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The tryptophan metabolite kynurenic acid is a neuromodulator
mediating learning, brain aging, and neurodegeneration

by

Mihir Vohra

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Thank you to everyone I have known.

“Still, there are times I am bewildered by each mile I have traveled, each meal I have eaten, each person I have known, each room in which I have slept. As ordinary as it all appears, there are times when it is beyond my imagination.”

-Jhumpa Lahiri, *The Namesake*

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The tryptophan metabolite kynurenic acid is a neuromodulator mediating learning, brain aging, and neurodegeneration

Mihir Vohra

Abstract

The ability to change behaviors based on past experience is one of the most important and complex functions of the brain. Elucidating how activity of molecules, cells, and circuits all ultimately control learning and memory is difficult, and the simplicity of the nematode *C. elegans* offers an opportunity to connect these lower-level changes to full-scale changes in behavior. This allows for a depth of understanding of the brain's biology that is largely impossible in other model organisms. Here we show that the tryptophan metabolite kynurenic acid (KYNA) functions as an inhibitory neuromodulator, communicating information about peripheral physiology to the nervous system to alter behavior. We have found that dietary restriction and many pathways involved in the body's responses to dietary restriction – long known to affect brain function – enhance learning in *C. elegans* because they reduce production of KYNA, releasing KYNA-induced inhibition of neuronal activity in a specific pair of interneurons required for learning. In contrast, KYNA production increases with age, accounting for a significant portion of the decline in learning that occurs as animals get older. Furthermore, a *C. elegans* model of neurodegeneration may also increase KYNA production to impair learning. These studies demonstrate that metabolism can regulate brain function, having profound effects on cellular activity, learning, and memory. They establish that KYNA production is a crucial physiological indicator of peripheral status to the nervous system, offering insight into how the brain integrates information to direct behavior.

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Chapter 1: Introduction

Learning and Memory

The brain must be able to faithfully store and communicate information so animals can survive. However, because circumstances and environments change, it must also be modulate an animal's behavior based on experience. This process of acquiring new information and changing behaviors in response to it is commonly referred to as learning. Learned information is stored in and retrieved from the brain in a process called memory (Zola-Morgan and Squire, 1993). In humans, memory is often classified as being declarative, meaning material that can be consciously recalled (such as lists of words), or nondeclarative, meaning material that cannot be consciously recalled (such as motor skills). Memory can also be classified temporally: from holding experiences for seconds (immediate memory), to hours (working memory), or up to years (long-term memory) (Zola-Morgan and Squire, 1993).

Historically, one way we have come to understand the physiology of learning and memory has been through human patients who have specific deficiencies in parts of these processes. For example, the patient H.M. suffered from debilitating and untreatable seizures, and underwent surgery to remove parts of his medial temporal lobes including most of his hippocampus. After the surgery he was found to have severe deficits in forming new short-term declarative memories, called anterograde amnesia. There were no changes to his IQ, language ability, emotion, or memory of events before surgery (Scoville and Milner, 1957), strongly suggesting a role for the hippocampus specifically in immediate memory. The patient R.B. exhibited similar deficits after recovering from anesthesia given to perform a cardiac bypass: anterograde amnesia with no changes to IQ or prior memories. After his death, an autopsy revealed lesions to the hippocampus without affecting nearby brain structures (such as the

amygdala and thalamus), establishing that loss of the hippocampus alone could prevent formation of declarative memories (Zola-Morgan et al., 1986).

The importance of the hippocampus in memory formation has been validated in mammalian systems as well. For example, a spatial learning and memory paradigm for rodents called the Morris Water Maze places animals in a pool of opaque water and has them use visual cues in the arena to find a hidden platform. At first, they search randomly for the platform, but after repeated trials they can recall where in the arena it is located. Animals given lesions to the hippocampus are unable to learn the platform's location (Schenk and Morris, 1985). Thus, just as the hippocampus is vital for encoding declarative memory in humans, it is required for encoding spatial and temporal memories in model organisms. Of course, the advantage of studying learning and memory in organisms other than humans is experimental tractability, and establishing the importance of the hippocampus presents a relevant neuroanatomical site to investigate some underlying cell and molecular processes that create memory.

Long-term potentiation: a synaptic correlate of learning

The anatomy of the hippocampus is such that sections of this region maintained alive *in vitro* maintain much of its circuitry intact. In particular, most studies focus on the excitatory synapses that axons from a region called CA3 make onto pyramidal neurons in a region called CA1. Excitatory post-synaptic potentials (EPSPs) can be recorded in CA1 neurons after electrical stimulation of CA 3 axons (called Schaffer collaterals). If Schaffer collaterals are given brief, high frequency stimulation, there is a long and stable increase in EPSPs; this increase in synaptic strength is called long-term potentiation (LTP). LTP is specific, only occurring at the synapses that are stimulated (Nicoll et al., 1988). It is also dependent on the state of the post-

synaptic cell: if a stimulus that is normally insufficient for inducing LTP is presented at the same time the post-synaptic CA1 neuron is depolarized, LTP will occur (Gustafsson et al., 1987). For these reasons and others, LTP may represent a synaptic correlate of learning.

Molecular underpinnings of LTP

LTP relies on two types of ionotropic glutamate receptors on post-synaptic neurons: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) and N-methyl D-aspartate receptors (NMDARs). These receptors are nonselective cation channels, producing excitatory responses in post-synaptic cells when engaged by the neurotransmitter glutamate. The function of NMDARs is particularly complex. In addition to glutamate, they also bind extracellular Mg^{2+} . When the post-synaptic cell is at rest, Mg^{2+} blocks the pore of the receptor, preventing ion flow. However, depolarization of the post-synaptic cell removes Mg^{2+} and allows the receptor to conduct ions (Nakanishi, 1992). Thus, because NMDARs require both glutamate from the pre-synaptic cell and depolarization of the post-synaptic cell in order to open, they are considered coincidence detectors. This accounts for many of the features of LTP described above. LTP is specific to the stimulated synapses because it is only these synapses where glutamate will be released. LTP is state-dependent because stimulation results in pre-synaptic glutamate release and post-synaptic depolarization removes NMDARs' Mg^{2+} block (Nicoll et al., 1988). This suggests that NMDAR activity may be important in information storage, and indeed, pharmacologic inactivation of NMDARs prevents LTP (Collingridge et al., 1983). Adding further complexity to how NMDAR function integrates multiple coincident signals to modulate neuronal activity is that fact that it requires co-activation: in addition to glutamate, they must also bind glycine at a separate modulatory site in order to mediate channel activation (Furukawa et al.,

2005; Kleckner and Dingledine, 1988). NMDAR activity can also lead to insertion of more AMPARs into the post-synaptic membrane, making the synapse more responsive to glutamate, thereby accounting for how LTP may be maintained (Shi et al., 1999).

Importantly, NMDARs conduct Ca^{2+} , which functions as a second messenger within the post-synaptic cell, activating Ca^{2+} -dependent protein kinases required to induce LTP (Malinow et al., 1989). These synaptic events be transduced into long-term changes (called late phase LTP) because Ca^{2+} -dependent protein kinases also activate cAMP response element-binding protein (CREB), a transcription factor regulating expression of synaptic genes (Silva et al., 1998), or remodeling of dendritic spines (Engert and Bonhoeffer, 1999). Thus, post-synaptic activity-dependent changes may provide a molecular underpinning of LTP, which itself may be a cellular underpinning of learning and memory. However, the complexity of mammalian brains makes it difficult to make mechanistic connections from molecules to cells to circuits to behaviors. For this reason, simpler invertebrate model organisms have been used to study learning and memory, and many aspects of the fundamental neurobiology have been found to be conserved in higher organisms (Barco et al., 2006).

The *C. elegans* nervous system

Several invertebrate model organisms have been used to understand how molecular changes in the nervous system can ultimately lead to learning behaviors, such as *Aplysia depilans* (Kandel and Tauc, 1965) and *Drosophila melanogaster* (Dudai et al., 1976). Even among invertebrate systems, the nematode *C. elegans*, provides a particularly elegant model for investigating the underpinnings of learning and memory. Transparent, 1mm long, and grown as hermaphrodites in the lab, *C. elegans* have an invariant number of somatic cells as adults with

each cell's shape, location, and lineage identified (Sulston and Horvitz, 1977). *C. elegans* hermaphrodites have 302 neurons that are each given a unique three-letter code to identify them. Furthermore, cells of the nervous system have not only been identified, but have also been mapped with an EM-level anatomical connectome that shows every electrical and chemical synapse these cells make (White et al., 1986).

This is the only organism for which a complete connectome exists, and along with the powerful genetic tools that exist in this organism, provides rich opportunities for exploring neurobiology and behavior. For instance, many genes have been found that are expressed in single neurons, which allows for genetic manipulation of individual neurons by using these genes' promoters. These techniques can be used to both observe and perturb activity of individual neurons to elucidate how their physiology affects behavior. As in mammals, in *C. elegans* Ca^{2+} is used both to depolarize neurons and as a second messenger. To observe activity of neurons, they can be made to express the Ca^{2+} indicator GCaMP, with its fluorescence signifying Ca^{2+} influx, and thus activation (Grienberger and Konnerth, 2012). To control activity of neurons, many chemical and optogenetic tools used in other systems can be used in *C. elegans* as well. One example of a genetic technique to control *C. elegans* neurons is by expressing constitutively active versions of proteins that will force neurons to be either hyperpolarized or depolarized. A constitutively active form of the Ca^{2+} -dependent protein kinase C (PKC) leads to depolarization, synaptic transmission, and neuropeptide release (Macosko et al., 2009). Alternatively, a constitutively active form of the potassium channel UNC-103 leads to hyperpolarization (Cho et al., 2016). These tools to observe and control neuronal activity, along with the connectome, allow for specific understanding of neuronal circuits, which is particularly useful for studying processes as complex as learning and memory.

Learning and memory in *C. elegans*

As with other animals, *C. elegans* have many sensory modalities, such as olfaction, gustation, touch, temperature sensation, and oxygen sensation, and each of these modalities has been used to probe *C. elegans* ability to learn based on prior experience. The first characterization of this was in a type of learning called habituation. This is a type of nonassociative learning (meaning the sensory cue presented to the animals is not paired with another cue) where animals decrease their response to a cue after it has been presented many times. Researchers found that *C. elegans* will move backwards in response to a mechanical stimulus, but that repeated presentation of this stimulus decreased the frequency and magnitude of this response, indicating habituation (Rankin et al., 1990). Using a laser to ablate specific sensory neurons and interneurons, they were able to identify several important neurons (Wicks and Rankin, 1995, 1996), some of which were involved specifically in the plasticity of this circuit (Wicks and Rankin, 1997). Cellular activity of relevant sensory neurons was characterized with Ca^{2+} indicators (Kindt et al., 2007; Suzuki et al., 2003) and *ex vivo* electrophysiology (O'Hagan et al., 2005). Later, genetic identification of a mechanosensory potassium channel (Cai et al., 2009) and glutamate transporter (Rankin and Wicks, 2000) required for this plasticity were identified. This habituation paradigm demonstrates how in *C. elegans*, a learned behavior can then be dissected and understood at the levels of circuits, cells, molecules, and genes in a way not possible in more complex organisms.

Similar strategies of establishing learning paradigms and discovering the underlying mechanisms at different levels have been used in *C. elegans* associative learning models as well. In general, these paradigms pair an odor, taste, oxygen, or temperature environment (called the

conditioned stimulus) with either the presence or absence of food (the unconditioned stimulus) (Cheung et al., 2005; Colbert and Bargmann, 1995; Hedgecock and Russell, 1975; Kauffman et al., 2010; Kunitomo et al., 2013; Saeki et al., 2001; Torayama et al., 2007; Tsui and van der Kooy, 2008). Presumably because keeping track of environments that do or do not have food is so evolutionarily important, *C. elegans* will then learn to associate the conditioned stimulus with nutrient status: stimuli presented in the absence of food will become aversive whereas those presented in the presence of food will become attractive. This learned aversion or attraction is measured with a chemotaxis assay, where animals are allowed to choose their preference between the conditioned stimulus and a control stimulus they have not previously been exposed to. The proportion of the population that chooses the conditioned stimulus can be compared to the chemotaxis behavior of naïve animals to provide a readout for the extent to which animals have learned the association between the conditioned and unconditioned stimuli. Many components of the circuitry, cellular physiology, and molecular mechanisms underlying these associative learning behaviors have been identified (Ardiel and Rankin, 2010). These include new mechanisms as well as ones known to be involved in mammalian learning such as CREB (Kauffman et al., 2010; Lau et al., 2013), AMPARs (Kauffman et al., 2010; Lau et al., 2013; Morrison and van der Kooy, 2001), and NMDARs (Kano et al., 2008; Lau et al., 2013).

The kynurenine pathway

The involvement of NMDARs in *C. elegans* learning paradigms was of particular interest to the Ashrafi lab. This is not only because it plays an important and complex role in LTP and memory in mammals (Collingridge et al., 1983; Morris et al., 1986), but also because the Ashrafi lab found a role for endogenous modulation of NMDAR function in *C. elegans* behavior

(Lemieux et al., 2015). This occurs via the tryptophan metabolite kynurenic acid (KYNA), which was originally considered to be a waste product, but has recently become appreciated as a neuromodulator. As the only known endogenous NMDAR antagonist, it antagonizes NMDAR activity by binding to their glycine modulatory sites (Kessler et al., 1989), and may also inhibit glutamatergic signaling through other mechanisms (Hilmas et al., 2001). Tryptophan is an essential amino acid, and while it can be used in protein synthesis or to make serotonin, over 95% of ingested tryptophan is degraded through the evolutionarily conserved kynurenine pathway (KP) (Bender, 1983; van der Goot et al., 2012; Stone et al., 2013). One arm of the KP produces KYNA, but the other converts KYNA's immediate precursor kynurenine into the NMDAR agonist quinolinic acid (QA) and NAD⁺ (Stone and Perkins, 1981; Stone et al., 2013) (Figure 1-1). Metabolism of tryptophan via the KP is distributed across different cells and tissues. For instance in mammals a majority of kynurenine in the brain is produced in the periphery and crosses the blood-brain barrier to then be converted into KYNA by astrocytes or QA by microglia (Cervenka et al., 2017; Guillemin et al., 2005). Production of KYNA occurs in *C. elegans* nervous systems as well (Lemieux et al., 2015).

Through its inhibition of glutamatergic transmission, KYNA has been shown to alter learning and memory in rodents. Deletion of the brain's primary KYNA-producing enzyme enhanced hippocampal-dependent learning in mice. At the circuit level, hippocampal slices from these mice had increased extracellular glutamate and enhanced LTP (Potter et al., 2010). In contrast, addition of excess KYNA to rat's brains impaired learning and decreased extracellular glutamate in the hippocampus (Pocivavsek et al., 2011). These observations are consistent with the idea that KYNA is an inhibitory neuromodulator, but do not necessarily elucidate the normal physiology of the KP.

Despite producing neuroactive metabolites, regulation of the KP remains largely unknown. The Ashrafi lab recently described a role for the KP, and KYNA specifically, in communicating nutrient status to the nervous system in *C. elegans*. Levels of KYNA were found to decrease, as measured by HPLC, when animals were given a brief fast and were slowly restored upon re-feeding. This fluctuation in KYNA levels is responsible for mediating the overactive feeding observed when fasted *C. elegans* are reintroduced to food (Lemieux et al., 2015). This is the first characterization of a role for endogenous fluctuations in the KP in modulating plasticity. While it makes intuitive sense that fasting may decrease KYNA levels by removing tryptophan as a substrate, KYNA concentration is many orders of magnitude lower than that of tryptophan, meaning tryptophan is not limiting for KYNA production (Lemieux et al., 2015). This indicates that while nutrient status can modulate flux through the kynurenine pathway, there must be active regulation of this process. One potential source of regulation is insulin signaling; RNAseq data from *C. elegans daf-2* mutants, which have impaired insulin signaling, found that compared to wild-type, one of the most upregulated transcripts was *kmo-1*, which takes kynurenine down the arm of the KP that does not produce KYNA (Zarse et al., 2012) (Figure 1-1). Other potential layers of regulation such as transport of KP metabolites, trafficking of KP enzymes, and post-translational modifications to KP enzymes remain unexplored.

The kynurenine pathway in disease

Despite the outstanding questions regarding how flux through the KP is regulated, this pathway has been implicated in many human diseases. For instance in the periphery it is involved in inflammation, largely through inflammation-induced expression of IDO, kynurenine-

induced activation of the aryl hydrocarbon receptor, and KYNA-induced activation of the immune receptor GPR35 (Cervenka et al., 2017). Thus dysregulation of the KP has recently been implicated in gastrointestinal diseases of inflammation such as irritable bowel disease and pancreatitis, as well as diabetes and cancer (Cervenka et al., 2017). However, the most well known instances of KP involvement in disease is in the nervous system. Dysregulation in humans has been implicated in Alzheimer's disease (Bonda et al., 2010; Guillemin et al., 2005), depression (Steiner et al., 2011), Huntington's disease (Beal et al., 1992; Pearson and Reynolds, 1992), Parkinson's disease (Ogawa et al., 1992), and schizophrenia (Schwarcz et al., 2001).

Animal models have elucidated some mechanisms by which KP modulation may ameliorate these pathologies. They take advantage of the fact that as mentioned above, kynurenine can cross the blood-brain barrier and be used to make KYNA once in the brain, but peripheral KYNA cannot cross the blood-brain barrier. For instance, a way exercise promotes resilience to stress-induced depression in mice is through stimulating conversion of kynurenine to KYNA in skeletal muscle. Thus, consumption of kynurenine by skeletal muscle reduces the amount available for KYNA production in the brain, and this reduction of brain KYNA mediates a protection from depression (Agudelo et al., 2014). In Alzheimer's Disease and Huntington's Disease, decreased KYNA levels are thought to contribute to excitotoxicity, and in mice a drug thought to increase peripheral kynurenine levels protected against models of these diseases by elevating brain KYNA production (Zwilling et al., 2011). These examples demonstrate that modulating flux through the KP in the periphery is a viable strategy for altering KYNA production in the brain, and that whether the pathology results in impaired or excessive glutamatergic transmission will determine whether therapies should increase or decrease peripheral kynurenine levels. Overall, these connections establish a role for tryptophan

metabolism in inter-organ communication – that KP metabolites are important signaling molecules circulating through the body to relay information between different cells, tissues, and organs.

Here we show that dietary restriction and modulation of pathways implicated in the physiological responses to dietary restriction – insulin signaling, TOR signaling, autophagy, and AMPK signaling – enhance associative learning in *C. elegans* because they reduce KYNA levels. While this occurs without changes to organismal lifespan, KYNA accumulation as animals age underlies a significant portion of the learning impairments associated with aging. Furthermore, a mutation associated with age-related neurodegeneration appears to impair learning partially through elevating KYNA. Overall, this demonstrates that many physiological pathways modulate nervous system function and dysfunction by converging on regulation of production of this neuroinhibitory metabolite.

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Figure 1-1.

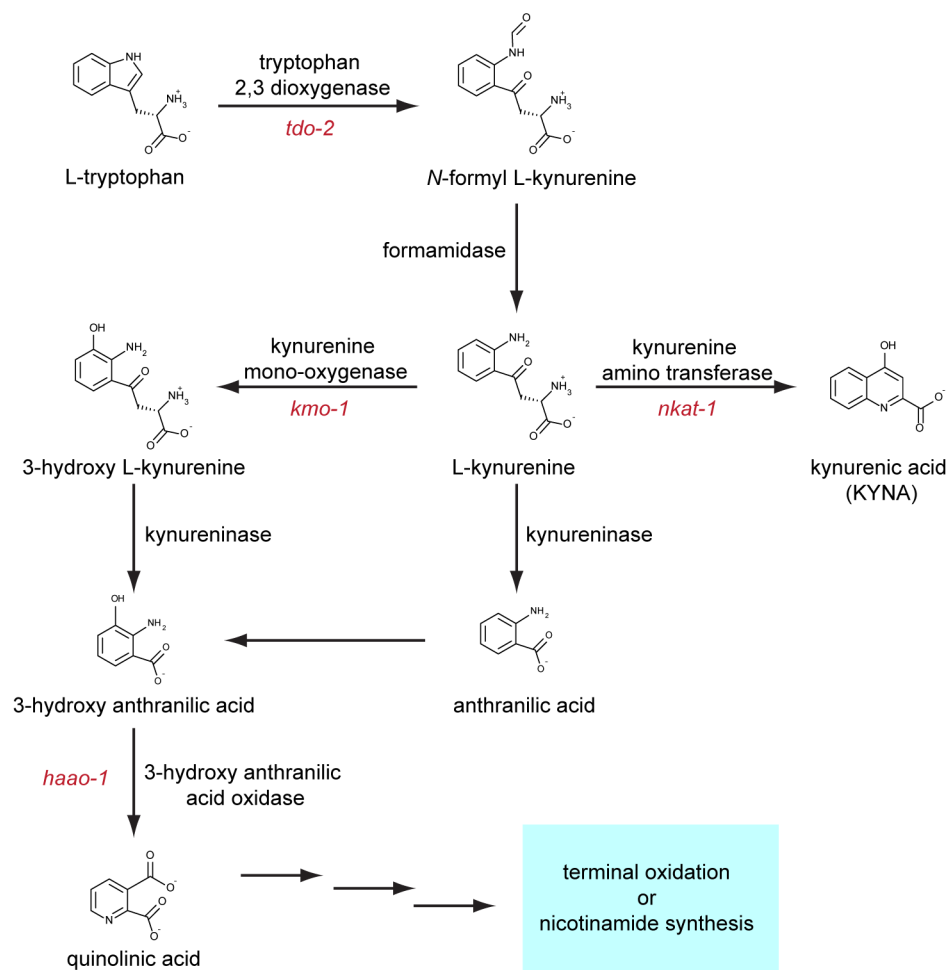


Figure 1-1. The kynurenine pathway. Schematic of the kynurenine pathway, with *C. elegans* genes relevant to these studies labeled in red.

