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The Effects of Glutamate Transporter Deletions on Lifespan in Caenorhabditis Elegans

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Abstract

Glutamate transporters (GLTs) remove glutamate from synapses to prevent excitotoxicity and maintain conditions necessary for glutamatergic signaling. Once transported into cells adjacent to the synapse, glutamate can be metabolized into α-ketoglutarate (α-KG) or glutamine. Since GLT deletions are often lethal in mammals, *C. elegans* were used to investigate the role of GLTs on metabolism and aging in this thesis. The lifespans of *C. elegans* GLT-knockouts (GLT-1;GLT-3, GLT-4, and GLT-5) were measured and compared to wildtype worms. A well-established method involving FUdR, a chemical that prevents egg-laying in *C. elegans*, was used to conduct the lifespan experiments. Results indicated that GLT-4 mutants had shorter lifespans, GLT-5 mutants had longer lifespans, and GLT-1;GLT-3 mutants had similar lifespan lengths compared to wildtype worms. Despite being the primary method for aging experiments in *C. elegans*, FUdR is costly and hazardous. We pursued an alternative lifespan technique using mesh-well plates to separate *C. elegans* from their laid eggs. While the mesh-well technique had multiple theoretical advantages, this technique did not consistently separate adult worms from laid-eggs. To determine the influence of glutamate metabolism on the lifespan differences between strains, we refined a protocol to analyze the levels of glutamine, glutamate, and α-KG metabolites using ^{1}H-NMR. While NMR could not be used to measure metabolite levels in *C. elegans* samples, metabolite experiments are on-going. These findings bring us closer to understanding how the effects of glutamate metabolism in cellular respiration and neurotransmission influence aging.

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Introduction

Introduction to Glutamate

Glutamate is an important molecule that acts as an amino acid in cellular metabolism and as a neurotransmitter responsible for the majority of excitatory neurotransmission in the mammalian brain (Vandenberg, 1998). While studying metabolism and neurotransmission individually can provide a wealth of information, understanding the connection between the processes may provide critical insights into health and aging.

Glutamate is considered a non-essential amino acid because it is produced by the body (Watford, 2015). Normal levels of glutamate in the body range from 50-200 μM (Zlotnik et al., 2011). For its role in signaling, however, glutamate in the brain must be tightly regulated with synaptic glutamate levels kept around 25 nM (Herman and Jahr, 2009). Since the higher concentration of glutamate in the blood would be toxic to neurons, glutamate cannot cross the blood-brain barrier and must be produced within the brain (Danbolt, 2001; Zhou and Danbolt, 2014). A primary focus of this thesis regards metabolites glutamine and α-ketoglutarate (α-KG), which are major precursors of glutamate in the brain (Waniewski and Martin, 1986; Bauer et al., 2012). Glutamine, once transported into the pre-synaptic terminal, is converted into glutamate by phosphate-activated glutaminase in the mitochondria (Platt, 2007; Bauer et al., 2012). α-Ketoglutarate, is converted into glutamate by glutamate dehydrogenase or transaminase in the mitochondria of the cells adjacent to the pre-synaptic neuron (Daikhin and Yudkoff, 2000; Platt, 2007; Bauer et al., 2012).

Glutamate Signaling and the Role of Glutamate

Once produced, glutamate is moved by presynaptic vesicular glutamate transporters into the vesicles in the presynaptic neuron that will release it into the synapse (Zhou and Danbolt, 2014). In response to an action potential, glutamate is released into the synapse via exocytosis of the synaptic vesicles (Zhou and Danbolt, 2014). Once released, glutamate acts at post-synaptic channels and receptors such as AMPA, NMDA, and kainate (Meldrum, 2000; Mano and Driscoll, 2009). For normal glutamate signaling, glutamate concentrations in the synapse must be reset to low levels around 25 nM. Glutamate transporters are responsible for removing this synaptic glutamate by clearing the molecule into cells adjacent to the synapse, where the glutamate is then degraded (Vandenberg, 1998).

Since glutamate mediates ample information critical in brain development, the formation of synapses, cellular survival, timing, location, and concentration of glutamate activity must be precise. Overactivation of the glutamate receptors on the postsynaptic cells can lead to excess depolarization and cell death (Danbolt, 2001). This phenomenon, excitotoxity, results from increased intracellular Ca^{2+} caused by the over-excitation of the neurons (Mano and Driscoll, 2009). Glutamatergic dysfunction and aberrations have been implicated in the pathogenesis of neurological diseases like ALS (Mano and Driscoll, 2009; Edwards et al., 2015), Alzheimer's disease (Danbolt, 2001), epilepsy (Tanaka et al., 1997; Vandenberg, 1998; Meldrum, 2000; Zhou and Danbolt, 2014), schizophrenia (Danbolt, 2001), and stroke (Platt, 2007). In this pathogenesis, glutamate excitotoxicity may result from excessive glutamate release, change in receptor function, or reduced glutamate uptake (Tanaka et al., 1997; Vandenberg, 1998; Platt, 2007; Tehrani et al., 2014).

The Function of Glutamate Transporters

Glutamate transporters (GLTs) are responsible for clearing the glutamate from the synapse and ending the signal. In mammals, the synapse is reset by glutamate transporters on the membrane of glial cells adjacent to the synapse. There are two glial and three neuronal glutamate transporters in the brain (Platt, 2007). The driving force for GLTs comes from the transmembrane gradients of Na^+ and K^+. One molecule of glutamate is transported into the cell with three Na^+ ions and one H^+ ion in exchange for one K^+ ion leaving the cell (Kanai et al., 1995). *C. elegans* is the model organism used in this research, and *C. elegans'* glutamate synapses differ from those of mammalian in certain regards. In *C. elegans*, transporters remove glutamate from the synapse into the presynaptic neuron, muscle cells, canal cells, and glial cells. GLT-4s are found on the presynaptic neuron in *C. elegans*, but in mammals, these transporters are found on the postsynaptic neuron (Mano et al., 2007). Additionally, the synapses of *C. elegans* are less tightly regulated than those in mammals.

Model Organism: C. elegans

GLTs are essential to mammalian health, making their study essential. GLT knockouts are lethal in mammalian models but can be achieved in *C. elegans*, allowing us to study GLTs in ways that cannot be executed in mammals (Tanaka et al., 1997; Rimmele and Rosenberg, 2017). We use four primary strains in our lab, one wild type (WT) strain and three mutant strains. Each mutant strain has a different type of GLT knock out (KO). The kinds of strains include a GLT-5 KO (GLT-5), GLT-4 KO (GLT-4), and a GLT-1;GLT- 3 double KO (GLT-1;GLT-3). Since *C. elegans* also have shorter lifespans than mammals, we can study the effects of these GLT mutations on health and longevity. Studying glutamatergic synapses in *C. elegans* may yield insights about the evolutionary transition of glutamate as a molecule only involved in

metabolism to the role of glutamate in neurotransmission as well. With these insights, we may develop ways to improve the length and quality of life in *C. elegans* and subsequently in mammals. We hypothesize that differences in lifespan between the *C. elegans* strains will stem from differences in the levels of glutamate and its metabolites between strains resulting from differences in the synthesis and breakdown of glutamate.

Glutamate Metabolism and Lifespan

Glutamate is important for key brain processes like cognition, learning, and memory (Danbolt, 2001). Within the scope of learning and memory, glutamate plays a role in long-term potentiation for neurons (Platt, 2007). As an amino acid, glutamate is also an important component of metabolic processes throughout the body such as maintaining a robust immune system (Du et al., 2016). In yeast, glutamate slows down cellular aging and increases lifespan (Kamei et al., 2011; Wu et al., 2013). In chickens, a supplement of glutamate and its metabolite glutamine reduces mortality rates (Zulkifli et al., 2016). In *C. elegans,* glutamate metabolite α-KG has been shown to extend lifespan (Chin et al., 2014). These findings that have demonstrated important connections between glutamate metabolism and longevity.

Evidence for the relationship between aging and metabolism can be found in studies that analyzed dietary restriction on the lifespan of rhesus monkeys (Colman et al., 2009; Mattison et al., 2017) and rats (Duffy et al., 1990). The results of these studies show increased healthspan and lifespan accompanying dietary restriction (Colman et al., 2009; Mattison et al., 2017). α-KG is a tricarboxylic acid cycle intermediate and is involved in different pathways related to cellular respiration (Wu et al., 2016). α-KG's involvement in these metabolic pathways has been used to explain the positive relationship between α-KG and longevity (Chin et al., 2014; Wu et al., 2016). Chin et al. (2014) demonstrated that supplemented α-KG can increase longevity in *C.*

elegans(Chin et al., 2014). These authors proposed that the mechanism behind the longer worm lifespan is α-KG inhibiting ATP synthase which leads to the inhibition of target of rapamycin (TOR) (Chin et al., 2014). Previous studies showed that decreased TOR activity delays the aging process (Wu et al., 2016). In *C. elegans*, this phenomenon was observed as the delay in decline of rapid, coordinated body movement (an age-related phenotype) (Chin et al., 2014). The TOR-aging phenomenon has also been observed in other organisms such as yeast, flies, and mice, where inhibition of TOR delayed aging and increased lifespan (Wu et al., 2016). α-KG's inhibition of ATP synthase is also associated with decreased oxygen consumption and increased autophagy in both *C. elegans* and mammalian cells, and both processes are implicated in increased longevity (Chin et al., 2014). α-KG and dietary restriction are not additive for lifespan increase, suggesting that there is a shared pathway (Chin et al., 2014). Due to these critical roles of glutamate metabolites, we hypothesize that information about strain metabolite levels will inform our understanding of strain differences in lifespan.

