100mM sodium cacodylate (CAB) buffer (pH7.2). Worm heads and tails were removed with a scalpel, and the bodies left overnight in fixative at 4^{o}C. Worms were washed twice with CAB and suspended in 2% low melting-point agarose in CAB. Worms were identified in agarose suspension by dissecting scope, excised and transferred to 7mL glass vials, where they were post-fixed in 1% osmium tetroxide in CAB for 1hr at room temperature. These were washed twice in Milli-Q (10 mins each wash), and dehydrated in an ethanol series (50%, 70%, 90% for 10 mins each) followed by 100% dry ethanol (3 times, 10 mins each). Finally, samples were washed 2 times (10 mins each) in propylene oxide. Agar scientific low viscosity (LV) resin was prepared fresh and mixed 1:1 with propylene oxide and added to the samples (30 mins RT). Samples were then incubated in fresh LV resin 2 times (2hrs each), embedded in LV resin by polymerising at 60^{o}C for 24hrs. Polymerised samples were identified under a dissecting scope and individual worms were cut out and orientated on a resin block for optimal sectioning. 70nm sections were cut on a Leica EM UC7 ultramicrotome, using a Diatome diamond knife and collected onto 400-mesh copper grids (Agar Scientific). Sections were counterstained with 4.5% uranyl acetate (45 mins) and Reynolds lead citrate (7 mins). Sections were imaged on a Jeol 1230 transmission electron microscope operated at an accelerating voltage of 80kV; images acquired using a Gatan One View 4x4K digital camera.

Chapter 3 - Investigating the role of *skn-1b* in environment perception and behaviour

Neuronal SKN-1B expression responds to specific dietary cues

C. elegans perceives the environment through the ASIs and other sensory neurons (Bargmann et al., 2006; Brenner, 1974; Corsi et al., 2015). Environmental cues such as the amount and type of food are detected by the worm through a complex network, leading to behavioural responses (Boris Borisovich Shtonda & Avery, 2006b). The perception of these cues begins at the openings of the amphid neuron projection and is relayed via peptides and hormones to the rest of the animal. Maintaining homeostatic, as well as behavioural adaptation to the environment, is crucial for organism survival. Thus, elucidating the molecular pathways and other elements involved in this process is essential.

In *C. elegans*, *skn-1b* contributes to DR lifespan extension and is possibly important for foraging behaviour (Bishop & Guarente, 2007; Wilson et al., 2017). *skn-1b/c::GFP* levels in the ASI neurons also respond to the amount of food in the environment and 5'RACE analysis suggested that *skn-1b* expression was driven from its own promotor (Bishop & Guarente, 2007). This data suggested that SKN-1B may act independently and play a role in food related processes. To examine SKN-1B independently from other SKN-1 isoforms, a SKN-1B::GFP translational reporter was generated (by Jennifer Tullet) (Tataridas-Pallas et al., 2021). This transgene contains the 2kb of the predicted promotor region upstream of *skn-1b* (Figure 7,8) followed by *skn-1b* genomic DNA and *gfp*. This allowed the examination of SKN-1B::GFP under the control of a putative *skn-1b* promotor.

GFP expression in WT animals carrying SKN-1B::GFP found in the ASI neurons [Figure 11 A]. Under normal conditions SKN-1B:GFP found exclusively to the ASI neurons with no detectable

expression in other tissues. This comes in agreement with other observations about neuronal SKN-1 expression and ASIs with no *skn-1b* expression observed in the intestine. To confirm the expression patterns of SKN-1B in ASIs, a strain generated by crispR (Kundra transgenics) COP1835 *[skn-1b::wrmScarlet]*. A scarlet reporter was inserted at the *skn-1b* transcriptional start site, leading to endogenous *skn-1b* being tagged with *wrmScarlet*. The same expression pattern observed with the endogenous Scarlet::SKN-1B reporter [Figure 11 B]. These results indicate that SKN-1B::GFP can be expressed independently from other SKN-1 isoforms and *skn-1b* is expressed exclusively in the ASI neurons.

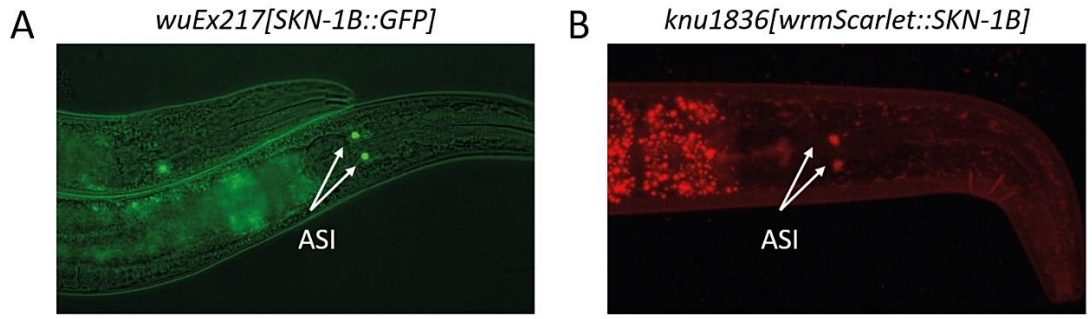


Figure 11: *skn-1b* is being expressed exclusively in the ASI neurons. **A)** SKN-1B::GFP expression in WT*[Pskn-1b::skn-1b::GFP]*. The SKN-1B::GFP translational reporter confirmed that SKN-1B::GFP can be expressed independently from other SKN-1 isoforms and *skn-1b* is expressed solely in the ASIs. **B)** This expression pattern was confirmed with an endogenous Scarlet::SKN-1B reporter. These images were generated by Max Thompson (PhD student in Tullet lab).

To investigate the interaction between environment and *skn-1b*, we examined the expression of SKN-1B under the influence of food-related cues. Our results showed that diluting bacteria in liquid culture leads to an increased expression of SKN-1B::GFP in the ASIs [Figure 12]. Similar results had previously been observed using a SKN-1B/C::GFP transgene (Bishop & Guarente, 2007). In addition, similar increase in SKN-1B::GFP levels compared to ad libitum fed controls

was observed when animals were fasted for 24 hrs [Figure 12]. These results indicate that neuronal SKN-1B levels respond selectively to the amount of food available.

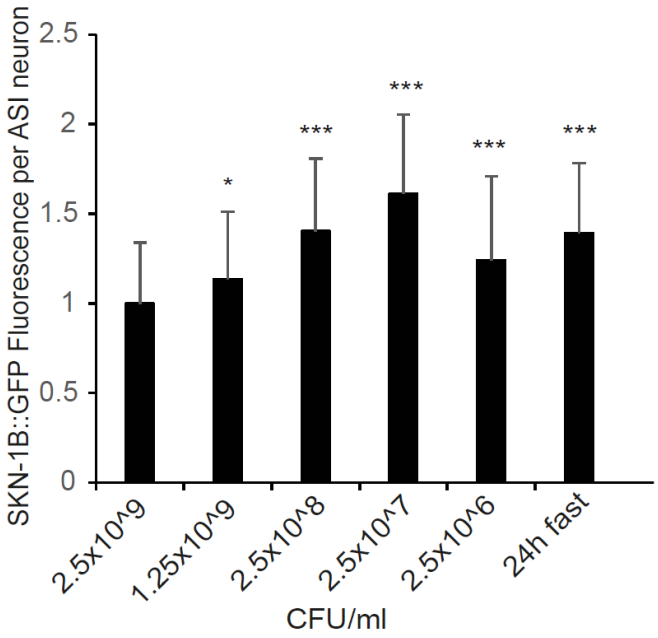


Figure 12: SKN-1B::GFP levels respond to the amount of food. Quantitative fluorescence microscopy of SKN-1B::GFP in response to bacterial dilution [169] and 24 hrs fasting. Imaged at 1-day adults, each bar is the mean of 3 biological replicates ± st. dev. Two-tailed *t-test *p<0.05, ***p<0.0001,* NS not significant.

To explore further the SKN-1B response to different food cues, worms were fed with different bacterial diets and SKN-1B::GFP levels measured. *C. elegans* carrying the SKN-1B::GFP reporter were fed with the following bacterial diets: *E. coli* B (OP50), *E. coli* B/K-12 hybrid (HB101), *E. coli* K-12 (HT115), *B. subtilis* (PY79), and *Pseudomonas aeruginosa* (PA14). Animals were allowed to feed with the appropriate bacteria from hatching to L4 stage under standard

conditions. The, SKN-1B::GFP levels in these animals was then compared with the levels of OP50 fed animals.

Compared with OP50, SKN-1B::GFP levels were not altered when worms were fed with HT115 or HB101. In contrast, SKN-1B::GFP levels increased in response to *Bacillus Subtilis* or the pathogen *Pseudomonas aeruginosa*, in comparison with OP50 [Figure 13 A]. To test whether this induction of expression occurs dynamically, animals were grown on OP50 until the L4 stage. Then, animals were placed onto PY79 bacterial diet for 16hrs and SKN-1B levels measured using quantitative fluorescence microscopy and compared with the animals left on the OP50 bacterial diet. The data showed a significant induction of SKN-1B expression on PY79, indicating that neuronal SKN-1B::GFP expression increases specifically and rapidly in response to different bacterial diets [Figure 13 B].

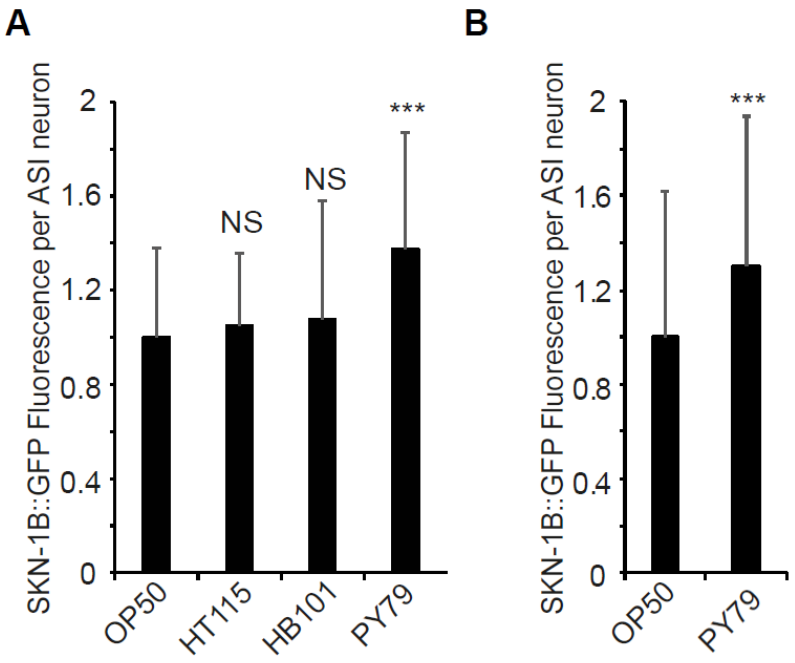


Figure 13: SKN-1B::GFP levels respond to different bacterial strains. A) Quantitative fluorescence microscopy of SKN-1B::GFP in response to different bacterial strains. **B)** Quantitative

fluorescence microscopy of SKN-1B::GFP. Animals fed with OP50 until L4 stage then allowed to feed with PY79 for 16 hrs. Imaged at 1 day adults, each bar is the mean of 3 biological replicates ± st. dev. Two-tailed *t-test *p<0.05, ***p<0.0001,* NS not significant.

skn-1b acts to promote food-related exploratory behaviour

Given the sensitivity of neuronal SKN-1B levels in response to the amount and type of food, we asked whether *skn-1b* is involved in fundamental food-related behaviours. In worms, there are three main food-related locomotory behaviours- roaming, dwelling, and quiescence. All three together comprise nematode's exploratory behaviour (Ben Arous et al., 2009). We examined the role of *skn-1b* in exploratory behaviour using a *skn-1b*-specific allele *(tm4241)*. To test that, an exploration assay was performed. Individual L4 stage animals placed onto uniformly seeded plates and allowed to explore for 16 hrs.

During the 16 hrs period, WT animals explored significantly more in comparison with the *skn-1b* mutants [Figure 14]. More specifically, WT animals explored ~82% of the plate, whereas *skn-1b* mutants explored only ~45% of the plate. These results indicate that *skn-1b* mutants have an impaired exploratory behaviour compared to WT. To explore this further, we examined the exploratory behaviour of animals carrying specific *skn-1* mutations. The exploratory behaviour of *skn-1(zu135)* mutants which lack all *skn-1* isoforms and *skn-1(zu67)* mutants which lack only *skn-1a* and *c* isoforms examined. During the 16 hrs period, *skn-1(zu135)* mutants explored only ~47% of the plate, whereas *skn-1(zu67)* mutants explored ~82% of the plate [Figure 14]. These results indicate that the absence of all *skn-1* isoforms impairs exploratory behaviour in a similar degree as in *skn-1(tm4241)* mutants. In addition, the absence of *skn-1a* and *c* does not affect exploratory behaviour as shown in *skn-1(zu67)* mutants. Thus, exploratory behaviour was found to be *skn-1b* dependent.

Finally, we examined whether the exploratory deficit of *skn-1b* mutants could be rescued. Microinjection experiments performed on *skn-1b(tm4241)* animals. A SKN-1B::GFP specific transgene used which drives *skn-1b* expression from its own promoter specifically in the ASI neurons. The following strains generated and used for exploration assay: JMT66 (*skn-1b(tm4241), ukcEx15 [Pskn-1b::skn-1b::GFP; myo-3::mcherry]*) and JMT67 (*skn-1b(tm4241); ukcEx16 [Pskn-1b::skn-1b::GFP; myo-3::mcherry]*). These animals performed exactly as the WT animals, showing no exploratory impairment [Figure 14].

These results indicate that restoring the lost SKN-1B in the ASI neurons is sufficient to rescue the impaired exploratory behaviour of *skn-1b* mutants. In conclusion, *skn-1b* acts to promote food-related exploratory behaviour in *C. elegans*.

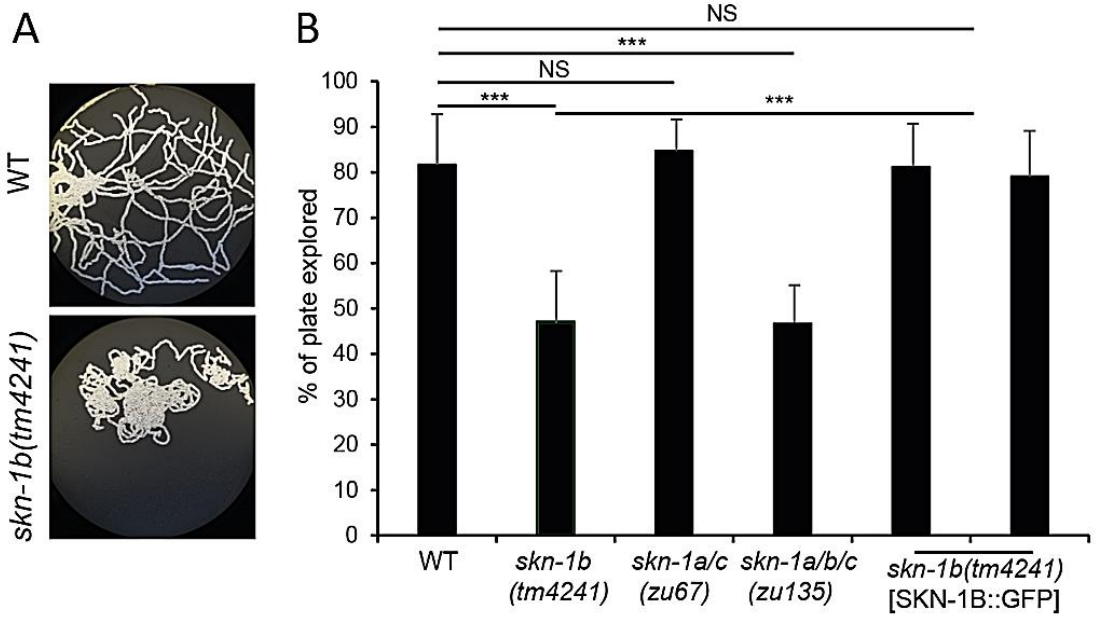


Figure 14: *skn-1b* is required for normal exploratory behaviour. **A)** Agar plates showing exploratory behaviour of a single WT and *skn-1b* animal over the 16 hrs period. **B)** Quantification of exploratory behaviour. WT animals explore significantly more, they cover almost the whole surface of the plate. In contrast, *skn-1b* mutants explore significantly less and remain in a restricted area of the plate. Mean plate coverage of n>11 animals per group ± st. dev. One representative experiment of 3 biological replicates shown. Two-tailed *t-test *p<0.05, **p<0.001, ***p<0.0001,* NS not significant.