Chapter 2: The beneficial effects of dietary restriction on learning are distinct from its effects on longevity and mediated by depletion of kynurenic acid

Introduction

Aging and various neurodegenerative disorders are characterized by progressive declines in learning capacity. There are well established but poorly understood connections between molecular mechanisms that regulate longevity and those that influence learning and memory (Murphy et al., 2014). For example, reductions in insulin signaling are associated with lifespan extensions in many species (Kenyon, 2010; López-Otín et al., 2013). In addition to its metabolic effects, dysregulation of insulin signaling has been implicated in cognitive defects such as Alzheimer's disease (Stanley et al., 2016). In turn, dietary restriction (DR), a dietary intervention that is known to reduce insulin signaling and extend lifespan, is associated with enhancements in learning and memory and delays in cognitive decline even in the context of neurodegenerative disorders (Dal-Pan et al., 2011; Maalouf et al., 2009; Murphy et al., 2014; Riddle et al., 2013). However, given that DR or reductions in insulin signaling affect a wide range of cellular and organismal processes that can collectively promote longevity, it is unclear whether the beneficial effects elicited by these manipulations are due to direct effects on mechanisms of learning or due to myriad indirect consequences of lifespan extension and general improvements in neural maintenance and survival (Greer and Brunet, 2009; Maalouf et al., 2009).

C. elegans provides an opportunity for investigating the connections between metabolism, aging and learning. As in mammals, in *C. elegans* various forms of dietary and caloric restriction extend lifespan (Greer and Brunet, 2009; Kenyon, 2010). And many of the findings that helped solidify the beneficial effects of reduced insulin signaling on lifespan extension initially emerged from studies in *C. elegans* (Kenyon, 2010). Similarly, other manipulations that recapitulate some of the physiological responses to reduced food intake – for example reductions in certain components of mTOR signaling, activation of AMP-activated protein kinase (AMPK),

as well as activation of autophagy – promote longevity even when *C. elegans* have unlimited access to food (Kenyon, 2010).

C. elegans have also been used to investigate molecular underpinnings of learning and memory with paradigms for both short-term and long-term memory (Colbert and Bargmann, 1995; Kano et al., 2008; Kauffman et al., 2010; Torayama et al., 2007). Many of the key molecular components that function in mammalian learning are evolutionarily conserved in *C. elegans* including N-methyl D-aspartate receptors (NMDARs), which are required for spatial memory and long term potentiation in mice (Morris et al., 1986) and certain forms of associative learning in *C. elegans* (Kano et al., 2008). One paradigm for studying short-term associative learning in *C. elegans* is to pair food with butanone, an odorant that in naïve animals is only mildly attractive. This pairing results in subsequent attraction to butanone, which can be scored in a chemotaxis assay (Kauffman et al., 2010; Torayama et al., 2007). The proportion of animals attracted to butanone is calculated as a chemotaxis index, and a learning index is the difference between the chemotaxis index of conditioned animals and that of naïve animals that have never been exposed to butanone, which allows for normalized comparisons between treatments or strains that may differ in innate responses to butanone (Kauffman et al., 2010; Torayama et al., 2007).

Using the butanone association assay, we investigated the effects of DR and perturbations of molecular mechanisms that change upon restricting nutritional intake – hereafter referred to as DR mimetics – on *C. elegans* learning capacity. Specifically, we investigated the effects of reductions in either insulin or mTOR signaling pathways as well as effects of pharmacological and genetic interventions that lead to activations of AMPK and autophagy. Here, we show that DR and these DR mimetics each result in learning enhancements. Despite their wide-ranging

cellular and organismal effects, we find that the beneficial effects of each of these interventions on learning are fully dependent on reductions in kynurenic acid (KYNA), a tryptophan-derived metabolite that can be a competitive antagonist of NMDARs (Kessler et al., 1989). We identify the neuronal sites of KYNA production and KYNA-responsive NMDAR-expressing neurons required for learning. Although each of DR, insulin, mTOR, AMPK, and autophagy has been under intensive investigation, their effects on the kynurenine pathway have remained largely unknown. We show that each of these interventions modulate transcription of a gene encoding a key enzyme of the pathway and thereby provide one potential explanation of how molecular mechanisms that function in the periphery of the animal can affect levels of a neuronal metabolite. Finally, we show that changes in KYNA levels do not alter lifespans in the context of any of the dietary, genetic, and pharmacological interventions tested, suggesting that the effects of DR and its mimetics on learning can be disentangled from their broad effects on cellular maintenance and lifespan.

Results

NMDAR-dependent activity of a single pair of interneurons promotes learning.

Associative learning helps animals predict outcomes based on environmental stimuli. We used the well-established butanone learning paradigm to investigate effects of various manipulations on short-term learning capacity of *C. elegans* (Figure 2-S1) (Kauffman et al., 2010; Torayama et al., 2007). The learned attraction to butanone was notable after 30 minutes of pairing butanone with the food *E. coli* (conditioning) and reached maximal levels by 1 hour (Figure 2-1A). This learned attraction was dependent on the previously identified ODR-1

receptor and function of the AWC neurons, the site of ODR-1 function in butanone sensation (Bargmann et al., 1993) (Figure 2-1B).

Associative learning to butanone can be enhanced if animals are given a brief fast before conditioning. This is not simply modulation of the attraction to butanone by fasting because fasted animals that are re-fed in the absence of butanone do not exhibit any enhanced attraction to butanone (Kauffman et al., 2010). Consistent with these prior results, we found that learned attraction to butanone was progressively enhanced by chronic dietary restriction, where animals were grown on decreasing concentrations of food for their entire lives prior to the assay (Figure 1C). The learning enhancing effects of chronic DR were also observed upon a short term fast or acute DR, where immediately before conditioning, animals were cultured with decreasing concentrations of food for one hour had a similar effect (Figure 2-1D). While there was a progressive enhancement of learning by increasing the duration of the fast, additional benefits did not accrue beyond a 1 hour fast (Figure 2-1E). The extent of the learning enhancement seen with a 1 hour acute DR was the same as that seen in animals that were exposed to chronic DR.

In mammals, members of the ionotropic glutamate receptors of both the NMDA and the non-NMDA classes are required for learning (Morris et al., 1986) and, in *C. elegans*, a requirement for *glr-1*, encoding a non-NMDA AMPA receptor (AMPA), in butanone learning has been previously reported (Kauffman et al., 2010). We found similar requirements for *nmr-1* and *nmr-2* which encode the two subunits of *C. elegans* NMDARs (Kano et al., 2008), and *glr-4* which encodes a putative kainate receptor homolog (Sprengel et al., 2001) (Figure 2-1F).

While AMPARs and kainate receptors are widely expressed in the *C. elegans* nervous system, expression of NMDARs is restricted to only a few neurons, including the RIM and AVA interneurons (Brockie et al., 2001a). Therefore, we chose to focus on the NMDAR-expressing

neurons as a strategy for identifying specific neurons that are involved in this learning circuit. We previously found that activity of NMR-1 in AVA but not RIM controls the elevated feeding behavior that animals exhibit post-fast (Lemieux et al., 2015). By contrast, reconstitution of wild-type *nmr-1* in RIM conferred learning ability to *nmr-1* mutants while reconstitution of *nmr-1* in AVA could rescue post-fast feeding behavior but not learning (Figure 2-1G and Figure 2-S2A). Thus, while the two behaviors both reflect NMDAR-dependent food-related plasticity, they are mediated through distinct circuits.

To better understand the function of RIM in learning, we used a genetically encoded calcium reporter, GCaMP3.0, specifically expressed in RIM to measure intracellular Ca^{2+} transients as a proxy for neuronal activity. We first assessed the basal state of RIM activity by measuring spontaneous transients over an extended period (250s). To compare average intensities of transients, we identified transients during the 250s imaging window and plotted fluorescence over the following 20s. Similar to other reports (Gordus et al., 2015), spontaneous transients were largely absent in naïve, *ad libitum* fed animals. However, spontaneous Ca^{2+} transient intensity increased substantially after *ad libitum* fed animals were conditioned on butanone, and this was even further increased when animals were fasted before conditioning (Figure 2-1H). We also analyzed Ca^{2+} transients by measuring the total intensity from all transients during the entire 250s imaging window and found that the results were similar (Figure 2-1I). In contrast, there was no change in transients when *nmr-1* mutants were similarly fasted and conditioned (Figure 2-S2B and S2C). As it is known that presence of both NMR-1 and NMR-2 is required for NMDAR function (Brockie et al., 2001b; Kano et al., 2008), *nmr-1* mutants lack functional NMDARs, indicating that learning requires NMDAR activity in RIM. Thus, RIM activity positively correlates with learned attraction to butanone.

To determine a causal relationship between RIM and learning, we genetically controlled RIM activity. We expressed a constitutively active protein kinase C (PKC) to chronically activate RIM (Macosko et al., 2009) and an overactive potassium channel, UNC-103, to chronically silence it (Cho et al., 2016). Activating RIM enhanced butanone learning while silencing RIM blocked it (Figure 2-1J) without changing naïve chemotaxis (Figure 2-S2D). Thus, not only do RIM's Ca^{2+} dynamics correlate with learning, but its activity determines the extent to which learning occurs.

KYNA acts as an NMDAR neuromodulator to regulate learning

Given the requirement of NMDARs in the butanone learning paradigm, we next examined kynurenic acid (KYNA) as a potential mechanism by which fasting may promote learning. This seemed possible because the tryptophan-derived KYNA (Figure 2-2A) is an endogenous NMDAR antagonist (Kessler et al., 1989) and we previously found that, in the context of *C. elegans* feeding regulation, KYNA is depleted during fasting which results in activation of NMDAR-expressing neurons (Lemieux et al., 2015). To demonstrate that the KYNA levels change in a timeframe consistent with the effects of acute fasting on learning enhancement, we extracted KYNA from animals fasted for 30 or 60 minutes and found it to be depleted to the same low levels as those reported for 2 hour fasted animals (Lemieux et al., 2015) (Figure 2-2B). This was consistent with our finding that fasting beyond 1 hour does not further improve learning.

KYNA, a terminal metabolite of the kynurenine pathway (KP), is synthesized from kynurenine by the action of kynurenine amino transferases, encoded by *nkat* genes in *C. elegans* (Lemieux et al., 2015). However, if kynurenine is acted upon by kynurenine monooxygenase,

encoded by *kmo-1*, and 3-hydroxyanthranilate 3,4-dioxygenase, encoded by *haao-1*, it is converted to the NMDAR agonist quinolinic acid (QA) (Stone and Perkins, 1981) (Figure 2-2A). We and others have previously found that inactivation of *nkat-1*, which is expressed in only a small subset of neurons, results in depleted levels of KYNA while *kmo-1(tm4529)* or *haao-1(tm5627)* mutants have elevated KYNA levels (van der Goot et al., 2012; Lemieux et al., 2015). Consistent with a role for KYNA in inhibition of NMDAR signaling, *kmo-1* and *haao-1* mutants had learning defects while *nkat-1(ok566)* mutants had enhanced learning that was not further elevated by fasting (Figure 2-2C and Figure 2-S3A). To ascertain whether the learning improvements of *nkat-1* were due to reduced KYNA or elevated QA, we examined *nkat-1;kmo-1* double mutants, which are predicted to be doubly deficient for KYNA and QA biosynthetic capacity. We found them to have enhanced learning, suggesting an inhibitory role for KYNA predominates in this behavior (Figure 2-2C).

We considered the possibility that the learning defects of KYNA replete animals may be due to altered development or damage to learning circuits. However, prolonged conditioning rescued learning defects in *kmo-1* and *haao-1* mutants (Figure 2-S3B). Similarly, fasting, which reduces KYNA levels even in *kmo-1* and *haao-1* mutants, partially reversed their learning deficits (Figure 2-S3A). Chronic DR had similar effects on *kmo-1* mutants (Figure 2-S3C) whereas it did not further elevate *nkat-1* mutants' learning (Figure 2-S3D). By contrast, neither depleting KYNA (via RNAi of *nkat-1*) nor prolonged conditioning conferred a similar increase in learning capacity to the *nmr-1* or *nmr-2* mutants (Figure 2-2D and Figure 2-S3B). Together, these findings support the notion that NMDARs play a critical role in this learning paradigm and that levels of KYNA modulate the activity of these receptors without being fundamentally required for learning.

In *C. elegans*, *nkat-1* and *nmr* genes are expressed in a small subset of neurons that are either in close proximity to each other or, as in the case of RIM, overlapping (Lemieux et al., 2015). To determine the relationship between sites of KYNA production and the NMDAR-dependent response of RIM neurons to this neuromodulatory metabolite, we reconstituted *nkat-1* in subsets of neurons in which *nkat-1* is normally expressed. Reconstitution of *nkat-1* in RIM was sufficient to blunt the enhanced learning of *nkat-1* mutants. In contrast, reconstitution in another pair of *nkat-1* expressing neurons, RID, had no effect on learning but could rescue *nkat-1* mutants' hyperactivated feeding (Figure 2-2E and Figure 2-S3E). Given that KYNA has been shown to directly bind to the glycine site of NMDARs to decrease their activity (Kessler et al., 1989), our findings are consistent with KYNA regulation of NMDAR activity in an autocrine fashion but do not rule out more complicated possibilities including indirect effects on NMDAR activity, for example through modulation of receptors, that in turn, affect release of glutamate or other agonists of NMDARs.

Finally, we found that kynurenine pathway metabolites affect Ca^{2+} influx into RIM in a manner consistent with behavioral results. As before, naïve, *ad libitum* fed animals on vector RNAi exhibited very little Ca^{2+} influx, which was increased upon conditioning (Figure 2-2F). Depletion of KYNA via *nkat-1* RNAi resulted in animals with already elevated Ca^{2+} transients that were further increased upon conditioning (Figure 2-2F and Figure 2-S3F). The elevated Ca^{2+} transients of *ad libitum* fed KYNA deficient animals was similar to those seen in wild type animals that were fasted and conditioned (Figure 2-2G). By contrast, elevation of KYNA via *kmo-1* mutation largely abrogated the elevation of Ca^{2+} transients seen upon conditioning of fasting animals (Figure 2-2G and Figure 2-S3G). Together with previous results, these data are consistent with a model whereby levels of KYNA modulate responsiveness of RIM neurons in

an NMDAR-dependent fashion.

KYNA also modulates NMDAR-dependent aversive learning behaviors

nmr-1 and *nmr-2* are known to mediate aversive learning when *C. elegans* are exposed to high NaCl concentrations without food (Kano et al., 2008). As with attractive butanone learning, the ability of animals to learn the aversive signal of high NaCl was diminished with excess levels of KYNA but promoted upon KYNA depletion (Figure 2-3A). Moreover, extending the conditioning period could enhance learning of wild-type animals to the level of *nkat-1* mutants but had no effect on *nmr-1* or *nmr-2* mutants (Figure 2-3B). Alternatively, fasting animals in a normal NaCl environment to deplete KYNA and subsequently conditioning them with high NaCl also enhanced learning in wild-type and *kmo-1* animals to the level of *nkat-1* mutants with no effect on *nmr-1* or *nmr-2* mutants (Figure 2-S4). Thus, KYNA-mediated modulation of learning is independent of the sensory modality or valence of the paradigm: elevated KYNA dampens and reduced KYNA enhances learning regardless of whether the stimulus is olfactory or gustatory and whether the learned association is attractive or aversive.

Further supporting an NMDAR-dependent mechanism for effects of KYNA on learning, we found that altering KYNA or QA levels had no effect on learning in two paradigms in which NMDAR activity is not required: learned attraction when food was paired either with high NaCl concentrations or the odor diacetyl (Hadziselimovic et al., 2014; Kunitomo et al., 2013) (Figure 2-3C and 3D). Thus, KYNA-mediated modulation of learning is a general phenomenon that occurs in NMDAR-dependent paradigms.

Genetic and pharmacological manipulations that mimic aspects of DR enhance learning

While many molecular components underlying learning are known and many mutations that impair learning have been studied, relatively few genetic manipulations are known to improve learning. One such manipulation is impairment of insulin signaling (Kauffman et al., 2010; Murakami et al., 2005). Consistent with this, we found downregulation of the *C. elegans* insulin receptor *daf-2* via RNAi was associated with improved learning in *ad libitum* fed animals with no further improvements upon fasting (Figure 2-4A). We reasoned that manipulating other nutritionally sensitive pathways may similarly identify molecular manipulations that improve learning. The mTOR complexes of proteins sense nutrient availability and regulate processes ranging from fat storage and protein synthesis to development and lifespan (Jones et al., 2009). We found that RNAi-mediated inactivations of *let-363* (encoding the *C. elegans* mTOR homolog), *daf-15* (encoding raptor), and *ric-1* (encoding rictor), each led to elevated levels of learning in *ad libitum* fed animals (Figure 2-4A). This finding was reminiscent of mammalian studies reporting that chronic mTOR inhibition has procognitive effects (Halloran et al., 2012; Majumder et al., 2012). Similarly, we found improved learning upon inactivation of the transcription factor *mxl-3*, which leads to autophagy and lipolysis in intestinal cells in response to nutrient deprivation (O'Rourke and Ruvkun, 2013), or upon treatment with phenformin, a biguanide compound that activates AMP-activated kinase (AMPK), a master regulator of energy homeostasis (Beale, 2008) (Figure 2-4A). Again, none of these enhanced learning capacities were further improved by fasting (Figure 2-4A). All of these learning enhancements, however, required *nmr-1* (Figure 2-4B).

Enhanced learning capacity was not a general feature of manipulating food-related signaling pathways, as mutations in various biogenic amine or peptidergic signaling pathways implicated in other food-related plasticity behaviors had minor or no effects on learning. These

included mutations in genes required for synthesis of serotonin (*tph-1*), that of a neuropeptide Y-like molecule (*flp-18*), octopamine (*tbh-1*), both tyramine and octopamine (*tdc-1*), and synthesis of a TGF β superfamily member ligand (*dbl-1*). Each of these pathways have been implicated in various food related behaviors: for example, reductions in serotonin and the TGF β ligand DAF-7 are associated with reduced food levels or increased population density (Horvitz et al., 1982; Ren et al., 1996). Similarly, tyramine, octopamine, and neuropeptide Y-like signaling pathways are thought to be active when *C. elegans* are food deprived (Alkema et al., 2005; Lemieux et al., 2015; Suo et al., 2006). Moreover, while DR or changes in insulin, mTOR, AMPK, and autophagy can extend lifespan, not all long-lived mutants had enhanced learning. Interestingly, *eat-2* mutants, which are frequently used as a model of DR because of their defects in food intake and prolonged lifespan (Avery, 1993; Lakowski and Hekimi, 1998; Raizen et al., 1995), failed to learn (Figure 2-4C). The reason for this failure is not known, however, given that *eat-2* mutants have defects in cholinergic signaling (Raizen et al., 1995), it is possible that cholinergic signaling plays roles in the development or function of the learning circuit in addition to its role in promoting pharyngeal neuromuscular contractions required for feeding. Of note, *eat-2* mutants were previously shown to have defective long-term memory but normal learning capacity (Kauffman et al., 2010). The reason for the discrepancy between our results and the previous publication is not known, although both of these studies indicate that mutations in *eat-2* do not confer enhancements on short-term learning.

The elevated learning capabilities of DR mimetics are KYNA dependent

We next sought to better understand the relationship of KYNA to learning enhancements caused by DR mimetics. As in fasting or depletion of KYNA, the learning enhancements seen in

DR mimetics even in the presence of plentiful food supplies correlated with increases in Ca^{2+} transient intensity in RIM similar to control animals that had been fasted before conditioning (Figure 2-4D and Figure 2-S5). In each case, elevation of KYNA levels using either *haao-1* or *kmo-1* mutants caused a significant reduction in learning capacity (Figure 2-4E). We next exposed *nkat-1* mutants to various DR mimetic treatments. However, given that *nkat-1* mutants already have elevated learning, we decreased the time animals were conditioned with butanone to avoid being confounded by a ceiling in our ability to measure learning. Under these conditions, wild-type animals did not learn but fasted or KYNA deficient animals still did so, albeit to a lesser degree than when the conditioning was for the standard one hour period used elsewhere in this study (Figure 2-4F). Treatment of *nkat-1* mutants with fasting or exposure to any of the DR mimetics did not lead to further improvements in learning (Figure 2-4F), suggesting that DR mimetics and *nkat-1* mutants function in the same pathway to enhance learning.