Measuring Glutamate Metabolites

My project aims to create a sample from *C. elegans* that can be analyzed in a nuclear magnetic resonance (NMR) machine. By using the NMR spectroscopy technique, we hope to obtain information regarding the relative quantities for each strain of our three metabolites of interest, glutamate, glutamine, and α-KG. Part of our analysis will involve NMR, which is a technique used to obtain the molecular structure of organic compounds (Rabi et al., 1938; Bloch et al., 1946; Purcell et al., 1946). NMR spectroscopy can give information about the purity and content of an unknown sample by comparing the sample's spectrum with spectral libraries. For known samples, NMR can also be used to determine relative quantities of molecules within and between samples.

The way NMR works is that a nucleus with an odd atomic number has a nuclear spin that can be detected by a NMR spectrometer (Hornback, 2006). A proton has an odd atomic number of 1 and therefore the charge of ^{1}H "spins" on its nuclear axis, which generates a small magnetic field that can be thought of like a small bar magnet (Hornback, 2006).

In the absence of an external field, spins of the hydrogen nuclei are randomly oriented (Hornback, 2006). When an external magnetic field is applied, the spinning nuclei of the protons will align either with or against the external field, leading to two possible spin states with different energies (Hornback, 2006). Irradiation equal to the difference in energy can cause the spinning proton to flip its spin state, and this photon absorption is detected by the NMR spectrometer (Hornback, 2006). The exact difference in energy for a proton depends on the local environment (Hornback, 2006). Electrons shield the proton from the external magnetic field (Hornback, 2006). Since electron density varies throughout a compound, different protons will appear at different positions on the NMR spectrum (Hornback, 2006). Spectrums that tell us about the protons in a molecule are referred to as ^{1}H-NMR (Hornback, 2006). The ^{1}H-NMR spectrum gives information that can be used to determine the number of hydrogens, information that describes the kind of atom the proton is bonded with, and information that describes how those protons in the same chemical environment are influenced by adjacent protons that are in different chemical environments.

Measuring Lifespan – with FUdR

We are also interested in measuring lifespan. A common method to determine worm lifespan uses Floxuridine. Floxuridine (FUdR) is a chemical developed as a chemotherapy drug in cancer treatment (Ko and Karanicolas, 2014). FUdR inhibits DNA synthesis and is used by the worm community to prevent *C. elegans* from reproducing (Mitchell et al., 1979). FUdR is popular, because it does not interfere with post-maturational development and aging of the worms (Mitchell et al., 1979). FUdR was created to enhance the activity of 5-fluorouracil (5-FU), another chemotherapy drug, and functions similarly (Avendaño et al., 2008).

As a chemotherapy agent, 5-FU is a pyrimidine analogue antimetabolite that affects cells only when they are dividing, specifically during the S phase of cell growth (Aitlhadj and Stürzenbaum, 2010). Antimetabolites have similar structures to normal molecules in nucleic acid, so antimetabolites can be incorporated into DNA and RNA (Gandhi et al., 1980). As an antimetabolite, 5-FU mimics the naturally occurring pyramidine uracil and inhibits DNA synthesis. These 5-FU metabolites interfere with the enzymatic activity of thymidylate synthetase, leading to faulty DNA and RNA and cell death of rapidly dividing cancer cells (Avendaño et al., 2008).

As an enhancer of 5-FU, FUdR has been used in *C. elegans* experiments to prevent egg laying. In our project, we use FUdR to ascertain lifespan of different *C. elegans* strains. Synchronized *C. elegans* populations will be transferred at pre-maturation stage (L4) to agar plates containing low levels of FUdR. These worms will age normally but will not be able to reproduce, enabling us to track the lifespan of the individual worms.

While FUdR is an established technique that can successfully determine the lifespan of a synchronized population without affecting development and aging markers of the worms, there are some significant drawbacks of this technique. One limiting aspect of using FUdR is that the chemical is expensive to work with; 1 gram of the substance costs $1,000+ (Anon, n.d.). FUdR also has severe health hazards and must be handled in a chemical fume hood with gloves, goggles, and a coat worn as personal protective equipment. Everything exposed to FUdR must be disposed of with caution. Lastly, the current technique is cumbersome in many ways. Worms must be handled in the dark and transferred onto fresh FUdR plates one week after beginning the procedure, because FUdR is light-sensitive and loses potency over time. This transfer poses risk for harming the worms in the process and requiring censoring of damaged worms.

Measuring Lifespan – without FUdR

My last project aims to pilot a method to measure *C. elegans* longevity without using FUdR. If successful, using this mesh-well plate technique would eliminate all of the disadvantages of FUdR mentioned above. This method allows the worms to age and reproduce naturally and uses a mesh platform to separate the eggs from the tracked worms. The worms will live in liquid media above the mesh and any laid eggs will fall through to the receiver plate below. With the eggs removed and the liquid media replaced regularly, the lifespan of the individual worms can be tracked. This technique will reduce the costs and eliminate the health hazards associated with using FUdR. The mesh-well procedure will also make tracking the worms easier, because fewer worms may be censored due to disappearance. This procedure will also reduce counting-based error, because there will only be one worm per well rather than 15 worms scattered around a 60 mm plate.

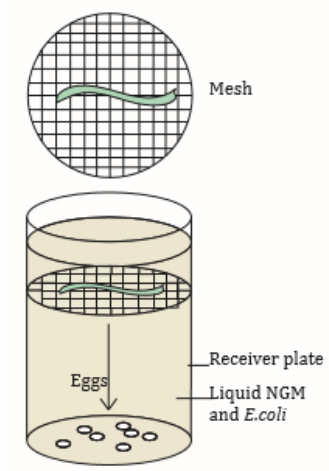


Figure 1: **A schematic of the mesh-well strategy keeping the worms separated from the laid egg**s. The well is filled with liquid media that contains *E. coli*. A single *C. elegans* is placed on top of the mesh. The *C. elegans* is large enough to live on top of the mesh, but the laid eggs are small enough to pass through the mesh and collect at the bottom of the well.

Methods

Maintenance Protocols

Seeding: An unopened tube of Luria-Bertani (LB) broth was obtained. The broth was checked for contamination, and only clear broth with an orange-yellow color were used. The broth was then inoculated with OP50 *E. coli* by using a thawed aliquot of OP50 and pouring it into the new LB broth tube. This newly inoculated tube was then placed on a shaker overnight at 38˚C. The tube was removed the next day after the OP50 proliferated and the tube became a cloudy yellow color. Once removed from the warm room, the tube was vortexed. Using a transfer pipette, seven drops of the OP50 mixture were placed onto an agar plate for the food source lawn.

Chunking: A scalpel was sterilized using an electric sterilizer heated to 150˚C. Using the scalpel, a section of agar containing many worms was cut and transferred to the freshly seeded plate. The section of agar was placed upside down on the edge of the OP50 lawn of the fresh plate; worms from this section can then populate the new plate. This process was repeated for all plates of each strain.

Age Synchronization: The decontamination solution was prepared in a 10mL Eppendorf tube by combining 4 mL of M9 buffer, 5 mL of 1M NaOH, and 1 mL of 5% bleach. Worms were displaced from the agar plate by pipetting 1.5 mL of M9 buffer onto the agar and swirling the plate. The M9 with the worms was then removed via a transfer pipette and placed in to a 2 mL microcentrifuge tube. This tube was centrifuged, and the M9 supernatant was removed, taking care not to disrupt the worm pellet at the bottom. The microcentrifuge tube was then filled up to the 1.5mL line with decontamination solution and inverted every few minutes for 10 minutes. The centrifugation step was repeated, and the supernatant was removed from the tube

and more decontamination solution was added until the 1.5mL line was reached. The tube was then inverted every minute for 5 minutes during this time. After the 5 minutes, the centrifugation and supernatant removal was done 4 more times with M9 buffer washes for the last 3 repetitions. To wash the worms, M9 buffer was added, and the tube was then shaken, centrifuged, and the supernatant removed. After the third and final wash with M9, the supernatant was removed so that only about 0.1mL of the combined supernatant and pellet remained. A transfer pipette was then used to remove the pellet and place it onto the fresh, seeded plate on the agar outside of the *E. coli* lawn. Eggs hatched overnight.