The *skn-1b* exploratory phenotype is not due to SKN-1's role in development

From published work we know that some *skn-1* isoforms are important for normal embryogenesis (Blackwell et al., 2015). Since we have no clear evidence about *skn-1b* and developmental disruption, we examined that possibility. More specially, we asked whether the lack of *skn-1b* disrupts ASI development and function, which in turn leads to impaired exploratory behaviour.

WT animals exposed to *skn-1* RNAi post embryonically from L1 or L4 larval stages and their exploratory behaviour examined. We examined progeny production on the *skn-1* RNAi maintenance plates, for the effectiveness of RNAi treatment (data not shown). In *C. elegans*, the absence of *skn-1a* or *c* is embryonic lethal, thus no progeny is being produced and there is an accumulation of dead eggs. Plates seeded with control RNAi showed a robust production of progeny and a population of animals varying from L1 larval stage to adults. In contrast, plates seeded with *skn-1* RNAi showed no progeny production and only the population of the selected adults was present. These results indicate that RNAi treatment is knocking down the isoforms of *skn-1*. However, standard RNAi method in worms has limitations, especially when used for neuronal-targeted experiments. We did not obtained results proving the efficiency, or the complete knock down of *skn-1* in the neurons of these animals. We examined GFP expression in WT animals carrying SKN-1B::GFP fed with *skn-1* RNAi. We have found that *skn-1* RNAi reduces the SKN-1B levels in the ASI neurons [Figure 15].

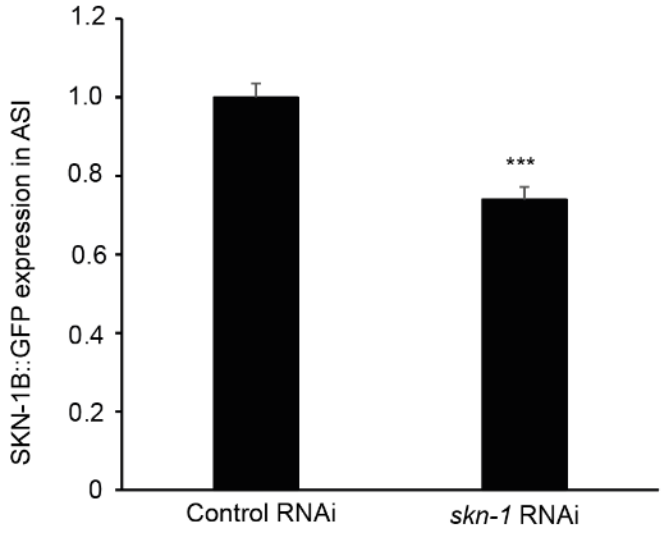


Figure 15: *skn-1* RNAi effectively reduces SKN-1B::GFP levels in ASI neurons. Neurons are relatively resistant to RNAi (Timmons et al., 2001), but quantitative fluorescence microscopy shows that *skn-1* RNAi from the L1 larval stage reduces SKN-1B::GFP in ASIs by ~30%. Pooled data from 3 biological replicates shown, n>100 individual worms per group. Two-tailed *t-test *p<0.05, **p<0.001, ***p<0.0001,* NS not significant.

We performed an exploration assay to examine the impact of *skn-1* RNAi treatment on behaviour. *skn-1* RNAi treatment from either the L1 or L4 larval stage effectively impaired WT animals' exploratory behaviour [Figure 16]. WT animals fed with control RNAi from L1 stage and explored 75% of the plate in a 16hr period. In contrast, WT animals fed with *skn-1* RNAi from L1 stage explored only 46% of the plate in a 16hr period [Figure 16 B]. Likewise, WT animals fed with control RNAi from L4 stage explored 69% of the plate, whereas WT animals fed with *skn-1* RNAi from L4 stage explored 57% of the plate in a 16hr period [Figure 16 C]. These results indicate that *skn-1* RNAi effectively impairs exploration behaviour in post-embryonically WT animals.

RNAi treatment of L1 stage animals showed a stronger suppression of exploratory behaviour compared to L4 stage treated animals [Figure 16 B, C]. This is presumably due the duration of RNAi treatment. L1 stage animals have already *skn-1* knocked down by the time of exploration

assay is performed in contrast with the L4 stage animals where *skn-1* is being knocked down at the same time as the exploration assay is performed. In conclusion, *skn-1* phenotype is not due a developmental impairment of the ASI neurons.

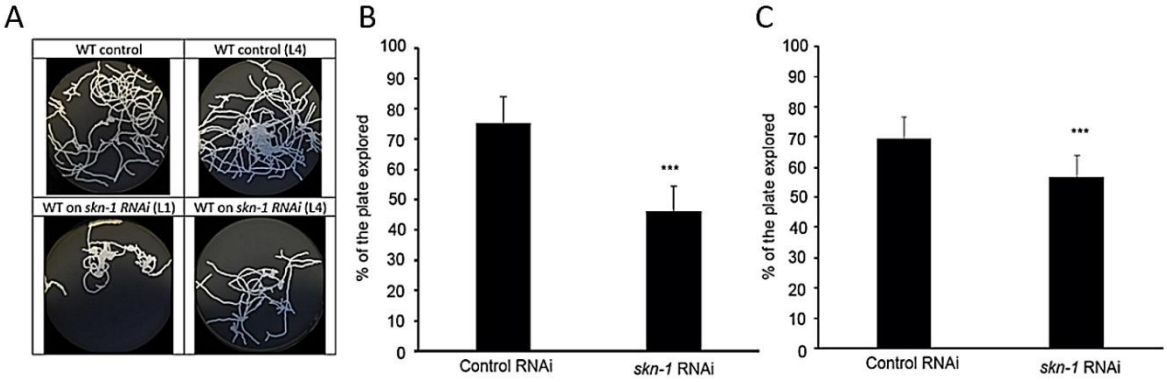


Figure 16: *skn-1* RNAi effectively impairs exploratory behaviour of wild-type animals. A) Agar plates showing the exploratory behaviour of WT animals treated with control or *skn-1* RNAi from L1 or L4 larval stage. **B, C)** Quantification of exploratory behaviour. WT animals fed with *skn-1* RNAi, similar to *skn-1a/b/c (zu135)* mutants, demonstrate impaired exploratory behaviour. Mean plate coverage of n>11 animals per group ± st. dev. One representative experiment of 3 biological replicates shown. Two-tailed *t-test *p<0.05, **p<0.001, ***p<0.0001,* NS not significant.

The *skn-1b* exploratory phenotype is not due to impaired movement

As *skn-1b* mutants did not move as much as WT animals we asked whether this was due to impaired motility e.g. in the case of motor neuron disruption exploratory behaviour is restricted. To exclude that possibility a thrashing assay was performed. The Nemametrix microtracker uses laser beams to measure the thrashing ability of animals in liquid. Beam disruption by the animals' moving body permits the accurate identification and measurement of thrashing ability. Using the microtracker, the movement of L4 WT and *skn-1b* animals was compared and found to have similar thrashing rates [Figure 17]. *skn-1b* mutants perform as well as WT in liquid, with regards to thrashing ability.

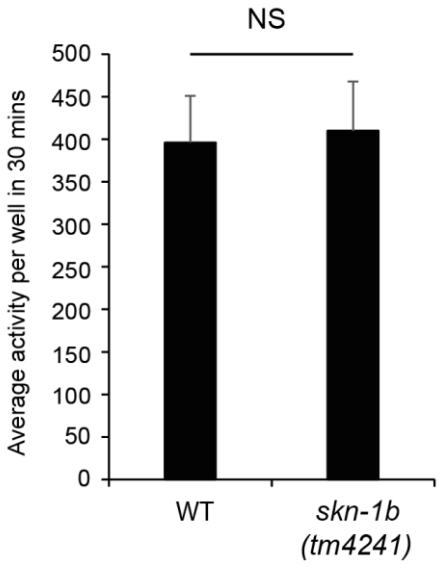


Figure 17: *skn-1b* mutants demonstrate normal thrashing ability. Quantification of WT and *skn-1b* animals thrashing activity in liquid. *skn-1b* mutants demonstrate normal thrashing activity. Average of 3 biological replicates shown, n>33 individual worms per group. Two-tailed *t-test *p<0.05, **p<0.001, ***p<0.0001*, NS not significant.

In addition, motility of WT and *skn-1b* animals was examined on solid media plates [Figure 18] (Magnitude Biosciences). A camera recorded the activity of WT an *skn-1b* animals under standard conditions for 20 hours. Then, software was used to analyse the speed, distance covered, and area explored of animals. *skn-1b* mutants performed as well as WT animals in all assays regarding speed and distance covered. However, again, *skn-1b* mutants were found to explore less and restricted themselves to a small area of the plate. These results combined indicate that *skn-1b* reduced exploratory behaviour is a matter of environment perception and not due a movement impairment.

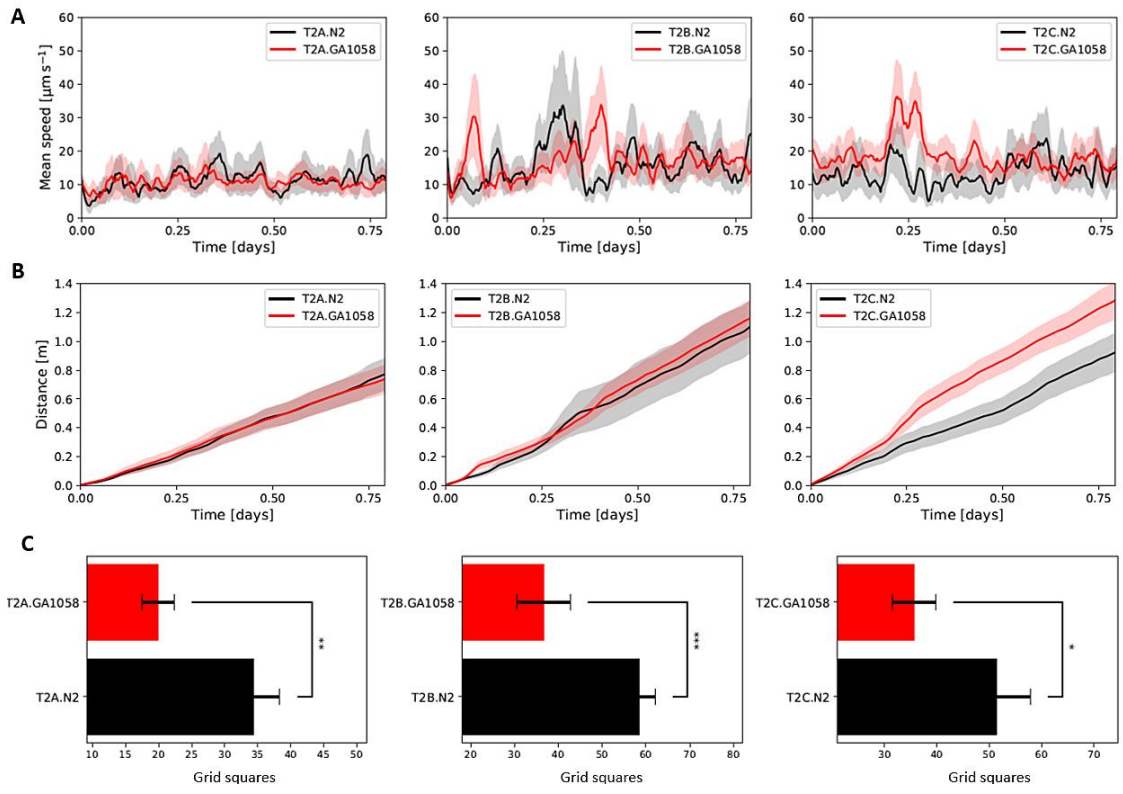


Figure 18: Motility assays using WT and skn-1b animals. A) Mean speed, **B)** Distance covered, **C)** Exploratory behaviour. *skn-1b* mutants performed as well as WT animals in speed and distance tests. Exploratory behaviour of *skn-1b* mutants showed an impairment compared to WT animals. Two-tailed *t-test *p<0.05, **p<0.001, ***p<0.0001*, NS not significant. These data were generated by Magnitude Biosciences.

daf-11 and *skn-1b* interact to perceive the environment

The ASI neurons comprised by two cells that found anterior to the large bulb of the pharynx. Projections from these cells reach forward to the amphid openings (the worm's nose). ASI neurons express *daf-11*, a transmembrane receptor-type guanylate cyclase, which communicates signals from the environment to the cell body [Figure 5] *daf-11* mutants demonstrate sensory impairments, including chemoattraction towards known attractants such as NaCl and diacyl. In addition, *daf-11* is required for normal dauer entry and exit. SKN-1B is expressed in the ASI neurons and responds to environmental cues such as the amount and type of food [Figure 11, 12, 13]. In addition, SKN-1B is required for normal exploratory behaviour [Figure 14]. I then examined the relationship between *daf-11* and *skn-1b*, hypothesizing that they communicate in order worms to perceive environmental cues, which can modify behaviour.

The epistatic relationship between of *daf-11* and *skn-1b* was examined using exploration assay [Figure 19]. WT, *skn-1b*, *daf-11*, and *daf-11; skn-1b* exploratory behaviour analysed. *daf-11* mutants showed reduced exploratory behaviour similar to *skn-1b* mutants. In addition, *daf-11; skn-1b* double mutants showed reduced exploratory behaviour similar to *daf-11* and *skn-1b* single mutants. The absence of an additive effect of these two mutations indicates that *daf-11* and *skn-1b* operate in the same pathway to regulate behaviour.

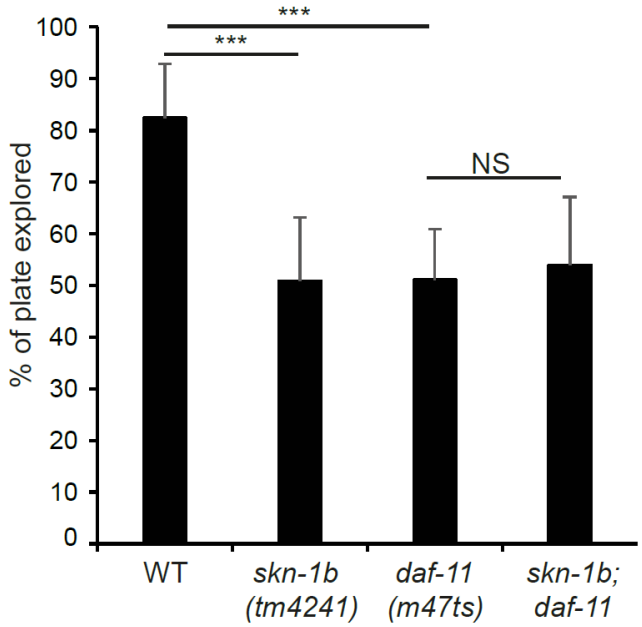


Figure 19: *daf-11* and *skn-1b* act on the same pathway to control behaviour. Quantification of exploration. *skn-1b* and *daf-11* mutants demonstrate reduced exploratory activity compared to WT animals. Interestingly, *skn-1b; daf-11* mutants explore same as *skn-1b* and *daf-11* mutants. Mean plate coverage of n>11 worms per group ± st. dev. One representative experiment of 3 biological replicates shown. Two-tailed *t-test *p<0.05, **p< 0.001, ***p<0.0001,* NS not significant. The *daf-11; skn-1b* double mutant was generated by Max Thompson.

Our behavioural data showed an interaction between *daf-11* and *skn-1b*. We examined their relationship further, by looking the SKN-1B::GFP levels in *daf-11* mutants [Figure 20]. We have previously shown that the absence of food for 24 hours is sufficient to increase SKN-1B::GFP levels compared to fully fed conditions [Figure 12]. However, the lack of a functional *daf-11* significantly reduces the SKN-1B::GFP levels and completely diminishes the effect of fasting, with SKN-1B::GFP levels no longer being elevated [Figure 20]. These results indicate that SKN-1B requires functional *daf-11* to respond to the environment. Taken together behavioural and expression experiments [Figure 19, 20] and given the ASI expression patterns of DAF-11 (amphid opening) and SKN-1B (nucleus), an epistatic relationship for these molecules

revealed. This relationship supports the integration of the external environment to SKN-1B levels and subsequent behaviours.