We next employed direct biochemical measurements of KYNA levels extracted from populations of whole animals exposed to each of the DR mimetics. As in the case of fasted animals, we found that with each of the DR mimetics, KYNA levels were already depleted, even under *ad libitum* fed conditions (Figure 2-4G). While there was a trend of decreased KYNA levels in animals exposed to *daf-2* RNAi, the reduction was not statistically significant. Since these metabolite measurements were conducted on extracts from populations of animals and we could not be certain of the efficacy of *daf-2* RNAi throughout the population, we used *daf-2(e1370)* mutants. KYNA levels of *ad libitum* fed *daf-2* mutants were substantially reduced compared to those wild type animals (Figure 2-4H).

DR mimetics alter expression levels of kynurenine pathway genes.

Although each of insulin, mTOR, AMPK, and autophagy are extensively studied, mechanisms through which these manipulations could result in changes in the kynurenine pathway have not been established. Moreover, while molecular components of insulin, mTOR and AMPK signaling pathway are broadly expressed in *C. elegans*, the transcriptional regulator MXL-3 acts in the intestine (O'Rourke and Ruvkun, 2013) yet its inactivation leads to reduced KYNA levels and enhanced learning. Thus, there must be mechanisms that regulate KYNA levels even when working at sites distant from the neurons involved in KYNA production.

To better understand mechanisms that regulate flux through the kynurenine pathway, we first considered sites of expression of key enzymes that are required for generation or utilization of kynurenine, the substrate from which KYNA is produced. It has been previously reported that the *tdo-2* gene encoding the first enzyme in the kynurenine pathway is expressed in the body wall muscle and skin-like epidermis of *C. elegans* (van der Goot et al., 2012). Indeed, using a transcriptional fusion of the *tdo-2* promoter to GFP, we observed robust expression in the epidermis (Figure 2-5A and 5C). We found that *kmo-1* shows epidermal expression as well (Figure 2-5B). Neither *tdo-2* nor *kmo-1* appeared to be expressed in the neurons that express *nkat-1* (Figure 2-5B and 5C). This suggests that kynurenine must be transported across tissues in order for RIM to produce KYNA.

We next examined *tdo-2*, *kmo-1*, and *nkat-1* transcripts via qPCR in animals given DR mimetics. Compared to *ad libitum* fed animals on vector RNAi, fasting and DR mimetics caused no significant changes in either *tdo-2* or *nkat-1* expression. In contrast, each of the mimetics resulted in a significant increase in *kmo-1* expression (Figure 2-5D). Given coincident tissue expressions of *tdo-2* and *kmo-1* in a large tissue such as the epidermis, these data indicate that

upregulation of *kmo-1* could compete with KYNA production by shunting kynurenine, the common substrate between NKAT-1 and KMO-1, down a different branch of the kynurenine pathway, resulting in reduced KYNA levels (Figure 2-2A).

To further understand the relationship between *kmo-1* expression and DR, we set out to investigate the effects of several transcription factors with key roles in DR on *kmo-1* expression. It is well established that insulin signaling causes functional inactivation the FOXO transcription factor DAF-16 and that many of the consequences of reduced insulin signaling seen in *daf-2* mutants require *daf-16* (López-Otín et al., 2013). Similarly, mTOR and AMPK signals can be transduced via the NRF2 master regulator SKN-1 (López-Otín et al., 2013). Additionally, mTOR activity affects HLH-30, TFEB orthologue that competes with MXL-3 for binding sites and exerts opposing effects, and fasting increases *hlh-30* expression (Lapierre et al., 2013; O'Rourke and Ruvkun, 2013). Finally, cross talk among many of these pathways has been shown. Thus, we sought to determine the effects of *daf-16*, *skn-1*, and *hlh-30* reduction-of-function mutations on learning. We found that *skn-1* mutants failed to chemotax, so they could not be properly assayed. Although other phenotypes resulting from loss of *let-363* and *mxl-3* are known to involve *hlh-30* (O'Rourke and Ruvkun, 2013), *hlh-30* mutants had no learning phenotype (Figure 2-5E), suggesting it may not play a role in regulating KYNA production relevant for this behavior. In contrast, *daf-16* mutants had a learning defect that blocked the enhancements of fasting (Figure 2-5F). Moreover, loss of *daf-16* not only blocked the enhanced learning of insulin deficient animals, it abrogated the enhanced learning of various DR mimetics (Figure 2-5F), raising the possibility that insulin signaling pathway serves as a major link between various nutritionally sensitive pathways and the kynurenine pathway. Consistent with this, we found that, compared to wild-type, *daf-16* mutants had significantly reduced *kmo-1* transcript levels (Figure

2-5G). It is currently unknown whether *kmo-1* is a direct target of DAF-16.

Effects of KYNA on learning are independent of lifespan effects

We considered the possibility that the effects of KYNA levels on learning may be secondary consequences of overall improvement of animal health and lifespan. Despite dramatically increased or reduced levels of KYNA respectively, *kmo-1* and *nkat-1* mutants had wild-type lifespans. Furthermore, while reduction in insulin signaling, inactivation of various components of mTOR, activation of autophagy, or treatment of phenformin had a variety of effects on lifespan, none of their lifespans were altered by *kmo-1* or *nkat-1* mutations (Table 2-1 and Figure 2-S6-S7). Our results indicate that the beneficial effects of DR on learning in *C. elegans* can be accounted for by reductions in KYNA, and that this is independent of the effects of DR or its mimetics on lifespan.

Discussion

We have identified several pathways that enhance learning in *C. elegans*. In addition to previously reported effects of impaired insulin signaling, we found that decreased mTOR signaling, activated intestinal autophagy, and AMPK activation each lead to learning enhancements in well-fed animals that are similar in magnitude to the levels seen when animals are exposed to dietary restriction or a short fast. Since changes in insulin, mTOR, autophagy, and AMPK are all features of the physiological changes induced by DR, we termed them DR mimetics and predicted that they may share common mechanisms of modulating learning. This was indeed the case, as all of these DR mimetics enhanced learning via depletion of KYNA in the nervous system and consequent activity of a specific NMDAR-expressing neuron, RIM.

Simply depleting KYNA mimics the effects of DR on learning while KYNA elevation completely abrogates the learning enhancements caused by DR mimetics. Importantly, while the changes in KYNA were necessary and sufficient to account for the improvements in learning, KYNA levels did not alter lifespan in any of the conditions tested. Thus, the effects of DR on learning can be ascribed to a very specific mechanism rather than myriad benefits associated with DR.

Unlike the NMDARs, which were absolutely required for learning in this model, KYNA levels modulated the speed at which learning occurred rather than being critically required for the occurrence of learning at all. KYNA depletion did not result in naïve attraction to butanone or aversion to NaCl, but accelerated the rate at which the associations were formed. This is supported by the observation that *nkat-1* RNAi and DR mimetics that depleted KYNA could still elicit learning even with a shortened conditioning period that was insufficient for learning in wild-type animals (Figure 2-4F). Furthermore, the learning deficits of *kmo-1* or *haao-1* mutants with high KYNA could be rescued with a longer conditioning period (Figure 2-S3B).

Our data are consistent with a model whereby signals that promote activity of NMDARs in RIM neurons compete with locally produced KYNA, which antagonizes these receptors. This signaling balance determines the timeframe in which learning occurs. Genetic, pharmacological, or nutritional manipulations that enhance learning did so by depleting the antagonizing effects of KYNA on NMDAR signaling (Figure 2-6).

The precise mechanisms by which KYNA antagonizes NMDARs remains unsettled. Two competing yet not mutually exclusive mechanisms for antagonistic effects on glutamatergic signaling have been proposed. One is based on the ability of KYNA to directly bind to the glycine binding site of NMDARs (Kessler et al., 1989), albeit with low affinity, making KYNA

the only known potential endogenous antagonist of NMDARs. Another proposed mechanism is that KYNA reduces glutamate release via antagonism of $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ nAChRs) (Carpenedo et al., 2001; Hilmas et al., 2001). While the combination of genetic, direct metabolite measurement, and neuronal imaging studies strongly indicated that KYNA exerts an inhibitory effect on NMDARs, our data cannot distinguish between direct and indirect effects. However, the finding that RIM is both the site of production of KYNA and KYNA-responsive NMDARs is consistent with KYNA being a direct, low-affinity noncompetitive antagonist of NMDARs.

We found that KYNA depletion enhanced both attractive and aversive learning behaviors. Moreover, we found that reconstitution of *nmr-1* in RIM was sufficient to confer learning to *nmr-1* mutants in the butanone learning paradigm, and it has been reported that *nmr-1* activity in RIM is required for aversive NaCl learning (Kano et al., 2008). Butanone is sensed by the AWC neurons while salt is sensed by ASE neurons. The fact that NMDAR function in RIM directs both attractive and aversive learning suggests that RIM activity generally enhances the ability of the *C. elegans* nervous system to form associations in response to environmental stimuli even when those stimuli are sensed by distinct sensory neurons. Furthermore, we demonstrated the requirement of RIM in learning by artificially silencing it while artificial activation of RIM in the presence of butanone resulted in enhanced learning but did not confer any naïve attraction to this odor. Thus, activity of RIM promotes learning but environmental cues dictate what the learned behavior is.

By modulating activity of RIM-localized NMDARs, KYNA levels provide a general strategy for linking metabolic state to learning capacity. Despite the highly localized requirement for KYNA in learning, its production could be affected by factors that act in tissues

distant from the neuronal sites of KYNA production. Our findings raise the possibility that substrate availability can serve as mechanism for linking peripherally acting pathways to neuronal KYNA production. In support of this, we found that *tdo-2* and *kmo-1*, encoding for enzymes that are required for generation or utilization of kynurenine, respectively, have coincident expression patterns in the *C. elegans* epidermis, a relatively large tissue. In contrast, we found no evidence of expression of these genes in the few neurons that express *nkcat-1*. Surprisingly, while each of DR, insulin, mTOR, AMPK, and autophagy has been intensely investigated, their effects on flux through the kynurenine pathway have remained largely unexplored. DR as well as each of the DR mimetics that enhance learning caused a significant upregulation of *kmo-1*, potentially through the transcription factor FOXO/DAF-16. Since KMO-1 and NKAT-1 both utilize kynurenine as a substrate, the anatomical site of function of *kmo-1*, as well as its upregulation, deprive NKAT-1 of access to kynurenine to produce KYNA. Of course, additional layers of regulation including those that directly impinge on the activity of NKAT-1 or those that function in transport of metabolic intermediates across tissues are also potential mechanisms by which distally acting factors could regulate KYNA levels. Reminiscent of the *C. elegans* findings, data in mammalian systems also indicates that manipulation of the kynurenine pathway in peripheral tissues can affect brain levels of the metabolites (Agudelo et al., 2014).

There is compelling evidence that, in mammals as in *C. elegans*, the KP has significant effects on mechanisms of neural plasticity and learning (Schwarcz et al., 2012). Mice lacking the primary KYNA-producing enzyme in the brain have enhanced hippocampal-dependent learning; hippocampal slices from these animals have increased extracellular glutamate and exhibit greater long-term potentiation amplitude in an NMDAR-dependent manner (Potter et al., 2010). In contrast, adding exogenous KYNA to the brain impairs spatial learning and lowers

extracellular glutamate levels in rats (Pocivavsek et al., 2011). Additionally, dysregulation of the KP in humans has been implicated in Alzheimer's disease (Bonda et al., 2010; Guillemín et al., 2005), Huntington's disease (Beal et al., 1992; Pearson and Reynolds, 1992), Parkinson's disease (Ogawa et al., 1992), depression (Steiner et al., 2011), and schizophrenia (Schwarcz et al., 2001). Since changes in KP metabolite levels can slow progression of neurodegeneration in experimental animal systems, a potential direct link between the KP and diverse neurological disorders has been suggested (Campesan et al., 2011; van der Goot et al., 2012; Savvateeva et al., 2000; Zwillig et al., 2011). It remains to be seen whether in mammals, as in *C. elegans*, KYNA levels fluctuate with nutrient status and if changes in the KP contribute to learning improvements associated with DR or genetic and pharmacological interventions that mimic aspects of DR.

We found that a short-term fast was as effective at enhancing learning as chronic DR mimetics such as decreased insulin or mTOR signaling. In mammals, long term exposure to DR activates additional mechanisms, such as increased BDNF expression and enhanced neurogenesis, that also contribute to learning (Lee et al., 2000, 2002). Because the levels of KYNA are exquisitely sensitive to nutrient status, its role may be in making the nervous system quickly responsive to DR. We speculate that the effects that are sensed by KYNA may then be maintained via other mechanisms involved in DR-mediated plasticity: intermediate-term changes such as translation of synaptic proteins and long-term changes such as dendritic spine remodeling or neurogenesis.

Overall, this work points to KYNA as an evolutionarily conserved inhibitory neuromodulator whose levels directly link the metabolic state of the periphery to neuronal functions involved in learning. As such, they reveal a specific mechanism that underlies the

beneficial effects of DR on learning independent of the effects on lifespan.

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Figure 2-1.

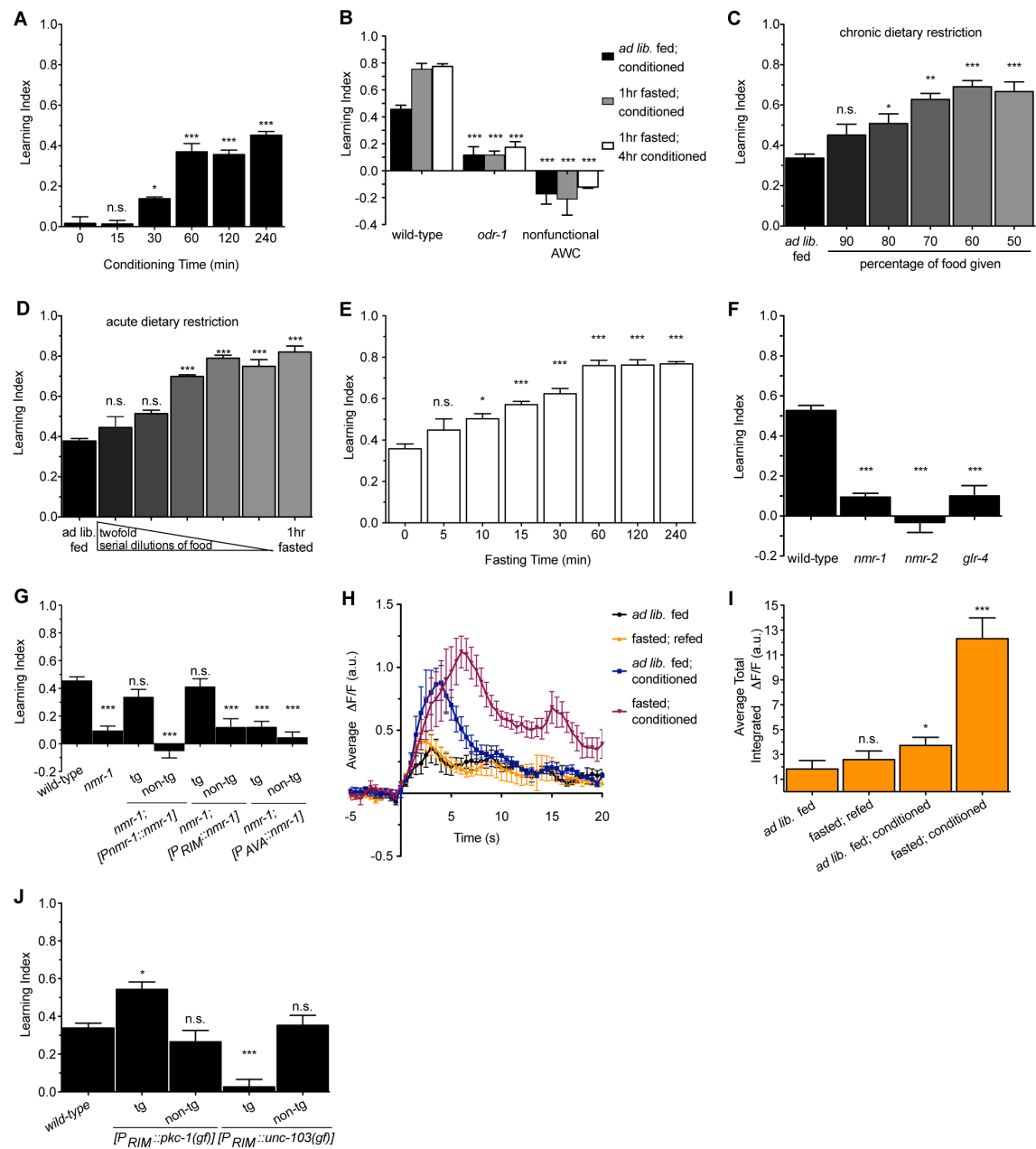


Figure 2-1. Dietary restriction and activation of RIM interneurons enhance learning.

(A) Effects of various durations of conditioning on the learning index of wild type animals. $n = 6$, $*p < 0.05$, $***p < 0.001$ by one-way ANOVA (Tukey). (B) Animals mutant in *odr-1* or with nonfunctional AWC neurons fail to learn in response to butanone. $n = 6$, $***p < 0.001$ by one-way ANOVA (Tukey). (C) Chronic DR (50-90% of *ad lib.* food levels for animals' entire lives) enhance learning. $n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by one-way ANOVA (Tukey). (D) Acute DR (DR for 1hr) as well as a 1hr fast enhance learning. $n = 3-6$, $***p < 0.001$ by one-way ANOVA (Tukey). (E) Effects of various durations of fasting prior to a one-hour conditioning period on learning. $n = 3-10$, $*p < 0.05$, $***p < 0.001$ by one-way ANOVA (Tukey). (F) Animals mutant in glutamatergic receptors fail to learn. $n = 6-10$, $***p < 0.001$ by one-way ANOVA (Tukey). (G) Reconstitution of *nmr-1* in only the RIM neurons is sufficient to restore learning capacity to *nmr-1* mutants. Learning index values for *nmr-1* mutants with *nmr-1* reconstituted under its own promoter, an RIM-specific promoter, or an AVA-specific promoter are shown. tg denotes transgenic animals; non-tg denotes non-transgenic siblings. $n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by one-way ANOVA (Tukey). (H) Average intensity of spontaneous GCaMP transients in RIM from the 250s imaging window aligned to a -5s to 20s time axis. (I) Average total intensity of RIM GCaMP fluorescence over the entire 250s imaging window shows that conditioning significantly increases transient intensity and fasting before conditioning has an even greater effect. $n = 6-10$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by one-way ANOVA (Tukey). (J) Chronic activation of RIM by using a constitutively active protein kinase C encoded by *pkc-1(gf)* promotes learning even in *ad libitum* fed animals while silencing of RIM using an overactive potassium channel encoded by *unc-103(gf)* abolishes learning capacity. tg denotes transgenic animals; non-tg denotes non-transgenic siblings. $n = 4-9$, $*p < 0.05$, $***p < 0.001$ by one-way ANOVA (Tukey). Animals in panels F, G, and J were *ad libitum* fed and conditioned for 1hr. All data are represented as mean $\pm$ SEM.

Figure 2-2.

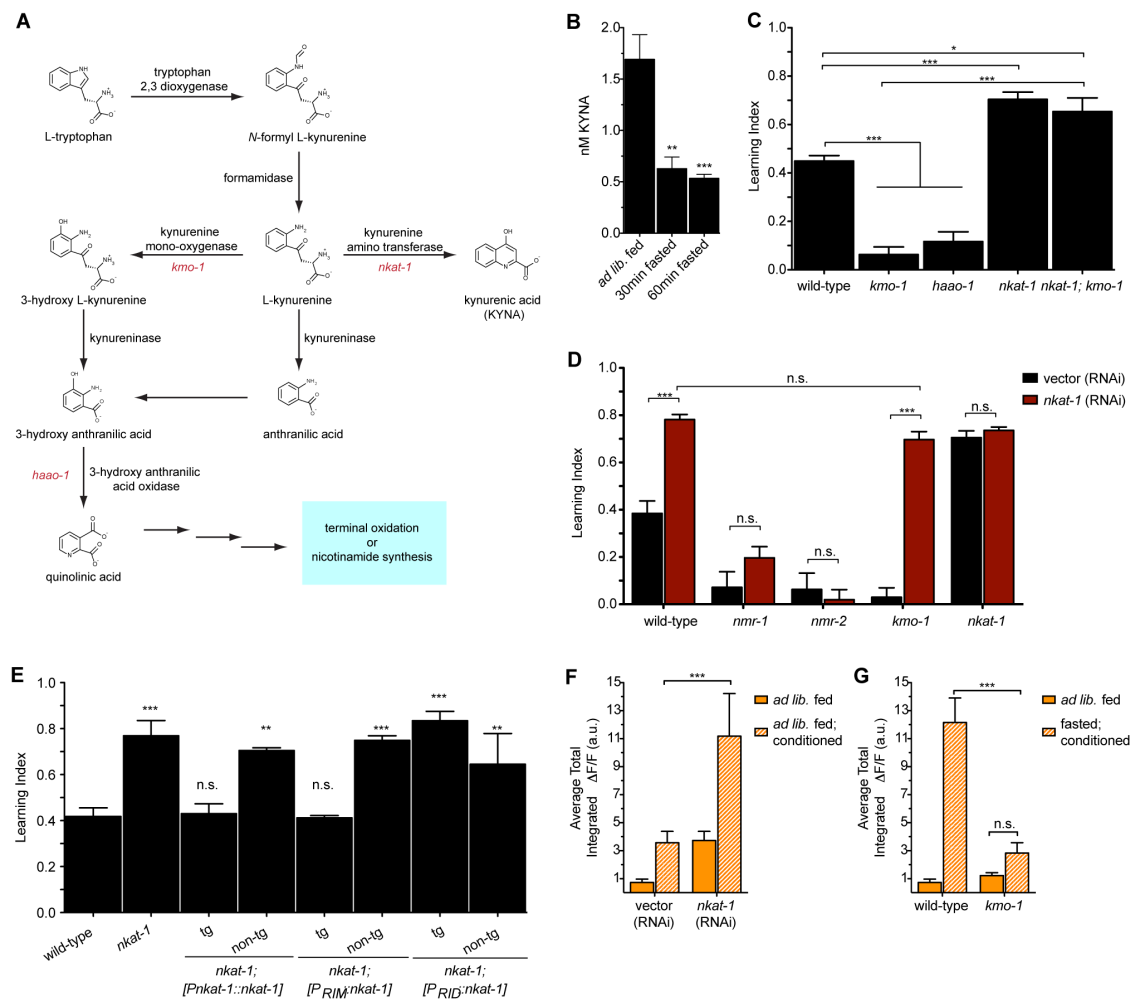


Figure 2-2. Reduction of neuronally produced KYNA mimics the effects of DR on learning.

