2017

Genetic Regulators Of Toxicity In A Mouse Model Of Amyotrophic Lateral Sclerosis And A Worm Model Of Anoxic Injury

Shachee Doshi
University of Pennsylvania, doshis@alumni.upenn.edu

Genetic Regulators Of Toxicity In A Mouse Model Of Amyotrophic Lateral Sclerosis And A Worm Model Of Anoxic Injury

Abstract
The equilibrium between energy consumption and energy production defines the metabolic rate of an organism. This homeostatic balance is tightly regulated by a variety of sophisticated processes that occur within and between cells and tissues. These processes also allow animals to tolerate some deviation from baseline by recruiting adaptive mechanisms to bring cells and organisms back to homeostasis. Temporary changes in the organism's environment, such as alterations in ambient temperature, oxygen levels and infections are examples of conditions where animals must have a healthful adaptive metabolic response, allowing them to sustain the duration of the stress. However, in conditions of chronic disease or long-term stress, these adaptive mechanisms can no longer be protective and may even contribute to the damage incurred by the animal. Therefore, metabolism can either be healthful and adaptive to stressors, or stressors can induce pathogenic metabolic changes in an organism. In this body of work, I explore this bidirectional relationship between external stresses and organismal metabolism. In chapter 2, I investigate the contribution of hypermetabolism (energy production > energy consumption) in a mouse model of the disease amyotrophic lateral sclerosis (ALS). While hypermetabolism is a feature of ALS, it is not known if it contributes to disease pathogenesis. In a mouse model of ALS, I genetically induce hypometabolism to determine if this change alters disease progression. In chapter 3, I study the role of neuropeptides in regulating hypometabolic tolerance to extreme oxygen deprivation. Here, I employ C. elegans, a genetically tractable soil-dwelling nematode, as a model. Worms can tolerate long durations of anoxia by lowering their metabolic rate, and loss of neuropeptide signaling can further increase its ability to tolerate this stress. I investigate various aspects of neuropeptide regulation of this phenotype. Together, these projects demonstrate the role of metabolism in health, disease and stress, and suggest that inter-cellular and inter-tissue communication is a critical aspect of metabolic homeostasis.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Neuroscience

First Advisor
Robert G. Kalb

Keywords
ALS, C. elegans, hypoxia, MC4R, metabolism, neuropeptides

Subject Categories
Genetics | Neuroscience and Neurobiology

GENETIC REGULATORS OF TOXICITY IN A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS AND A WORM MODEL OF ANOXIC INJURY

Shachee Doshi

A DISSERTATION

In

Neuroscience

Presented to the Faculties of the University of Pennsylvania

In

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2017

Supervisor of Dissertation

_____

Robert G. Kalb, MD
Professor of Neurology

Graduate Group Chairperson

_____

Joshua I. Gold, PhD
Professor of Neuroscience

Dissertation Committee

Thomas A. Jongens, PhD, Associate Professor of Genetics

David M. Raizen, MD, PhD, Associate Professor of Neurology

Amita Sehgal, PhD, John Herr Musser Professor of Neuroscience

Virginia M. -Y. Lee, PhD, John H. Ware 3rd Endowed Professor of Alzheimer's Research

Anne C. Hart, PhD, Professor of Neuroscience

Dedication page

To my father, Bimal, who made me acutely aware of the sensitivity, complexity and beauty of the brain.

ACKNOWLEDGMENT

Thank you mom for always prioritizing my education and opening up my world to infinite possibilities.

Thank you Shantanu for the unwavering love, support and commitment to my success.

Thank you mom, Rahul, Shantanu and Shinobi for the food, clothes, snuggles and for the warmth of home.

Thank you Sarah, Noam, Morgan, Isaac, Leo, Chris and Russ for making grad school feel like family, and for reaffirming that neuroscientists may be the most fun of all the scientists.

Thank you Bob, Preetika, Chia-Yen, Lei, Jelena, Ria, John, Rahul, Matt, Ogul, Urva, Heather and Sean-Patrick for making lab a home away from home.

Thank you Emma and Justin for your incredible love and dedication to the worms, and for teaching me to teach.

Thank you NGG for allowing me to grow in ways I never thought I could while doing a PhD.

Thank you Akshay, Andrea and Monica for giving me a reason to escape lab.

Thank you, worms and mice, for all that you unknowingly do for science.

Thank you tardigrades, for being invincible and giving me hope that nuclear holocaust may not be the end of life.

ABSTRACT

GENETIC REGULATORS OF TOXICITY IN A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS AND A WORM MODEL OF ANOXIC INJURY

Shachee Doshi

Robert G. Kalb

The equilibrium between energy consumption and energy production defines the metabolic rate of an organism. This homeostatic balance is tightly regulated by a variety of sophisticated processes that occur within and between cells and tissues. These processes also allow animals to tolerate some deviation from baseline by recruiting adaptive mechanisms to bring cells and organisms back to homeostasis. Temporary changes in the organism's environment, such as alterations in ambient temperature, oxygen levels and infections are examples of conditions where animals must have a healthful adaptive metabolic response, allowing them to sustain the duration of the stress. However, in conditions of chronic disease or long-term stress, these adaptive mechanisms can no longer be protective and may even contribute to the damage incurred by the animal. Therefore, metabolism can either be healthful and adaptive to stressors, or stressors can induce pathogenic metabolic changes in an organism. In this body of work, I explore this bidirectional relationship between external stresses and organismal metabolism. In **chapter 2**, I investigate the contribution of hypermetabolism (energy production > energy consumption) in a mouse model of the disease amyotrophic lateral sclerosis (ALS). While hypermetabolism is a feature of ALS, it is not known if it contributes to disease pathogenesis. In a mouse model of ALS, I genetically induce hypometabolism to determine if this change alters disease progression. In **chapter 3**, I

study the role of neuropeptides in regulating hypometabolic tolerance to extreme oxygen deprivation. Here, I employ *C. elegans*, a genetically tractable soil-dwelling nematode, as a model. Worms can tolerate long durations of anoxia by lowering their metabolic rate, and loss of neuropeptide signaling can further increase its ability to tolerate this stress. I investigate various aspects of neuropeptide regulation of this phenotype. Together, these projects demonstrate the role of metabolism in health, disease and stress, and suggest that inter-cellular and inter-tissue communication is a critical aspect of metabolic homeostasis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

General Introduction

Metabolic homeostasis is essential to health and physiology. It operates at cellular and organismal levels to coordinate and balance energy intake and expenditure. However, it can deviate from this homeostatic balance in conditions of stress and disease. Here, I will explore two different contexts of metabolism - one in which metabolic adaptation is detrimental to a particular neurodegenerative disease, and another in which metabolic adaptation is essential to survival against an environmental stress.

Metabolic dysfunction at the organism level is a feature of many neurodegenerative diseases such as Alzheimer's disease (AD), Amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) and Parkinson's disease (PD) (Dupuis et al., 2011; Cai et al., 2012). These changes are not identical across these diseases, but it is interesting to note that many of the same markers such as circulating hormones and body weight are commonly altered in all of them. A brief outline of these abnormalities follows below.

In Alzheimer's disease, the most widely occurring neurodegenerative disease and the leading cause of dementia and cognitive impairment, increased body weight in midlife (35-65 years) has a significant correlation with onset of dementia in later life (Albanese et al., 2017). Type 2 diabetes is strongly correlated with AD, brain glucose metabolism is decreased in patients with AD compared to controls, and abnormalities in

glucose metabolism manifest decades before cognitive symptoms of AD (Mosconi, 2005; Chen and Zhong, 2013; Duran-Aniotz and Hetz, 2016). AD patients can manifest neuronal insulin resistance and have increased brain insulin receptor expression (Frölich et al., 1998; Stanley et al., 2016). Metabolic hormones such as leptin and ghrelin are also altered in AD – higher circulating leptin correlates with higher brain volume and reduced incidence of dementia and AD while ghrelin mRNA is decreased in cortex of AD patients (Lieb et al., 2009; Gahete et al., 2010).

HD patients have increased weight loss and increased fasting energy expenditure than controls (Pratley et al., 2000; Aziz et al., 2010). Patients also have increased leptin secretion with increased CAG repeat length (Ahmad Aziz et al., 2009). Total cholesterol, high density lipoprotein and low density lipoprotein levels are all decreased in HD patients compared to familial controls (Wang et al., 2014).

In PD, patients have increased serum levels of insulin-like growth factor 1 compared to healthy controls (Godau et al., 2010). Patients also demonstrate decreased body weight (Chen et al., 2003), and patients with decreased weight loss also have an unrelated decrease in circulating leptin (Evidente et al., 2001).

ALS patients also present with a hypermetabolic phenotype, measured as a higher resting energy expenditure, that manifests early, is persistent and is correlated with survival (Desport et al., 2001; Bouteloup et al., 2009; Funalot et al., 2009a). A higher premorbid BMI and higher fat diets are associated with better disease progression in ALS (Ngo et al., 2014). Patients have impaired glucose tolerance (Pradat et al., 2010), and the level of total cholesterol is two-fold higher in ALS patients compared to controls (Dupuis et al., 2008).

While there is a growing body of work highlighting a wide range of organism level deficits in metabolic homeostasis in neurodegenerative diseases, it is unknown if this

altered metabolic state contributes to worsening disease progression. It is, therefore, important to gain a deeper understanding of how perturbations in metabolic homeostasis contributes to the progression of various neurodegenerative diseases in order to devise effective treatment plans. In the current work, we focus on ALS to highlight and address whole organism metabolic changes observed in this disease.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis, also known as Lou Gehrig's disease, Charcot disease and motor neuron disease, is a devastating neurodegenerative disease characterized by progressive loss of upper and lower motor neurons leading to muscle wasting, paralysis and ultimately death due to respiratory failure. It has a worldwide incidence of 1.9 in every 100,000 people (Arthur et al., 2016), and affects more men than women. It is also more prevalent in non-Hispanic populations, and affects military veterans and sports people at a higher rate than the rest of the population (Hardiman et al., 2011; Kiernan et al., 2011). ALS is the most common adult onset motor neuron disease, with an average age of onset around 55-60 (Ferraiuolo et al., 2011). It progresses rapidly, and most patients die within 3-5 years of symptom onset. In addition to motor symptoms, a subpopulation of patients also manifests deficits in cognitive functions such as decision-making and language processing (Phukan et al., 2007). As a result, ALS has significant clinical and biological overlap with frontotemporal dementia, and the two are thought to be on a pathophysiological continuum (Ling et al., 2013). Ling *et al* carefully describe the clinical features that set up the ALS-FTD spectrum as well as underlying genes and molecular mechanisms that may regulate ALS-FTD. For example, mutations in some genes such as SOD1 and TDP-43 are thought to predominantly result in ALS, while others in VCP and Tau are predominant in causing FTD, and still others

like C9orf72 can cause either ALS or FTD or both. It is expected that the number of people living with ALS will increase from 222,801 in 2015 to 376,674 in 2040, representing an increase of 69% (Arthur et al., 2016), and the disease will thus place a significant economic burden on society. There is currently no cure for the disease and Riluzole, one of two drugs approved for treatment of ALS, provides a very modest improvement in patient outcome (Renton et al., 2014). The FDA approved a second drug, Radicava, earlier this year, 22 years after Riluzole. However, it too, was only able to slow symptoms in a 6-month clinical trial without significantly altering disease course (Hardiman and van den Berg, 2017).

The majority of ALS cases are sporadic (sALS), with no known single genetic abnormality but 5-10% of cases are familial (fALS) and result from single gene mutations with a family history of the disease (Ferraiuolo et al., 2011). The most common genetic cause of fALS is C9orf72, and the pathogenic expansion of a hexanucleotide repeat in the gene accounts for 25-40% of familial cases (Allen et al., 2014; Tan et al., 2014). While the exact function of the native protein encoded by the C9orf72 locus remains unclear, the disease-associated allele of C9orf72 (containing the GGGGCC repeat expansion) disrupts many aspects of cellular function. Cells with expanded C9orf72 have RNA accumulations in the nucleus with improper RNA processing and mis-splicing (Prudencio et al., 2015) and dysfunctional trafficking between the nucleus and cytoplasm (Zhang et al., 2015).

The second most common causative gene in fALS is the Cu/Zn superoxide dismutase 1 (SOD1), accounting for 12-20% of familial cases (Renton et al., 2014). SOD1 is a cytosolic enzyme and is responsible for scavenging free radicals and converting superoxide to hydrogen peroxide. A large number of point mutations (>100) in SOD1 have been associated with ALS, and these mutations cause a toxic gain of

function in the enzyme, which leads to pathology (Mattiazzi, 2002; Lin and Beal, 2006). Mutations in TAR DNA-binding protein of 43kDa molecular weight (TDP-43), fused in sarcoma (FUS), optineurin (OPTN), valosin-containing protein (VCP), among others, are also known to cause fALS (Ferraiuolo et al., 2011; Renton et al., 2014).

Generally, patients with fALS have an earlier age of onset than those with sALS, however, disease progression and pathological features of sALS and fALS share commonalities (Kiernan et al., 2011; Ngo et al., 2014). The most widely used mouse models of the disease are based on fALS-linked mutations in SOD1 and TDP-43. These models share many of the features of the disease, including death of motor neurons, muscle wasting, limb paralysis and short lifespan (Picher-Martel et al., 2016). Studies in these models of disease have revealed many cellular features and pathogenic characteristics associated with disease progression. Some of these include dysregulated RNA metabolism/trafficking, endoplasmic reticulum (ER) stress, oxidative stress, protein misfolding and aggregation, autophagy, mitochondrial dysfunction, impaired axonal transport and inflammation (Tefera and Borges, 2017). Adding to this list is a growing body of work implicating the role of altered metabolism to ALS pathophysiology.

Metabolic dysfunction in ALS

Studies in ALS patients and animal models have revealed a range of organism-level and cellular metabolic abnormalities, some of which are highlighted below:
(i) ALS patients and mouse models display hypermetabolism, a state in which energy expended by the organism is greater than the energy consumed (Dupuis et al., 2004; Bouteloup et al., 2009; Dupuis et al., 2011; Ngo et al., 2014). There is a 20% increase in resting energy expenditure measured by indirect calorimetry in a significant subpopulation (48%) of ALS patients, even after normalizing for fat-free mass

(Bouteloup et al., 2009). In a small study, 100% of patients with fALS were hypermetabolic, as compared to 52% of sALS patients (Funalot et al., 2009a).