NMR Protocol

C. elegans were cultured on nematode growth medium (NGM) agarose plates according to standard chunking methods; the worms were maintained at 20˚C. The strains WT, GLT-4, GLT-1;3, and GLT-5 were used in this study. A pellet of *C. elegans* was formed by washing plates of the given strain until a 0.2mL volume was collected in a 1.5mL microcentrifuge tube. After the worms were washed, 1.0mL of -20˚C methanol was added to the pellet, killing the worms. To elute the metabolites from the tissues, the pellet was transferred to a 15mL Eppendorf tube and sonicated. For the sonication process, the tube containing the pellet was placed in a beaker of 30 mL -40˚C ethanol and secured using clamps and a ring stand. The sonicator was placed inside the tube such that the tip was submerged in the pellet but not touching the sides of the tube. The worm pellet was sonicated (50% duty cycle with the maximum output power of 5) for 20 cycles of 1 minute with 15 seconds rest between each minute. The pellet was then centrifuged for 3 minutes at -9˚C. The supernatant was removed and placed into a beaker in a -20˚C freezer. To extract the metabolites, 1.0mL of warm methanol was added to the remaining

pellet, vortexed for 15 seconds, and incubated on a hot plate for 3 minutes. After incubation, the sample was again vortexed for 15 seconds. This extraction process was performed a total of 3 times and supernatants combined. To clean the sample, the supernatant was filtered through a glass pipette containing a kimwipe layer into a fresh 25mL beaker. To reduce water contamination in the NMR spectrum, the sample was dried in a hood using tubing to direct airflow on the sample. Once the supernatant was visibly dry, airflow was removed, and the sample was left in the hood overnight to further dry. The following day, the dried sample was resuspended in 0.7mL of D_2O, and filtered again. The sample was then placed in an NMR tube for ^{1}H-NMR data acquisition. 200 scans were run on the NMR.

Longevity Protocol

Two plates were chunked from each strain on Day 1. After worms reached adult stage (about 3 days), the decontamination was performed. 5-fluoro-2'-deoxyuridine (FUdR) plates were poured on Day 4. The worms were then monitored until they reached L4 stage. Worms typically reached L4 stage between Day 6-7. An inoculated OP50 was put on a shaker at 38˚C the night before the expected transfer day (~ Day 5). Worms were identified as having reached L4 stage if the developing vulva – a white oval in the middle of the body – was present (Corsi et al., 2015).

Fifteen L4 worms were transferred to each of five FUdR plates, so that each strain had total 75 worms. For every day following the transfer, worms were counted and recorded. If any worms were found dead or injured the day following the transfer, those worms were censored as they may have been injured during the transfer process. The following information was recorded for each strain:

	Plate #
Strain	- # of alive worms - # of dead worms - # of censored worms - presence of any eggs/small worms - # of worms with protrusions and the size of the protrusions

Plate Pouring

The agar was prepared for 5 plates per strain with each plate allotted about 10mL per plate. The recipe resulted in 2-3 extra plates, which were needed if any FUdR plates became contaminated towards the end of the experiment. On the bottom of each plate, a cross was drawn to make counting easier. On the sides of each plate (except for the extra plates which only had the cross), the strain, date, FUdR, and the plate number were written. The concentration of agar needed was 29g/1000mL. The amount of FUdR needed was 0.1mg/mL. For the first trial of the FUdR experiment, each strain was included, so a total of 20 plates were prepared. 200 mL of agar was autoclaved with the stir bar on Liquid cycle 4. From this point on, most lights in the room were turned off since the strength of the FUdR diminishes upon light exposure. While the agar was in the autoclave, 0.02g FUdR was measured. Since anything that contacts FUdR must be disposed of as toxic chemical waste, the FUdR was measured on folded weighing paper and transferred to the disposable 250mL plastic bottle from which the FUdR was poured. A funnel

was then put on top of the entrance to the bottle to enable agar to be added to the bottle. Once removed from the autoclave, the agar was mixed slowly for about 30 seconds, making sure no air bubbles were introduced. The stir bar was removed and the agar was then poured into the bottle containing the FUdR. The funnel was removed, the bottle capped, and the bottle was gently swirled and inverted. This procedure was cautiously done to prevent air bubbles from forming in the agar. Once the FUdR was incorporated with the agar, the mixture was poured into the petri dishes until the agar sealed, forming a thin layer. Once all the plates were poured, a piece of aluminum foil was placed over all the plates to further prevent exposure to light while they dried.

Worm transfer

The OP50 was removed from the 38 ˚C and vortexed. Seven drops of OP50 were placed on the plate before any worms were transferred. The OP50 lawn was kept in the center of the plate. This technique minimizes the number of worms along the plate edge, where counting is more difficult. The 15 worms were then transferred to the plates before the OP50 dried. On some plates, more than 15 worms were added in case worms were removed from the study (censored) for any reason.

Determining if a worm has died

If a worm stopped moving, the head was monitored to check for slow but visible nodding. If no head nodding was observed, then the head was gently poked with a pick and observed for movement. The worm was poked three times, and if there was no movement, then the worm was marked as dead, removed from the plate, and sterilized in the chemical fume hood.

Cases for censoring worms: Any instance where the lifespan of the worm was influenced by unnatural factors external to strain type resulted in the removal of those worms from the study.

- Injured from handling: If the worms were injured or killed during the transfer process or during counting, the worm was censored.
- Ruptured vulva: If the vulva ruptured and the intestines spilled onto the plate, the worm was censored even if the worm was not yet dead.
- Contamination: Sterile technique was always used. FUdR helped prevent the growth and spread of contamination, but as the experiment progressed, the strength of the FUdR diminished and contamination was possible. Worms were moved to one of the extra plates at the first sign of contamination. Otherwise, the worms were censored.
- Missing: If a worm could not be found, the worm was censored.
- If a worm strayed to the edge of the plate and died, the worm was censored.

Statistical Analysis

All statistical analyses were performed in the free statistical software R. Survival curves were produced using the survfit (Kaplan-Meier) function in the survival package in R. The log-rank (*G-rho*) test was used to examine the survival differences between strains with the survdiff function.

Mesh-well Plates

Worms were chunked and decontaminated in the same way as the preparation for the longevity experiment. After the decontamination, the worms grew up until they reached adult stage and began producing eggs. Because we had witnessed the population of adult worms

diminishing in the presence of many small worms, 2 days after the worms began laying eggs, 20 of the oldest generation of adult worms from each strain were picked onto a fresh plate. Seven days after the decontamination, one worm from each strain was tested in the 40 µm mesh well plate. The wells were filled with liquid media, and the worm was placed on top of the mesh and monitored to see how the width of the worm compared to the mesh and if the worm fell through. This process was repeated every subsequent day until the worms stayed above the mesh. Then, all the worms were transferred to the mesh plate – one worm per well so that each strain filled one row. The liquid media was replaced every 3rd day to prevent starvation and confusion between the original generation of worms and worms hatched from the eggs at the below the mesh. Worm lifespan was tracked the same way as in the longevity experiments with FUdR. The worms were counted every day, and each worm was marked as either alive or dead.

Results

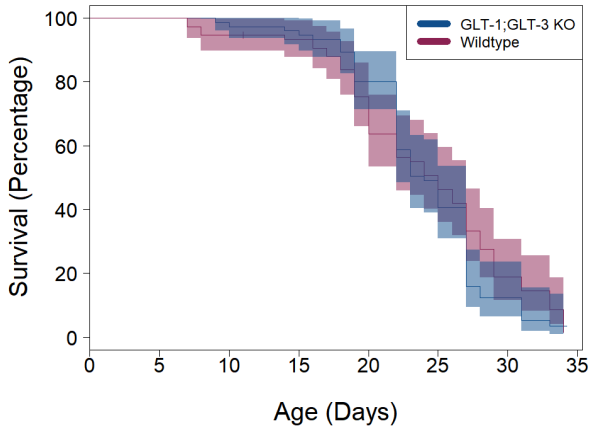
FUdR

FUdR experiments were conducted to assess and compare the lifespan of each mutant strain to the wildtype strain. *C. elegans* were placed on FUdR plates to prevent egg-laying and enable tracking of the same generation of worms for the entirety of their lifespan. Worms from each strain were age-matched and transferred to FUdR plates when they reached L4 stage. Worms were transferred to new FUdR plates after a week so that the food source and strength of the FUdR was maintained. Worms were counted every day and kept on the plate until death, when they were removed. This experiment was the third cycle executed to collect longevity data using this technique. This experiment was performed during March 2018.