(A) Schematic of the kynurenine pathway. (B) KYNA levels are reduced upon fasting as indicated by HPLC measurements of whole animal extracts. $n = 5-18$, $**p < 0.01$, $***p < 0.001$ by one-way ANOVA (Tukey). (C) Mutants with reduced KYNA levels have enhanced learning while those with elevated levels have learning deficits. $n = 6-10$, $*p < 0.05$, $***p < 0.001$ by one-way ANOVA (Bonferroni). (D) The learning deficits of *kmo-1* mutants but not those of *nmr* mutants is fully reversed by *nkat-1* RNAi. $n = 3$, $***p < 0.001$ by two-way ANOVA (Bonferroni). (E) Learning index values for *nkat-1* mutants with *nkat-1* reconstituted under its own promoter, an RIM-specific promoter, or an RID-specific promoter. tg denotes transgenic animals; non-tg denotes non-transgenic siblings. $n = 3$. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by one-way ANOVA (Tukey). (F) Average total intensity of RIM GCaMP fluorescence over the entire 250s imaging window shows that conditioning and exposure to *nkat-1* RNAi result in increased transient intensity. $n = 6-10$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by one-way ANOVA (Tukey). (G) Average total intensity of RIM GCaMP fluorescence over the entire 250s imaging window shows that fasting and conditioning does not increase transient intensity in RIM in *kmo-1* mutants. $n = 6-10$, $***p < 0.001$ by two-way ANOVA (Bonferroni). Animals in panels C, D, and E were *ad libitum* fed and conditioned for 1hr. All data are represented as mean $\pm$ SEM.

Figure 2-3.

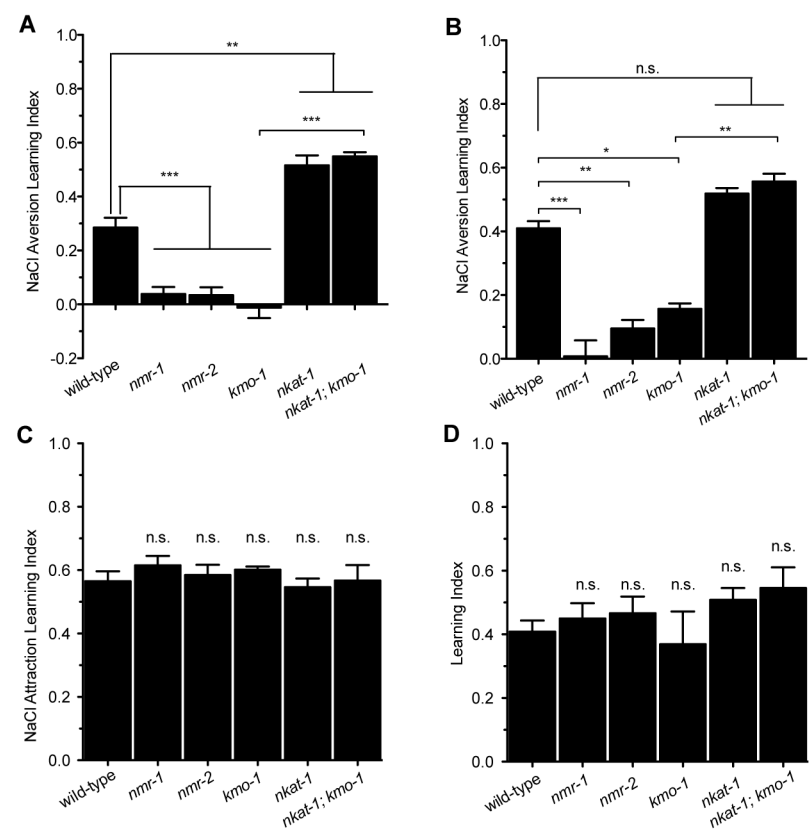


Figure 2-3. KYNA depletion enhances learning only in paradigms that require NMDARs.

(A) NaCl aversion short-term training: learning index values for animals conditioned with high NaCl without food for 3 hours. $n = 3-6$, $*p < 0.05$, $***p < 0.001$ by one-way ANOVA (Bonferroni). (B) NaCl aversion long-term training: learning index values for animals conditioned with high NaCl without food for 6 hours. $n = 3-6$, $*p < 0.05$, $***p < 0.001$ by one-way ANOVA (Bonferroni). (C) NaCl attraction short-term training: Learning index values for animals conditioned with high NaCl with food for 6 hours. $n = 3-6$, significance measured by one-way ANOVA (Tukey). (D) Diacetyl short-term training: Learning index values for animals conditioned with the odor diacetyl with food. $n = 3$, significance measured by one-way ANOVA (Tukey). All data are represented as mean $\pm$ SEM.

Figure 2-4.

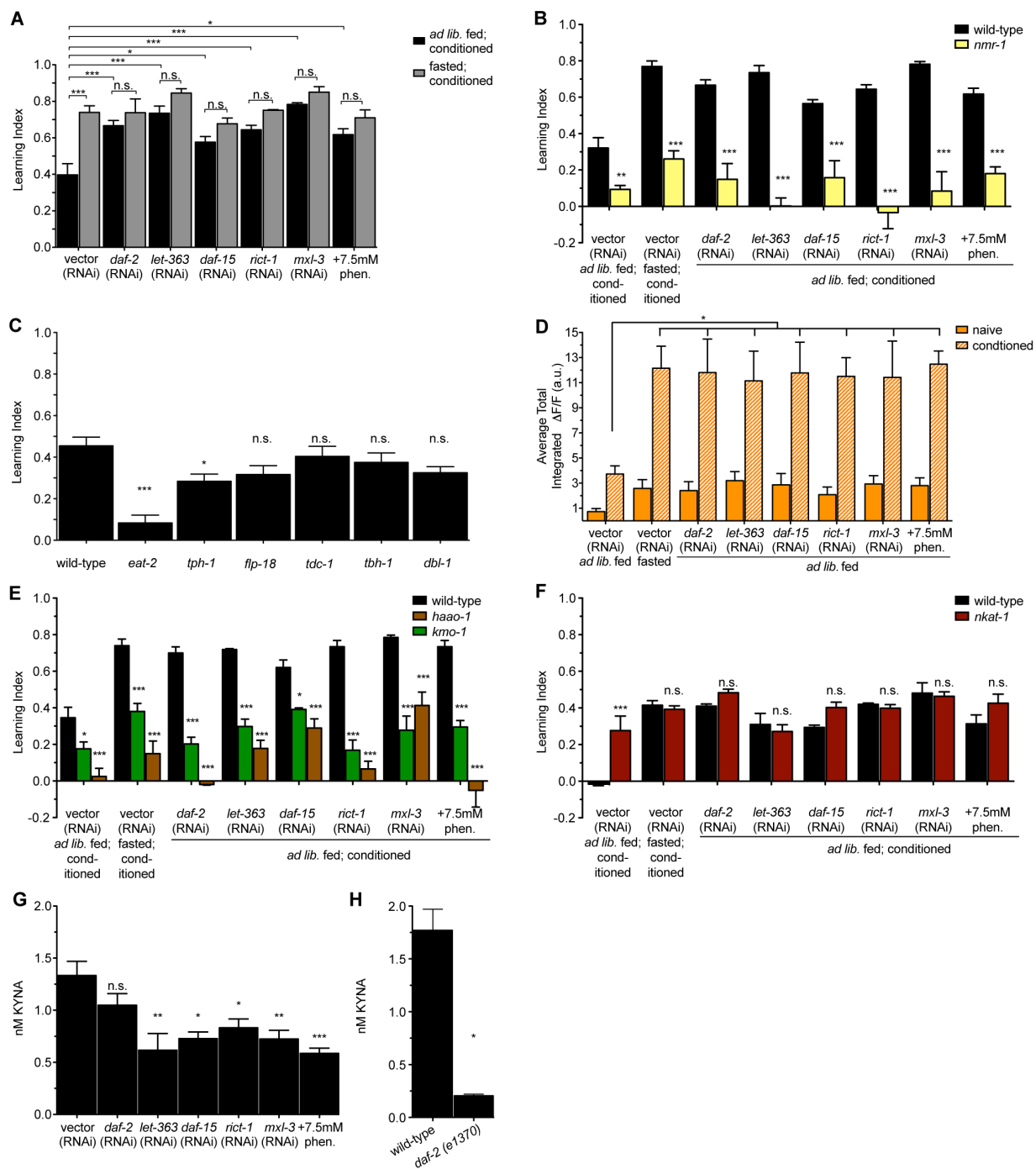


Figure 2-4. Genetic and pharmacological manipulations that mimic DR enhance learning by depleting KYNA.

(A) RNAi-mediated reductions in the insulin receptor (*daf-2*), the mTOR kinase (*let-363*), Raptor (*daf-15*), Rictor (*rict-1*), and a negative regulator of autophagy (*mxl-3*), as well as animals treated with an activator of AMPK (phenformin) have enhanced learning capacity even when fed *ad libitum*. n = 3-6, *p<0.05, ***p<0.001 by two-way ANOVA (Bonferroni). (B) The elevated learning capacities of genetic and pharmacological mimetics of DR are dependent on NMDAR signaling. n = 3, *p<0.05, **p<0.01, ***p<0.001 by two-way ANOVA (Bonferroni). (C) Learning index values for additional mutants in various neural nutrient sensing pathways: *eat-2* mutants have a pharyngeal pumping defect, *tph-1* mutants do not produce serotonin, *flp-18* mutants lack a neuropeptide Y-like peptide, *tdc-1* mutants do not produce tyramine or octopamine, *tbh-1* mutants do not produce octopamine, and *dbl-1* mutants lack a TGF- β . n = 3-6, *p<0.05, ***p<0.001 by one-way ANOVA (Tukey). (D) Average total intensity of RIM GCaMP fluorescence over the entire 250s imaging window in animals exposed to genetic and pharmacological DR mimetics. n = 6-10, *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA (Tukey). (E) Learning index values for mutants with high KYNA exposed to genetic and pharmacological DR mimetics. n = 3, *p<0.05, **p<0.01, ***p<0.001 by two-way ANOVA (Bonferroni). (F) Learning index values for wild-type and *nkat-1* animals given DR mimetics. N.B. To ensure that effects of DR mimetics in the context of KYNA depletion can be observed, animals were conditioned for only 15 minutes. n = 3, ***p<0.001 by two-way ANOVA (Bonferroni). (G) HPLC measurements of steady-state KYNA levels for animals exposed to genetic and pharmacological DR mimetics. n = 5-18, *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA (Tukey). (H) HPLC measurements of steady-state KYNA levels for wild-type and *daf-2(e1370)* mutant animals. n = 2, *p<0.05 by two-tailed Student's t-test. Animals in panels B, C, E, and F were *ad libitum* fed and conditioned. All data are represented as mean $\pm$ SEM.

Figure 2-5.

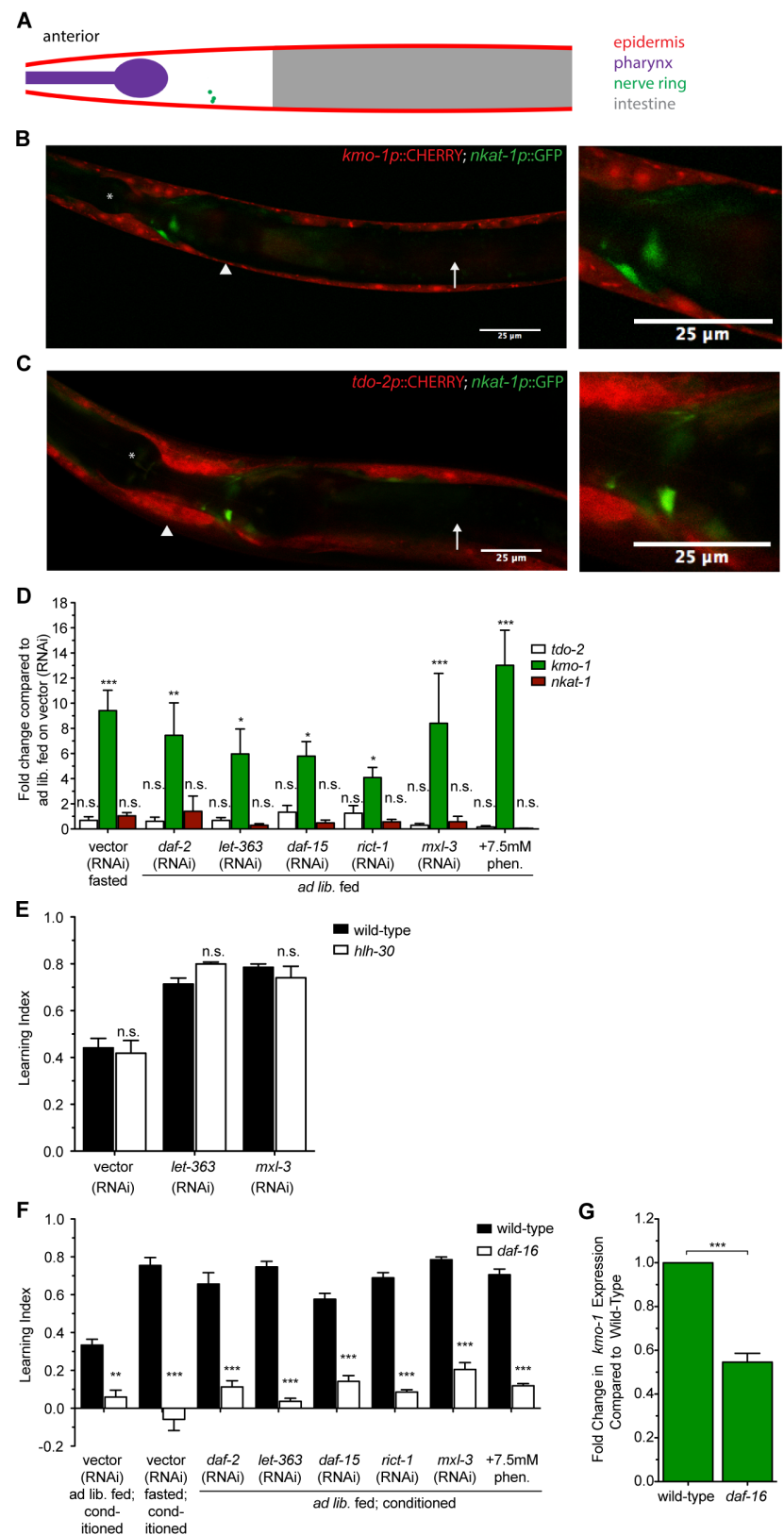


Figure 2-5. *kmo-1* and *nkat-1* have distinct tissue expression patterns and exhibit distinct transcriptional patterns of regulation.

(A) Cartoon representation of the anterior portion of adult *C. elegans* comparable to images in panels B and C. Labeled anatomical structures correspond to those in panels B and C. (B) 5µm thick z-projection of an adult animal with mCherry under control of the *kmo-1* promoter and GFP under control of the *nkat-1* promoter. Left: anterior portion of the animal. Right: magnified view of the nerve ring showing no overlap of GFP and mCherry. Asterisk indicates pharynx; arrowhead indicates epidermis; arrow indicates intestine. (C) 5µm thick z-projection of an adult animal with mCherry under control of the *tdo-2* promoter and GFP under control of the *nkat-1* promoter. Left: anterior portion of the animal. Right: magnified view of the nerve ring showing no overlap of GFP and mCherry. Asterisk indicates pharynx; arrowhead indicates epidermis; arrow indicates intestine. (D) Change in transcript levels of KP genes in animals treated with DR or DR mimetics as determined by real-time qPCR. Data are represented as fold change compared to *ad libitum* fed animals on vector (RNAi). n = 3 biological replicates, *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA (Tukey). (E) Learning index values for *hlh-30* mutants on *let-363* and *mxl-3* RNAi. n = 3, significance measured by two-way ANOVA (Bonferroni). (F) Learning index values for *daf-16* mutants on DR mimetics. n = 3, **p<0.01, ***p<0.001 measured by two-way ANOVA (Bonferroni). (G) Change in *kmo-1* transcript levels in *daf-16* mutants as determined by real-time qPCR. Data are represented as fold change compared to wild-type. n = 3 biological replicates, *p<0.05, ***p<0.001 by one-way ANOVA (Tukey). All data are represented as mean ± SEM.

Table 2-1.

RNAi	Strain	Replicate 1 (Figure 2-S6)		Replicate 2 (Figure 2-S7)	
		n	Median lifespan	n	Median Lifespan
Vector	wild-type	86	17	58	17
	<i>kmo-1</i>	88	17 (n.s.)	60	19 (n.s.)
	<i>nkat-1</i>	73	17 (n.s.)	58	17 (n.s.)
<i>daf-2</i>	wild-type	83	27	60	29
	<i>kmo-1</i>	75	29 (n.s.)	59	29 (n.s.)
	<i>nkat-1</i>	77	27 (n.s.)	60	29 (n.s.)
<i>let-363</i>	wild-type	81	22	60	24
	<i>kmo-1</i>	76	24 (n.s.)	58	24 (n.s.)
	<i>nkat-1</i>	78	22 (n.s.)	62	24 (n.s.)
<i>daf-15</i>	wild-type	75	20	60	24
	<i>kmo-1</i>	78	20 (n.s.)	60	24 (n.s.)
	<i>nkat-1</i>	81	20 (n.s.)	60	24 (n.s.)
<i>rict-1</i>	wild-type	83	13	61	15
	<i>kmo-1</i>	79	13 (n.s.)	57	15 (n.s.)
	<i>nkat-1</i>	82	13 (n.s.)	60	15 (n.s.)
mxl-3	wild-type	75	20	77	20
	<i>kmo-1</i>	80	20 (n.s.)	74	20 (n.s.)
	<i>nkat-1</i>	72	17 (n.s.)	69	20 (n.s.)
vector + 7.5mM phen.	Wild-type	76	22	60	24
	<i>kmo-1</i>	76	20 (n.s.)	60	26 (n.s.)
	<i>nkat-1</i>	79	22 (n.s.)	68	24 (n.s.)

Table 2-1. Median lifespans of animals on DR mimetics. Median lifespans of animals given DR mimetics are not affected by *kmo-1* or *nkat-1* mutations. Significance was measured by logrank tests. See also Figure 2-S6-S7 for full survival curves.

Figure 2-6.

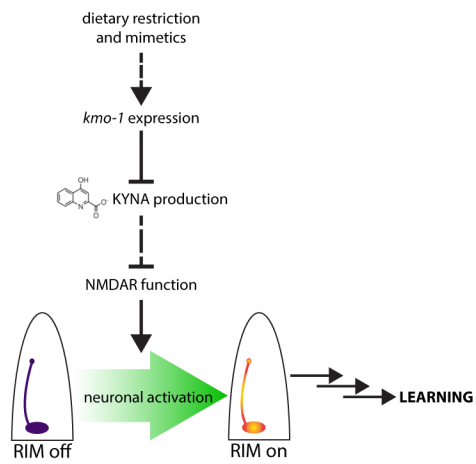


Figure 2-6. Model for how DR and DR mimetics enhance learning.

DR and DR mimetics decrease production of KYNA. This acts as a release of inhibition on NMDAR function. The result is a greater influx of Ca^{2+} into the RIM interneurons after conditioning, ultimately leading to learning.

Figure 2-S1.