(ii) There is a strong correlation between premorbid BMI and ALS risk. Patients with low BMI have decreased survival compared to those with higher BMI (Jawaid et al., 2010a). Additionally, individuals with higher BMI (overweight or obese) earlier in life had a lower risk of developing ALS. There was a 21% decrease in rate of ALS with every 5-unit increase in BMI (O'Reilly et al., 2013).

(iii) ALS patients display dyslipidemia, where the ratio of low density lipoproteins (LDL) to high density lipoproteins (HDL) is high (Dupuis et al., 2008; Jawaid et al., 2010b).

(iv) ALS patients present with a higher prevalence of hyperglycemia and glucose intolerance compared to healthy controls. Additionally, ALS patients and mouse models have lower glucose uptake in certain regions of the brain and the spinal cord (Miyazaki et al., 2012; Cistaro et al., 2014). This demonstrates impaired glucose metabolism in patients and animal models of ALS.

(v) ALS patients and mouse models have mitochondrial dysfunction. Motor neurons from patient spinal cords display morphologically abnormal mitochondria, appearing aggregated, swollen and vacuolated (Sasaki and Iwata, 2007). Similarly, SOD1 and TDP-43 mouse models of ALS are also similar in their morphology (Magrané et al., 2014; Stribl et al., 2014). In addition, mitochondrial function is compromised in ALS as evidenced by decreased electron transport chain activity and decreased ATP production in spinal cords from patients and animal models (Mattiazzi, 2002; Wiedemann et al., 2002; Kirkinezos et al., 2005). The work in mice shows that these mitochondrial abnormalities present earlier than motor symptoms, and may indicate a role for cellular energy dysfunction in disease pathophysiology.

Together, these data indicate significant perturbations in organism-wide as well as cellular changes in metabolism associated with ALS. Is organismal metabolic dysfunction a result of underlying cellular pathology in ALS, or does it contribute to causing disease pathology? If the latter is true, these metabolic parameters could be potential therapeutic targets to alter course of disease. Data from mouse studies suggest that the latter may be true. For example, mitochondrial dysfunction as well as altered expression of lipid metabolism genes occurs at presymptomatic stages in ALS (Ferraiuolo et al., 2011). Certain dietary interventions, all involving different types of high caloric diets have benefited mutant SOD1 mice (Dupuis et al., 2004; Zhao et al., 2006; Ari et al., 2014). In patients, similar dietary manipulations have also proven successful, albeit the size of the study was small. Patients fed a high carbohydrate, high calorie diet had an increased lifespan with delayed weight loss and fewer adverse events compared with patients fed a regular diet (Wills et al., 2014a). These studies indirectly suggest that metabolic dysfunction, specifically hypermetabolism, could be a targeted for therapeutic intervention in ALS. However, the question that remains is whether directly altering metabolic dysfunction can be beneficial to disease progression.

Previous studies from our lab have also tried to address hypermetabolism in SOD1 mice bearing the G93A point mutation by genetically inducing a lower metabolic state (Lim et al., 2014). This was achieved by generating G93A mutant SOD1 mice that had a deficiency in the leptin hormone. Leptin is a circulating hormone released by adipocytes. It crosses the blood brain barrier and acts on leptin receptors in the hypothalamus to suppress hunger and appetite. Leptin deficient animals are obese and hypometabolic, as they are worse at suppressing hunger and inducing satiety. In their study, Lim *et al* found that in agreement with the literature, G93A SOD1 single mutant mice were hypermetabolic. Leptin deficient (ob/+) single mutant mice, on the other hand,

were overweight and hypometabolic. G93A SOD1 and ob/+ double mutant mice mimic the weight gain and hypometabolic phenotype of ob/+ mice, demonstrating that genetically altering leptin levels is sufficient to alter the hypermetabolism of G93A SOD1 animals. Interestingly, the double mutant mice had improved limb strength and a female specific increase in median lifespan compared to G93A SOD1 mice alone. This indicates that hypermetabolism contributes to ALS pathophysiology in the G93A SOD1 mouse model.

Since leptin is a circulating hormone, it has functions in the periphery as well as the brain. Additionally, there is emerging evidence for non-hypothalamic actions of leptin in the brain (Ahima et al., 1999; Figlewicz et al., 2006; Van Doorn et al., 2017). This raises a few questions: 1) does leptin act on its receptor targets in the hypothalamus to achieve this benefit, or are its peripheral targets also important? 2) since heterozygous leptin deficient mice showed improvement in the G93A SOD1 background, could stronger hypometabolism be even more beneficial in this model? To answer these, we focused our studies on deletion of the melanocortin-4 receptor (MC4R), a downstream target of leptin in the arcuate nucleus of the hypothalamus. If leptin acts on the hypothalamus for its hypometabolic benefit in G93A SOD1 mice, MC4R should mimic leptin's effects in the G93A SOD1 mouse, and enhance the effect because it is a complete null rather than a heterozygote. We hypothesized that loss of MC4R would induce hypometabolism and increase motor output and survival in G93A SOD1 mice. This work is detailed in **chapter 2** of my dissertation.

Metabolic adaptation to adverse environmental stresses

ALS serves as an example that lowering metabolic rate can be neuroprotective in certain neurodegenerative diseases where hypermetabolism is a feature of disease. In

certain acute injuries and stresses, lowering metabolic rate by therapeutic hypothermia is also neuroprotective. In the clinic, therapeutic hypothermia is used to treat patients with cardiac arrest and in newborn infants with hypoxic ischemic encephalopathy to prevent resultant anoxic brain damage (Nagel et al., 2014). It is also being tested as a therapy for other acute conditions such as traumatic brain injury and stroke (Han et al., 2015). In therapeutic hypothermia, the core body temperature is reduced from 37°C to 32-35°C (mild), 25-32°C (moderate) or < 25°C (severe/deep) (Lyden et al., 2006). While the exact mechanism for the neuroprotective effects of hypothermia remain murky, lowering metabolic rate in cells helps to prevent the inflammatory response and resulting apoptosis or necrosis in neurons.

Despite the potential neuroprotective benefits of lowering metabolic rate in certain cases of disease and acute injury/trauma, humans (and many other mammals) do not generally alter their metabolic rate physiologically in response to disease, injury, or environmental stress. Many other species, however, have evolved hypometabolic mechanisms to adapt to environmental stresses such as extreme temperatures and low oxygen levels. A large number of birds and mammals hibernate to conserve energy during winter months. The western jumping mouse can hibernate for upto 9 months of the year, and edible dormice can hibernate upto 11 months in years they are non-reproductive (Cranford, 1983; Hoelzl et al., 2015). During hibernation, animals suspend feeding, locomotion and reproduction. This is achieved by lowering metabolic rate leading to slow breathing, decreased heart rate and lower core temperature. Seasonal hibernation can thus help animals reduce energy expenditure by as much as 85% (Dark, 2005). Some animals enter a state of torpor where they decrease energy utilization on shorter timescales – hours or days Hummingbirds can lower their core temperature and

metabolic rate during the night (Hargrove, 2005). Similarly, when oxygen availability is reduced, many animals adapt by lowering their metabolic rate. Worms, flies, zebrafish, turtles and even mammals, such as the naked mole-rat, are able to survive extreme oxygen deprivation (anoxia) by reducing metabolic demand (Ultsch and Jackson, 1982; Krishnan et al., 1997; Van Voorhies and Ward, 2000; Padilla and Roth, 2001; Park et al., 2017).

Humans too adapt to certain environmental stresses on the evolutionary timescale. For example, populations of people that live in high altitude regions such as Tibet have differentially expressed genes in order to efficiently utilize limited oxygen in that environment (Simonson et al., 2010). They have a normal metabolic rate despite severe arterial hypoxia, potentially representing an adaptation to extreme environments over thousands of years. The authors found genes in the hypoxia-inducible factor signaling pathway such as EGLN1, EPAS1 and PPARA were positively selected in Tibetan highlanders. Additionally, high altitude-dwelling Tibetans with lower levels of hemoglobin also have increased cardiac output and greater exercise capacity (Simonson et al., 2015).

These adaptive advantages are present in select human groups because they evolved over long timescales. Therefore, not all humans can adopt these strategies to survive environmental stresses on the order of days or months. Many mammals, however, including primates like the Madagascan lemur, can hibernate to adapt to environmental stressors (Dausmann et al., 2004). The western painted turtle survives oxygen deprivation and freezing temperatures, and has many differentially expressing genes in anoxia that have human orthologs such as the glucose transporter GLUT1 and an apolipoprotein encoding gene APOLD1, among others (Shaffer et al., 2013). Studying metabolic adaptations in these animals can thus be utilized to understand if similar

changes would benefit humans, who have similar genes and molecular pathways, when challenged with similar environmental stresses. Gaining a better understanding of adaptations to environmental stresses can also have potential benefits for acute injury-related challenges or chronic challenges such as space travel. In fact, NASA is studying torpor to allow humans to survive long journeys in space (www.nasa.gov).

Then nematode *Caenorhabditis elegans* (*C. elegans*) is a great animal model to study response and adaptation to changes in environment (described in detail below). In **chapter 3** of my dissertation, I will describe work in *C. elegans* to focus on severe oxygen deprivation (anoxia) as one such environmental stress. The goal of my project was to expand on previous data from our lab and specifically explain how neuropeptide signaling regulates the worm response to anoxia.

Oxygen deprivation in C. elegans – hypoxia vs. anoxia

C. elegans is a 1mm long, transparent nematode that normally dwells in soil and rotting vegetable matter. It was established as a powerful organism by Sydney Brenner a few decades ago (Brenner, 1974) and has since been used to study genetics, cell biology, circuit function and basic behaviors. It feeds on bacteria and is easily cultivated in the lab on agar plates seeded with a lawn of the *E. coli* strain OP50, although other strains of feeding bacteria are used depending on the study question. There are many advantages to studying worms as a model organism. *C. elegans* has a short life cycle – it grows from egg to adulthood in 3 days when raised at 20°C, and lives for 2-3 weeks, so experiments can be done relatively rapidly. It is primarily a self-fertilizing hermaphrodite and each animal can lay upto 300 eggs, allowing for high throughput experiments. It is transparent, allowing for ease of microscopy and use of fluorescent genetic markers. Its anatomy is well studied – it has exactly 959 somatic cells and 302

neurons and its neuronal wiring diagram is known. Its genome has been sequenced and a large number of *C. elegans* genes have homology with mammals and even humans (www.wormbook.org).

Since they live in the soil and rotting fruit, worms frequently encounter oxygen-depleted microenvironments in their natural habitat and can tolerate low oxygen well. They generally prefer 5-12% O_2 environments (Gray et al., 2004) but are able to survive hypoxia (<0.5 - 1% O_2) without altering feeding, locomotion or reproduction (Nystul et al., 2003; Miller and Roth, 2009). This requires the activation of the hypoxia induced transcription factor *hif-1* and its downstream gene targets. Their response to anoxia (<0.1% O_2), however, is markedly different and is *hif-1*-independent (Padilla et al., 2002; Ghose et al., 2013). In anoxia, animals enter a reversible state of 'suspended animation' in which they cease all movement, feeding and reproduction. Larvae and young adults can tolerate 24 hours of suspended animation under anoxia at 20°C, but lethality increases with increasing temperature and increasing duration of anoxia exposure (Scott et al., 2002; Mendenhall et al., 2006a). Those that survive extended anoxia have striking morphological defects and tissue damage (Scott et al., 2002). There are fewer neurons, suggesting cell death; there is a pronounced cell-swelling phenotype in the pharynx and across the body wall muscle, reminiscent of necrosis; there is an axonal beading phenotype indicating damage to neuronal integrity; nearly all myocytes display nuclear fragmentation.

Besides temperature and duration of insult, other factors contributing to anoxia tolerance are developmental stage, diet, genotype and growth conditions (solid agar or liquid culture). Of the developmental stages, dauer larvae and embryos are the most tolerant to anoxia (Padilla et al., 2002). Exposure to anoxia for shorter durations (upto 4 hours), also known as anoxic preconditioning, can induce changes in gene expression

that provide a protective advantage against long term anoxia. This can occur via changes in the apoptosis pathway (*ced-4*) (Dasgupta et al., 2007) and induction of the unfolded protein response pathway (Mao and Crowder, 2010). While subpopulations of worms can survive long-term anoxia under certain conditions, it does lead to cellular injury such as loss of tissue integrity, presence of large vaucoles, distortion of the pharynx and necrotic-like cell swelling (Mendenhall et al., 2006b; Sun et al., 2014).

A note about techniques: Worm biology under anoxic conditions is a relatively young field of investigation, and it is essential to point out that studies do not have a consistent method of inducing anoxia. Some studies (including ours) take advantage of commercially available biobags that generate an anoxic environment in a sealed bag via catalytic consumption of O_2 in the bag. Others use gloveboxes or chambers that are outfitted to carefully control O_2 concentration inside them by replacing it with N_2. Some studies are done on solid agar culture plates and others are done in liquid media. Some are conducted at standard laboratory 20°C and others are conducted at a range of different temperatures (25-28°C). Finally, some are conducted in embryos, others at larval stages of development and yet others in adult worms. As a result of these variations in different labs for studying anoxia, there are many differences in similar outcome measures such as percent survival. Some basic observations that have been repeated by different groups have been consistent across these different techniques, but there is not enough data to know if every observation is similarly consistent. However, it is important to remain mindful of these technical differences when evaluating the results of each study.

Metabolic regulation of anoxia

Under anoxic stress, worms enter a state of suspended animation and resume regular physiological activity when re-exposed to normoxic environments. Early studies of worms in anoxia established that their metabolic rate during anoxia falls to as low as 5% of normoxic levels (Van Voorhies and Ward, 2000). Presumably, this low-activity, hypometabolic state is an adaptive mechanism to preserve precious energy stores since reproduction, locomotion and tissue maintenance are energetically costly. Adult worms will cease all movement and appear 'stunned' within 8 hours of anoxia onset (Mendenhall et al., 2006b). While there are certain mutants (*daf-2*, the insulin receptor homolog in worms) that take longer to suspend activity, no studied mutant is capable of avoiding it completely.

