AMPK-dependent regulation of foraging behaviours in *Caenorhabditis elegans*

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August 2016

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of PhD in Biology

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Abstract

Energy homeostasis is of paramount importance for metabolic health and survival during energy stress and this must occur at both the cellular and organismal level. Starvation is therefore often accompanied by compensatory behaviours that are believed to increase the probability of encountering a food source. But how hunger results in changes in the neural activity to trigger specific adaptive behavioural responses has yet to be elucidated. AMP-activated protein kinase (AMPK) is a key metabolic sensor that acts as a regulator of adaptive behavioural responses during acute starvation, although the mechanisms by which this kinase affects such outcomes remain unclear. We observed that the starved AMPK-deficient *C. elegans* larvae display a locomotory defect that reveals their inability to appropriately respond to resource depletion. We observed that reconstitution of AMPK in the AIB and AIY interneurons that are involved in the various locomotory responses associated with reduced food availability completely rescues the locomotory defects of AMPK mutants. Using a combination of classic genetic analysis, optogenetic approaches and calcium imaging techniques, we found that although AMPK is not involved in essential aspects of neural function, it does play a key role in the modulation of neuronal activity upon starvation which in turn ensures adaptive behavioural outcomes in response to acute starvation. Furthermore, we discovered that AMPK mediates this effect by regulating the levels of AMPA-type glutamate receptor GLR-1 and Metabotropic glutamate receptor MGL-1 in the AIB and AIY interneurons respectively, which ultimately modulates synaptic strength under conditions of starvation. Overall, our study suggests that besides its well-known function in metabolic control at the cellular level, AMPK also acts as a molecular trigger

at the organismal level to regulate neuronal activity and eventually to elicit adaptive behavioural outputs in response to hunger. .

Résumé

L’homéostasie énergétique est d'une importance capitale pour la santé métabolique et la survie au cours d’un stress énergétique et cela doit se produire à la fois au niveau cellulaire et organismal. Inanition est donc souvent accompagnée de comportements compensatoires qui, pense-t-on, accroîent la probabilité de rencontrer une source d'alimentation. On ne fait que commencer à élucider comment les conséquences de la faim en termes de changements dans l'activité neuronale peut déclencher des réponses comportementales spécifiques d'adaptation. La protéine kinase activée par l'AMP (AMPK) est un capteur métabolique qui a été également proposé comme un régulateur clé de réponses comportementales d'adaptation à la privation de nourriture, bien que les mécanismes par lesquels cette kinase affecte ces résultats restent incertains. Nous avons observé que les larves de *C. elegans* affamées qui n’ont pas d'AMPK affichent un défaut locomoteur qui révèle leur incapacité à répondre de manière appropriée à l'épuisement des ressources. Nous avons observé que la reconstitution de l'AMPK dans les interneurones AIB et AIY qui sont impliqués dans les différentes réponses locomotrices associés à la disponibilité alimentaire réduite sauve complètement les défauts locomoteurs des mutants AMPK. En utilisant une combinaison d'analyse génétique classique, des approches optogénétiques et des techniques d'imagerie de calcium, nous avons constaté que, bien que l'AMPK ne participe pas à des aspects essentiels de la fonction neuronale, il joue un rôle clé dans la modulation de l'activité neuronale lors de la famine qui garantit à son tour des comportements adaptatifs en réponse à ce stress énergétique aiguë. De plus, nous avons découvert que l'AMPK médie cet effet en régulant les niveaux de récepteur type AMPA du glutamate GLR-1 et

du récepteur métabotropique du glutamate MGL-1 dans les interneurones AIB et AIY respectivement, qui module en fin de compte la force synaptique dans des conditions de famine. Dans l'ensemble, notre étude suggère que, outre sa fonction bien connue dans le contrôle métabolique au niveau cellulaire, l'AMPK agit également comme un déclencheur moléculaire au niveau organismal pour réguler l'activité neuronale et éventuellement susciter des comportements adaptatifs en réponse à la faim.

Acknowledgements

First and foremost, I would like to thank my doctorate supervisor Professor Richard Roy. Under his guidance, I was given the freedom to pursue my research in my own way and I greatly appreciate that freedom. I greatly appreciate all his contributions of time, ideas, and funding which profoundly made my Ph.D. experience productive and stimulating. The joy and enthusiasm he has for science was contagious and motivational for me, even during tough times.

Next, I would like to thank our lab manager Shaolin Li, who always was available to solve the technical issues and provided many useful suggestions to my projects. I would like to specially thank Dr. Michael Hendricks for the substantial influence his support and continuous help have had on my Ph.D. project. I would also like to thank Dr. Sreekanth Chalasani for his support and critical points during the LA worm meeting. I gratefully acknowledge my supervisor committee members Dr. Joseph Dent, Dr. Alanna Watt and Dr. Michael Hendricks for their support and helpful comments during my committee meetings.

In addition, I would like to thank all the present and former lab members, Dr. Emilie Demoinet, Dr. Yu Lu, Dr. Meng Xie, Dr. Sebastian Schmeisser , Anna Kazanets, Pratik Kadekar, Rita Chaouni for their scientific input and all the great times we have shared. I would also like to thank Dr. Zatka and her lab members for the discussions and useful suggestions. Thanks to Camille Delouche for the French translation of my abstract. My special thanks to Anna Kazanets, Sebastian Schmeisser, Alexander Vujin, Azam Aslemarz and Zahra Jalali who were great colleagues and remain wonderful friends.

I am deeply thankful to my parents who raised me with a love of science and supported me in all my pursuits. I am also thankful to my sister and brothers for their love and support from across the seas during my Ph.D. studies. I have saved the last word of appreciation for my loving, supportive, encouraging husband, Dr. Mohammad Raeesi, who has been with me all these years and has made them the best years of my life. Without him I would not write this thesis at all.

Preface

This thesis is prepared in accordance with the manuscript-based thesis guidelines. It consists of 3 chapters: Chapter 1 includes the literature review that is divided into 8 subchapters to provide a thorough review of the relevant literature and states the rationale and objectives of the thesis; The second chapter includes the research manuscripts which is published in elife; and 3rd chapter is the general discussion. Each chapter also contains its own reference sections.

Chapter 1.7 is published as part of AMP-activated Protein Kinase book cited as: Ahmadi, M. and Roy, R. 2016. AMPK signaling in *C. elegans*. EXS. 107:375-388

Chapter 2 is published in elife. Ahmadi, M. and Roy, R. 2016. AMPK acts as a molecular trigger to coordinate glutamatergic signals and adaptive behaviours during acute starvation. elife.19:5. doi: 10.7554/eLife.16349.

. The research chapter is formatted as research manuscripts composed of the following sections: Abstract, Introduction, Materials and Methods, Results, Discussion, Figures and Tables, References.

This thesis has been entirely written by the candidate in collaboration with the candidate's thesis supervisor. This thesis has been written according to the "Guidelines for thesis preparation" from the Faculty of Graduate Studies and Research.

Contributions of Co-Authors

Chapter 1.7: This chapter is published in AMP-activated Protein Kinase book as follow:

Ahmadi, M. and Roy, R. 2016. AMPK signaling in *C. elegans*. EXS. 107:375-388

This chapter has been entirely written by Moloud Ahmadi in collaboration with the Dr. Richard Roy.

Chapter 2: This chapter is published in elife as follow:

Ahmadi, M. and Roy, R. 2016. AMPK acts as a molecular trigger to coordinate glutamatergic signals and adaptive behaviours during acute starvation. elife.19:5. doi: 10.7554/eLife.16349.

All the experiments and subsequent analysis were performed by Moloud Ahmadi and the article was written by Moloud Ahmadi in collaboration with Dr. Richard Roy.

Chapter 1: Literature Review

Chapter 1.1: General introduction

Homeostatic functions are often linked to modifiable adaptive behaviours such as food intake (feeding) or food-seeking that have an important influence both on fitness and survival of individuals in a fluctuating environment. These behaviours are rapidly triggered upon depletion of an animal's energy stores leading to physiological changes that result in the sensation of hunger and the activation of a set of starvation-sensitive neural circuits (Atasoy et al., 2012; Gray et al., 2004; Wang et al., 2005). Due to the overwhelming complexity of mammalian nervous system our understanding of how hunger evokes changes in the activity of neural circuits to elicit adaptive behaviours has been challenging (Dietrich et al., 2015). However, molecular genetic approaches along with the recent advances in optogenetic tools and technologies that allow investigators to enhance or perturb neuron function have enabled the description and characterisation of the neural circuits that underlie such behavioural responses to changes in physiology (Bernstein and Boyden, 2011).

Given the relative simplicity of neural circuits and behavioural outputs in invertebrates, better understanding of how neural circuits and genes function to elicit adaptive food-seeking behaviours could benefit from investigating how genes and gene products govern the development and function of neural networks in such model organisms (Dietrich et al., 2015). Deriving key principles of the function of starvation-sensitive neural circuits in a depleted environment holds promise for revealing fundamental features that may be extended to more complex animals.

Chapter 1.2: Neural basis of feeding and food-seeking behaviours in mammals

1.2.1. Brain regulation of feeding and food-seeking behaviours.

Appetite is partly modulated by the opposing actions of the "hunger" and "satiety" hormones ghrelin and leptin, respectively. Leptin is released by the adipocytes and enables the communication between the brain and energy stores to induce satiety. Conversely, ghrelin is a 28–amino acid peptide mainly released by the endocrine cells in the stomach and may serve as a critical signal to induce hunger during fasting and to consequently stimulate appetite. Both hormones are secreted into the blood and pass the blood/brain barrier to bind specific receptors in the hypothalamus. These two hormones have specific neuronal targets that express various neuropeptides that in turn selectively alter appetite (Begg and Woods, 2013; Paspala et al., 2012; Marić et al., 2014; Farr et al., 2016).

Leptin regulates the arcuate nucleus (ARC) in the hypothalamus directly by binding to the long leptin receptor (LRb), resulting in activation of JAK-STAT3 signaling and modulation of neuropeptide expression (Bjørbæk, 2009; Varela and Horvatha, 2012; Villanueva and Myers, 2009). Decreased leptin levels during fasting induces hyperphagia and reduces energy expenditure through increasing neuropeptide Y (NPY)/agouti related protein (AGRP), while also suppressing α-melanocyte stimulating hormone (α-MSH) and cocaine and amphetamine-regulated transcript (CART) (Bjørbæk, 2009; Varela and Horvatha, 2012). Moreover, this reduction also stimulates the production of orexins in the lateral hypothalamic area. Conversely, increased leptin

levels in the sated animals suppresses food intake by inhibiting NPY/AGRP and increasing the production of the anorexigenic peptides, e.g. α-MSH and corticotropin-releasing hormone (CRH) (Bjørbæk, 2009; Varela and Horvatha, 2012; Villanueva and Myers, 2009).

The effects of leptin on neurotransmission can be very rapid. Upon its application to hypothalamic slices, leptin almost immediately increases the frequency of action potentials in the anorexigenic Pro-opiomelanocortin (POMC) neurons. This effect is induced by depolarization of a non-specific cation channel, decreased inhibitory tone of γ-aminobutyric acid (GABA) released from NPY terminals in the arcuate nucleus (ARC), and hyperpolarization of the NPY-expressing neurons (Cowley et al., 2001; Hill et al., 2008). Importantly, consistent with its potent anorexigenic action, applying leptin decreases the frequency of action potential spikes in fasted wild-type mice and leptin signaling deficient $Lep^{ob/ob}$ mice (Takahashi and Cone, 2005). The hyperphagic phenotype of $Lep^{ob/ob}$ mice is characterised by an increased ratio of excitatory/inhibitory synapses in the hypothalamus (Pinto et al., 2004), while it was rapidly reversed by leptin treatment within 6 hours, suggesting that leptin-mediated synaptic plasticity preceded the appetite-suppressing effect of the hormone (Pinto et al., 2004). In contrast, the induced food intake mediated by ghrelin has been associated with an increase in synaptic activity in the hypothalamus (Abizaid et al., 2006). These results suggest that these peripheral hormones that respond to food intake mediate brain function at least partly through their modulation of synaptic function (Abizaid et al., 2006; Pinto et al., 2004).

Hunger involves interoceptive sensory neurons that monitor metabolic signals to coordinate food-seeking and/or food consumption behaviours. The AGRP-expressing neurons in the hypothalamic arcuate nucleus are sensory neurons that are activated by circulating signals of energy deficit, such as ghrelin (Atasoy et al., 2012). AGRP neurons are organized into distinct projection populations and their axon projections into the bed nucleus of the stria terminalis (aBNST), lateral hypothalamic area (LHAs), and paraventricular hypothalamic nucleus (PVN) reveal brain regions with a capacity to orchestrate feeding and food-seeking behaviours. Increased AGRP neuron activity rapidly evokes voracious eating and food-seeking-behaviours, even in well-fed mice (Atasoy et al., 2012; Balthasar et al., 2005), while their ablation results in aphagia (Belgardt et al., 2009), and suppressing their electrical activity reduces food intake. Therefore, these well-defined neurons that sense energy demand are sufficient to orchestrate complex counter-regulatory behavioural responses to hunger (Aponte et al., 2011 and van de Wall et al., 2008). POMC neurons that are intermingled with AGRP neurons in the hypothalamic ARC are inhibited by AGRP neurons and have an important functional role in suppressing food consumption. POMC neurons send their projections to most of the same target regions as the AGRP neurons. Both of these neuronal populations transduce circulating metabolic signaling molecules such as leptin, ghrelin and glucose into electrical activity to control feeding behaviour, and may contribute to the internal representation of hunger (Atasoy et al., 2012; Aponte et al., 2011 and van de Wall et al., 2008).

Hypothalamic AMP-activated protein kinase (AMPK) is a central mediator of the adaptive responses to physiological stress partly through its roles in the regulation of

feeding and food-seeking behaviour through the integration of hormonal and nutritional cues (Blanco Martinez de Morentin et al., 2011; Yang et al., 2011). For instance, orexigenic signals such as fasting, hypoglycemia, and circulating ghrelin can activate AMPK in the hypothalamus, while anorexigenic signals such as feeding, hyperglycemia, insulin, leptin exert the opposite effect (Minokoshi et al., 2004; Blanco Martinez de Morentin et al., 2011). Therefore, it is of interest to better understand how this protein kinase modulates the activity of neural circuits that are engaged in food-seeking and food intake behaviours.

Chapter 1.3: Neural control of food-seeking behaviour in *C. elegans*

1.3.1. Nervous system in *C. elegans*

The *C. elegans* nervous system is relatively simple yet it still represents the most complex organ in this organism. The hermaphrodite nervous system is composed of 302 neurons and 56 glial cells, and together they account for 37% of the somatic cells (Ward et al., 1975; Sulston and Horvitz, 1977; Sulston et al., 1983; White et al., 1986). A large number of neurons belong to the somatic nervous system (282 neurons) whereas a small population form the pharyngeal nervous system (20 neurons) (Varshney et al., 2011). The pharyngeal nervous system is largely independent of the extra-pharyngeal nervous system and accounts for all chemical synapses onto pharyngeal muscle to modulate pharyngeal pumping (Avery and Horvitz, 1989). These two nervous systems communicate through a single pair of interneurons known as RIP (Ward et al., 1975; Sulston and Horvitz, 1977; Sulston et al., 1983; White et al., 1986).

The hermaphrodite neurons fall into 118 classes, which are based on topology and patterns of synaptic connection (White et al., 1986). The majority of the *C. elegans* neurons have simple monopolar or bipolar morphology, which are mostly unbranched. The majority of cell bodies are clustered in ganglia in the head or tail. The neurons communicate through approximately 6400 chemical synapses, 900 gap junctions, and 1500 neuromuscular junctions (NMJs). The name of each neuron consists of either two or three uppercase letters and in some cases a number indicating class and the neuron number within one class, respectively. If neurons are radially symmetrical, the three-letter name is followed by L (left), R (right), D (dorsal), or V (ventral) (Ward et al., 1975; Sulston and Horvitz, 1977; Sulston et al., 1983; White et al., 1986).

Despite its relatively simple nervous system, *C. elegans* is capable of complex behaviours, such as chemotaxis, thermotaxis, and even social feeding behaviours (Sengupta and Samuel, 2011). Most of these behaviours are plastic and therefore subject to learning and memory (Giles et al., 2006). Importantly, food is a significant regulator of many *C. elegans* behaviours such as egg-laying, feeding, locomotion, and olfactory behaviour which are regulated through different pathways (Zhang et al., 2005). Of note, the neural circuits that mediate each of these behaviours communicate via interneurons to produce hierarchies in their execution. For example, when an animal is faced with a depleted environment, egg-laying behaviour is suppressed and locomotory behaviour is induced, whereas upon encountering a food source, locomotory behaviour becomes suppressed allowing the animal to feed accordingly (Kang et al., 2010; Kindt et al., 2007; Hills et al., 2004; Sawin et al., 2000). This coordinated regulation of different behaviours in response to food availability is critical for dynamic adjustment to fluctuating energy levels at time scales that range from seconds to hours.

In most neurons, an electrical signal is propagated via all-or-none action potentials. These regenerative depolarizations ensure that the transmission of signal is robust and reliable over long distances. Conversely, some neurons, including the majority of neurons in *C. elegans,* are isopotential, and rely on passive propagation and graded release of neurotransmitter rather than action potentials. Graded synaptic transmission depends on two attributes: passive propagation of depolarization along the axon, and non-saturating calcium influx at the synaptic bouton (Liu et al., 2009). Therefore, graded transmission is associated with the absence of the axonal voltage-gated ion channels, and incomplete activation of synaptic voltage-gated calcium

channels. Therefore, neurotransmitter release from neurons is tonic (Davis and Stretton, 1989) and they fire graded potentials whose amplitude and waveform are sensitive to the amplitude and waveform of the stimulus (Davis and Stretton, 1989). This graded transmission implies that the circuit outputs may be a shifting integration of analog inputs. Moreover, activity-dependent plasticity will change the character of synapses during behaviour.

Overall, the relatively simple and well-characterized nervous system, along with simple and quantifiable behavioural outcomes, together with the ability to perform rigorous genetic analysis, make *C. elegans* an attractive model to investigate how various environmental stressors, such as starvation, trigger changes in the activity of neural circuits to induce adaptive behavioural outputs which are critical to survival.

1.3.2. *C. elegans* behaviours

Behaviour is among the most obvious outputs of neural activity and is dependent on multiple factors such as external stimuli, past experience, neuronal structure and changes in the internal milieu of the animal. Cellular or functional alterations can profoundly change the basal and evoked activity (Hart, 2006).

C. elegans senses a variety of mechanical and sensory stimuli that modify its behavioural outputs, accordingly. Locomotory behaviour is the most prominent behaviour in *C. elegans*, and it can be modified by a multitude of different effectors including food availability, mechanical stimuli, addition or removal of attractants or repellents, or changes in temperature (Hart, 2006).

1.3.3. The neural basis of locomotory behaviour in *C. elegans*

On solid surfaces and during conditions of abundant food, *C. elegans* will execute exploratory behaviour that includes a predominant tendency toward forward locomotion. This tendency to move in a forward direction is occasionally interrupted by turns and reversals that occur at predictable frequencies. However once faced with a gradient of a chemical attractant or repellent, *C. elegans* displays a dramatic modification of this predictable behaviour by increasing reversal frequency to the detriment of forward locomotion in the case of an attractant, or conversely, a dramatic suppression of reversals to enhance forward locomotion if it encounters a repellent (Wakabayashi et al., 2015).

The locomotory behaviour of *C. elegans* is characterized by its sinusoidal movement propagated by alternating dorsal-ventral contractions of body wall muscles. There are 95 body wall muscle cells arranged in interleaved patterns of two dorsal and two ventral rows. These body wall muscle cells are innervated by 113 motor neurons which, based on their function and position, fall into eight distinct classes (AS, DA, DB, DD, VA, VB, VC, and VD). While DA, DB, DD, and AS innervate the dorsal body wall muscles, VA, VB, VD, VC innervate the ventral body muscles. Dorsal motor neurons reside in the ventral nerve cord (VNC), while extending commissures dorsally to establish the dorsal nerve cord. Each class is constituted from multiple members that are aligned along the anterior-posterior axis of the ventral nerve cord in repeating units (*i.e.* VA-VA6) (White et al., 1986).

Alternate contraction of the dorsal and ventral longitudinal muscle rows generates the characteristic undulatory movement of the animal; a feature that is

modulated by the VNC motor neurons. The VNC motor neurons synapse onto either both dorsal or both ventral muscle quadrants, thereby restricting the body's flexures to the dorsoventral plane. Upon activation of the dorsal muscles, the ventral muscles are reciprocally inhibited and vice versa allowing for generation of anterior to posterior body curvature along the animal, thus creating the typical sinusoidal waves as the animal lies on its lateral side on the substratum (Sulston and Horvitz, 1977; Von Stetina and Miller, 2006).

The D-type motor neurons (VD, DD) are GABA(ɣ-aminobutyric acid)ergic and are inhibitory. They form the most important neuronal population involved in resetting the posture of the animal, for example when animals reverse or initiate rapid movement (Jorgensen and Nonet, 1995). They send their GABAergic synaptic outputs onto diametrically opposite muscles, so that upon activation of a ventral or dorsal muscle group by a cholinergic motor neuron, the opposite group of muscles is inhibited and relaxed. GABA inputs affect the amplitude of the body waves, although it does not interfere with wave propagation (McIntire et al., 1993).

A- and B-type motor neurons (VA, VB, DA, DB, AS) are cholinergic and postulated to be stimulatory. The A-type motor neurons (DA and VA neurons) are dedicated to backward movement, while B-type motor neurons (DB and VB neurons) promote forward locomotion (White et al., 1986; Chalfie et al., 1985). The outputs of these motor neurons are governed by distinct classes of command interneurons, whose roles in direction of locomotion have been elegantly mapped and characterized using laser ablation (Chalfie et al., 1985; Haspel et al., 2010; Leifer et al., 2011). A-type motor neurons receive signals predominantly from the command interneurons AVA, AVD, and

AVE, whereas the AVB and PVC interneurons form the predominant presynaptic partners of B-type motor neurons (Chalfie and White, 1988; Driscoll and Kaplan, 1997; Von Stetina et al., 2006). The direction of locomotion is established by the synaptic innervation of the motor neurons that are promoted by command interneurons throughout the length of the VNC (Jorgensen and Nonet, 1995). Reversals require the backward command interneurons AVA and AVD (Chalfie et al., 1985), with minor cross-talk from the forward command interneurons AVB and PVC (Zheng et al., 1999).

1.3.4. Command interneurons

The command interneurons (AVA, AVD, AVE, AVB, and PVC) are unique in that they are the only sets of interneurons that synapse onto the VNC motor neurons and also span the length of the ventral nerve cord (White et al., 1986; Chalfie et al., 1985). Their role in directing forward or backward movement was first discovered through laser ablation of their precursors. Ablation of the AVA and AVD interneurons rendered animals incapable of reversal behaviour. Conversely, ablation of the AVB and PVC shortened the duration of forward movement (Chalfie et al., 1985).

Subsequent calcium imaging studies demonstrated that during backward movement calcium transients increase in the AVA, further supporting its role in the regulation of backward movement (Kawano et al., 2011; Ben Arous et al., 2010; Faumont et al., 2011; Piggott et al., 2011). Consistent with this, the channelrhodopsin-mediated stimulation of AVA and AVD triggers robust backward movement (Guo et al., 2009; Stirman et al., 2011). In contrast, calcium transients increase in the AVB interneurons during forward movement, further suggesting its involvement in the

regulation of forward locomotion (Kawano et al., 2011; Faumont et al., 2011), while channelrhodopsin-mediated stimulation of PVC results in increased speed of movement (Stirman et al., 2011).

AVA and AVB receive input from the navigation circuit as described before (Gray et al., 2005) that includes the AIB, RIB, RIM, and the SMB interneurons. Information from this navigation circuit is then transmitted to the muscles through the command interneurons and their synaptic partners in the ventral cord, or those in the head, including symmetrical groups of two (RIM and RIV), four (SMD, SMB, SIA, and SIB) or six (RMD) neurons (Figure 1.1). These ventral cord and head motor neurons innervate three different sets of muscles in the head (White et al., 1986). The generation of these probabilistic behaviours is therefore likely a consequence of transmission of information between the sensory neurons, interneurons, and motor neurons in this circuit (Figure 1.2).

1.3.5. The transition between forward and backward movement

The role of command interneurons in modulating forward and backward movement was supported largely by a study by Brockie and colleagues, in which they found that a constitutively open GLR-1(A/T) glutamate receptor driven in the command interneurons resulted in a quick transition from forward to backward movement (Zheng et al., 1999). This suggests that the feedback for direction of movement occurs under the control of glutamatergic inputs into these command interneurons. Furthermore, *unc-7* and *unc-9,* both of which encode *C. elegans* innexins and are required for formation of gap junctions in invertebrates, establish the balance between forward and backward

movement (Kawano et al., 2011). Combined expression analysis and behaviour suggest an important role for *unc-7*, *unc-9,* and other innexins in the suppression of backward movement (Kawano et al., 2011) although, their role in modulating forward movement is unclear. Given the importance of glutamatergic signaling in the modulation of forward and backward locomotion we have briefly outlined the various *C. elegans* glutamate receptors that mediate glutamatergic signaling and their effects in the following section.

Chapter 1.4: Glutamatergic signaling

1.4.1. General introduction

Food intake and food-seeking behaviours are mainly modulated by the ionotropic glutamate receptors. The genes that comprise the mammalian ionotropic glutamate receptor family are encoded by 18 gene products that mediate most of the rapid excitatory synaptic signaling in the central nervous system (Traynelis et al., 2010). These receptors are expressed in neuronal and non-neuronal cells and their co-assembly results in the formation of functional ligand-gated ion channels that contain a transmembrane ion binding site, an agonist recognition site, and gating elements that couple agonist-induced conformational changes to the opening or closing of the pore. Glutamate receptors modulate a broad spectrum of processes in the brain, spinal cord, peripheral nervous system and retina. In addition to their function in numerous behaviours and learning and memory, these receptors may play important roles in several neurological diseases thereby attracting intense scrutiny (Traynelis et al., 2010).

Like in the mammalian nervous system, glutamatergic signaling is an important component of nervous system function in *C. elegans*. A brief overview will be presented below describing different types of glutamate receptors encoded by *C. elegans* genome.

1.4.2. Ionotropic glutamate receptors (iGluRs)

Ionotropic glutamate receptors (iGluRs) are a major class of heteromeric ligand-gated ion channels that mediate the majority of the excitatory neurotransmission in the vertebrate central nervous system (CNS).

The eighteen receptor subunits identified in rat belong to the N-methyl-d-aspartate receptor (NMDA) class (NR1, NR2A-D, NR3A-B), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA; GluR1-4/GluRA-D), or kainate (KA; GluR5-7, KA1-2). A functional ligand-gated receptor can be formed by the co-assembly of four subunits from a single class, e.g., NMDA subunits assemble with other members of the NMDA subfamily (Laube et al., 1998; Mano and Teichberg, 1998; Rosenmund et al., 1998).

The *C. elegans* genome encodes four classes of glutamate receptors: AMPA-type glutamate-gated cation channels (encoded by eight genes *glr-1-glr-8*); NMDA-type glutamate-gated cation channels (encoded by *nmr-1* and *nmr-2* genes); Metabotropic G-protein-coupled glutamate receptors (encoded by *mgl-1* and *mgl-2*, *mgl-3* genes); and glutamate-gated chloride channels (encoded by 10–20 *glc/avr* genes) which are found only in protostome invertebrate phyla and are closely related to glycine receptors in mammals (Brockie et al., 2001a; Brockie and Maricq, 2003; Dent et al., 1997; Dent et al., 2000).

AMPA, NMDA, kainate and mGLURs show high sequence similarities with their vertebrate counterparts (Brockie et al., 2001a). For example, the extracellular ligand binding domains, S1 and S2, the 4 hydrophobic domains TM1-TM3 and the pore region are conserved in *C. elegans* iGluRs. Furthermore, amino acid residues within the "Q/R" site of non-NMDA receptors (Seeburg et al., 1998) and the conserved asparagine (N) residue in NMDA receptors (Burnashev et al., 1992b), which are important to modulate calcium permeability, are also conserved in *C. elegans*. Similarly, the SYTANLAA amino acid stretch, which is the iGluR signature at the C-terminus of TM2 and is postulated to

influence receptor gating, is also conserved in the *C. elegans* orthologues. It has been shown that a single nucleotide conversion (A/T) in the δ2 subunit in this region causes the receptor to remain constitutively open. Introducing this (A/T) mutation into the *C. elegans* GLR-1 subunit causes a dramatic change in locomotion, presumably due to the increased ion flow through the receptor channel (Zheng et al., 1999).

The NMDA (*nmr-1* and *nmr-2*) and non-NMDA (*glr-1*, *glr-2*, *glr-4* and *glr-5*) subunit genes are highly expressed in the command interneurons - AVA, AVB, AVD, AVE and PVC which modulate forward and backward movement. Both *glr-3* and *glr-6* are expressed in a pair of interneurons called the RIA neurons that are involved in the neural circuit that controls thermotaxis (Mori and Ohshima, 1995). The remaining non-NMDA subunits, *glr-7* and *glr-8*, are expressed in a set of neurons in the pharyngeal nervous system. The broad expression pattern of iGluR subunits suggests their likely involvement in numerous behaviours as diverse as the avoidance of noxious stimuli to the control of pharyngeal activity (Brockie et al., 2001a; Brockie and Maricq, 2003).

1.4.3. Glutamate gated chloride channels (GluCls)

In *C. elegans* both excitatory and inhibitory glutamate receptors have been shown to control activity within neural circuits and consequently regulate behaviour. The inhibitory glutamate receptors appear to be unique to the invertebrate nervous system and some members of this family have also been identified and characterized in *Drosophila* and the parasitic nematodes *Haemonchus contortus*, *Ascaris suum* and *Dirofilaria immitis* (Cully et al., 1996; Forrester et al., 1999; Jagannathan et al., 1999; Cheeseman et al., 2001; Forrester et al., 2002; Yates and Wolstenholme, 2004). These inhibitory GluCls

channels are permeable to chloride ions and are sensitive to the anthelmintic drug avermectin (Cully et al., 1994).

The *C. elegans* genome encodes at least 6 GluCl subunits (*glc-1*, *glc-2*, *glc-3*, *glc-4*, *avr-14/gbr-2* and *avr-15*) (Cully et al., 1994; Dent et al., 1997; Vassilatis et al., 1997; Laughton et al., 1997a; Dent et al., 2000; Horoszok et al., 2001). Based on the subunit membrane topology these receptors are placed in the ligand-gated ion channel superfamily (LGICS) that also includes the inhibitory GABA and glycine receptors and the excitatory acetylcholine and serotonin receptors (Ortells and Lunt, 1995).

The expression pattern of a subset of *C. elegans* GluCl subunits has been indicated using the GFP fusion reporters. GluCls are widely expressed in both the pharyngeal muscle cells and neurons suggesting their potential role in regulation of behaviour in worms. *avr-14* (GluClα3) is exclusively expressed in the extrapharyngeal nervous system such as in the mechanosensory neurons ALM, PLM and PVD (Dent et al., 2000). *avr-15* (GluClα2) is expressed in the extrapharyngeal nervous system as well as the pm4 (metacorpus) and the pm5 (isthmus) pharyngeal muscle cells (Dent et al., 1997). Unlike *avr-14* and *avr-15,* both of which show broad expression, *glc-2* (GluClβ) expression is specific to the pm4 muscle cells (Laughton et al., 1997b) where it likely forms functional heteromeric channels with AVR-15/GluClα2 (Vassilatis et al., 1997).

1.4.4. Metabotropic glutamate receptors (mGluRs)

Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors that play an important neuromodulatory role in glutamatergic signaling within the mammalian nervous system (Dillon et al., 2015; Awad et al., 2000; Heidinger et al., 2002). The

importance of this class of receptor is highlighted by their involvement in numerous neurological diseases including anxiety disorder, some autism spectrum disorders, and schizophrenia (Herman et al., 2012; Oberman, 2012), which may manifest as a consequence of the imbalance between cellular inhibition and excitation within the context of discrete microcircuits (Yizhar et al., 2011; Rubenstein and Merzenich, 2003; Kehrer et al., 2008).