March Experiment Data

Combined Data

A



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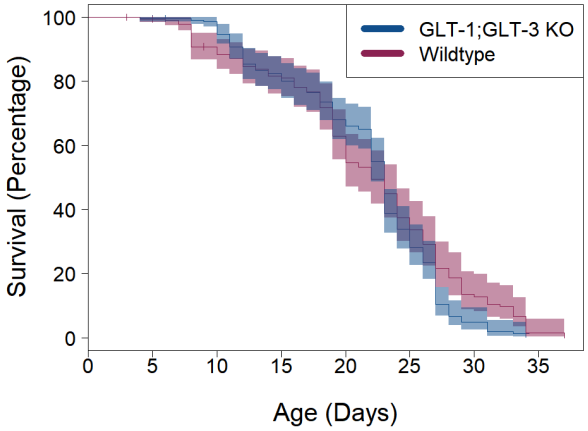

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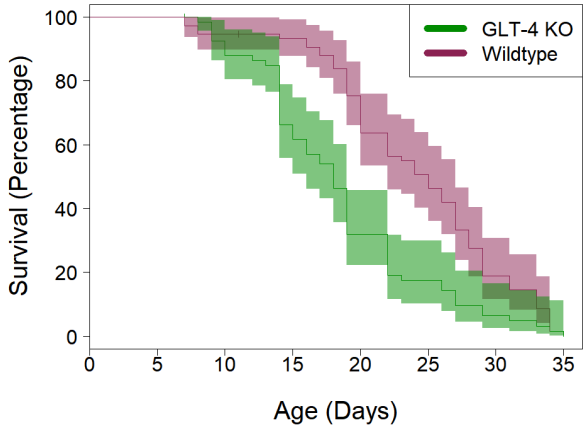

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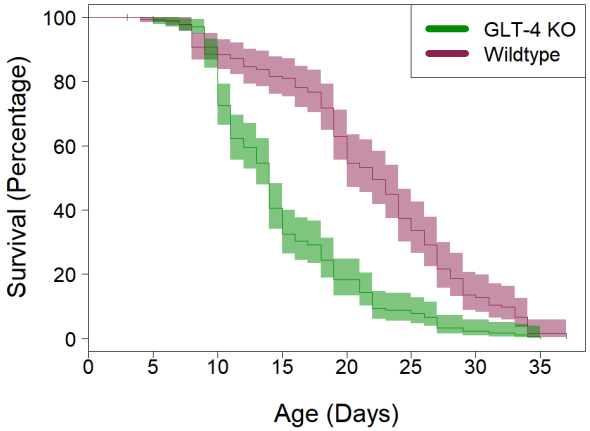

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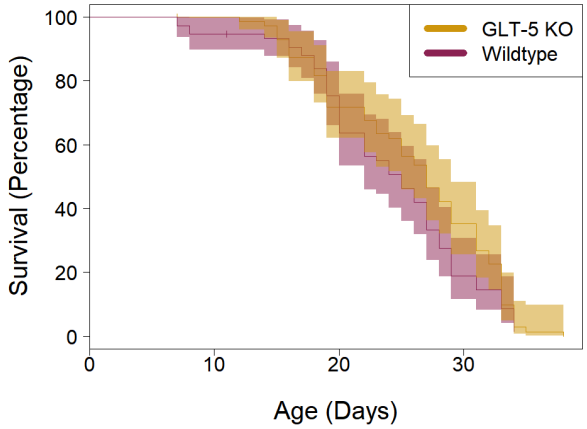

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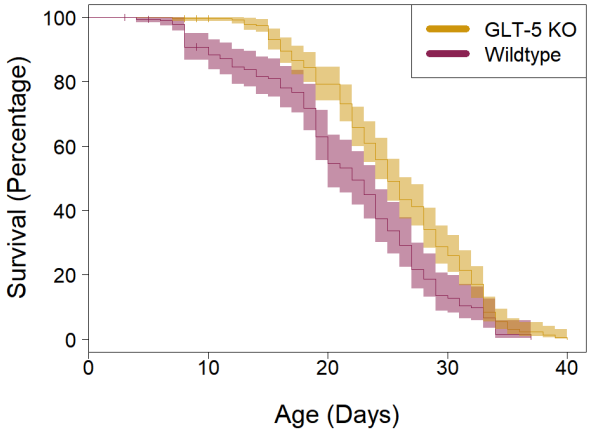

Figure 2: **The Lifespan curve for the GLT-1;GLT-3, GLT-4, and GLT-5 mutants compared to wildtype worms.** Worms were placed on FUdR plates and counted each day. Worms were marked as either dead, alive, or censored. Dead or censored worms were removed from the plate and flamed. Only non-censored worms appear in the curve. Lifespan curve was plotted using Kaplan-Meier plot in R. Shaded areas indicate 95% confidence intervals. Left column are plots with data from the experiment ran in March 2018. Right column are plots with combined data from all experiments. **A)** Wildtype worms (n = 69) are indicated by the pink curve and GLT-1;GLT-3 worms (n = 68) are indicated by the blue curve. A log-rank test indicated the lifespan curve of GLT-1;GLT-3 worms was not different from that of the wildtype worms, $p = 0.434$. **B)** All wildtype worms (n = 143) are indicated by the pink curve and all GLT-1;GLT-3 worms (n = 125) are indicated by the blue curve. A log-rank test indicated the lifespan curve of GLT-1;GLT-3 worms was not different from that of the wildtype worms, $p = 0.151$. **C)** Wildtype worms (n = 69) are indicated by the pink curve and GLT-4 worms (n = 64) are indicated by the green curve. A log-rank test indicated the lifespan of GLT-4 worms was shorter from that of the wildtype worms, $p < 0.01$. **D)** All wildtype worms (n = 143) are indicated by the pink curve and all GLT-4 worms (n = 123) are indicated by the green curve. A log-rank test indicated the lifespan of GLT-4 worms was shorter from that of the wildtype worms, $p < 0.01$. **E)** Wildtype worms (n = 69) are indicated by the pink curve and GLT-5 worms (n = 71) are indicated by the yellow curve. A log-rank test indicated the lifespan curve of GLT-5 worms was not different from that of the wildtype worms, $p = 0.157$. **F)** Wildtype worms (n = 143) are indicated by the pink curve and GLT-5 worms (n = 155) are indicated by the yellow curve. A log-rank test indicated the lifespan curve of GLT-5 worms was greater than that of the wildtype worms, $p < 0.01$.

GLT-1;GLT-3 mutant vs Wildtype

Using FUdR to prevent egg-laying in *C. elegans* is an established method to measure longevity. The GLT-1; GLT-3 lifespan curve was plotted over the wildtype lifespan curve to compare both strains' lifespans (Figure 2A). GLT-1;GLT-3 was not different from the wildtype curve in either the March 2018 experiment ($p = 0.434$) or in the combined experiment ($p = 0.151$) curves, and both Figures 2A and 2B showed many points of overlap between their curves. Figure 2A and B showed worms with a GLT-1;GLT-3 knockout lived about the same length of time as wildtype worms.

GLT-4 mutant vs Wildtype

The GLT-4 lifespan curve was compared to the wildtype lifespan curve in Figures 2C and 2D. The lifespan curve of the GLT-4 worms was lower than the wildtype curve in this trial, and there was a very low amount of overlap between the two curves in both the March 2018 experiment ($p < 0.01$) (Figure 2C) and the combined experiments ($p < 0.01$) (Figure 2D). The GLT-4 *C. elegans* had shorter lifespans than the wildtype *C. elegans.*

GLT-5 mutant vs Wildtype

The GLT-5 lifespan curve was plotted over the wildtype lifespan curve to compare lifespans (Figure 2E and 2F). While the lifespan curve of the GLT-5 worms was not different from the wildtype curve in the March 2018 trial (Figure 2E), the GLT-5 worms outlived the wildtypes in the plot of the combined data ($p < 0.01$) (Figure 2F).

NMR

To complement the lifespan experiments and determine the relative amounts of glutamine, glutamate, and α-ketoglutarate (α-KG) metabolites in each strain, a protocol for extracting and visualizing the metabolites using ^{1}H-NMR was adapted from Lourenço et al., (2015). After steps were carried out following the Lourenço paper, several changes were made to the protocol to reduce the spectrum's noise to signal ratio and identify peaks representing the three metabolites.