Studies in adult worms show that under anoxia, animals switch their primary energy source from fat to glycogen/glucose stores. Over a 24h anoxic exposure, animals can lose up to 2/3rds of their carbohydrate reserve (Föll et al., 1999). In anoxia-tolerant mutant (*daf-2*) worms, there is an increase in fat and glycogen production in intestinal and hypodermal cells (Kimura et al., 1997). Worm homologs of the glycoytic enzyme glyceraldehyde-3 phosphate dehydrogenase isoforms, *gpd-2* and *gpd-3*, are also involved in anoxia tolerance (Mendenhall et al., 2006b). RNAi against these genes led to impaired worms after anoxia. However, these results were confounded because other glycolytic genes did not similarly induce anoxia sensitivity.

ATP production, as expected, is also markedly reduced during anoxia (Föll et al., 1999; Padilla et al., 2002). AMP-kinase (AMPK) is the master cellular sensor of the ATP/AMP ratio. AMPK is generally activated when cellular ATP levels fall, and its activity leads to an increase in energy producing anabolic processes and a decrease in energy consuming catabolic processes. Given that ATP levels decline during anoxia, it is

perhaps to be expected that AMPK activation might be upregulated to allow cells to cope with this energy deficit appropriately. Worms lacking *aak-2*, the *C. elegans* homolog for the catalytic alpha subunit of AMPK, are in fact hypersensitive to oxygen deprivation (LaRue and Padilla, 2011). They have decreased survival and those that survive have defects in motility and/or tissue morphology. Additionally, in one anoxia-tolerant mutant (*daf-2*), there is a decrease in stored carbohydrate consumption compared to wild type worms, and this is partially dependent on AMPK. These data suggest that carbohydrate availability can impact worm tolerance of anoxia, and AMPK has a role to play in regulating its consumption.

Mitochondrial changes in anoxia

As the primary site for aerobic ATP synthesis in the cell, it is not surprising that mitochondria are sensitive to changes in available oxygen. In *C. elegans* at the L4 stage of development, 24 hours anoxia induces a stress response wherein neuronal mitochondria undergo fission, and upon reoxygenation, they refuse and restore their original shape and size (Ghose et al., 2013). This study shows that, in the suspended animation state, worms prefer smaller, fewer mitochondria, perhaps as a mechanism for limiting reactive oxygen species production. Oxidative stress is, however, induced in anoxia, and successful recovery from anoxia requires activity of the oxidative factor SKN-1/Nrf to allow proper refusion.

In other studies conducted in adults and in liquid media, mitochondrial damage is observed after just 12h of extreme oxygen deprivation (<0.2% O_2). They have severely altered morphology, compromised membrane potential as well as accumulation of protein aggregates (Kaufman and Crowder, 2015). Consistent with protein accumulation, the authors found that the mitochondrial unfolded protein response (UPR^{mt}), was

induced in these animals even as early as 4h anoxia, when animals recovered completely. This indicated that proteostasis in the mitochondria is extremely sensitive to changes in ambient oxygen. When UPR^{mt} was experimentally induced by doxycycline or constitutive activation of *atfs-1* (the transcription factor required for UPR^{mt}), animals were protected from anoxic insult as measured by healthier mitochondria, decreased protein accumulation in the mitochondria and improved survival. Additionally, upregulated UPR^{mt} was protective both before and after the anoxic insult, suggesting a potential priming mechanism to protect mitochondria and improve whole animal survival from anoxic stress.

An independent study from a different group also found that *atfs-1* loss of function mutants were sensitive to 20h anoxia in day 1 adults, and that gain of *atfs-1* function was protective as measured by improved survival and decreased axonal damage (Peña et al., 2016). However, inducing *atfs-1* specifically in the nervous system was able to rescue neurons from damage but was not able to improve survival of the animal, suggesting either a cell non-autonomous mechanism for *atfs-1* or a requirement of *atfs-1* function in different tissues for protection of *C. elegans* against anoxia.

Cell non-autonomous features of anoxia in C. elegans

Oxygen deprivation is known to cause cell death via cell autonomous and non-autonomous pathways. Cell autonomous mechanisms lead to induction of apopotosis and necrosis in injured cells (Lipton, 1999). Cell non-autonomous pathways are less well understood, but lead to injury of secondary cells due to signals released from primary injured cells. Release of glutamate and potassium from neurons exposed to hypoxia causes cell death in surrounding neurons (Choi and Rothman, 1990; Broughton et al., 2009). Another example is that in focal ischemic stroke, there is neuronal death in the

area of the brain most exposed to the hypoxic insult, but the ischemic penumbra region surrounding the stroke is initially viable but experiences delayed cell death presumably due to non cell-autonomous factors from the directly injured neurons (Lo, 2008). As mentioned above, necrotic-like cell death is observed in worms after an anoxic insult (young adult worms exposed to 21h of ≤0.3% O_2 in liquid culture) (Sun et al., 2014). To study cell autonomous vs non-autonomous cell death in worms after anoxia, these authors generated transgenic worm lines that were protected from anoxic death due to a loss of function mutation in the gene *rars-1* (discussed in detail below), with *rars-1* rescue in specific cell types – either pharyngeal myocytes or GABA-ergic neurons. In this manner, just these specific cells were vulnerable to oxygen deprivation while the rest of the cells in the organism remained protected. They found that these lines had necrotic-like swelling and damage in cells of the pharynx and the tail, suggesting a cell non-autonomous mechanism for cellular injury after anoxia. They also found that loss of function in *daf-2*, the insulin-like receptor gene in worms, rescued the pharyngeal cell-swelling phenotype observed after anoxia, and that this required the *C. elegans* FOXO transcription factor homolog *daf-16*. Interestingly, *daf-16* expression in the intestine was able to rescue survival as well as pharyngeal cell-swelling after extreme oxygen deprivation, further suggesting a cell-autonomous regulation of anoxia-related injury and response.

Worms have distinct sets of sensory neurons in their head to detect changes in ambient oxygen. Upshifts in oxygen are detected by URX, AQR and PQR neurons, while downshifts in oxygen are detected by the BAG neuron. Indeed, worms deficient in BAG neurons due to a genetic ablation are unable to sense downshifts in oxygen (Zimmer et al., 2009). Interestingly, loss of BAG neurons confers a survival advantage in L4-staged worms exposed to anoxia (Flibotte et al., 2014). These worms, similar to wild type

animals, enter suspended animation when exposed to anoxia, but have a much higher rate of survival after 24 hours of normoxic recovery compared to wild type animals. While 45% of wild type animals survived 48 hours of anoxia, 67% of BAG-deficient animals survived the same insult (Flibotte et al., 2014). A single neuron is therefore capable of regulating whole animal survival when exposed to anoxia, providing yet another example of a cell non-autonomous mechanism for regulating the response to anoxia.

Gene regulation of anoxia tolerance

In order for cell non-autonomous regulation of anoxic stress to occur, molecular communication between cells is necessary. Neuropeptides are soluble factors widely used in *C. elegans* to communicate between cells and tissues. Our lab recently identified that loss of function mutations in *egl-3* and *unc-31* protected developing *C. elegans* from anoxia (Flibotte et al., 2014). The *egl-3* gene encodes a proprotein convertase required for the processing and maturation of neuropeptides (Thacker and Rose, 2000; Kass et al., 2001), and the *unc-31* gene encodes the *C. elegans* homolog of the calcium-dependent activator protein for secretion (CAPS), required for the secretion of neuropeptide-carrying dense core vesicles (Speese et al., 2007). Hence, loss of neuropeptide signaling is protective against anoxic stress in developing worms. However, neuropeptide signaling is not necessary in BAG neurons for this protective phenotype. Restoring *egl-3* function specifically in BAG neurons in an *egl-3* null background does not rescue *egl-3*-mediated increase in anoxic survival (Flibotte et al., 2014). It remains to be understood which cells neuropeptides might act in for this benefit, what mechanistic pathways neuropeptides employ to regulate the response to anoxia and which specific neuropeptides might be involved in extending survival under the

hypometabolic conditions induced by anoxia. This is the primary focus in chapter 3 of this dissertation.

Other genes known to affect *C. elegans* survival against anoxia are enumerated below. In chapter 3, we explore the contribution of some of these genes to neuropeptide-mediated regulation of anoxia survival.

daf-2: Loss of function mutants in *daf-2*, the worm homolog of the insulin-like growth factor 1 receptor, leads to strong anoxia resistance in worms. Mutants are able to survive anoxia (upto 72h) induced in different ways significantly better than wild type worms (Scott et al., 2002; Mendenhall et al., 2006a). This survival benefit is dependent on the activation of the transcription factor *daf-16*, the worm homolog of FOXO, downstream of *daf-2*. Further, *daf-2* expression in muscle and neurons, but not intestine, is necessary for this effect. These data implicate the insulin signaling pathway in regulating worm response to anoxia.

rars-1: Loss of function mutations in *rars-1*, the worm homolog of the arginyl aminoacyl t-RNA synthetase (AAR), significantly protects worms from anoxia-induced death (Anderson et al., 2009). This effect was partially due to induction of the unfolded protein response. Interestingly, RNAi against almost all other AARs in *C. elegans* also protected them from anoxia, suggesting that translational repression plays a critical role in surviving this stress.

nsy-1: Loss of function mutation in *nsy-1*, the worm homolog for apoptosis signal-regulating kinase (ASK), protects worms against long-term anoxia (Hayakawa et al., 2011). *nsy-1* encodes a MAP kinase kinase kinase (MAP3K), and its activity is required in many cell types such as hypodermis, neurons and intestine, for this phenotype. Additionally, a loss of function mutation in MAP2K (*sek-1*) also improved survival under

anoxic conditions. Together, these data point to a global role for the MAPK pathway in regulating anoxia in worms.

hyl-2: Loss of *hyl-2*, the worm homolog for a ceramide synthase, makes worms hypersensitive to anoxia (Menuz et al., 2009). In worms, *hyl-2* encodes a ceramide synthase responsible for the *de novo* synthesis of specific ceramides ranging 20-22 carbon chain lengths. *hyl-2* does not depend on apoptosis to induce anoxia sensitivity in worms, and loss of *daf-2* in *hyl-2* mutants partially rescues survival of animals suggesting a potential interaction between these two genes in anoxia survival. Overall, these data suggest that synthesis of specific ceramides contributes to the worm response against anoxic insult.

skn-1: Loss of function in *skn-1*, the worm ortholog for nuclear factor-erythroid-related factor (Nrf), prevents recovery of worms after anoxic exposure (Ghose et al., 2013). Compared to wild type worms, *skn-1* mutants had significantly impaired recovery of movement 10 minutes post-anoxia (24h exposure). This lack of behavioral recovery correlated with increased mitochondrial fission in *skn-1* mutant worms, suggesting a role for *skn-1*-mediated response to oxidative stress in regulating mitochondrial dynamics under anoxic stress.

Overall, I will explore the bidirectional role of metabolism in disease and stress by a) studying how manipulating organismal metabolism can alter progression of a neurodegenerative disease (ALS) (**Chapter 2**) and b) studying the contribution of neuropeptide signaling in a physiological state driven by metabolic adaptation to an environmental stress (anoxia) (**Chapter 3**).

CHAPTER 2

Genetic induction of hypometabolism by ablation of MC4R does not suppress ALS-like phenotypes in the G93A mutant SOD1 mouse model

Shachee Doshi[1,2], Preetika Gupta[1,2], Robert G. Kalb[1,2,3]

[1]Division of Neurology, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA 19104,
[2]Neuroscience Graduate Group and [3]Department of Neurology, University of Pennsylvania, Philadelphia, PA 19104

Scientific Reports (in press)

ACKNOWLEDGEMENTS

We would like to express deep gratitude to Dr. Maria Lim for help with grip strength and CLAMS experiments and Dr. Lei Zhang for help with mouse husbandry. We would also like to thank Dr. Virginia Lee, Dr. David Raizen, Dr. Thomas Jongens and Dr. Amita Sehgal for valuable ongoing feedback on the project. This work is supported by NIH grants NS087077 and NS052325 to RGK.

Abstract

Dysfunction and death of motor neurons leads to progressive paralysis in Amyotrophic lateral sclerosis (ALS). Recent studies have reported organism-level metabolic dysfunction as a prominent, but poorly understood feature of the disease. ALS patients are hypermetabolic with increased resting energy expenditure, but if and how hypermetabolism contributes to disease pathology is unknown. We asked if decreasing metabolism in the mutant superoxide dismutase 1 (SOD1) mouse model of ALS (G93A SOD1) would alter motor function and survival. To address this, we generated mice with the G93A SOD1 mutation that also lacked the melanocortin-4 receptor (MC4R). MC4R is a critical regulator of energy homeostasis and food intake in the hypothalamus. Loss of MC4R is known to induce hyperphagia and hypometabolism in mice. In the MC4R null background, G93A SOD1 mice become markedly hypometabolic, overweight and less active. Decreased metabolic rate, however, did not reverse any ALS-related disease phenotypes such as motor dysfunction or decreased lifespan. While hypermetabolism remains an intriguing target for intervention in ALS patients and disease models, our data indicate that the melanocortin system is not a good target for manipulation. Investigating other pathways may reveal optimal targets for addressing metabolic dysfunction in ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease characterized by loss of upper and lower motor neurons leading to paralysis and death (Ferraiuolo et al., 2011; Hardiman et al., 2011; Kiernan et al., 2011; Taylor et al., 2016). Work from a variety of models implicates dysfunctional RNA metabolism, protein homeostasis, altered mitochondrial function and oxidative stress in disease pathophysiology (Ferraiuolo et al., 2011; Robberecht and Philips, 2013; Taylor et al., 2016). In addition to cell-autonomous factors (i.e., the accumulation of toxic misfolded proteins in motor neurons), there is compelling evidence for non-cell autonomous processes contributing to disease progression, such as the participation of microglia and astrocytes (Wang et al., 2010; O'Rourke et al., 2016). At present these insights into ALS have not translated into drugs that substantively influence the course of disease.