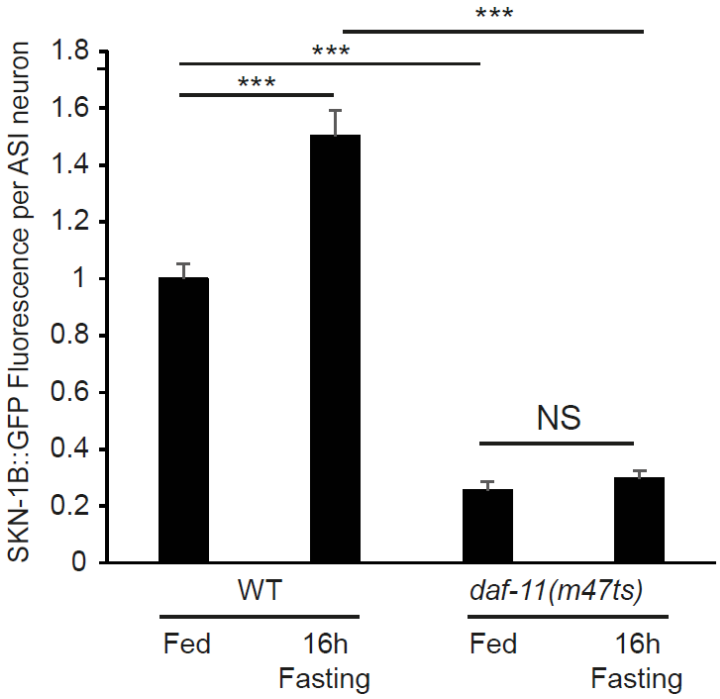


Figure 20: *daf-11* mutation alters SKN-1B::GFP levels in response to food. Quantitative fluorescence microscopy of SKN-1B::GFP, in response to a combination of *daf-11* mutation and fasting was used. Imaged at 1 day adults, each bar is the mean of 3 biological replicates ± st. dev. Two-tailed t-test *p<0.05, ***p<0.0001, NS not significant. These data were generated by Max Thompson.

skn-1b mutants demonstrate disrupted food avoidance behaviour

A common response of animals against stressful conditions is to attempt escape. *C. elegans* demonstrates aversive behavioural responses to a wide range of stimuli associated with physiological stress such as high temperature, pathogenic bacteria, specific RNAi clones/bacteria, hypoxia, hyperoxia, and ultraviolet light (Bargmann et al., 2006; Ben Arous et al., 2009; Shtonda & Avery, 2006a). In the wild, *C. elegans* is exposed to a wide range of bacteria with some of these can be used as food sources and others can be pathogenic. For this reason, it is crucial the nematodes to be able to distinguish and avoid pathogenic food sources. This response can be learned, and it is called aversive response (Boris Borisovich Shtonda & Avery, 2006a). Memory and perception of the nutritional value of the diet are both crucial for aversive response in *C. elegans*.

The exploration assay measures the exploratory behaviour of animals on a uniformly seeded plate. The continuous lawn of *E. coli* allows us to observe the worm tracks and quantify them. As *skn-1b* mutants demonstrate an impaired exploratory behaviour, we reasoned that they may spend less time away from food than WT animals. To test this, we performed food avoidance assays. NGM plates seeded with a small lawn of OP50 bacteria in the centre of an otherwise empty plate. Adult animals were allowed to lay eggs for 4 hours and the resulting progeny allowed to develop to L4 stage. The number of worms on and off the bacteria lawn were then quantified.

At any given time, approximately 25% of WT animals were found away from a standard OP50 lawn, in contrast with *skn-1b* mutants which were all found on the lawn [Figure 21]. Similar mild avoidance of bacteria lawns in WT animals, but not in *skn-1b* mutants was observed for other bacteria, including another four *E. coli* strains, *Comamonas aquatica* and a *Pseudomonas species* encountered in the wild [Figure 21].

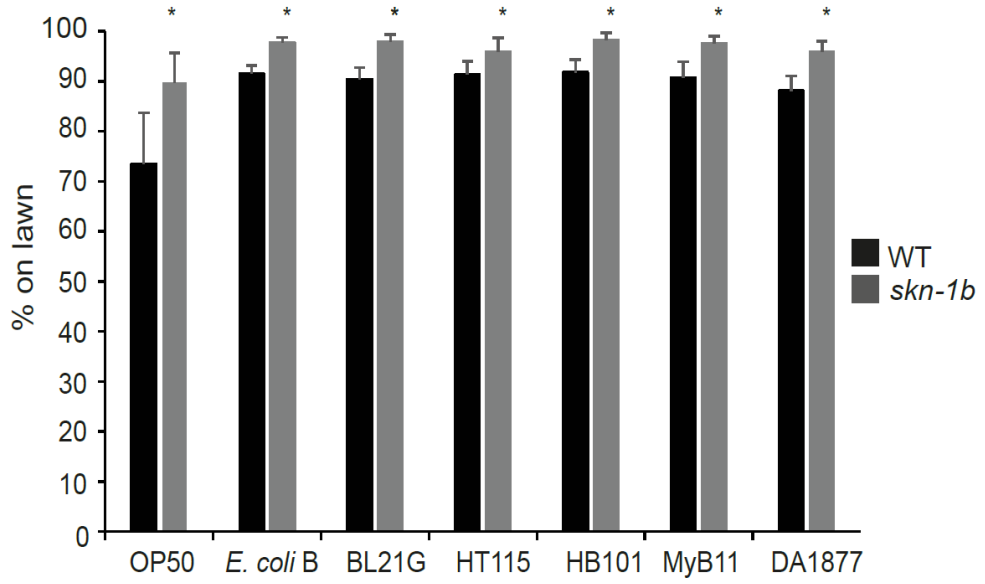


Figure 21: SKN-1B in the ASI neurons is required for optimal food avoidance response. Quantification of animals being on the bacterial lawn. *skn-1b* mutants found to remain on the bacterial lawn in comparison with WT animals. Each bar represents a mean of 3 biological replicates ± st. dev. Two-tailed *t-test *p<0.05, **p< 0.001, ***p<0.0001,* NS not significant.

Surprisingly, when WT animals were fed with *B. subtilis* (PY79), the proportion on the lawn increases compared to OP50, whereas that of *skn-1b* mutants remains the same [Figure 22 A]. No difference in lawn avoidance was observed on *E. coli* K-12 (W3110 or MG1655) [Figure 22 B]. In conclusion, almost all *skn-1b* mutants are present on an OP50 lawn, implying that while WT animals adapt to preferentially consume some food, *skn-1b* mutants do not.

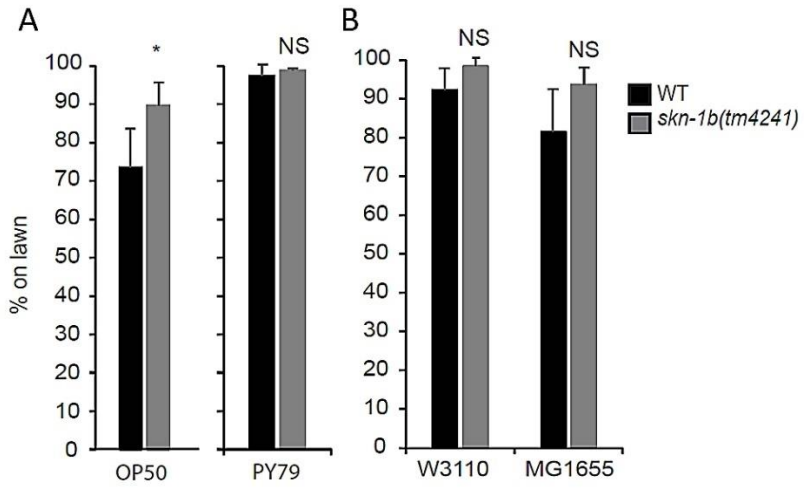


Figure 22: Food avoidance response of WT and *skn-1b* mutants. Quantification of animals being on the bacterial lawn. No significant difference between WT and *skn-1b* animals food avoidance behaviour on A) PY79, B) W3110, MG1655. Each bar represents a mean of 3 biological replicates ± st. dev. Two-tailed *t-test *p<0.05, **p< 0.001, ***p<0.0001,* NS not significant.

SKN-1B::GFP levels were increased in response to pathogenic bacteria *P. aeruginosa* compared to SKN-1B::GFP levels on OP50 [Figure 23 A]. More specifically, SKN-1B::GFP levels increase after feeding with *P. aeruginosa* and we hypothesised that *skn-1b* might contribute to pathogen avoidance response. To test this, we examined food avoidance behaviour of WT and *skn-1b* animals fed with *P. aeruginosa* [Figure 23 B]. However, both WT and *skn-1b* animals avoided the pathogen to a similar degree [Figure 23 B]. This indicates that *skn-1b* is not involved in pathogen avoidance behaviour. In conclusion, *skn-1b* acts to sense food types rather than pathogenicity and subsequently controls behaviour.

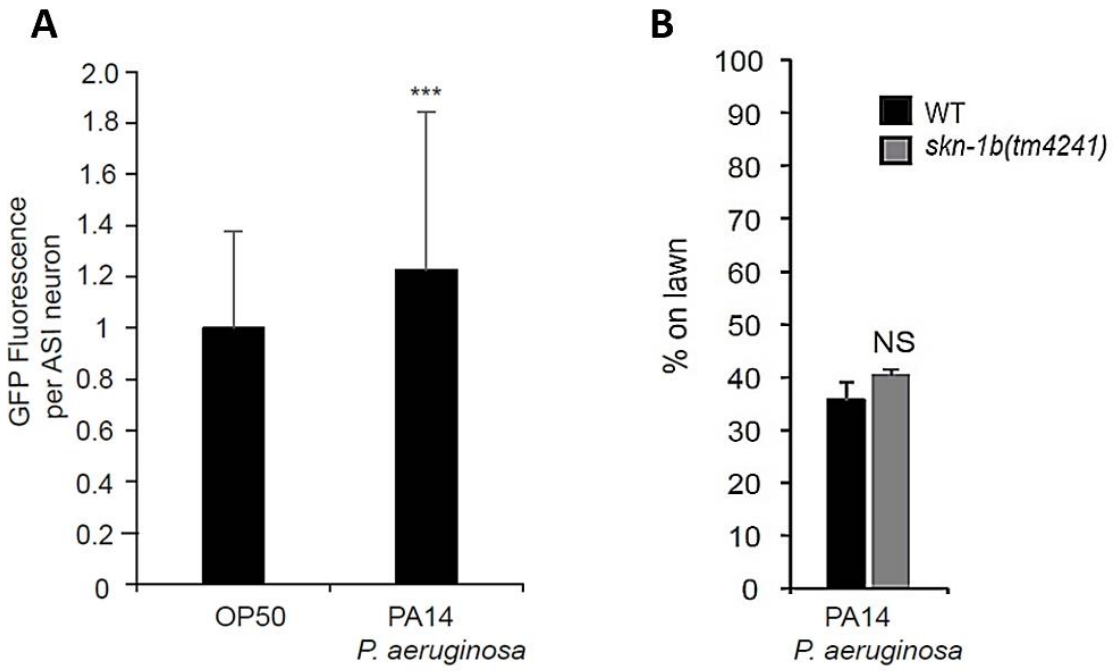


Figure 23: SKN-1B in the ASI neurons and response to *P. aeruginosa*. A) SKN-1B::GFP levels in the ASI neurons. **B)** Food avoidance assay on *P. aeruginosa*. Each bar represents a mean of 3 biological replicates ± st. dev. Two-tailed *t-test *p<0.05, **p< 0.001, ***p<0.0001,* NS not significant.

skn-1b regulates food-related behaviours in response to fasting

C. elegans exploratory behaviour plays an important role in locating food sources (Ben Arous et al., 2009). The absence of food known as fasting, alters worms' behaviour upon re-feeding. More specifically, worms during re-feeding demonstrate increased food consumption and spent more time in dwelling state (Ben Arous et al., 2009). Roaming and quiescence periods are diminished, in order the animal to refuel its energy stores. After that, there is a change in the behavioural states of the worm and increased quiescence periods appear, related to mammalian satiety.

Exploratory behaviour

SKN-1B::GFP levels respond to the absence of food (fasting for 24hrs) [Figure 12]. Therefore, the absence of *skn-1b* may dysregulate behaviours associated with fasting in worms. To investigate the role of *skn-1b* in fasting, exploration assay performed with animals being fasted for 1 to 16hrs prior to the experiment. Whilst WT animals demonstrated reduced exploratory behaviour under these conditions, *skn-1b* mutants did not [Figure 24 A]. Fasting of WT animals for 16hrs severely affected exploratory behaviour compared to 1 hr fasting but had no effect on *skn-1b* mutants [Figure 24 B]. These results indicate that *skn-1b* is essential for behavioural regulation in response to fasting and re-feeding.

From our previous experiments we have found that *daf-11* mutants demonstrate similar exploratory behaviour to *skn-1b* mutants [Figure 19]. We wanted to examine whether *daf-11* was required for *skn-1b* mediated behavioural changes in response to fasting and re-feeding. *daf-11* mutants like *skn-1b* mutants, did not respond to fasting and re-feeding [Figure 24 A]. In addition, the combined effect of *daf-11; skn-1b* mutants was non-additive [Figure 24 A]. These results indicate that the combined function of DAF-11 and SKN-1B are needed for

C. elegans to modulate their exploratory behaviour in response to fasting and re-feeding. This supports the conclusion that these two proteins operate in the same pathway.

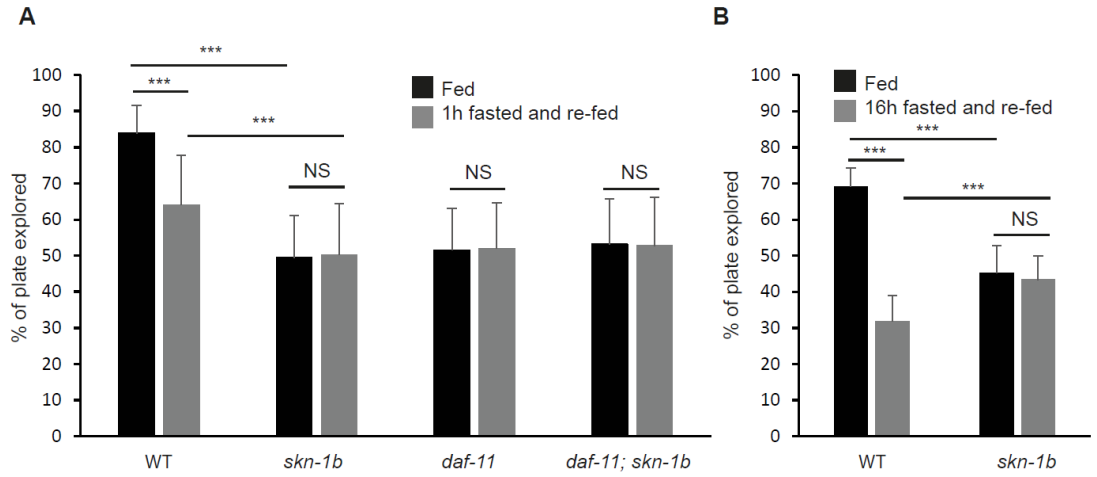


Figure 24: *daf-11* and *skn-1b* interaction and response to fasting. A) Quantification of exploration in fed vs fasted/re-fed conditions, worms fasted for 1hr. WT animals respond to fasting and demonstrate reduced exploratory activity compared to their fed counterparts. In contrast, *skn-1b*, *daf-11*, and *daf-11; skn-1b* mutants do not respond to fasting and demonstrate similar exploratory activity as their fed counterparts. Mean plate coverage of n>35 individual worms per group ± st. dev., 3 combined experiments shown. **B)** Quantification of exploration in fed vs fasted/re-fed conditions, worms fasted for 16hrs. WT respond to this extended period of fasting and demonstrate a more severe reduction in exploratory behaviour in contrast with *skn-1b* mutants where they do not alter their exploratory behaviour. Mean plate coverage of n>7 worms per group ± st. dev., one representative experiment of 3 trials shown.

Satiety induced quiescence behaviour

C. elegans demonstrates three main food-related behaviours, roaming, dwelling, and quiescence (Ben Arous et al., 2009). Quiescence resembles satiety in mammals. Our results showed that fasting and re-feeding impacts exploratory behaviour of WT animals but not of *skn-1b* mutants [Figure 24]. This can be attributed to either increased time spent dwelling or in quiescence. To investigate the quiescence behaviour of WT and *skn-1b* animals, fasting and re-feeding of animals performed and then, their quiescence periods measured. As *skn-1b* mutant's exhibit reduced exploration we asked whether they also differ in these other behaviours.