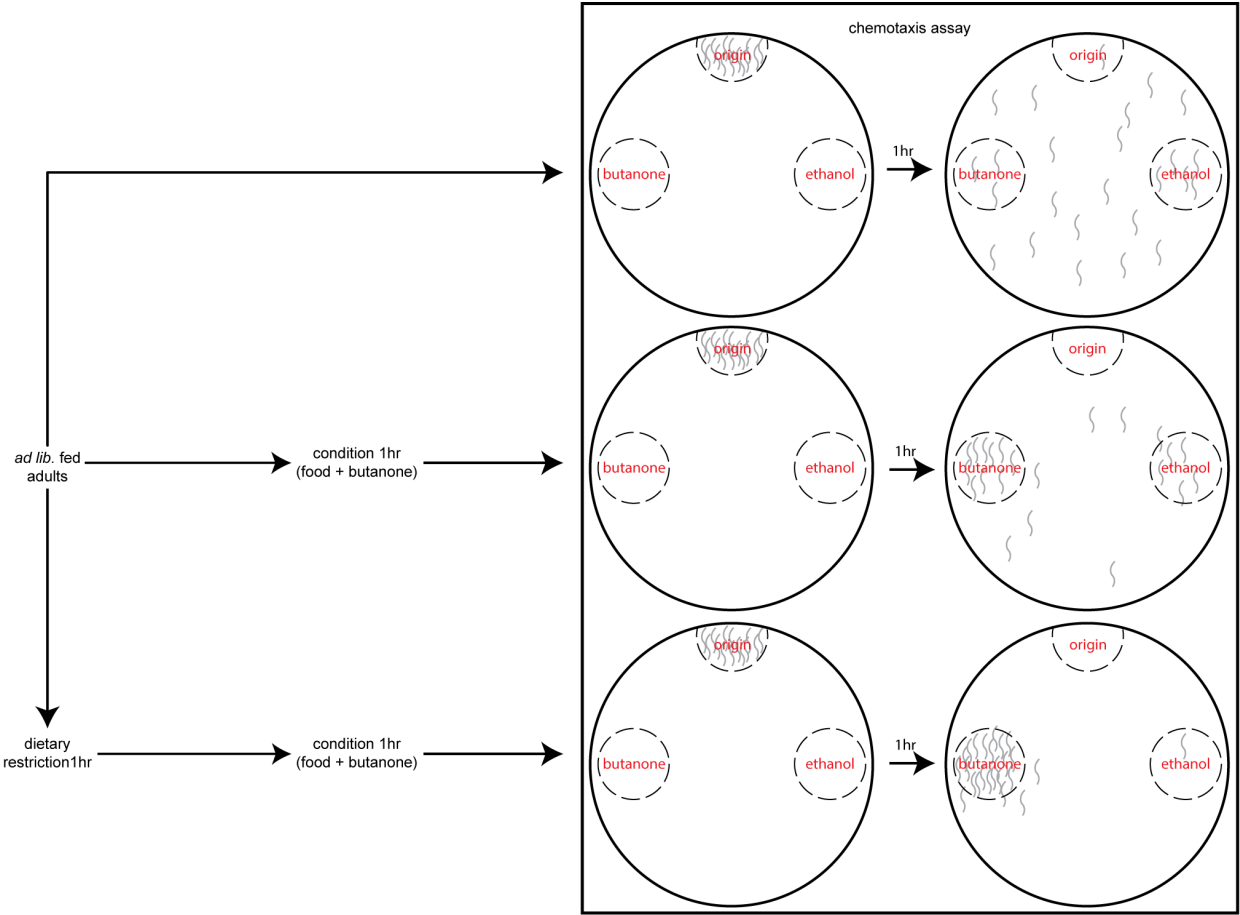


Figure 2-S1. Short term associative learning assay, related to Figure 2-1.

Ad libitum fed animals at day 1 of adulthood were put through one of three conditioning manipulations before being assessed in a chemotaxis assay.

Figure 2-S2.

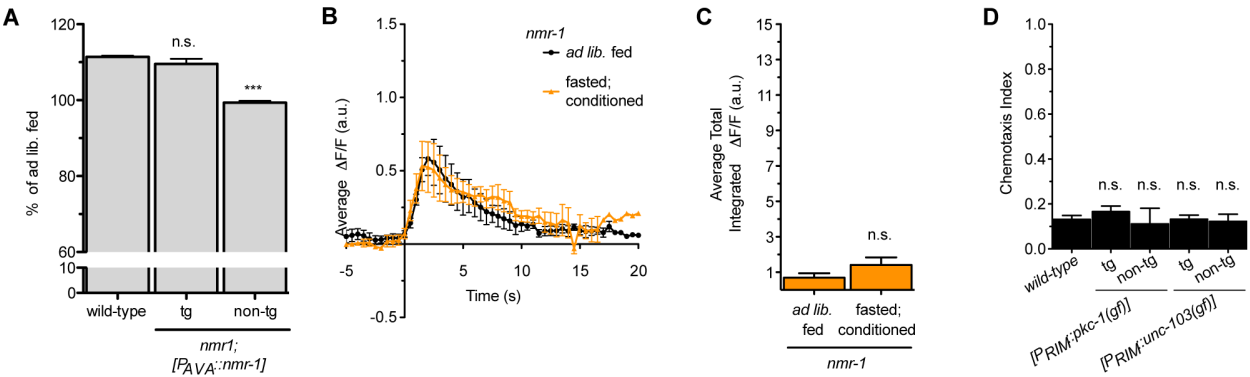


Figure 2-S2. Requirements of NMDAR and RIM function, related to Figure 2-1.

(A) Reconstitution of *nmr-1* in AVA rescues its post-fast feeding phenotype. Pharyngeal pumping rate was measured when fasted animals were reintroduced to food. Values are represented as percentage of *ad libitum* fed pumping rate. tg denotes transgenic animals; non-tg denotes non-transgenic siblings. n = 10, *p<0.05, ***p<0.001 by one-way ANOVA (Tukey). (B) Average intensity of *nmr-1* mutants' spontaneous GCaMP transients in RIM from the 250s imaging window aligned to a -5s to 20s time axis. n = 6-10. (C) Average total intensity of RIM GCaMP fluorescence in *nmr-1* mutants over the entire 250s imaging window shows no significant change when fasted and conditioned. n = 6-10, by one-way ANOVA (Tukey). (D) Chronic activation or inactivation of RIM has no effect on naïve chemotaxis to butanone. n = 3-6, by one-way ANOVA (Tukey). All data are represented as mean ± SEM.

Figure 2-S3.

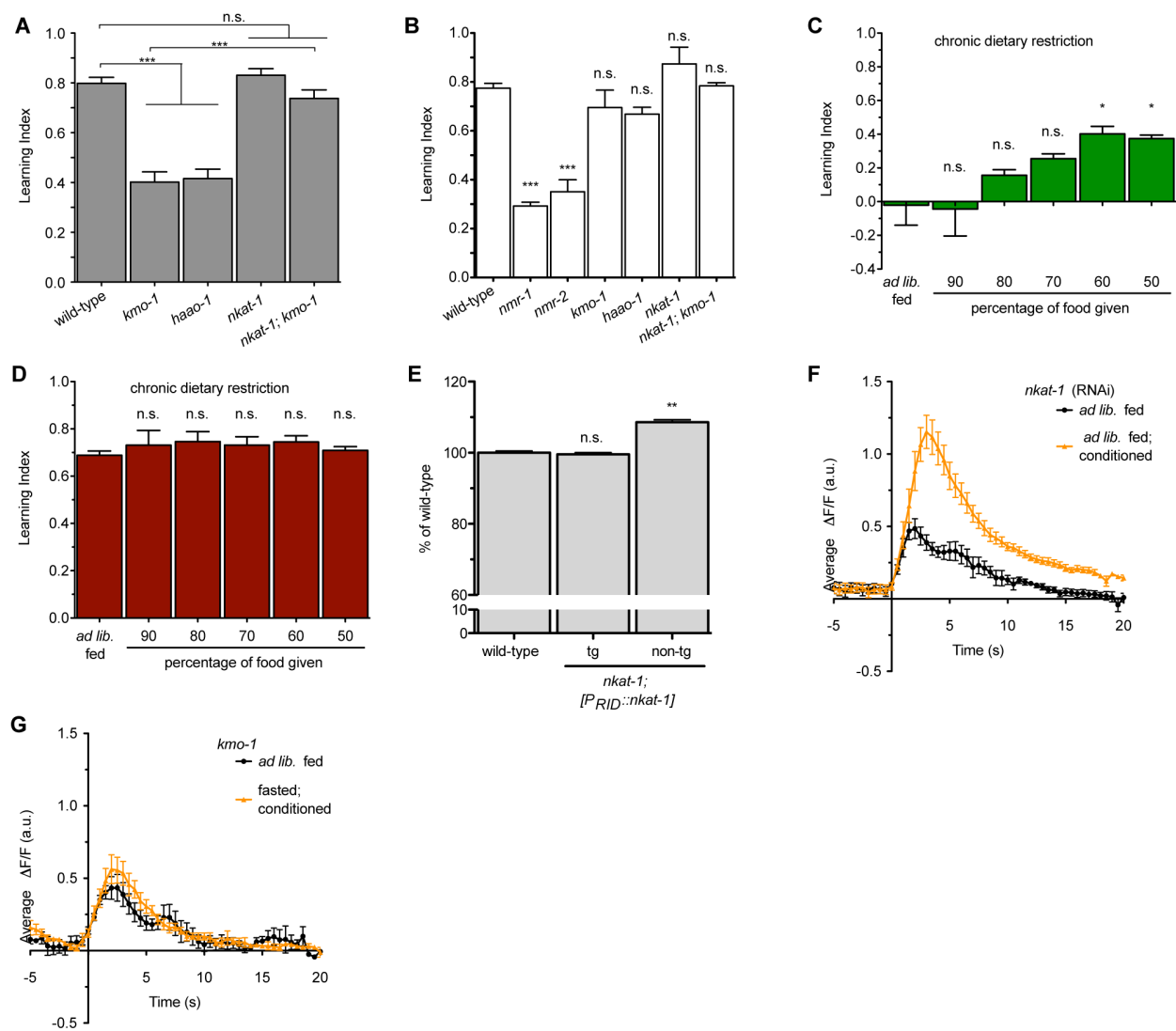


Figure 2-S3. The beneficial effects of KYNA depletion on learning require NMDARs, related to Figure 2-2.

(A) Learning index values for animals that have been fasted for one hour and then conditioned for one hour. $n = 6-10$, *** $p < 0.001$ by one-way ANOVA (Bonferroni). (B) Learning index values for animals that have been fasted for one hour and then conditioned for 4 hours. $n = 6$, *** $p < 0.001$ by one-way ANOVA (Tukey). (C) Learning index values for *kmo-1* mutants given chronic DR (50-90% of *ad lib.* food levels for their entire lives). $n = 3$, * $p < 0.05$ by one-way ANOVA (Tukey). (D) Learning index values for *nkat-1* mutants given chronic DR (50-90% of *ad lib.* food levels for their entire lives). $n = 3$, significance measured by one-way ANOVA (Tukey). (E) Reconstitution of *nkat-1* in the RID interneuron is sufficient to restore *nkat-1* mutants' hyperactivated feeding to wild-type levels. tg denotes transgenic animals; non-tg denotes non-transgenic siblings. $n = 10$, ** $p < 0.01$ by one-way ANOVA (Tukey). (F) Average intensity of spontaneous GCaMP transients in RIM of animals on *nkat-1* RNAi from the 250s imaging window aligned to a -5s to 20s time axis. $n = 6-10$. (G) Average intensity of *kmo-1* mutants' spontaneous GCaMP transients in RIM from the 250s imaging window aligned to a -5s to 20s time axis. $n = 6-10$. All data are represented as mean $\pm$ SEM.

Figure 2-S4.

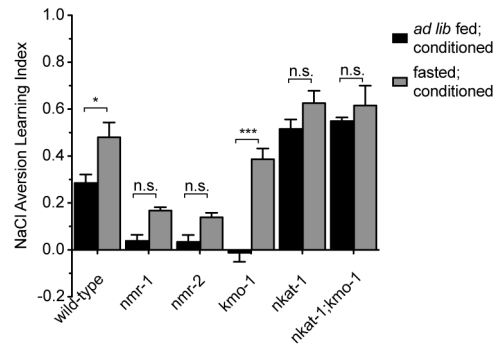


Figure 2-S4. Fasting enhances NaCl aversion learning, related to Figure 2-3.

NaCl aversion short term training: learning index values for animals fasted in normal NaCl for 3 hours and subsequently conditioned with high NaCl without food for 3 hours. $n = 3$, $*p < 0.05$ by one-way ANOVA (Bonferroni). All data are represented as mean $\pm$ SEM.

Figure 2-S5.

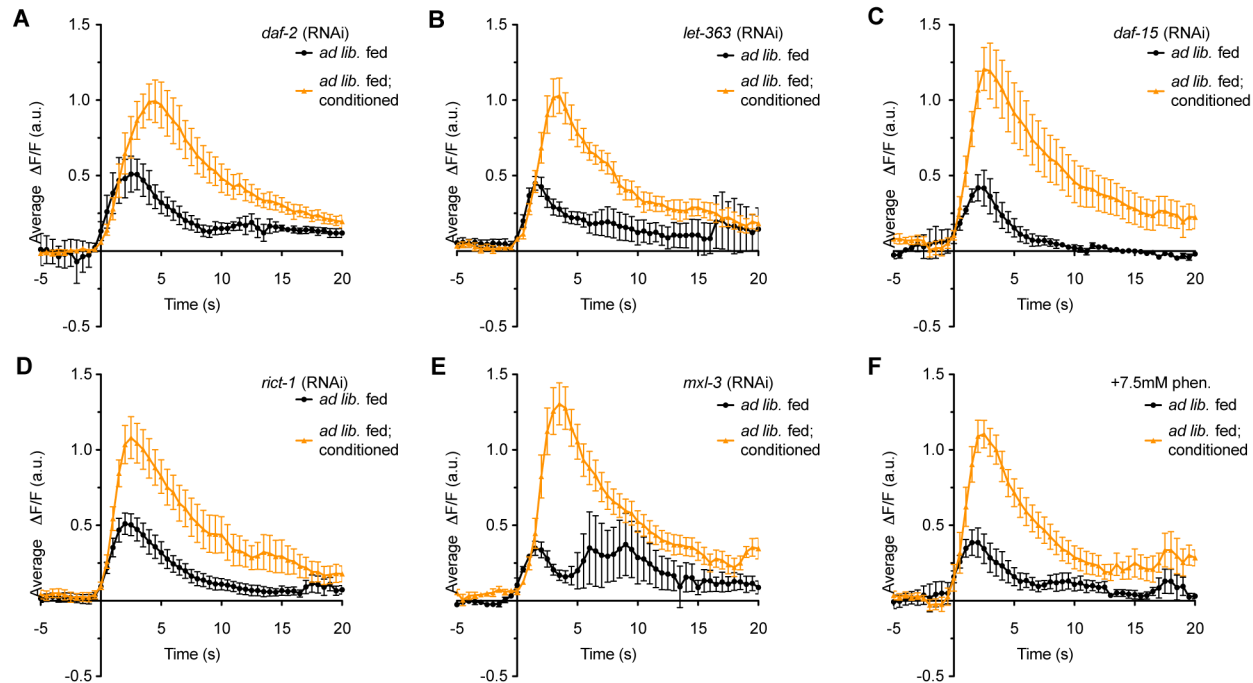


Figure 2-S5. GCaMP traces of animals given DR mimetics, related to Figure 2-4.
(A-F) Average intensity of spontaneous GCaMP transients in RIM of animals on DR mimetics from the 250s imaging window aligned to a -5s to 20s time axis. n = 6-10.

Figure 2-S6.

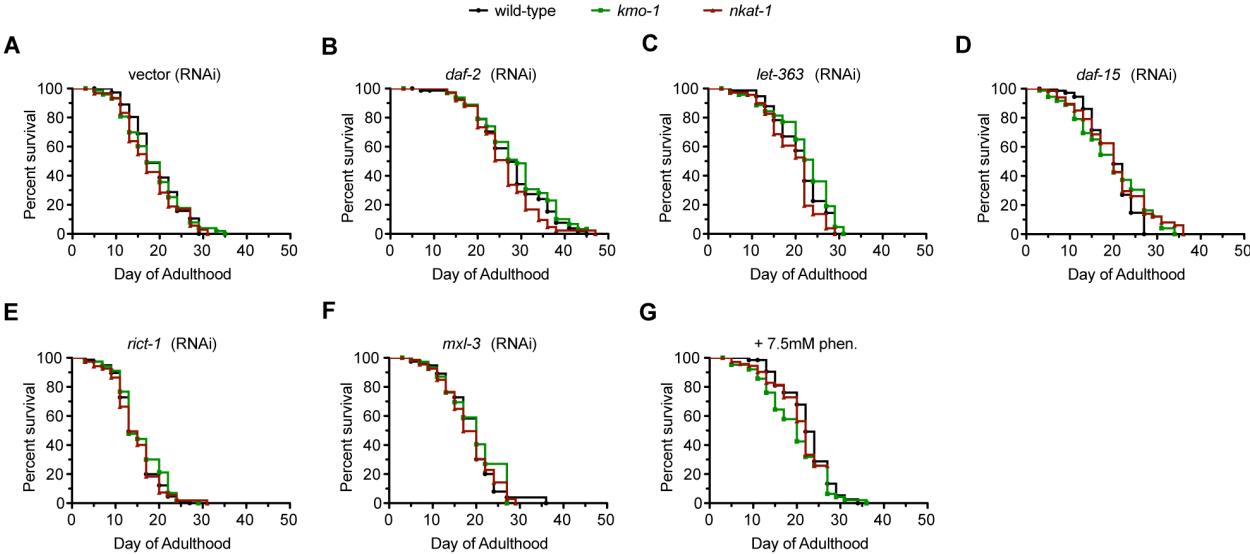


Figure 2-S6. KP modulation does not affect lifespan, related to Table 2-1.

(A-G) Survival curves for wild-type, *kmo-1*, and *nkaf-1* animals given DR mimetics. Significance measured by logrank test. Median lifespans and n can be found in Table 2-1.

Figure 2-S7.

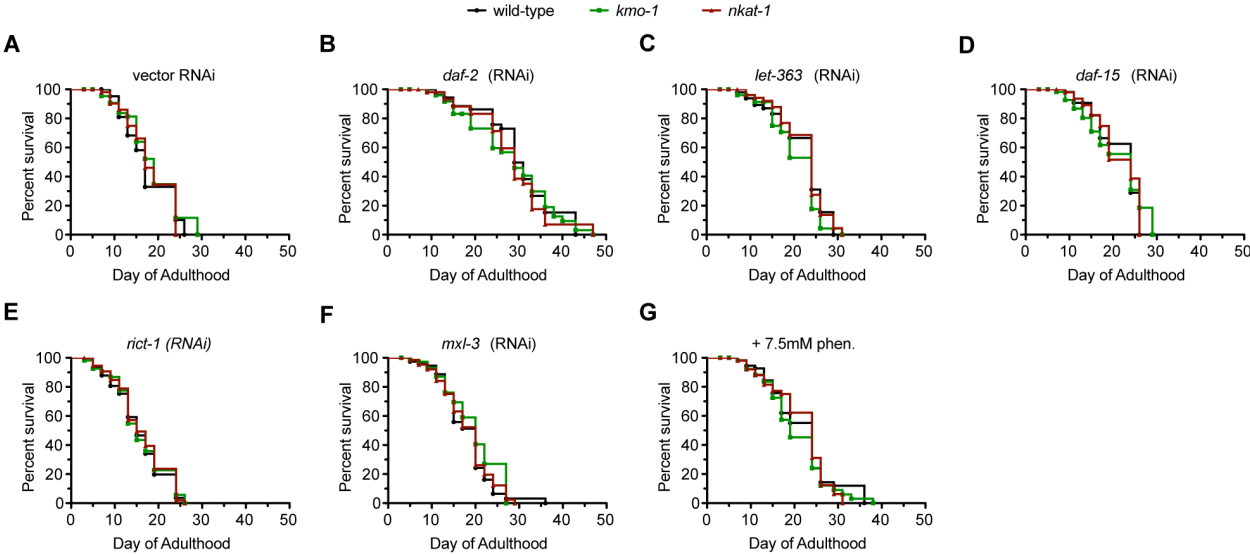


Figure 2-S7. KP modulation does not affect lifespan, related to Table 2-1.

(A-G) Survival curves for second replicates of wild-type, *kmo-1*, and *nkat-1* animals given DR mimetics. Significance measured by logrank test. Median lifespans and n can be found in Table 2-1.

Chapter 3: Accumulation of kynurenic acid underlies learning impairments associated with aging

Introduction

One of the most devastating effects of aging is impaired cognitive capacity. Aging is the biggest risk factor for neurodegenerative diseases like Alzheimer's Disease, Huntington's Disease, and Parkinson's Disease, but learning declines with age even in the absence of other neurological pathologies (López-Otín et al., 2013; Wyss-Coray, 2016). Few interventions are known to protect against age-induced learning impairment (Dubal et al., 2014; Villeda et al., 2011; Yu et al., 2017), and even in these cases, the precise mechanisms by which age-dependent deficits are ameliorated remain unclear. The kynurenine pathway (KP) is an evolutionarily conserved pathway that degrades the essential amino acid tryptophan and produces several neuroactive compounds including kynurenic acid (KYNA), a noncompetitive antagonist of N-methyl-D-aspartate receptors (NMDARs) (Kessler et al., 1989), which are vital for learning in many organisms (Kano et al., 2008; Morris et al., 1986). Here, using *C. elegans* to investigate the effects of aging on learning and memory, we show that aging is associated with accumulation of KYNA, which blunts activity of specific NMDAR-expressing neurons in *C. elegans*. KYNA-deficient animals exhibit learning capacity when aged, and importantly, reducing the rate of decline in learning has no effect on the overall rate of aging. In addition to its effects on learning, depletion of KYNA allows for a profound retention of memory. We demonstrate that increased insulin signaling is one factor that underlies the age-associated increase in KYNA. Finally, we demonstrate that depletion of KYNA is a viable strategy for restoring learning capacity to aging animals even when the intervention is initiated late in adulthood.