All eight mammalian mGluRs exhibit a heptahelical domain organization and are subclassified into three distinct groups based on conserved structural and functional features. Moreover, Group 1 (mGluRs 1 and 5), Group 2 (mGluRs 2 and 3) and Group 3 (mGluRs 4, 6, 7 and 8) receptors are postulated to play distinct neuromodulatory roles in the mammalian brain (Conn et al., 2009; Pin and Acher, 2002). Like vertebrate mGluRs, this class of receptor plays an important role in glutamatergic signaling and adaptive behaviours such as context-dependent feeding behaviour in *C. elegans* (Dillon et al., 2015).

The *C. elegans* genome encodes at least three mGluRs known as *mgl-1*, *mgl-2*, and *mgl-3*. Similar to their mammalian counterparts *mgl-1, mgl-2, and mgl-3,* are organized into three subgroups,. *mgl-1* encodes the *C. elegans* group II Metabotropic glutamate receptor and is predicted to function as a pre-synaptic G protein-coupled receptor (GPCR) that inhibits adenylyl cyclase activity in response to glutamate binding (Conn et al., 2009; Pin and Acher, 2002). By contrast *mgl-2* and *mgl-3* encode the *C. elegans* group I and group III Metabotropic glutamate receptors respectively, which have been found to function as post-synaptic G protein-coupled receptors that stimulate phospholipase C activity resulting in an increase in neuronal excitation (Conn et al.,

2009; Pin and Acher, 2002). Metabotropic glutamate receptors have a widespread expression pattern in the *C. elegans* nervous system. *mgl-1*, *mgl-2* and *mgl-3* are exclusively expressed in the pharyngeal nervous system, suggesting their potential involvement in the modulation of pharyngeal network activity (Dillon et al., 2015). Moreover, their expression in the numerous interneurons and motor neurons suggest a likely role for them in regulation of animal behaviour.

1.4.5. Mechanisms of iGluR localization, stabilization and function at the synapse

The subcellular localization of iGluRs is visualized using functional fusion proteins (Rongo et al., 1998; Brockie et al., 2001b; Mellem et al., 2002). These iGluR::GFP chimeras are distributed along the neuronal processes. These puncta closely appose presynaptic markers and therefore are likely to represent postsynaptic sites (Rongo et al., 1998). Like in mammals, the *C. elegans* AMPARs are assembled as homo- or hetero-tetramers.

In vertebrates PDZ-domain proteins interact with NMDA and non-NMDA subunits and are postulated to mediate the localization and stabilization of iGluRs at the cell membrane of postsynaptic puncta (Sheng and Sala, 2001; Bredt and Nicoll, 2003; Kim and Sheng, 2004). During development, the density of GLR-1::GFP puncta along the ventral cord remains unchanged (Rongo et al., 1998).

Synaptic AMPA iGluRs are derived from a rapidly recycling pool of receptors that undergo repeated cycles of insertion by exocytosis and removal by endocytosis. This dynamic process is of paramount importance for synaptic plasticity (Malinow and Malenka, 2002; McGee and Bredt, 2003; Contractor and Heinemann, 2004). In

vertebrates agonist-induced endocytosis of AMPA receptors has been coupled to the ubiquitin-proteasome pathway (Colledge et al., 2003; Patrick et al., 2003). Similarly, studies in *C. elegans* have demonstrated that iGluR ubiquitylation together with clathrin-mediated endocytosis modulate iGlutRs levels at postsynaptic elements (Burbea et al., 2002). *unc-11,* which encodes the mammalian orthologue of the AP180 clathrin adaptin protein, mediates clathrin-mediated endocytosis of GLR-1 containing receptors as mutations in *unc-11* cause defects in GLR-1 endocytosis resulting in the accumulation of GLR-1::GFP at the ventral cord puncta (Nonet et al., 1999).

Several studies in *C. elegans* have shown that increased glutamatergic signaling biases locomotion toward backward locomotion. For example, the “lurcher” mutation which is a spontaneous, semidominant mouse neurological mutation is speculated to encode a constitutively open receptor in *C. elegans*. In *C. elegans,* a similar mutation in GLR-1 results in both increased backward locomotion rate and decreased duration of forward movement, which consequently affects food-seeking behaviour (Zheng et al., 1999). Glutamatergic signaling has recently been demonstrated to play an important role in the modulation of compulsive behaviours in starved mice (Dietrich et al., 2015), while AMPA-type mediated regulation of glutamatergic signaling in a specific set of neurons was shown to influence the rate of food intake in both *C. elegans* and mammals in an AMP-activated protein kinase (AMPK) dependent manner (Lee et al., 2008; Cunningham et al., 2014; Cunningham et al., 2012; Yang et al., 2011). However the mechanism through which this kinase modulates adaptive feeding and food-seeking behaviour remains poorly understood.

Chapter 1.5: Sensory and environmental influences on *C. elegans* feeding and exploratory behaviour

Natural isolates of *C. elegans* display two distinct solitary or social feeding behaviours. Social foragers move rapidly on bacterial lawn and aggregate together whereas solitary foragers move slowly on bacteria and disperse across the food. It was found that a loss-of-function mutation in the *C. elegans* ortolougue of neuropeptide Y receptors known as *npr-1*, which encodes a predicted G protein-coupled receptor results in social feeding behaviour (de Bono M and Bargmann CI, 1998).

C. elegans has distinct exploratory states in which the overall pattern of locomotion is modified based on the animals' recent experience with food. These locomotory behaviours are modulated by a well-defined navigation circuit. In the following section I outline the effects of food availability on a subset of behavioural paradigms. This information will provide a framework for the neural circuits that function to trigger food-seeking behaviour under different conditions of food availability.

1.5.1. Effect of food availability on behaviour

The stereotyped locomotory behaviour of *C. elegans* is sensitive to food availability. Under laboratory conditions *C. elegans* spends the majority of its time foraging through an abundant lawn of *E. coli* on minimal nematode growth medium (NGM) plates. Once on food, animals move slowly in a forward direction, while they exhibit frequent short reversals. Upon removal from food, well-fed animals reverse and turn frequently resulting in the efficient exploration of the surrounding area (Figure 1.3). Sensory inputs contribute to the behavioural changes observed after removal from food as seen in

osm-6 mutants, which are defective in the development of all ciliated chemosensory and mechanosensory neurons (Gray et al., 2005). Similarly, *che-2* mutants, whose cilia are also defective, continue to execute frequent short reversals after even several hours of continued starvation (Gray et al., 2005). In contrast, starvation suppresses reversals and turns while triggering forward movement (runs) that allow animals to more effectively explore distal areas; a strategy that is referred to as dispersal behaviour or alternatively, distal exploration (Figure 1.3). The transition from local search behaviour to dispersal behaviour occurs after 30 minutes off food as animals become starved (Gray et al., 2005).

1.5.2. The neural basis of adaptive locomotory behaviour in response to food availability

The increased reversal frequency during local search behaviour reflects a sensory memory of food that is expressed by the navigation circuit. This may be partially dependent on the activity of the AWC olfactory and the ASK gustatory neurons, both of which contribute to the execution of this exploratory behaviour. AWC and ASK neurons stimulate the AIB interneurons in response to removal from food, and AIB in turn triggers long reversals and omega turns. The transition from local search to dispersal behaviour requires the ASI sensory neurons and the AIY interneurons. While AIB activation triggers reversals and elicits local search behaviour, the AIY interneurons suppress reversals and promote forward locomotion and eventually dispersal behaviour. Serotonin and the TGFβ orthologue DAF-7 released from the ASI, both affect a variety

of neuronal responses associated with starvation, including this transition (Ren et al., 1996; Sze et al., 2000).

C. elegans also displays an experience-dependent response that may be an adaptive mechanism that increases the amount of time animals spend in the presence of food. These experience-dependent responses are modulated by the distinct serotonergic and dopaminergic signals that contribute to basal and enhanced slowing response evoked in well-fed and starved animals that are reintroduced to food, respectively. These paradigms modulate behavioural plasticity in response to food availability and are consistent with the notion that a well-fed animal would be more likely to risk exploring distant locations for high quality food sources, whereas a starved animal would be less likely to stray far from a recently discovered vital food supply (Sawin et al., 2000).

The sensory neurons involved in chemotaxis and thermotaxis such as AWC, AFD, ASE, synapse primarily onto the interneurons AIY, AIB, AIZ, and RIA-all of which are required for thermotaxis (Mori and Ohshima, 1995). Therefore, the regulation of reversal frequency by these sensory neurons and their downstream interneurons may play a role in the biased random walk component of various types of taxis.

Chapter 1.6: AMP-activated protein kinase mediated signaling

The many signaling networks that are triggered throughout the nervous system in response to the action of small molecules, hormones and nutrients on energy balance likely mediate the mechanism through which hunger changes the activity of neural circuits and their behavioural outputs. One of the key factors for metabolic homeostasis and survival is the highly conserved heterotrimeric protein kinase 5' adenosine monophosphate-activated protein kinase (AMPK). Of note, in both *C. elegans* and mammals this protein kinase plays an important role in the modulation of glutamatergic signaling and consequently feeding behaviour. Moreover, some evidence suggests its involvement in regulation of food- seeking behaviour in *Drosophila* (Johnson et al., 2010; Braco et al., 2012) and *C. elegans* (Cunningham et al., 2014), although the mechanism through which this enzyme functions remains unknown.

1.6.1. AMPK signaling

The SNF1/AMPK family of protein kinases is required for energy homeostasis in mammals, plants, and fungi. SNF1 was originally identified in the budding yeast *Saccharomyces cerevisiae* by genetic analysis in a screen to identify genes involved in growth during sub-optimal nutrient conditions. Cloning the SNF1 gene revealed that it encoded the catalytic subunit of a serine/threonine kinase (Celenza and Carlson, 1984; Celenza and Carlson, 1986). It was subsequently shown that SNF1 modulates transcription, modifies the function of metabolic enzymes, and controls various nutrient-responsive cellular developmental processes. In 1994, a cDNA encoding the catalytic

subunit of AMPK was cloned and was identified as the mammalian ortholog of Snf1 (Carling et al., 1994; Mitchelhill et al., 1994).

AMPK is an enzyme that plays a critical role in cellular energy homeostasis. AMPK consists of a catalytic α-subunit and regulatory β- and γ-subunits that together form a functional enzyme. Genes encoding the AMPK catalytic and regulatory subunits are found in essentially all eukaryotes from nematodes to humans. The catalytic activity of the αβγ-complexes, is increased by increases in the intracellular AMP/ATP ratio and through the activity of its upstream activating kinases (Hardie, 2008; Hardie and Ashford, 2014; Hardie et al., 2012).

The AMPK catalytic subunits contain a conventional Ser/Thr kinase domain at the N-terminus. The activity of the complex increases more than 100-fold upon phosphorylation of a conserved Thr residue in the activation loop (which is referred to as Thr172 owing to its position in the original rat sequence) by upstream kinases (Hawley et al., 1996). In mammals, the major upstream AMPK activating kinases are the LKB1–STRAD–MO25 complex (Hawley et al., 2003; Woods et al., 2003; Shaw et al., 2004) (whose mutation results in Peutz-Jeghers syndrome) and the CaMKKβ (also known as CaMKK2) (Hawley et al., 2005; Woods et al., 2005; Hurley et al., 2005).

A high basal level of phosphorylation at Thr172 of AMPK can be achieved by the LKB1–STRAD–MO25 complex that is modulated by the binding of AMP to the AMPK γ-subunit: increasing the rate of phosphorylation while decreasing dephosphorylation (Figure 1.4) (Kim et al., 2013; Hawley et al., 1995; Davies et al., 1995). Although AMP efficiently promotes the allosteric activation of AMPK, it has recently been found that the effects on phosphorylation and dephosphorylation can also be triggered by ADP (Xiao

et al., 2011; Oakhill et al., 2011). The effects of both AMP and ADP on phosphorylation/activation depend on myristylation of N-terminus of the β-subunit (Oakhill et al., 2011; Oakhill et al., 2010). AMP and ADP bind to the γ-subunits of AMPK with similar affinity to ATP (Xiao et al., 2011) (which does not cause activation). Therefore, given the higher concentrations of ADP compared to AMP in cells, ADP may be the physiological activating signal that triggers phosphorylation of Thr172 during moderate energy stress. However, allosteric activation of AMPK by AMP would further amplify its activation during a more severe stress. This complex mechanism provides a graded response of AMPK activity over a wide range of stress levels (Hardie et al., 2012).

Alternatively, AMPK can be phosphorylated by CaMKKβ in response to increased cellular Ca2+ without necessarily requiring any change in the AMP or ADP levels. CAMKK2 appears to particularly contribute in AMPK activation in neurons and T cells (Anderson et al., 2008; Tamas et al., 2006).

In the hypothalamus CaMKK2 stimulates NPY expression by functioning as the AMPKK linking ghrelin-dependent Ca2+ signaling to AMPK activation (Anderson et al., 2008). These findings suggest that CaMKK2 association with the AMPKα/β heterodimer provides an alternative means of AMPK stabilization, conferring Ca2+ sensitivity and AMP independence to the kinase which is fundamentally different from the AMP-dependent LKB1-AMPK signaling (Anderson et al., 2008). Similarly, in T lymphocytes, AMPK is activated via a Ca2+–CaMKK-dependent pathway upon triggering of the T cell antigen receptor (TCR), which is not a response to energy stress. Thus, in T cells, AMPK function is not solely limited to restoring energy balance after the depletion of

energy stores. Rather, increased [Ca2+], which activates CaMKKs, triggers AMPK activation enhancing the conservation and the accelerated production of ATP in anticipation of a decrease in energy supplies (Tamas et al., 2006).

This ability would be advantageous for cells that need to rapidly respond to an increased demand for ATP. In this context, activation of the TCR leads to the initiation of an energy-demanding program that is only successful under the conditions in which the overall capacity for cellular energy production satisfies the biosynthetic demands of an immune response. T cells use various mechanisms to cope with the energy depletion, and Ca2+-mediated activation of AMPK would allow the rapid activation of ATP production before the onset of cell proliferation and differentiation (Tamas et al., 2006). Therefore, Ca2+-mediated activation of AMPK in the nervous system and T cells may serve as an important player of energy conservation under specific conditions (Anderson et al., 2008; Tamas et al., 2006).

1.6.2. AMPK structure

The structures of various combinations of AMPK subunits have been determined by X-ray crystallography. The α-subunit contains a conventional kinase domain at the N-terminus that is immediately followed by an auto-inhibitory domain (AID), so-called because its presence results in a less active form of the kinase (Chen et al., 2009). The AID is then followed by an extended 'linker peptide' that links the AID to the α-subunit carboxy-terminal domain (α-CTD) (Pang et al., 2007; Chen et al., 2009).

The β-subunits contain a carbohydrate-binding module (CBM), which mediate the association of mammalian AMPK to glycogen particles, the significance of which is still

unclear (Hudson et al., 2003; Polekhina et al., 2003). The C-terminal domain (β-CTD) of β-subunit is connected to both the α-CTD and the γ-subunit forming the core of the complex. The γ-subunits contain 4 sequence motifs called the CBS repeat (numbered as CBS1 to CBS4) (Bateman et al., 1997). These tandem repeats form a Bateman domain, which contain ligand-binding sites in the cleft between the repeats. Most Bateman domains bind to adenosine-containing ligands which usually includes ATP, but also S-adenosyl Met (Kemp et al., 2004), and mutations in these domains have been shown to be associated with several human diseases such as heart disease (familial hypertrophic cardiomyopathy) (Ignoul et al., 2005; Scott et al., 2004) which has been linked to mutations affecting the AMPK γ2-subunit (Ignoul et al., 2005; Scott et al., 2004). In AMPK, the four CBS repeats in the γ-subunits form four potential ligand-binding sites in the center and each of them possess a conserved aspartate residue involved in ligand binding (Oakhill et al., 2012).

1.6.3. Regulation of AMPK in intact cells

In mammalian cells, AMPK is activated by various stressors, drugs and xenobiotics through the mechanisms that involve increases in cellular AMP, ADP or Ca2+. These can be referred as the 'canonical' AMPK activation mechanisms. However, recent studies have demonstrated that activation of AMPK can be achieved via mechanisms that do not require any change in the levels of AMP, ADP and Ca2+. This mechanism can therefore be referred as 'non-canonical' pathway. A brief introduction on these distinct types of mechanism is outlined below.

1.6.4. Canonical activation by metabolic stresses, drugs and xenobiotics

The canonical mechanisms of AMPK activation in mammals involve increases in AMP/ATP and ADP/ATP ratios, where AMPK is activated by stressors that inhibit the catabolic production of ATP, such as glucose starvation (Salt et al., 1998), oxygen deprivation (Marsin et al., 2000), or addition of a metabolic poison (Corton et al., 1994), as well as by stresses that increase rapid ATP consumption, such as muscle contraction (Winder and Hardie, 1996). AMPK is also activated by numerous drugs and xenobiotics such as metformin, phenformin and thiazolidinediones (Zhou et al., 2001; Fryer et al., 2002), resveratrol from grapes and red wine (Baur et al., 2006), and capsaicin from peppers (Hwang et al., 2005).

Metformin, which is the most widely prescribed antidiabetic drug in the world, activates AMPK most likely through its role as a mitochondrial poison, although this activation of AMPK may not explain all of the positive therapeutic effects of metformin (Hardie et al., 2012). Mice that lack both AMPKα1 and AMPKα2 in the liver show a normal hypoglycaemic response to metformin, and the acute effect of metformin on gluconeogenesis in isolated hepatocytes is also unaffected in the absence of AMPK function (Foretz et al., 2010). The effect of metformin on glucose production may be a consequence of an increase in the AMP levels that directly inhibit the gluconeogenic enzyme fructose-1,6-bisphosphatase. One caveat in the interpretation of these findings is that the decreased ATP levels (and hence the increased AMP levels) caused by metformin was larger in cells lacking AMPK signaling than in wild-type cells (Foretz et al., 2010) suggesting that the inhibition of fructose-1,6-bisphosphatase by AMP may be accentuated upon depletion of AMPK. Thus, although not completely excluding a role of

AMPK in metformin action, these findings do suggest that other targets, such as fructose-1,6-bisphosphatase, are also important. In addition, it has been shown that metformin may activate AMPK in L6 skeletal muscle cells through inhibition of AMP deaminase (the enzyme that breaks down AMP), resulting in accumulation of AMP (Ouyang et al., 2011).

Intriguingly, some of the AMPK activators such as resveratrol and metformin have been revealed to extend lifespan in *C. elegans*, and genetic studies have shown that the AMPK is required for these effects. Various studies in *C. elegans* and mammalian cells have shown that AMPK upregulates many genes involved in oxidative metabolism and oxidative stress resistance by regulating the transcription factors of the abnormal dauer formation 16 (DAF-16)/forkhead box O (FOXO) family (Greer et al., 2007; Greer et al., 2007).

One important question is how these drugs and xenobiotics, many of which have dramatically different structures, all contribute to the activation of AMPK. Since most of these activators modulate AMPK in intact cells but not in cell-free assays, they may activate AMPK indirectly. One study has shown that many of these drugs and xenobiotics, including metformin and resveratrol, activate AMPK indirectly by increasing the cellular AMP and ADP levels, which is usually caused by inhibition of mitochondrial ATP synthesis (Hawley et al., 2010).

Many of these natural products that activate AMPK appear to be defensive compounds, which are produced by plants to deter infection by pathogens or grazing by insects or mammalian herbivores. Given the large multiprotein complexes in mitochondrial respiratory chain and ATP synthase, they might contain many potential

binding sites for hydrophobic xenobiotics that result in complex inhibition and ultimately can decrease cellular energy status to a specific threshold that has favourable effects on lifespan through activation of AMPK.

1.6.5. Non-canonical activation of AMPK by oxidative stress and genotoxic treatments

AMPK can be activated by some types of cellular stresses through non-canonical mechanisms that may not involve increases in AMP, ADP or Ca2+ levels. AMPK can be activated by reactive oxygen species (ROS) such as hydrogen peroxide (H2O2) in cultured cells. However, at high ROS concentrations, AMPK activation is likely to be a secondary effect to the inhibition of mitochondrial ATP synthesis, with consequent increases in AMP and ADP levels (Hawley et al., 2010). Moreover, there is a more direct AMPK activation mechanism that involves oxidation or glutathionylation of two conserved Cys residues in the AMPK α-subunit (Zmijewski et al., 2010). In addition, AMPK activation by H2O2 may occur via a third mechanism involving a cytoplasmic form of the phosphoinositide 3-kinase-like kinase (PIKK) ataxia telangiectasia mutated (ATM) (Alexander et al., 2010); the product of the causative gene in human ataxia telangiectasia. Nuclear ATM is part of a key DNA damage-sensing pathway that is activated by DNA double-strand breaks. However, a cytoplasmic pool of ATM that plays an important role in the response to oxidative stress was recently revealed (Ditch et al., 2011). ATM-dependent activation of AMPK by oxidative stress seems to be dependent on LKB1, as this activation is attenuated in the cells lacking this upstream kinase (Alexander et al., 2010). Interestingly, ATM can phosphorylate LKB1, although it

remains unclear whether this phosphorylation affects LKB1 activity (Sapkota et al., 2002).

Similarly, Glucose deprivation has been demonstrated to induce the phosphorylation of p53 in Ser15 (Ser18 in mice) in an AMPK-dependent manner. This phosphorylation is required for initiation of AMPK-mediated cell-cycle arrest which occurs at the G_1/S boundary (Jones et al., 2005). Moreover, glucose deprivation activates the AMPK/p53 pathway through ATM kinase leading to increased expression of IFI16 and induction of autophagy (Duan et al., 2011). In low glucose conditions, AMPK-mediated phosphorylation of p53 results in inhibition of mTOR and induction of autophagy, through a mechanism that involves p53 translocation to the nucleus (Lee at al., 2009), where it binds to the promoter regions of different genes implicated in autophagy, e.g., SESTRIN 2 and DAPK-1 (death-associated protein kinase 1) to induce their expression (Moruno et al., 2012). Activation of a p53-dependent metabolic cell-cycle checkpoint and autophagy by AMPK is critical for cell survival under unfavorable growth conditions. To this end, the AMPK involvement in the regulation of senescence may at least partially explain its contribution as a tumor suppressor (Kato et al., 2002).

Another class of AMPK activators includes genotoxic, DNA-damaging treatments such as etoposide (Fu et al., 2009), doxorubicin (Ji et al., 2010) and ionizing radiation (Sanli et al., 2010), all of which activate AMPK initially in the nucleus (Sanli et al., 2010). There is some evidence to suggest that these effects may also be mediated by ATM while, surprisingly, LKB1 is not required because the pathway is functional in LKB1-null cells (Fu et al., 2009; Sanli et al., 2010). However, the detailed mechanism involved in this process remains unclear.

To summarize this section, AMPK is activated in intact cells by either canonical pathways that involve increases in AMP, ADP or Ca2+, and/or by non-canonical pathways that can be triggered by other stresses such as ROS and DNA-damaging agents, which do not require changes in AMP, ADP or Ca2+ levels.

1.6.6. AMPK coordinately modulates cell growth and autophagy

In conditions of resource scarcity, AMPK acts as a metabolic checkpoint that inhibits cellular growth. The most thoroughly described mechanism through which AMPK modulates cell growth is via inhibition of the mammalian target of rapamycin complex 1 (mTORC1) pathway. AMPK controls the mTORC1 partly by direct phosphorylation of the tumor suppressor TSC2 on serine 1387, a negative regulator of TOR signaling that acts through its GAP function (Zang et al., 2006).

In addition to its involvement in modulation of cell growth, mTORC1 also regulates autophagy; a self-degradative process leading to “self engulfment” during which the cell breaks down its own organelles (macroautophagy) and cytosolic components (microautophagy) to provide energy in response to nutrient and/or other stress. The core components of the autophagy pathway were initially discovered in genetic screens in budding yeast (Yang and Klionsky, 2010; Mizushima and Levine, 2010). The most upstream components of this pathway include the serine/threonine kinase Atg1 and its two associated regulatory subunits Atg13 and Atg17 (Yang and Klionsky, 2010; Mizushima and Levine, 2010). The activity of the mammalian orthologs of the Atg1 complex is suppressed by mTORC1 through a poorly defined mechanism which likely involves phosphorylation of the Atg1 orthologs ULK1 and ULK2, as well as

their regulatory subunits (Mizushima, 2010). In contrast to inhibitory phosphorylation by mTORC1, various studies have revealed that AMPK can activate the ULK1 complex via direct phosphorylation, which is critical for its function in autophagy and mitochondrial homeostasis (Hardie, 2011).

Moreover, in addition to the unbiased mass spectrometry studies that show the endogenous AMPK subunits as ULK1 interactors (Behrends et al., 2010; Lee et al., 2010), two recent studies have demonstrated that AMPK can directly phosphorylate several sites in ULK1 (Egan et al., 2011; Kim et al., 2011). As observed for other core autophagy proteins, ULK1 plays a critical role in cell survival following nutrient stress and this function requires the multiple consensus AMPK phosphorylation sites on ULK1 (Egan et al., 2011; Kim et al., 2011). Similarly, genetic studies have demonstrated that Atg1 is also required downstream of AMPK for appropriate autophagy in budding yeast (Wang et al., 2001) and in *C. elegans* (Egan et al., 2011). Collectively, these studies show that AMPK regulates autophagy through a double-pronged mechanism that involves the direct activation of ULK1 as well as inhibition of the negative regulatory effect of the mTORC1 complex1 on ULK1 function. However, many of the temporal and spatial details of the modulation of these three nutrient-sensitive kinases (AMPK, ULK1, mTOR) remain yet to be elucidated.

1.6.7. Modulation of metabolism via transcription and direct effects on metabolic enzymes

AMPK was described as the critical regulator of the key metabolic enzymes Acetyl-CoA carboxylase (ACC1 & ACC2) and HMG-CoA reductase, both of which serve as the rate-

limiting steps for fatty-acid and sterol synthesis in wide-variety of eukaryotes (Carling et al., 1987; Sato et al., 1993). In specialized tissues including muscle and fat, AMPK function is required for modulation of glucose uptake by regulating the RabGAP TBC1D1, which along with its homolog TBC1D4 (AS160), play critical roles in Glucose transporter 4 (GLUT4) trafficking following exercise (Sakamoto and Holman, 2008). Moreover, in fat tissue AMPK also directly phosphorylates lipases, including both hormone sensitive lipase (HSL) (Watt et al., 2006) and adipocyte triglyceride lipase (ATGL) (Ahmadian et al., 2011).

In addition to the acute effect of AMPK on these metabolic enzymes, it is also involved in an adaptive reprogramming of metabolism induced by transcriptional changes. AMPK-dependent transcriptional changes are triggered via phosphorylation and regulation of a number of transcription factors, coactivators, the acetyltransferase p300, a subfamily of histone deacetylases, and even histones themselves. In 2010, Bungard *et al*., reported that AMPK phosphorylates histone H2B on Serine36 and eventually effects transcriptional changes (Bungard et al., 2010). Moreover, chromatin immunoprecipitation studies detected AMPK at the promoters of genes at specific chromatin loci in mammalian cells (Bungard et al., 2010).

Given the important role of transcriptional programs in the physiology of metabolic tissues, several connections between AMPK and transcriptional control have been discovered in these systems. Importantly, many of the transcriptional regulators which are phosphorylated by AMPK in metabolic tissues are expressed more ubiquitously than initially speculated and therefore may be playing more central roles tying metabolism to growth. One recent example is the lipogenic transcription factor

Srebp1 which induces a gene expression program that includes upregulation of both ACC1 and FASN, both of which stimulate fatty acid synthesis in cells (Li et al., 2011). Despite its critical role in the regulation of lipids in liver and other metabolic tissues, Srebp1 mediated control of lipogenesis has been shown to be required in all dividing cells given its importance as a major cell growth regulator in *Drosophila* and mammalian cells (Porstmann et al., 2008). AMPK has been shown to phosphorylate a conserved serine near the cleavage site within Srebp1, thereby blocking its activation (Li et al., 2011). Therefore, while AMPK mediates long-term adaptive effects via Srebp1 phosphorylation and loss of expression of lipogenic enzymes, AMPK acutely modulates lipid metabolism by phosphorylating ACC1 and ACC2.

AMPK has also been reported to phosphorylate the glucose-sensitive transcription factor ChREBP (Kawaguchi et al., 2002) which together with Srebp1 modulates the expression of an overlapping lipogenic gene signature (Dentin et al., 2005). However phosphorylation of the histone acetyltransferase p300 by AMPK and its related kinases also affects the acetylation and activity of ChREBP which add more complexity to AMPK-mediated regulation of ChREBP (Bricambert et al., 2010).

Similarly, the CRTC family of transcriptional co-activators for CREB and its related family members, which have important roles in metabolic tissues, may also play roles in epithelial cells and cancer (Altarejos and Montminy, 2011; Mair et al., 2011). In addition to these highly conserved targets, AMPK has also been reported as the regulator of the nuclear receptors HNF4α (NR2A1) (Hong et al., 2003) and TR4 (NR2C2) (Kim et al., 2011), the coactivator PGC-1α (Jager et al., 2007) and the zinc-

finger protein AREBP (ZNF692) (Inoue et al., 2006), though additional studies are required to further define the functional roles of these events.

Another set of transcriptional regulators targeted by AMPK are the class IIa family of histone deacetylases (HDACs) (van der Linden et al., 2007; Chang et al., 2005; Dequiedt et al., 2006; Berdeaux et al., 2007; McGee et al., 2008; Mihaylova et al., 2011; Wang et al., 2011). Like CRTCs, AMPK-mediated phosphorylation of class IIa HDACs inhibits them through the phosphorylation-dependent formation of 14-3-3 binding and cytoplasmic sequestration (Mihaylova et al., 2011).

In the liver the class IIa HDACs are dephosphorylated in response to the fasting hormone glucagon, resulting in an increase in transcription that is normally opposed by AMPK. In the nucleus class IIa HDACs bind FOXO family transcription factors which subsequently stimulate their de-acetylation and activation (Mihaylova et al., 2011) leading to increased expression of gluconeogenesis genes such as G6Pase and PEPCK. Collectively, these findings suggest that AMPK inhibits glucose production via two transcriptional effects: reduced expression of CREB targets and FOXO target genes via CRTC inactivation and class IIa HDAC inactivation, respectively. However, while AMPK activation suppresses expression of FOXO gluconeogenic targets in the liver, AMPK stimulates a set of FOXO-dependent target genes in stress resistance via direct phosphorylation of novel sites in FOXO3 and FOXO4 in other cell types (Greer et al., 2007).

1.6.8. AMPK modulates cell polarity, migration, and cytoskeletal dynamics

In addition to the ample data providing evidence for AMPK involvement in cell growth and metabolism, studies have suggested a potential role for AMPK in the regulation of cell polarity and cytoskeletal dynamics (Mirouse and Billaud, 2011). LKB1 is well known to play a critical role in cell polarity during critical asymmetric cell divisions in *C. elegans* (Watts et al., 2000), *Drosophila* (Martin and Johnston, 2003) and mammalian cell culture (Baas et al., 2004). Not surprisingly, it was initially assumed that the AMPK-related MARKs (Microtubule Affinity Regulating Kinases), which play well-established roles in polarity, were the principal targets of LKB1 in polarity establishment (Jansen et al., 2009). However, some studies also suggest an important role for AMPK in cell polarity.

In mammalian MDCK cells, AMPK function is required for proper re-polarization and formation of tight junction following calcium switch (Zhang et al., 2006; Zheng and Cantley, 2007). Afadin (Zhang et al., 2011) and a Golgi-specific nucleotide exchange factor for Arf5 (GBF1) (Miyamoto et al., 2008), which are adherens junction proteins, have been reported to be regulated by AMPK and may be involved in this polarity (Zhang et al., 2011). In *Drosophila* AMPK deficiency results in alteration of multiple polarity markers, including loss of myosin light chain (MLC) phosphorylation (Lee et al., 2007). AMPK and its related family members have been identified as modulators of both kinases and phosphatases that regulate MLC (MLCK, MYPT1). Therefore, MLC phosphorylation may be indirectly controlled via one of these potential mechanisms.