C. elegans quiescence periods peak between 3 and 6 hours after fasting and re-feeding (Gallagher & You, 2014; You et al., 2008). WT and *skn-1b* L4 stage animals selected and fasted for 16 hrs. Then, they allowed to feed for 3-6 hrs and their quiescence behaviour determined. *skn-1b* mutants found to spent longer in quiescence state than WT animals in both 3 and 6hrs after re-feeding [Figure 25]. However, similar numbers of WT and *skn-1b* animals were found in quiescence state under these conditions (data not shown). In conclusion, SKN-1B acts to suppress satiety-induced quiescence, promoting exit from but not entry into quiescence. Despite that, this assay does not measure quiescence period in fed conditions and taken together with the inability of *skn-1b* mutants to alter their behaviour in response to fating and re-feeding, implies that the impaired exploration of *skn-1b* mutants is due to the increased time spend in quiescence.

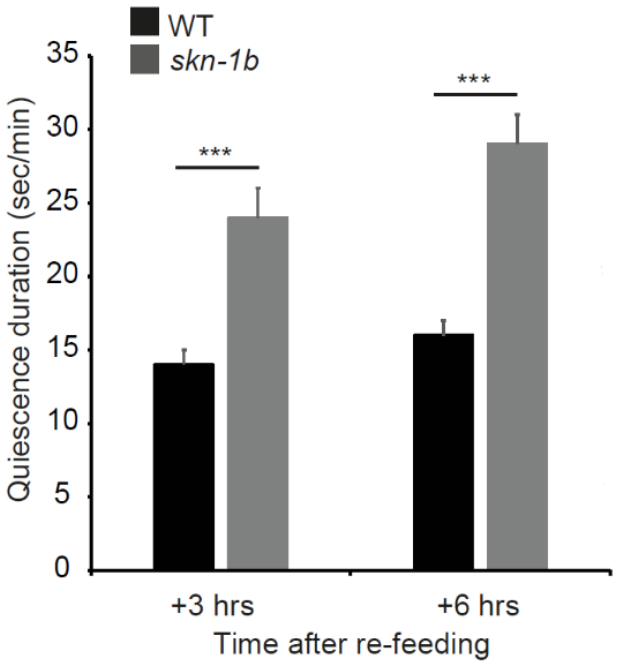


Figure 25: SKN-1B regulates satiety quiescence. Quantification of the time spent in quiescence after fasting and re-feeding. *skn-1b* mutants demonstrate higher quiescence periods compared to WT animals. Each bar represents a mean of 3 biological replicates ± SEM, n>40 worms per group. Two-tailed *t-test *p<0.05, **p< 0.001, ***p<0.0001,* NS not significant.

skn-1b mutation alters feeding behaviour

C. elegans modifies pharyngeal pumping rates according to its environment and other conditions (Ben Arous et al., 2009; Gallagher & You, 2014; You et al., 2008). Pharyngeal pumping and pharyngeal autophagy increase when worms being in a less desirable food source. Fasting also affects pharyngeal pumping. During the re-feeding of fasted worms, pharyngeal pumping is elevated, then, slowly decreases over time.

Quiescence is linked to satiety in mammals. Quiescent *C. elegans* demonstrate inhibition of pharyngeal pumping and thus, no ingestion of bacteria occurs (Gallagher & You, 2014; You et al., 2008). Since *skn-1b* mutants spend more time in quiescence after fasting, we wanted to examine their pharyngeal pumping behaviour under fed or fasted-refed conditions. In addition, *skn-1b* mutants spend more time on food regardless the type of bacterial diet. Measurement of pharyngeal pumping rates used to examine the amount of food that worms consumed.

Animals were fed *ad libitum* on HB101 bacteria until L4 stage and then transferred onto fresh plates seeded with HB101 or onto plates with no food for 16 hours. Then, animals transferred onto plates seeded with HB101 and allowed to feed for 5 minutes before measuring the pharyngeal pumping rates. *skn-1b* mutants in both fed and fasted-refed conditions showed significantly higher pumping rates compared to WT animals [Figure 26 A]. In addition, fasting influenced animal's pharyngeal pumping behaviour and promoted higher rates compared to fed conditions [Figure 26 A]. WT and *skn-1b* animals responded to fasting with regards to pumping rates.

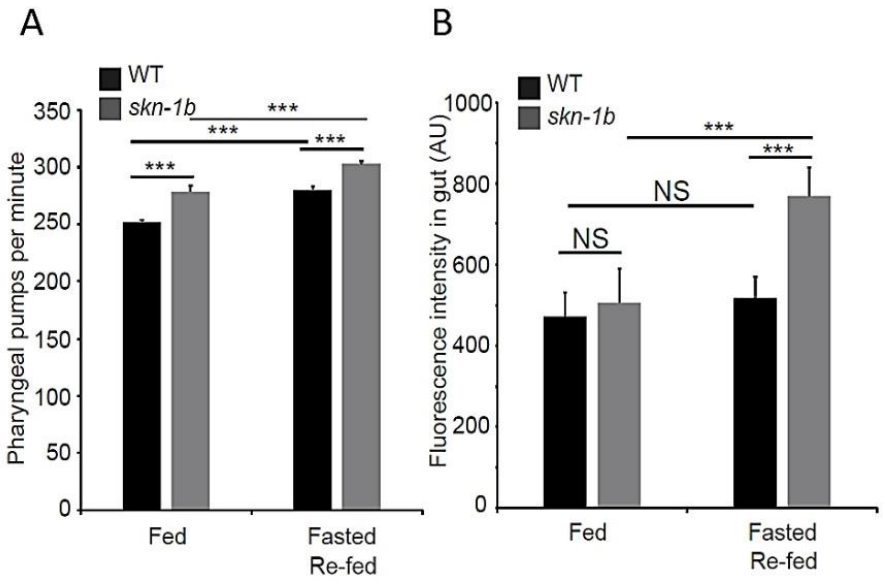


Figure 26: SKN-1B is required for optimal feeding rates. A) Quantification of pharyngeal pumping rates in fed and fasted re-fed conditions. *skn-1b* mutants demonstrate elevated pharyngeal pumping in both fed and fasted-refed conditions compared to WT. Each bar represents a mean of 3 biological replicates, ± st. dev., n=7 worms per group. **B)** Effect of *skn-1b* on intake of fluorescently labelled OP50. *skn-1b* mutants demonstrate elevated intake of OP50 in fasted and re-red conditions compared to WT. Each bar represents a mean of 3 biological replicates, ± st. dev., n>42 worms per group.

In *C. elegans* higher pharyngeal pumping is often translated to increased food intake. However, there are cases where this relationship is not true. To explore this further, food intake experiments performed using fluorescently labelled OP50 bacteria to measure bacteria ingestion. More specifically, animals which maintained on NGM plates seeded with OP50 allowed to reach L4 stage. Then, animals selected and transferred onto plates with or without food for 16 hrs. After that, fed and fasted animals transferred onto mcherry OP50 bacteria plates and allowed to feed for 5 minutes.

The fluorescent intensity of bacteria in the worm's gut was measured. Under fed conditions, WT and *skn-1b* animals contained similar amount of bacteria in their guts [Figure 26 B]. However, in response to fasting and re-feeding, *skn-1b* mutants accumulated more OP50 than WT animals, corresponding to a further increase in pumping rate under these conditions

[Figure 26 B]. Due to the increased pharyngeal pumping of *skn-1b* mutants, we asked whether this behaviour alters body composition and more specifically the size of the animals. Indeed, *skn-1b* mutants found to be 10% larger than WT animals [Figure 27]. In conclusion, *skn-1b* mutation alters feeding behaviour and response to fasting.

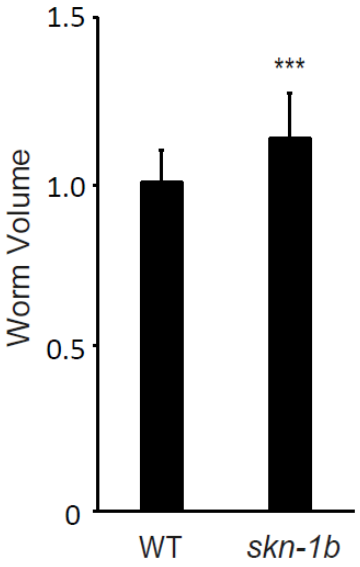


Figure 27: *skn-1b* mutants demonstrate increased body size. Quantification of animals' volume. *skn-1b* mutants appeared to be slightly larger compared to WT animals. Each bar represents a mean of 3 biological replicates, ± st. dev., n>63 worms per group.

SKN-1B requires TGF-β signalling to specify satiety-induced quiescence

Previous experiments showed that *skn-1b* function in the ASI neurons is essential for the regulation of food-related behaviours. This can be achieved through the transmission of chemosensory information from the ASI to the rest of the animals via neuropeptide secretion (Shtonda et al., 2006; You et al., 2008). DAF-7, the ligand of the canonical TGF-β signalling pathway considered as one of these neuropeptides which can regulate various functions in worms including behaviour (Nolan et al., 2002)[Figure 4]. However, the regulators upstream of DAF-7 are not yet known. Under favourable for growth and reproduction environmental conditions, ASI neurons secrete DAF-7 (You et al., 2008). In addition, DAF-7 expression reaches the highest levels when worms demonstrate high levels of quiescence. Expression of *daf-7* in ASI neurons has been shown to promote quiescence although *daf-7* mutants are unable to enter a satiety quiescence state (Gallagher & You, 2014; You et al., 2008).

Due to enhanced quiescence behaviour of *skn-1b* mutants we wanted to examine the possibility of *daf-7* acting as a contributing factor. To test this, we examined the ability of *daf-7; skn1b* mutants (stain was generated by Max Thompson) to undergo quiescence in response to fasting and re-feeding. In agreement with published work, WT animals demonstrated enhanced quiescence following re-feeding, in contrast with *daf-7* mutants [Figure 28]. Furthermore, *skn-1b* mutants showed longer quiescence periods compared to WT however, this effect was found to be completely *daf-7* dependent [Figure 28].

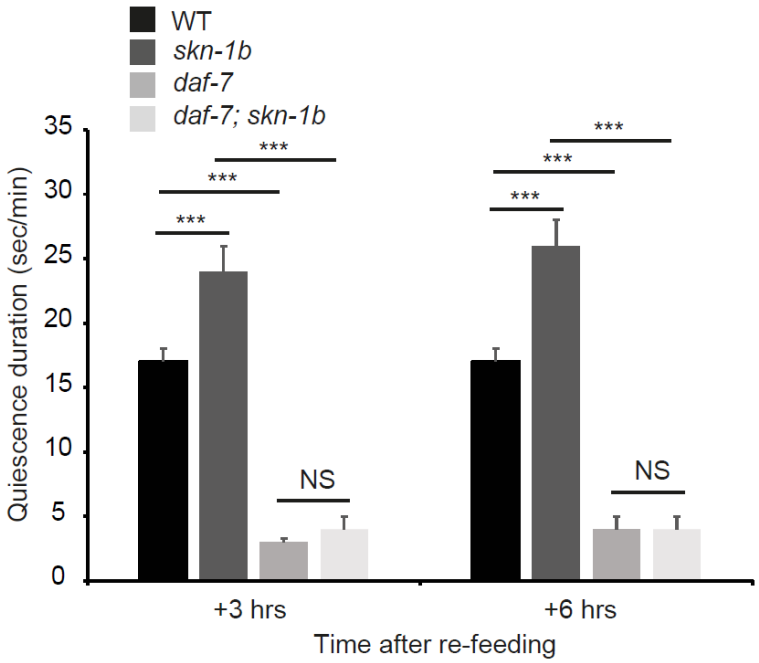


Figure 28: *daf-7* requirement for quiescence behaviour. Quantification of quiescence period of WT, *skn-1b*, *daf-7*, and *daf-7; skn-1b* animals. *daf-7* and *daf-7; skn-1b* mutants demonstrate reduced quiescence periods compared to WT and *skn-1b* animals. Each bar represents a mean of 3 biological replicates, ± SEM, n>9 worms per group. Two-tailed *t-test *p<0.05, **p< 0.001, ***p<0.0001,* NS not significant. The *daf-7; skn-1b* double mutant was generated by Max Thompson.

To investigate the interaction between *daf-7* and *skn-1b* further, we examined the expression of *Pdaf-7::Venus* reporter in WT and *skn-1b* mutants. Likewise to *skn-1b*, *daf-7* is only expressed in ASI neurons however, its expression increased in response to fasting and remained at high levels for at least 6 hrs, presumably promoting entrance into quiescence [Figure 29]. In fed conditions, *skn-1b* mutants demonstrated strongly enhanced *Pdaf-7::Venus* expression which barely changed in response to fasting or re-feeding [Figure 29]. These results indicate that SKN-1B inhibits satiety quiescence in response to fasting and re-feeding by suppressing *daf-7* expression and subsequently TGF-β signalling.

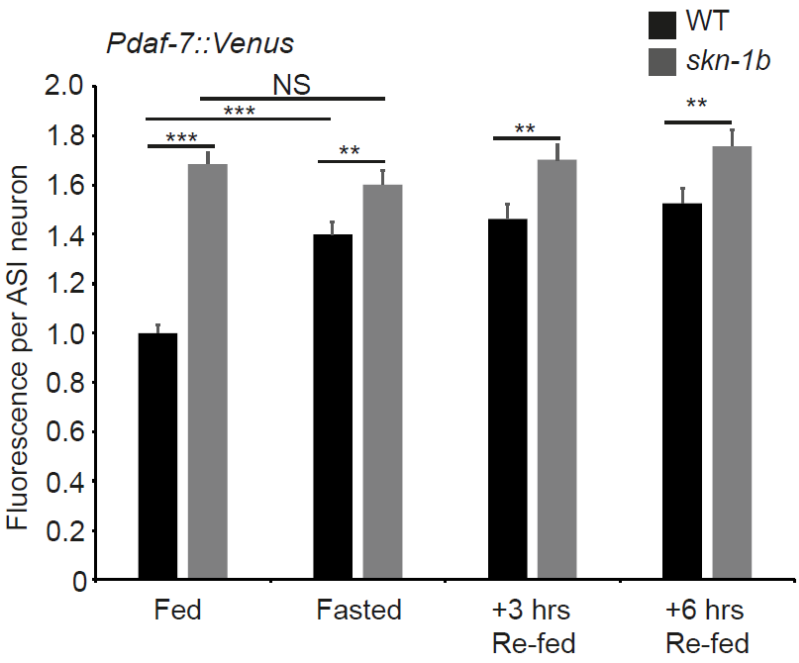


Figure 29: Fluorescence levels of *Pdaf-7::GFP* in ASIs responds to *skn-1b* mutation and food cues. No significant difference was found between WT samples in fasted vs re-fed conditions. No significant difference was found between *skn-1b* samples at any point. This regulation of *daf-7* is unlikely to be direct as there is no SKN-1 binding site within 3Kb of its transcriptional start site. Each bar represents a mean of 3 biological replicates ± st. dev., n>230 worms per group. Two-tailed *t-test* *$p<0.05$, **$p< 0.001$, ***$p<0.0001$, NS not significant.

Published work showed that *daf-7* mutants have deceased exploratory activity in comparison with WT animals (Ben Arous et al., 2009). This in some respects resembles the exploratory behaviour of *skn-1b* mutants. To examine further the behavioural epistasis relationship between *daf-7* and *skn-1b* mutants, exploration assay performed using *daf-7; skn-1b* double mutants. Our results showed that *daf-7* and *skn-1b* effects were non-additive [Figure 30].