Results

C. elegans exhibit associative learning, increasing their attraction to the odor butanone after it has been paired with food (Kauffman et al., 2010). This process uses molecular components also required for mammalian learning, such as NMDARs, AMPARs, and CREB (Kano et al., 2008; Kauffman et al., 2010; Vohra et al., 2017). The capacity to learn associations undergoes a progressive, age-induced impairment in *C. elegans* (Kauffman et al., 2010). For example, 7-day old adult animals only exhibit ~5% of the learning capacity seen in day 1 adults (Kauffman et al., 2010). Since many tissues deteriorate with age, it is possible that some irreparable damage to the nervous system underlies this age-induced learning impairment. But simply increasing the duration of conditioning from 1 to 4 hours is sufficient to counter, albeit partially, these learning impairments (Figure 3-S1A and S1B). Thus, a significant portion of age-induced impairment in learning is caused by reversible changes.

Consistent with the role of KYNA as a noncompetitive antagonist of NMDARs, and given that increasing the duration of conditioning confers a significant degree of learning capacity to aged animals, we considered the possibility that aberrant accumulation of KYNA may contribute to age-induced impairments. We found that as *C. elegans* age, the pattern of KYNA-mediated modulation reflects what may be expected from an antagonist of neuronal activity: elevating KYNA via mutation in *kmo-1* exacerbates age-induced learning impairment, and depleting KYNA via mutation in *nkat-1* is protective against it (Figure 3-1A and 1B). More importantly, reducing KYNA levels also slowed the rate of age-induced learning impairment while increasing its levels exacerbated it (Figure 3-1C). As with wild-type animals, increasing the duration of conditioning could partially decelerate age-induced learning impairments in *kmo-1* mutants, though it had no effect on *nkat-1* mutants (Figure 3-1D and 3-1E). Although by day 7

of adulthood wild-type animals show almost no learning capacity upon 1 hour of conditioning, their capacity for motility remains about the same as those of day 1 animals, they retain some fertility, and as a population they remain almost all alive. Furthermore, altering KYNA levels does not affect *C. elegans* lifespan (Figure 3-1F) (Vohra et al., 2017), indicating that the age-associated effects we observed are brain-specific, not simply the result of general impairments in cellular maintenance or organismal health.

To confirm that these brain-specific impairments were related to plasticity, not just sensorimotor systems more generally, we assessed naïve chemotaxis to butanone at various ages and found that it was unchanged regardless of KYNA levels (Figure 3-S1C). However, the magnitude of this chemotaxis was small, raising the possibility that subtle changes would not be detectable. Therefore, we examined the more robust naïve attraction animals exhibit to a lower concentration of butanone (Bargmann et al., 1993), and we found that even using this concentration, there were no age-related changes in chemotaxis regardless of KYNA levels (Figure 3-S1D). Because chemotaxis to butanone requires odor sensation and movement, this indicates that general sensorimotor circuits remains intact in aged animals, and that the age-induced changes we observed most likely represent an impairment in plasticity.

In *C. elegans*, NMDARs are expressed in only a few pairs of neurons (Brockie et al., 2001). Among these, activity of NMDARs in the RIM pair of interneurons is both sufficient and necessary for several types of learning behaviors, including the butanone associative learning assay (Kano et al., 2008; Lau et al., 2013; Vohra et al., 2017). We previously showed that activity of RIM neurons, as assessed by the intensity of spontaneous Ca^{2+} transients using GCaMP3.0 over an extended period of time (250s), is a cellular correlate of learning such that conditions that enhance or diminish learning cause elevated or reduced RIM transients,

respectively (Vohra et al., 2017). Thus, we investigated RIM transients upon conditioning as animals aged. To compare average intensities of transients, we aligned transients from 5s before their initiation to 20s later. Consistent with the behavioral phenotype, average intensity of RIM activity after conditioning decreased with age (Figure 3-1G). Totalling the average intensity of transients over the entire 250s imaging window demonstrated the same phenomenon, with no significant age-related changes to the number of transients observed during the imaging window (Figure 3-1H). The age-induced impairment in learning and RIM activity, along with the deceleration of this impairment observed in KYNA-depleted animals, supported the notion that KYNA accumulates with age, dampening RIM activity and learning capacity. Indeed, when we extracted KYNA from animals we found that levels increased with age as measured by HPLC (Figure 3-1I).

We found that KYNA levels modulate not just learning, but memory as well. In a short-term associative memory assay, young adult animals are conditioned with food and butanone and subsequently given food in the absence of butanone. Then at regular intervals, cohorts of animals are tested to determine their ability to remember the learned attraction to butanone (Kauffman et al., 2010). Wild-type animals could maintain this memory for 90 minutes after conditioning. *kmo-1* mutants (which have high KYNA) exhibited accelerated deterioration of this memory whereas *nkcat-1* mutants (which have low KYNA) showed enhanced memory maintenance (Figure 3-2A). Next, we tested the effects of KP metabolites in a long-term memory assay, where instead of a single conditioning period animals were alternatingly conditioned and fasted seven times (Kauffman et al., 2010). Under these conditions, maintenance of memory in wild-type animals increases from the 90 minutes seen in the short term assay to many hours. By about 40 hours, however, this extended memory is largely extinguished in wild type animals. Loss of

memory was accelerated in *kmo-1* mutants such that it is almost entirely lost in about 16 hours. By contrast, there was very little loss of memory at 40 hours in *nkat-1* mutants (Figure 3-2B). Thus, KYNA levels modulate both learned attraction to an odor and the memory of that attraction.

We next considered mechanisms that may underlie age-dependent accumulation of KYNA. We first considered the possibility that this phenomenon may be specific to hermaphrodites given age-onset changes of egg laying rate that may contribute to accumulation of KYNA. However, this was not the case as a similar age-dependent impairment of learning capacity was noted in males (Figure 3-S1E and S1F). We next considered the effects of insulin signaling, as this pathway regulates expression of kynurenine pathway enzymes (Vohra et al., 2017; Zarse et al., 2012) and that insulin signaling increases with age (Dues et al., 2016). Insulin levels can be elevated by reduction-of-function mutations in the Bardet-Biedl syndrome gene homolog *bbs-7* (also known as *osm-12*) (Lee et al., 2011) or the PTEN homolog *daf-18* (Ogg and Ruvkun, 1998). These high insulin-signaling mutants all had a learning impairment at day 1 of adulthood that could be partially rescued by depleting KYNA via *nkat-1* RNAi (Figure 3-3A). These mutants were measured to have high KYNA levels compared to wild-type animals (Figure 3-3B).

To better understand how increases in insulin signaling contribute to increased accumulation of KYNA, we examined expression patterns of genes that encode for various enzymes of the kynurenine pathway. We found that increasing insulin signaling decreased expression of *kmo-1*, which competes with NKAT-1 for the same substrate and thus its downregulation would be expected to allow for greater production of KYNA (Figure 3-1A and Figure 3-3C). Increasing insulin signaling had little effect on expression of other enzymes of the

KP (Figure 3-3C). Mimicking the effects of elevated insulin signaling, wild-type animals at day 5 and 7 of adulthood also had decreased expression of *kmo-1* (Figure 3-3D). Given that decreasing insulin signaling elevates *kmo-1* expression (Vohra et al., 2017; Zarse et al., 2012), these data suggest that insulin-mediated regulation of *kmo-1* may represent a critical point of regulation of KYNA production during the course of aging.

If age-induced accumulation of KYNA is indeed regulated by insulin signaling, then reducing insulin signaling should restore learning in old animals. We treated animals with *daf-2* RNAi to impair insulin signaling and observed a deceleration of age-induced learning impairment (Figure 3-4A and 4B), consistent with results previously reported for *daf-2(e1370)* mutants at day 5 of adulthood (Kauffman et al., 2010). However, insulin signaling has broad effects, and *daf-2* RNAi extends median lifespan (Figure 3-4C), making it difficult to discern whether the effects on age-induced learning impairment are a result of specific changes in circuitry responsible for learning. For a more specific approach, we reasoned that if KYNA accumulates with age and increasingly antagonizes neuronal function, we should be able to bypass this accumulation and restore learning by artificially stimulating relevant neurons in the circuit. As RIM activity correlates with learning capacity in this paradigm, we chronically activated RIM neurons using a constitutively active protein kinase C (PKC) (Macosko et al., 2009). Similar to *nkat-1* mutants, transgenic animals with activated RIM showed a deceleration of age-induced learning impairment (Figure 3-4D and 4E). Also similar to *nkat-1* mutants, this was a brain-specific effect and did not alter lifespan (Figure 3-4F).

While lifelong interventions such as *nkat-1* mutation, *daf-2* RNAi, and chronic neuronal activation were effective at protecting against some of the age-induced learning impairment, we were particularly interested in whether KYNA depletion in adult animals that were already aging

could change the trajectory of this impairment and be protective as well. We treated animals with *nkat-1* RNAi beginning at day 3 of adulthood and found that this was still protective to about the same extent (Figure 3-4G, Figure 3-4H, and Figure 3-S2A), indicating that intervention later in life to deplete KYNA could rejuvenate brain function. Similarly, this intervention restored some of the Ca^{2+} activity in RIM (Figure 3-4I, Figure 3-S2B, and Figure 3-S2C). Thus, altering KYNA levels or neuronal responsiveness to KYNA are viable strategies to decelerating age-induced learning impairment, even in aged animals.

Discussion

C. elegans display rudimentary forms of neural plasticity as illustrated by their ability to form associations between an odorant and food. There is a progressive decline in this ability as animals age. We found that this decline has at least two components: a component that is subject to reversal upon increasing the conditioning period or artificially activating the specific neurons that are known to be required for formation of these associations. Our data here show that this reversible decline is due to age-dependent accumulation of KYNA, which blunts activity of NMDARs that are critically required for learning capacity across phylogeny. While it remains unknown why this accumulation occurs, our findings suggest elevated insulin signaling is likely to be at least one factor that drives it. Thus, age-related accumulation of KYNA may not be a program per se, but a consequence of various physiological changes, including elevated insulin signaling that collectively alter the flux through the kynurenine pathway resulting in KYNA accumulation.

Interestingly, KYNA levels are higher in day 5 adults than in day 7 adults (Figure 3-1I). Given the nature of KYNA as a competitive antagonist of NMDAR signaling, it is likely that

once KYNA levels increase beyond a certain concentration, additional accrual of KYNA may not cause additional detrimental effects to learning capacity. The precise levels at which KYNA becomes saturating for its effects on learning capacity are not currently known. This, along with KYNA-independent deterioration of the nervous system between days 5 and 7 may explain why day 5 adults learn better than day 7 adults despite their relative KYNA concentrations. And one possible explanation for these differences in KYNA is that *nkx-1* transcript levels decrease by day 7 of adulthood (Figure 3-3D), which could decrease KYNA production between days 5 and 7 of adulthood.

Our data also suggest that by blunting accumulation of KYNA it is possible to restore a significant portion of learning capacity to animals even when the protective measures are initiated late in adulthood. Moreover, there is an intriguing correlation between conditions or treatments that impact lifespan generally – and neural health more specifically – and levels of KYNA. For example, conditions that are generally considered detrimental to aging and neural functions such as inflammation and elevated insulin signaling are associated with accumulation of KYNA (Cervenka et al., 2017) while beneficial treatments such as dietary restriction and exercise reduce KYNA (Agudelo et al., 2014; Cervenka et al., 2017; Lemieux et al., 2015). Each of the detrimental and beneficial regimens are certain to exert their effects through a multitude of processes, nevertheless, KYNA is emerging as a key link between each of these interventions and neural functions. Importantly, modulation of KYNA does not appear to affect lifespan itself, thus its effects on neural functions are specific and not simply consequences of overall improved health. Our data also indicate that while accumulation of KYNA significantly contributes to age-onset learning impairments, it is not the only reason for this decline. After all, even the

treatments that have the most robust effects on animal lifespan do not prevent aging, they simply delay onset of various impairments or extend median and maximum lifespans.

The importance of KYNA production for learning and neuronal function is likely to be relevant in other species as well, given that changes in KYNA levels are associated with many neurodegenerative disorders in humans such as Parkinson's Disease , Alzheimer's Disease (Bonda et al., 2010; Guillemin et al., 2005), and Huntington's Disease (Beal et al., 1992; Pearson and Reynolds, 1992). The biggest risk factor for these diseases is age, and consistent with our findings that KYNA accumulates with age in *C. elegans*, it is reported to accumulate in rat brains as well (Gramsbergen et al., 1992; Moroni et al., 1988). There has long been an appreciation for the link between metabolism and neurological decline, and our findings suggest KYNA may be a major mechanism by which this communication occurs. Our ability to decrease KYNA production in aging adults to decelerated age-induced learning impairment provides encouraging evidence that later in life interventions may be effective in ameliorating neurological decline.

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Figure 3-1.

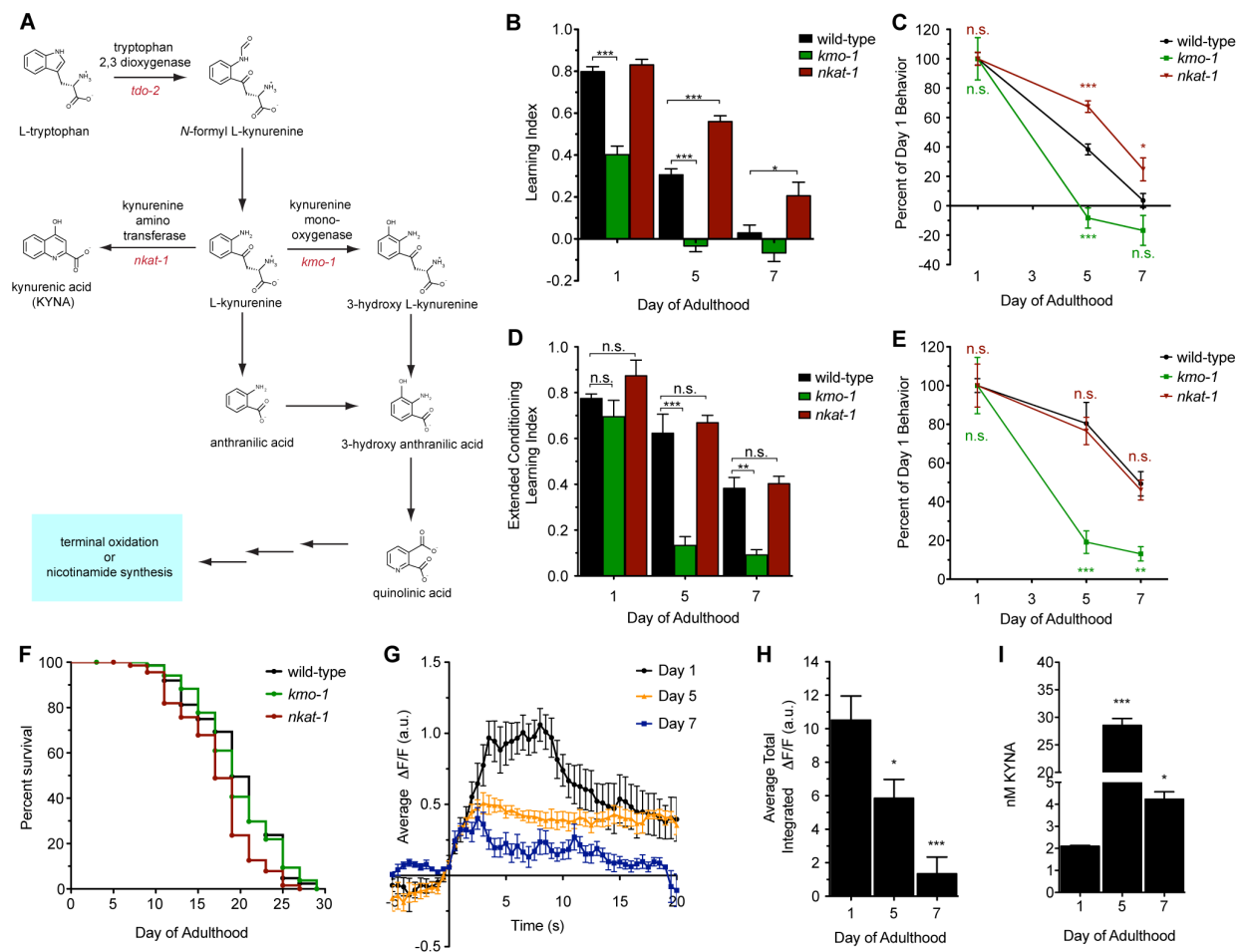


Figure 3-1. KYNA negatively modulates age-induced learning impairment.

(A) Schematic of the kynurenine pathway. (B) Elevating KYNA (via *kmo-1* mutation) exacerbates age-induced learning impairment whereas depleting KYNA (via *nkat-1* mutation) protects against it. $n = 6-12$, $*p < 0.05$, $***p < 0.001$ by two-way ANOVA (Tukey). See also Figure 3-S1. (C) Effects of KYNA on rates of decline in learning capacity upon aging. Data from panel B graphed as a percentage of learning at day 1 of adulthood for each strain. $n = 6-12$, $*p < 0.05$, $***p < 0.001$ by two-way ANOVA (Tukey). Asterisk colors denote statistical significance of a given strain compared to wild-type. (D) Conditioning animals for a longer period of time (4 hours) protects against age-induced learning impairment. $n = 6-12$, $**p < 0.01$, $***p < 0.001$ by two-way ANOVA (Tukey). (E) Increasing the conditioning time (4 hours) prevents age-induced decline in learning to the same extent as loss of KYNA. Data from panel D graphed as a percentage of learning at day 1 of adulthood for each strain. $n = 6-12$, $**p < 0.01$, $***p < 0.001$ by two-way ANOVA (Tukey). Asterisk colors denote statistical significance of a given strain compared to wild-type. (F) Survival curves for wild-type and kynurenine pathway mutant animals. $n = 80-100$ animals per strain. There were no significant differences in median survival based on logrank tests. (G) Aging blunts average intensity of spontaneous GCaMP transients in the RIM interneuron in wild-type animals. Transients from the 250s imaging window are aligned to a -5s to 20s time axis. $n = 5$. (H) Aging reduces average total intensity of RIM GCaMP fluorescence in wild-type animals over the entire 250s imaging window. $n = 5$, $*p < 0.05$, $***p < 0.001$ by one-way ANOVA (Dunnett). (I) KYNA levels increase with age as indicated by HPLC measurements of whole animal extracts. $n = 4$, $*p < 0.05$, $***p < 0.001$ by one-way ANOVA (Dunnett). All data are represented as mean $\pm$ SEM.

Figure 3-2.

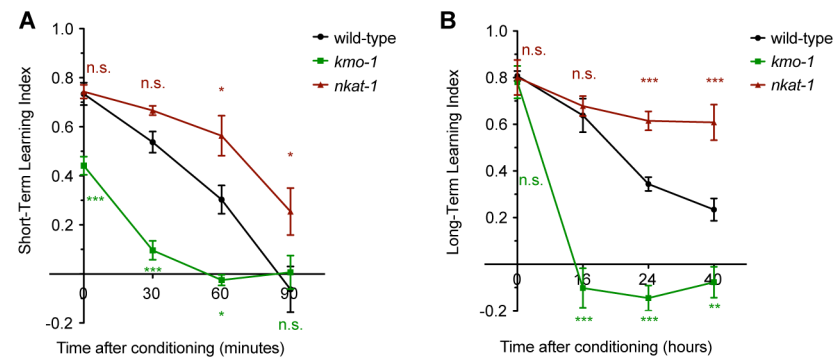


Figure 3-2. KYNA negatively modulates short- and long-term memory.

(A-B) Elevating KYNA (via inactivation of *kmo-1*) impairs (A) short-term and (B) long-term memory whereas depleting KYNA (via inactivation of *nkaf-1*) enhances it. n = 3, *p<0.05, **p<0.01, ***p<0.001 by two-way ANOVA (Tukey). Asterisk colors denote statistical significance of a given strain compared to wild-type. All data are represented as mean $\pm$ SEM.

Figure 3-3.