Another study has shown that mutation of the AMPK site in the microtubule plus end protein CLIP-170 (CLIP1) causes slower microtubule assembly, suggesting a role for AMPK in the dynamics of CLIP-170 dissociation from the growing end of

microtubules. It is noteworthy that mTORC1 was also previously reported as a modulator of CLIP-170 (Choi et al., 2002), suggesting that like ULK1, CLIP-170 may also be a convergence point in the cell for AMPK and mTOR signaling. Consistent with this, besides its effects on the cell growth, LKB1/AMPK control of mTOR has been suggested as the modulator of cilia (Boehlke et al., 2010) and neuronal polarization under conditions of energy stress (Williams et al., 2011).

Finally, an independent study suggested that AMPK mediates neuronal polarization via control of PI3K localization (Amato et al., 2011). AMPK was indicated as the inhibitor of axonal growth by directly phosphorylating Kinesin Light Chain 2 (KLC2) and preventing PI3K localization to the axonal tip. Interestingly, a previous study has shown that the related protein KLC1 is not a real substrate in vivo (McDonald et al., 2009). Therefore, further experiments are required to clarify whether AMPK is a bona fide kinase for KLC1 or KLC2 in vivo.

1.6.9. AMPK; a regulator of ion channels and gap junctions

Beside its well-established role in cell growth, metabolism and cell polarity recent studies suggest a potential role for AMPK in the modulation of ion channel function as well (Andersen and Rasmussen, 2012). This concept emerged with the discovery by Hallows and coworkers who showed that AMPK inhibits the cystic fibrosis transmembrane conductance regulator (CFTR), the ATP-gated chloride channel mutated in cystic fibrosis (Rommens et al., 1989; Preston et al., 2010). By conducting a yeast two-hybrid screen, they demonstrated the interaction between AMPK α1 subunit and CFTR (Hallows et al., 2000). Subsequent functional studies in *Xenopus* oocytes

showed that AMPK reduces the open probability of the CFTR, thereby resulting in inhibition of channel activity. Two subsequent studies have identified S768 which was initially described as inhibitory PKA site, as the primary site of AMPK phosphorylation (King et al., 2009; Kongsuphol et al., 2009).

Subsequent studies revealed that CFTR is not the only ion channel that is targeted by AMPK. In 2005, AMPK was reported as a negative modulator of the epithelial sodium channel ENaC in *Xenopus* oocytes and polarized mouse collecting duct mpkCCDC cells (Carattino et al., 2005). Interestingly, ENaC is unlikely to be a direct target of AMPK since as it was not phosphorylated by AMPK in vitro and any direct interaction between the kinase and ENaC could not be detected.

The Liddle's syndrome ENaC β-subunit mutant was unresponsive to AMPK activation. This mutant is defined by its inability to interact with the E3 ubiquitin ligase Nedd4-2, which is involved in ubiquitylation of ENaC marking it for endocytosis and degradation. As this mutant is insensitive to Nedd4-2 regulated endocytosis, it can be proposed that AMPK inhibits ENaC through Nedd4-2. The interaction between AMPK and Nedd4-2 was further confirmed in a subsequent study that demonstrated a direct role of AMPK in the modulation of Nedd4-2, thereby increasing the interaction between the ENaC β-subunit and Nedd4-2 (Bhalla et al., 2006). However it is still not entirely clear how AMPK influences the interaction between Nedd4-2 and ENaC. Similarly, the two potassium channels Kir2.1 and Kv7.1 have been reported to be inhibited through the AMPK-Nedd4-2 pathway (Andersen et al., 2012; Alesutan et al., 2011).

Intriguingly, there are increasing number of ion channels emerging as targets of Nedd4-2 including Nav1.5, Kv1.3, Kv1.5 and Kv7.2/3 raising the possibility that these

channels may be also targeted by AMPK activation through Nedd4-2 (Andersen et al., 2011; Rotin and Staub, 2011; Light et al., 2003; Ikematsu et al., 2011). Therefore, AMPK mediated Nedd4-2 activation could be speculated as general mechanism involved in removal of ion channels from the membrane during cellular stress.

Chapter 1.7: AMPK signaling in *C. elegans*

Like other organisms, in *C. elegans* AMPK targets a wide spectrum of substrates involved in diverse cellular processes that all contribute to appropriate adaptation during conditions of stress, particularly energy stress. The *C. elegans* genome encodes two catalytic α subunits, *aak-1* and *aak-2*; two β regulatory subunits, *aakb-1* and *aakb-2*; and five γ regulatory subunits called *aakg-1 aakg-2, aakg-3, aakg-4 and aakg-5* (Apfeld et al., 2004; Beale, 2008). As is the case in most metazoa, AMPK activation is mainly achieved through phosphorylation of Thr^{243} by PAR-4; the LKB-1 ortholog in *C. elegans* (Narbonne et al., 2006). AMPK has been shown to enhance lifespan, autophagy, and to modify the feeding and forging behaviour of starved animals (Mair et al., 2011; Egan et al., 2011; Mihaylova et al., 2011; Lee et al., 2008; Lemieux et al., 2015; Cunningham et al., 2012).

The α subunits of *C. elegans* AMPK are striking in that 30% of residues are identical and 78% are similar to the human AMPK catalytic subunits. Notably, the β subunit alignment also shows 30% identity and 84% similarity to human AMPK β subunit. Interestingly, the *C. elegans* β subunits are most similar to human γ 1 subunits with the maximum similarity in the AMP/ATP binding Bateman domains. Because of this high degree of similarity shared between the AMPK subunits of *C. elegans* and human, and that *C. elegans* mutants that lack all AMPK signaling are viable, many laboratories have turned to *C. elegans* as a model to identify and characterize the molecular targets and the associated cellular roles of AMPK in animal development and physiology (Beale, 2008).

A functional AMPK/GFP fusion protein is expressed in several tissues during hermaphrodite development including the pharynx, the head and ventral cord neurons, hermaphrodite-specific neuron (HSN), body wall muscles, the vulva, the excretory canal, distal tip cells, spermatheca, sheath cells and intestine (Lee et al., 2008). This broad expression pattern suggests that AMPK may have unforeseen roles in the correct execution of several important signaling pathways that range from organogenesis and tissue maintenance to stem cell integrity, metabolism and behaviour.

1.7.1. Gonadal function of AMPK

C. elegans germline is supported by two gonadal arms in which germline stem cells (GSCs) proliferate at the distal ends and differentiate more proximally into mature gametes. The entire reproductive apparatus arises from a gonadal primordium that consists of two somatic gonad precursors and two primordial germ cells (PGCs) present at the first larval (L1) stage (Hirsh et al., 1976; Kimble et al., 1979). The PGCs are born during embryogenesis at the 100 cell stage and remain quiescent thereafter until the animal emerges from the egg.

Upon exposure to food, these four cells that have remained quiescent for much of embyrogenesis resume mitotic cell divisions and initiate development (Hirsh et al., 1976; Kimble et al., 1979). Upon initiation of postembryonic development cell divisions will begin but will arrest again in the L1 stage and then resume proliferation and differentiation later in the L3 stage (Killian et al., 2004).

Upon the initiation of differentiation, the proliferative zone of the germ line is restricted to the area distal to the mitosis/meiosis border. All the germ cells proximal to

this border are differentiated, (in prophase of meiosis I) (Hubbard et al., 2013). Of note, many factors have been revealed to influence the expansion of the germ line proliferative zone making these physiologically-sensitive progenitor cells an interesting model for understanding developmental and physiological control of cell proliferation (Killian et al., 2004; Killian et al., 2005).

The germ line is subjected to environmentally regulated diapause points that allow worms to survive the environmental conditions range from deserts to the Antarctic. AMPK signaling has been revealed as one of the conserved mechanisms that mediates the sensitivity of germline development in response to such challenges.

The formation of long-lived larval stage called the dauer diapause, which animals enter under harsh conditions results in the establishment of a G2 phase cell cycle arrest and ultimately the inhibition of germline proliferation (Narbonne et al., 2006). In a genetic screen conducted to identify factors required for germ line stem cell quiescence, AMPK signaling was identified as one of the genetic mechanisms that limit the capacity of germline proliferation. Notably, AMPK function was found to be crucial for the establishment of GSC quiescence as the removal of any one of the AMPK subunit orthologues namely *aak-1*, *aak-2* or *aakb*-1 leads to supernumerary germ cell divisions during the dauer stage. AMPK therefore ensures that GSC proliferation is restricted during periods when inadequate resources are available to fuel critical cellular processes (Narbonne et al., 2006; Narbonne et al., 2010).

Like most organisms, the optimal activation of AMPK in *C. elegans* requires PAR-4(LKB1)/STRD-1-dependent phosphorylation at Thr^{243}. In the dauer germ line AMPK acts downstream of PAR-4 to coordinate proliferation with somatic development

in response to all three dauer signaling pathways, although compound mutant analysis suggests that PAR-4/LKB1 may have additional AMPK-independent effects on germ line quiescence (Narbonne et al., 2006). Studies performed in mammalian systems suggest that TSC1/2/mTOR pathway may contribute to quiescence in various tissues (Avruch et al., 2006). However, based on bioinformatic analyses there is no clear TSC1/2 ortholog annotated in the *C. elegans* genome, suggesting that alternative mechanisms must exist that control quiescence downstream of AMPK in the GSCs during the dauer stage in *C. elegans* that are independent of TSC1/2 (Gwinn et al., 2008).

Like the GSCs that escape quiescence in the dauer stage, compromise of either of the two catalytic subunits results in an abnormal PGC proliferation defect and reduced viability during L1 diapause. AMPK signaling is therefore also required to maintain quiescence and presumably to reallocate energy resources in response to food deprivation upon hatching (Fukuyama et al., 2012). Similarly, many of these phenotypes are mirrored by the *C. elegans* PTEN ortholog *daf-18* (Baugh et al., 2006; Sigmond et al., 2008). Although intestinal and pan-neuronal promoters restored the survival of AMPK mutant animals, neither of these tissue-specific transgenes could suppress the abnormal proliferation of PGCs during the L1 diapause consistent with AMPK acting in distinct tissues for survival and to establish and maintain PGC quiescence during the L1 diapause (Fukuyama et al., 2012).

The mechanisms by which AMPK regulates germline quiescence remain unknown (Fukuyama et al., 2012). Previous findings from mammalian studies suggest that AMPK may function to promote the activity of p53 homolog *cep-1* or the cyclin-

dependent kinase inhibitor orthologs *cki-1* and *cki-2* to ultimately modulate cell cycle arrest (Tobin et al., 2012). However since both *cep-1/*p53/ and *cki-1/-2* modulate their targets predominantly at the G1-S transition it is difficult to account their role in maintaining the typical G2 arrest that is observed in the PGCs and the GSCs during periods of quiescence based on their canonical mode of action. To elucidate the mechanism(s) by which AMPK regulates cell cycle quiescence, the identification of additional AMPK substrates that may ultimately link energy stress and cell cycle arrest will be essential.

1.7.2. AMPK regulates fat rationing during the dauer stage

In situations of cyclical climate change, many organisms will accumulate fuel resources during the periods of abundance that precede a long cold or dry season. By altering its metabolism accordingly prior to long periods of resource scarcity the *C. elegans* dauer can augment its triglyceride stockpile to act as an energy source during periods when the animal cannot feed. The utilization of this resource must therefore be tightly managed as premature exhaustion of the fuel stockpile could jeopardize survival.

Unlike most organisms, animals that lack all AMPK signaling are viable in *C. elegans*. However, AMPK must affect critical pathways to ensure L1 survival through phosphorylation of targets that have yet to be determined. On the other hand, AMPK mutant dauer larvae die prematurely despite the transient accumulation of fat upon dauer entry. AMPK mutant dauers hydrolyze and deplete their accumulated fat reservoirs in less than 48 hr after dauer formation (Narbonne et al., 2009). LKB-1/AMPK signaling mediates the survival of dauer animals by functioning within both the skin-like

hypodermis, which is the major site of *C. elegans* fat storage, and in the kidney-like excretory cell. At the time of dauer entry, *aak-2* mutants display abundant fat accumulation and normal osmoregulation. However, animals quickly deplete their fat reservoirs due to the increased activity of the lipase, ATGL-1, the ortholog of mammalian adipose triglyceride lipase. Moreover, they also become osmosensitive resulting in a rapid decline in viability. Taken together, AMPK ensures long term dauer survival by limiting fat utilization by downregulation of ATGL-1 activity all the while ensuring appropriate osmoregulation (Narbonne et al., 2009).

AMPK directly phosphorylates serine 303 on adipose triglyceride lipase (ATGL-1), to release a single free fatty acid (Narbonne et al., 2009; Cunningham et al., 2009). It therefore regulates the rate-limiting step in triglyceride hydrolysis and hence energy production from this source. This ATGL-1 inactivation following AMPK phosphorylation is triggered by the generation of 14-3-3 binding sites on ATGL-1, which are recognized by the *C. elegans* 14-3-3 protein orthologue PAR-5, resulting in sequestration of ATGL-1 away from the lipid droplets and subsequent proteasome-mediated degradation (Xie et al., 2015).

This AMPK-dependent block in lipid hydrolysis is somewhat counterintuitive considering its role in mammalian cells that have been subjected to energy stress. Activation of AMPK is reflected by a direct phospho-inhibition of ACC1/2 which both affect lipid biosynthesis indirectly by relieving the malonyl CoA-mediated inhibition of CPT. The result of this is an increase in fatty acid transport to the mitochondria, subsequent β-oxidation and increased energy production from lipid (Mihaylova et al., 2011). Although the differences are striking, they may be justified based on the

timescales associated with of each of the two physiological responses; one being activated on a very short timescale (minutes/hours) following starvation, and the other dominating during the comparatively longer timescales typical of dauer survival (several months-up to 10 times the normal lifespan of the animal).

The survival of AMPK mutant dauers is highly dependent on generating novel energy sources. One such mechanism includes regulation of the abundance and the nature of the fatty-acid content by increasing the HIF-1-dependent expression of the enzymes that are critical for fatty-acid biosynthesis. This phenomenon can occur downstream of reduced catalase activity which allows cellular levels of hydrogen peroxide to progressively increase during dauer. This metabolic by product is often vilified because of its ROS-generating capacity although at low levels it ensures readjustment of lipid biosynthetic capacity downstream of HIF-1 activation to compensate for cellular energy deficiencies in the AMPK mutants (Xie et al., 2012). Similar metabolic re-adjustments can also occur in tumour cells and are often associated with the Warburg effect, where energy consuming pathways are modified towards a growth condition of these rogue cells, while consequently compromising their energetic efficiency (Deberardinis et al., 2008).

If tumor cells can generate their own energy through activation of HIF-1 they can become less dependent on nutrient delivery. This property could potentially enhance their capacity for unscheduled growth. As AMPK has been show to play an important role in blocking cell growth in response to nutrient/energy stress or during oxidative stress and in certain cell types loss of AMPK does lead to increased HIF levels (Shackelford et al., 2009), it is paramount to investigate whether activation of HIF-1 is

associated with the loss of AMPK or its upstream kinase LKB-1 in various tumours, or in patients with tumour predisposing diseases such as Peutz-Jeghers Syndrome.

1.7.3. AMPK; a modulator of anti-aging signaling network

The lifespan of most animals is organism-specific and it appears to be selected for some optimal time duration that does not necessarily correspond to the maximal capacity of the organism in question. Through studies in model organisms it has since become clear that our lifespan is by no means definite in length, and it can be extended under specific genetic and metabolic conditions. This can be achieved by impinging on a number of genetic pathways. First, by limiting caloric intake or by altering the activity of single genes within the defined pathways that modulate metabolism, mutant organisms can live significantly longer than wild type animals (Schulz et al., 2007; Hansen et al., 2008; Mair et al., 2011). Various regimens of calorie restriction in which macronutrient sources of protein, carbohydrate and lipid are scarce can consequently extend life span through mechanisms that are as of yet unclear. As a consequence of calorie restriction, physical aging is slowed and there is an increased resistance to disease (Longo et al., 2003; Bordone et al., 2005). Alternatively, modification of the insulin signaling pathway also results in significant lengthening of lifespan, while mitochondrial homeostasis also influences lifespan, although whether this arises through increased or decreased generation of ROS is still somewhat controversial (Yee et al., 2015)

C. elegans has proven to be an excellent model to test many of these concepts, while its genetic tractability has provided particularly valuable information concerning

the molecular genetic mechanisms that underlie our genetically encoded lifespan. Given the importance of energy homeostasis in the genetic regulation of aging, it is not surprising that AMPK has surfaced as a key regulator of lifespan determination by converging on an integrated signaling network that intersects with multiple genetic pathways including insulin-like signaling, enhanced SIR-2.1 deacetylase activity and/or mitochondrial homeostasis.

1.7.4. Insulin/IGF-1/FOXO signaling

Mutations in the *C. elegans* dauer formation abnormal gene *daf-2*/InR provided the first genetically-characterized long-lived mutants where loss of function mutations conferred a greater than 2-fold extension to the wild-type lifespan (Kenyon et al., 1993). Molecular analyses revealed that many of the genes that extended lifespan were indeed implicated in insulin signaling: DAF-2 was identified as the *C. elegans* ortholog of the mammalian insulin/IGF-1 receptor (IGFRs), AGE-1 *C. elegans* ortholog of PI 3-kinase (PI3K) and DAF-16/ *C. elegans* ortholog of forkhead box protein O (FOXO) and together they form the components of a conserved insulin-like signaling pathway (Morris et al., 1996; Kimura et al., 1997; Lin et al., 1997; Ogg et al., 1997; Paradis et al., 1998).

Insulin/IGF-1 signaling is one of the key regulators of metabolism and energy homeostasis stimulating glucose uptake and growth signaling pathways thereby coordinating food intake and cellular energy homeostasis by promoting anabolic processes. The DAF-16/FOXO transcription factor modulates growth, metabolism and aging in *C. elegans*. The serine/threonine protein kinases AKT-1/2, and SGK-1 respond to activation of the insulin receptor DAF-2 and phosphorylate and inactivate the DAF-

16/FOXO by preventing its translocation to the nucleus and thereby blocking transcription of its target genes. As expected, abolished insulin/IGF-1 signaling (e.g., using mutations in *daf-2*/InR) relieve this block resulting in DAF-16/FOXO translocation into the nucleus where it induces the expression of genes that are involved in resistance to various stresses that ultimately affect lifespan determination (Paradis et al., 1998; Hertweck et al., 2004).

Exposure of *daf-2*/InR mutants and wild-type animals to starvation or heat stress and mitochondrial poisoning increases the intracellular AMP to ATP ratios resembling a condition that activates AMPK (Apfeld et al., 2004). Consistent with this, *aak-2* functions both in a linear pathway and in parallel with *daf-16*/FOXO to extend lifespan of *daf-2*/InR mutants, at least partially through inhibition of the CREB-regulated transcriptional coactivator CRTC-1 (Apfeld et al., 2004; Mair et al., 2011). Interestingly, it was revealed that *crtc-1* specifically mediates the longevity output of AMPK through the directly phosphorylation of CRTC-1 in the nervous system resulting in 14-3-3 binding and cytosolic sequestration and inactivation of CRTC-1 leading to longevity and systemic changes in metabolic transcription (Burkewitz et al., 2015).

1.7.5. TOR signaling

The TOR (target of rapamycin) pathway plays a key role in the regulation of growth and reproduction in response to nutrient and energy availability. As such, blocking its activity has been shown to extend lifespan in a variety of species (Kapahi et al., 2010). Inhibition of TOR signaling and reduced insulin/IGF-1 signaling both converge on common transcriptional targets and non-transcriptional effectors and these likely

function together since blocking TOR signaling does not further extend the increased lifespan of *daf-2*/InR mutants (Vellai et al., 2003; Hansen et al., 2007).

In mammals, TOR exists in the form of TORC1 and TORC2 complexes, which contain DAF-15/Raptor and RICT-1/Rictor coactivators, respectively. These two complexes act in independent genetic pathways to affect distinct cellular processes; TORC1 controls cell growth and proliferation in response to nutrient signals largely by regulating protein synthesis, whereas TORC2 modulates cell morphology. In *C. elegans* TORC1 modulates longevity at least partially via the GTPases RAGA-1/RAGC-1 (Robida-Stubbs et al., 2012; Schreiber et al., 2010), RHEB-1/Rheb (Honjoh et al., 2009), and DAF-15/Raptor (Jia et al., 2004). In addition TOR may also exert its role in the modulation of longevity by non-transcriptional mechanisms such as TORC1-mediated phosphorylation of S6 kinase (S6K), which is one of the principal growth-promoting targets of TOR through its role in promoting translation. Interestingly, the combination of mutations in the insulin signaling *daf-2*/InR and the *rsks-1* (S6K) pathways in *C. elegans* produces a synergistic lifespan extension that requires the positive feedback regulation of DAF-16/FOXO which is AMPK dependent (Greer et al., 2007; Chen et al., 2013). This positive feedback is most likely achieved by the direct phosphorylation of DAF-16/FOXO via AMPK (Greer et al., 2007), which eventually leads to regulation of *aakg-4* (a gene encoding an atypical γ subunit of AMPK) by DAF-16/FOXO promoting AMPK activation (Tullet et al., 2014).

1.7.6. Autophagy

Autophagy has recently been identified as a significant player in the genetic determination of lifespan and it is also affected by activated AMPK in *C. elegans*. Autophagy is a self-degradative process that has emerged as a modulator of longevity in both the TOR and the insulin-IGF-1 signaling pathways as well as in the dietary-restriction paradigm (Lapierre et al., 2011). *daf-2*/InR mutants display enhanced autophagy that is AMPK-dependent and which contributes significantly to its effects on lifespan extension (Egan et al., 2011). Although somewhat speculative, autophagy may extend lifespan through the inhibition of protein aggregation thereby delaying the collapse of proteostasis observed during aging (Florez-McClure et al., 2007; Ben-Zvi et al., 2009). In mammals, AMPK directly phosphorylates the autophagy initiating kinase, UNC-51 (ATG-1/ULK-1) which consequently induces autophagy. Consistent with this, both AMPK and ULK1 are necessary for inducing autophagy upon reduced insulin signaling in *C. elegans* (Egan et al., 2011).

Chapter 1.8: Thesis rational

Adaptive food-seeking behaviours influence fitness through effects on both reproductive efficiency and survival. During starvation, when energy reserves are low, foraging strategies are modified to enhance the possibility of identifying new resources and such behaviours become then prioritized over other energy-consuming behaviours such as mating. These starvation-inducible behavioural modifications are a consequence of changes in the activity of neural circuits that govern these behavioural outcomes. Therefore, the signaling networks that are evoked throughout the nervous system to mediate the effect of small molecules, hormones and nutrients on energy homeostasis are of major interest due to their implication in, or treatment of various disorders.

Energy stress has been demonstrated to induce adaptive behaviours in a neuronal AMPK-dependent manner (Lee et al., 2008; Cunningham et al., 2014; Yang et al., 2011). Anorexic and orexigenic signals regulate feeding behaviour in mammals partly through regulation of AMPK in the nervous system (Minokoshi et al., 2004; Seo et al., 2008). More specifically, the anorexigenic hormone leptin, insulin in multiple hypothalamic regions, high glucose and refeeding (Minokoshi et al., 2004), resistin (Vazquez et al., 2008), alpha-melanocyte-stimulating hormone (Minokoshi et al., 2004; Tanaka et al., 2007), ciliary neurotrophic factor (Steinberg et al., 2006), and glucagon-like-peptide-1 (Seo et al., 2008) all have been shown to inhibit AMPK activity in the arcuate nucleus (ARC) and paraventricular hypothalamus (PVH), whereas the orexigenic stomach-derived hormone ghrelin, hypoglycemia (McCrimmon et al., 2004; Han et al., 2005), glucocorticoids (Shimizu et al., 2008), thyroid hormones (Ishii et al., 2008), cannabinoids (Kola et al., 2005), adiponectin (Kubota et al., 2007) increase its

activity in the hypothalamus and other regions of CNS. In addition, AMPK function is also important for glucose sensing in the AgRP and the POMC neurons, which modulate feeding and food-seeking behaviours (Claret et al., 2007; Dietrich et al., 2015).

In *C. elegans* AMPK modulates feeding behaviour by acting in a pair of neurons that express the orthologue of the *D. melanogaster* and mammalian single-minded 1 (SIM1) genes, which encode a transcription factor required for development of the paraventricular nucleus (PVN) (Michaud et al., 2001).

In addition to the documented role of hypothalamic AMPK in responding to peripheral signals of energy status in mammals, it has also been shown that neuronal AMPK activity plays an important role in the modulation of locomotory behaviour in *C. elegans* in a resource depleted environment (Lee et al., 2008), although the mechanism is poorly understood. Given the common regulatory framework that is apparent in the neural circuitry between *C. elegans* and mammalian models, significant advances can be made using *C. elegans* to investigate the mechanism/s through which AMPK regulates food-seeking behaviour in response to hunger. Therefore, using calcium imaging analysis, cell type–specific optogenetic techniques, and classic genetic analysis, we investigated the mechanism/s through which AMPK functions to trigger changes in the activity of neural circuits to affect their outputs leading to appropriate responses to hunger.

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Figures

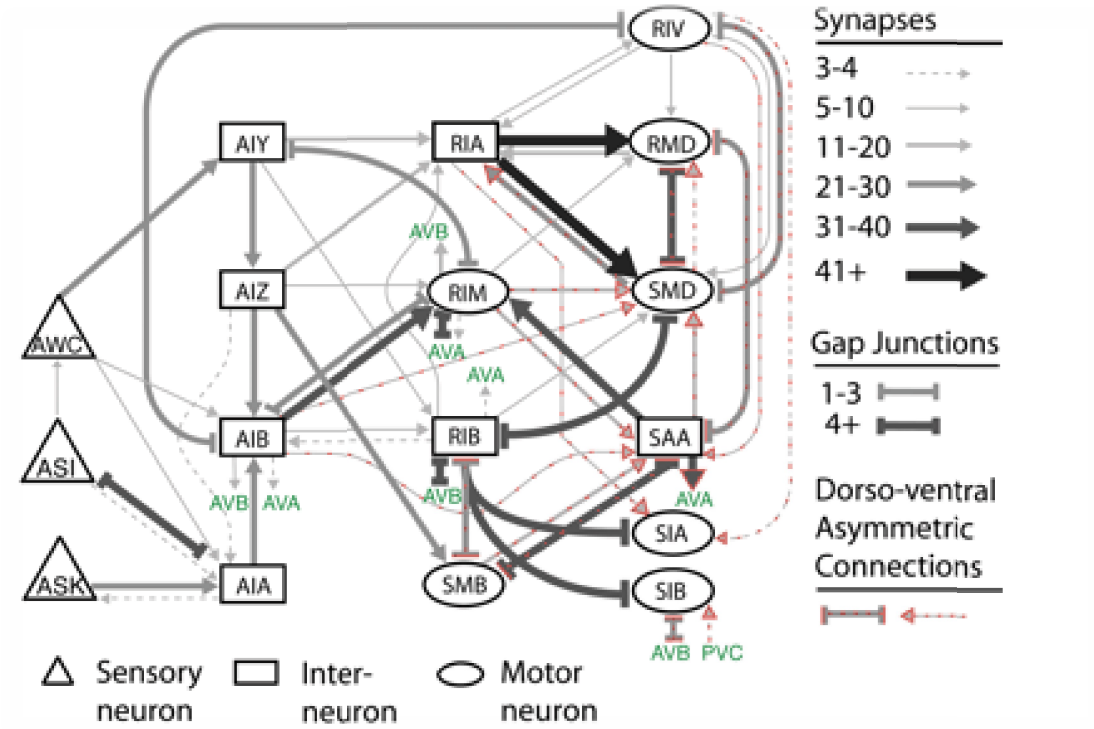

Figure 1.1: A predicted navigation circuit in *C. elegans*. Data is obtained from serial section reconstructions of electron micrographs. Each neuron in the following circuit represents a bilaterally symmetric left–right pair: AWC, ASI, ASK, AIY, AIZ, AIB, AIA, RIA, RIM, RIB, and RIV. The SMD, SIA, SMB head neurons and SIB neck motor neurons each have four members that innervate muscle quadrants. The connections that were asymmetric in the dorsoventral direction are indicated by the red dotted lines. The command interneurons are demonstrated in green. This fiqure is opted with permission from Gray, J. M., Hill, J. J. and Bargmann, C. I. 2005. A circuit for navigation in Caenorhabditis elegans. *Proc Natl Acad Sci*. 102: 3184-3191.

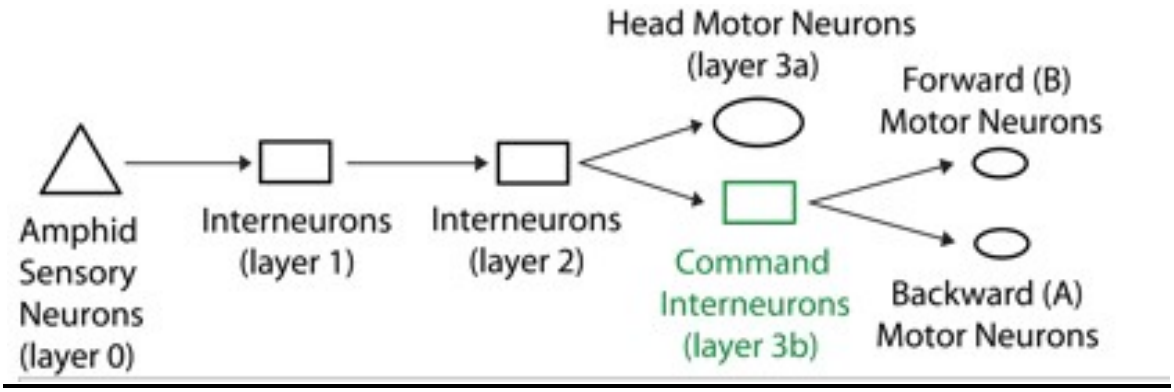

Figure 1.2: A schematic representing the transmission of information from sensory neurons to motor neurons. This fiqure is opted with permission from Gray, J. M., Hill, J. J. and Bargmann, C. I. 2005. A circuit for navigation in Caenorhabditis elegans. *Proc Natl Acad Sci*. 102: 3184-3191.