Studies of patients with ALS have provided intriguing evidence for whole organism metabolic derangements. For example, glucose intolerance is present in 33% of sporadic ALS patients vs. 9.5% in controls (Pradat et al., 2010), hypermetabolism is present in 50-60% of ALS patients (Funalot et al., 2009b) and dyslipidemia, measured by increased LDL/HDL ratio, is present in 45.4% of ALS patients vs 16.1% in controls (Dupuis et al., 2008). ALS patients have lower body mass index (BMI) and lower lean body mass compared to healthy controls (Ahmed et al., 2014), and many of these perturbations present early in disease and are progressive. There is an inverse correlation between premorbid BMI and risk of ALS; in overweight or obese individuals, the risk of ALS was reduced 30-40% (O'Reilly et al., 2013). Mouse models of ALS, based on known familial mutations such as SOD1, TDP43, FUS and C9ORF72, similarly show energetic abnormalities including mitochondrial dysfunction, decreased body weight and hypermetabolism (Mattiazzi, 2002; Dupuis et al., 2004; Chiang et al., 2010;

Stribl et al., 2014; Perera and Turner, 2015). ALS patients fed a high calorie, high carbohydrate diet showed fewer adverse events, delayed weight loss and longer lifespan than those fed a control diet (Wills et al., 2014b). Various types of high calorie diets have been shown to provide benefits to the mutant SOD mouse model of the disease as well (Dupuis et al., 2004; Zhao et al., 2006; Ari et al., 2014). Together, these observations provide evidence that organismal metabolism is likely to contribute to the pathogenesis of ALS.

One approach to investigating the contribution of hypermetabolism (an increase in resting energy expenditure) to ALS (Bouteloup et al., 2009; Funalot et al., 2009b) is to genetically decrease metabolic rate in mouse models of disease. For example, placing the G93A SOD1 mice on a leptin deficient background (G93A;$ob^{+/-}$) lowers organism level metabolism (Lim et al., 2014). These mice gain more weight, became hypometabolic, have improved motor function as well as longer lifespan compared to G93A SOD1 mice alone. However, this study has several inherent limitations. For technical reasons, it was not possible to study G93A;ob/ob animals and thus the degree of hypometabolism achieved was rather modest. In addition beyond its role in energy homeostasis (by controlling feeding and promoting satiety) leptin has myriad additional actions, including effects on neuronal development (Ahima et al., 1999; Figlewicz et al., 2006; Van Doorn et al., 2017). These limitations led us to consider if a different genetic manipulation that led to a more pronounced hypometabolic phenotype, and obviated the complexities of leptin deficiency, might lead to a greater improvement in the G93A SOD1 model.

To this end, we crossed G93A SOD1 mice, henceforth referred to as G93A, to those lacking the melanocortin-4 receptor (MC4R), henceforth referred to as $MC4R^{-/-}$, generating double mutant G93A;$MC4R^{-/-}$ mice (see methods and Fig. 2.1a for breeding

strategy). We chose this gene since MC4R null mice are shown to be hypometabolic and hyperphagic (Huszar et al., 1997; Ste Marie et al., 2000). Additionally, MC4R is a good candidate because of its central role in energy homeostasis (Krashes et al., 2016). It is expressed in the anterior bed nucleus of the stria terminalis, paraventricular nucleus of the hypothalamus and lateral hypothalamus and regulates food intake, metabolic rate and body weight. Mice lacking this receptor cannot promote energy expenditure, leading to hypometabolism, hyperphagia and weight gain. Our goal was to genetically induce hypometabolism in G93A mice by placing them in the MC4R null background, thus altering a central component of energy balance, and test the effect of this specific manipulation on metabolism, disease progression and survival.

Results

G93A mice lacking the melanocortin-4 receptor are obese, hyperphagic and lethargic

Body weight. Beginning at P40, WT mice (C57Bl/6) of both sexes gained weight over the subsequent 40 weeks (Fig. 2.1b, c). $MC4R^{-/-}$ mice also gained weight over the same period, however, they were heavier than WT mice at every recorded time point. For example, at week 10 of measurements (P110), female and male WT mice weighed an average of 28.3g and 34.9g respectively, while $MC4R^{-/-}$ mice weighed 58.6g and 57.9g respectively. G93A mice of both sexes also gained the same amount of weight as WT mice until about P103 (9-11 weeks after P40), after which they progressively lost weight until they were moribund. G93A;$MC4R^{-/-}$ mice of both sexes gained the same amount of

weight as MC4R$^{-/-}$ mice until about P124 (11-12 weeks after P40), after which they progressively lost weight until they were moribund. At P110, female and male G93A mice weighed an average of 24.9g and 29.2g respectively, while G93A;MC4R$^{-/-}$ mice weighed 55g and 53.4g respectively. These results indicate that, as previously reported (Huszar et al., 1997; Balthasar et al., 2005), MC4R$^{-/-}$ mice gain significantly more weight compared to WT mice as they age, and G93A mice too gain significant weight when MC4R function is ablated, in comparison to WT and G93A animals.

Feeding. Next, we used the comprehensive lab animal monitoring system (CLAMS, see methods) to study food consumption in these mice over a 24h period at P60, P90 and P120 (Fig. 2.1d, e). When all four genotypes (WT, G93A, MC4R$^{-/-}$, G93A;MC4R$^{-/-}$) were compared, group differences were found by one-way ANOVA for females (P60: $F_{(3,25)}$ = 4.476, p = 0.012; P90: $F_{(3,21)}$ = 5.924, p = 0.0043; P120: $F_{(3,21)}$ = 1.56, p = 0.2287) and males (P60: $F_{(3,19)}$ = 1.311, p = 0.2998; P90: $F_{(3,22)}$ = 3.579, p = 0.0302; P120: $F_{(3,20)}$ = 1.552, p = 0.232). Female WT mice consumed 3-4g of food over 24h at P60, P90 and P120, while male WT mice consumed 4-5g of food at the same times. Female and male MC4R$^{-/-}$ mice, in comparison, consumed more food than WT mice (4-5g and 6-7g respectively) at these time points but this did not achieve significance in *post hoc* analyses. Female and male G93A mice had similar amounts of food consumption compared to WT mice (3-4g and 4-5g respectively), and female and male G93A;MC4R$^{-/-}$ mice had similar amounts of food consumption compared to MC4R$^{-/-}$ mice (5g and 5-6g respectively). Importantly, female G93A;MC4R$^{-/-}$ mice had significantly greater food consumption than G93A mice at P60 and P90 (*post hoc*, $p<0.05$ at P60 and $p<0.01$ at P90), but not at P120. Male G93A;MC4R$^{-/-}$ mice consumed more food than G93A mice at all age points, but this did not reach statistical significance. These results demonstrate

that WT and G93A mice have similar daily food consumption, and MC4R$^{-/-}$ and G93A;MC4R$^{-/-}$ mice have similar daily food consumption that is greater than WT and G93A mice.

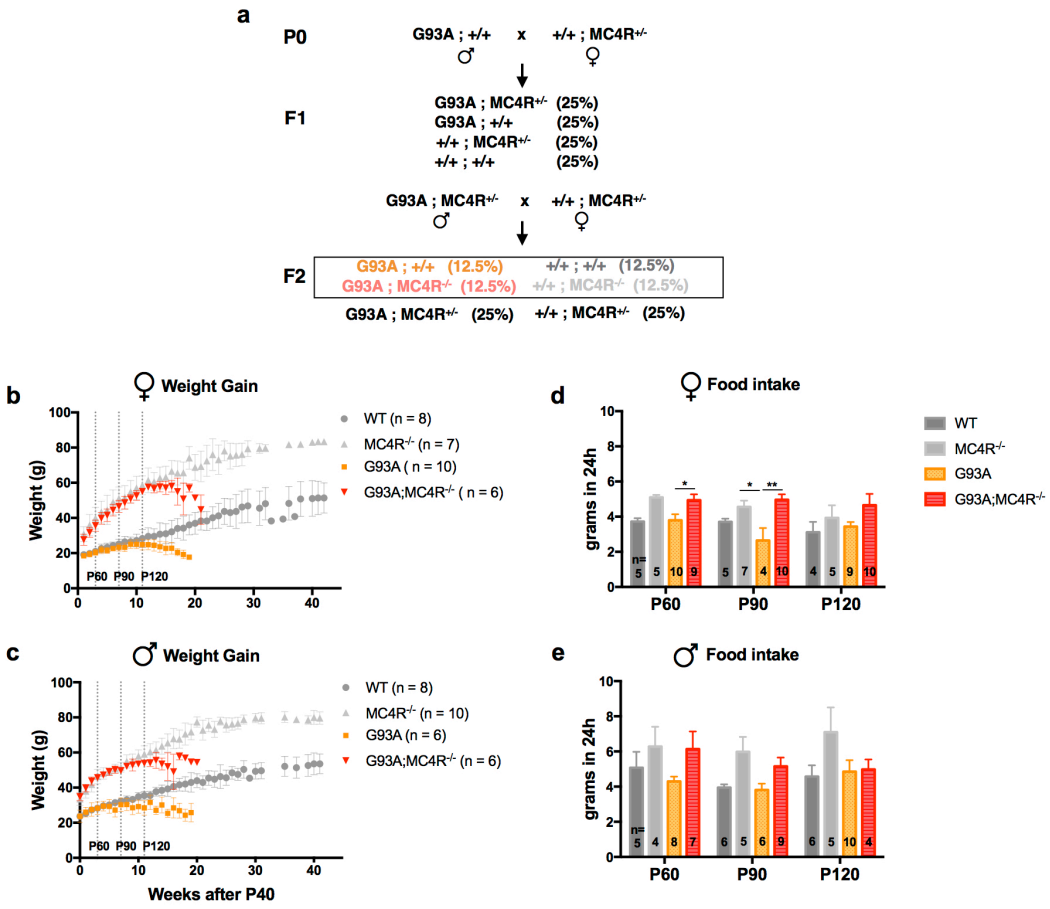


Figure 2.1. Breeding strategy, body weight and food intake in study mice.
(a) Two step breeding strategy for generating study mice. Parentheses indicate the expected genotype frequencies of the progeny in each cross. The relevant animals used in this study are represented inside the box in the F2 generation. Body weight measurements in female (b) and male (c) WT, G93A, MC4R-/- and G93A;MC4R-/- mice, recorded weekly after P40. The vertical dashed lines represent P60, P90 and P120 when measurements of food intake, ambulatory activity, metabolic rate and motor function were made. Food intake measurements over a 24h period at P60, P90 and P120 in female (d) and male (e) mice. Significance measured by one-way ANOVA among genotypes at each time point, followed by Tukey's test for multiple comparisons: p<0.05 (*), p<0.01 (**), p,0.001 (***), p<0.0001 (****).

Ambulatory activity. We also used the CLAMS to look at ambulatory (non-grooming) activity of these mice over 24h at P60, P90 and P120 (Fig. 2.2a, b). Three parameters

were measured at each age: total activity over 24h, activity during the 12h light period and activity during the 12h dark period. Group differences within the 4 genotypes (WT, G93A, MC4R$^{-/-}$ and G93A;MC4R$^{-/-}$) were determined using one-way ANOVA at each age, followed by *post hoc* analysis with Tukey's test for multiple comparisons.

a) Total Activity. Group differences were found in total activity over 24h at P60, P90 and P120 for females (P60: $F_{(3,26)} = 9.546$, p=0.0002; P90: $F_{(3,24)} = 36.55$, p<0.0001; P120: $F_{(3,23)} = 34.36$, p<0.0001) and males (P60: $F_{(3,20)} = 7.346$, p=0.017; P90: $F_{(3,22)} = 15.31$, p<0.0001; P120: $F_{(3,21)} = 8.289$, p=0.0008). MC4R$^{-/-}$ mice of both sexes were significantly less active than WT mice at all three ages (*post hoc*, p<0.05). G93A mice of both sexes were as active as WT mice at all three ages. G93A;MC4R$^{-/-}$ mice of both sexes were as active as MC4R$^{-/-}$ mice, and were significantly less active compared to G93A mice at all three ages for females and at P90 and P120 for males (*post hoc*, females p<0.01, males p<0.05).

b) Light Period Activity. Group differences were found in activity of the mice during the 12h light period at P60, P90 and P120 in females (P60: $F_{(3,25)} = 5.328$, p=0.0056; P90: $F_{(3,22)} = 20.76$, p<0.0001; P120: $F_{(3,23)} = 19.32$, p<0.0001) and males (P60: $F_{(3,20)} = 7.939$, p=0.0011; P90: $F_{(3,22)} = 4.413$, p=0.0142; P120: $F_{(3,21)} = 19.51$, p<0.0001). MC4R$^{-/-}$ mice of both sexes were significantly less active than WT mice at all three ages for females and at P60 and P120 for males (*post hoc*, p<0.05). G93A mice of both sexes had similar activity compared to WT mice at all three ages. G93A;MC4R$^{-/-}$ mice of both sexes were as active as MC4R$^{-/-}$ mice, and were significantly less active compared to G93A mice at P90 and P120 (*post hoc*, females p<0.001, males p<0.05).

c) Dark Period Activity. Group differences were found in the activity of the mice during the 12h dark period at P60, P90 and P120 in females (P60: $F_{(3,25)} = 9$, p=0.0003; P90: $F_{(3,22)} = 28.5$, p<0.0001; P120: $F_{(3,23)} = 32.57$, p<0.0001) and males (P60: $F_{(3,20)} = 6.436$,

p=0.0032; P90: $F_{(3,22)}$ = 17.46, p<0.0001; P120: $F_{(3,20)}$ = 6.302, p=0.0035). MC4R$^{-/-}$ mice of both sexes were significantly less active than WT mice at all three ages (*post hoc*, p<0.05). G93A mice of both sexes had similar activity compared to WT mice at all three ages. G93A;MC4R$^{-/-}$ mice of both sexes were as active as MC4R$^{-/-}$ mice, and were significantly less active compared to G93A mice at P90 and P120 for females and at P90 for males (*post hoc*, females p<0.01, males p=0.0001).