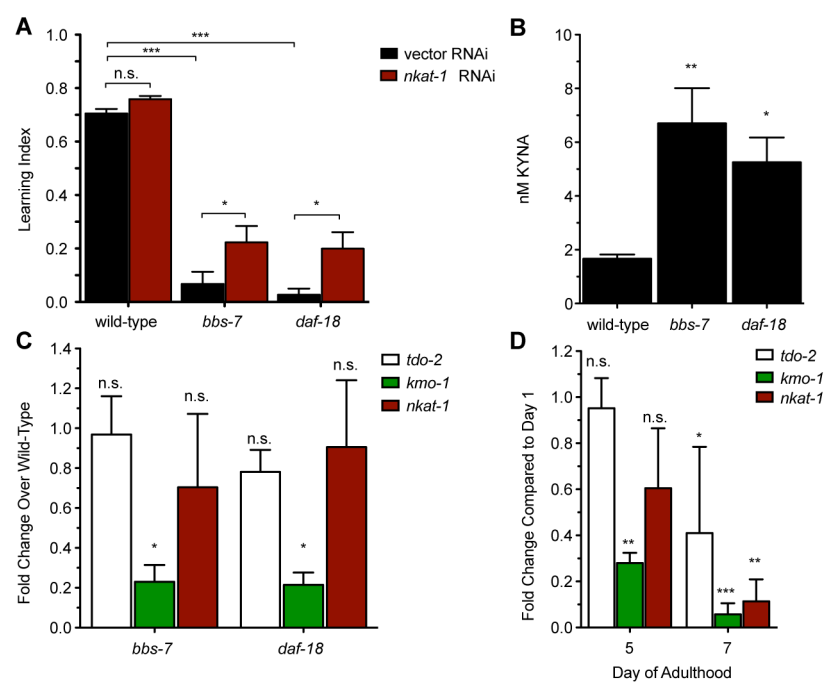


Figure 3-3. Effects of increased insulin signaling on learning and KYNA levels.

(A) Animals with elevated insulin signaling have learning impairments even as young adults that can be partially rescued by *nkx1-1* RNAi. $n = 3-6$, $*p < 0.05$, $***p < 0.001$ by two-way ANOVA (Tukey). (B) Mutants with elevated insulin signaling have high KYNA levels as indicated by HPLC measurements of whole animal extracts. $n = 4$, $*p < 0.05$, $**p < 0.01$ by one-way ANOVA (Dunnett). (C) Change in transcript levels of kynurenine pathway genes in mutants with elevated insulin signaling as determined by real-time qPCR. Data are represented as fold change compared to wild-type. $n = 4$ biological replicates, $*p < 0.05$ by two-way ANOVA (Bonferroni). (D) Change in transcript levels of kynurenine pathway genes in wild-type animals at day 5 and 7 of adulthood as determined by real-time qPCR. Data are represented as fold change compared to wild-type at day 1 of adulthood. $n = 4$ biological replicates, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by two-way ANOVA (Bonferroni). All data are represented as mean $\pm$ SEM.

Figure 3-4.

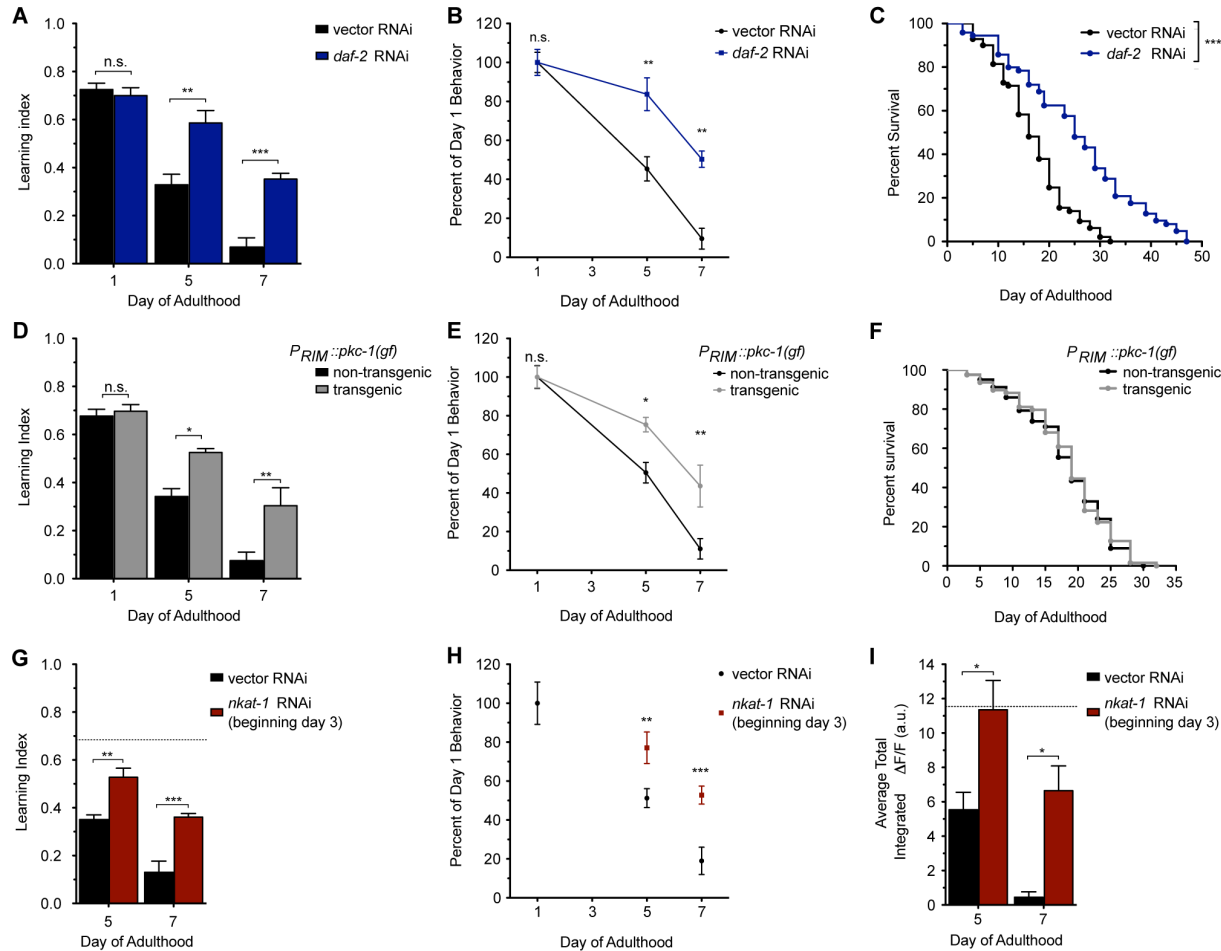


Figure 3-4. Adult depletion of KYNA protects against age-induced learning impairment.

(A) Lifelong impairment of insulin signaling via *daf-2* RNAi protects against age-induced learning impairment. $n = 3$, $**p < 0.01$, $***p < 0.001$ by two-way ANOVA (Tukey). (B) Data from panel A graphed as a percentage of learning index at day 1 of adulthood shows that *daf-2* RNAi decreases the rate at which age-induced learning impairment occurs. $n = 3$, $**p < 0.01$, $***p < 0.001$ by two-way ANOVA (Tukey). (C) *daf-2* RNAi extends median lifespan. $n = 80-100$ animals per genotype. $***p < 0.001$ by logrank test. (D) Chronic activation of RIM by using a constitutively active protein kinase C protects against age-induced learning impairment. $n = 3-6$, $*p < 0.05$, $**p < 0.01$ by two-way ANOVA (Tukey). (E) Data from panel D graphed as a percentage of learning index at day 1 of adulthood shows that RIM activation decreases the rate at which age-induced learning impairment occurs. $n = 3-6$, $*p < 0.05$, $**p < 0.01$ by two-way ANOVA (Tukey). (F) Chronic activation of RIM does not affect lifespan. $n = 80-100$ animals per genotype. Significance measured by logrank test. (G) Depletion of KYNA in aging adults via *nkat-1* RNAi beginning at day 3 of adulthood protects against age-induced learning impairment. Dotted line indicates the learning index of day 1 adults on vector RNAi (see also Figure 3-S2A). $n = 3-9$, $*p < 0.05$, $**p < 0.01$ by two-way ANOVA (Tukey). (H) Data from panel G graphed as a percentage of learning index at day 1 of adulthood shows that depletion of KYNA in aging adults decreases the rate at which age-induced learning impairment occurs. $n = 3-9$, $*p < 0.05$, $**p < 0.01$ by two-way ANOVA (Tukey). (I) Depletion of KYNA in aging adults via *nkat-1* RNAi beginning at day 3 of adulthood protects against age-induced decreases in Ca^{2+} transient intensity upon conditioning. Average total intensity of RIM GCaMP fluorescence over the entire 250s imaging window in wild-type animals are shown. Dotted line indicates the average intensity of day 1 adults (see also Figure 3-S2B and S2C). $n = 5-7$, $*p < 0.05$ by two-way ANOVA (Tukey). All data are represented as mean $\pm$ SEM.

Figure 3-S1.

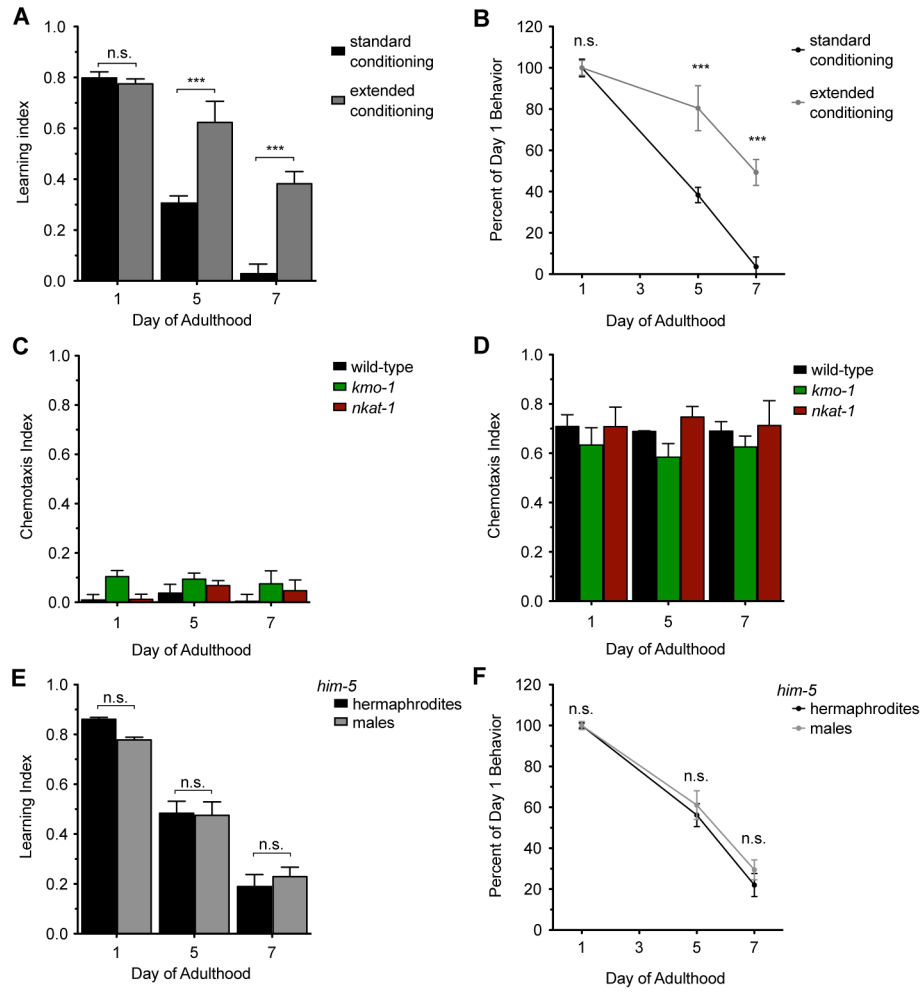


Figure 3-S1. Learning declines with age, related to Figure 3-1.

(A) Wild-type animals exhibit age-induced learning impairment which can be partially rescued by extended conditioning (4 hours) compared to standard conditioning (1 hour). $n = 3-6$, *** $p < 0.01$ by one-way ANOVA (Dunnett). (B) Extended conditioning reduces the rate of decline in learning that occurs with age. Wild-type learning index values from panel A are shown as a percentage of learning at day 1 of adulthood. $n = 3-6$, *** $p < 0.01$ by one-way ANOVA (Dunnett). (C) Chemotaxis index to 10% butanone. Animals were *ad libitum* fed and had never been exposed to butanone before. $n = 6-12$, no significant differences across all genotypes and ages as measured by two-way ANOVA (Tukey). (D) Chemotaxis index to 0.01% butanone. Animals were *ad libitum* fed and had never been exposed to butanone before. $n = 3-6$, no significant differences across all genotypes and ages as measured by two-way ANOVA (Tukey). (E) Learning index of hermaphrodites and males. In order to obtain enough males to assay, *him-5* mutants, which have a defect causing increased X chromosome disjunction and a greater percentage of male progeny, were used. Learning index values for *him-5* hermaphrodites and males are comparable to those of wild-type animals at all ages (See Figure 3-1B). $n = 3-4$, no significant differences as measured by two-way ANOVA (Tukey). (F) Data from panel E graphed as a percentage of learning index at day 1 of adulthood. $n = 3-4$, no significant differences as measured by two-way ANOVA (Tukey). All data are represented as mean $\pm$ SEM.

Figure 3-S2.

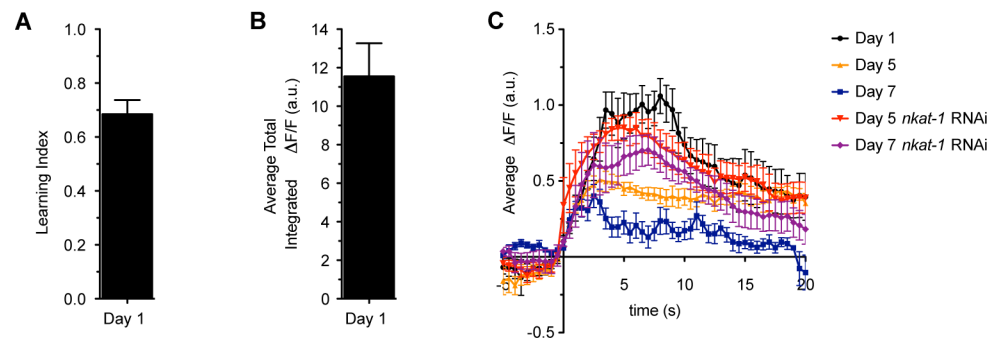


Figure 3-S2. Performance of animals on vector and *nkat-1* RNAi, related to Figure 3-4.

(A) Learning index of wild-type day 1 adults on vector RNAi, used to create the dotted line in Figure 3-4G. $n = 3$. (B) Average intensity of spontaneous Ca^{2+} transients in RIM of wild-type day 1 adults upon conditioning on vector RNAi, used to create the dotted line in Figure 3-4I. $n = 5$. (C) Average intensity of spontaneous GCaMP transients in the RIM interneuron from the 250s imaging window aligned to a -5s to 20s time axis. Wild-type animals were given *nkat-1* RNAi on day 3 of adulthood and assayed at days 5 and 7. Average total intensity over the entire 250s imaging window is shown in Figure 3-4I. $n = 5-8$. All data are represented as mean $\pm$ SEM.

**Chapter 4: A disease-associated mutation in tau may impair learning through elevating
kynurenic acid**

Introduction

The microtubule-associated protein tau (MAPT) functions to stabilize microtubules, which is a particularly important role in neurons because of their polarized morphology (Weingarten et al., 1975; Witman et al., 1976). The network of microtubules is crucial for the development and maintenance of neuronal morphology as well as transport of molecules and organelles across axons (Ballatore et al., 2007), making tau-mediated regulation of the assembly of microtubules vital to neuronal function. Tau proteins exist in dynamic equilibrium, binding and unbinding microtubules. This process is thought to be controlled by serine/threonine phosphorylation of tau, though other post-translational modifications may play a role as well (Ballatore et al., 2007). Tau phosphorylation causes detachment from microtubules, and excess cytosolic tau can promote misfolding of the protein leading to soluble oligomers. These can become insoluble aggregates of hyperphosphorylated and misfolded tau called neurofibrillary tangles (NFTs). NFTs are associated with neurodegenerative diseases such as Alzheimer's Disease (AD) and frontotemporal dementia (Ballatore et al., 2007).

Recently, a human variant of tau has been identified to increase risk for these diseases. It is an alanine to threonine mutation at position 152 (A152T) and it decreases binding to microtubules, possibly because it increases phosphorylation at position 152. This both impairs microtubule assembly and results in increased soluble tau oligomers (Coppola et al., 2012). Mice expressing A152T tau exhibit excitotoxicity and cognitive impairments (Decker et al., 2016; Maeda et al., 2016). Similarly, *C. elegans* expressing A152T show neuronal dysfunction, shortened lifespan, and mislocalization of synaptic proteins and mitochondria (Pir et al., 2016). As alterations in the kynurenine pathway are associated with AD (Bonda et al., 2010; Guillemín et al., 2005), and modulation of the kynurenine pathway can ameliorate mouse models of AD

(Zwilling et al., 2011), we sought to determine if dysregulation of the kynurenine pathway may mediate some pathological effects of A152T tau in *C. elegans*. Indeed, animals expressing A152T tau likely have high kynurenic acid (KYNA) levels, and decreasing KYNA can partially rescue their learning defects.

Results

C. elegans learn an attraction to the odor butanone after it has been paired with food (Kauffman et al., 2010). Expression of wild-type human tau in *C. elegans* neurons had no effect on animals' ability to learn, demonstrating that although artificial, this may be a valid and useful model for understanding how tau biology affects plasticity. Consistent with mammalian models, expression of A152T tau caused a complete inability to learn (Figure 4-1A). As this mutation may allow for increased phosphorylation at position 152, we assessed the effects of a phosphomimetic A152E mutation. Animals expressing A152E had a similar though less severe learning deficit (Figure 4-1A), suggesting that phosphorylation at this site may contribute to the learning phenotype. Confirming that these phenotypes were a result of defects in plasticity and not sensorimotor function more generally, none of these versions of human tau caused defects in chemotaxis to butanone when expressed in *C. elegans* (Figure 4-1B). It was previously established that a brief fast before conditioning with food and butanone enhances learning (Kauffman et al., 2010; Vohra et al., 2017), and while this had a modest but significant effect on A152T-expressing animals' learning capacity, they still exhibited a deficit (Figure 4-1C). Furthermore, conditioning for an extended period of time still could not fully rescue the A152T-expressing animals' phenotype (Figure 4-1C). We previously found that extended conditioning could fully rescue the learning phenotype of other mutants (Vohra et al., 2017), which may

suggest that A152T causes learning phenotypes via more severe dysfunction of the nervous system. This would be consistent with previous reports of neuronal dysfunction caused by the A152T tau mutation (Pir et al., 2016).

Does this learning impairment arise from specific effects of A152T tau or from more general changes to proteostasis? To distinguish between these possibilities, we treated wild-type animals with tunicamycin, a drug that induces ER stress and activates the unfolded protein response (UPR) (Calton et al., 2002). Tunicamycin had no effect on learning (Figure 4-1D) even though it was efficient in inducing expression of the UPR-related transcript *hsp-4* (Figure 4-1E). This suggests that while A152T expression may result in soluble oligomers and NFTs which activate the UPR, UPR activity alone is not sufficient to impair learning. Thus, something more specific about A152T tau must be causing this phenotype.

As dysregulation of the kynurenine pathway (Figure 4-2A) is associated with Alzheimer's Disease (Bonda et al., 2010; Guillemin et al., 2005), and we found KYNA depletion to be effective at promoting learning in many contexts (see chapters 2 and 3), we reasoned that it may rescue A152T-induced learning deficits. Depleting KYNA via *nkat-1* RNAi partially rescued learning (Figure 4-2B), suggesting that A152T tau may cause neuronal dysfunction by decreasing neuronal transmission. Again, this would be consistent with the observation that A152T causes defects in neuronal morphology and localization of synaptic proteins (Pir et al., 2016). Insulin signaling is implicated in AD (Stanley et al., 2016), and we previously found that impaired insulin signaling depleted KYNA levels (Vohra et al., 2017). We reasoned that impairing insulin signaling via *daf-2* RNAi may be able to rescue A152T-expressing animals' learning defects through both KYNA-dependent and KYNA-independent mechanisms. However,

this could only partially rescue learning (Figure 4-2B), further suggesting that A152T tau causes severe nervous system dysfunction.

These results suggest that KYNA depletion – through fasting, impaired insulin signaling, or *nkat-1* inactivation – may be a strategy for restoring some learning capacity to A152T-expressing animals. It is possible that this is simply because KYNA acts as an inhibitory neuromodulator, not because KYNA production is related to A152T-induced pathology. We assessed transcript levels of key enzymes in the kynurenine pathway that could be responsible for regulating KYNA production and found that A152T-expressing animals have low levels of *kmo-1* (Figure 4-2C). As we found previously, *kmo-1* expression seems to be a common point of regulation of KYNA production: expression is increased in dietary restriction mimetics which reduce KYNA (see chapter 2), and expression is decreased in aging and high insulin signaling models which elevate KYNA (see chapter 3). Thus, *kmo-1* transcript levels may indicate that A152T causes an increase in KYNA, and this may be partially responsible for the learning defects induced by this mutation in tau.