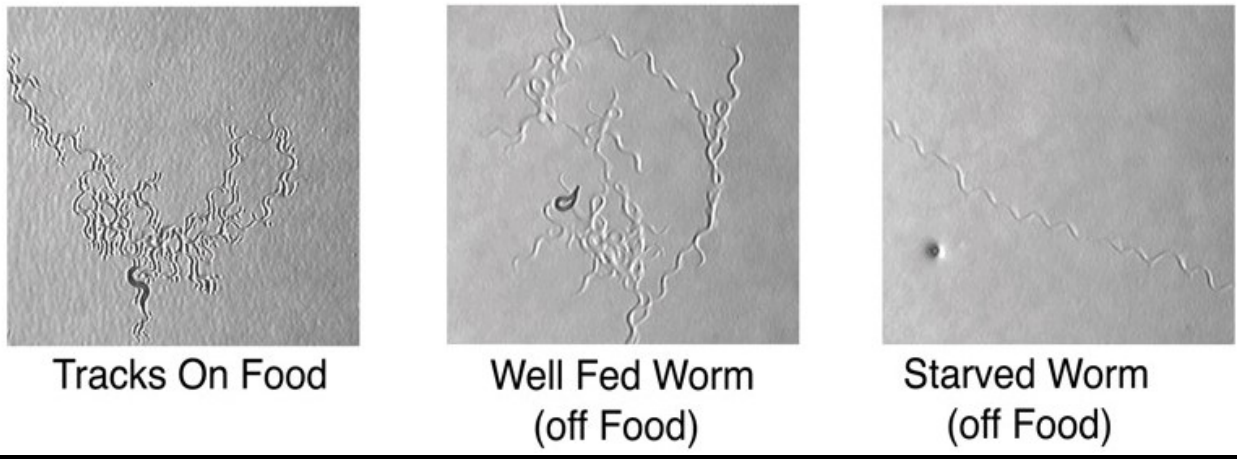

Figure 1.3: Locomotory behaviour of *C. elegans* in response to food availability. *C. elegans* display frequent reversals in the presence of food while moving slowly in forward direction. Upon removal from food the frequency of reversals increases whereas animals still move in forward direction slowly resulting in efficient local exploration for food sources. Once starved, reversals become suppressed and forward locomotion (runs) is induced allowing animals to efficiently explore more distant locations. This fiqure is opted with permission from Gray, J. M., Hill, J. J. and Bargmann, C. I. 2005. A circuit for navigation in *Caenorhabditis elegans*. *Proc Natl Acad Sci*. 102: 3184-3191

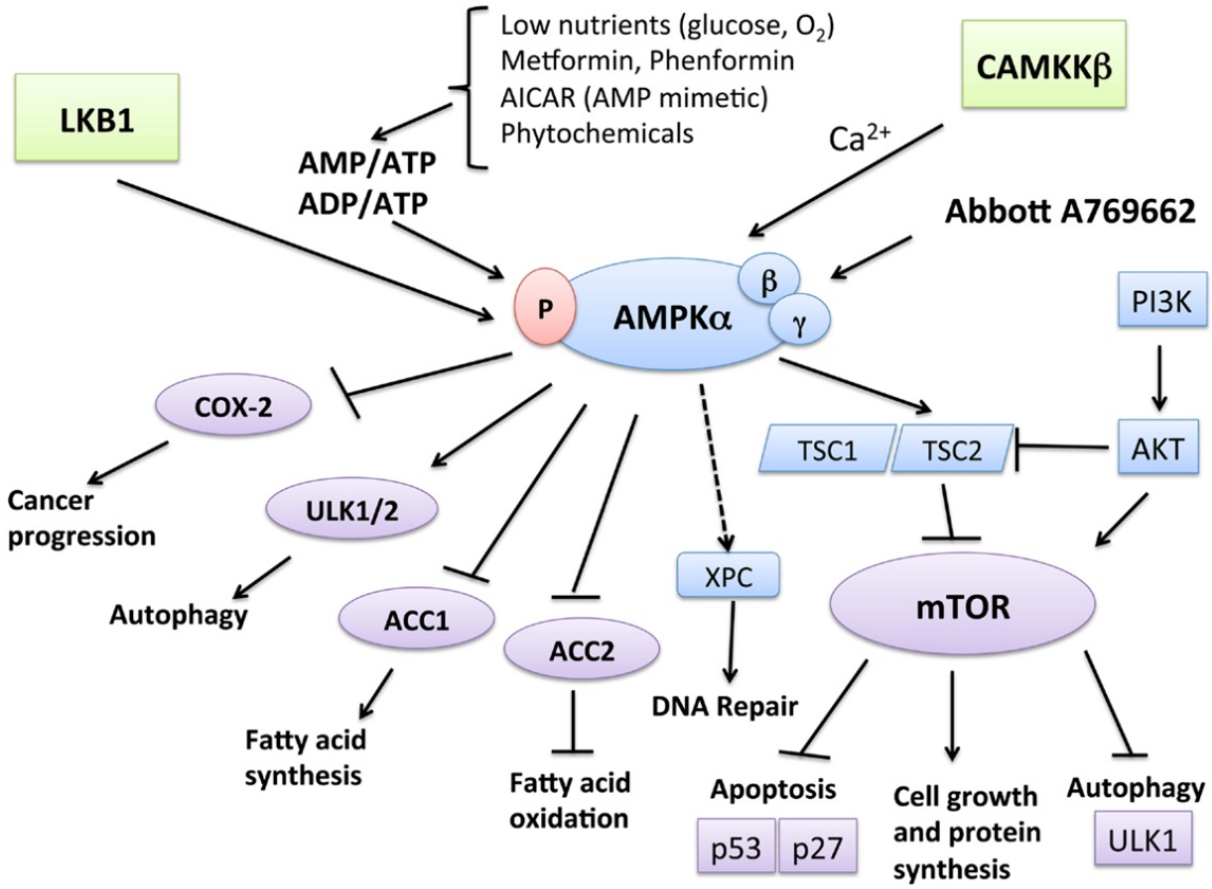
Low nutrients (glucose, O_2)
Metformin, Phenformin
AICAR (AMP mimetic)
Phytochemicals
LKB1
CAMKKβ
AMP/ATP
ADP/ATP
Ca^{2+}
Abbott A769662
P
AMPKα
β
γ
PI3K
COX-2
TSC1
TSC2
AKT
Cancer progression
ULK1/2
Autophagy
ACC1
ACC2
XPC
mTOR
Fatty acid synthesis
Fatty acid oxidation
DNA Repair
Apoptosis
p53
p27
Cell growth and protein synthesis
Autophagy
ULK1

Figure 1.4: AMPK signaling. AMPK activation is induced once AMP/ATP or ADP/ATP ratios increases in the cells due to various physiological stresses, such as hypoxemia and hypoglycemia, resulting in the activation of LKB1. Metformin and phenformin can also activate AMPK in a LKB1-dependent manner. AMPK also becomes activated by CaMKKβ in response to increased calcium levels. AMPK activation then induces catabolic pathways, such as fatty acid oxidation. For instance, AMPK phosphorylation leads to the inactivation of acetyl CoA carboxylase (ACC2). On the other hand, AMPK activation leads to inhibition of anabolic pathways, such as fatty acid synthesis, mediated by ACC1. One of the most well-known pathways in which AMPK is involved is the TSC1/TSC2 complex, which can also can be activated downstream of the PI3K-AKT and Ras-Raf-MEK-ERK signaling pathways. The mTOR pathway suppresses apoptosis by effecting the tumor suppressors p53 and p27 and inhibits autophagy by suppressing UNC-51-like kinase 1 (ULK1) and ULK2. AMPK activation results in downregulation of these effects of mTOR, thus leading to increased apoptosis and autophagy-mediated cell death. Moreover, independent of mTOR, AMPK also phosphorylates ULK1 and ULK2, whose activation triggers autophagy. AMPK has been also demonstrated to downregulate cyclooxygenase (COX)-2 expression, which contributes to the pathophysiological progression of certain human cancers and inflammatory disorders. This figure is opted with permission from Kim, I., He, Y. Y. 2013. Targeting the AMP-Activated Protein Kinase for Cancer Prevention and Therapy. *Front Oncol*. 3: 175.

Chapter 2: AMPK acts as a molecular trigger to coordinate glutamatergic signals and adaptive behaviours during acute starvation

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This article is published in elife.

Abstract

The stress associated with starvation is accompanied by compensatory behaviours that enhance foraging efficiency and increase the probability of encountering food. However, the molecular details of how hunger triggers changes in the activity of neural circuits to elicit these adaptive behavioural outcomes remains to be resolved. We show here that AMP-activated protein kinase (AMPK) regulates neuronal activity to elicit appropriate behavioural outcomes in response to acute starvation, and this effect is mediated by the coordinated modulation of glutamatergic inputs. AMPK targets both the AMPA-type glutamate receptor GLR-1 and the Metabotropic glutamate receptor MGL-1 in one of the primary circuits that governs behavioural response to food availability in *C. elegans*. Overall, our study suggests that AMPK acts as a molecular trigger in the specific starvation-sensitive neurons to modulate glutamatergic inputs and to elicit adaptive behavioural outputs in response to acute starvation.

Keywords- AMPK, foraging behaviour, glutamatergic signaling, GLR-1, MGL-1, *C. elegans*

Introduction

Most organisms are faced with unpredictable fluctuations in their natural environment that often lead to periods of limited food resources. Their ability to adapt to these changes in resource availability is critical for survival and is often a driving force in evolution (Gray et al., 2004; Wang et al., 2005). When resources are scarce, pathways associated with energy conservation at both the organismal and the cellular levels become activated, and these are often complemented by behavioural modifications that simultaneously enhance foraging efficiency (Wang et al., 2005; Ashrafi, 2006).

The mammalian central nervous system (CNS) integrates internal and external cues that signal energy demand and availability and coordinately regulates outputs ranging from energy expenditure to feeding and associated locomotory behaviours (Cone, 2005; Balthasar et al., 2005; Belgardt et al., 2009; Dietrich and Horvath, 2011; Yang et al., 2011; Aponte et al., 2011; Sternson et al., 2013; Dietrich et al., 2015). In mice, the Agouti-related protein (AGRP)- and Pro-opiomelanocortin (POMC)-expressing neurons in the arcuate nucleus of the hypothalamus form a core circuit to regulate food intake and energy expenditure through the modulation of their neuronal activity in response to hormonal signals linked to metabolic status (Cowley et al., 2001; Bewick et al., 2005; Yang et al., 2011). The activity of both of these neuronal populations is mediated by engaging signaling pathways that control the strength and/or plasticity of rapid, excitatory glutamatergic transmission (Bito et al., 2010; Collingridge et al., 2010; Liu et al., 2012), but how energy stress results in changes in neuronal activity to elicit adaptive, or even compulsive behaviours is just now beginning to be elucidated (Dietrich et al., 2015).

The many signaling networks that are triggered throughout the nervous system that mediate the action of small molecules, hormones and nutrients on energy balance are of major interest due to their implication in, or treatment of various disorders. One of the key factors of paramount importance for metabolic homeostasis and survival is a highly conserved heterotrimeric protein kinase called AMP-activated protein kinase (AMPK). AMPK is regulated by the ratio of cellular AMP/ATP and by upstream activating kinases (Hardie, 2008; Hardie et al., 2012). It functions as a 'fuel gauge' to monitor cellular energy status by inhibiting anabolic pathways and activating catabolic pathways so as to generate sufficient levels of metabolic substrates required to maintain a minimal threshold of basal cellular activities (Hardie, 2008; Hardie et al., 2012; Hardie and Ashford, 2014)

Energy stress has been demonstrated to induce adaptive behaviours in a neuronal AMPK-dependent manner (Lee et al., 2008; Cunningham et al., 2014). Moreover, accumulating evidence has implicated AMPK in the hypothalamic regulation of metabolic rate and food intake behaviour (Kola, 2008; Lopez et al., 2010; Lim et al., 2010; Yang et al., 2011; Schneeberger and Claret, 2012). However, our understanding of how starvation influences adaptive foraging behaviours in an AMPK-dependent manner is still largely unknown, mostly due to the overwhelming complexity of the response in higher animals (Dietrich et al., 2015).

In *C. elegans* these foraging behaviours are comparatively simple, consisting of a series of forward or backward movements, specific turns, or changes in direction (Gray et al., 2005; Piggott et al., 2011; Chen et al., 2013; Hendricks, 2015). Food availability has been demonstrated to affect various aspects of these key elements in *C*. *elegans*

locomotion (Sawin et al., 2000; Gray et al., 2005; Chalasani et al., 2007; Flavell et al., 2013). In the absence of food, well-fed animals reverse frequently, a behavioural pattern that reflects a sensory memory of food that is expressed by the navigation circuit and results in efficient exploration of a limited area. In contrast, starvation suppresses reversals and induces forward movement (runs) that allow animals to explore distal areas; a strategy that is referred to as dispersal behaviour or alternatively, distal exploration (Gray et al., 2005).

The simplicity of the neural circuits and locomotory behaviours in conjunction with its amenability to genetic manipulation makes *C. elegans* an ideal model to investigate the mechanisms through which AMPK regulates neuronal activity and adaptive locomotory behaviour (searching for food) in response to hunger. Unlike most other organisms studied to date, *C. elegans* mutants that lack all AMPK signaling are viable, but show clear phenotypes when subjected to energy stress (Narbonne and Roy, 2006; Narbonne and Roy 2009). Therefore, using calcium imaging, cell type–specific optogenetic techniques, and classic genetic analysis, we identified and characterized the neural circuit in which AMPK functions as a molecular switch. This circuit includes the AIB and AIY interneurons; two neurons that form one of the primary circuits that dictate appropriate food- and odour-evoked behaviours (Gray et al., 2005; Chalasani et al., 2007). We discovered that AMPK modulates AIB and AIY activity through two distinct mechanisms to ultimately ensure that adaptive foraging behaviours are appropriately triggered during periods of starvation. In the AIB interneurons AMPK regulates the abundance of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor GLR-1 in the postsynaptic elements, presumably by phosphorylation

of serine 907 and 924 resulting in changes in synaptic strength and specific behavioural outputs. In addition, we also demonstrate that AMPK modulates a key Metabotropic glutamate receptor called MGL-1 in the AIY interneuron at both mRNA and protein levels leading to an increase in AIY neuronal activity in starved animals. Together, our results indicate that AMPK acts as a starvation-inducible molecular trigger in the nervous system that modulates glutamatergic neuronal activity by at least two distinct mechanisms to modify behavioural outcomes in response to energy stress.

Results

Neuronal AMPK signaling triggers distal exploratory behaviour in starved animals

AMPK has been implicated in the regulation of feeding behaviours in higher animals (Minokoshi et al., 2004; Yang et al., 2011) although its essential role in development and cellular homeostasis has made it very difficult to study its role outside this closed circuit. Accordingly, the identification of the neuronal targets of this protein kinase that may be important for these behaviours has been virtually impossible to interrogate. Fortunately, *C. elegans* mutants that completely lack AMPK signaling are viable, thus providing us with an opportunity to investigate how AMPK regulates characteristic foraging behaviours in response to acute periods of starvation.

We and others found that removal of *aak-2*, the more prominent of two catalytic subunits of AMPK present in *C. elegans*, disrupts most cellular AMPK signaling and affects various aspects of *C. elegans* foraging behaviour (Lee et al., 2008; Cunningham et al., 2014). More specifically, *aak-2* mutants exhibit a behavioural profile in response to acute starvation that is more typical of satiated animals, where animals tend to explore locally rather than foraging in more distant locations. This is reflected by the frequency of body bends; which is considered as a representative readout for locomotory movement away from a nutrient-depleted environment in search of new resources, and reversals; a behaviour most often associated with local exploration (Gray et al., 2005). Both of these behaviours are affected in starved *aak-2* mutants (Figure 2.1A). These behaviours appear to be regulated predominantly by *aak-2* activity since removal of both catalytic subunits and hence eliminating all AMPK signaling

(*aak(0)*) only modestly enhanced the phenotype of *aak-2* mutants in all our behavioural assays suggesting that *aak-2* mutants recapitulate a severe loss of AMPK function.

Since *aak-2* is a major regulator of foraging behaviour in *C. elegans*, we transferred mid-fourth larval stage (L4) *aak-2* mutant larvae to bacteria-free plates and monitored their movements for 20 hours to delineate sub-behaviours underlying *aak-2* deficiency. We found that starved *aak-2* mutants show defects in their rate of forward locomotion measured in the absence of food, which is compounded by their inability to appropriately suppress reversal behaviour during periods of more prolonged starvation (Figure 2.1B). Because we observe changes in each of these AMPK-dependent parameters in starved animals, and given the antagonistic relationship between forward and backward locomotion where increased reversal frequency affects the onset and/or duration of forward movement (Burbea et al., 2002; Juo et al., 2007), we chose to assess both behaviours in starved animals throughout our study.

The *aak-2*-dependent foraging defects we observed in starved animals are not likely to be due to a more general role of AMPK in the regulation of appropriate motor neuron development or function since AMPK mutants were comparable to wild type controls when reversal frequency and forward locomotion were quantified during conditions when food was abundant (Figure S2.1). Moreover, *aak-2* mutants displayed a normal fleeing speed in response to a mechanical stimulus that was applied to the posterior (Figure S2.1D). In addition, we observed that *aak-2* (RNAi) performed at the late L2 stage, when the majority of neurons have already been generated, resulted in a reduction in forward locomotion compounded with an increase in reversal frequency in starved animals. This suggests that the reduced locomotory speed is unlikely to be the

consequence of a general defect in motor function or neuromuscular development (Figure S2.2).

To discern whether *aak-2* is specifically required in neurons, muscle, or both tissues to modulate distal exploratory behaviours we used tissue-specific promoters to drive *aak-2* expression and test its ability to correct the behavioural defects typical of AMPK mutants. While an *aak-2* cDNA (Narbonne and Roy, 2009) expressed under the control of a pan neuronal promoter [P*unc-119*::*aak-2*] rescued the distal exploratory defect of *aak-2* mutants, *aak-2* mutants continued to exhibit prolonged local exploration when the same *aak-2* cDNA was reconstituted in body wall muscle using [P*unc-54*::*aak-2*] transgene (Figure 2.1C). Consistent with these results, we observed that a rescuing translational fusion reporter [P*aak-2*::*aak-2*::GFP] was broadly expressed throughout the nervous system (Figure 2.1D, 2.1E, 2.1F). These data are consistent with previous observations indicating that AMPK is required in the nervous system to regulate locomotory behaviour in response to food availability (Lee et al., 2008; Cunningham et al., 2014).

AMPK is not required for all starvation-related behaviours

The ability of AMPK mutants to appropriately respond to starvation could result from a global AMPK-dependent defect in their nervous system rendering them incapable of responding to multiple cues in addition to starvation. However this seems unlikely since two other food-related behaviours namely, basal slowing response and enhanced slowing response, which are triggered in well-fed and starved animals that are reintroduced to food, respectively, remain unaffected in AMPK mutants. In its natural habitat, a well-fed animal would be more likely to risk exploring distant locations for high

quality food sources, whereas a starved animal would be less likely to stray far from a recently discovered vital food supply. These paradigms regulate behavioural plasticity in response to starvation in *C. elegans* and are mediated by distinct dopaminegic and serotonergic signaling (Sawin et al., 2000). When we re-introduced well-fed or starved *aak-2* mutants to food we did not detect any significant difference in either the basal slowing response or the enhanced slowing response (Figure S2.1B, S2.1C) suggesting that AMPK is not involved in the behavioural plasticity that occurs after re-introduction of animals to food and that the neural circuitry that mediates such behavioural plasticity is presumably intact and functional in animals that lack AMPK.

AAK-2 is required in both the AIY and the AIB interneurons to mediate the transition from local to distal exploration in response to starvation

Since pan neuronal *aak-2* expression rescued the distal exploration defect of *aak-2* mutants, we next sought to identify the individual neurons that require *aak-2* activity to trigger this behaviour. The neural circuitry that dictates these simple behavioural outcomes has been well described (White et al., 1976; Chalfie et al., 1985; White et al., 1986; Wicks et al., 1996; Gray et al., 2005). In particular, five pairs of command interneurons are required for the control of coordinated movement. The PVC and AVB interneurons are primarily required for the initiation of forward movement, while the AVA, AVD, and AVE interneurons control reversals (White et al., 1976; Chalfie et al., 1985; Wicks et al., 1996; Gray et al., 2005). Sensory inputs and serotonergic signaling together contribute to the behavioural changes that occur following removal from food (Gray at al., 2005). Moreover, several studies have underscored a critical role for both

the AIY and the AIB interneurons, which are independently required to suppress and enhance reversals, respectively, resulting in longer or shorter durations of forward movement (Tsalik and Hobert, 2003; Wakabayashi et al., 2004; Gray et al., 2005; Chalasani et al., 2007; Luo et al., 2014). The RIM interneurons have also been determined as regulators of reversal frequency (Gray et al., 2005; Gordus et al., 2015).

To map the precise sites of AMPK function in its regulation of distal exploratory behaviour, we introduced *aak-2* in different subsets of neurons using neuronal sub-type-specific promoters: *tph-1* (serotonergic neurons) (Cunningham et al., 2012), *glr-1* (command interneurons) (Zheng et al., 1999), *che-2* (chemosensory neurons) (Gray et al., 2005), *ttx-3* (AIY) (Chalasani et al., 2007), *npr-9* (AIB) (Piggott et al., 2011), *tdc-1* (RIM) (Cunningham et al., 2012) and *rig-3* (AVA) (Marvin et al., 2013). Reconstitution of AMPK (*aak-2*) using the *glr-1, tdc-1, ttx-3, npr-9* promoters was sufficient to partially rescue the defective distal exploration typical of *aak-2* mutants suggesting that AMPK functions within these neurons to phosphorylate targets involved in regulating these behavioural responses. Conversely, introducing AMPK (*aak-2*) in the sensory neurons, the serotonergic neurons, or the command interneurons involved in triggering reversals failed to correct the defect (Figure 2.2A). As AMPK expression within the GLR-1-expressing neurons partially rescued the locomotory defect of *aak-2* mutants, it is very likely that AMPK may be independently required in the GLR-1-expressing neurons AIB and RIM, although this does not exclude a role for *aak-2* in other GLR-1-expressing neurons.

The AIY and AIB interneurons receive synapses from the AWC sensory neurons and act in parallel to suppress and enhance reversals, respectively, in response to food

availability (Figure S2.3A, S2.3B) (Chalasani et al., 2007). The AIB interneurons relay their signals to the RIM and AVA, which in turn send their outputs to the muscles (Figure S2.3C) (Gordus et al., 2015). As AMPK expression within the AIY, AIB and RIM partially restored the defective distal exploration of starved *aak-2* mutants, it is likely that AMPK functions in multiple neurons in a parallel circuit to regulate locomotory behaviour in response to starvation.

When we expressed *aak-2* in both the AIY and the AIB interneurons, we completely restored the defective distal exploration of *aak-2* mutants, while expression of *aak-2* within the RIM and the AIB interneurons, or in RIM and AIY, did not improve the distal exploration defect of starved *aak-2* mutants beyond the rescue observed in AMPK mutant animals expressing *aak-2* within the AIB or the AIY interneurons alone (Figure 2.2B). The RIM neurons form chemical and electrical synapses with AIB and AIY respectively (White at al., 1986). Of note, it has been shown that chemical synapses from the RIM neurons enhance the variability in response to odour in the AWC-AIB-RIM olfactory circuit (Gordus et al., 2015). Therefore, *aak-2* expression within the RIM neuron might influence this variability, whereby expression of *aak-2* within the AIB and RIM neurons biases the circuit towards either a more probabilistic or reliable output. Taken together, our data highlight a critical role of AMPK in the AIY and AIB interneurons to regulate the appropriate transition from local to distal exploration in animals subjected to acute starvation.

AMPK functions within the AIB and AIY interneurons to integrate sensory signals in response to starvation

AMPK mutants demonstrate reduced forward movement accompanied by an increased frequency of reversals. If these defects disrupt distal exploratory foraging behaviour then starved *aak-2* mutants could also be defective in finding food at a distance. We therefore placed starved animals on plates with a point food source (*E. coli)* and monitored the time required for each animal to encounter the bacteria. We noted that *aak-2* mutants were substantially less efficient than wild type controls in their ability to track to the food source (Figure 2.2C). However, from this assay alone we were unable to distinguish if this defect is a consequence of a deficiency in their ability to sense food or to integrate the detection of nutritional signals to appropriately modify locomotory behaviour since we also observed a significant defect in the ability of AMPK mutants to chemotaxis toward the attractant isoamyl alcohol (IAA) (Figure 2.2D). However, since the expression of *aak-2* within the sensory neurons failed to restore the defective distal exploratory behaviour in *aak-2* mutants, while this defect was fully rescued by driving *aak-2* expression specifically within the AIB and AIY interneurons, we conclude that AMPK is critical for the integration of sensory cues and not with chemosensation *per se* (Figure 2.2D). Taken together, our results indicate that AMPK is required in the AIB and AIY interneurons to trigger appropriate exploratory behaviour in response to acute starvation.

AMPK functions upstream of *mgl-1*, *glr-1* and *eat-4* to modulate distal exploration

To position how and where AMPK functions within the AIY and AIB interneurons to modulate the transition between local and distal exploration, we performed optogenetic experiments to manipulate AIB and AIY activity in starved *aak-2* mutants (Kocabas et al., 2012). ChR2-mediated depolarization of the AIY interneurons in *aak-2* mutant animals resulted in a significant decrease in reversal frequency that was persistent throughout the illumination time. In contrast, AIY activation in WT animals slightly altered the forward locomotion and reversal rate which is consistent with previous studies demonstrating the activation of the AIY interneurons in starved animals (Gray et al., 2005; Flavell et al., 2013) (Figure 2.3A, S2.6A).

In a complementary experiment, we assessed the behaviour of starved *aak-2* mutants following inactivation of AIB induced by the light-gated chloride pump archaerhodopsin-3 (Arch) (Kocabas et al., 2012). The Arch-induced hyperpolarization of AIB was sufficient to suppress reversals in the *aak-2* mutants, while also promoting normal distal exploratory behaviour during starvation. In contrast, Arch-induced AIB inactivation in WT animals altered their locomotory behaviour slightly which can be explained by inactivation of AIB during acute bouts of starvation as the locomotory behaviour of starved animals has been shown to remain unchanged upon AIB ablation (Gray et al., 2005) (Figure 2.3B). We interpret these observations to indicate that although AMPK activity is required within the AIB and AIY interneurons for appropriate distal exploration in response to acute starvation, it does so without negatively impacting normal neurotransmission, neuronal development or connectivity within the interneurons.

Having identified the interneurons in which *aak-2* is required to suppress reversals, promote forward locomotion, and eventually trigger distal exploration, we then set out to identify AMPK targets within these interneurons that mediate its effects in response to starvation. Using bioinformatic tools we scanned the *C. elegans* proteome for proteins that possessed consensus AMPK phosphorylation sites and were known to be expressed in the AIB or AIY interneurons, providing us with a list of potential neuronal phosphorylation targets of AMPK (Figure S2.4A, S2.10).

Among the candidates that we identified through our bioinformatic analysis GLR-1, EAT-4, and MGL-1 emerged as highly predicted phosphorylation targets of AMPK (Figure 2.3C, 2.3D, 2.3E, S2.4A). *eat-4,* an ortholog of a mammalian brain-specific sodium-dependent inorganic phosphate co-transporter I (BNPI), encodes a vesicular glutamate transporter and is required for glutamatergic neurotransmission in the AIB (Lee et al., 1999; Piggott et al., 2011). Interestingly, we found that *eat-4* is epistatic to AMPK function during the response to acute starvation; single *eat-4* mutants behave like starved WT animals by decreasing reversal frequency in contrast to the *aak-2* mutants that exhibit an increased number of reversals. The *aak-2; eat-4* double mutants behave more like starved WT (or *eat-4*) animals than *aak-2* mutants (Figure 2.3C) indicating that *eat-4* acts downstream of, or in parallel to AMPK, in the genetic pathway controlling this starvation-induced behaviour.

The AMPA-type GluR GLR-1 is expressed in both the command interneurons and in some motor neurons, and is implicated in memory formation and the behavioural responses to light nose touch and to sensory cues such as food (Hart et al., 1995; Rose et al., 2003; Chalasani et al., 2007). Moreover, GLR-1 function is required for

glutamatergic activity in the AIB (Chalasani et al., 2007). Similar to *eat-4*, mutations in *glr-1* suppress the defective distal exploratory behaviour of AMPK mutants suggesting that like *eat-4*, *glr-1* is epistatic to *aak-2* (Figure 2.3D). Taken together, these results indicate that blocking the glutamatergic inputs to the AIB through the elimination of *glr-1*, or blocking its glutamatergic neurotransmission by disrupting *eat-4*, can compensate for the behavioural defects in starved *aak-2* mutants. Our data are therefore consistent with *aak-2* acting upstream of, or in parallel to these two glutamatergic effectors to modulate neuronal activity in the AIB interneuron during periods of acute starvation.

As initially determined in mammals, the Metabotropic glutamate receptors (mGluRs) also have an important neuromodulatory role in glutamatergic transmission within the *C. elegans* nervous system (Dillon et al., 2006; Dillon et al., 2015). *mgl-1* encodes one of the three Metabotropic glutamate receptors in *C. elegans* and is expressed throughout the AIY interneurons. Based on sequence analysis, MGL-1 is a group II Metabotropic glutamate receptor that acts as both pre- and postsynaptic detectors of glutamate to reduce neuronal excitation at least partly by inhibition of adenylyl cyclase activity (Dillon et al., 2015; Niswender and Conn, 2010). Previous studies have identified *mgl-1* as an essential component for the appropriate regulation of fat accumulation (Greer et al., 2008) and the starvation responses mediated by AIY (Kang and Avery, 2009). Our genetic analyses indicate that mutations in *mgl-1* could also significantly, but not completely, suppress the prolonged local exploration typical of *aak-2* mutants whereby compromise of *mgl-1* in the *aak-2* background results in reduced reversals and increased distal runs (Figure 2.3E). These results suggest that AMPK may negatively regulate MGL-1 activity in the AIY in response to starvation.

Our data are consistent with AMPK acting as a potential modulator of glutamatergic inputs in both the AIB and the AIY, but it cannot distinguish whether AMPK affects glutamatergic inputs in a subset of glutamatergic neurons (ie…the AIB and AIY) or in all glutamatergic neurons. Furthermore, if AMPK does have a specific role in the AIB and the AIY, then what determines its functionality in this subset of AMPK-sensitive neurons? To test if AMPK affects other mediators of glutamatergic inputs we assessed whether AMPK compromise could modify the phenotypes associated with: *nmr-1*, which encodes a NMDA-type ionotropic glutamate receptor subunit; the glutamate-gated chloride channels *avr-14 and mgl-2* which encodes a group I Metabotropic glutamate receptor (Chalasani et al., 2007; Piggott et al., 2011; Dillon et al., 2015). Double mutant combinations with *aak-2* did not modify any of the *aak-2* phenotypes we tested (Figure S2.4B) suggesting that *aak-2* does not affect glutamatergic transmission generally, but rather it seems to only affect a subset of glutamatergic neurons critical for starvation-dependent functions and/or behaviours.

Parallel and opposing function of AMPK in the modulation of AIB and AIY outputs

Given the epistatic relationship between *aak-2* and genes involved in glutamatergic signaling we wanted to clarify if AMPK regulates the AIB and AIY neural activity through these targets. We monitored calcium transients using the genetically-encoded calcium sensor G-CaMP individually in both interneurons as a proxy for neuronal activity (Figure 2.4A, 2.4B, 2.4C, 2.4D, 2.4E, 2.4F). We observed a decrease in AIY activity in starved *aak-2* mutants that was rescued by AIY-specific *aak-2* expression, consistent with *aak-2* regulating AIY activity/response during starvation (Figure 2.4A, 2.4B, 2.4C, 2.4G,

S2.5A). Previous studies have shown that calcium spikes in the AIY interneurons correlate positively with forward runs and negatively with the initiation of reversals with gradual increases preceding forward run initiation (Flavell et al., 2013; Luo et al., 2014). Our results suggest that the decreased AIY activity in starved *aak-2* mutants at least partially contributes to their defective distal exploratory behaviour. To further investigate whether the increased reversal rate correlates with decreased AIY activity in starved *aak-2* mutants, we monitored the calcium levels in the AIY interneuron of freely behaving animals. The AIY calcium levels were consistently highest upon the termination of reversals, and during the subsequent forward runs with gradual increase preceding forward run initiation suggesting that the calcium peaks in the AIY correlate with the suppression of reversals and the observed acute increase in forward speed (Figure S2.6A). Furthermore, consistent with our previous results, we found that starved *aak-2* mutants display increased reversal frequency and shorter durations of forward locomotion, both of which correlated with decreased calcium influx and associated AIY activity (Figure S2.6C, S2.6E, S2.6F).

The epistatic relationship between *mgl-1* and *aak-2* in distal exploration was further corroborated by the total integrated fluorescence intensity demonstrated by *aak-2; mgl-1* animals. These readings resemble both starved WT and/or *mgl-1* mutants, reinforcing our data indicating that *mgl-1* is epistatic to *aak-2* function in the AIY interneuron during acute starvation, where MGL-1 potentially acts as a postsynaptic receptor in the AIY (Figure 2.4H, S2.5A). AIY-ablated animals are defective in transitioning between local and distal exploration (Gray et al., 2005), these results reveal an additional, albeit critical, role of AMPK within the AIY interneurons to modulate AIY

neuronal activity autonomously, thus contributing to the behavioural transition associated with the decision to explore more distal environments when animals are subjected to starvation.

Unlike the AIY interneurons, activity in AIB increased in starved *aak-2* mutants and could be rescued by AIB-specific *aak-2* expression (Figure 2.4D, 2.4E, 2.4F, 2.4I). Calcium peaks in the AIB have been shown to correlate with reversal frequency (Piggott et al., 2011; Gordus et al., 2015), but during our recording the animals are restrained therefore we presume that each calcium peak is representative of a reversal attempt. Given the increased calcium levels in the AIB interneurons in starved *aak-2* mutants, our data indirectly suggest that the increased reversal frequency and the abnormally prolonged local exploration is at least partly a consequence of increased AIB neuronal activity. To further examine if the increased AIB activity in starved *aak-2* mutants correlates with their increased reversal frequency, we examined the behaviour of freely moving animals during acute starvation. The calcium peaks in the AIB were well correlated with reversal frequency, further suggesting that the increased reversal frequency in starved *aak-2* mutants is at least partially due to the increased AIB activity (Figure S2.6B, S2.6D, S2.6G, S2.6H).