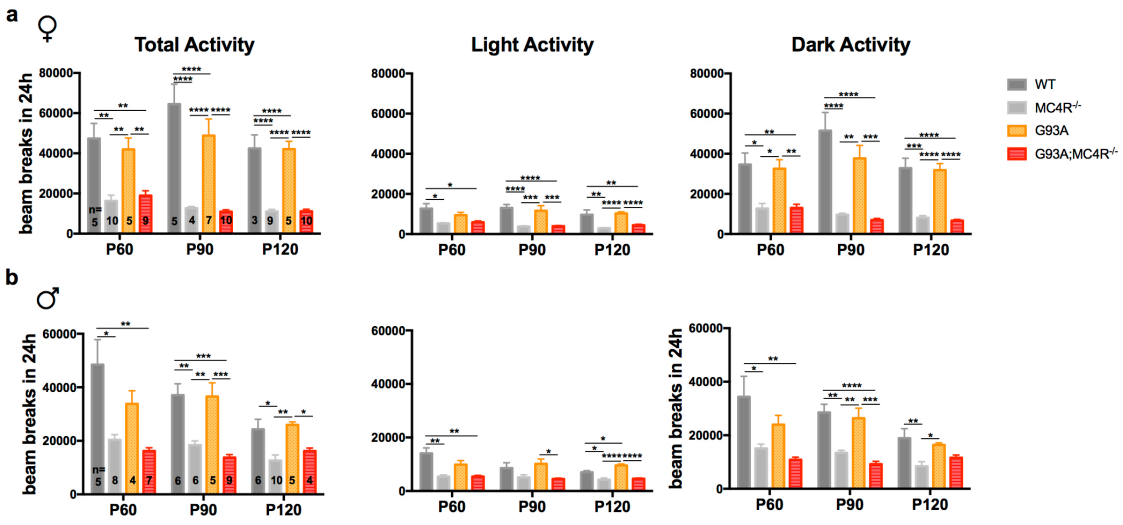


Figure 2.2. Ambulation in study mice.
Ambulatory activity measured by beam breaks recorded at P60, P90 and P120 in female (a) and male (b) mice. Significance measured by one-way ANOVA among genotypes at each time point, followed by Tukey's test for multiple comparisons: p<0.05 (*), p<0.01 (**), p,0.001 (***), p<0.0001 (****).

These data indicate that WT and G93A mice have similar activity levels at the measured time points both in the light and the dark cycles. During the same periods, MC4R$^{-/-}$ mice are significantly less active than WT mice and G93A;MC4R$^{-/-}$ mice have similar activity levels as MC4R$^{-/-}$ mice, significantly less than G93A mice alone.

In summary, G93A mice begin to lose weight compared to WT mice as they age. However, their food intake and activity levels are similar to WT mice at all recorded ages and even as they are losing weight, indicating a hypermetabolic phenotype. In all three measures - weight gain, food intake and activity level - G93A;MC4R$^{-/-}$ mice are indistinguishable from MC4R$^{-/-}$ mice. They are overweight, feed more and have greatly reduced activity compared to G93A mice, indicating that ablation of MC4R leads to unambiguous and significant phenotypic changes in G93A mice. Manipulating the activity of MC4R is thus a reasonable way to induce changes at the whole organism level in this mutant SOD1 background.

G93A mice lacking the melanocortin-4 receptor are hypometabolic

In order to determine metabolic flux in test mice over time, we measured average total oxygen consumption and carbon dioxide production per hour, per kg over a 24h period at the three different ages (P60, P90 and P120) using indirect calorimetry by the CLAMS (Fig. 2.3a, b). We also looked at the O_2 consumption and CO_2 production during the 12h light period (Fig. 2.4a, b) and 12h dark period (Fig. 2.5a, b) within the 24h recording. Group differences between the genotypes (WT, G93A, MC4R$^{-/-}$ and G93A;MC4R$^{-/-}$) were determined using one-way ANOVA at each age, followed by *post hoc* analysis with Tukey's test for multiple comparisons.

Total O_2 consumption (Fig. 2.3a). Group differences in O_2 consumption over 24h were found at P60, P90 and P120 for females (P60: $F_{(3,25)}$ = 5.259, p=0.0060; P90: $F_{(3,22)}$ =

13.61, p<0.0001; P120: $F_{(3,24)}$ = 17.07, p<0.0001) and males (P60: $F_{(3,20)}$ = 3.3916, p=0.0238; P90: $F_{(3,22)}$ = 2.434, p=0.0919; P120: $F_{(3,21)}$ = 9.607, p=0.0003). Female MC4R$^{-/-}$ mice had significantly less O_2 consumption than WT mice at P90 and P120 (*post hoc*, p<0.05), while male MC4R$^{-/-}$ mice had decreased O_2 consumption compared to WT mice at all three ages but this did not achieve significance. Female G93A mice had significantly more O_2 consumption than WT mice at P90 (*post hoc*, p<0.05), whereas male G93A mice had increased O_2 consumption compared to WT mice at P60 and P120 but this did not achieve significance. G93A;MC4R$^{-/-}$ mice of both sexes were similar compared to MC4R$^{-/-}$ mice, and had significantly less O_2 consumption compared to G93A mice at all three ages for females and at P60 and P120 for males (*post hoc*, females p<0.01, males p<0.05).

Total CO_2 production (Fig. 2.3b). Group differences in CO_2 production over 24h were found at P60, P90 and P120 for females (P60: $F_{(3,25)}$ = 4.258, p=0.0147; P90: $F_{(3,22)}$ = 10.67, p=0.0002; P120: $F_{(3,24)}$ = 15.85, p<0.0001) and males (P60: $F_{(3,20)}$ = 3.852, p=0.0252; P90: $F_{(3,22)}$ = 1.681, p=0.2002; P120: $F_{(3,21)}$ = 6.699, p=0.0024). Female MC4R$^{-/-}$ mice had significantly less CO_2 production than WT mice at P120 (*post hoc*, p<0.05), while male MC4R$^{-/-}$ mice had decreased CO_2 production than WT mice at all three ages but this was not statistically significant. Female G93A mice had significantly greater CO_2 production than WT mice at P90 (*post hoc*, p<0.05), whereas male G93A mice had increased CO_2 production compared to WT mice at P60 and P90 but this was not statistically significant. G93A;MC4R$^{-/-}$ mice of both sexes were similar compared to MC4R$^{-/-}$ mice, but had significantly less CO_2 production compared to G93A mice at all three ages for females and at P60 and P120 for males (*post hoc*, p<0.05 for females and males).

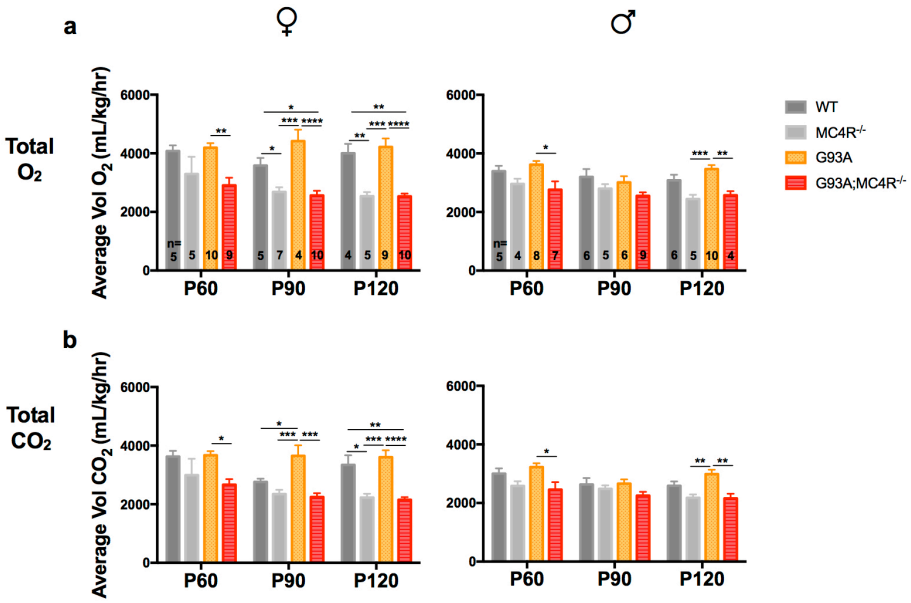


Figure 2.3. Oxygen consumption and carbon dioxide production over one day (24h) in study mice.
Total O_2 consumption (a) and CO_2 production (b) in female and male WT, G93A, MC4R$^{-/-}$ and G93A;MC4R$^{-/-}$ mice at P60, P90 and P120 measured in mL, per hour, per kg mass of the animal over 24 hours of recording, including light and dark cycles. Significance measured by one-way ANOVA among genotypes at each time point, followed by Tukey's test for multiple comparisons: p<0.05 (*), p<0.01 (**), p,0.001 (***), p<0.0001 (****).

Light Period O_2 consumption (Fig. 2.4a). Group differences in oxygen consumption during the12h light period were found at P60, P90 and P120 for females (P60: $F_{(3,25)}$ = 3.271, p=0.0378; P90: $F_{(3,22)}$ = 7.161, p=0.0016; P120: $F_{(3,24)}$ = 10.16, p=0.0002) and males (P60: $F_{(3,20)}$ = 1.16, p=0.3498; P90: $F_{(3,22)}$ = 0.747, p=0.5357; P120: $F_{(3,21)}$ = 5.867, p=0.0045). Female MC4R$^{-/-}$ mice had significantly less O_2 consumption than WT mice at P120 (*post hoc*, p<0.05), while males had decreased O_2 consumption compared to WT mice at P60 and P120, but this was not significant. Female G93A mice had significantly greater O_2 consumption than WT mice at P90 (*post hoc*, p<0.05), whereas male G93A mice had increased O_2 consumption compared to WT mice at P120, but this was not

significant. G93A;MC4R$^{-/-}$ mice of both sexes were similar compared to MC4R$^{-/-}$ mice, but had significantly less O_2 consumption compared to G93A mice at all three ages for females and at P120 for males (*post hoc*, females $p<0.05$, males $p<0.05$).

Light Period CO_2 production (Fig. 2.4b). Group differences in CO_2 production during the 12h light period were found at P60, P90 and P120 for females (P60: $F_{(3,25)} = 3.337$, $p=0.0354$; P90: $F_{(3,22)} = 5.136$, $p=0.0076$; P120: $F_{(3,24)} = 10.79$, $p=0.0001$) and males (P60: $F_{(3,20)} = 1.326$, $p=0.2939$; P90: $F_{(3,22)} = 0.4866$, $p=0.6951$; P120: $F_{(3,21)} = 5.126$, $p=0.0081$). MC4R$^{-/-}$ mice of both sexes had decreased CO_2 production than WT mice at P60 and P120 but this was not statistically significant. G93A mice had increased CO_2 production compared to WT mice at P90 and P120 in females and P120 in males but this was not statistically significant. G93A;MC4R$^{-/-}$ mice of both sexes were similar compared to MC4R$^{-/-}$ mice, but had significantly less CO_2 production compared to G93A mice at all three ages for females and at P120 for males (*post hoc*, $p<0.05$ for females and males).

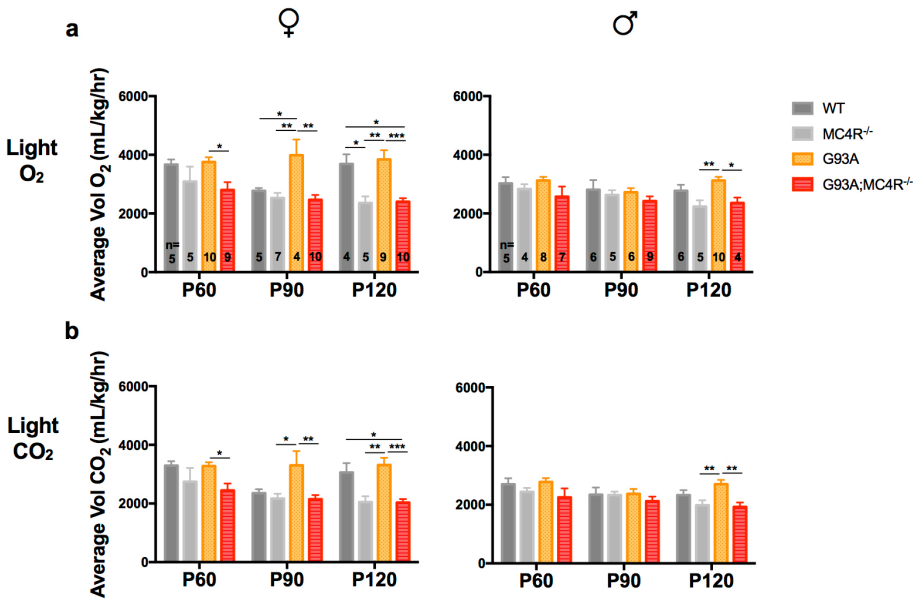


Figure 2.4. Oxygen consumption and carbon dioxide production during the 12h light cycle in study mice.
Light cycle O_2 consumption (a) and CO_2 production (b) in female and male WT, G93A, MC4R$^{-/-}$ and G93A;MC4R$^{-/-}$ mice at P60, P90 and P120 measured in mL, per hour, per kg mass of the animal over 12 hours of recording during the light cycle of the day. Significance measured by one-way ANOVA among genotypes at each time point, followed by Tukey's test for multiple comparisons: p<0.05 (*), p<0.01 (**), p,0.001 (***).

Dark Period O_2 consumption (Fig. 2.5a). Mice of all genotypes had increased O_2 consumption in the dark compared the light period, consistent with their increased nocturnal activity. Group differences in O_2 consumption during the 12h dark period were found at P60, P90 and P120 for females (P60: $F_{(3,25)}$ = 6.772, p=0.0017; P90: $F_{(3,22)}$ = 29.86, p<0.0001; P120: $F_{(3,24)}$ = 24.78, p<0.0001) and males (P60: $F_{(3,20)}$ = 9.646, p=0.0004; P90: $F_{(3,22)}$ = 9.709, p=0.0003; P120: $F_{(3,21)}$ = 11.98, p<0.0001). MC4R$^{-/-}$ mice of both sexes had significantly less O_2 consumption than WT mice, at P90 and P120 for females and at P120 for males (*post hoc*, females p<0.01, males p<0.05). Female G93A mice had significantly greater O_2 consumption than WT mice at P90 (*post hoc*, p<0.05), whereas male G93A mice had increased O_2 consumption compared to WT mice at P60

and P120, but this was not statistically significant. G93A;MC4R$^{-/-}$ mice of both sexes were similar compared to MC4R$^{-/-}$ mice, and had significantly less O_2 consumption compared to G93A mice at all three ages (*post hoc*, p<0.01).

Dark Period CO_2 production (Fig. 2.5b). Similar to O_2 consumption, mice of all genotypes had increased CO_2 production in the dark compared the light period, consistent with their increased nocturnal activity. Group differences in CO_2 production during the 12h dark period were found at P60, P90 and P120 for females (P60: $F_{(3,25)}$ = 4.646, p=0103; P90: $F_{(3,22)}$ = 20.56, p<0.0001; P120: $F_{(3,24)}$ = 19.56, p<0.0001) and males (P60: $F_{(3,20)}$ = 8.061, p=0.0010; P90: $F_{(3,22)}$ = 3.994, p=0.0206; P120: $F_{(3,21)}$ = 6.135, p=0.0037). Female MC4R$^{-/-}$ mice had significantly less CO_2 production than WT mice at P90 and P120 *(post hoc*, p<0.05), whereas male MC4R$^{-/-}$ mice had decreased CO_2 production compared to WT mice at all ages but this was not statistically significant. Female G93A mice had significantly increased CO_2 production compared to WT mice at P90 (*post hoc*, p<0.05), while male G93A mice had increased CO_2 production compared to WT mice at P60 and P120 but this was not statistically significant. G93A;MC4R$^{-/-}$ mice of both sexes were similar compared to MC4R$^{-/-}$ mice, but had significantly less CO_2 production compared to G93A mice at all three ages (*post hoc*, p<0.05 for females and males).