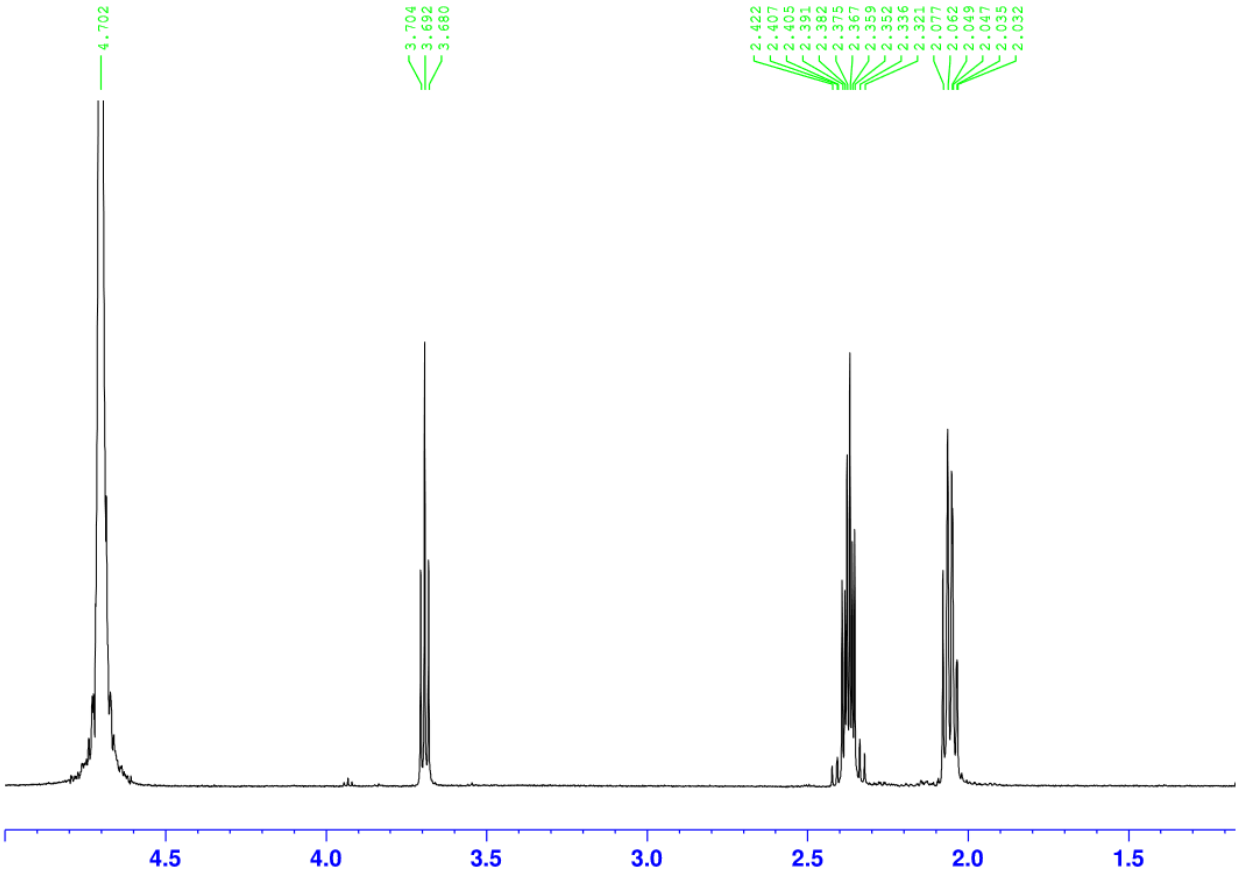

Figure 3: **A standard sample of glutamine ^{1}H-NMR spectrum – 200 scans.** An ^{1}H-NMR spectrum for glutamine in a purified chemical form was obtained to use as a reference for interpreting the ^{1}H-NMR spectra for our *C. elegans* samples. The primary peaks of interest are the triplet at 3.7 ppm and a peak around 2.3 ppm. These peaks overlap with those of glutamate.

To identify the peaks representing glutamine, the ^{1}H-NMR spectrum for a sample of purified glutamine was obtained to use for reference (Figure 3). From this spectrum, we found that peaks observed around 2.3 ppm (multiplet) and 3.7 ppm (triplet) could indicate the presence of glutamine and glutamate (Figure 3).

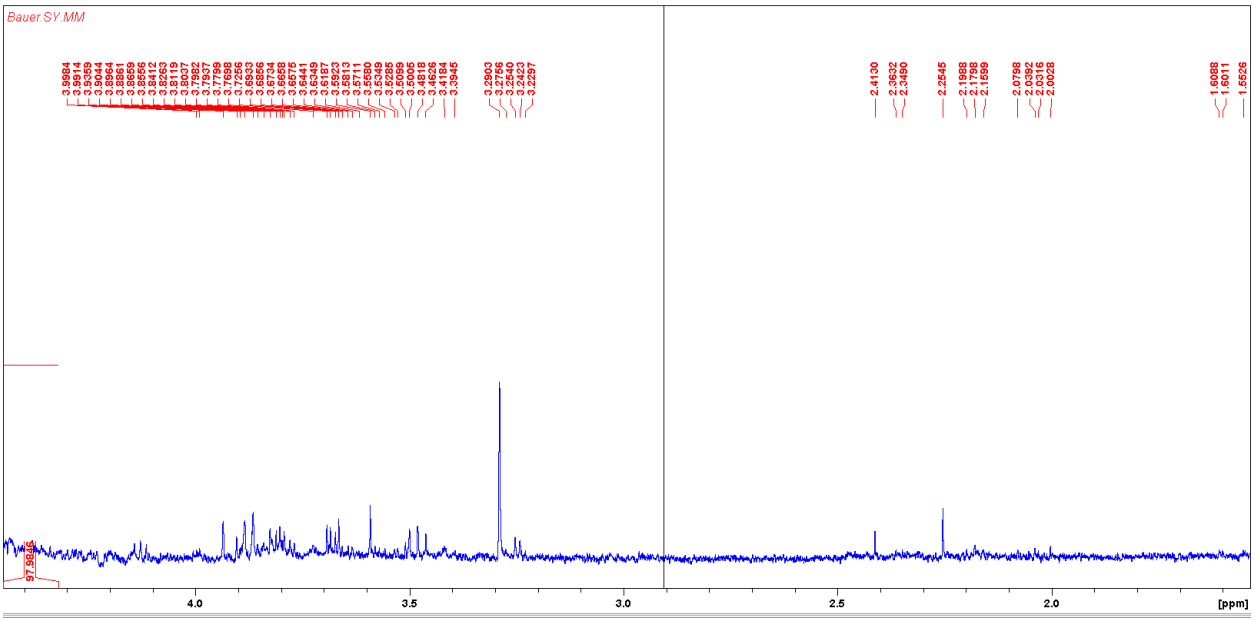

Figure 4: **^{1}H-NMR spectrum from the first protocol run through – 200 scans**. The sample for the first trial was prepared from WT worms using ethanol, sonication, a hot ethanol extraction, and phosphate buffer. For this first ^{1}H-NMR spectrum profile, the spectrum contained high amounts of noise, and the water contamination was also high (peak ~4.5 not shown). Peaks indicating glutamate, glutamine, and α-ketoglutarate could not be identified from this spectrum.

A spectrum was obtained from the first trial using a *C. elegans* sample following the protocol described by Lourenço et al., (2015) (Figure 4). To reduce the noise signals for the following trials, it was decided that the phosphate buffer would be excluded from the protocol since it did not have a necessary role.

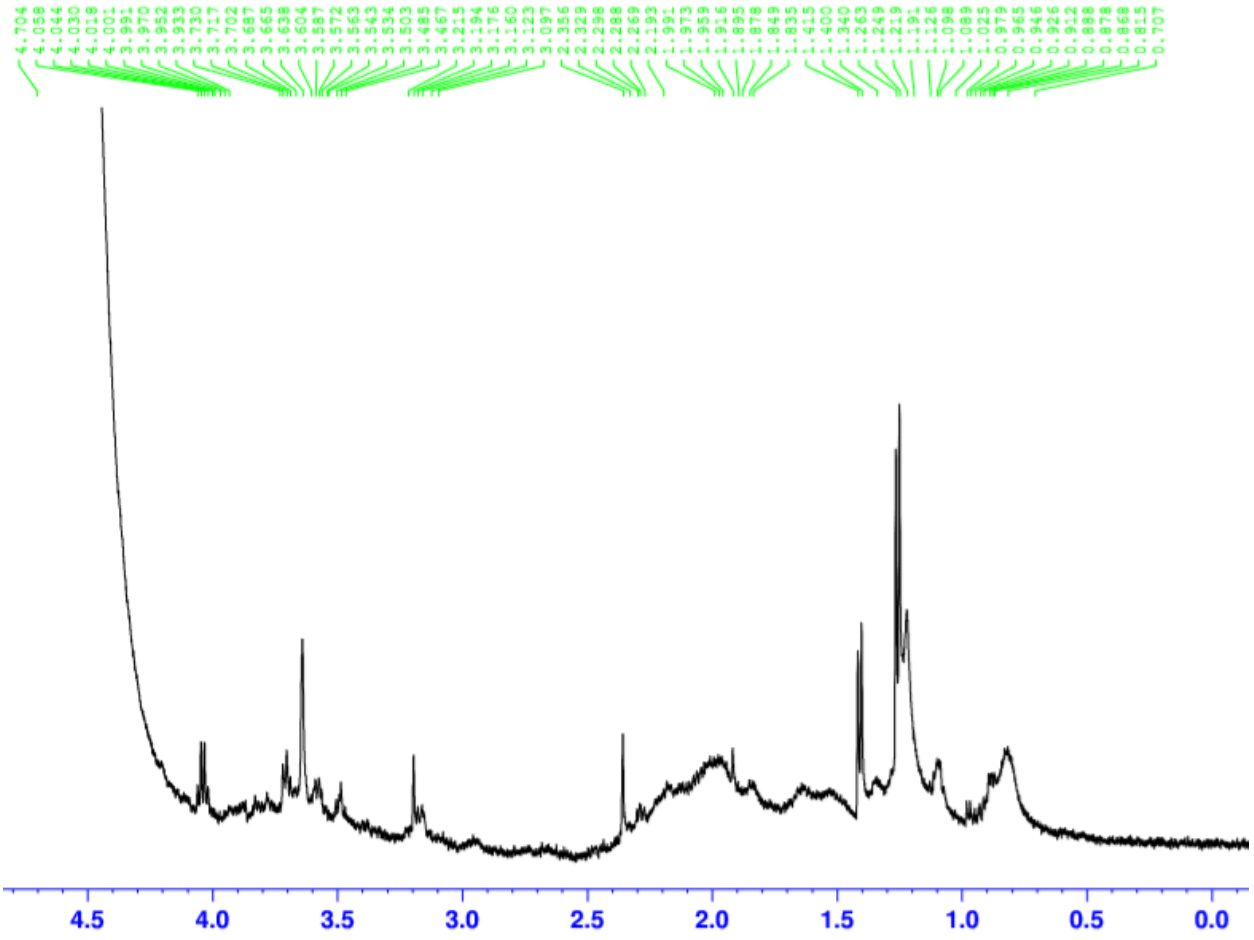

Figure 5: **NMR spectrum of sample prepared from same protocol without the phosphate buffer – 200 scans.** Sample was prepared from WT worms using ethanol, sonication, and a hot extraction, and no phosphate buffer. The large peak beginning around 4.1ppm indicates water contamination. Peaks present around 2.3 and 3.7 ppm may indicate glutamate and/or glutamine, but the two cannot be distinguished by this spectrum. There is no indication of α-ketoglutarate from this spectrum.