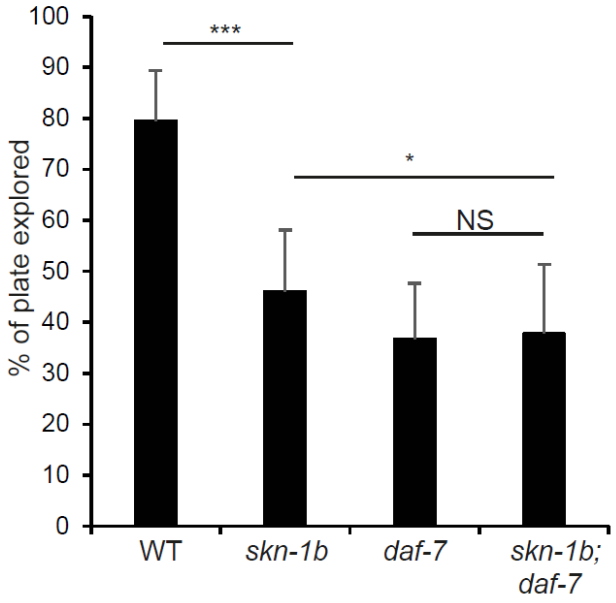


Figure 30: Quantification of exploratory behaviour of WT, *skn-1b*, *daf-7*, and *daf-7; skn-1b* animals. Each bar is a mean of 5 biological replicates, n>44 worms per group ± st. dev. There is no additive effect between *skn-1b* and *daf-7*. Two-tailed *t-test *p<0.05, **p< 0.001, ***p<0.0001,* NS not significant.

SKN-1B modulates IIS to alter food-responsive behaviour

In *C. elegans* there are around 40 insulin-like peptides (ILPs), which are being expressed and secreted (Kimura et al., 1997; You et al., 2008b). Some of them bind to the DAF-2 insulin/IGF-1-like receptor, which is found in various tissues. rIIS leads to the de-phosphorylation and nuclear-localization of its downstream target, the FOXO transcription factor DAF-16 [Figure 3]. Active DAF-16 in *C. elegans* has been implicated in a range of phenotypes including behaviour, longevity, and immunity with the majority of these being mediated by the DAF-16 operation in the gut (Tullet et al., 2008; Tullet, 2015).

Insulin signalling is a conserved pathway for detecting food (K. D. Kimura et al., 2011). In worms, several food-related behaviours are controlled by the nervous system together with the insulin signalling pathway. An example of this is demonstrated when reduced insulin signalling, in *daf-2* mutants, leads to induced behavioural quiescence, which is depended on DAF-16 (Gaglia & Kenyon, 2009). Another example is showed when reduced insulin signalling (*daf-2* mutants), leads to feeding cessation phenotypes, depended on DAF-16 (Mccloskey et al., 1911). *skn-1b* also found to play a role in food-related behaviours, such as fasting-induced quiescence and pharyngeal pumping. Due to its important role in food perception and behaviour, insulin signalling pathway will be examined for interactions with *skn-1b*.

To investigate the involvement of *skn-1b* on this pathway, we examined the cellular localization of a gut-specific DAF-16a::GFP reporter in both WT and *skn-1b* mutants. These experiments were done in collaboration with Alex Howard, a Masters by Research student in the lab. *skn-1b* did not affect nuclear localization of DAF-16 under fed conditions [Figure 31]. In contrast, under a 16 hrs fasting period, DAF-16a::GFP accumulates in both WT and *skn-1b* gut nuclei [Figure 31]. Interestingly, animals lacking *skn-1b* could not maintain DAF-16::GFP in their gut nuclei after re-feeding as WT animals do, reverting to WT levels of nuclear DAF-

16::GFP within 3 hrs of being fed [Figure 31]. These results indicate that *skn-1b* is required to maintain DAF-16 in the nucleus in response to fasting and re-feeding.

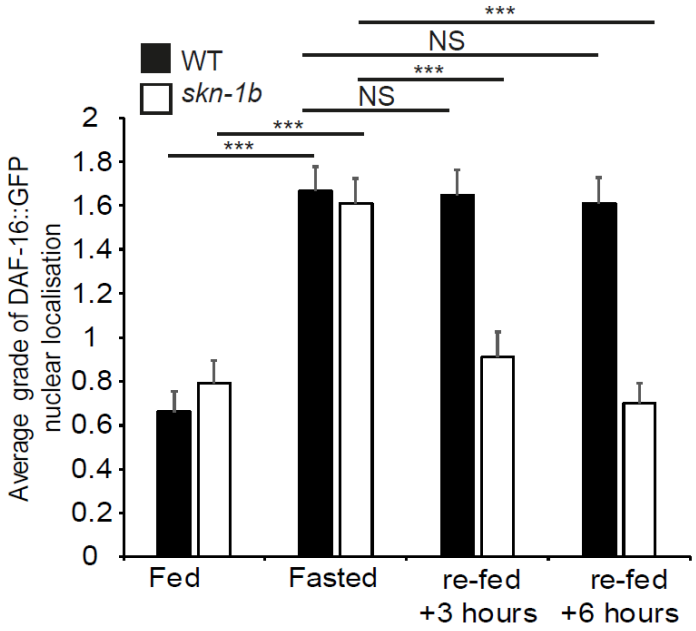


Figure 31: Quantification of nuclear localisation, WT and *skn-1b* worms expressing *ges-1p::GFP::daf-16.* Average grading shown. Combined data from 3 biological replicates shown ± SEM, n>48 worms per group. Two-tailed *t-test *p<0.05, **p< 0.001, ***p<0.0001,* NS not significant. The JMT82 strain used in this experiment was generated by Alex Howard (Masters student in the Tullet lab).

Published work showed that IIS is involved in food-related behaviour (Artan et al., 2016; Ben Arous et al., 2009; You et al., 2008). In agreement with this, I found that *daf-2 (e1370)* mutants demonstrated decreased exploratory activity similarly to *skn-1b* mutants, and dependent on *daf-16* [Figure 32]. To examine the regulatory relationship between SKN-1B and IIS we tested the relationship between *skn-1b* and *daf-16*. I reduced *daf-16* mRNA expression using RNAi in WT, *daf-2 (e1370)*, *skn-1b*, and double *daf-2 (e1370); skn-1b* mutants and carried out the exploration assay. We have found that knocking down *daf-16* had no effect on the exploratory behaviour of either WT or *skn-1b* mutants alone [Figure 32]. In contrast, *daf-16* RNAi

effectively suppressed the exploratory deficit of *daf-2* mutants back to WT levels [Figure 32]. Interestingly, *daf-16* RNAi had no effect on the exploratory activity of *daf-2; skn-1b* mutants [Figure 32].

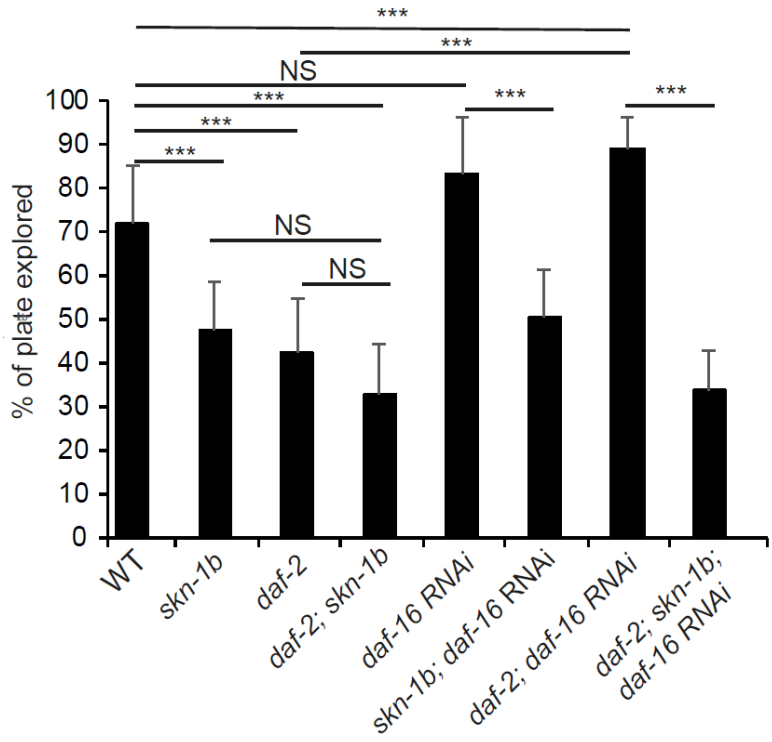


Figure 32: SKN-1B regulates IIS to control exploratory behaviour. *daf-16* RNAi had no effect on WT and *skn-1b* animals' exploratory behaviour however, it rescued the exploratory deficit of *daf-2* mutants back to wild-type levels. Surprisingly, *daf-16* RNAi had no effect on *daf-2; skn-1b* mutants' exploratory behaviour. One representative of 3 biological replicates shown ± st. dev., n>10 worms per group. Two-tailed *t-test *p<0.05, **p< 0.001, ***p<0.0001,* NS = not significant.

In addition to that, we examined the relationship between *daf-2* and *skn-1b* in response to food. More specifically, with the presence of food, the reduced exploratory activity of *daf-2* and *skn-1b* mutants was non-additive, indicating that they operate in the same pathway [Figure 33]. Nevertheless, *skn-1b* and *daf-2* mutants respond in a different way to fasting and re-feeding: *skn-1b* mutants behaviour found to be completely unresponsive; in contrast with

daf-2 mutants which respond like WT, reducing their exploration upon re-feeding, a phenotype that seems independent of *skn-1b* [Figure 33]. The class 1 allele *daf-2 (e1370)* demonstrates exceptionally low exploratory behaviour. For that reason, we have tested a 'weaker' class 2 allele *daf-2 (e1368)*, which demonstrates a milder exploratory deficit. Similar to *e1370* results, *daf-2 (e1368)* and *skn-1b* exploratory deficits were non-additive in both fed and fasted and re-fed conditions (data not shown). These results possibly indicate that for rIIS conditions, DAF-16 acts to reduce exploration and SKN-1B acts to promote it.

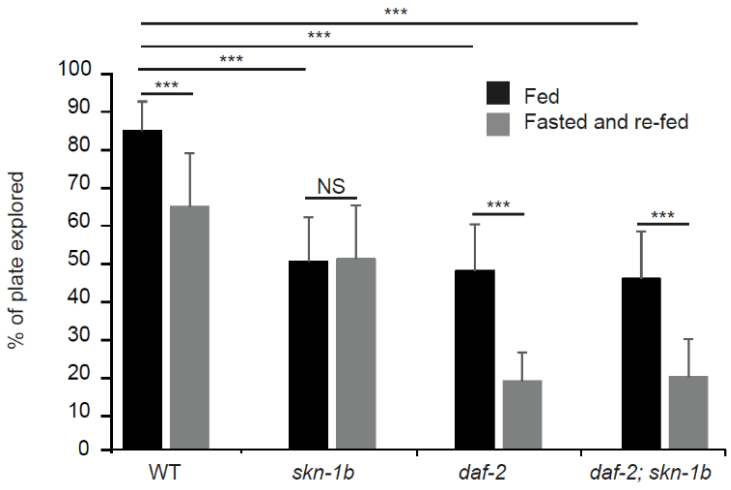


Figure 33: Animals' exploratory behaviour in fed and fasted re-fed conditions. Animals fasted for 1hr. WT, *daf-2*, *daf-2; skn-1b* animals respond to fasting and demonstrate reduced exploration behaviour compared to fed animals. In contrast, fasting had no effect in exploratory behaviour of *skn-1b* mutants. Both fed and fasted *skn-1b* animals demonstrate similar exploratory behaviour. One representative of 3 biological replicates shown ± st. dev., n>10 worms per group. Two-tailed t-test *p<0.05, **p< 0.001, ***p<0.0001, NS = not significant.

Taken together these data indicate that *skn-1b* impacts on DAF-16 regulation in response to fasting and re-feeding and *skn-1b* mutants are incapable of maintaining DAF-16 in gut nuclei under these conditions. Satiety quiescence periods are extended under rIIS conditions and

dependent on DAF-16. For that reason, we examined whether *daf-16* contributes to the high levels of quiescence in *skn-1b* mutants under rIIS condition.

We observed that whilst *daf-16* RNAi had no effect in quiescence behaviour of either WT or *skn-1b* mutants [Figure 34 A], *daf-2* mutation enhanced quiescence in comparison to WT, an effect suppressed by *daf-16* RNAi [Figure 34 B]. These results indicate the importance of *daf-16* in quiescence when IIS is absent. Nevertheless, the addition of *skn-1b* mutation increases further the quiescence period of *daf-2* mutants and this effect was not suppressed by *daf-16* RNAi [Figure 34 B].

daf-16 RNAi treatment in *daf-2; skn-1b* mutants increased quiescence periods in comparison with *daf-2; daf-16* RNAi [Figure 34 B]. The fact that *daf-16* RNAi did not alter the satiety quiescence behaviour of WT or *skn-1b* animals indicates that IIS must be reduced for this interaction to occur. Taken together these results indicate that SKN-1B operates to modulate both TGF-β and IIS in response to food which permit the outputs of these pathways to control behaviour. This highlights SKN-1B as a new central node in ASI behavioural response pathways.

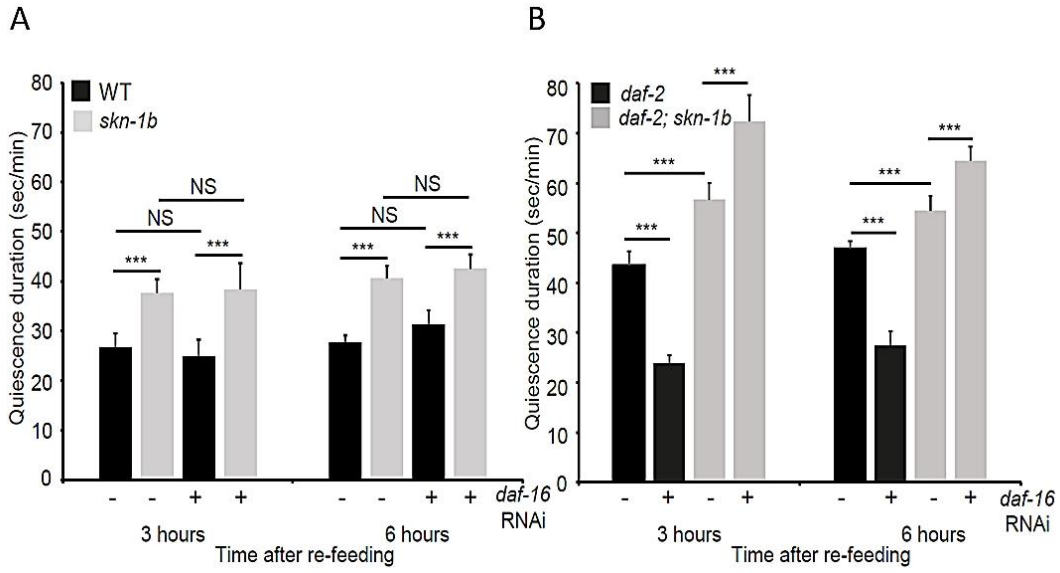


Figure 34: SKN-1B regulates IIS to control satiety-induced quiescence. A) Quiescence periods of WT and *skn-1b* animals fed with control or *daf-16* RNAi. *skn-1b* mutants spent significantly more time in quiescence after fasting and refeeding compared to WT. *daf-16* RNAi had no effect on WT or *skn-1b* animals' quiescence behaviour. **B)** Quiescence periods of *daf-2* and *daf-2; skn-1b* animals fed with control or *daf-16* RNAi. *daf-16* RNAi rescued the extended quiescence periods of *daf-2* mutants back to wild-type levels. In contrast, *daf-16* RNAi had no effect on *daf-2; skn-1b* mutants quiescence behaviour. *daf-2; skn-1b* mutants on *daf-16* RNAi demonstrate even more pronounced quiescence behaviour. Each bar represents a mean of 3 biological replicates ± SEM, total of n>36 worms per group. Two-tailed *t-test* *$p<0.05$, **$p< 0.001$, ***$p<0.0001$, NS = not significant.

Chapter 3 - Discussion

Animals including *C. elegans*, modulate their behaviour by integrating information about their external environment with internal cues. Our study indicates SKN-1B as novel and crucial regulator of food-related behaviour. In fed conditions, SKN-1B levels respond to food availability and fasting, promoting exploration, and suppressing quiescence in response to fasting and re-feeding. The data suggests that SKN-1B is acting as a molecular switch which allows behaviour fine-tuning in response to environmental change.

Surprisingly, the response of SKN-1B::GFP expression to diet and the behavioural responses which are manifested with, are not always constant. This is demonstrated when similar increase in SKN-1B::GFP expression levels occurs, in response to *B. Subtilis* (PY79) and *Pseudomonas* (PA14), however leading to opposite behavioural responses. It has been reported that the transcriptional outputs of other SKN-1 isoforms differ, depending on the stimulus, which is possible to be the case for SKN-1B as well. On the other hand, responses to pathogens, require additional immune signals which may override any satiety behaviour in the *skn-1b* mutants.