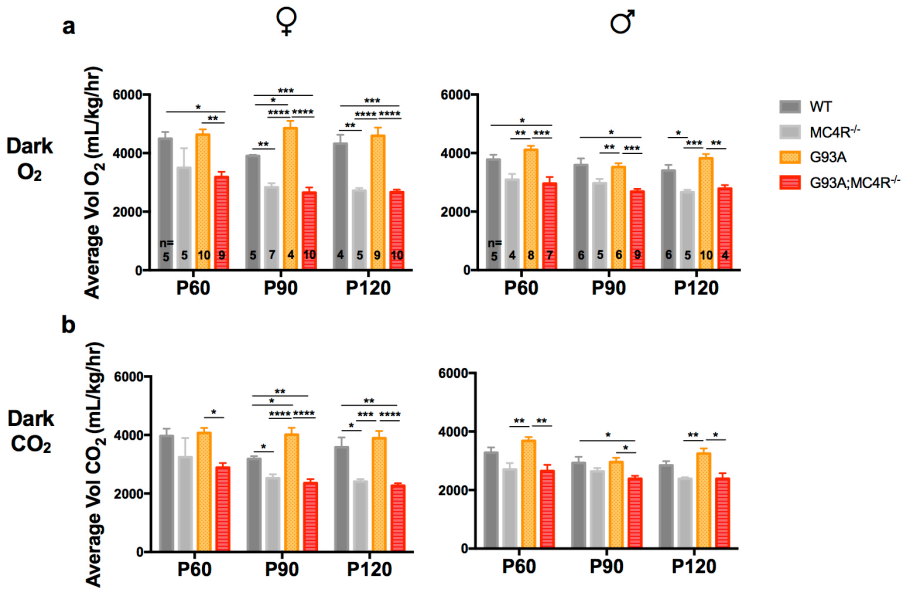


Figure 2.5. Oxygen consumption and carbon dioxide production during the 12h dark cycle in study mice.
Dark cycle O_2 consumption (a) and CO_2 production (b) in female and male WT, G93A, MC4R$^{-/-}$ and G93A;MC4R$^{-/-}$ mice at P60, P90 and P120 measured in mL, per hour, per kg mass of the animal over 12 hours of recording during the dark cycle of the day. Significance measured by one-way ANOVA among genotypes at each time point, followed by Tukey's test for multiple comparisons: p<0.05 (*), p<0.01 (**), p,0.001 (***), p<0.0001 (****).

Together, these data from indirect calorimetry suggest that compared to WT animals, G93A mice display a moderately higher metabolic rate and that MC4R$^{-/-}$ mice are hypometabolic. Interestingly, both the G93A hypermetabolism and the MC4R$^{-/-}$ hypometabolism is more striking in females than in males, and when the animals are in the dark. When G93A mice are placed in the MC4R$^{-/-}$ background, their metabolic profile is similar to MC4R$^{-/-}$ mice alone, for both males and females. G93A;MC4R$^{-/-}$ mice are significantly more hypometabolic compared to G93A mice in both dark and light periods. This suggests that MC4R ablation, in addition to affecting weight, food intake and activity, can also rescue the hypermetabolic phenotype in this mutant SOD1 model of ALS.

G93A mice lacking the melanocortin-4 receptor do not have improved motor function

Motor function of G93A mice deteriorates over time (Feeney et al., 2001). We asked if induction of hypometabolism blunted this age dependent phenotype. To this end, we measured forelimb and hindlimb grip strength in male and female study mice at P60, P90 and P120.

Forelimb Strength (Fig. 2.6a). Group differences in forelimb grip strength among genotypes (WT, MC4R$^{-/-}$, G93A, G93A;MC4R$^{-/-}$) were found using one-way ANOVA at each age point for females (P60: $F_{(3,27)} = 1.724$, p=0.1856; P90: $F_{(3,29)} = 12.77$, p<0.0001; P120: $F_{(3,30)} = 50.14$, p<0.0001) and males (P60: $F_{(3,24)} = 9.174$, p=0.0003; P90: $F_{(3,26)} = 15.69$, p<0.0001; P120: $F_{(3,29)} = 66.43$, p<0.0001).

MC4R$^{-/-}$ mice of both sexes had similar forelimb grip strength compared to WT mice at all three ages. G93A mice of both sexes had significantly decreased forelimb grip strength compared to WT mice, at P90 and P120 for females and at all three ages for males (*post hoc*, females p<0.001, males p<0.01). G93A;MC4R$^{-/-}$ mice of both sexes had significantly decreased forelimb grip strength than MC4R$^{-/-}$ mice at P90 and P120 (*post hoc*, females p<0.001, males p<0.01), and had similar forelimb grip strength compared to G93A mice at all three ages. The only exception was that female G93A;MC4R$^{-/-}$ mice had significantly improved grip strength compared to G93A mice at P120 (*post hoc*, p<0.05).

Hindlimb Strength (Fig. 2.6b). Group differences in hindlimb grip strength among genotypes (WT, MC4R$^{-/-}$, G93A, G93A;MC4R$^{-/-}$) were found using one-way ANOVA at each age point for females (P60: $F_{(3,27)}$ = 12.66, p<0.0001; P90: $F_{(3,29)}$ = 25.69, p<0.0001; P120: $F_{(3,30)}$ = 57.73, p<0.0001) and males (P60: $F_{(3,24)}$ = 7.494, p=0.0010; P90: $F_{(3,26)}$ = 26.7, p<0.0001; P120: $F_{(3,29)}$ = 68.35, p<0.0001).

MC4R$^{-/-}$ mice of both sexes had similar hindlimb grip strength compared to WT mice at all three ages, with one exception: male MC4R$^{-/-}$ mice had significantly increased hindlimb strength compared to WT mice at P120 (*post hoc*, p=0.0051). G93A mice of both sexes had progressively worsening hindlimb grip strength with age, and this was significantly decreased compared to WT mice at all three ages (*post hoc*, females p<0.001, males p<0.05). This difference was larger as the animals aged, at P90 and P120. G93A;MC4R$^{-/-}$ mice of both sexes had significantly decreased hindlimb grip strength than MC4R$^{-/-}$ mice all three ages (*post hoc*, p<0.05). Although female G93A;MC4R$^{-/-}$ mice had increased hindlimb grip strength compared to G93A mice at all ages, this did not achieve significance. Male G93A;MC4R$^{-/-}$ mice had similar hindlimb grip strength compared to G93A mice at all three ages.

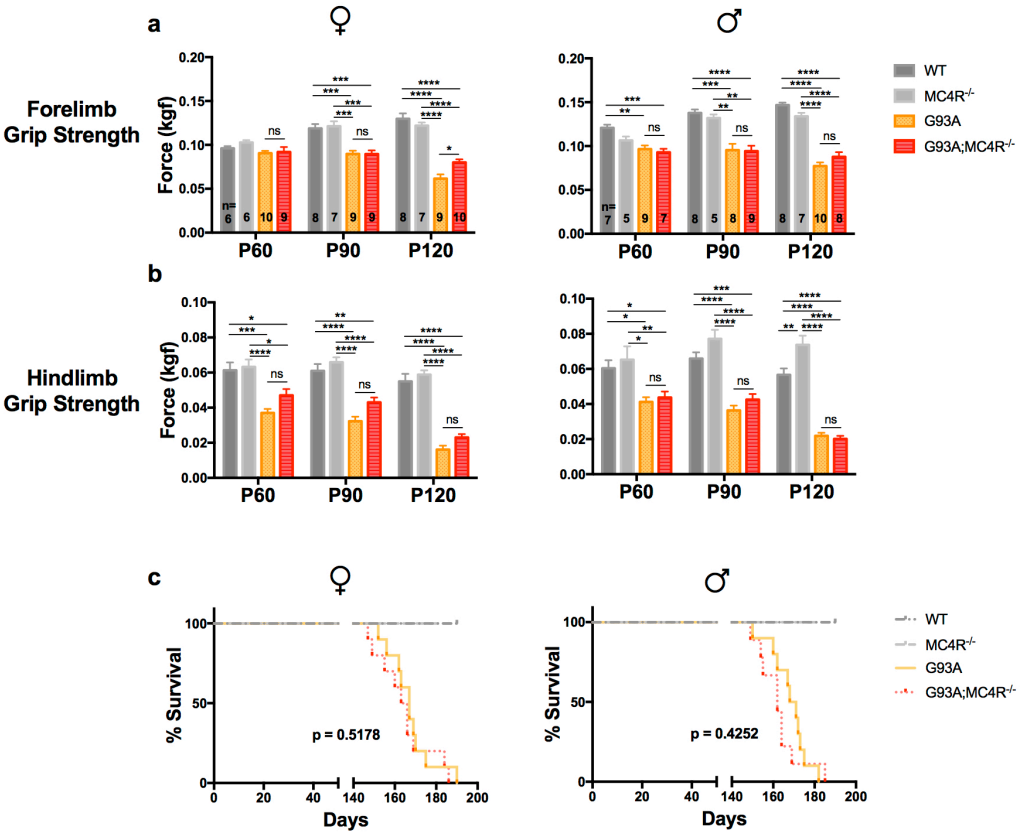


Figure 2.6. Motor function and survival of study mice.
Forelimb (a) and hindlimb (b) grip strength of female and male WT, G93A, MC4R$^{-/-}$ and G93A;MC4R$^{-/-}$ mice measured at P60, P90 and P120. Significance measured by one-way ANOVA among genotypes at each time point, followed by Tukey's test for multiple comparisons: p<0.05 (*), p<0.01 (**), p,0.001 (***), p<0.0001 (****). Kaplan-Meier survival plots (c) of female and male study mice. Significance was calculated using the log-rank (Mantel-Cox) test for survival curve comparison between G93A and G93A;MC4R$^{-/-}$ mice, and was set to p<0.05.

These experiments show that G93A mice have significantly less forelimb and hindlimb grip strength compared to WT mice, indicating progressively impaired motor function. Importantly, although G93A;MC4R$^{-/-}$ mice are similar to MC4R$^{-/-}$ mice in their hypometabolic phenotype, they are significantly worse than MC4R$^{-/-}$ mice in grip strength. In fact, male G93A;MC4R$^{-/-}$ mice are indistinguishable from G93A mice in grip

strength. In females, there is significantly improved forelimb strength at P120 in G93A;MC4R$^{-/-}$ mice compared with G93A mice, and there is a consistent trend in improved hindlimb strength at all time points. Therefore, MC4R ablation has no effect on the motor function of male G93A mice and has a modest effect in female G93A mice.

G93A mice lacking the melanocortin-4 receptor do not have extended lifespan

Finally we looked at the lifespan of our mice. WT and MC4R$^{-/-}$ mice had similar longevity and no animals of these genotypes died within 1 year of birth. In agreement with published work, we found male and female G93A mice had a severely shortened lifespan compared to WT mice (Gurney et al., 1994), with a median survival of 169.5 days and 167 days respectively (Fig. 2.6c). There was no significant difference in the lifespan of G93A;MC4R$^{-/-}$ mice compared to G93A mice. Median survival in these mice was 162 days and 164.5 days for males and females respectively (Fig. 2.6c). Although the double mutant G93A;MC4R$^{-/-}$ mice reach the pre-determined euthanasia criteria at the same time as G93A mice, they continue to have significantly higher body weight than the G93A mice at end stage (Fig. 2.1b, c). Together, these data suggest that ablation of melanocortin-4 receptor does not significantly prolong the life span of the G93A mouse.

The leptin-MC4R pathway is altered in mice lacking the melanocortin-4 receptor

In addition to their role in controlling food intake and organismal metabolism, peptide hormones that impinge upon the MC4R signaling pathway can display beneficial

neuronal activities. In its canonical role in regulating metabolism, leptin acts on two populations of neurons in the arcuate nucleus of the hypothalamus: the proopiomelanocortin (POMC)-producing neurons and the agouti-related peptide (AgRP)-producing neurons (Krashes et al., 2016). The POMC derived α-melanocyte stimulating hormone (α-MSH) is an agonist and AgRP is an antagonist of the MC4R. When calories are replete, α-MSH activates MC4R to promote satiety, feeding suppression, and weight loss by energy expenditure. When calories are depleted AgRP antagonizes MC4R to promote energy consumption and blunt energy expenditure. With regard to beneficial neuronal activities, α-MSH can promote cognitive recovery in a mouse model of Alzheimer's disease (Ma and McLaurin, 2014) and demonstrates general anti-inflammatory properties (Caruso et al., 2007; Brzoska et al., 2008). Similarly, higher circulating leptin is associated with decreased risk of Alzheimer's disease and protection against cognitive decline (Holden et al., 2009). Due to their varied actions, we asked if the expression of serum leptin and α-MSH might be altered in G93A and $MC4R^{-/-}$ animals in order to clarify the role of these peptides in our experiments.

We measured plasma concentrations of leptin and α-MSH in adult male WT, $MC4R^{-/-}$ and G93A mice. α-MSH levels were decreased in both $MC4R^{-/-}$ and G93A mice compared to WT (Fig. 2.7a), but this did not achieve significance ($p = 0.08$ for $MC4R^{-/-}$ and $p = 0.16$ for G93A). Leptin levels are moderately decreased in G93A mice (Fig 3.7b) and markedly increased in $MC4R^{-/-}$ mice compared to WT animals ($p < 0.0001$), consistent with the increased body weight and adiposity of these animals (Fig. 2.1). As seen in previous work (Dupuis et al., 2004; Lim et al., 2014), leptin levels were decreased in G93A mice compared to WT mice. The modest difference in α-MSH levels among the genotypes may indicate relatively little contribution of the extra-metabolic

effects of this hormone on neuronal health. The markedly elevated leptin levels in the absence of MC4R may indicate a healthful compensatory hormonal response, although if so, it does not improve motor function or extend life of the G93A mice. In light of the benefits of haploinsufficiency of leptin demonstrated by Lim *et al* (Lim et al., 2014), it is conceivable that increased leptin in the absence of MC4R actually mitigates any potential benefits of hypometabolism.