Discussion

These results suggest a previously unappreciated connection between tau mutation and regulation of flux through the kynurenine pathway. Even though it has been difficult to develop interventions to combat soluble tau oligomers or insoluble NFTs, modulating production of this single metabolite may be a way to restore some cognitive function in models of tauopathy. Interestingly, our findings that depleting KYNA is beneficial in a context analogous to AD differ from other reports that elevating KYNA is beneficial in a mouse AD model (Zwilling et al., 2011). These studies used overexpression of human amyloid precursor protein to model AD, so

they may not be directly comparable to the work presented here. However, it does indicate that protein misfolding events may interact with metabolic systems like the kynurenine pathway differently even though they cause similar cognitive effects. Thus, modulating KYNA levels to ameliorate disease must be done with an understanding of whether excessive or deficient neurotransmission is responsible for behavioral phenotypes.

How does A152T tau interact with the kynurenine pathway to mediate learning? Because tau's primary known role is in maintaining the microtubule network, and the A152T mutation impairs trafficking of proteins, vesicles, and mitochondria in *C. elegans* (Pir et al., 2016), it is possible that it causes mislocalization of kynurenine pathway enzymes. In fact, the mammalian KMO enzyme is known to be localized to the mitochondrial outer membrane (Hirai et al., 2010; Okamoto et al., 1967), so altering mitochondrial trafficking may indeed affect utilization of kynurenine pathway metabolites and production of KYNA. However, we found that neuronal A152T tau decreases *kmo-1* transcript levels, but *kmo-1* expression has only been observed in non-neuronal tissues (van der Goot et al., 2012; Vohra et al., 2017). Thus, if A152T tau increases KYNA by decreasing *kmo-1*, it is likely a cell non-autonomous effect communicated to the periphery from mutant tau-affected neurons. This potential axis of communication raises many questions: What specific pathways does A152T engage to initiate this signal? Why does not arise simply from activating UPR? What is the nature of this signal? Is it communicating specifically to the kynurenine pathway or to the periphery more generally and incidentally changing *kmo-1* expression? Additionally, non-transcriptional regulation of flux through the kynurenine pathway probably exists as well. As subcellular localization of other kynurenine pathway enzymes remains largely unknown, it is still possible that tau-induced trafficking defects within neurons may alter flux through the kynurenine pathway. Overall, these results provide promising

therapeutic directions for A152T-induced tauopathy and present many questions of how tau physiology interacts with metabolism to cause cognitive effects.

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Figure 4-1.

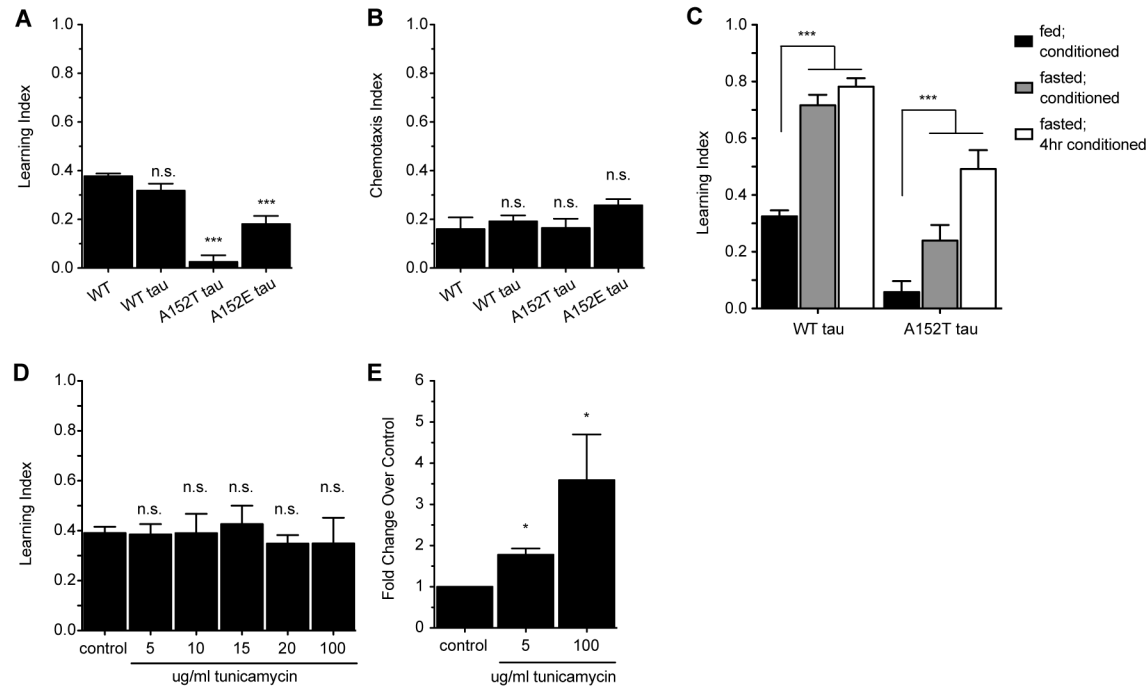


Figure 4-1. A152T tau expression impairs learning.

(A) The disease-associated A152T tau mutation and phosphomimetic A152E tau mutation impair learning. $n = 6-12$, *** $p < 0.001$ by one-way ANOVA (Dunnett). (B) No tau-expressing strains exhibit phenotypes in naïve chemotaxis to butanone. $n = 3-6$, significance measured by one-way ANOVA (Dunnett). (C) Fasting before conditioning or fasting and extended conditioning (4 hours) partially rescues A152T tau mutants' learning defects. $n = 3-6$, *** $p < 0.001$ by two-way ANOVA (Bonferroni). (D) Activating the unfolded protein response (UPR) via the drug tunicamycin has no effect on learning. $n = 3-6$, significance measured by one-way ANOVA (Dunnett). (E) Tunicamycin increases expression of the UPR-related transcript *hsp-4* as determined by real-time qPCR. Data are represented as fold change compared to control. $n = 3$ biological replicates, * $p < 0.05$ by one-way ANOVA (Dunnett). All data are represented as mean $\pm$ SEM.

Figure 4-2.

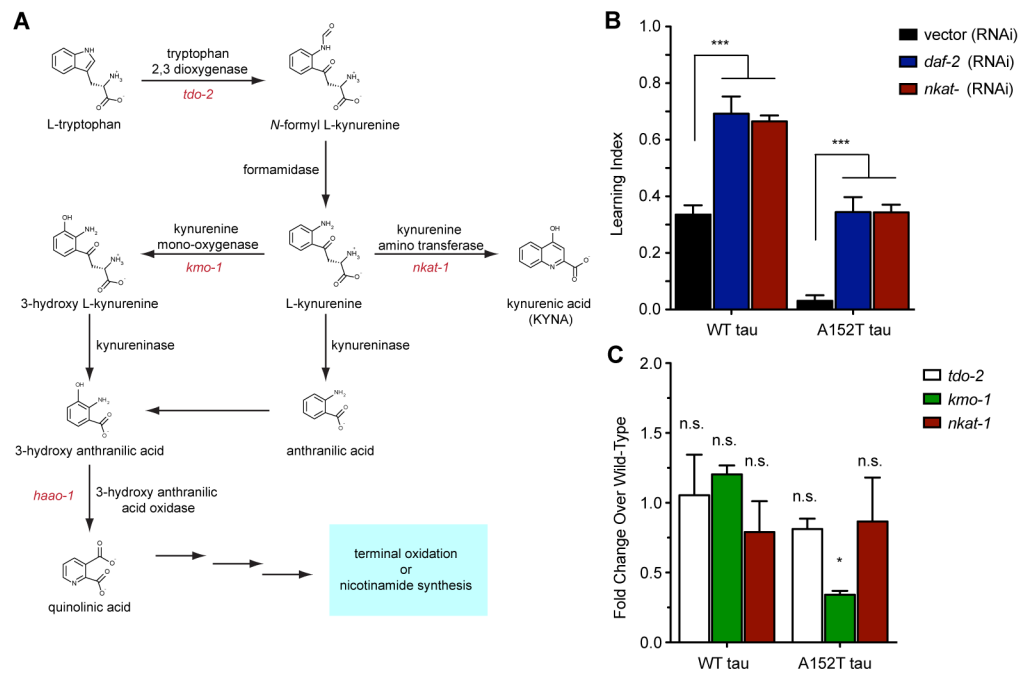


Figure 4-2. The learning deficits caused by A152T tau expression can be partially rescued by KYNA reduction.

(A) Schematic of the kynurenine pathway. (B) Impairing insulin signaling via *daf-2* RNAi or depleting KYNA via *nkat-1* RNAi partially rescues A152T tau mutants' learning defects. $n = 3$, *** $p < 0.001$ by two-way ANOVA (Bonferroni). (C) Change in transcript levels of kynurenine pathway genes in tau-expressing animals as determined by real-time qPCR. Data are represented as fold change compared to wild-type. $n = 4$ biological replicates, * $p < 0.05$ by two-way ANOVA (Bonferroni). All data are represented as mean $\pm$ SEM.

Chapter 5: Concluding Remarks

Concluding Remarks

The work presented here demonstrates that KYNA plays an important physiological role as a signaling molecule. First, we showed that it functions as a point of convergence for energy homeostasis pathways: dietary restriction and many pathways implicated in the physiological responses to dietary restriction – insulin signaling, TOR signaling, autophagy, and AMPK signaling – enhance learning in *C. elegans* by reducing KYNA. Although each of these interventions has systemic effects, KYNA seems to be the mechanism by which they specifically modulate brain function. In particular, these brain-specific effects can be uncoupled from lifespan effects, as modulating KYNA levels has no effect on longevity. Next, we found that even though KYNA itself does not modulate lifespan, it does make a major contribution to the impairment in learning that occurs as animals age. KYNA accumulates as animals get older, possibly because of increased insulin signaling. Importantly, depleting KYNA in aging animals can decelerate age-induced learning impairment, suggesting it may have therapeutic potential for treating age-related cognitive decline. Finally, KYNA accumulation may also underlie some of the plasticity defects that arise from the disease-associated A152T mutation in human tau, further demonstrating the therapeutic relevance of KYNA reduction. This establishes that KYNA-mediated NMDAR inhibition is physiological mechanism controlling many phenomena.

Until now, few regulators of KYNA production were known. Surprisingly, every context we investigated that modulates KYNA levels was accompanied by a corresponding change in *kmo-1* transcript levels, suggesting that transcriptional regulation at this point may be a way to regulate KYNA production via use of its immediate precursor kynurenine. As KYNA levels can fluctuate within as little as 30 minutes (Vohra et al., 2017), transcriptional regulation seems unlikely to be the only node of regulation of the kynurenine pathway. Post-translational

modifications to kynurenine pathway enzymes to alter their activity remain a possibility to be explored.

Another possibility is that enzyme localization may play a role in regulating flux through the kynurenine pathway. This is a particularly interesting idea because mammalian KMO is localized to the mitochondrial outer membrane (Hirai et al., 2010; Okamoto et al., 1967) and dietary restriction, dietary restriction mimetics, and aging all affect energy homeostasis and therefore mitochondrial function (López-Otín et al., 2013). Further implicating mitochondria in flux through the kynurenine pathway is the fact that the A152T tau mutation causes mitochondrial mislocalization (Pir et al., 2016). Thus, mitochondrial biology may play a key role in determining KYNA production, functioning as a crucial site of metabolic integration, taking nutritional, aging, energy homeostasis, and proteostasis signals and using KYNA production to communicate this information to the nervous system so it can direct behavior accordingly. Thus, understanding more about *kmo-1* expression and localization, as well as how mitochondrial function is translated into changes in KYNA production, may be the most important questions to answer regarding the role of KYNA as a physiological signal.

Finally, this idea put forth by the Ashrafi lab – that KYNA is not merely a waste product but a physiologically relevant neuromodulator – presents an interesting framework for considering the evolution of nervous systems. It may not represent a single example of a nutrient-derived metabolite incidentally being neuroactive. The more well studied biogenic amines such as serotonin, dopamine, norepinephrine, tyramine, and octopamine, are all also biosynthesized from amino acids. The most common excitatory neurotransmitter, glutamate, is itself an amino acid, and the most common inhibitory neurotransmitter, GABA, is a metabolite of glutamate. It seems that the advent of nervous systems took advantage of existing metabolic

pathways as a way of acquiring a large complement of molecules to use for different signaling purposes. KYNA may simply be one of these molecules that retained a connection to nutrient status as it was repurposed as a nervous system signaling molecule. Considering KYNA as another player in the realm of neuronal signaling will be important to more fully understanding how the nervous system stores and communicates information to direct behaviors.

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Appendix 1: Materials and methods

Strains.

Mutant and transgenic strains used in this study were: *nmr-1(ak4)*, *nmr-2(tm3785)*, *glr-4(tm3239)*, *kmo-1(tm4529)*, *haao-1(tm4627)*, *nkat-1(ok566)*, *nkat-1(ok566);kmo-1(tm4529)*, *odr-1(n1936)*, *N2;Ex[Pceh-36:ced-3; Podr-1::GFP; Pmyo-2::mcherry] Is[Pmex-5::GFP::his58:odr1.7::tbb2]*, *eat-2(ad465)*, *tph-1(mg280)*, *flp-18(gk3063)*, *tdc-1(ok914)*, *tbh-1(ok1196)*, *dbl-1(nk3)*, *daf-2(e1370)*, *nmr-1(ak4);Ex[nmr-1p::nmr-1::bcsGFP;unc-122p::GFP]*, *nmr-1(ak4);Ex[cex-1p::nmr-1::bcsGFP;unc-122p::GFP]*, *nmr-1(ak4);Ex[rig-3p::nmr-1::bcsGFP;odr-1p::CHERRY]*, *N2;Ex[cex-1p::pkc-1(gf)::bcsGFP;odr-1p::CHERRY]*, *N2;Ex[cex-1p::unc-103(gf)::bcsCHERRY;unc-122p::GFP]*, *nkat-1(ok566);Ex[nkat-1p::nkat-1::bcsGFP;unc-122p::GFP]*, *nkat-1(ok566);Ex[cex-1p::nkat-1::bcsGFP;unc-122p::GFP]*, *nkat-1(ok566);Ex[exp-1::nkat-1::bcsCHERRY;unc-122p::GFP]*, *N2;Ex[cex-1p::GCaMP3.0; tdc-1p::CHERRY]*, *nmr-1(ak4);Ex[cex-1p::GCaMP3.0; tdc-1p::CHERRY]*, *kmo-1(tm4529);Ex[cex-1p::GCaMP3.0; tdc-1p::mCherry]*, *N2;Ex[nkat-1p::GFP; kmo-1p::CHERRY]*, *N2;Ex[nkat-1p::GFP; tdo-2p::CHERRY]*, *daf-16(mu86)*, *skn-1(EU1)*, *hlh-30(tm1978)*, *him-5(e1490)*, *bbs-7(n1606)*, *daf-18(ok480)*, CF3810, CF3827, and AWK63. Transgenic lines were prepared via microinjection of DNA plasmids. Unless described otherwise, *C. elegans* were cultured on agar plates with OP50 *E. coli* or HT-115 *E. coli* encoding relevant double-stranded RNAi.

Plasmids.

Plasmids were constructed using standard techniques. *cex-1p* was used as an RIM-specific promoter. *exp-1p* was used to express genes in RID and *rig-3p* was used for AVA expression.

Materials.

Chemicals were purchased from Sigma. HT-115 *E. coli* encoding double-stranded RNAi were from existing libraries (Dillin et al., 2002; Kamath et al., 2003).

Butanone associative learning and memory assays.

Short- and long-term associative learning and memory were performed as previously described (Kauffman et al., 2010). Animals were grown, conditioned, and assayed at 20°C. When RNAi was used, animals were grown on vector RNAi until the L4 stage and transferred to the appropriate RNAi bacteria until adulthood; animals were conditioned on the corresponding RNAi bacteria. To assay older animals, at day 1 of adulthood, they were transferred to plates with 100uM FUDR to prevent development of progeny and then assayed at day 5 or 7 of adulthood.

Diacetyl learning assay.

Diacetyl learning was performed as previously described (Hadziselimovic et al., 2014). Animals were grown, conditioned, and assayed at 20°C.

NaCl learning assay.

NaCl learning was performed as previously described (Kunitomo et al., 2013). Animals were grown on plates with 50mM NaCl and conditioned on plates with 150mM NaCl with or without food for 3 or 6 hours. Animals were grown, conditioned, and assayed at 20°C.

Culture of *C. elegans* for metabolite determination.

Synchronized wild-type L1 animals were grown in 15ml liquid cultures of 20,000 animals in S-Medium and vector RNAi bacteria at an OD of 9 and allowed to shake at 150RPM at 20°C. At the L3 stage, animals were collected, washed, and placed in new cultures with corresponding RNAi bacteria. (*daf-2(e1370)* mutants were cultured on OP50 bacteria at 15°C until L3 and then shifted to 20°C to prevent them from entering the dauer stage.) At the L4 stage animals were collected, placed at 4°C, washed 3 times in S-basal, washed once in 10mM pH 6.0 potassium citrate buffer, frozen in liquid nitrogen, and placed at -80°C until metabolite extraction. For metabolite determination of aging adults, L4 animals were transferred to S-Medium and OP50 bacteria that contained 100uM FUDR to prevent development of progeny and were transferred to new culture liquids every day to prevent starvation until they were collected for extraction.

Metabolite extraction.

Metabolite extraction and determination were performed as previously described (Lemieux et al., 2015). Samples were thawed on ice and 5g/ml ZrO beads (0.5mm diameter) were added. Samples were lysed using a bead beater at 4°C (6 cycles of 20s on followed by 1min off). Lysed samples were separated from beads using a 200µl pipet and then centrifuged. 20µl 50% w/v trichloroacetic acid in water was added to supernatants to precipitate proteins and incubated at 4°C for 30min. They were then centrifuged at 4°C and the deproteinized supernatants were collected. Extracts were frozen at -80°C until metabolite determination.

Metabolite determination.

Extracts (100 µl per sample) were separated on a Zorbax Eclipse-plus C18 reverse-phase column (4.6 x 150 mm, 100 Å pore size, 3.5 µm particle size) equipped with a 4.6 x 10 mm C18 guard column using an isocratic elution of a mobile phase containing 250 mM zinc acetate/acetic acid pH 6.2 and 4% (v/v) acetonitrile at a flow rate of 1 ml/min. Kynurenic acid was detected with an inline fluorescence detector set at 350 nm excitation and 415 nm emission wavelengths. The concentration of kynurenic acid in the extracts was determined by comparison of peak areas to that of standard samples of known concentration prepared in 5% trichloroacetic acid, eluted and detected under identical conditions.

Spontaneous Ca²⁺ transients.

Day 1 adult *Ex[cex-1p::GCaMP3.0; tdc-1p::CHERRY]* animals were conditioned as in the short term butanone associative learning assay, mounted on agarose pads, and immobilized with cover slips. They were imaged for 250s using a 60x objective on a microscope with green epifluorescence acquiring 4x4 binned images at 2Hz. A square 6x6 pixel region of interest was drawn around RIM and integrated fluorescence intensity was measured in each frame. An average baseline intensity was calculated, and any values that were at least 30% larger than baseline were scored as Ca²⁺ transients. The average total integrated intensity for each imaging period was then calculated.

Real-time qPCR.

RNA extraction, cDNA preparation, and real-time qPCR were performed as previously described (Van Gilst et al., 2005) from three biological replicates of each condition. Data were

standardized to actin (*act-1*). *act-1* primer F: CCCACTCAATCCAAAGGCTA; R: ATCTCCAGAGTCGAGGACGA. *tdo-2* primer F: TCTCACAGCCATATCAGATCC; R: GAAGCACGTGGTTGTATCTC. *kmo-1* primer F: GAAGCTGACCTCATTCTTGC; R: AAAGTCAAATCTCGGAATGGTC. *nkat-1* primer F: AGGAGTCGATTTGGGTAGAG; R: GTGAATCTGGGAATCCTTGG. *hsp-4* primer F: AGATGCCGCCAAGAATCAGC; R: TCGACGATCTTGAACGGCCAG.

Lifespan assay.

Lifespan assays were performed at 20°C according to standard protocols. Animals were grown on vector RNAi until the L4 stage and then transferred to the appropriate RNAi. Assays began at day 1 of adulthood with no more than 30 animals per plate. Every second day animals were poked three times with a platinum wire. Those that did not move were scored as dead. Those that did move were scored as alive and moved to a new plate. Animals that could not be found were scored as censored.

Pharyngeal pumping.

The number of contractions of the posterior pharyngeal bulb of L4 stage animals grown at 20°C was counted for 10s as previously described (Srinivasan et al., 2008). To assay post-fast pharyngeal pumping, L4 animals were fasted on NGM plates for 2 hours and then contractions of the pharyngeal bulb were counted 5 minutes after they were returned to NGM plates with food.

Confocal microscopy.

C. elegans were immobilized on 2% agarose pads containing NaN₃ and imaged under a coverslip through a 60X (1.0 NA) water immersion objective using a Nikon C1si point scanning confocal microscope equipped with 488 nm and 561 nm lasers as excitation sources for GFP and mCherry respectively. Images presented are the average intensity projections of 5 serial, 1 µm spaced Z-sections.

Statistical analyses.

On all bar graphs, bars represent mean + SEM. Two-tailed Students t-tests were used for comparisons between two conditions. One- or two-way ANOVAs were used for comparisons between multiples conditions and appropriate post-tests were used. When all conditions were compared to one control, a Tukey correction was used. When conditions were compared among themselves, a Bonferroni correction was used. Logrank tests were used for comparisons of survival curves.

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