Following the Lourenço et al., (2015) protocol with the phosphate buffer omitted, the spectrum in Figure 5 was obtained. This spectrum still had both contamination and background noise, but peaks around 2.3 ppm and 3.7 ppm were still distinguishable within the noise of the sample. These peaks may indicate the presence of glutamine and glutamate, but α-KG was still not identified, requiring further refinement of the protocol. To further reduce the background noise, all of the steps containing ethanol were replaced with methanol in the same volume.

Figures 6 and 7 are different sections of the same NMR spectrum.

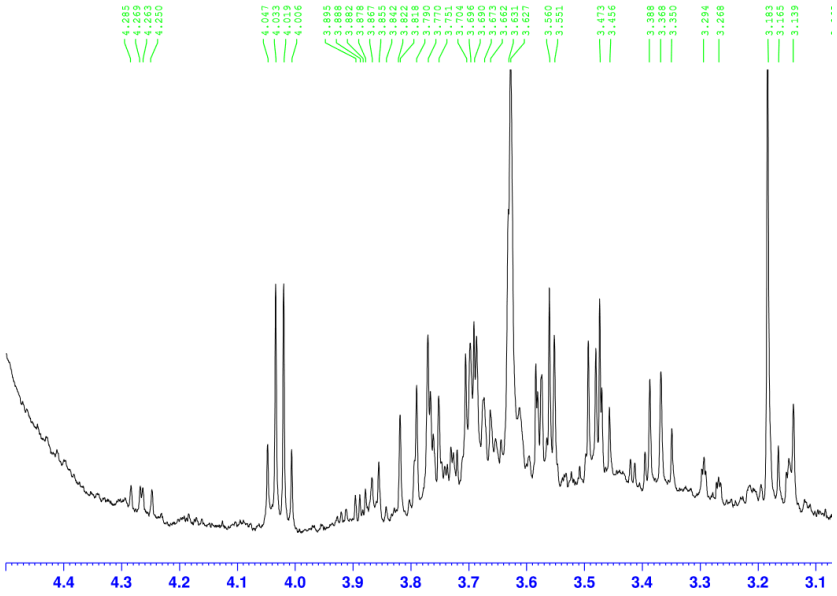

Figure 6: **NMR spectrum of sample prepared from same protocol but replacing ethanol with methanol and using GLT-5 mutant worms – 500 scans.** Sample was prepared from GLT-5 worms using methanol, sonication, and a hot extraction, and no phosphate buffer. The series of peaks that would correspond to glutamine/glutamate's series of peaks around 3.7 ppm is present on this spectrum. α-KG could not be identified.

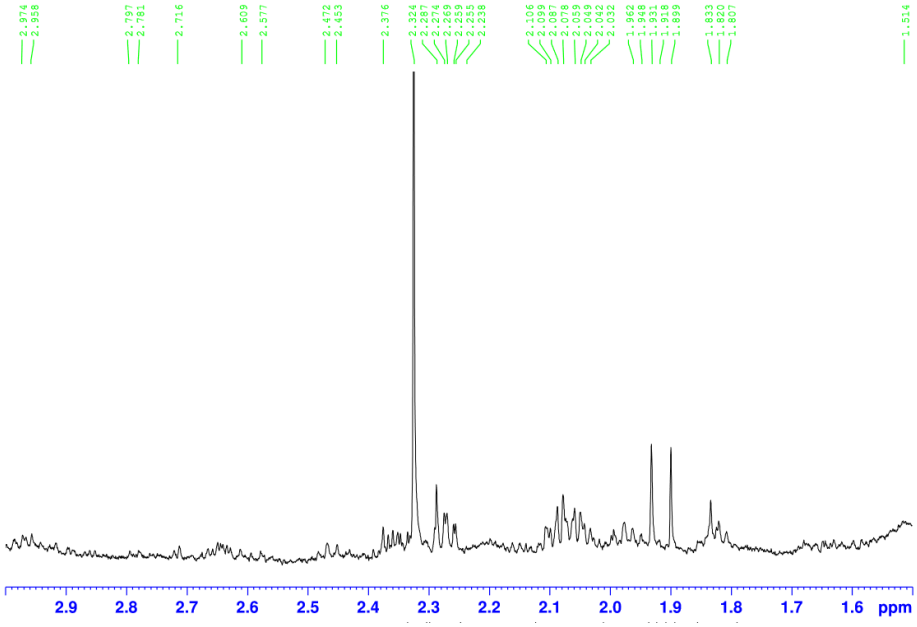

Figure 7: **NMR spectrum of sample prepared from same protocol replacing ethanol with methanol and using GLT-5 mutant worms – 500 scans.** Sample was prepared from GLT-5 worms using methanol, sonication, and a hot extraction, and no phosphate buffer. The series of

peaks that would correspond to glutamine/glutamate's series of peaks around 2.3 ppm is present on this spectrum. α-KG could not be identified.

The spectrum depicted by Figures 6 and 7 was produced using GLT-5 worms, replacing all the ethanol steps with methanol, and running ^{1}H-NMR at 500 scans rather than 200. All of these aspects were changed, because we thought this combination of variables would yield the highest amount of α-KG possible, providing the best chance for identifying α-KG in the ^{1}H-NMR spectrum. If α-KG was not identifiable in this spectrum, it would be unlikely to find α-KG in a ^{1}H-NMR spectrum with any other combination of conditions. Methanol was used to reduce the noise to help us find a peak for α-KG. 500 scans enhanced the resolution of the spectrum to identify α-KG signals that may have been too sensitive to appear in a spectrum with fewer scans. GLT-5 worms were used, because they have the highest predicted concentration of α-KG. Therefore, the increased amount of α-KG would strengthen the visibility of the peak in the ^{1}H-NMR spectrum.

From the spectrum shown in Figures 6 and 7, no α-KG peaks were identifiable and the glutamate signal was still indistinguishable from the glutamine signal. From these findings, we decided that this ^{1}H-NMR method would not quantify the metabolites in our strains in a way we could interpret.

Ultimately, the final protocol developed consisted of collecting and washing the worms, adding methanol and sonicating the worms to burst the cuticle, drying the sample over air to reduce the water content, resuspending the dried sample in D_2O, straining the sample through a glass pipette with a layer of kimwipe into the NMR tube, running the sample through an NMR machine and obtain the spectrum.

Mesh-well Plate

C. elegans from each strain were placed in the 60 µm mesh-well – one worm per well. Immediately after being placed into the wells, the worms crawled through the mesh and fell to the bottom of the well. After repeating this process, it was determined a smaller mesh-size would be needed. Before ordering new mesh plates, the diameter of several worms from each strain was measured using a microscope to gauge the appropriate size mesh for the order.

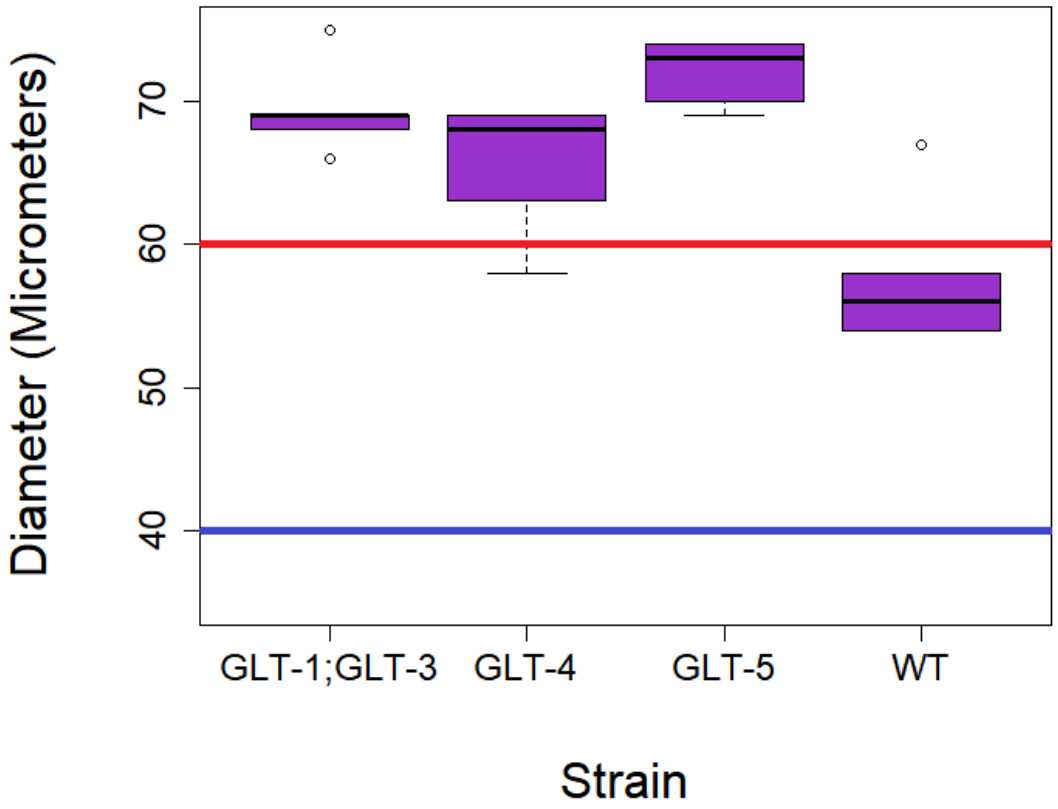

Figure 8: **Diameter of adult GLT-1;GLT-3, GLT-4, GLT-5, and WT strains.** The median diameter for the GLT-1;GLT-3 worms is 69 µm (n=5). The median diameter for the GLT-4 worms is 68 µm (n=5). The median diameter for the GLT-5 worms is 73 µm (n=5). The red line indicates the 60 µm mesh size. The blue line indicates the 40 µm mesh size. Note that all diameters fall above blue 40 µm.