The genetic requirement for *aak-2* in the AIB interneurons to modulate neuronal activity, along with its epistatic relationship with *eat-4* and *glr-1* in the modulation of locomotory behaviour, are consistent with at least two models: During acute starvation *aak-2* might regulate excitatory synaptic inputs onto the AIB interneurons through modulation of *glr-1* activity, or *aak-2* might affect glutamatergic neurotransmission from the AIB interneurons through, or in parallel with *eat-4*. To distinguish between these

possibilities, we monitored calcium transients in *aak-2; glr-1* and *aak-2;* [P*npr-9::eat-4*(RNAi)] double mutants. Of note, we observed that although blocking the AIB neurotransmission in *aak-2*; [P*npr-9*::*eat-4* (RNAi)] animals resulted in normal distal exploratory behaviour during starvation (Figure 2.3C), the total integrated signal was significantly higher compared to both WT and [P*npr-9::eat-4* (RNAi)] animals (Figure 2.4J, S2.5B). This suggests that *aak-2* acts independently of *eat-4* to modulate neural activity in the AIB.

Taken together, our genetic analyses indicate that AMPK is required within the AIY and the AIB interneurons to modulate their neuronal activity through its direct or indirect regulation of the glutamatergic receptors GLR-1 and MGL-1 during starvation-induced exploratory behaviour.

AMPK controls GLR-1 abundance in the neuronal postsynaptic membranes

Increased GLR-1-mediated glutamatergic inputs have been shown to bias the locomotory circuit toward reversals (Zheng et al., 1999). Consistent with this, overexpression of GLR-1 specifically within the AIB interneurons increases reversal frequency and turning rate in the absence of food (Chalasani et al., 2007). Because the abundance of GLR-1 within the AIB influences reversal behaviour, we tested whether the increased reversal frequency in *aak-2* mutants is a consequence of changes in GLR-1 levels. We quantified *glr-1* mRNA levels in WT and *aak-2* mutants under well-fed and starved conditions and we did not detect any differences (Figure S2.7A). However we did notice that total GLR-1 protein levels in starved WT animals were reduced and this effect was less pronounced in starved *aak-2* mutants (Figure S2.7B) suggesting

that AMPK may modulate GLR-1 protein levels in starved animals either at the level of synthesis or stability.

Studies have demonstrated that disruption of GLR-1 endocytosis results in increased GLR-1 abundance in the postsynaptic elements which results in increased synaptic strength and reversal frequency (Burbea et al., 2002; Juo et al., 2007). Given the epistatic relationship between *aak-2* and *glr-1* we next asked if the increased reversal frequency in starved *aak-2* mutants occurs as a result of decreased GLR-1 turnover linked to a change in its post-translational regulation due to the absence of AMPK.

To examine this possibility, we used a previously described chimaeric receptor tagged with the green fluorescent protein (GLR-1::GFP) that localizes to discrete punctate structures and that can be used to visualize central glutamatergic synapses in living animals (Burbea et al., 2002). We measured the density of GLR-1::GFP-containing puncta in the AIB neuronal process in a strain that expresses GLR-1::GFP driven by the AIB-specific promoter *npr-9*. Notably, we observed that the GLR-1 levels in the AIB neuronal process in the nerve ring where the AIB interneurons form their synapses (Figure 2.5A) was significantly greater in *aak-2* mutants than WT animals during starvation (Figure 2.5B, 2.5C). Moreover, this difference could be rescued by introducing AMPK specifically within the AIB interneurons (Figure 2.5C), highlighting the importance of AMPK in modulating GLR-1 abundance within the AIB interneurons.

Our data suggest that AMPK regulates GLR-1 abundance in the AIB interneurons to potentially modulate synaptic inputs and consequently affect reversal behaviour. But *aak-2* mutants have additional behavioural defects that may also

manifest due to inappropriate glutamatergic signaling (Cunningham et al., 2012). Therefore, to further investigate if the effect of AMPK on GLR-1 abundance is limited to the AIB interneurons or alternatively AMPK is globally required for the modulation of GLR-1 steady state levels throughout the nervous system, we measured the density of GLR-1::GFP-containing puncta in the anterior region of the ventral nerve cord between the RIG neuron cell bodies, and in the vulva using the [P*glr-1::glr-1::gfp*] transgene (Burbea et al., 2002). Interestingly, we observed a significant increase in the GLR-1 levels and the size (diameter) of GLR-1-expressing puncta in starved *aak-2* mutant animals compared to starved WT controls (Figure 2.5D, 2.5E, 2.5F). Furthermore, we noted that the GLR-1::GFP signal in *aak-2* mutants overlapped with that of EAT-4 which marks the majority of glutamatergic synapses (Figure S2.7C, S2.7D, S2.7E). To summarize, the removal of *aak-2* results in an increase in GLR-1 abundance in starved animals and a large fraction of the GLR-1::GFP puncta seen in the mutants correspond to postsynaptic elements.

As AIB interneurons form their synapses in the nerve ring, the increased GLR-1 abundance in the ventral nerve cord of *aak-2* mutants must be a consequence of AMPK compromise in additional GLR-1-expressing neurons. Since the quantification of fluorescent intensity and size/width of the puncta are two determinants of total receptor abundance at each synapse, these results collectively suggest that GLR-1 levels at the postsynaptic elements are globally changed in *aak-2* mutants. Therefore, AMPK compromise is associated with increased steady state GLR-1 protein levels and GLR-1::GFP fluorescence, and that most of the GLR-1 receptors may be degraded in an

AMPK-dependent manner, or alternatively, that GLR-1 synthesis may also be affected in starved *aak-2* mutants.

AMPK acts with UNC-11/AP180 to regulate GLR-1 levels within the postsynaptic elements and consequently modulate synaptic strength

Mammalian AMPA receptors are removed from postsynaptic membranes by clathrin-mediated endocytosis (Carroll et al., 1999; Man et al., 2000). In *C. elegans*, the ubiquitylation of conserved cytoplasmic lysine residues on GLR-1 constitutes an endocytic signal to remove the receptors from postsynaptic elements and which subsequently targets them for degradation; a process that is mediated by the *unc-11*/AP180 clathrin adaptin protein (Burbea et al., 2002). To further investigate if AMPK modulates GLR-1 abundance by affecting endocytosis in response to starvation, we measured GLR-1 abundance in *unc-11* mutants that are unable to internalize GLR-1. The GLR-1 levels in *aak-2*; *unc-11* double mutants were indistinguishable from those found in either *unc-11* single mutants (Figure 2.6A, 2.6B, 2.6C). Similarly, the increased GLR-1 levels and size/width of GLR-1 puncta measured in the *aak-2* mutants was not further enhanced when all four cytoplasmic lysine residues required for GLR-1 ubiquitylation and endocytosis were mutated to arginine [GLR-1(4KR)::GFP], further suggesting that *aak-2* acts in a linear pathway with an *unc-11*/clathrin-mediated mechanism to regulate GLR-1 endocytosis (Figure 2.6A, 2.6B, 2.6C).

Activation of AMPK during periods of starvation could result in the regulation of GLR-1 abundance by its direct phosphorylation through its consensus phosphorylation sites. To determine the importance of these sites we used the [P*npr-9::glr-1::gfp*] fusion

protein as a template to generate a GLR-1::GFP variant with non-phosphorylable AMPK sites. The cytoplasmic domain of GluR has already been described as an important phosphoregulatory target, mostly by kinases such as PKA and PKC (Roche et al., 1996). Since two conserved AMPK phosphorylation sites were identified within the cytoplasmic domain of GLR-1 (Figure S2.7F), we mutated both S907 and S924 to non-phosphorylable alanine residues to evaluate how these sites contribute to the stability of GLR-1 within the AIB neuronal process during periods of starvation. Wild type animals bearing the compound (S907A, S924A) variant transgene demonstrated defective exploratory behaviour that was comparable to *aak-2* mutants (Figure 2.6D). To further examine if the GLR-1 receptors that accumulate in AMPK mutants act as functional synaptic receptors we examined the locomotory behaviour of *eat-4; glr-1* double mutants expressing the non-phosphorylable variant (S907A, S924A). We noticed that increased reversal frequency rate accompanied by reduced forward locomotion in animals expressing the (S907A, S924A) variant was largely suppressed in the absence of *eat-4* indicating that the compromise of AMPK signaling results in an increased abundance of functional synaptic GLR-1 receptors (Figure 2.6D).

Furthermore, the GLR-1 (S907A, S924A) variant accumulated in the AIB neuronal process and this accumulation was not enhanced upon removal of *unc-11* (Figure 2.6E), further suggesting that AMPK regulates GLR-1 abundance directly by phosphorylating the protein to mediate UNC-11/AP180-dependent GLR-1 endocytosis in response to starvation.

Elevated glutamatergic transmission within the command interneurons is associated with a hyper-reversal phenotype and a dramatic decrease in the duration of

forward movement (Zheng et al., 1999). It would therefore be intuitive that an increase in synaptic strength, or input, would result in a consequential increase in reversal frequency. Although we were unable to directly examine the role of AMPK in the regulation of synaptic strength, taken together, our data suggest that AMPK modulates synaptic strength by regulating GLR-1 abundance in the postsynaptic puncta to bias the behavioural readout in response to starvation.

AMPK modulates MGL-1 in the mRNA and protein levels in the AIY interneurons to modify distal exploratory behaviour in response to starvation

The results of our epistasis analysis between *mgl-1* and *aak-2* are consistent with *mgl-1* acting downstream of *aak-2* to regulate the transition from local to distal exploration in parallel with *glr-1* in response to starvation. Therefore, to further explore the mechanism by which AMPK regulates MGL-1 activity in AIY, we determined the fluorescence intensity of a [P*mgl-1*::*mgl-1*::GFP] translational fusion protein in both well-fed and starved animals. Starved animals all showed reduced MGL-1 levels, which was completely reversed in *aak-2* mutants (Figure 2.7A, 2.7B, S2.8A). Moreover, expressing *aak-2* specifically within the AIY interneurons was sufficient to restore MGL-1 levels to those of starved WT animals suggesting that AMPK activity modulates MGL-1 abundance in the AIY in response to starvation.

To determine how AMPK affected MGL-1 abundance in the AIY we first measured the mRNA levels in well-fed and starved animals using semi quantitative RT-PCR, while in parallel we also monitored *glc-3* levels which are expressed in the AIY where it acts as a glutamate-gated chloride channel for local search (Chalasani et al., 2007).

Although we did not observe any change in the levels of *glc-3* mRNA in well-fed or starved animals, we did observe an slight increase in *mgl-1* mRNA levels in well-fed and a more significant increase in starved *aak-2* mutants (Figure S2.8B, S2.8C) suggesting that AMPK regulates MGL-1 levels by either blocking its transcription or affecting the stability of *mgl-1* transcripts. The former possibility is less plausible given the short time delay that occurs between the response to food deprivation and the consequent behavioural changes. Since we did not detect any significant change in the *mgl-1* mRNA levels in WT animals, it is likely that AMPK controls the abundance of *mgl-1* transcripts in the AIY and possibly other *mgl-1*-expressing neurons in response to starvation, typical of its function in adapting to energy stress. These results however do not exclude the possible role of AMPK in regulating MGL-1 protein levels directly through phosphorylation, as has been described for some ion channels (Lang and Föller, 2014), although this would be in addition to its observed impact on *mgl-1* transcript abundance.

To further investigate this possibility, we mutated the conserved potential AMPK phosphorylation site in MGL-1 (Figure S2.8D) to examine if AMPK also regulates MGL-1 through direct phosphorylation. We noted that mutating serine 234 to alanine (S234A) resulted in a modest, but nevertheless significant reduction in forward locomotion (Figure 2.7C), although this mutation did not result in increased MGL-1 levels (Figure S2.8E). This suggests that AMPK regulates *mgl-1* activity by impinging on both mRNA and protein function. The AMPK-mediated phosphorylation of serine 234 in MGL-1 may be required for the modulation of distal exploratory behaviour independent of its regulation of MGL-1 levels. We currently cannot exclude that this post-translational

modification could affect some aspect of MGL-1 receptor function. Taken together, our results are consistent with a role for AMPK in modulating *mgl-1* function in the AIY interneurons whereby *mgl-1* activity is attenuated in response to starvation-induced AMPK signaling resulting in increased AIY activity and the consequent suppression of reversals.

Our results so far suggest that increased GLR-1 and MGL-1 abundance in the AIB and AIY interneurons, respectively, cause the defective distal exploration in starved *aak-2* animals. To further test whether indeed this may be the case we overexpressed GLR-1 and MGL-1 in the AIB and AIY interneurons, respectively, in WT animals to verify if this misexpression could recapitulate the defective distal exploration of starved *aak-2* mutants. The overexpression of MGL-1 in the AIY resulted in a phenotype that was quite similar to that seen in starved *aak-2* mutants whereas overexpression of GLR-1 in the AIB resulted in a more modest, but nevertheless significant, defect in transition between local to distal exploration. This difference can be explained by the fact that the overexpressed GLR-1 is most likely still targeted for endocytosis and degradation while AMPK is present and active, while in *aak-2* mutants there is a defect in GLR-1 endocytosis allowing it to accumulate to functionally critical levels in the puncta. Nevertheless, the overexpression of either of these transgenes in results in a behavioural defect which phenocopies that of starved *aak-2* mutants (Figure S2.9).

Discussion

In most free-living organisms energy stress is often accompanied by characteristic compensatory behaviours that are triggered to increase the probability of encountering a food source (Wang et al., 2005; Gray et al., 2005; Dietrich et al., 2015). The spectrum of these responses however varies dramatically and naturally reflects both the environment and the physiology of the organism. Humans are characteristically hypersensitive and/or rapidly irritable during periods of acute food deprivation providing a familiar example of how a collection of neural circuits become reproducibly activated or blocked in response to this specific acute stress, culminating in highly predictable behavioural responses. These behavioural changes are achieved in part through synaptic plasticity, which is required for adaptation to varying circumstances in an experience-dependent manner (Bito et al., 2010; Collingridge et al., 2010; Liu et al., 2012). During our work we have taken advantage of a common physiological response to an acute environmental stress in order to delineate the genetic pathway, the corresponding neuronal circuitry, and the biochemical targets that drive adaptive exploratory behaviours in response to starvation in *C. elegans*.

AMPK function within the AWC-AIB-AIY circuitry is required for modification of distal exploratory behaviour in response to acute starvation

We and others found that the disruption of AMPK signaling resulted in marked changes in exploratory behaviour partly by reduced forward locomotion (Lee et al., 2008; Cunningham et al., 2014). However, we also noticed that starved *aak-2* animals execute

frequent reversals, which might contribute significantly to their defective distal exploration given the antagonistic relationship between the well-characterized forward and backward locomotory circuits. We were able to improve forward locomotion by the targeted expression of *aak-2* in the AIB and AIY interneurons, while consequently reducing the abnormally frequent reversal behaviour typical of AMPK mutants, consistent with the involvement of AIB and AIY in regulating both reversal frequency and the duration of forward movement (Tsalik and Hobert, 2003; Wakabayashi et al., 2004). Notably, unlike *aak-2* expression in the sensory neurons, its expression within the AIB and AIY interneurons also improved the chemotaxis defect of *aak-2* mutants suggesting that AMPK may be critical for the integration of sensory signals received by AIB and AIY interneurons.

A recent study showed that *aak-2* compromise mimics the effect of elevated serotonin on movement (Cunningham et al., 2014). The AIB and AIY interneurons synapse directly or indirectly onto the command interneurons to control whether *C. elegans* moves forward or backward (Gray et al., 2005; Chalasani et al., 2007; Piggott et al., 2011; Chen et al., 2013). Moreover, these neurons have been identified as potential targets of serotonergic signaling to mediate the effects of serotonin on locomotion (Harris et al., 2009). It is likely therefore that *aak-2* acts within the AIB and AIY interneurons to modulate the effects of serotonergic signaling on the resulting movement rather than functioning directly within the serotonergic neurons *per se*.

AMPK acts as a molecular trigger in the AWC-AIB-AIY circuitry to modify their neuronal activity and the resulting behavioural outputs

The very short time delay that occurs between the response to the lack of food and the observed behaviours makes it unlikely that the effects of AMPK within the AIB and AIY interneurons are mediated by transcription. Alternatively, AMPK has been demonstrated to act directly upon ion channels and gap junctions (Hallows et al., 2000; Lang and Föller, 2014). Our calcium imaging data is consistent with a model whereby starvation-induced AMPK activation leads to the inhibition of neuronal activity within the AIB, while promoting activity in the AIY interneuron, thereby altering behavioural outputs in a coordinated manner.

How then can we account for the AMPK-dependent starvation-induced changes in calcium levels within the AIB and AIY interneurons and the effects on their respective neural activity? Neuronal activity results from the interplay between synaptic excitation and inhibition. In the brain, excitation is mainly achieved through glutamatergic transmission (Bito et al., 2010; Collingridge et al., 2010; Liu et al., 2012). Remarkably, our results delineate a novel role for AMPK in the modulation of neuronal activity in response to starvation through the simultaneous regulation of glutamatergic inputs mediated by two different receptors: GluR/GLR-1 in the AIB and MGL-1 in the AIY. Of note, unlike *mgl-1* null mutations that resulted in partial rescue of locomotory behaviour of *aak-2* mutants, we noticed a complete rescue upon removal of *eat-4* or *glr-1*. As *eat-4* is the only identified vesicular glutamate transporter in *C. elegans*, inhibition of *eat-4* is expected to result in a complete inactivation of both *mgl-1*- and *glr-1*-dependent outputs that are both potentially dependent on glutamate release. In the case of *glr-1*, AIY may

require either direct or indirect synaptic inputs from an additional *glr-1*-expressing neuron(s), or alternatively may signal to a *glr-1*-expressing neuron(s) so that eliminating *glr-1* not only results in reduced AIB activity, but also directly or indirectly blocks AIY synaptic inputs or outputs.

AMPK coordinates glutamatergic signals through distinct mechanisms

In addition to the plethora of processes that are regulated downstream of AMPK activation, AMPK has been shown to modulate synaptic aging (Samuel et al., 2014). In mice, starvation increases the activity of the AgRP neurons by affecting their firing rate, ultimately inducing intense food-seeking behaviour and increased feeding (Dietrich et al., 2015). The enhanced firing rate of the AgRP neurons has been shown to be largely dependent on glutamatergic inputs mediated by glutamatergic ionotropic AMPA and NMDA receptors (Bito, 2010; Liu et al., 2012). AMPK modulates this food intake behaviour through the regulation of glutamate release in both mice and in *C. elegans,* although the precise mechanism has not been elucidated in either case (Yang et al., 2011; Cunningham et al., 2012).

Our data demonstrate that AMPK *(aak-2)* regulates GLR-1 and MGL-1 abundance postsynaptically. The AWC is sensitive to food availability and is inhibited during starvation, resulting in the inactivation of AIB and the activation of AIY (Gray et al., 2005; Chalasani et al., 2007). Our data demonstrate that the loss of AMPK signaling results in an increase in AIB neuronal activity and a concomitant decrease in AIY activity. This may be rationalized by the fact that *C. elegans* neurons release neurotransmitter in a graded fashion. Like vertebrate rod and cone photoreceptors

which show tonic activity in the dark, and are hyperpolarized or depolarized by light, and removal of light respectively, AWC olfactory neurons have been shown to display basal activity in the absence of odour, but are inhibited or stimulated by odour or odour removal, respectively (Chalasani et al., 2007). Therefore, the increased GLR-1 and MGL-1 abundance in the AIB and AIY interneurons could potentially result in responses of longer duration and increased strength upon tonic neurotransmitter release at rest, or upon activation of AWC consequently increasing reversal frequency and shortening runs (Figure 8).

In *C. elegans* an increase in glutamatergic inputs mediated by GLR-1 results in enhanced reversal frequency making this simple behaviour a faithful determinant of increased synaptic strength (Zheng et al., 1999; Burbea et al., 2002; Chalasani et al., 2007). We provide several lines of evidence that demonstrate that AMPK regulates GLR-1 abundance, by affecting its ubiquitylation status during conditions of acute starvation. First, the total GLR-1 level is decreased upon starvation. Second, the amplitude and size/width of GLR-1 puncta that are two determinants of GLR-1 abundance are increased in the AIB neuronal process and in the ventral nerve cord in *aak-2* mutants. Third, this elevated GLR-1 accumulation cannot be further enhanced upon the disruption of the various processes required to remove GLR-1 from the membrane suggesting that AMPK and the ubiquitin-mediated internalization of GLR-1 may therefore function in a linear genetic pathway. Fourth, mutating the AMPK phosphorylation sites in GLR-1 results in increased GLR-1 abundance and defective distal exploratory behaviour. Finally, this defect is comparable to that observed in *aak-2* mutants, while crossing the GLR-1 (S907A, S924A) variant into the *aak-2* mutant

background did not further exacerbate the distal exploratory behavioural defect. Therefore, consistent with its well-described role in the mouse where it regulates glutamatergic neurotransmission (Yang et al., 2011), AMPK is required to regulate the abundance of GLR-1 in specific glutamatergic neurons. In doing so it modulates glutamatergic inputs and synaptic strength to elicit appropriate behavioural outcomes in response to starvation.

AMPA receptors are either inserted or removed from postsynaptic membranes in an activity-dependent manner leading to the potentiation or depression of synaptic transmission, respectively (Carroll et al., 1999). Our findings might be further extended to implicate AMPK in the control of synaptic plasticity that underlies learning and memory as the appropriate regulation of glutamate receptor abundance is critical for long-term memory through habituation in *C. elegans* (Rose et al., 2003). Any increase in GluR abundance, and hence signaling, that arises in AMPK-compromised animals is very likely to result in irregular plasticity and abnormal behavioural outcomes, consistent with what is observed in starved AMPK mutants.

In addition to its regulation of GLR-1 abundance in the AIB, we show that AMPK also regulates glutamatergic inputs by regulating MGL-1 in the AIY. A key function of group II mGlu receptors is to modulate neuronal excitability and synaptic transmission at least partly by inhibiting adenylate cyclase (Dillon et al., 2006; Niswender and Conn, 2010). Group II mGlu receptors are required in both cognitive and emotional processes, and have been linked to various neuropsychiatric conditions, including anxiety, stress-related disorders, schizophrenia and substance misuse (Niswender and Conn, 2010). In mammals, activation of mGluR II induces long term depression at GABAergic synapses

in the cochlear nucleus magnocellularis neurons where they have been shown to modulate synaptic plasticity (Lu, 2007).

In *C. elegans*, *mgl-1* regulates a systemic starvation response and fat accumulation (Greer et al., 2008; Kang et al., 2009). Consistent with the inhibitory role of group II mGlu receptors in the vertebrate nervous system, *mgl-1* was recently demonstrated to coordinate environmental sensory cues to modulate the activity of the pharyngeal neural network (Dillon et al., 2015). Although some studies have indicated that Metabotropic glutamate receptors can be phosphoregulated by kinases such as PKC (Kim et al., 2005; Ko et al., 2012), our work has uncovered an AMPK-mediated modulation of the MGL-1 in response to acute food deprivation in *C. elegans*. This effect is at least partly achieved by influencing the regulation of *mgl-1* mRNA levels in starved animals. Considering the brief delay between stimulus and response it is most likely that AMPK mediates its effect on *mgl-1* transcript levels through the phosphorylation of key regulators of *mgl-1* mRNA stability as described previously (Yun et al., 2005). Therefore, in addition to its well-described role in the regulation of ionic channels by effecting their degradation, AMPK may regulate its downstream neuronal effectors by affecting mRNA stability to regulate protein levels.

In summary, our findings suggest a simple model, in which AMPK coordinately regulates the activity of the AIB and AIY interneurons by modulating their glutamatergic synaptic inputs through two distinct mechanisms. AMPK modulates glutamatergic inputs in both interneurons by impinging on AMPA-type glutamate receptor GLR-1 and Metabotropic glutamate receptor MGL-1 which consequently regulate distinct downstream behavioural outputs (Figure 2.8). The most salient feature of our study is

our demonstration that AMPK acts as a molecular switch in the nervous system that is most likely not limited to the circuitries engaged in regulation of feeding and locomotory behaviours. Given the conserved AMPK phosphorylation sites in GLR-1 and MGL-1, we speculate that AMPK regulates the activity of neural circuits in various organisms at least partly by affecting these two receptor types to modulate a wide range of neuronal outputs that respond to physiological or developmental contexts involving various stresses. Alternatively, its starvation-inducible or pharmacological activation could provide a highly effective, non-invasive means of modifying behavioural outputs.

Materials and methods

Strains

Caenorhabditis elegans strains were maintained under standard conditions (Brenner, 1974). The Bristol isolate (N2) was used as wild type. The following alleles were used in this study: *aak-1(tm1944)*, *aak-2(ok524), eat-4(ky5), glr-1 (n2461), lite-1*(*ce314*), *unc-11(e48), mgl-1(tm1811), osm-6(p811), avr-14(ad1305), nmr-1(ak4), mgl-2(tm355), sid-1(pk3321).* Please see supplementary file 1 for the complete list of strains used in this study.

Plasmid Constructs and Transgenes

Genomic *aak-2* and *mgl-1* and their 2kb and 3kb upstream sequences were amplified and cloned upstream of GFP to generate [P*aak-2::aak-2::gfp*] and [P*mgl-1::mgl-1::gfp*]. *unc-119, unc-54, glr-1, tph-1, rig-3, ttx-3, npr-9, che-2* promoters were amplified from genomic DNA and were cloned upstream of *aak-2* cDNA (Narbonne and Roy, 2009) to drive *aak-2* expression globally throughout the nervous system, body wall muscle and in different neuronal subtypes. GLR-1::GFP was amplified from *nuIs24* strain and was inserted downstream of *npr-9* promoter to generate [P*npr-9::glr-1::gfp*]. The resulting constructs were injected at 10-50 ng/ul and either *Punc-122::gfp, Pelt-2::gfp, Pmyo-2::gfp* or *Punc-122::dsred* were used as the co-injection marker. To generate the GLR-1 variants including (S907A) and (S924A), PCR-mediated site-directed mutagenesis was performed using Gene-Tailor site-directed mutagenesis (Invitrogen) on [P*npr-9::glr-1::gfp*] and transgenic lines were generated after microinjecting plasmids at 50 ng/ul.

We used CRISPR/Cas9 to mutagenize the AMPK phosphorylation site serine 234 to alanine in *mgl-1* as described previously (Paix et al., 2014).

Quantitative Analysis of Exploratory Behaviour

Well-fed adult animals were scored for exploratory behaviour following 2 hours starvation as described previously (Gray et al., 2005; Calhoun et al., 2015). For our time course experiment mid-L4 animals were scored for exploratory behaviour either immediately after removal from food or following longer durations off food. To minimize the behavioural modifications caused by variability in their environment, animals were grown on the similar size and amount of bacterial patches (Calhoun et al., 2015). All body bends and reversals were scored by eye, by an investigator blind to the genotype of the animals.

L4 animals of *sid-1*(*pk3321*); *uIs69 [pCFJ90* (*pmyo-2*::mCherry) + *unc-119p::sid-1*] and *aak-2*(*ok524*); *sid-1*(*pk3321*); *uIs69 [pCFJ90* (*pmyo-2*::mCherry) + *unc-119p::sid-1*] which are nervous system RNAi hypersensitive were transferred to the RNAi plates seeded with overnight cultures of HT-115 *E. coli* clones expressing double stranded RNAi targeting *glr-1, glr-2, eat-4, mod-1, ser-2, inx-7, unc-7, inx-19, gar-2, gcy-1, flp-1, mgl-1, npr-11*. The exploratory behaviour of the resultant progeny was monitored upon reaching to adulthood.

Locomotory behaviour in the presence of food

The basal and enhanced slowing response was measured by transferring well-fed animals to plates with no bacteria, and transferring them to an assay plate with a thin

layer of bacteria immediately or after 30 minutes off food as described previously (Sawin et al., 2000).

Chemotactic Analysis

Chemotaxis assays towards a bacterial lawn or isoamyl alcohol (IAA) were performed on 10 cm nematode growth medium (NGM) plates incubated at room temperature (22°C) overnight as previously described. Animal behaviour was scored by eye, by an investigator blind to the genotype of the animal. 10 and 300 animals were used for each chemotaxis assay, either toward the bacterial lawn and IAA, respectively (Hart, 2006).

Optogenetics

C. elegans grown on NGM plates supplied with 5 µM all-trans retinal (ATR) were starved for 2 hours and their exploratory behaviour was monitored on retinal-free NGM plates. A 1 minute pulse of blue (480 nm) or yellow light (540 nm) was delivered from an Arc lamp (EXFO) by a 10× objective (Zeiss M2Bio) to the head of an individual animal to activate ChR2 or Arch, respectively as described previously (Piggott et al., 2011; Kocabas et al., 2012). In order to eliminate intrinsic phototaxis responses, we used *lite-1* mutants that are defective in UV light phototaxis. *lite-1* mutants did not show any obvious defect in the locomotory behaviour in our assays. To depolarize the AIY interneurons or hyperpolarize the AIB interneurons, we used previously described strains expressing channelrhodopsin-2 (ChR2) or archaerhodopsin-3 (Arch), respectively (Kocabas et al., 2012).

In vivo Imaging

For the calcium imaging adult transgenic animals expressing [P*npr-9::*G-CaMP3+ P*npr-9*::DsRed2B] (Piggott et al., 2011) or [P*ttx-3*::G-CaMP1+ unc-122::GFP] (Chalasani et al., 2007) were transferred to a 10% agarose pad following 2 hours starvation. A coverslip was then applied and the calcium transients were monitored in the cell body or neuronal process of the AIB and AIY interneurons, respectively as previously described (Lemieux et al., 2015). Briefly, to measure calcium transients in the AIB interneurons fluorescence images (green channel: G-CaMP3, red channel: DsRed2B) were acquired sequentially over 120s window at a frequency of 2Hz through a 63X objective on a Zeiss microscope. To measure calcium transients in the AIY interneurons fluorescence images (Green channel) were acquired from the neuronal process in the nerve ring over a 65s window at a frequency of 3Hz through a 63X objective. The resulting time-point series obtained from the fluorescence images for the AIB and AIY were then analyzed using ImageJ (RRID:SCR_001935).

To calculate the baseline values (F^b) averages of global minima for 5-10 frames over the duration of each time-lapse sequence were obtained and the difference between a given each time point's fluorescence and F^b (ΔF) was normalized by dividing ΔF to F^b to plot $\Delta F/F^b$ for each individual. In our analyses, we report the total integrated signal of all the observed peaks during the 65s or 120s recording window that consists of an unambiguously defined initiation of a spontaneous transient in the neural process or cell body for the AIY and AIB, respectively. To measure the total integrated fluorescence the $\Delta F/F$ for each time point were summed over each time-lapse

sequence to obtain a measurement for each time series sequence as described previously (Lemieux et al., 2015).

Calcium imaging in freely behaving animals was performed on a Zeiss microscope equipped with a Hamamatsu camera as previously described (Luo et al., 2014; Flavell et al., 2013). To prevent behavioural responses to blue light, the imaging was performed in the *lite-1* background (Flavell et al., 2013). Animals were placed on 10% agarose pads to slow down their locomotion and the pad was sealed in a small chamber to prevent evaporation. The elapsed time from placing animals on the agarose pads to initiation of imaging was <5 minutes. Fluorescence time-lapse imaging was performed over 90 seconds at a frequency of 2Hz using a 10x objective by an experimenter blind to the genotype. The images were processed as described before (Lemieux et al., 2015; Luo et al., 2014).

Microscopy

All images were acquired using a Zeiss AX10 microscope equipped with a Hamamatsu camera as previously described (Burbea et al., 2002). Starved GLR-1::GFP-expressing strains were immobilized with 10 mM levamisole. Images were captured and processed using AxioVision software. Maximum intensity projections of Z series stacks were obtained. GLR-1 levels were calculated using imageJ and normalized relative to WT. Puncta widths were estimated as the peak width at half-maximal amplitudes as described previously (Burbea et al., 2002). At least three transgenic lines were examined for these experiments and all the imaging and subsequent quantification was performed by an experimenter who was blind to the genotype.