SKN-1B found to be constitutively nuclear expressed, in the ASI neurons. It requires the receptor type guanylate cyclase *daf-11*, which is expressed at the amphid opening of the ASI, to sense the environment. The expression pattern of *daf-11* and *skn-1b* in the ASI, the requirement of *daf-11* for SKN-1B::GFP expression, and the non-additive behavioural effects of *daf-11* and *skn-1b,* indicates that these molecules operate in the same pathway.

Studies indicate that *daf-11* operates upstream of both IIS and TGF-β pathways. Our data suggest a new mode of *daf-11* action. Despite that both *daf-11* and *skn-1b* act to regulate quiescence, *daf-11* mutants demonstrate decreased quiescence, in contrast with the increased quiesce behaviour of *skn-1b* mutants, always in comparison with WT.

The interaction of *daf-11* and *skn-1b* is important for environmental perception however, their roles in behaviour with regards to fasting and re-feeding are independent.

Complete ablation of the ASI neurons had the opposite effect to *skn-1b* mutants, leading to a reduced satiety-induced quiescence. Therefore, genetic removal of the SKN-1B does not physiologically damage the neurons. Instead, we suggest that specific and rapid changes in SKN-1B levels provide sensitivity for modulating behaviour and physiology.

Our results indicate that SKN-1B operates specifically to suppress satiety induced quiescence. Movement analysis in fed conditions showed that *skn-1b* mutants move slightly more than WT. Taken together with the reduced exploratory behaviour of fed *skn-1b* mutants, we could extrapolate that in fed conditions they spend more time dwelling. Therefore, SKN-1B acts to regulate different behaviours depending on food status.

Similar to mammals, *C. elegans* IIS and TGF-β hormone signalling are nutritionally controlled and essential to many processes. They are regulated by ILPs and NLPs, including the TGF-β ligand DAF-7. In *C. elegans* they are known to regulate development, growth, immunity, lifespan, and age-related decline. Our work shows that SKN-1B acts as a sensory switch in the ASI neurons, operating upstream to modulate both IIS and TGF-β signalling. That allows the animal to accurate perceive the environment and to regulate its behaviour accordingly. By regulating DAF-7 in the ASI neurons and DAF-16 in the gut, SKN-1B integrates the external environment with the internal state of the worm.

IIS is a conserved pathway for detecting food. Reduced IIS, in case of *daf-2* mutants, induces quiescence dependent on DAF-16. Nevertheless, the contribution of *daf-16* to quiescence is being abolished in the absence of *skn-1b*. Therefore, under normal conditions *skn-1b* allows *C. elegans* to achieve appropriate levels of quiescence for its environment. *skn-1b* and IIS/*daf-16* interact genetically only under rIIS conditions to regulate behaviour. This indicates to us,

that WT *C. elegans* ILP signalling needs to be "programmed" to downregulate IIS, for this relationship to be important.

One mechanism via which DAF-16 can regulate quiescence is via food consumption. *daf-2* mutation renders worms to consume less food, in contrast with the *daf-2; daf-16* mutation where they consume more food.

skn-1b mutants demonstrate reduced levels of nuclear DAF-16::GFP in their gut, which could mimic a condition comparable to *daf-16* knockdown. Nuclear localisation of DAF-16 in *skn-1b* mutants is short lived. SKN-1B is not required for changes in DAF-16 localization under fed or fasted conditions. A modification of the original fasting protocol (described in Materials and Methods) was made, to include re-feeding, post-fasting, to investigate the longer effects of DAF-16 localisation. More specifically, after 16 hours of fasting, animals were transferred onto plates with food and allowed to re-feed for 6 hours in total. DAF-16 nuclear localization examined and compared between fully fed, fasted, fasted and re-fed (3hrs or 6hrs in refeeding) animals. It has been reported that DAF-16 levels remain high after re-feeding to regulate a quiescence behavioural state. After 6 hours of re-feeding, DAF-16 localisation was reduced almost to the level of fed control in *skn-1b* mutants, whereas in WT worms this was consistently elevated.

Under fasted and re-fed conditions that promote satiety quiescence behaviour, *skn-1b* mutants demonstrated elevated pharyngeal pumping rates, accumulated more *E. coli* in their guts, and were slightly larger than WTs suggesting that under these conditions, they might be eating more. Furthermore, DAF-7 levels found to be increased in well fed conditions. Therefore, modified feeding parameters in *skn-1b* mutants promote the increase in *daf-7* reporter expression and satiety induced quiescence behaviour.

Chapter 4 - Investigating the role of SKN-1B in the mitochondria

Our data suggest that SKN-1B acts cell-non-autonomously to regulate behaviour. As food perception and consumption is linked to physiological and metabolic homeostasis, *skn-1b* dysregulation may cause physiological and metabolic disruption to the organism. We have shown that *skn-1b* is required for optimal behavioural responses to food and fasting. Despite that *skn-1b* mutants are being well-fed, they demonstrate behaviours similar to starved animals.

In the wild, *C. elegans* found in environment with low oxygen concentration, where they usually locate food (Shtonda, 2006; Zhao et al., 2018). Bordering or social feeding is a common behaviour of worms in nature, where they tend to aggregate together. This behaviour is often demonstrated in laboratory settings where worms accumulate at the edge/thickest part of the lawn. That region found to be higher in nutrients and characterised as the bacteria active growth region. In addition, the outer part of the lawn considered to have reduced oxygen levels. Bordering behaviour is demonstrated in both fed and starved animals. During food source depletion, worms tend to form large clumps onto the remained small patches of bacteria. Usually, *C. elegans* found evenly distributed in a bacteria lawn, with animals being in and out of the thickest part, as well as in the centre (Shtonda et al., 2006; Zhao et al., 2018).

skn-1b mutants demonstrated a strong preference for the thicker-outer part of the bacterial lawn [Figure 35]. It has been reported that the edge of the lawn has reduced levels of O_2 (~8%) and bordering has been associated with social and food preference behaviours, memory, temperature, and starvation (Zhao et al., 2018).

skn-1b mutants demonstrate high preference for the outer part of the bacteria lawn. This can be the result of disrupted environment perception, since worms usually search for new food sources, despite being on a highly nutritious one. The case of food-depletion, which makes animals to remain on small bacterial patches at the edge of the lawn is not considered since our experiment used fully grown bacterial lawns [Figure 35 A]. *skn-1b* mutants possibly exhibit signs of starvation, regardless of being well fed, due to their perceived state of starvation. Taken together with the previous results, which indicate that *skn-1b* mutants are unable to perceive and respond to food cues properly, we asked whether the physiological state of *skn-1b* mutants differ from WT.

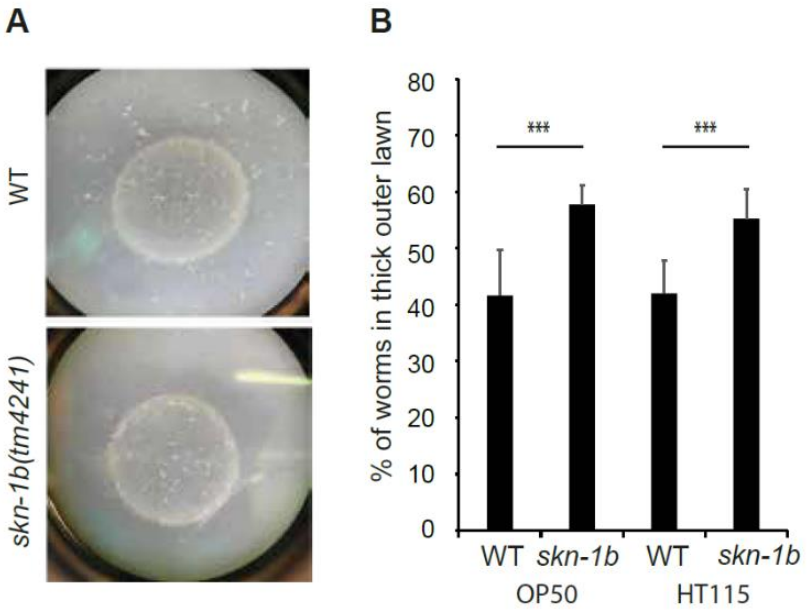


Figure 35: *skn-1b* mutants demonstrate enhanced bordering behaviour. A, B) Images (OP50 plates shown here) and quantification of bordering behaviour. Each bar represents a mean of 3 biological replicates ± st. dev. Two-tailed *t-tests *p<0.05, **p< 0.001, ***p<0.0001,* NS not significant.

skn-1b contributes to mitochondrial network integrity

The dynamic nature of mitochondria allows them to change their network morphology and balance fission and fusion events to maximize energy production. *C. elegans* mitochondria morphology has been shown to change in response to starvation, as well as in various DR protocols. This can be linked with animal's physiological state. Previous studies have shown that *skn-1* is important for maintaining mitochondrial networks in the muscle, as well as for anoxia-induced mitochondrial dynamics (Blackwell et al., 2015). This raises the question of whether these phenomena are potentially mediated by *skn-1b*. To answer this question, we examined the mitochondrial networks of WT and *skn-1b* mutants expressing an outer mitochondrial membrane marker in muscle, *myo-3::GFP(mit)*.

Published work showed that mitochondria coverage area can be used as a read-out of mitochondria volume (Byrne et al., 2019). This also demonstrates the condition of mitochondrial networks. We compared the coverage of mitochondria in the body wall muscle of WT and *skn-1b* animals under fed and fasted conditions. Despite the variability between body-wall muscle cells and individual animals, consistent results were obtained. The mitochondria of *skn-1b* mutants appear to have disorganized networks and cover significantly less surface area than that of WT animals [Figure 36]. This mitochondria appearance resembles that one of fasted WT animals, implying that *skn-1b* mutants are at least as their mitochondria are concerned, in a starvation state [Figure 36]. Fasting enhanced the already disorganised appearance of *skn-1b* mutants' mitochondria, indicating that there are also other factors involved to this mitochondrial morphology phenotype [Figure 36].

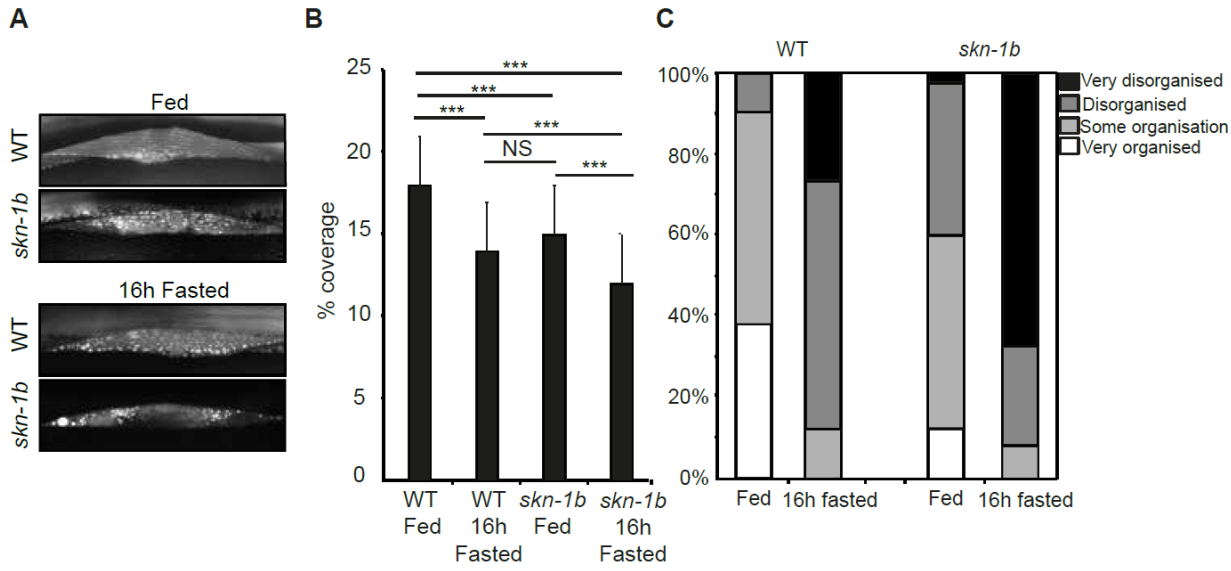


Figure 36: Disorganised mitochondrial network and volume loss in *skn-1b* mutants, using *myo-3::GFP(mit)* reporter. A-C) Expression and quantification of WT and *skn-1b* mutant worms expressing *myo-3::GFP(mit)*. This muscle specific reporter expresses an outer mitochondrial membrane protein and hence marks all mitochondria, delineating their shape. In **B** and **C)** Each bar represents a mean of 3 biological replicates ± SEM, n>62 day 1 adult worms per group. The qualitative scoring system used in C) is shown in [Figure 10]. Two-tailed *t-tests *p<0.05, **p< 0.001, ***p<0.0001,* NS not significant.

A similar pattern was also observed with a second mitochondrial reporter *tomm20::GFP* [Figure 37] when it was studied by Alex Howard. Again, *skn-1b* mutants demonstrated disorganized mitochondria networks and cover significantly less surface area compared to WT animals in both fed and fasted conditions. In addition, the mitochondria of *skn-1b* mutants under fed conditions demonstrate phenotype similar to WT under fasted conditions [Figure 37]. Taken together, these results indicate that *skn-1b* contributes to maintaining the mitochondrial networks in the muscle.

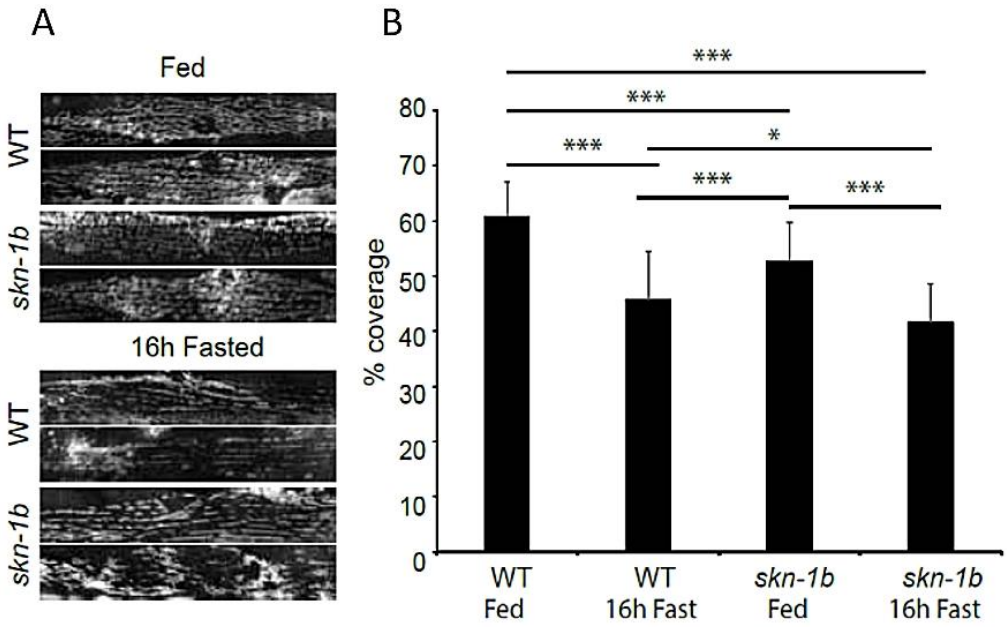


Figure 37: Disorganised mitochondrial network and volume loss in *skn-1b* mutants, using *tomm20::GFP* reporter A, B) Expression and quantification of WT and *skn-1b* mutant worms expressing *tomm20::GFP* reporter. This reporter expresses a peptide of tomm20, an outer mitochondrial membrane protein and hence marks all mitochondria, delineating their shape. Each bar represents a mean of 3 biological replicates ± SEM, n>49 day 1 adults worms per group. Two-tailed *t-tests *p<0.05, **p< 0.001, ***p<0.0001,* NS not significant. These data were generated by Alex Howard.

Under fed conditions, mitochondrial reporters showed that *skn-1b* mutants have disorganised mitochondrial network compared to WT [Figure 36, 37]. Fasting negatively affected mitochondria area coverage and levels of organisation, with a more severe impact on *skn-1b* mutants [Figure 36, 37]. Mitochondria area coverage in both WT and *skn-1b* animals was being decreased under fasted conditions compared to their fed counterparts. Finally, mitochondria of fasted *skn-1b* mutants showed pronounced volume reduction and levels of disorganisation compared to WT.