When measuring the diameter of adult worms under the microscope, all the strains observed were above 40 µm (Figure 8), which is the next smallest mesh size below the 60 µm mesh. The median diameter for each strain was 69 µm for GLT-1;GLT-3, 68 µm for GLT-4, 73 µm for GLT-5, and 56 µm for wildtype. The WT worms were the smallest, but even at their smallest (54 µm), they were 14 µm larger than the 40 µm, so we proceeded to order the 40 µm mesh-well plates.

After the plates arrived, the process of placing worms individually into the wells at young adult stage was repeated. All of these worms, however, also fell through the 40 µm mesh. We decided to age match the worms and then transfer a few worms from each strain for each day after the worms became young adults to see if there was a certain age when they were large enough to remain above the mesh.

Three days after the worms entered adult stage, the GLT-5 strain was large enough to stay above the mesh and never fall through. One day later (4 days post young adult), the GLT-4 worms were also able to remain above the mesh. The wildtype and GLT-1;GLT-3 worms, however, never remained above the mesh without falling through. Occasionally the GLT-1;GLT-3 worms would fall through the mesh and swim back up and stay above the mesh for several minutes, but eventually they would fall through again.

Contamination and Evaporation Issues

The liquid media in each well could exchange with liquid media in adjacent wells. Using colored water, different volumes of liquid media per well were tested to find the maximum volume of liquid media that did not overspill into adjacent wells. 300 µL was the volume that achieved this goal.

When a new trial was conducted with the revised volume, a different problem was observed – the liquid media would evaporate from the plate and the volume would fall below the mesh. To combat this issue, the top of the plate was sealed with parafilm and a rubber band. While the parafilm reduced the loss of media, we decided that crossover of media was less important than having enough media per well. To address future crossover of media, an empty row of wells would separate each strain.

We observed that worms from laid-eggs were capable of swimming from the bottom of the well to above the mesh a few days after hatching. It was decided that the liquid media should be replaced every 3rd day to prevent confusion between the original worms above the mesh and worms that hatched from the eggs at the bottom of the well.

Adult population Issue

Since the GLT-4 and GLT-5 worms were not big enough to transfer to the mesh-well plate until a few days after they reached adult age, many small worms would populate the plate by the time the adult worms were the right size. Very few adults remained from the original generation by the time they were large enough. This lack of worms would be problematic for any large-scale lifespan test like the FUdR experiments.

Because of these issues, development of the mesh-well plate protocol was discontinued as a method for measuring lifespan alternative to using FUdR.

Discussion

The projects within this thesis aim to address a gap in the literature regarding the effects of glutamate metabolism on lifespan in *C. elegans*. The FUdR and mesh-well plate experiments focused on characterizing the longevity of our mutant GLT-1;GLT-3, GLT-4, and GLT-5 strains, and identifying any differences between the longevity of these strains and that of wildtype worms. The NMR experiments were developed to investigate connections between glutamate metabolites and any lifespan differences observed between strains.

FUdR

In the project using FUdR to measure lifespan, the lifespan of each of our mutant strains, GLT-1;GLT-3, GLT-4, and GLT-5, were compared to the lifespan of the wildtype *C. elegans*. These FUdR experiments were conducted based on a well-established and widely used method. *C. elegans* typically have a lifespan of 25 days at temperature of 20 degrees Celsius (Sutphin and Kaeberlein, 2009). Roughly 50% of the wildtype worms died before day 23, and 50 % of our worms died after day 23. This finding was encouraging as these numbers correspond to the literature values of wildtype lifespan. Lifespans of the mutant strains could not be verified since those values have not yet been reported. This gap in the literature was our primary motivation for conducting these experiments.

The GLT-1;GLT-3 mutants lived the same length of time as the wildtype worms (Figure 2B). The GLT-4 mutants had shorter lifespans compared to the wildtype (Figure 2D), while the GLT-5 worms outlived their wildtype counterparts (Figure 2F). Observing the shortened lifespan of GLT-4 mutants was unsurprising considering GLT-4s worms' numerous behavioral abnormalities such as poor performance in chemotaxis, reversal to smell on a stick, and

sensitivity to nose touch, learning, and memory (Mano et al., 2007; Harling, 2017). Possible explanations for GLT-4's shortened lifespan may result from lower levels of α-ketoglutarate (α-KG), increased synaptic glutamate levels leading to excitotoxicity, or disruption of glutamate recycling since GLT-4s are localized to the presynaptic terminal. Additionally, high mortality rates and shortened lifespans have been documented in mammals with GLT knockouts due to the neurotoxic and seizure-inducing effects of the knockout (Tanaka et al., 1997; Rimmele and Rosenberg, 2017). For these reasons, these findings regarding the unchanged lifespan of GLT-1;GLT-3 and longer lifespan of GLT-5 worms are intriguing. Our prediction is that the different mutants have different levels of the metabolites, particularly α-KG. Different GLT knockouts shift the cells that the glutamate is entering, therefore shifting the metabolic pathway glutamate follows and the metabolite formed depending on the enzymes present in that cell.

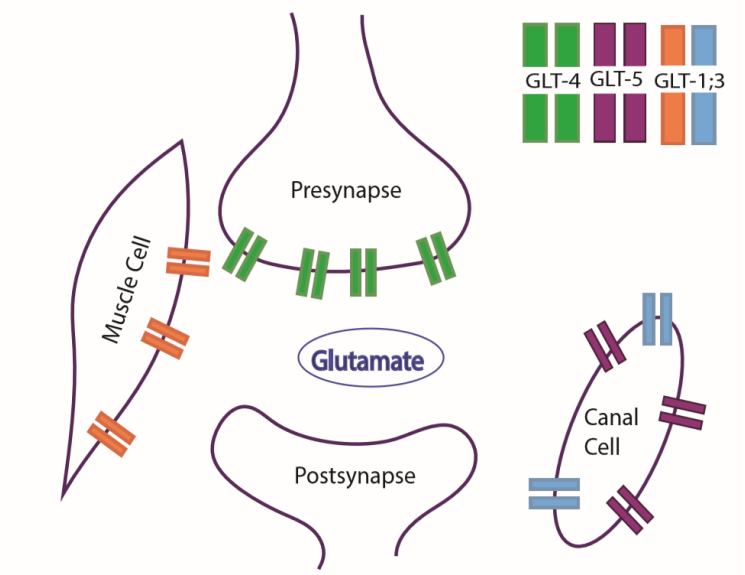

Figure 9: **A diagram of the glutamate transporter (GLT) distribution in the *C. elegans* synapse.**

A possible explanation may be the mutation in the GLT-5 and either GLT-3 worms promotes the formation of α-KG, a metabolite that has been shown to increase *C. elegans* lifespan. Both α-KG and dietary restriction prevent aging and age-related diseases by deactivating the target of rapamycin (TOR) pathway that is thought to inhibit longevity and health (Chin et al., 2014). This mechanism may confer an evolutionary advantage by prioritizing maintenance rather than growth of the organism during times of low nutrient availability.

Both Mitchell et al., 1979 and Gandhi et al., 1980 provide some of the earliest descriptions of FUdR as a DNA synthesis inhibitor to prevent the hatching of eggs and maintain the synchrony of the original generation. Both papers describe the use of FUdR to test lifespan in *C. elegans* without altering the typical post-maturational aging characteristics of FUdR-treated worms. While our FUdR lifespan experiments were successful, protrusions were observed developing around the vulva region of many of the worms from all strains. The protrusion appeared as a round bump in the mid-section of the worm. Since we had never noted the presence of these protrusions in our normal (untreated) maintenance populations, it was uncertain if the emergence of these protrusions was a result of the FUdR treatment. This observation was additionally confusing since the Mitchell et al., 1979 and Gandhi et al., 1980 papers explicitly mentioned that there were no other changes to the development of the worm other than reproduction. After noting the presence of protrusions in our FUdR treated worms, we began looking at our maintenance plates and noticed some untreated worms with protrusions as well.