The extent of signal overlap between GLR-1 and the presynaptic marker EAT-4 was indicated by imaging transgenic animals expressing GLR-1::GFP and EAT-4::mCherry. Maximum intensity projections of sequential images from fluorescent green and red filters were obtained. The local maximum in each punctum was used to determine the spatial position of the GFP and mCherry peaks. The percentage of GLR-1 puncta co-localizing with a EAT-4 punctum was assessed by the fraction of GLR-1 puncta peaks that were less than 1 μm (or the width of an average punctum) from a presynaptic peak as previously described (Burbea et al., 2002).

For quantification of MGL-1 abundance in the AIY, the fluorescence intensity was measured in at least 3 transgenic lines expressing [*Pmgl-1::mgl-1::gfp*] which were starved for two hours. The exposure time was adjusted in the well-fed wild-type background and images were acquired by an experimenter who was blind to the genotype. Pixel intensity was measured in ImageJ (NIH) by calculating the mean pixel intensity for the entire region of interest.

Western blot

To compare GLR-1 levels, proteins were extracted from well-fed and starved WT and *aak-2* mutants by sonication in lysis buffer (50mM Hepes pH7.5, 150mM NaCl, 10% glycerol, 1% Triton X-100, 1.5mM MgCl2, 1mM EDTA and protease inhibitors). Protein concentration was then determined by NanoDrop 2000c spectrophotometer (Thermo Scientific). Similar amount of protein for each condition was then subjected to 8% SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad) and blotted by anti-GFP antibody (RRID:AB_162553) as described previously (Xie and Roy, 2015).

Semi-qRT-PCR

RNA extraction was performed in well-fed and starved animals as described previously (Lu and Roy, 2015). Similar quantities of RNA from each sample were used to generate comparable amplicon levels for each gene tested. PCR was performed using gene-specific primers for each query gene for 15 cycles with ProtoScript M-MuLV *Taq* RT-PCR Kit (NEB, E6400S).

Acknowledgements

We would like to thank Cori Bargmann, Sreecanth Chalasani and Joshua Kaplan for reagents, along with Michael Hendricks for both reagents and very helpful discussion. We are grateful to our fellow lab members, and I am particularly indebted to my colleagues Francois Fagotto and Monique Zetka for their support. We also thank Mohammad R. Raeesi N for his help with image processing and analysis and also the Caenorhabditis Genetic Center for *C. elegans* strains. This work was supported by funds awarded by the Canadian Institutes of Health Research (CIHR).

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Figures

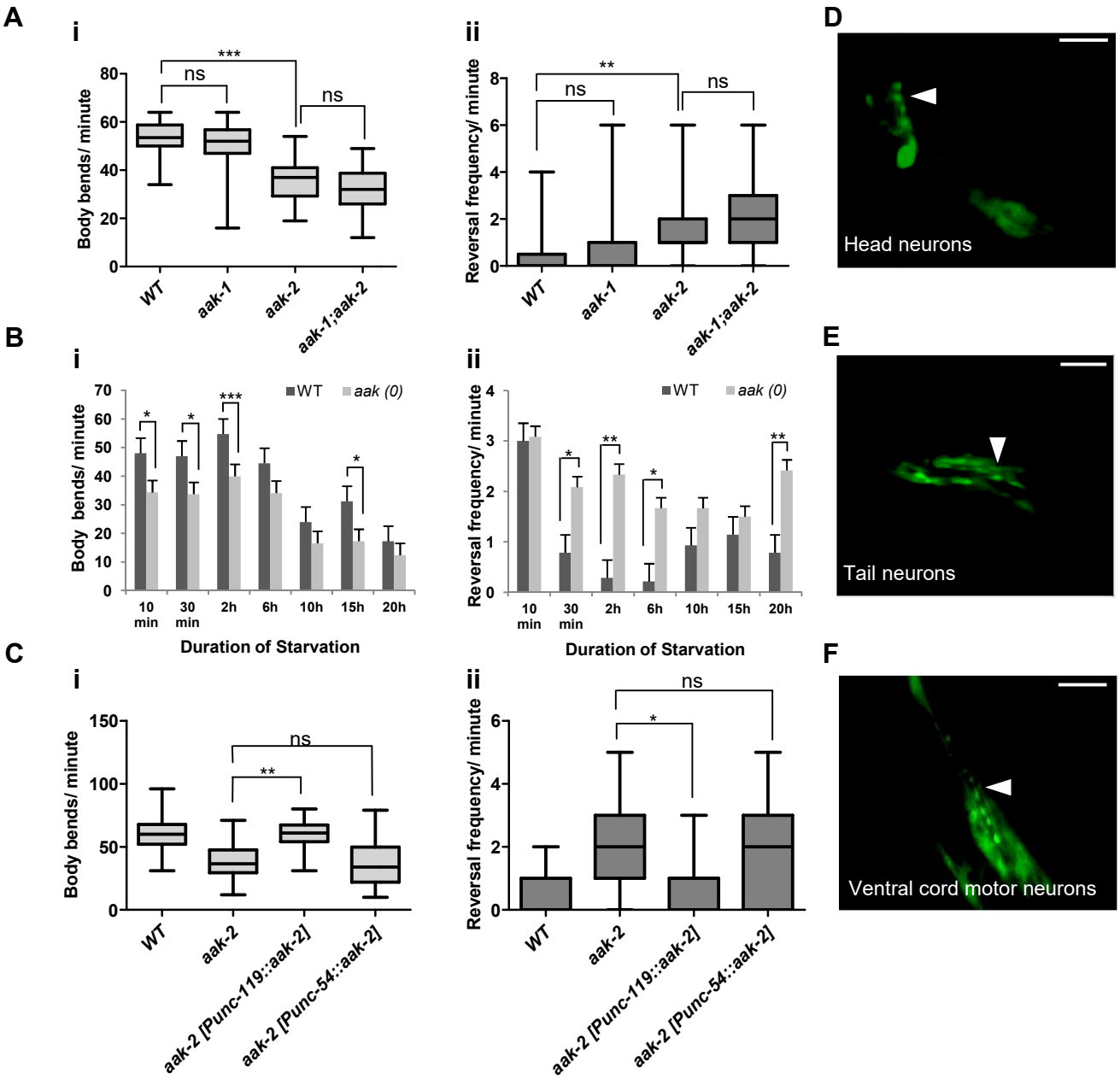

A
i
Body bends/ minute
***
ns
ns
WT
aak-1
aak-2
aak-1;aak-2
ii
Reversal frequency/ minute
**
ns
ns
WT
aak-1
aak-2
aak-1;aak-2
B
i
Body bends/ minute
WT
aak (0)
10 min
30 min
2h
6h
10h
15h
20h
Duration of Starvation
ii
Reversal frequency/ minute
WT
aak (0)
10 min
30 min
2h
6h
10h
15h
20h
Duration of Starvation
C
i
Body bends/ minute
ns
**
WT
aak-2
aak-2 [Punc-119::aak-2]
aak-2 [Punc-54::aak-2]
ii
Reversal frequency/ minute
ns
*
WT
aak-2
aak-2 [Punc-119::aak-2]
aak-2 [Punc-54::aak-2]
D
Head neurons
E
Tail neurons
F
Ventral cord motor neurons

Figure 2.1: Neuronal AMPK regulates distal exploratory behaviour in starved animals

(A) Starved *aak-2* mutants display defective transition between local and distal exploration indicated by (i) decreased forward locomotion and (ii) increased reversal rate during acute bouts of starvation. These phenotypes are not dependent on *aak-1* (n>40), (one-way ANOVA **p<0.001, ***p<0.0001).

(B) AMPK mutants display persistent decreased forward locomotion rate (i) and increased reversal frequency (ii) during periods of acute starvation (n>10), Error bars represent +/− SEM (Student's t-test, *p<0.05, **p<0.001, ***p<0.0001).

(C) *aak-2* reconstitution within the nervous system using the pan-neural [P*unc-119::aak-2*] transgene rescues the defective distal exploratory behaviour in starved *aak-2* mutants while its reconstitution within the body wall muscle using [P*unc-54::aak-2*] transgene does not improve the distal exploratory behaviour of *aak-2* mutants (n>15), (one-way ANOVA *p<0.05, **p<0.001).

(**D, E, F)** *aak-2* is highly expressed throughout the nervous system. Scale bars are 40 um.

In the box and whisker plots (A, C) the central line is the median, the edges of the box are the 25th and 75th percentiles, and the whiskers extend to the most extreme data points.

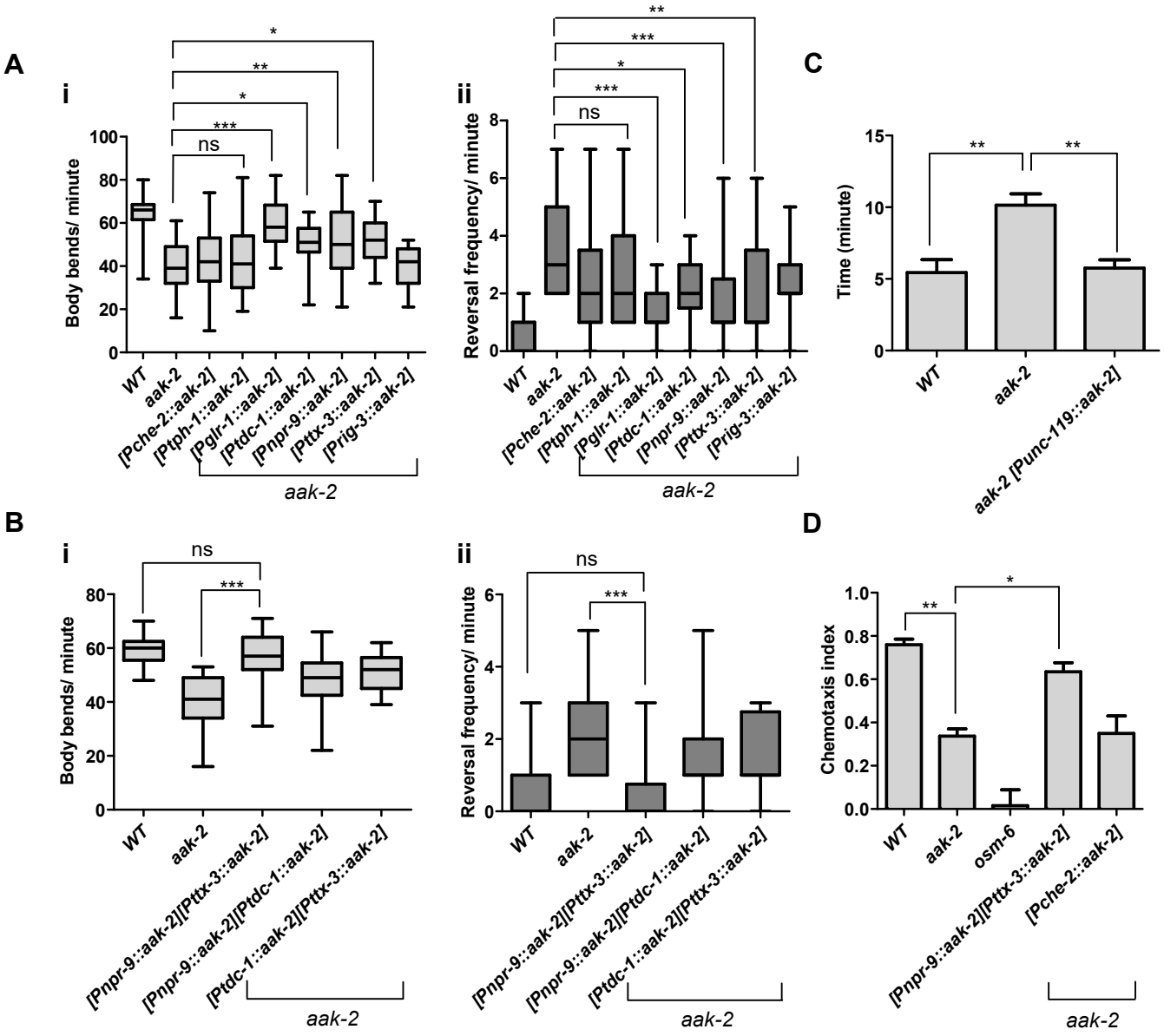

A
i
Body bends/ minute
ns
*
**
***
WT
aak-2
[Pche-2::aak-2]
[Ptph-1::aak-2]
[Pglr-1::aak-2]
[Ptdc-1::aak-2]
[Pnpr-9::aak-2]
[Pttx-3::aak-2]
[Prig-3::aak-2]
aak-2
ii
Reversal frequency/ minute
C
Time (minute)
WT
aak-2
aak-2 [Punc-119::aak-2]
B
i
Body bends/ minute
[Pnpr-9::aak-2][Pttx-3::aak-2]
[Pnpr-9::aak-2][Ptdc-1::aak-2]
[Ptdc-1::aak-2][Pttx-3::aak-2]
ii
Reversal frequency/ minute
D
Chemotaxis index
osm-6
[Pnpr-9::aak-2][Pttx-3::aak-2]
[Pche-2::aak-2]

Figure 2.2: AMPK acts in the AIB and AIY interneurons to integrate chemosensory signals and trigger distal exploratory behaviour in starved animals

(A) Targeted expression of *aak-2* within the GLR-1-expressing neurons [P*glr-1::aak-2*], RIM neurons [P*tdc-1::aak-2*], AIB interneurons [P*npr-9::aak-2*] and AIY interneurons [P*ttx-3::aak-2*], but not chemosensory neurons [P*che-2::aak-2*], serotonergic neurons [P*tph-1::aak-2*] or AVA neurons [P*rig-3::aak-2*] partially restores the distal exploratory defect in starved *aak-2* mutants by affecting both forward locomotion (i) and reversal frequency (ii) (n>20), (one-way ANOVA *p<0.05, **p<0.001, ***p<0.0001).

WT is significantly different from [P*che-2::aak-2*], [P*tph-1::aak-2*] and [P*rig-3::aak-2*] (one-way ANOVA ***p<0.0001).

WT is significantly different from [P*npr-9::aak-2*] and [P*tdc-1::aak-2*], (one-way ANOVA **p<0.001).

WT is significantly different from [P*glr-1::aak-2*] and [P*ttx-3::aak-2*], (one-way ANOVA *p<0.05).

(B) Simultaneous rescue of *aak-2* in the AIB and AIY using [P*npr-9::aak-2*][P*ttx-3::aak-2*] transgenes is sufficient to completely restore the defective exploratory behaviour typical of starved *aak-2* mutants (n>20), (one-way ANOVA ***p<0.0001).

(C) Starved worms were placed 1.5 cm away from a spot of food (fresh OP50) and the time required to encounter the food was monitored for the worms that found food within 16 minutes. *aak-2* mutants display defective food detection indicated by increased time spent to find food and this defect can be rescued by specific expression of *aak-2* throughout the nervous system using [P*unc-119::aak-2*] transgene (n>10).

(D) Targeted expression of *aak-2* within the AIB and AIY interneurons, but not the chemosensory neurons rescue the defective chemosensation of *aak-2* mutants toward IAA (n>300). Error bars in (C, D) represent +/− SEM (one-way ANOVA *p<0.05, **p<0.001).

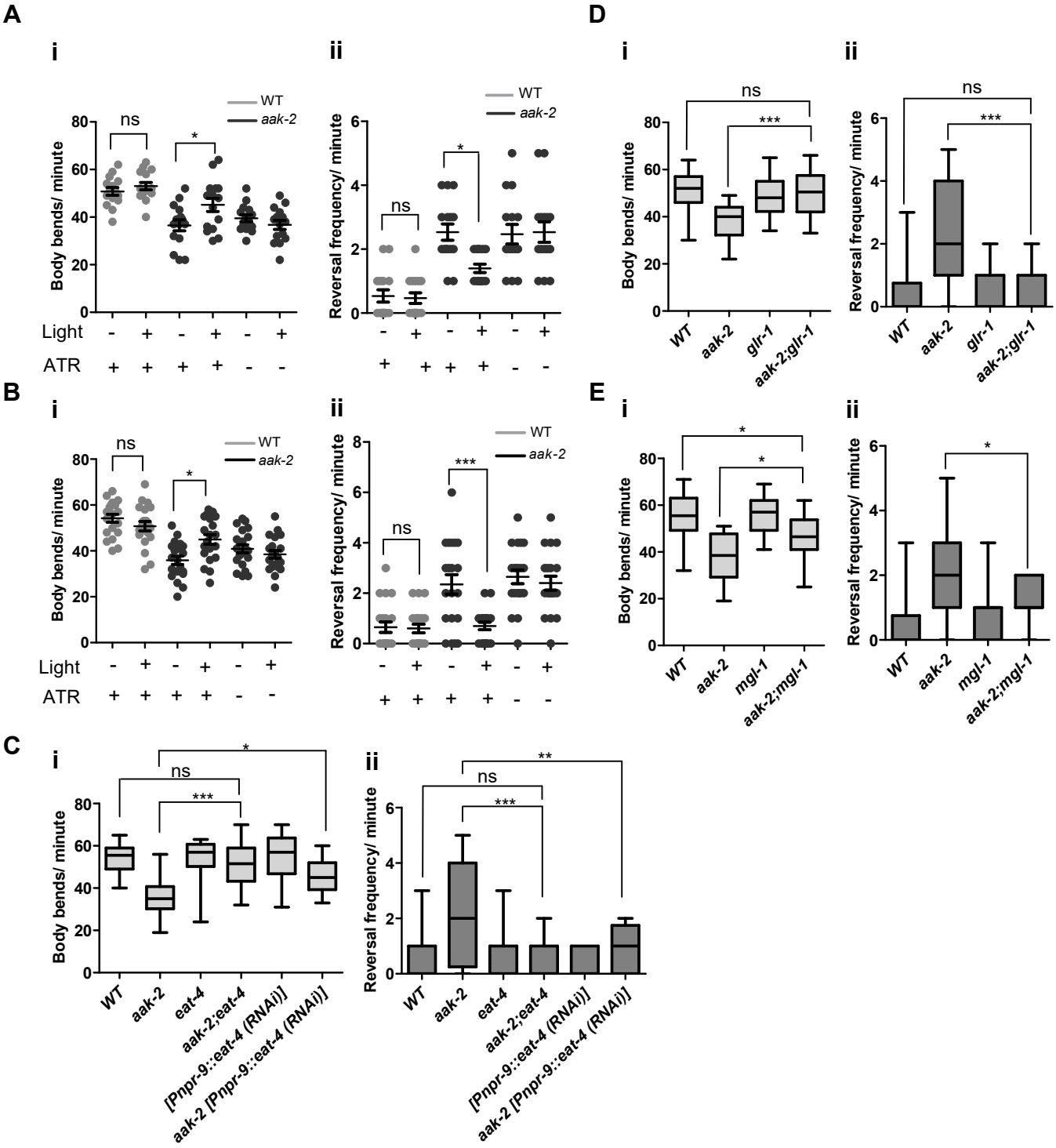

A
i
ii
WT
aak-2
Body bends/ minute
Reversal frequency/ minute
ns
*
Light
ATR
B
***
C
**
D
E
glr-1
aak-2;glr-1
mgl-1
aak-2;mgl-1
eat-4
aak-2;eat-4
[Pnpr-9::eat-4 (RNAi)]
aak-2 [Pnpr-9::eat-4 (RNAi)]

Figure 2.3: AMPK regulates glutamatergic signaling

(A, B) ChR2-mediated activation of AIY interneurons (A), or ARCH -mediated silencing of AIB interneurons (B) in starved *aak-2* mutants grown in the presence of all-trans retinal (ATR) induces distal exploration by suppressing reversals and inducing forward locomotion (n>15), Error bars represent +/− SEM (two-way ANOVA *p<0.05, ***p<0.0001).

(C, D, E) Compromised *eat-4*, *glr-1* or *mgl-1* function rescues the defective exploratory behaviour of starved *aak-2* mutants by inducing forward locomotion (i) and suppressing reversals (ii) (n>20), (one-way ANOVA *p<0.05, **p<0.001, ***p<0.0001). In the box and whisker plots (C, D, E) the central line is the median, the edges of the box are the 25th and 75th percentiles, and the whiskers extend to the most extreme data points.

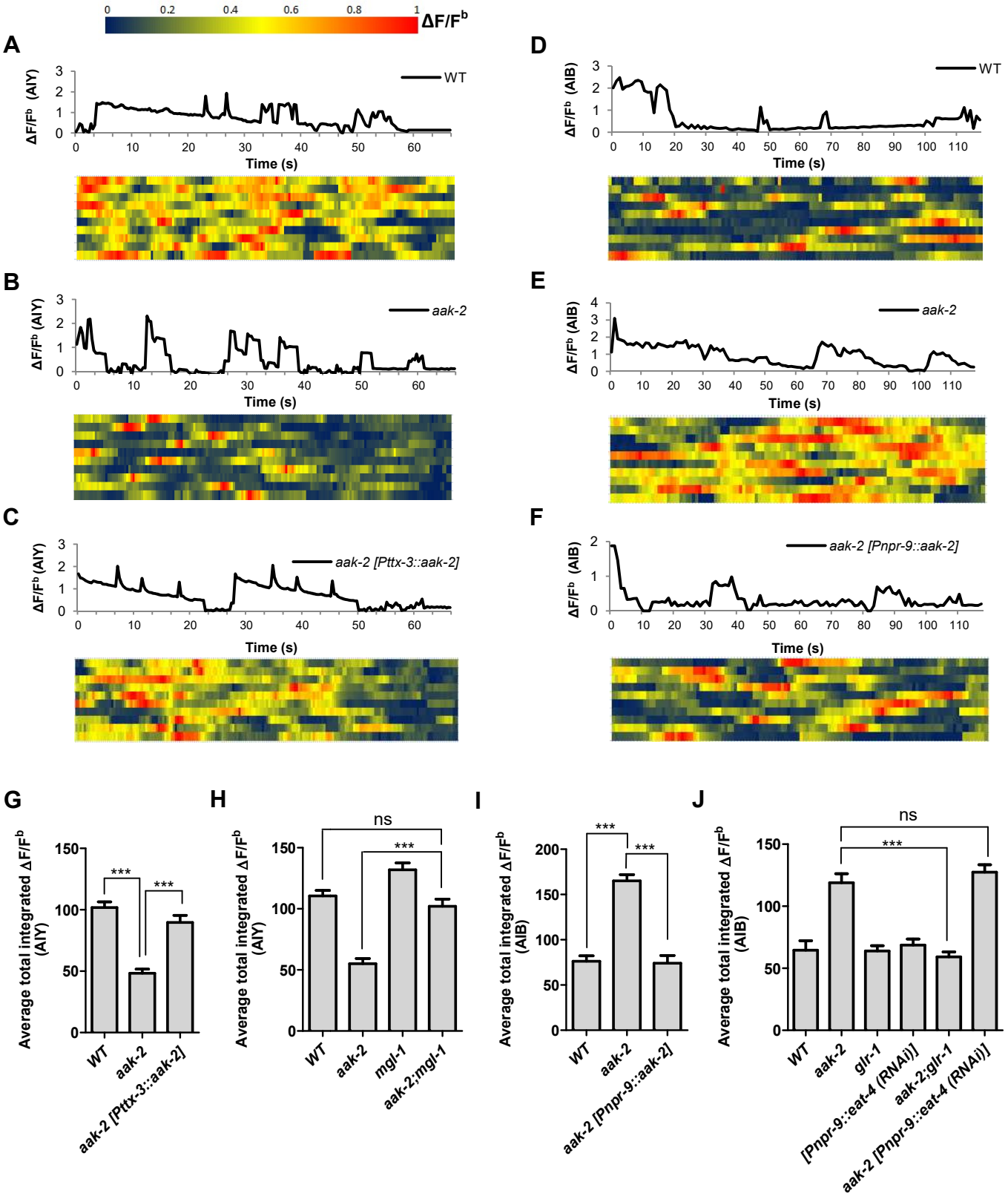

0 0.2 0.4 0.6 0.8 1 ΔF/F^b
A
ΔF/F^b (AIY)
WT
Time (s)
D
ΔF/F^b (AIB)
WT
Time (s)
B
ΔF/F^b (AIY)
aak-2
Time (s)
E
ΔF/F^b (AIB)
aak-2
Time (s)
C
ΔF/F^b (AIY)
aak-2 [Pttx-3::aak-2]
Time (s)
F
ΔF/F^b (AIB)
aak-2 [Pnpr-9::aak-2]
Time (s)
G
Average total integrated ΔF/F^b (AIY)
WT
aak-2
aak-2 [Pttx-3::aak-2]
H
Average total integrated ΔF/F^b (AIY)
ns
WT
aak-2
mgl-1
aak-2;mgl-1
I
Average total integrated ΔF/F^b (AIB)
WT
aak-2
aak-2 [Pnpr-9::aak-2]
J
Average total integrated ΔF/F^b (AIB)
ns
WT
aak-2
glr-1
[Pnpr-9::eat-4 (RNAi)]
aak-2;glr-1
aak-2 [Pnpr-9::eat-4 (RNAi)]

Figure 2.4: AMPK regulates AIB and AIY neuronal activity

(A, B, C, D, E, F) A sample $\Delta F/F^b$ plot showing spontaneous changes in the AIY and AIB neuronal activity in starved individuals during the course of a 65 and 120 seconds imaging window, respectively. These plots are the basis of the data in G, H, I and J. The heatmaps show normalized G-CaMP1 and G-CaMP3 traces in AIY and AIB, respectively in multiple animals.

(G) The average total integrated fluorescence intensity ($\Delta F/F^b$) over the course of a 65 seconds window. AIY neuronal activity is reduced in starved *aak-2* mutants and this reduction in the AIY neuronal activity is restored by targeted expression of *aak-2* within the AIY interneurons using the AIY-specific [P*ttx-3::aak-2*] transgene (n>10).

(H) Removal of *mgl-1* in *aak-2* mutants rescues the reduced AIY neuronal activity observed in starved *aak-2* mutants (n>10).

(I) The average total integrated fluorescence intensity ($\Delta F/F^b$) in AIB is increased upon removal of *aak-2* in starved animals and this increase is reversed by expression of *aak-2* within the AIB using the AIB-specific [P*npr-9::aak-2*] transgene (n>10).

(J) Removal of *glr-1,* but not *eat-4,* restores the increased AIB neuronal activity in starved *aak-2* mutants (n>10).

Error bars represent +/− SEM (one-way ANOVA ***p<0.0001).

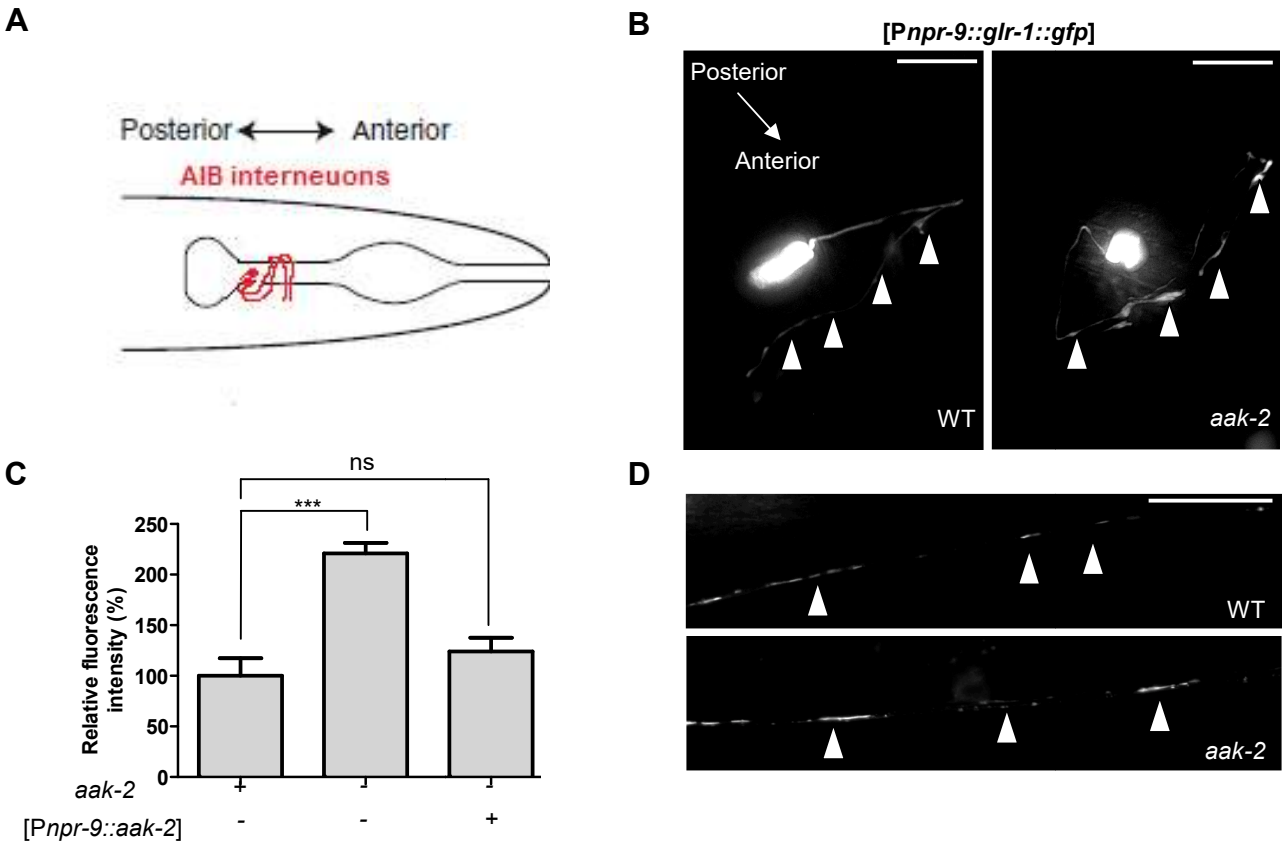

A
Posterior
Anterior
AIB interneuons
B
[Pnpr-9::glr-1::gfp]
Posterior
Anterior
WT
aak-2
C
ns
***
Relative fluorescence intensity (%)
250
200
150
100
50
0
aak-2
+
-
[Pnpr-9::aak-2]
-
+
D
WT
aak-2
E
***
Relative fluorescence intensity (%)
300
200
100
0
aak-2
+
-
F
***
Width (um)
1.5
1.0
0.5
0.0
+
-

Figure 2.5: AMPK regulates GLR-1 abundance in the AIB neuronal process and the ventral nerve cord

(A) Schematic representation showing AIB interneurons that extend their processes into the nerve ring.

(B, C) Representative images of [P*npr-9::glr-1::gfp*] in the AIB neuronal processes in starved WT and *aak-2* mutants (B). GLR-1::GFP level is significantly increased in the AIB neuronal processes in the *aak-2* mutants compared to WT animals under starvation condition. Targeted expression of *aak-2* within the AIB interneurons rescues the increased GLR-1::GFP level in starved *aak-2* mutants (C). At least 3 [P*npr-9::glr-1::gfp*] transgenic lines were separately examined for this experiment (n>20), Error bars represent +/− SEM (one-way ANOVA ***p<0.0001). Scale bars are 10 um.

(D, E, F) Representative images of [P*glr-1::glr-1::gfp*] in the ventral nerve cord (D). GLR-1::GFP level (E) and puncta width (F) are significantly increased in the ventral nerve cord of starved *aak-2* mutants compared to WT animals. (n>20), Error bars represent +/− SEM (Student's 2-tailed t test ***p<0.0001). Scale bars are 10 um.