Despite the conclusive results regarding disorganised mitochondria networks, mitochondrial reporters do not provide clear evidence about fission or fusion events. To observe abnormal mitochondria structures, a higher level of detail is needed. For that reason, transmission

electron microscopy used to examine mitochondria structure morphology, as well as fission and fusion events.

Muscle wall mitochondria of WT and *skn-1b* animals examined under fed and fasted conditions. Sections (transverse and longitudinal) were taken from whole worms and the mitochondria assessed. In fed conditions, the mitochondria of WT animals appeared rounded, but in contrast skn-1b mutants exhibited elongated and irregular shaped mitochondria [Figure 38, 39]. This phenotype was rescued by re-introducing SKN-1B::GFP into the *skn-1b* mutants [Figure 38]. This suggests that *skn-1b* is required for maintaining mitochondrial dynamics.

I then tested the effect of fasting on mitochondrial networks. As expected, fasting caused the mitochondria of WT animals to become fused (also observed in TEM sections). This was similar to the situation in the fed *skn-1b* mutants, supporting the idea that the mitochondria of *skn-1b* mutants respond to a perceived state of starvation [Figure 38, 39]. However, fasting *skn-1b* mutants, further amplified their mitochondrial deterioration implying that additional factors may contribute to this phenotype [Figure 38, 39].

From the fluorescent mitochondria images, we obtained evidence about mitochondria disruption however, electron microscopy provided a more precise look of this disruption. These results support a model whereby *skn-1b* operates directly in the regulation of mitochondrial homeostasis in response to food levels, balancing mitochondria fission and fusion.

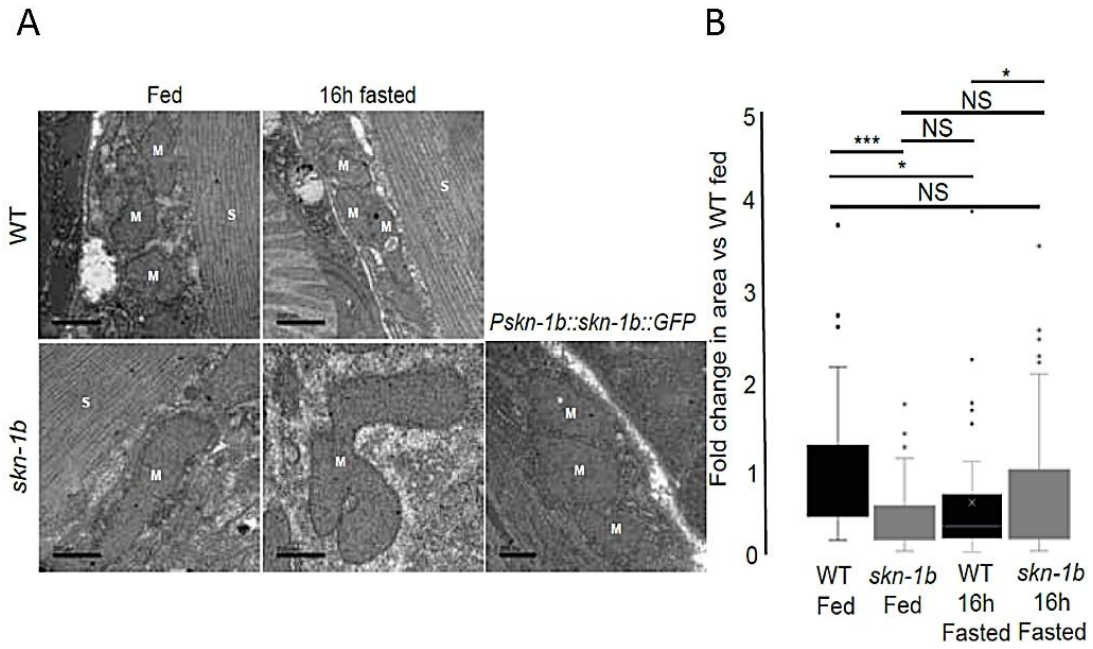
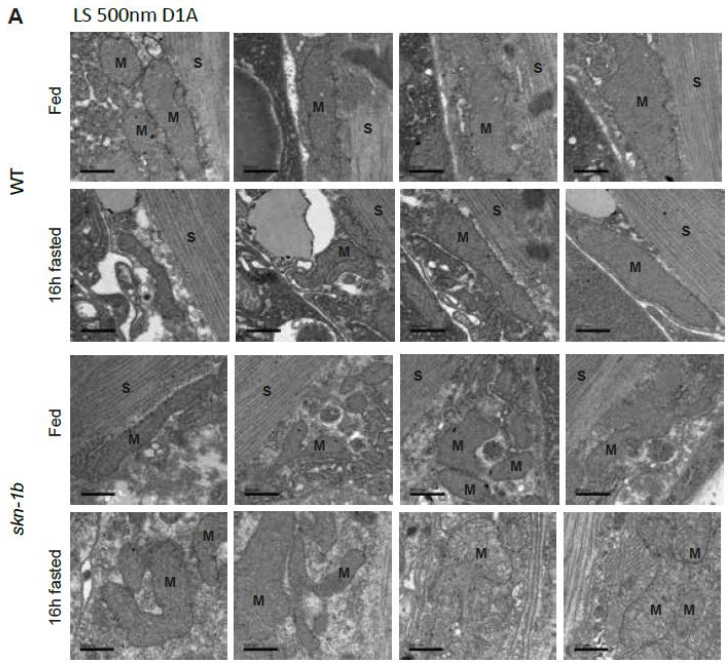


Figure 38: TEM results showing abnormal morphology of mitochondria in *skn-1b* mutants
A) Longitudinal sections imaged by Transmission Electron Microscopy (TEM). M=mitochondria, S=sarcomere. Scale bar = 500nm. **B)** Quantification of TEM: Mitochondrial area compared to WT control. Each bar represents a mean of 2 biological replicates, n>47 images per group ± SEM. For all graphs: Two-tailed *t*-tests *p<0.05, **p< 0.001, ***p<0.0001, NS not significant.



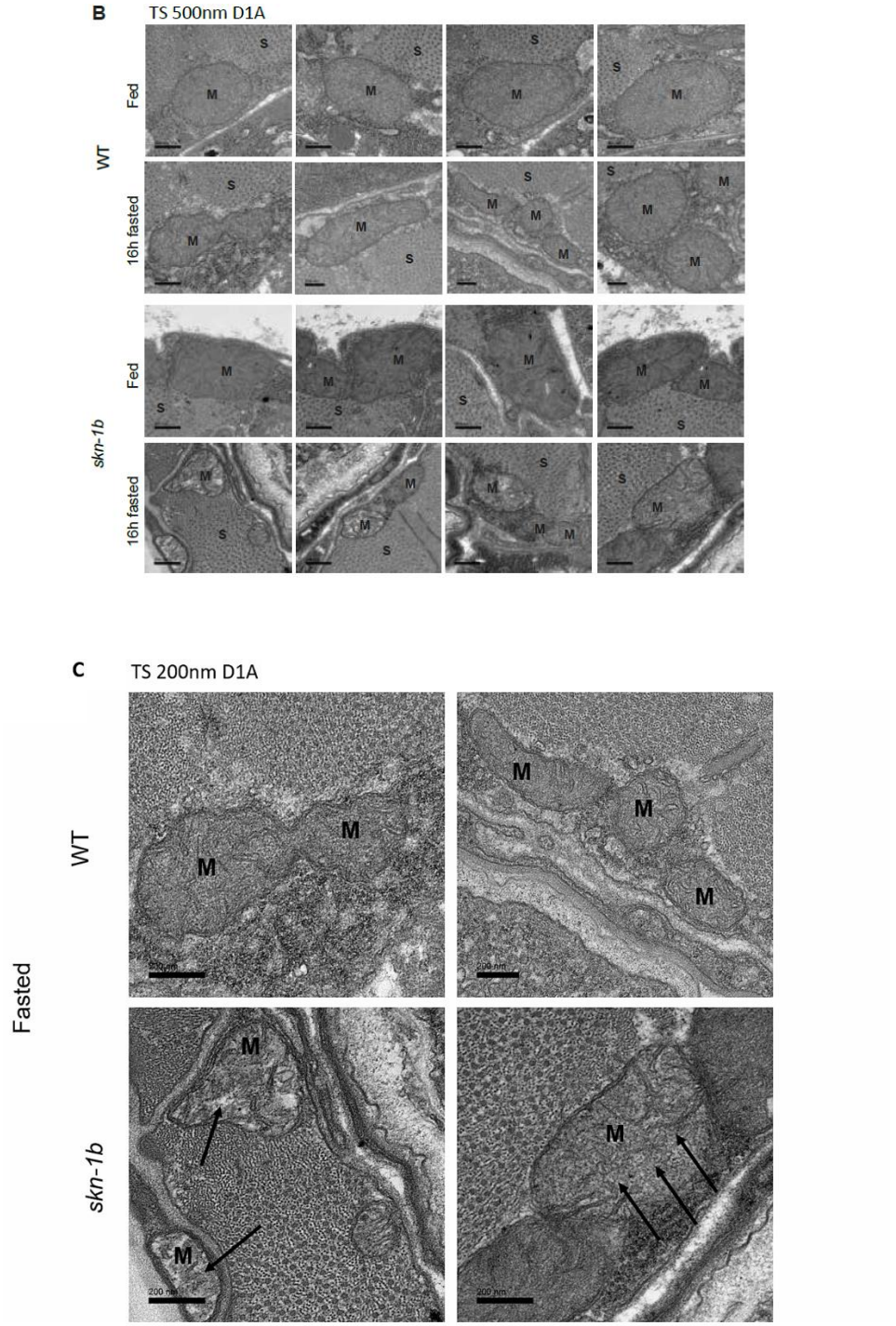


Figure 39: TEM of WT and *skn-1b* mutants in fed and fasted conditions. A) Longitudinal sections and **B, C)** Transverse sections. Scale bars = 200-500nm, M=mitochondria, S=sarcomere. Fasting disrupts mitochondrial networks in both WT and *skn-1b* animals. *skn-1b* mutants demonstrate increased mitochondria fusion. In response to fasting, *skn-1b* mutants' mitochondria appear much worse than WT, with disrupted membranes and cristae structures (Figure 39 C).

SKN-1B maintains mitochondrial networks to control food-related behaviour

Our data suggest an involvement of *skn-1b* in mitochondrial morphology and dynamics. To investigate this further, we examined the interaction of *skn-1b* with mitochondrial genes which are known to regulate mitochondrial dynamics. More specifically, mitochondria dynamics are regulated by the function of mitochondrial fusion and fission proteins (Byrne et al., 2019). *eat-3*/Opa1 and *fzo-1*/Mfn1 promote fusion and *drp-1*/Drp1 promotes fission. We examined mitochondria networks in the muscle of worms fed with either *eat-3*, *fzo-1* or *drp-1* RNAi, using both fluorescent (*myo-3::GFP(mit)*) and electron microscopy.

Results using the reporter strains showed that each RNAi effectively altered the mitochondria morphology in both WT and *skn-1b* animals [Figure 40 A]. Treatment with *eat-3* or *fzo-1* RNAi resulted to a fission-like appearance of mitochondria network, with dotty-like mitochondria, evenly distributed within muscle cell and not being interconnected [Figure 40 A]. Treatment with *drp-1* RNAi resulted to a fused/hyperfused-like mitochondria network with mitochondria accumulating in a specific region of the muscle cell, forming clusters, and being interconnected [Figure 40 A].

Results using electron microscopy showed mitochondria structures similar to what we have observed using the mitochondria reporter strain [Figure 40 B]. More specifically, the mitochondria of animals fed with *eat-3* or *fzo-1* RNAi were smaller and more disjointed (mitochondria were unable to fuse), in contrast with the mitochondria of animals fed with *drp-1* RNAi which were more elongated (mitochondria were unable to divide-impaired fission).

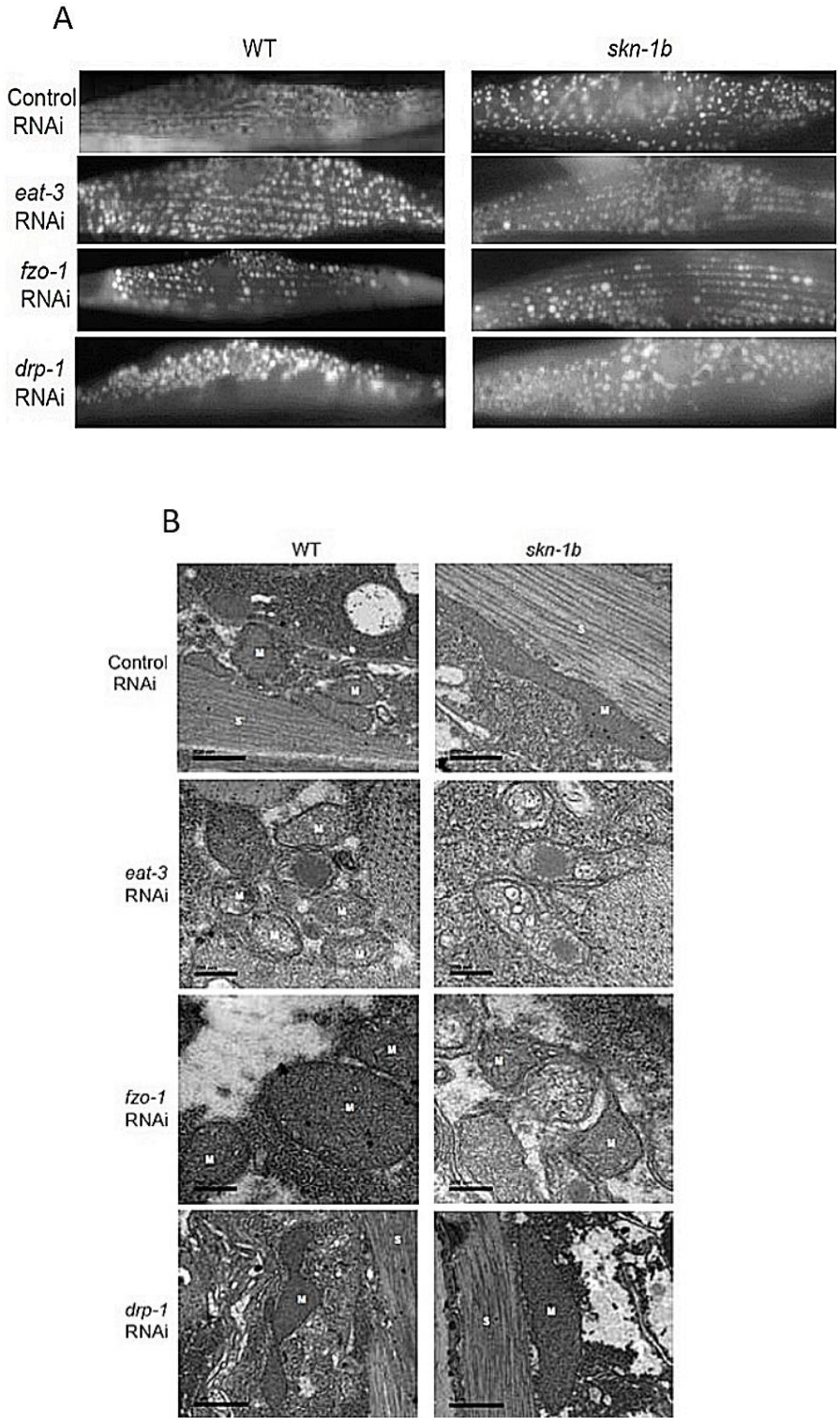


Figure 40: Comparing mitochondrial networks in WT and *skn-1b* mutants using fluorescent and Electron microscopy. Images of *myo-3::mitoGFP* **(A)** and TEM images **(B)** in WT and *skn-1b* mutant *C. elegans* fed control, *eat-3, fzo-1 or drp-1* RNAi. TEM shows Longitudinal sections 200nm. Note that although the fluorescent images in A clearly show signs of mitochondrial network disruption, it is only when examining the TEM images that the precise network structures can be seen e.g. both *eat-3* and *drp-1* RNAi show a "spotty" pattern on the fluorescent images, but this translates to a very different TEM image with *eat-3* RNAi causing fission and *drp-1* RNAi fusion (as expected).