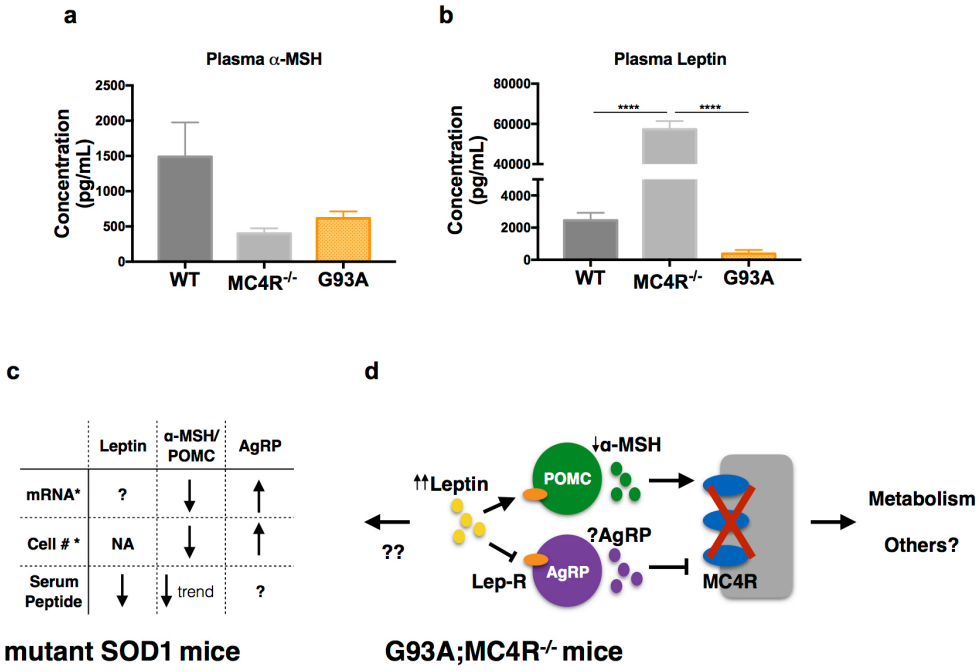


Figure 2.7. The leptin-MC4R pathway in G93A SOD1 mice.
Plasma α-MSH (a) and leptin (b) concentrations in adult male WT, MC4R$^{-/-}$ mice (n=3 for each genotype). Significance measured by one-way ANOVA among genotypes, followed by Tukey's test for multiple comparisons: p<0.0001 (****). (c) Summary of alterations in leptin, α-MSH/POMC cells and AgRP/AgRP cells in *G86R SOD1 mice (Vercruysse *et al*, 2016) and G93A SOD1 mice. (d) Speculative model of the leptin-MC4R pathway in G93A;MC4R$^{-/-}$ mice based on data from MC4R$^{-/-}$ mice.

Discussion

An increase in resting energy expenditure, or hypermetabolism, is a distinct organism-level feature of ALS patients and mouse models (Dupuis et al., 2004; Funalot et al., 2009a; Dupuis et al., 2011; Tefera and Borges, 2017). Our goal was to generate an ALS model mouse that was hypometabolic, and test the effect of this manipulation on key features of disease. In order to induce hypometabolism, we ablated MC4R function because it is a central regulator of energy metabolism. We successfully generated and studied mice that are null for MC4R and bear the ALS-linked G93A mutation in the SOD1 gene. We make two principal observations. First, the G93A;MC4R$^{-/-}$ animals are markedly hypometabolic as reported by weight gain and indirect calorimetry. Thus the G93A mutation does not impede the central regulation of metabolism via MC4R. Second, despite evoking hypometabolism in the G93A animals, the most important measures of disease (e.g., motor function and longevity) are unaffected. This argues that genetic blunting of the hypermetabolic phenotype by manipulation of MC4R is an ineffective intervention for slowing disease progression.

Different mechanisms of inducing hypometabolism in ALS

Prior studies in the G93A mouse model have shown that hypermetabolism does contribute to disease progression, and we highlight two of them here. In 2004, Dupuis *et al* studied male mice in two different ALS-related mutant SOD1 mouse models, G86R and G93A (Dupuis et al., 2004). They found that in both models, mice had lower body weight and increased energy expenditure by indirect calorimetry compared to age matched littermate controls. When the G86R mice were fed a high fat diet consisting of regular chow supplemented with 21% butterfat and 0.15% cholesterol, they gained

significantly more weight and had increased fat deposits than those fed regular chow alone. The high fat diet was associated with slowed motor neuron loss, slowed muscle denervation and a 20% improvement in mean lifespan. In 2014, Lim *et al* showed that the G93A mice were hypermetabolic and decreasing leptin levels (by placing them in a heterozgous $ob^{+/-}$ background), led to hypometabolism and weight gain in these mice (Lim et al., 2014). Leptin deficiency also led to improved hindlimb grip strength and led to a female-specific increase in median lifespan. These studies demonstrate that ameliorating the hypermetabolic phenotype, either by diet or by a specific genetic manipulation, can blunt the progression of ALS-like phenotypes in mutant SOD1 models of ALS.

What does the difference between our current results with MC4R and prior work by Dupuis *et al* and Lim *et al* tell us about the contribution of hypometabolism to ALS? To address this, it is worth considering leptin and its interaction with MC4R. Leptin is a circulating hormone that plays a prominent role in controlling food intake and satiety (Cone, 2005; Krashes et al., 2016). It is released by adipocytes and acts on specific neurons in the arcuate nucleus of the hypothalamus. These first order neurons then act on second order neurons in the lateral hypothalamus and the paraventricular nucleus of the hypothalamus. MC4R is expressed on the second order neurons that receive opposing inputs based on upstream leptin signaling. When calories are replete, leptin-mediated MC4R activation promotes satiety and energy expenditure. When calories are depleted, circulating leptin is reduced and MC4R is inhibited leading to food consumption and suppression of energy expenditure. On the surface, the leptin deficient G93A;$ob^{+/-}$ mice Lim *et al* studied and the G93A;$MC4R^{-/-}$ mice we studied are similar; they are both heavier than G93A mice and are hypometabolic. Despite this similarity, it is surprising that ablating MC4R, leptin's downstream target, does not confer the same

benefits on motor function as reduced leptin signaling does. One possibility is that the beneficial effects of reduced leptin signaling acts via MC4R independent pathways. Indeed, leptin receptors are expressed in extrahypothalamic neurons in the midbrain and the cerebellum and in peripheral tissue such as the liver (Cohen et al., 2005; Oldreive et al., 2008; Davis et al., 2011; Forero-Vivas and Hernández-Cruz, 2014) although the function of leptin signaling in extrahypothalamic tissues is not well understood. The reduction in circulating leptin that Lim *et al* achieved in the $ob^{+/-}$ background may point to important effects of leptin not captured by MC4R ablation. In light of this, we show here that mice lacking MC4R have a 20-30 fold increase in circulating leptin and perhaps this mitigates any potential benefit of hypometabolism induced by loss of MC4R.

The hypothalamic melanocortin pathway in ALS

Recent work indicates intrinsic abnormalities in hypothalamus in ALS. Gorges and colleagues find significant atrophy of the hypothalamus in sporadic and familial ALS patients (Gorges et al., 2017). Atrophy presents in presymptomatic stages in familial mutation carriers and is more severe in patients with lower BMI. This suggests that changes in the hypothalamus precede changes in metabolism in ALS patients. In a different study, Vercruysse and colleagues looked specifically at the hypothalamic melanocortin system in ALS patients and the G86R mutant SOD1 mouse model (Vercruysse et al., 2016). In the G86R SOD1 mice, there are significantly fewer first order, MC4R-activating (POMC-expressing) neurons and significantly more first order, MC4R-inhibiting (AgRP containing) neurons compared to WT mice. These differences were observed at pre-symptomatic stages and suggest that signaling in the melanocortin system is downregulated in this ALS model. We too find changes in the MC4R signaling pathway (e.g., leptin and α-MSH are decreased in G93A mice, Fig. 2.7a and b), although

we studied the G93A SOD1 model. To test the idea that evoking a hypometabolic state would be beneficial, Vercruysse *et al* administered an inhibitor of the melanocortin system, pioglitazone. This did not lead to weight gain in a clinical trial of ALS patients and did not lead to increased food intake by the G86R SOD1 mice. Vercruysse *et al* attributed the lack of efficacy of pioglitazone to the intrinsic abnormalities in melanocortin system. On the other hand we show that deletion of MC4R in the G93A mouse can clearly evoke hypometabolism, regardless of the state of the melanocortin system in ALS. Vercruysse *et al* did not study G93A mice, but our data suggest that even if Vercruysse *et al* could pharmacologically inhibit melanocortin system and evoke a hypometabolic state, it may not lead to a meaningful effect on weakness in these models.

Together, this study, Vercruysse *et al* (2016), Dupuis *et al* (2004) and Lim *et al* (2014) show that leptin levels are lower in mutant SOD1 mice, there are fewer POMC neurons, more AgRP neurons and lower trending α-MSH (Fig. 2.7c). Given that loss of MC4R leads to a large increase in leptin levels, we speculate that contrary to G93A mice, leptin levels are also upregulated in the G93A;MC4R$^{-/-}$ mice while α-MSH levels are lowered (Fig. 2.7d). If this is in fact the case, our study also suggests that upregulating leptin in mutant SOD1 mice may not be beneficial in altering disease course.

Sex, metabolism and ALS

Differences between males and females may contribute to the effectiveness of metabolic manipulations in ALS. We find a small but significant female-specific improvement in forelimb grip strength at P120 in G93A;MC4R$^{-/-}$ mice. In the leptin study,

Lim *et al* showed that the increase in median lifespan in G93A;ob$^{+/-}$ mice compared to G93A mice was female-specific. Dupuis *et al* only used male mice in their high-fat diet experiments, but a recent study showed that a diet with low unsaturated fatty acids led to female-specific decrease in median lifespan and a worsening of disease progression in G93A mice (Cacabelos et al., 2014). Similarly, metabolic manipulations are shown to affect male and female animals differently in other ALS studies (Kaneb et al., 2011; Kim et al., 2012; Eschbach et al., 2013; Lim et al., 2014).

In our study, male MC4R$^{-/-}$ mice have 22% lower O_2 consumption and 16% lower CO_2 production at P120 compared to WT mice in the active, dark period. In contrast, female MC4R$^{-/-}$ mice at the same age and time of day have 37% lower O_2 consumption and 33% lower CO_2 production than WT mice. This clearly shows a stronger MC4R mediated hypometabolic phenotype in females. The results are similar in the G93A background. Male G93A;MC4R$^{-/-}$ double mutant mice have 18% lower O_2 consumption and 16% lower CO_2 production than WT mice at P120 in the dark. In contrast, female G93A;MC4R$^{-/-}$ mice have 38% lower O_2 consumption and 37% lower CO_2 production than WT mice at the same age and phase of day. This indicates that even in the G93A background, loss of MC4R has a stronger hypometabolic effect in females, which coincides with improved forelimb strength in females at the same age. Lim *et al* found that G93A females had similar circulating leptin as WT mice while G93A males had decreased circulating leptin compared to WT (Lim et al., 2014). Higher baseline leptin in female mice may contribute to their differential response to metabolic manipulations in the Lim *et al* study and this study. These observations highlight sex-specific perturbations in metabolism and ALS.

There are well-documented metabolic differences between males and females, including effects of sex hormones such as estrogen and testosterone (Salehzadeh et al.,

2011; Mauvais-Jarvis et al., 2013), differences in fat storage (Fried et al., 2015) and food intake (Hallam et al., 2016), to name a few. As highlighted above with leptin, metabolic differences are also present between males and females in ALS. In addition, there are sex-specific differences in ALS presentation and progression. The overall incidence of ALS is higher in males than in females, and spinal cord onset is more likely in males while bulbar onset is more likely in females (McCombe and Henderson, 2010). In G93A mice, females have later onset and longer survival in different genetic backgrounds

(Heiman-Patterson et al., 2005), females have preferentially upregulated proteasomal activity (Riar et al., 2017) and loss of certain genes like PGC-1α accelerates disease progression in males but not females (Eschbach et al., 2013). The complex interaction of metabolic differences with pathophysiology and the differences in metabolism and disease progression in ALS are just beginning to be studied. It is therefore important to address male and female subjects differently in any ALS study, and especially those that perturb metabolism.

The G93A SOD1 mouse model of ALS

Different features of ALS in patients may not be recapitulated in every model of the disease. Here, we chose the G93A SOD1 mouse since it is the most widely studied model of ALS. These mice mimic many of the clinical features of the disease in patients, including hypermetabolism. Unfortunately, there is no systematic study of the level of hypermetabolism specifically in patients with SOD1 mutations. Hence, it is not known whether the hypermetabolism in the SOD1 mouse model mimics patient symptoms. However, a small study showed that patients with familial ALS have a higher incidence of hypermetabolism than those with sporadic ALS (Funalot et al., 2009a), and SOD1 mutations comprise up to 20% of familial ALS cases. Detailed analysis of the metabolic

abnormalities in different SOD1 mutations both in patients and in mouse models will help in designing and interpreting studies of the contribution of metabolism in ALS.

Further, the mutant SOD1 strain we used expresses high levels of the human G93A SOD1 transgene, leading to a 4-fold increase in SOD1 activity (Gurney et al., 1994). Compared to other mouse models with different mutations in SOD1, this line has earlier symptom onset (13-17 weeks) and shorter lifespan (17-26 weeks). In contrast, the G85R mutant SOD1 mouse model has a later symptom onset (35-43 weeks) and longer lifespan (37-45 weeks) (Bruijn et al., 1997). It is conceivable that the more aggressive presentation of ALS-like symptoms in the G93A mouse blunts the potential therapeutic benefit of hypometabolism. Dupuis *et al* described a hypermetabolic phenotype in G93A and G86R mutant SOD1 mice, both of which have earlier onset of symptoms and short lifespans (Dupuis et al., 2004), but it is unknown if the G85R mice have a hypermetabolic phenotype. It is thus important to determine the hypermetabolic phenotype in the G85R SOD1 model, and if it exists, to test the effect of lowering organismal metabolism in that model.