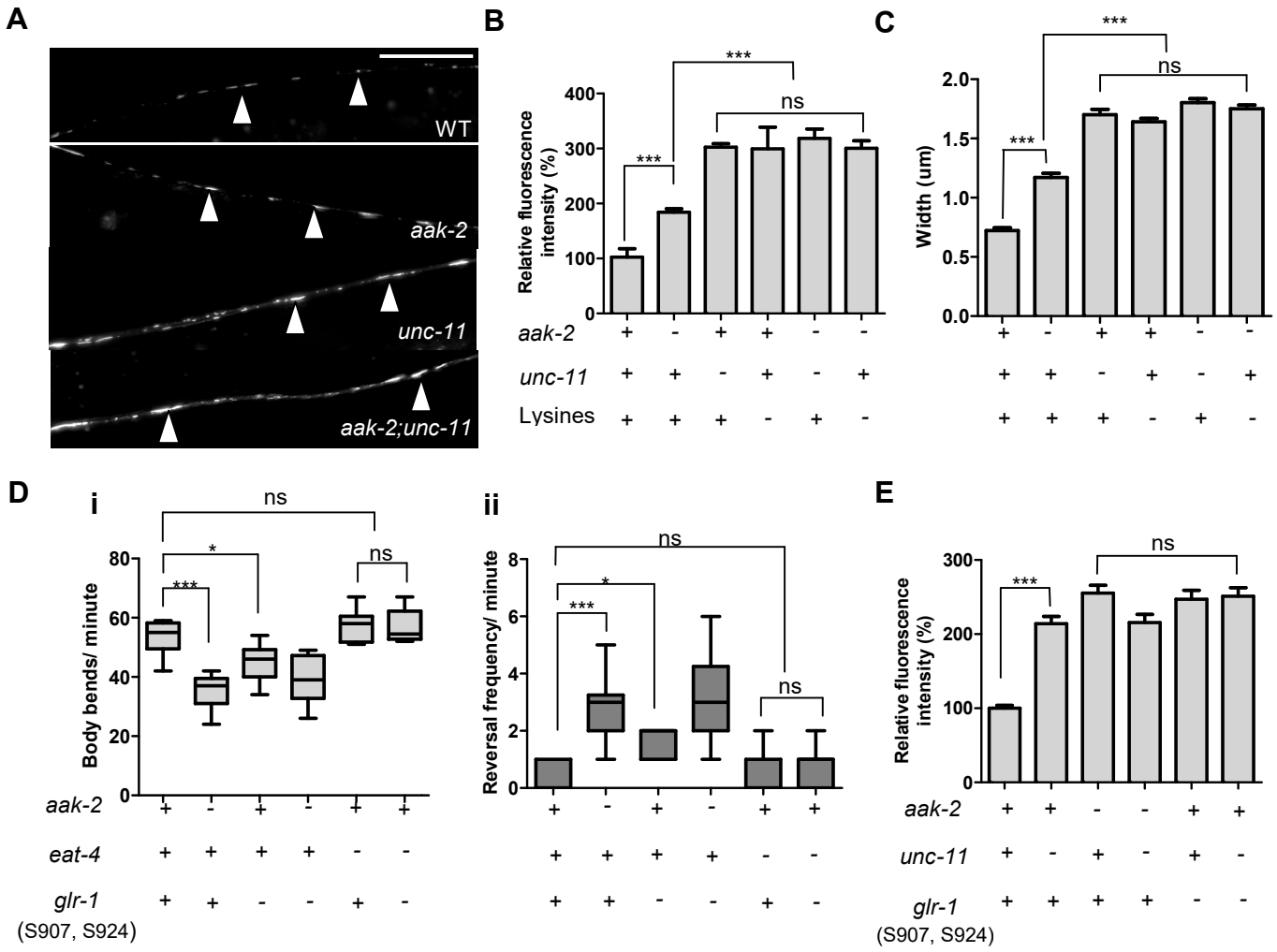

A
WT
aak-2
unc-11
aak-2;unc-11
B
Relative fluorescence intensity (%)
400
300
200
100
0
***
ns
aak-2 + - + + - -
unc-11 + + - + - +
Lysines + + + - + -
C
Width (um)
2.0
1.5
1.0
0.5
0.0
***
ns
+ - + + - -
+ + - + - +
+ + + - + -
D
i
Body bends/ minute
80
60
40
20
0
ns
*
***
ns
aak-2 + - + - + +
eat-4 + + + + - -
glr-1 + + - - + -
(S907, S924)
ii
Reversal frequency/ minute
8
6
4
2
0
ns
*
***
ns
+ - + - + +
+ + + + - -
+ + - - + -
E
Relative fluorescence intensity (%)
300
200
100
0
***
ns
aak-2 + + - - + +
unc-11 + - + - + -
glr-1 + + + + - -
(S907, S924)

Figure 2.6: AMPK directly regulates GLR-1 abundance through endocytic pathway

(A) Representative images of [P*glr-1::glr-1::gfp*] in the ventral nerve cord in starved WT, *aak-2*, *unc-11* and *aak-2; unc-11* mutants. Scale bars are 10 um.

(B, C) Similar to starved *aak-2* mutants, GLR-1::GFP level (B) and puncta width (C) are increased upon removal of *unc-11* or mutation of 4 lysines required for GLR-1 ubiquitination and endocytosis [P*glr-1::glr-1(4KR)::gfp*]. Increased GLR-1::GFP level and enhanced puncta width are not further enhanced upon depletion of *aak-2* in GLR-1 endocytosis defective mutants (n>25), Error bars represent +/− SEM (one-way ANOVA ***p<0.0001).

(D) Mutating AMPK phosphorylation sites S907 and S924 to non-phosphorylable alanine residues present in the cytoplasmic domain of GLR-1 (S907A, S924A) results in increased reversal frequency compounded with reduced forward locomotion and this defect can be rescued by introducing *eat-4* mutations (n>10), (one-way ANOVA *p<0.05, **p<0.001, ***p<0.0001). In the box and whisker plots the central line is the median, the edges of the box are the 25th and 75th percentiles, and the whiskers extend to the most extreme data points.

(E) Expression of the non-phosphorylable variant of [P*npr-9*::*glr-1*(S907A, S924A)::*gfp*] results in increased GLR-1::GFP level in the AIB neuronal process and it is not further increased upon disruption of endocytosis in *unc-11* mutants. At least 3 transgenic lines expressing non-phosphorylable variant of GLR-1 (S907A, S924A) were separately examined for this experiment (n>15), Error bars represent +/− SEM (one-way ANOVA ***p<0.0001).

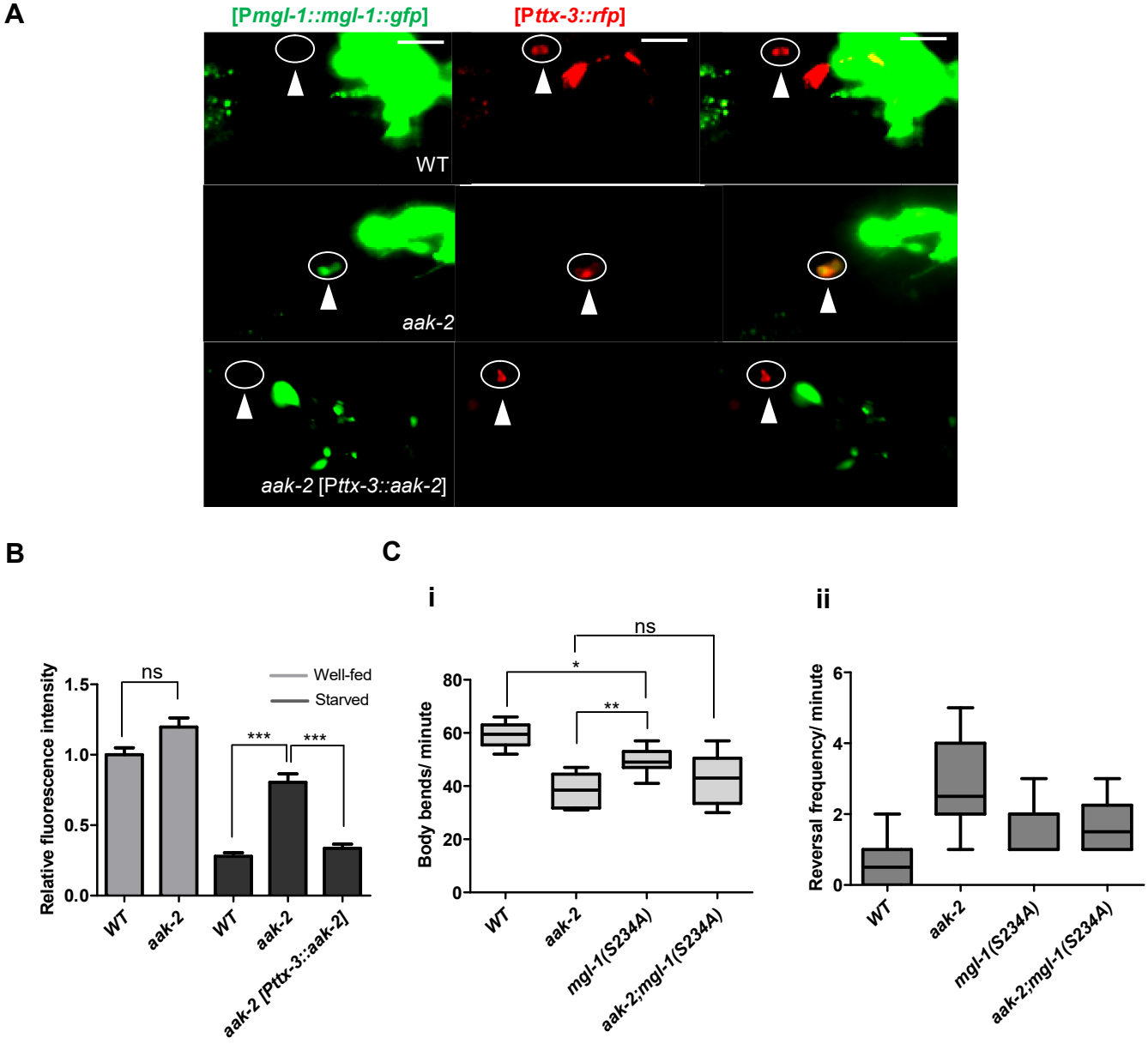

A
[Pmgl-1::mgl-1::gfp]
[Pttx-3::rfp]
WT
aak-2
aak-2 [Pttx-3::aak-2]
B
Relative fluorescence intensity
Well-fed
Starved
ns
***
***
WT
aak-2
WT
aak-2
aak-2 [Pttx-3::aak-2]
C
i
Body bends/ minute
ns
*
**
WT
aak-2
mgl-1(S234A)
aak-2;mgl-1(S234A)
ii
Reversal frequency/ minute
WT
aak-2
mgl-1(S234A)
aak-2;mgl-1(S234A)

Figure 2.7: AMPK regulates the abundance of MGL-1 levels, while direct phosphorylation may affect MGL-1 function.

(A, B) Starvation reduces [MGL-1::GFP] levels significantly in the AIY [*Pttx-3*::RFP] of WT animals and to a lesser extent in *aak-2* mutants (AIY neuron is shown in the circle). Increased [MGL-1::GFP] levels in starved *aak-2* mutants can be reversed by targeted expression of *aak-2* in the AIY interneurons using [P*ttx-3::aak-2*] transgene (n>15), Error bars represent +/− SEM (two-way ANOVA ***p<0.0001). Scale bars are 20 um. **(C)** Mutating the consensus AMPK phosphorylation site at serine 234 to a non-phosphorylable alanine in MGL-1 (S234A) resulted in a reduced forward locomotion rate that is not further reduced in *aak-2; mgl-1* (S234A) double mutants (i) (n>10), (one-way ANOVA *p<0.05, **p<0.001). In the box and whisker plots the central line is the median, the edges of the box are the 25th and 75th percentiles, and the whiskers extend to the most extreme data points.

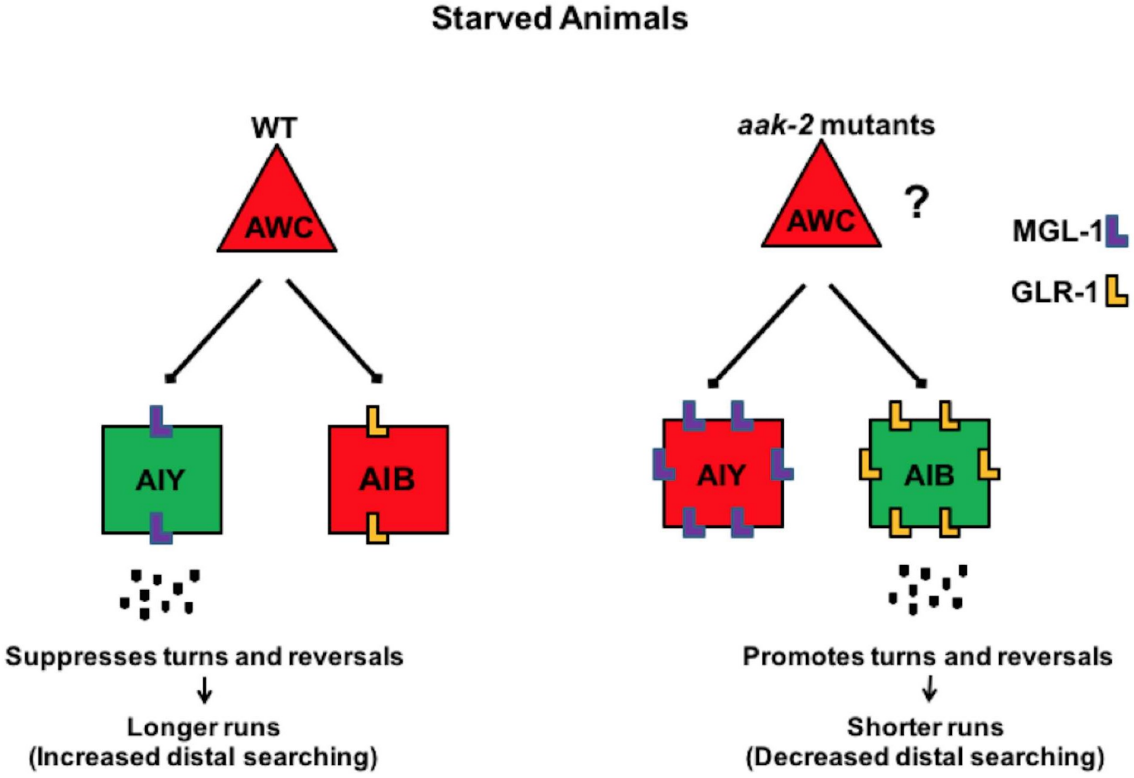

Starved Animals
WT
AWC
AIY
AIB
Suppresses turns and reversals
Longer runs
(Increased distal searching)
aak-2 mutants
AWC
?
AIY
AIB
Promotes turns and reversals
Shorter runs
(Decreased distal searching)
MGL-1
GLR-1

Figure 2.8: AMPK coordinates neuronal activity of the AIB and AIY interneurons by regulating their glutamatergic synaptic inputs in response to acute starvation

The schematic showing the proposed role of AMPK in the AIB and AIY interneurons for regulation of appropriate transition between local to distal exploration during starvation conditions. Green and red colors show depolarized and hyperpolarized neurons, respectively. AMPK regulates GLR-1-mediated synaptic inputs into AIB interneurons by directly phosphorylating GLR-1 and subsequently modulating its endocytosis and potentially its degradation leading to decreased AIB activity in starved animals. AMPK also regulates the MGL-1-dependent synaptic inputs into the AIY interneurons, not only through its effects on *mgl-1* mRNA levels but also by direct phosphorylation of MGL-1 protein. These functions of AMPK within the AIB and AIY interneurons collectively result in the transition between local exploration that occurs in well fed animals to distal exploration that occurs in starved animals, thus allowing them to explore their environment more extensively for energy resources.

Figure supplements

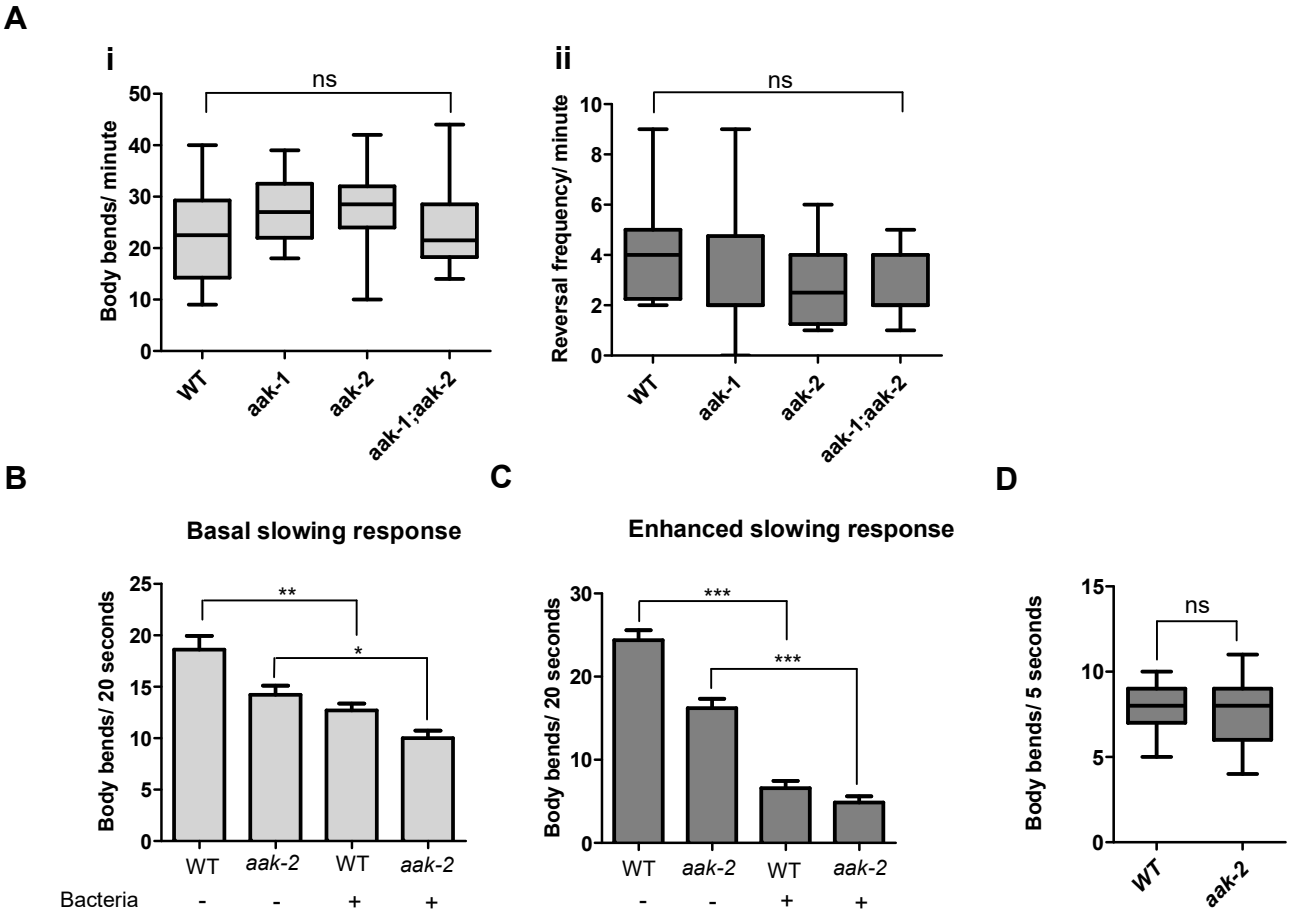


Figure S2.1: AMPK function is not required for other food-related behaviours rather than distal exploratory behaviour

(A) AMPK mutants display normal locomotory behaviour in the presence of food (i and ii) (n>20), (one-way ANOVA). In the box and whisker plots the central line is the median, the edges of the box are the 25th and 75th percentiles, and the whiskers extend to the most extreme data points.

(B) Well-fed *aak-2* mutants display normal basal locomotory behaviour once transferred to food (n>10).

(C) Starved *aak-2* mutants show normal enhanced slowing response once returned to food after 30 minutes of starvation (n>15).

(D) *aak-2* mutants display normal locomotory speed upon exposure to a gentle touch in the posterior part of their body in the absence of food (n>15). In (B, C) Error bars represent SEM (two-way ANOVA *p<0.05, **p<0.001, ***p<0.0001).

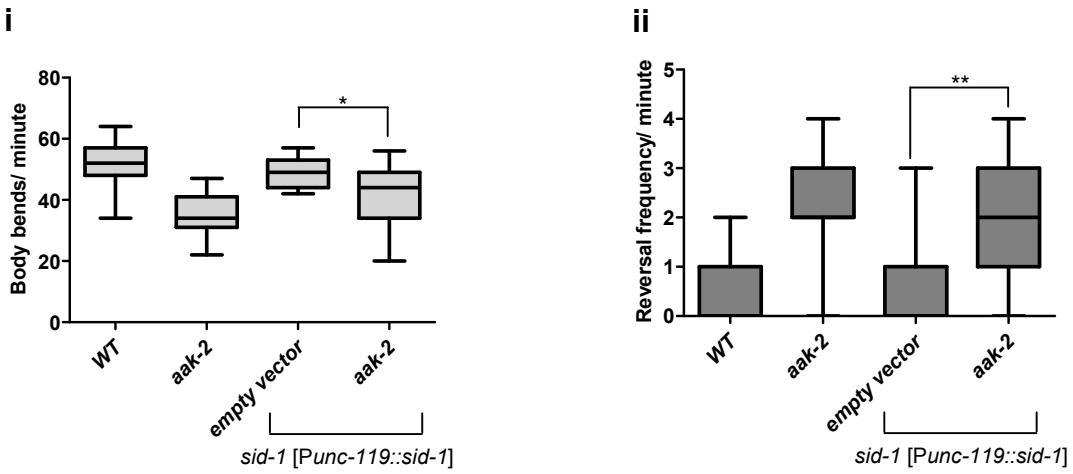


Figure S2.2: Reduced forward locomotion rate in *aak-2* mutants is not a consequence of developmental defect in their nervous system

AMPK depletion in the late L2 stage by knocking down *aak-2* in the nervous system resulted in decreased forward locomotion rate and increased reversal frequency under starvation condition (n>15), (one-way ANOVA *p<0.05, **p<0.001). In the box and whisker plots the central line is the median, the edges of the box are the 25th and 75th percentiles, and the whiskers extend to the most extreme data points.

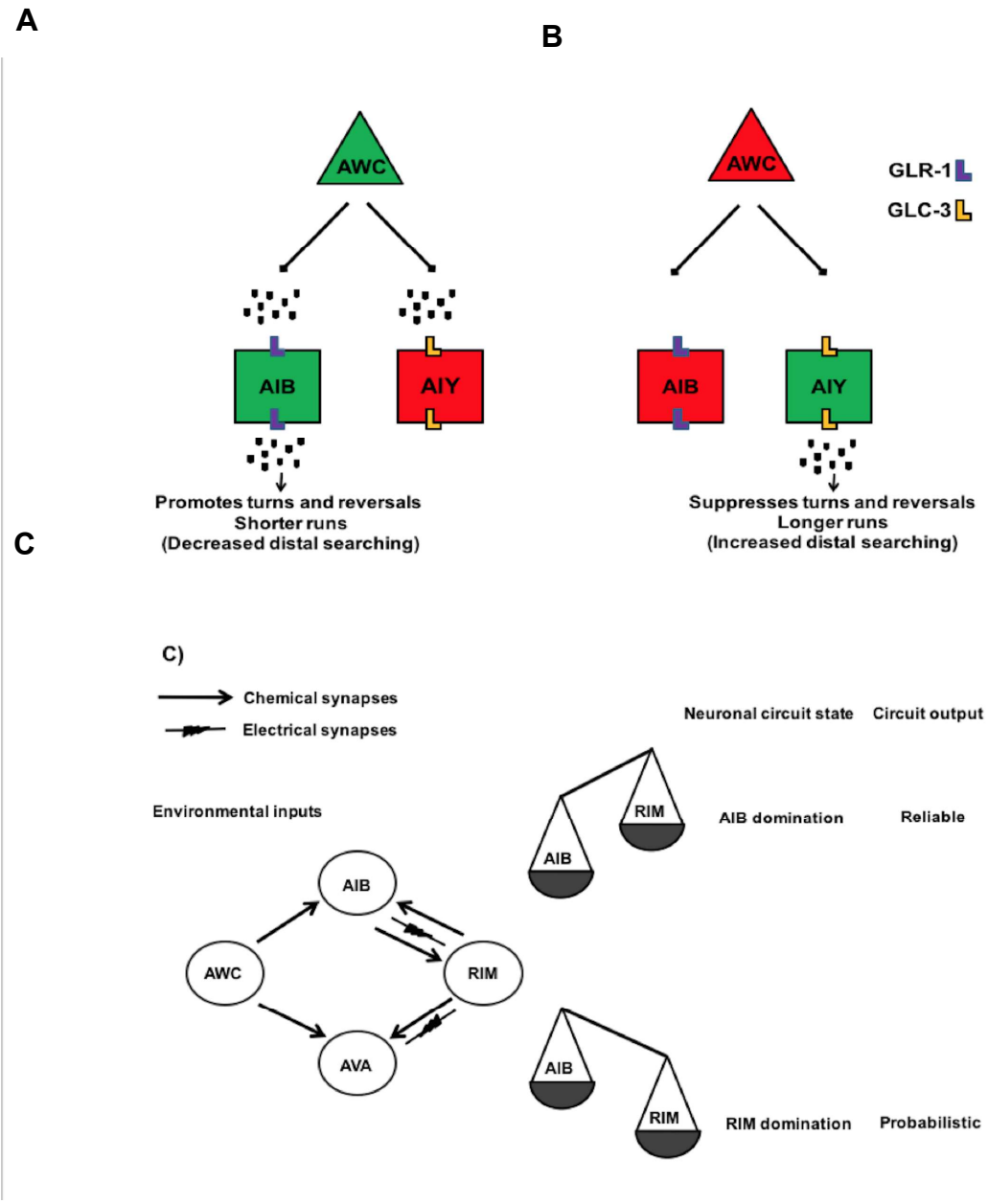

A
AWC
AIB
AIY
Promotes turns and reversals
Shorter runs
(Decreased distal searching)
B
AWC
GLR-1
GLC-3
AIB
AIY
Suppresses turns and reversals
Longer runs
(Increased distal searching)
C
C)
Chemical synapses
Electrical synapses
Environmental inputs
AIB
AWC
RIM
AVA
Neuronal circuit state
Circuit output
RIM
AIB
AIB domination
Reliable
AIB
RIM
RIM domination
Probabilistic

Figure S2.3: Neural circuits engaged in regulation of locomotory behaviour in *C. elegans*

(A) Schematic showing the proposed circuitry that regulates locomotory behaviour upon food removal. Green and red colors show depolarized and hyperpolarized neurons, respectively. This circuitry consists of AWC sensory neurons and AIB and AIY interneurons. Food or odour removal results in AWC depolarization. Upon its depolarization, AWC releases glutamate at AWC/AIB and AWC/AIY synapses. The released glutamate then eventually acts on AMPA-type glutamate receptor GLR-1 in the AIB or glutamate-gated chloride channel GLC-3 in the AIY resulting in their depolarization and hyperpolarization, respectively. This neuronal pattern ultimately promotes reversals and turns resulting in shorter runs and increased local exploration (Chalasani et al., 2007).

(B) Once AWC neuronal activity reduces, it however results in a reduction in the AIB neuronal activity and AIY depolarization that collectively leads to suppression of reversals and turns and inducing longer runs and distal exploration (Chalasani et al., 2007; Gordus et al., 2015).

(C) Simplified wiring diagram showing synaptic connection between AWC sensory neurons and three AIB, RIM and AVA interneurons in a circuit linking AWC to reversal behaviour. RIM, AIB and AVA participate in the overall network states. Once RIM is dominated, the circuit output is toward more probabilistic and upon domination of AIB the circuit output is more reliable (Model proposed by Gordus et al., 2015).

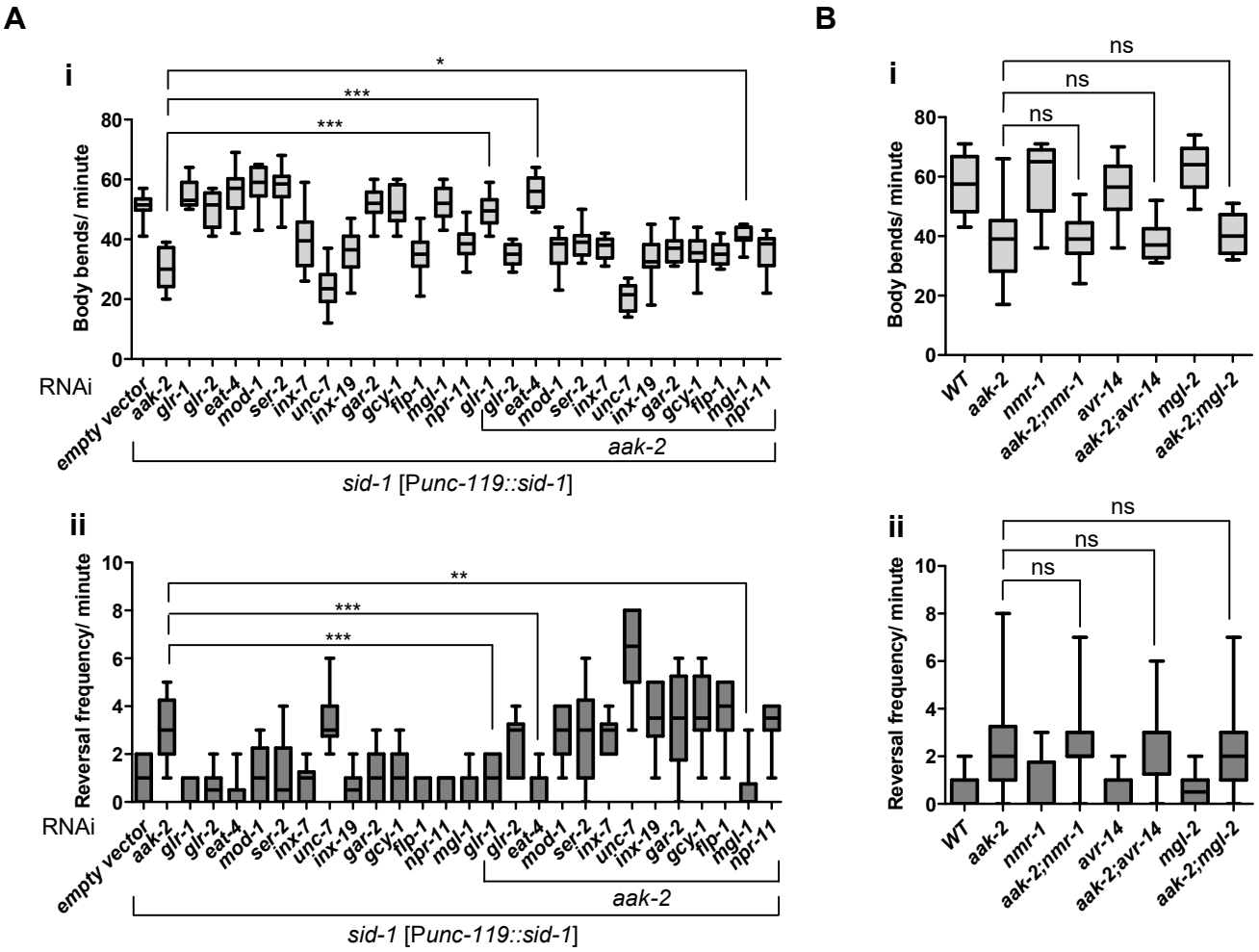

A
i
Body bends/ minute
RNAi
empty vector
aak-2
glr-1
glr-2
eat-4
mod-1
ser-2
inx-7
unc-7
inx-19
gar-2
gcy-1
flp-1
mgl-1
npr-11
aak-2
sid-1 [Punc-119::sid-1]
ii
Reversal frequency/ minute
B
i
ns
WT
aak-2
nmr-1
aak-2;nmr-1
avr-14
aak-2;avr-14
mgl-2
aak-2;mgl-2
ii

Figure S2.4: *glr-1*, *eat-4* and *mgl-1* display epistatic relationship to *aak-2*

(A) *sid-1* [P*unc-119::sid-1+ Pmyo-2::mCherry*] or *aak-2*; *sid-1* [*Punc-119::sid-1+ Pmyo-2::mCherry*] young adult animals grown on HT-115 *E. coli* clones expressing double stranded RNAi targeting various RNAi clones were starved for 1 hour and their locomotory behaviour was assessed (n>10). Among all the potential phosphorylation targets of AMPK within the AIB and AIY interneurons, *glr-1*, *eat-4* and *mgl-1* displayed epistatic relationship with *aak-2* in both forward (i) and backward locomotion (ii).

(B) Depletion of *nmr-1*, *avr-14* and *mgl-2* in *aak-2* background did not result in any additive or epistatic relationship with *aak-2* (n>10). Error bars represent SEM (one-way ANOVA *p<0.05, **p<0.001, ***p<0.0001).