It has been reported that mitochondria dynamics can influence behavioural responses (Byrne et al., 2019). Our study indicates that *skn-1b* plays an important role in *C. elegans* behaviour, as well as in the maintenance of mitochondrial networks. For these reasons, we examined whether mitochondria dynamics and *skn-1b* are linked. We found that whilst neither *eat-3* or *fzo-1* RNAi had any effect on WT exploratory behaviour, both completely rescued *skn-1b* mutant exploration to normal levels [Figure 41]. However, *drp-1* RNAi had no effect on either WT or *skn-1b* behavioural patterns [Figure 41]. These results support our model where SKN-1B acts to control mitochondrial networks, in particular mitochondria fission, and that in turn controls food related behaviour.

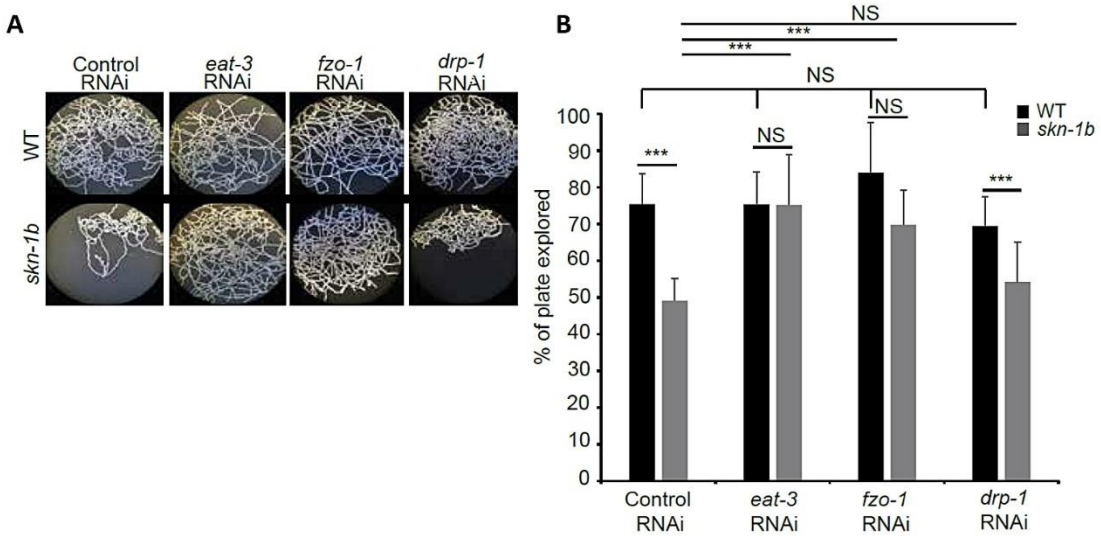


Figure 41: Effect of mitochondrial fission and fusion on mitochondrial networks and behaviour in WT and *skn-1b* mutants. Two-tailed *t*-tests *p<0.05, **p< 0.001, ***p<0.0001, NS not significant.

Chapter 4 - Discussion

skn-1b mutation caused behavioural responses which are found in animals with a disturbed nutritional status or with a perceived state of starvation. *skn-1b* mutants showed a bordering phenotype, which is a characteristic of starved animals, despite being well-fed. This disproportional accumulation of *skn-1b* worms at the outer part of the bacterial lawn, may be linked with physiological and metabolic disruption of the organism.

To investigate whether changes in signalling by *skn-1b* mutation contribute to any physiological differences, we examined mitochondria morphology. This is a reliable read-out regarding the integration of nutritional stratus and physiological responses of an organism. Mitochondrial reporters used to examine the mitochondrial network morphology within the body wall muscle of WT and *skn-1b* animals. This also included fasting condition, as it is known to contribute to a disorganised mitochondrial network morphology.

The area coverage of mitochondria in muscle fibers was measured. Here, we found a significant decrease in the area coverage of *skn-1b* mutants compared to WT, in both fed and fasted conditions. Fasting also had the effect on reducing coverage in *skn-1b* mutants independently. Fed *skn-1b* mutants showed a modest but significant decrease in average area, as the networks seen were not so sparsely distributed but exhibited a much greater degree of disorganization, and therefore decrease in average mitochondrial area. The mechanism through which mitochondrial volume is being reduced under fed or fasted conditions and the contribution of *skn-1b* to that remains to be determined. An interesting hypothesis is that mitochondria volume reduction occurs by a reduced mitochondrial biogenesis. It seems that *skn-1b* mutation alters the nutritional status of the animal according with previous experiments. Comparison between WT and *skn-1b* fasted animals reveals a very disorganized mitochondrial morphology for *skn-1b*. This gives the impression that *skn-1b* fed animals are in

a perceived state of starvation and additional fasting has a detrimental effect on mitochondria network morphology.

Mitochondrial reporters provided a clear image of the *skn-1b* mutants' disorganized mitochondria network. However, to have a more in-depth look, transmission electron microscopy used to examine mitochondrial network morphology within the body wall muscle of WT and *skn-1b* animals. This allowed not only to examine mitochondria morphology but also the dynamics between fission and fusion events. Fasting condition included, which is known to influence mitochondria dynamics, allowing the animal to maintain homeostasis.

In fed conditions, *skn-1b* mutants had elongated mitochondria structures in comparison with the more spherical appearance of WT mitochondria. In many cases, mitochondria of *skn-1b* mutants appeared dysmorphic with compromised membranes. Fasting exacerbated the mitochondria morphology of *skn-1b* mutants. They appeared even more dysmorphic. Mitochondria fusion observed mostly in *skn-1b* mutants and especially under fasted conditions.

This data suggests that SKN-1B is required for normal mitochondrial network morphology, and it is still able to adapt to changes. In addition, SKN-1B maintains an organised mitochondrial network and maintains the balance between fission and fusion events to support energy homeostasis in both fed and fasted, re-fed conditions. Maintaining mitochondrial homeostasis is crucial for a range of processes, including ageing and behaviour. In addition, balance between fission and fusion events is necessary for DR to extend lifespan. The enhanced mitochondrial fusion in *skn-1b* mutants resembles that of fasted or DR worms however, it is unlikely that *skn-1b* mutants are physically starved. We propose that this occurs via endocrine factors deriving from the ASI neurons, leading to a perceived state of malnourishment, with knock-on effects for mitochondrial physiology.

It has been reported that mitochondria dynamics can influence behavioural responses. Our data suggested an involvement of *skn-1b* in mitochondria morphology and dynamics. To investigate this further, we examined the interaction of *skn-1b* with mitochondrial genes which are known to regulate mitochondrial dynamics.

Mitochondrial reporters and TEM showed that RNAi treatment can effectively knock down mitochondrial genes by altering mitochondria morphology. *eat-3*, *fzo-1*, and *drp-1* RNAi altered mitochondria morphology in both WT and *skn-1b* animals. Mitochondria fission appeared in animals treated with *eat-3* or *fzo-1* RNAi in contrast with *drp-1* treated animals where mitochondrial fusion occurred. Disturbing the fused mitochondrial network of *skn-1b* mutants by promoting fission, using *eat-3*/Opa1 or *fzo-1* RNAi, we were able to rescue their exploratory behaviour impairment. Therefore, we conclude that correct behavioural responses to food requires mitochondria network control by SKN-1B. Our results indicate that SKN-1B acts cell non-autonomously in the gut to alter IIS, and in muscle to alter mitochondrial physiology [Figure 42].

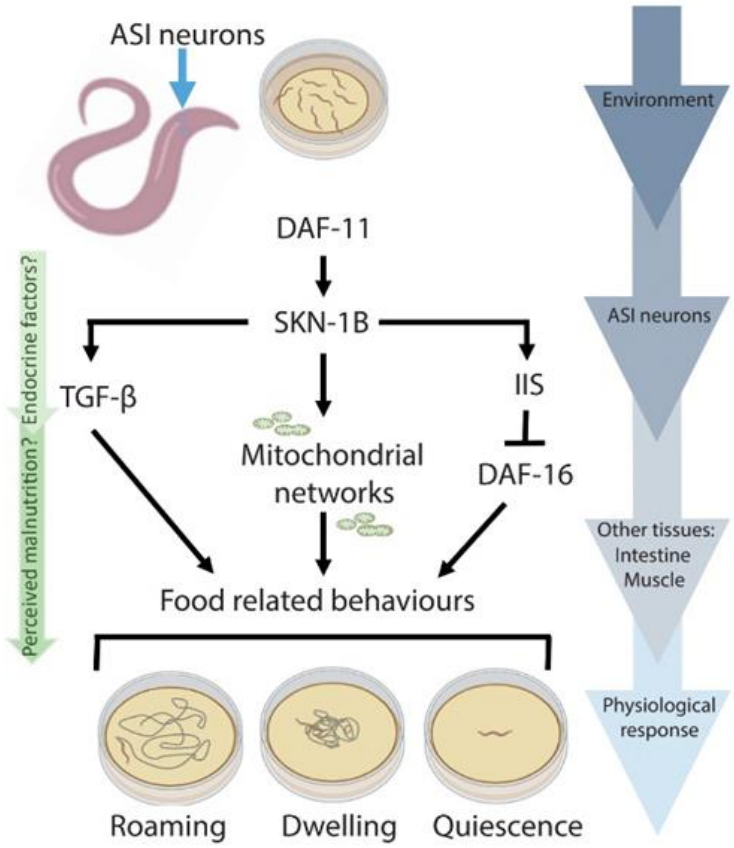


Figure 42: SKN-1B interacts with nutritional signalling pathways, regulates mitochondria networks, and controls satiety-related behaviour. Food-related behaviour is regulated by interactions between food cues, SKN-1B downstream signalling pathways (cGMP, TGF-β and IIS), and mitochondria. SKN-1B perceives food cues through cGMP signaling (DAF-11). SKN-1B plays an important role in fasting and re-feeding conditions, by controlling satiety quiescence. SKN-1B suppresses *daf-7* expression in the ASI neurons, which in turn downregulates TGF-β signaling and suppresses quiescence **[Figure 28, 29]**. DAF-16 nuclear localization is induced by fasting and maintained after re-feeding to promote quiescence. SKN-1B is needed for this response, possibly by operating upstream of both pathways **[Figure 31, 34]**. SKN-1B regulates food-related behavior by maintaining mitochondrial networks **[Figure 38, 39, 41]**. This study presents neuronal SKN-1B as novel factor in regulating satiety behavior in response to dietary signals.

Research relevance and future work

Mitochondria dynamics and animal behaviour is an exciting topic of research. There are not many studies showing a direct control of animal behaviour in a mitochondria-dependent manner. This study has enriched the field of *C. elegans* research in many ways. First of all, it supports previous studies regarding the effects of fasting and starvation on mitochondria morphology. In agreement with published work, mitochondria respond to fasting by increasing fusion events. In addition to that, high quality images acquired using electron microscopy, capture not only mitochondria structures, but also the precise moments of mitochondria fusion and fission events (*dpr-1, fzo-1, eat-3* RNAi). The images provide valuable knowledge, since only a few studies have showed something similar with this level of detail. An important result of this study is the effect of *skn-1b* on mitochondria morphology and behaviour. For the first time, *skn-1b* mutants are shown to have abnormal mitochondria morphology, which resembles mitochondria of animals in fasted conditions. Under fed conditions, the mitochondria of *skn-1b* mutants found to be fused, forming elongated and abnormal structures. Fasting exacerbates the already abnormal mitochondrial structure of *skn-1b* mutants. The lack of *skn-1b*, causes the animals to be in a perceived state of starvation which is reflected in both behavioural and physiological results. *skn-1b* mutants do not respond to starvation in terms of exploratory behaviour, with their levels being similar to fed *skn-1b* mutants. Manipulation of mitochondria dynamics of *skn-1b* mutants was able to restore exploratory behaviour defects. By forcing mitochondria fission (*fzo-1, eat-3* RNAi) on the abnormally fused mitochondria of *skn-1b* mutants, we were able to restore exploratory behaviour back to wild-type levels. This is something completely new in the field, not only highlighting the effect of *skn-1b* on exploration, but also how manipulation of mitochondrial dynamics affects exploratory behaviour in worms.

Despite the valuable results obtained, using mitochondrial reporters and electron microscopy, there are still limitations. Crucial characteristics of dysfunctional mitochondria, such as pronounced mitochondrial fusion and membrane/cristae disruption, are hard to reveal and analyse. Mitochondria reporters do not actually inform about mitochondrial fusion or fission in detail. There is indeed demonstration of mitochondrial networks which are found to display enhanced fusion or fission (using *drp-1* and *fzo-1*/*eat-3* RNAi), however, reporter fluorescence at this level of magnification and viewing angle does not represent reality. It is challenging to investigate the disrupted mitochondria membranes or cristae using this technique. In addition, a debate nowadays challenges the use of mitochondria reporters, stating that fluorescence does not accurately show mitochondria features. Electron microscopy also did not cover some important characteristics of dysfunctional mitochondria. Observation of individual mitochondria is shown, though a longitudinal and transverse view is not a 3D representation, which would provide a more complete picture of these events. The actual fused appearance of mitochondria is often not detected by standard electron microscopy. In addition, the orientation of fused mitochondria and the number of mitochondria structures connected cannot be presented accurately in two dimensions.

Future work must include advanced microscopy techniques, in order to strengthen the initial results, but also to reveal new aspects of the disrupted *skn-1b* mitochondria morphology. Confocal microscopy using the mitochondrial reporters can provide 3D models (Z-stack) of the fused *skn-1b* mitochondria. This method can demonstrate how fused mitochondria appear in a three-dimensional space. In addition, time-lapse microscopy (confocal) can be used to investigate the effects of fasting in both WT and *skn-1b* mutant mitochondria. That will provide information regarding the mitochondrial fusion and fission dynamics, in response to nutrient availability. The degree of change in mitochondria morphology of WT and *skn-1b* animals in real-time, under fasting or fasting-re-fed conditions, will reveal if *skn-1b* mutants have impaired mechanisms for maintaining mitochondrial dynamics. That could play a

significant role in DR lifespan extension, due to the fact that mitochondrial adaption is key element in this. Another method which would complement the use of confocal microscopy the usage of serial-section electron microscopy. A selected region of the animal can be used to produce a series of sections, which will help to generate a three-dimensional model. This sectioning method will provide a realistic EM representation of individual mitochondria, their orientation, structure, and fusion. Future work must also investigate the involvement of *skn-1b* in mitochondrial respiration. Published work showed that *skn-1* in the ASI neurons is required for DR mediated lifespan extension. A key element affecting this is increased mitochondrial respiration which is found under DR conditions. Experiments measuring mitochondrial respiration of WT and *skn-1b* animals under fed, fasted, and DR conditions are needed. Among other results, this study has shown that *skn-1b* is important for environment perception and foraging behaviour, two factors associated with DR mediated lifespan extension. In addition, *skn-1b* influences mitochondria dynamics which may impact mitochondrial respiration. It is possible that worms' behaviour, mitochondria dynamics, and DR lifespan are all associated.

The signalling pathway aspect of this study, as well as the molecules that are involved, for *skn-1b* to mediate physiological and behavioural changes requires more investigation. Future work will aim to answer questions regarding the precise mechanism by which mitochondrial volume is reduced in *skn-1b* mutants under fed conditions, as well as how fasting exacerbates this reduction. More work is needed to characterise the endocrine factors derived from the ASI neurons which alter the nutritional status of the animal. In the case of *skn-1b* mutants, which elements are disrupted or promote this perceived state of starvation require investigation. Neurotransmitters such as dopamine, serotonin, and octopamine play important roles in foraging behaviour of the worms. Future work must examine the interplay of these neurotransmitters with *skn-1b* in regulation of behaviour and mitochondrial networks. Roaming, dwelling, and quiescence, all three behavioural stages which comprise

exploratory behaviour in worms, are influenced by neurotransmitter signalling. Future work must include automated image acquisition with software responsible for analysing worm behaviour. The techniques used in this study provided valuable information about behavioural state, but there are some limitations. The amount of time animals spend in quiescence roaming and dwelling must be determined in future work.

Concluding Remarks

The ability of an organism to correctly identify a felling of satiety, has a direct impact on its health. For instance, perception of hunger when food is in abundance can lead individuals overeat and gain excess weight. This can have detrimental implications for their metabolic status and long-term health. Here, we show that in *C. elegans*, the transcription factor SKN-1B, regulates satiety behaviour. SKN-1B operates in two hypothalamus-like chemosensory neurons to detect and communicate nutritional status to the rest of the organism. Then, SKN-1B controls the animal's behavioural responses by modulating key nutritional signalling pathways and maintaining mitochondrial networks [Figure 42].

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