Research conducted by the Kaeberlein Lab provided some insight regarding the protrusions. The Kaeberlein Lab described a phenomenon called age-related vulval integrity defects (Avid). The Kaeberlein researchers are interested in studying this phenomenon, because

whether Avid is a natural part of *C. elegans* aging or not remains uncertain, and issues of censoring these worms have arisen for other researchers conducting lifespan and aging experiments (Leiser et al., n.d.). In our FUdR experiments, we decided to not censor worms with vulva protrusions unless the vulva had severely ruptured. The Kaeberlein Lab's website has an image depicting the levels of Avid and the accompanying differences between an unruptured, protruding, mildly ruptured, and severely ruptured.

Finding a *C. elegans* mutant that enhances lifespan is an exciting result. While some lifespan-enhancing mutants such as *daf-2* insulin-receptor mutants have been characterized in *C. elegans*, a mutation that confers longevity is a novel and rare discovery (Pinkston et al., 2006). These mutants can help us expand our understanding of the relationship between glutamate metabolism and aging and health. It is also fortunate that we can build from the literature that has shown the lifespan-enhancing effects of α-KG and dietary restriction, which has informed our hypotheses.

One caveat of our FUdR experiment was the appearance of the protrusions during the experiment, since we are still unsure how this phenotype affects the lifespan. However, this observation is not too concerning since the protrusions did not often rupture, and the protrusions were present in all strains. Another caveat regards the interpretation of the data from these GLT deletion experiments. Comparisons with these findings are limited with rodents and other mammalian models, because GLT knockouts often lead to premature death in those animals. Since *C. elegans* are unique in their ability to live with these GLT mutations, caution should be taken when extrapolating our results to other models.

NMR

To ascertain relative quantities of glutamate, glutamine, and α-KG metabolites, a protocol compatible with ^{1}H-NMR analysis was refined and developed from a *C. elegans* sample as described in Lourenço et al., (2015). After many iterations of the protocol, we determined that NMR was not a technique that could provide us the information we need, as the spectra contained a large amount of noise (non-important peaks), because the sample was developed from biological material. Because of the noise, α-KG was not detectable and glutamate and glutamine were not distinguishable within the spectra. Few researchers have used NMR in *C. elegans* samples. Studies by Blaise, Wong and Lourenço were helpful in guiding the process of developing a protocol where metabolites could be successfully extracted from worm tissue.

Wong et al., (2014) and Blaise et al., (1993) used solid-state NMR (1H high resolution magic angle spinning NMR spectroscopy) to obtain metabolic phenotypes of their strains from highly resolved spectra. Since we did not have access to the technology described in Blaise et al., (1993) and Wong et al., (2014), we relied on the work of Lourenço et al., (2015) to structure our method with the equipment available to us. Spectra in all three studies were analyzed via multivariate data analysis: principle component analysis and orthogonal partial least-squares discriminant analysis.

Despite not being able to move forward with the NMR protocol, we developed a method to extract metabolites from a *C. elegans* sample. This technique will be valuable in follow-up studies to identify metabolite levels within our strains. Even though we could not use our NMR technology to gain information about the metabolic phenotypes of our worms, multivariate analysis and other NMR technology such as ^{1}H high resolution magic-angle spinning NMR can provide rich metabolic profiles for *C. elegans* strains.

Mesh-well Plates

We endeavored to develop a method of measuring *C. elegans*' lifespan alternative to using FUdR. We pursued a technique using a 96-well plate with mesh layer in each well, because the mesh-wells could theoretically provide all the lifespan information of the FUdR method in a less, expensive, less hazardous, and more convenient way. Additionally, the mesh plates would not interfere with the normal development of the worm, whereas FUdR prevents the hermaphrodites from laying eggs. The replication of a liquid media recipe that enabled the worms to age and live normally in the mesh-wells was successful. The project began with 60 µm mesh-well plates, because our initial research showed fully grown *C. elegans* reach about 80 µm in diameter and eggs are 50 µm in length and 30 µm in diameter (Riddle et al., 1997). We determined that these dimensions made the 60 µm mesh-well plates the best fit since the worms would be large enough to live above the mesh but the eggs would be small enough to pass through the mesh to the receiver well after being laid. When we began testing our method, however, we were surprised to find that the worms also fell through the mesh.

After observing the worms falling through, further research revealed a variety of values regarding worm diameter – another article reported the average diameter of their worms as 49 µm. Due to the variation in the literature, we decided to measure the diameter of our worms with a microscope. We are still uncertain why our diameter is smaller than the diameter reported in Wormbook, but potential reasons may involve differences in feeding and/or maintenance practices. However, we ultimately decided to discontinue the development of this protocol, because even with the 40 µm, the worms were able to fall through the mesh to the bottom of the well. The ability of worms to fit through the mesh with diameters larger than that of the mesh may be due to the pliability of the mesh or the worms may be flexible enough to fit their bodies

through smaller openings. Something we can try would be to make the mesh layer out of a different, more rigid material to see if that prevents the worms from passing through the mesh.

We pursued the mesh-well plates because the theoretical advantages are numerous. If mesh-well plates could be used as a substitute for FUdR in lifespan experiments, these experiments would become safer, more cost-effective, and easier to execute. The mesh-well plate protocol would also complement any data obtained from FUdR experiments, because we would see if the findings are consistent with data collected from FUdR experiments. There are currently several obstacles preventing us from continuing with the mesh-well plates – not only the sizing of our worms. If we can come up with solutions to these current problems, this alternative method would be incredibly useful.

Conclusions

The results from the FUdR experiments suggest that different GLT deletions have different effects on *C. elegans* lifespan. Compared to wildtype *C. elegans*, GLT-4 mutants have decreased lifespans and GLT-5 mutants have increased lifespans. The unique deleterious effect of the GLT-4 deletion may occur because GLT-4 is the only GLT located on a neuron in the synapse. We hypothesize that the localization of the GLTs within the *C. elegans* synapse may result in different α-ketoglutarate (α-KG) levels for different strains. Based on previous studies implicating α-KG in lifespan extension, we predict α-KG levels are elevated in the GLT-5 worms, conferring enhanced longevity (Chin et al., 2014). While the mesh-well project was promising, we found that GLT-1;3 and wildtype worms could not be separated using the 40 µm mesh despite all strains growing larger than the 40 µm in width. Regarding the NMR project, we

decided it would not be feasible to use this technique to compare levels of α-KG between our strains. However, metabolite-supplementing experiments and α-KG assays show promise as alternative ways to test our hypothesis.

Future directions

NMR

We collaborated with the Huang Lab to develop the NMR protocol and interpret the spectra. Through our collaboration with the Huang lab, we determined that NMR would not answer our questions, and we were advised to pursue liquid chromatography – mass spectrometry (LC-MS) to continue investigating the relative amounts of our glutamate metabolites in each strain. Fortunately, we will still need the protocol we developed for the NMR sample preparation to extract metabolites from the tissue of *C. elegans* and prepare the sample for LC-MS and the α-KG assays.

Additionally, we began researching assays to measure α-ketoglutarate (α-KG) levels in *C. elegans*. In addition to Chin et al., (2014), which describes a method to extract and measure the α-KG in worm samples, we have been considering purchasing an assay kit designed to measure α-KG, because many of the kits would be sensitive enough to detect α-KG levels in *C. elegans* (0.01 to 10 nmoles - https://www.biovision.com/alpha-ketoglutarate-colorimetric-fluorometric-assay-kit.html) while being similar in expense to purchasing all the materials to perform the assay described by Chin et al., (2014).

FUdR

For the FUdR experiments, there are several other follow-up tests that I would like to conduct to build on our current findings. We currently have a double knockout strain (GLT-1;GLT-3), and we are uncertain if both GLT deletions are effecting the worm's longevity the same way versus one GLT deletion enhancing while the other reduces lifespan, in which case the effects would negate one another, explaining the lifespan of the worms to appear unchanged compared to wildtype. For these reasons, we are planning to obtain separate GLT-1 and GLT-3 strains. Once we have these two strains, we will run more FUdR experiments to test the lifespans of the strains individually.

Lastly, we are interested testing additional conditions in our FUdR experiments such as supplementing the glutamate metabolites and running dietary restriction trials with the FUdR plates. For the experiments supplementing the glutamate metabolites, we can follow the protocol of Chin et al., (2014). α-KG is the first metabolite I would want to supplement, because that experiment could provide important insights to our question of whether increases α-KG can explain the enhanced lifespan of the GLT-5 strain – even if we do not have the assay for α-KG levels running yet. According to Chin et al., (2014), the effects of endogenously increasing and exogenously supplementing α-KG cannot have additive effects on enhancing lifespan. Therefore, if α-KG levels are indeed increased in GLT-5s and GLT-1;3s, we would expect to see exogenous supplementation of α-KG enhance the lifespan of the WTs (and perhaps GLT-4s) but not the GLT-5s or GLT-1;GLT-3s. Including a dietary restriction test within the FUdR experiments would also help answer the same questions since dietary restriction leads to an endogenous increase in α-KG. If GLT-5 and GLT-1;GLT-3 already have increased α-KG levels, we would expect dietary restriction to not increase lifespan further but increase the lifespan of wildtypes.

These future experiments would build upon the information collected from my projects and past projects to provide important information towards identifying and explaining the processes that mediate the differences in lifespan between our *C. elegans* strains.

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