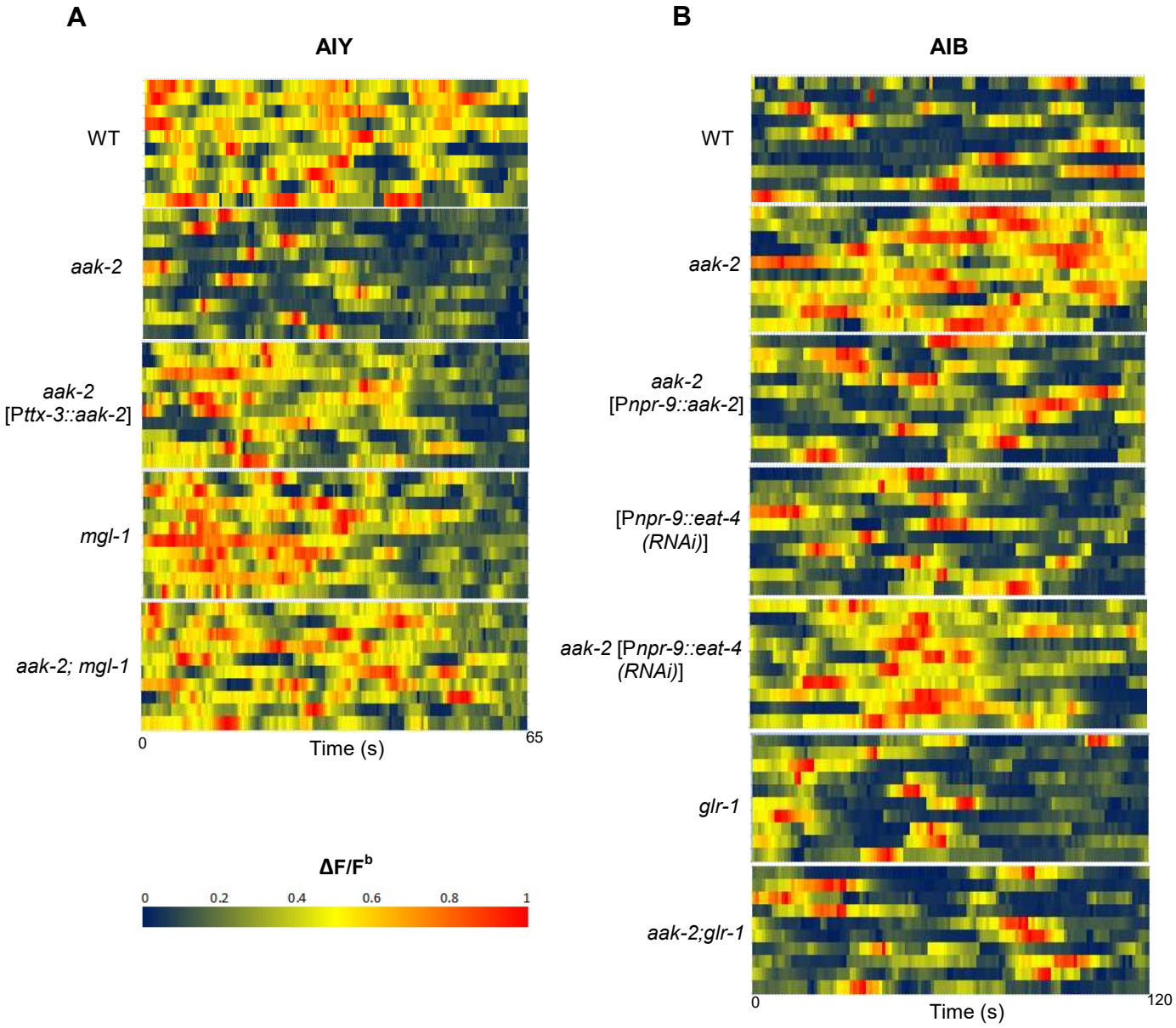

A
AIY
WT
aak-2
aak-2 [Pttx-3::aak-2]
mgl-1
aak-2; mgl-1
0
65
Time (s)
ΔF/F^b
0
0.2
0.4
0.6
0.8
1
B
AIB
WT
aak-2
aak-2 [Pnpr-9::aak-2]
[Pnpr-9::eat-4 (RNAi)]
aak-2 [Pnpr-9::eat-4 (RNAi)]
glr-1
aak-2;glr-1
0
120
Time (s)

Figure S2.5: Heat maps showing AIY and AIB spontaneous neuronal activity in starved animals

(A, B) Starved animals were placed on 10% agarose pads and the AIY and AIB neuronal activity was measured for 65 and 120 seconds respectively (n>10). G-CaMP imaging was performed on a Zeiss microscope. Images were captured by AxioVision software at 3Hz for AIY and 2Hz for AIB and were analyzed by imageJ. Baseline F (F^b) was measured as the global minima for 5-10 frames over the duration of each time-lapse sequence and the $\Delta F/F^b$ results were normalized between 0 and 1 for each animal.

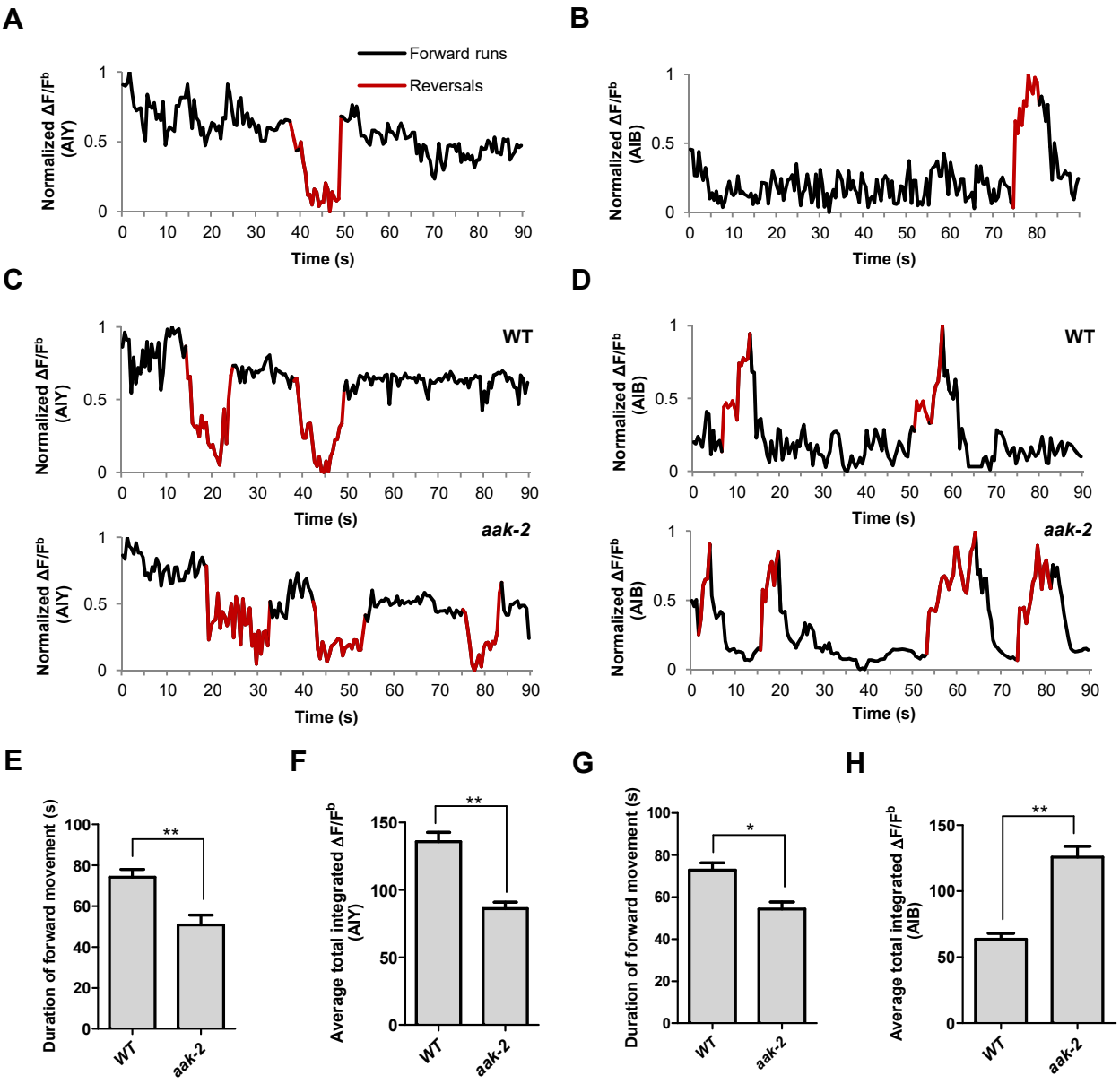

A
Forward runs
Reversals
Normalized ΔF/Fb (AIY)
Time (s)
B
Normalized ΔF/Fb (AIB)
Time (s)
C
WT
aak-2
Normalized ΔF/Fb (AIY)
Time (s)
D
WT
aak-2
Normalized ΔF/Fb (AIB)
Time (s)
E
Duration of forward movement (s)
WT
aak-2
F
Average total integrated ΔF/Fb (AIY)
WT
aak-2
G
Duration of forward movement (s)
WT
aak-2
H
Average total integrated ΔF/Fb (AIB)
WT
aak-2

Figure S2.6: AIY and AIB spontaneous neuronal activity in starved freely behaving animals

(A, B) A sample normalized $\Delta F/F^b$ plot showing spontaneous changes in the AIY and AIB neuronal activity in starved individuals during the course of a 90 seconds imaging window. AIY calcium levels decreases at the onset of reversals and reach the maximum levels at the onset of forward locomotion and it remains consistently high during forward runs (n>6) (A). AIB calcium levels increases during reversals and it gradually decreases and return to baseline values with the resumption of forward movement (n>6) (B).

(C, D) *aak-2* mutants display higher reversal frequency and decreased duration of forward runs which is correlated with increased AIB calcium levels and decreased AIY neuronal activity (n>6).

(E, F) The decreased forward locomotion rate and increased reversal frequency in *aak-2* mutants is consistently correlated by increased AIB neuronal activity and decreased AIY activity (n>6).

Error bars represent +/− SEM (two-way ANOVA *P<0.05, **p<0.001).

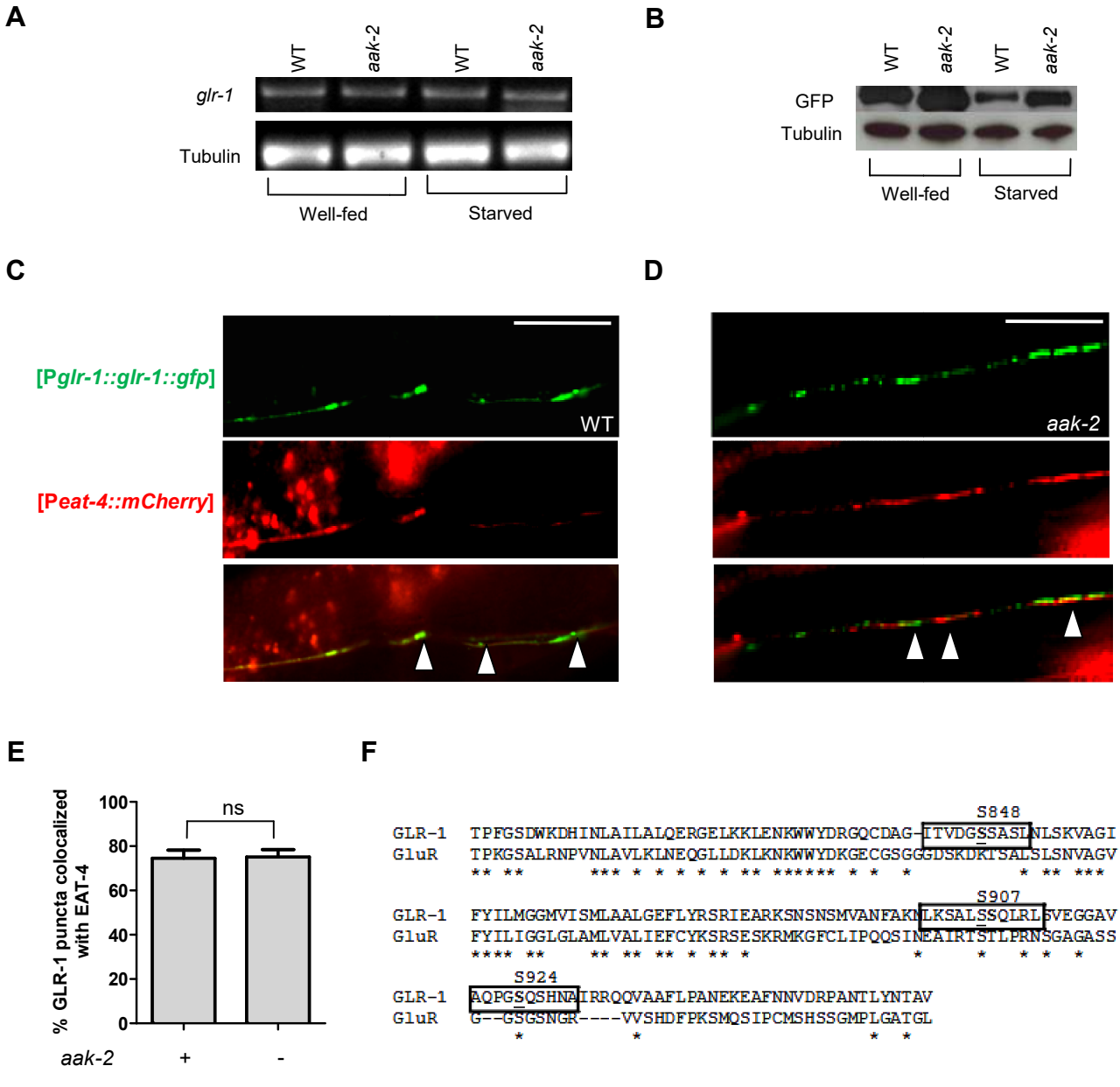

A
WT
aak-2
WT
aak-2
glr-1
Tubulin
Well-fed
Starved
B
WT
aak-2
WT
aak-2
GFP
Tubulin
Well-fed
Starved
C
[Pglr-1::glr-1::gfp]
[Peat-4::mCherry]
WT
D
aak-2
E
% GLR-1 puncta colocalized with EAT-4
100
80
60
40
20
0
ns
aak-2
+
-
F
S848
GLR-1 TPFGSDWKDHINLAILALQERGELKKLENKWWYDRGQCDAG-ITVDGSSASLNLSKVAGI
GluR TPKGSALRNPVNLAVLKLNEQGLLDKLKNKWWYDKGECGSGGGDSKDKTSALSLSNVAGV
S907
GLR-1 FYILMGGMVISMLAALGEFLYRSRIEARKSNSNSMVANFAKNLKSALSSQLRLSVEGGAV
GluR FYILIGGLGLAMLVALIEFCYKSRSESKRMKGFCLIPQQSINEAIRTSTLPRNSGAGASS
S924
GLR-1 AQPGSQSHNAIRRQQVAAFLPANEKEAFNNVDRPANTLYNTAV
GluR G--GSGSNGR----VVSHDFPKSMQSIPCMSHSSGMPLGATGL

Figure S2.7: AMPK regulates GLR-1 abundance by affecting steady state protein levels

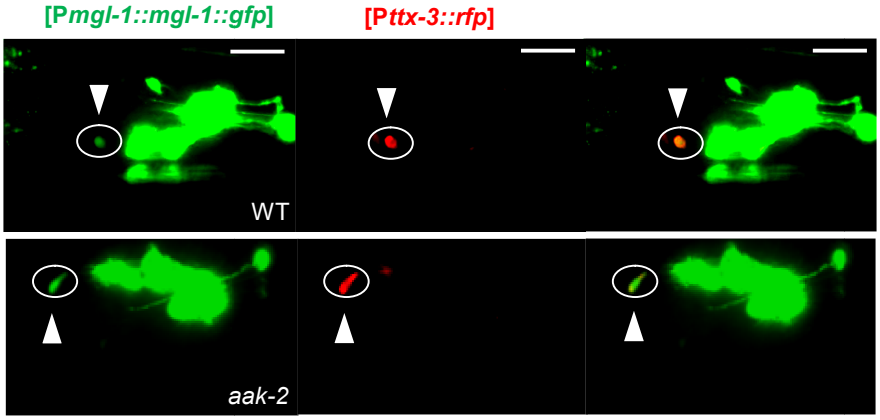
(A) Relative messenger RNA levels were analyzed with semi-quantitative RT-PCR in well-fed and starved animals. *glr-1* mRNA levels were similar in well-fed and starved WT and *aak-2* mutants.

(B) Western blot analysis of GLR-1 levels in Well-fed and starved WT and *aak-2* mutants expressing GLR-1::GFP using GFP antibody. GLR-1 protein levels decrease upon starvation in WT animals and this is less pronounced in *aak-2* mutants.

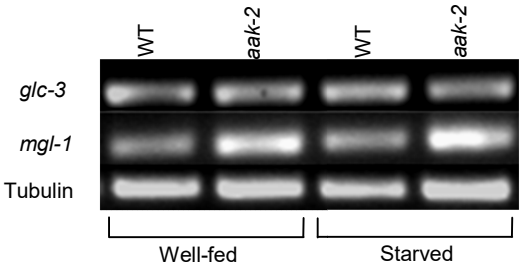
(C, D, E) Co-localization (arrows) of EAT-4 with GLR-1 is shown in the merged images. GLR-1 puncta were closely opposed to EAT-4 puncta in both WT **(C)** and *aak-2* mutants **D)** (n>7), Error bars represent +/− SEM (Student's 2-tailed t test).

(F) A schematic showing the 3 AMPK phosphorylation sites in the cytoplasmic tail of GLR-1 protein two of which are conserved between *C. elegans* and rat glutamate receptor GluR1.

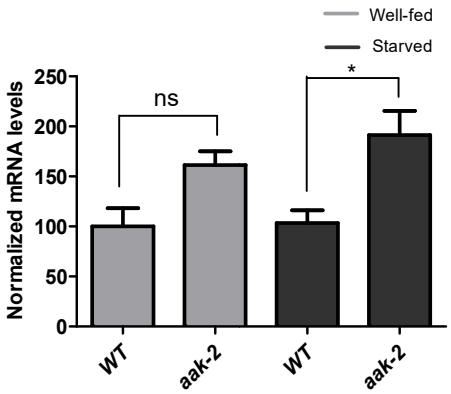
A



B



C



D

```
MGL-1    PAYALNQSLDFVRDMIGSS-EASDYVCLDGSDPNLKKQSQKKNVAAVVGGSYSSVSVQLA
GRM2     DTHALEQALDFVRASLSRGADGSRHICPDGSYATHG--DAPTAITGVIGGSYSDVSIQVA
GRM3     DTYALEQSLEFVRASLTKV-DEAEYMCPDGSYAIQE--NIPLLIAGVIGGSYSSVSIQVA
            ** * * ***             * ***               * ***** ** * *

                                                          S234
MGL-1    NLLRLFRIAQVSPASTNADLSDKNRFEYFARTVPSDDYQAMAMVEIAVKFKWSYVSLVYS
GRM2     NLLRLFQIPQISYASTSAKLSDKSRYDYFARTVPPDFFQAKAMAEILREFNWTYVSTVAS
GRM3     NLLRLFQIPQISYASTSAKLSDKSRYDYFARTVPPDFYQAKAMAEILREFNWTYVSTVAS
         ****** * * * *** * **** *  ******* *  ** ** **    * * *** * *
```

E

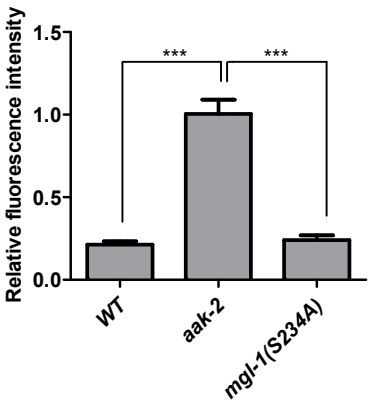


Figure S2.8: AMPK regulates MGL-1 by modulating its steady state mRNA levels while also affecting MGL-1 function through direct phosphorylation

(A) [MGL-1::GFP] levels are unchanged in well fed WT and *aak-2* mutants in the AIY interneurons (shown in the circle) indicated by [TTX-3::RFP]. Scale bars represent 20um.

(B, C) Relative messenger RNA levels were analyzed with semi-quantitative RT-PCR in well-fed and starved animals. *mgl-1* mRNA levels (normalized to WT) are significantly higher in starved *aak-2* mutants compared to starved WT animals. Error bars represent +/− SEM (two-way ANOVA *p<0.05).

(D) A schematic showing the conserved AMPK phosphorylation site in *C. elegans* MGL-1 and human GRM2 and GRM3 group II metabotropic glutamate receptors.

(E) Normalized [MGL-1::GFP] levels are unchanged upon mutating serine 234 in *mgl-1* (n>10), Error bars represent +/− SEM (one-way ANOVA ***p<0.0001).

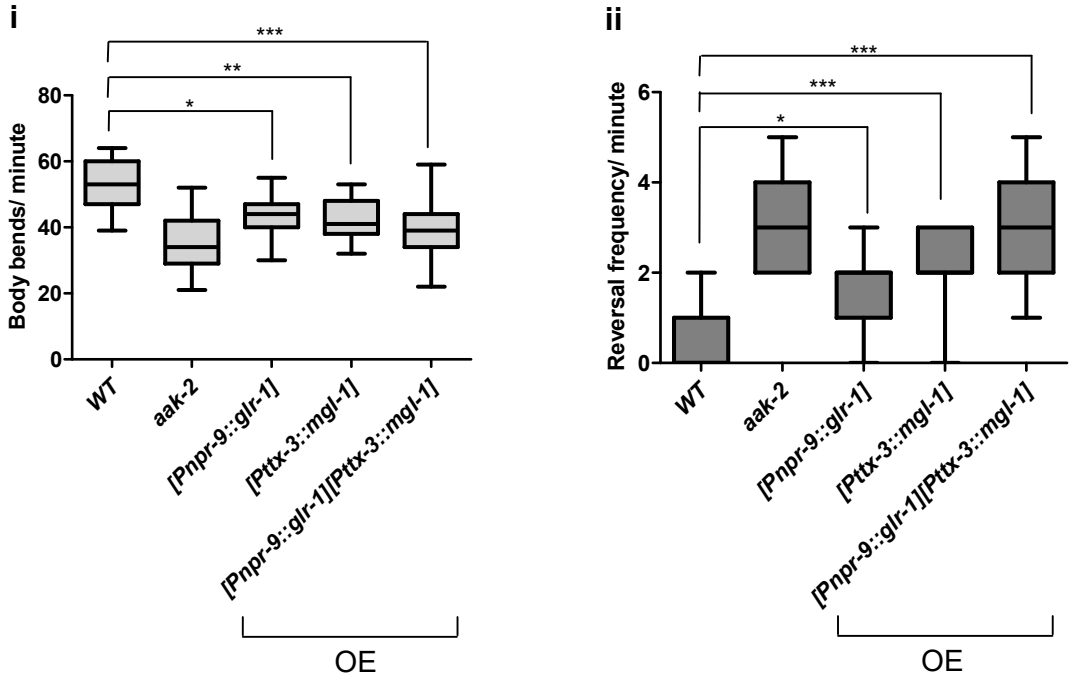


Figure S2.9: Overexpression of *glr-1* and *mgl-1* in the AIB and AIY, respectively results in the similar defect in distal locomotory behaviour as *aak-2* mutants.

Overexpression (OE) of *glr-1* or *mgl-1* in the AIB and AIY interneurons, respectively results in decreased forward locomotion rate and increased reversal frequency similar to starved *aak-2* mutants (n>15), (one-way ANOVA *p<0.05, **p<0.001, ***p<0.0001). In box and whisker plots, the central line is the median, the edges of the box are the 25th and 75th percentiles, and the whiskers extend to the most extreme data points.

Gene name	Function	AIB and/or AIY expression
glr-1	Glutamate receptor family (AMPA)	AIB
glr-2	Glutamate receptor family (AMPA)	AIB
eat-4	Vesicular glutamate transporter	AIB
mod-1	Serotonin-gated chloride channel	AIB/AIY
ser-2	Serotonin/octopamine receptor family	AIY
inx-7	Innexin	AIY
unc-7	Innexin	AIY
inx-19	Innexin	AIY
gar-2	G-protein-linked acetylcholine receptor	AIY
gcy-1	Guanylyl cyclase	AIY
flp-1	FMRF-like peptide	AIY
npr-11	Pyroglutamylated RFamide peptide receptor	AIY
mgl-1	Metabotropic glutamate receptor	AIY

Figure S 2.10: Potential AMPK phosphorylation targets expressed within the AIB and AIY interneurons

Chapter 3: Discussion

Most organisms have evolved a highly optimized food-seeking strategy to cope with dramatic fluctuations in food availability. Hunger exerts a systemic influence that initiates a broad array of adaptive behaviours. For example, starvation results in more tolerance to stressful foraging conditions and in engaging in aggressive behaviours (Cabanac, 1985; Dethier, 1976). Moreover, food-deprived animals display increased appetite and a lower threshold of food acceptance to accommodate less palatable energy sources (Carr, 1996; Wong, 1996; Wong, 1995). The prominent mechanisms engaged in modulation of feeding and food-seeking behaviours in vertebrates have been mapped to the regions of the CNS, especially the hypothalamus which is also known as the “feeding center” (Anand and Brobeck, 1951, Hillebrand et al., 2002). This was most strikingly verified by the observation that electrical stimulation of the lateral parts of the hypothalamus caused self-sustained food intake (Hess, 1954).

There has been increasing evidence suggesting an important role for the “metabolic master switch” AMP-activated protein kinase (AMPK) in the modulation of adaptive behaviours in response to hunger. Hypothalamic AMPK is inhibited by leptin (Andersson et al., 2004, Dagon et al., 2012 and Minokoshi et al., 2004) and becomes activated by fasting (Minokoshi et al., 2004), ghrelin (Andersson et al., 2004, Andrews et al., 2008 and López et al., 2008), and neuronal activity (Hawley et al., 2005 and Kawashima et al., 2012). Furthermore, manipulation of hypothalamic AMPK activity affects energy balance (Andersson et al., 2004, Claret et al., 2007 and Minokoshi et al., 2004). Of note, one recent study has shown that AMPK activity increases in the AgRP neurons in the hypothalamus resulting in fasting-induced spinogenesis and excitatory synaptic activity and this increase is dependent on p21-activated kinase (Kong et al.,

2016). This mechanism describes one of the ways through which AMPK modulates synaptic plasticity in response to hunger stress to evoke intense food intake. However, how AMPK activity triggers changes in the activity of neural circuits to trigger food-seeking behaviours in response to hunger is still largely unknown mainly due to the overwhelming complexity of neural circuits and resulting behavioural outputs in higher animals. Given the simplicity of nervous system and a comparatively simple foraging behaviour in invertebrates, we decided to further investigate the role of AMPK in modulation of signaling networks which integrate hunger and trigger adaptive food-seeking behaviour in *C. elegans.* Such studies have provided more detailed understanding of how hunger stimuli can evoke changes in the activity of neural circuits and consequently their resulting behavioural outputs that are critical to survival.

In this study we found that the loss of AMPK in starved animals led to increased local searching, which is normally triggered when animals are removed from food, but not when they become starved. This local searching for food is normally suppressed in starved animals, which allows them to more efficiently explore new distant locations for better food resources. Based on this result, we reconstituted AMPK in different subsets of neurons, which are known as regulators of food-seeking behaviours in well-fed and starved animals to investigate its site of action. By doing so, we mapped AMPK function to a neural circuit (AWC-AIY-AIB) that regulates adaptive food-seeking behaviour in response to different states of food availability. The AIB interneurons trigger local exploration in well-fed animals removed from food, whereas AIY suppresses local exploration and induces distal food-seeking behaviour in starved animals.

To further investigate how AMPK control food-seeking behaviour we analyzed different gene products expressed in the AWC-AIY-AIB circuitry that contain the consensus AMPK phosphorylation sites. We found that AMPK is responsible for the regulation of glutamatergic signaling in the AIB and AIY interneurons. AMPK has been previously reported as a regulator of glutamatergic neurotransmission (Yang et al., 2011; Cunningham et al., 2012) although the mechanism/s has been poorly understood. Of note, we found that removal of either AMPA-type glutamate receptor *glr-1* or Metabotropic glutamate receptor *mgl-1* in the *aak-2* background resulted in normal distal food-seeking behaviour suggesting that these receptors act in the same genetic pathway as AMPK to modulate behavioural responses to starvation. These results was further confirmed by mutating the consensuses AMPK phosphorylation sites in the GLR-1 and MGL-1 both of which resulted in similar defective distal food-seeking behaviour phenotype to *aak-2* mutant animals. Removal of *eat-4*, which is the only known vesicular glutamate transporter in *C. elegans* and is required for glutamatergic neurotransmission, in the *glr-1* and *mgl-1* mutant variants rescue the defective food-seeking behaviours further confirming the involvement of AMPK in regulation of synaptic GLR-1 and MGL-1 receptors in starved animals.

Recent studies have suggested both pre- and post-synaptic roles for AMPK in AGRP neurons in starved mice (Yang et al., 2011; Kong et al., 2016). In this study we revealed that AMPK affects the AIB and AIY synaptic inputs in different ways, thereby altering behavioural outputs in a coordinated manner. AMPK targets at least two conserved sites in the cytoplasmic tail of GLR-1, which has been shown as the target domain for other kinases in its counterpart in higher animals (Roche et al., 1996).

Previous studies have shown the differential role of GluRs phosphorylation. For example phosphorylation of S845 and S831 has been involved in modulation of channel function whereas their dephosphorylation is associated with receptor internalization and degradation (Roche et al., 1996; Lee, 2006). However, in our study we found that phosphorylation of this cytoplasmic tail at two conserved serines target the receptor for endocytosis and potentially degradation. GLR-1 endocytosis and degradation results in decreased GLR-1-mediated synaptic inputs into the AIB interneurons leading to their inactivation, which is ultimately required for modification of behavioural changes upon starvation. AMPK has been also demonstrated to phosphorylate the E3 ubiquitin ligase Nedd4-2 resulting in the degradation of specific ionic channels. In our study we however discovered that AMPK exerts its role in modulating GLR-1 through its direct phosphorylation (Bhalla et al., 2006; Andersen et al., 2012; Alzamora et al., 2010).

In parallel to its role in regulating GLR-1 levels within the AIB neuronal process, we found that AMPK also modulates MGL-1 abundance/function in the AIY interneurons. Group II mGlu receptors are involved in both cognitive and emotional processes, and have been linked to various neuropsychiatric conditions, including anxiety, stress-related disorders, schizophrenia and substance misuse (Niswender and Conn, 2010). Therefore, AMPK mediated changes to mGlu receptor function might provide a novel and potentially promising therapeutic approach for any of these psychiatric disorders.

Most types of learning and memory have been shown to involve plasticity at glutamatergic synapses. Phosphorylation of GluRs has been proposed as one of the mechanisms that is required for long term potentiation (LTP) and in long term

depression (LTD) whereas phosphorylation of group two mGlu is involved in long term depression (LTD) (Niswender and Conn, 2010). Therefore, given the involvement of AMPK in the regulation of glutamate receptor abundance, our findings might be therefore further extended to implicate AMPK in the control of LTP and LTD that underlie learning and memory.

Final Statement and Perspective

Overall, our work dissects the mechanism through which AMPK modulates neuronal activity to regulate the resulting adaptive food-seeking behaviours in *C .elegans*. We first revealed that AMPK modulates food-seeking behaviour by modulating the glutamatergic synaptic inputs through regulation of glutamate receptors. Given the conserved AMPK phosphorylation sites in GLR-1 and MGL-1, we speculate that AMPK regulates the activity of neural circuits in other organisms at least partly by modulating these two glutamate receptor types to affect a wide range of neuronal outputs that respond to physiological or developmental contexts involving various stresses. Future studies should focus on the potential role of AMPK in regulating adaptive food-seeking behaviours in response to hunger in higher animals, while the role of AMPK in additional stress-based adaptive behaviours may also be fruitful and enlightening. Moreover, AMPK starvation-inducible or pharmacological activation may provide us with a highly effective, non-invasive means of modifying these and potentially other behavioural outputs.

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