Molecular mechanisms of hypermetabolism in mutant SOD1-linked ALS

Despite decades of effort, the mechanism by which mutant SOD1 causes motor neuron death remains unclear. However, accumulating data suggests that soluble, and not aggregated, misfolded mutant SOD1 could lead to cellular damage by promiscuous interactions with different molecular pathways, including mitochondrial dysfunction and reactive oxygen species production (Parone et al., 2013). Damaged mitochondria can thus affect cellular metabolism, and by extension organismal metabolism. It is plausible that misfolded, soluble mutant SOD1 in the G93A mice is not affected by ablating MC4R and is therefore unable to change disease progression in these mice. Indeed, it is not

known if metabolic changes are caused by disease-linked mutations or if metabolism is distinct from underlying mutations given the prevalence of aberrant metabolism in sporadic ALS patients.

Conclusion

While experiments have shown that weight gain, increasing BMI, and decreasing energy expenditure are associated with lower risk for developing ALS (Paganoni et al., 2011; O'Reilly et al., 2013; Ngo et al., 2014), not all genetic manipulations for inducing weight gain and altering metabolism are equal in their effectiveness. The findings presented here add to the growing understanding of how energetic dysfunction is coupled to ALS. It is critical to understand the metabolic profile in ALS thoroughly, and to detail exactly how different dietary and genetic changes influence metabolism in ALS in a sex-specific way in order to design appropriate therapeutic interventions.

Methods

Mouse strains and husbandry

Male hemizygous G93A mutant SOD1 mice ($G93A^{+/-}$) on the C57BL/6 background (Strain #004435, Jackson Laboratories, Bar Harbor, ME), referred to as G93A, were crossed with female heterozygous loxTB MC4R mice ($MC4R^{+/-}$) in the C57BL/6 background (Balthasar et al., 2005) (Strain #006414, Jackson Laboratories, Bar Harbor, ME). The G93A mice carry one copy of the human G93A mutant SOD1 transgene, and the $MC4R^{+/-}$ mice contain a transcriptional block cassette before the MC4R start codon. From the F1 progeny, G93A;$MC4R^{+/-}$ males were crossed with $MC4R^{+/-}$ females. From the F2 generation, the following groups were used for study purposes: WT (+/+), G93A, $MC4R^{-/-}$, G93A;$MC4R^{-/-}$ (Fig. 2.1a). Both males and females from each group were used for all study parameters. Genotypes were determined by PCR using tail snip DNA using the following primers – MC4R: GCAGTACAGCGAGTCTCAGG (wild type forward), CTCCCACAGGCTTATGACACC (wild type reverse), GTGCAAGTGCAGGTGCCAG (mutant), and SOD1: CTAGGCCACAGAATTGAAAGATCT (genomic forward), GTAGGTGGAAATTCTAGCATCATCC (genomic reverse), CATCAGCCCTAATCCATCTGA (transgene forward), CGCGACTAACAATCAAAGTGA (transgene reverse).

Mice were housed at 22°C with a 12-hour light dark cycle. They were fed standard diet (23% protein, 22% fat, 55% carbohydrates). Animals were weighed once a week at the same time of day starting at P40 for at least 20 weeks. Mice without the G93A mutation were measured for over 40 weeks. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the Children's Hospital

of Philadelphia, and animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Study Design

Mice of both sexes with four genotypes (WT, G93A, MC4R$^{-/-}$, G93A;MCR4$^{-/-}$) were studied. The following parameters were measured at P60, P90 and P120: oxygen consumption, carbon dioxide production, food consumption, ambulatory activity, forelimb strength and hindlimb strength. P60 is shown to be presymptomatic in G93A mice, while P90 is symptomatic and P120 is close to end stage (Saxena et al., 2009). We chose these time points in order to monitor progression of ALS-related and metabolic phenotypes as animals age. Animals were sacrificed when they were unable to right themselves within 30 seconds from being supine, and this was recorded as day of death for survival measurements.

Weight gain, food consumption, activity and metabolic measurements

Food consumption, ambulatory activity and metabolic measurements were made at the appropriate time points (P60, P90 and P120) using the Comprehensive Laboratory Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus OH). Animals were weighed and placed in individual chambers with food and water for 48 hours. Food consumption was measured in grams by recording the decrease in weight of the pre-measured food provided in the chambers. Ambulatory activity was measured in arbitrary units by the number of infrared beam breaks in the x-y plane of the cage every minute, thus ruling out grooming-related activity in the z-axis. Oxygen consumption and carbon dioxide production were measured by indirect calorimetry approximately every 10 minutes and reported in milliliters per hour, normalized by the body weight of the animal

(mL/hr/kg). Animals were allowed to acclimate to the chambers for the first 24 hours, and only the data from the final 24 hours was used for analysis.

Motor Output

Grip strength measurements were made at the same time of day at appropriate time points for the fore and hind paws using a digital grip strength meter (Columbus Instruments, Columbus, OH). For fore grip measurements, animals were allowed to grip a bar with their fore paws while being held by their tails, and gently pulled back until they let go of the bar. The meter recorded the force (in kilogram-force or kgf) with which they hold on to the bar as they are pulled back. Similarly, hind grip strength was measured as mice gripped a bar with their hind paws. For each animal, one set of trials comprised 3-5 consecutive trials, and each set was repeated three times, with approximately 3 minutes between sets for the animal to rest. The average strength for the 12-15 trials was reported for both the fore and the hind paws at each time point.

Plasma leptin and α-MSH measurements

Mice were anesthetized using carbon dioxide and cardiac extraction was immediately performed to collect blood from adult WT, G93A and $MC4R^{-/-}$ male mice. Blood was spun down at 2000g for 20 minutes and supernatant plasma was frozen at -80C until further use. Plasma concentrations of leptin and α-MSH were determined by the Radioimmunoassay and Biomarkers Core facility at the University of Pennsylvania using standard ELISA (Leptin: Cat # 22-LEPMS-E01 from ALPCO, Salem NH; α-MSH: Cat # MBS2516107 from MyBioSource, San Diego CA).

Statistics

Data were analyzed using Prism (GraphPad Software, La Jolla, CA). Unless otherwise noted, significant differences within groups were determined using one-way ANOVA followed by Tukey's test for multiple comparisons. Survival curves were analyzed using the log-rank (Mantel-Cox) test for significance. For all tests, the significance threshold was set to $p < 0.05$.

Author Contributions

S.D. and R.G.K. designed the experiments and wrote the manuscript. S.D. and P.G. conducted the experiments. S.D. analyzed the results. All authors reviewed the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

CHAPTER 3

A novel role for neuropeptide signaling in regulating *C. elegans* response to anoxia

Shachee Doshi[1,2], Urva Barot[1], Emma Price[1], Justin Landis[1], Hannes Lars[4], Robert G. Kalb[1,2,3]

[1]Division of Neurology, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA 19104,
[2]Neuroscience Graduate Group and [3]Department of Neurology, University of Pennsylvania, Philadelphia, PA 19104,
[4]Department of Genetics, Erasmus Medical Centre, Cancer Genomics Institute, Rotterdam 3015 CN, The Netherlands

ACKNOWLEDGEMENTS

We would like to thank Dr. Mei Zhen and Dr. Derek Seiburth for sharing constructs and some worm strains for this project, and Dr. John Flibotte for the initial observations of neuropeptide mutant worms. We would also like to thank Dr. David Raizen, Dr. Meera Sundaram, Dr. Chris Fang-Yen, Dr. John Murray and the entire worm group at the University of Pennsylvania for invaluable ongoing feedback. Additionally, Dr. Virginia Lee, Dr. Thomas Jongens and Dr. Amita Sehgal for insightful comments on the progress of this project. We also want to thank Elelbin Ortiz and Lisa Romero for assisting in generating some *C. elegans* lines. Finally, we would like to thank the CGC and the National BioResource Project in Japan for worm strains.

Abstract

Oxygen deprivation, even for short durations, can be extremely injurious to cells. Neurons are particularly vulnerable to changes in oxygen, and cerebral hypoxia has debilitating consequences including motor impairments, speech impairments and memory loss, and can be fatal. The molecular mechanisms underlying extreme oxygen deprivation are not well understood. Here we describe a novel role for neuropeptide signaling in regulating survival of the nematode *Caenorhabditis elegans* under anoxic conditions. We find that loss of neuropeptide signaling protects worms from extended anoxia and it does not rely on known pathways in stress tolerance and longevity for this effect. Loss of genes regulating cellular energy homeostasis and membrane lipid synthesis suppresses neuropeptide-mediated protection, indicating an important role for metabolism in surviving extended anoxia. Additionally, the nervous system is necessary for the secretion of neuropeptides mediating this phenotype, and a screen of neuropeptide genes identified *nlp-40* as a partial regulator of anoxic survival. Together, these data highlight a potential cell non-autonomous regulation of survival against extreme environmental stress.

Introduction

Molecular oxygen is essential to the function and survival of multicellular organisms. It is the final electron-acceptor of the electron transport chain during mitochondrial oxidative phosphorylation, and is thus critical for producing the vast majority of ATP to fuel cellular functions (Rich, 2003). Even short periods of oxygen deprivation cause cell and organismal damage and death. While all organs are sensitive to changes in oxygen, the brain is unique in that it consumes the most (20% of total oxygen consumed) of any organ (Erecińska and Silver, 2001). It is, therefore, also the most sensitive to oxygen deprivation. There are many injuries and disorders where the brain experiences decreased oxygen supply, such as sudden cardiac arrest, ischemic stroke, strangulation, choking, carbon monoxide poisoning and seizures (Hopkins and Haaland, 2004). Birth-related injuries lead to hypoxic-ischemic encephalopathy in newborn infants (Volpe, 2001). Depending on the age at injury and the area of brain affected, there are wide-ranging and severe consequences of oxygen deprivation such as changes in mood and personality, loss of motor function or speech, memory loss and cognitive impairment (Yogaratnam et al., 2013). Understanding the genetic and molecular regulation of cellular response to oxygen deprivation can help design therapies and drugs to combat its devastating effects.

C. elegans, the soil-living nematode, is a great model to study oxygen deprivation, and has been used as such across its lifespan (Van Voorhies and Ward, 2000; Padilla et al., 2002; Scott et al., 2002; Peña et al., 2016). It is a relatively simple, 1mm long organism with 959 somatic cells and 302 neurons (www.wormbook.org). It has a short life cycle (it grows from egg to adult in 3 days and lives for 2-3 weeks), feeds on bacteria and is transparent, all of which allow for ease of experimental manipulation.

Importantly, its neuronal connectivity is completely mapped, its genome shares significant homology with humans and is completely sequenced, and many molecular pathways are identical between *C. elegans* and mammals.

Since it lives in soil, *C. elegans* generally prefers lower environmental oxygen (5-12% O_2) (Gray et al., 2004). Interestingly, it has distinct responses to moderate and severe oxygen deprivation (Padilla et al., 2002). Worms can survive, develop and reproduce under low oxygen, hypoxic conditions (0.5-1% oxygen) while decreasing their oxygen consumption and locomotion (Jiang et al., 2001). However, under extreme oxygen deprivation or anoxia (<0.1% oxygen), worms become hypometabolic, and suspend development, feeding and reproduction (Van Voorhies and Ward, 2000; Padilla et al., 2002; Nystul et al., 2003). Depending on growth conditions, they can survive in this 'suspended animation' state for long periods (a few days), but eventually die if oxygen is not reintroduced. Upon reoxygenation, they resume normal activity including reproduction. While the hypoxic response in *C. elegans* is extensively studied and depends on the canonical hypoxia induced factor (*hif-1*) pathway, the anoxic response is *hif-1*-independent and is less well understood (Padilla et al., 2002).

Studies in the past decade have shed light on some features of the worm response to anoxia. Loss of function in *daf-2*, the worm homolog of the insulin-like growth factor 1 (IGF1) receptor, protects worms from anoxia, suggesting a role for insulin signaling in this response (Scott et al., 2002). This protective effect of *daf-2* loss of function requires activity of its downstream transcription factor *daf-16*, the worm homolog of the forkhead box protein O (FOXO). Similarly, loss of function mutation in *nsy-1*, a MAP3K, also protects worms from anoxia (Hayakawa et al., 2011). Mutations the AMPK pathway (LaRue and Padilla, 2011) and the ceramide synthesis pathway (Menuz et al., 2009) make worms hyper sensitvie to anoxia, suggesting a role for

metabolism in the worm anoxic response. Indeed, ATP levels decrease during anoxia, and increase upon re-exposure to air (Van Voorhies and Ward, 2000; Padilla et al., 2002).

Previous work from our lab shows that the BAG sensory neuron, which detects downshifts in environmental oxygen, makes worms sensitive to anoxia. In worms where the BAG neuron is genetically ablated, anoxic survival is significantly higher than wild type worms (Flibotte et al., 2014). Similarly, we found that in the absence of neuropeptide synthesis and secretion, worms survive anoxia significantly better (Flibotte et al., 2014). However, neuropeptide signaling is not required in BAG neurons for this effect. These experiments utilized a temperature sensitive allele of *egl-3* and were conducted at 25 degrees Celsius to enhance *egl-3* loss of function. At this higher temperature, the temperature sensitive *egl-3* mutant continued to be protective against anoxic insult. Restoring neuropeptide synthesis specifically in BAG neurons in a neuropeptide synthesis-deficient background did not rescue the survival benefit conferred by loss of *egl-3* mediated neuropeptide signaling. One interpretations of this result is that there was not sufficient rescue of *egl-3* in the BAG neurons. Alternatively, these data suggest that neuropeptide signaling is not required in BAG neurons, is not directly related to oxygen sensing, and perhaps works in a parallel, cell non-autonomous fashion to regulate worm response to anoxia.

How, then, does neuropeptide signaling regulate worm survival against anoxia? We hypothesize that discrete neuropeptides are secreted by neurons and lead to inhibition of stress-resistance and longevity pathways to make developing wild type worms specifically vulnerable to anoxia. To test this hypothesis, we sought to answer the following questions: Is the regulation of anoxia by neuropeptides specific to this stress? Which tissues do neuropeptides act in? What known mechanisms in stress resistance

and longevity, if any, impinge on the regulation by neuropeptides? Which specific neuropeptides are involved in this anoxic response? How does exposure to anoxic stress impact worm lifespan? We chose the L4 larval stage of development to address these questions, since it is known to be sensitive to anoxia (Padilla et al., 2002; Flibotte et al., 2014), and can approximate hypoxic ischemic encephalopathy experienced at birth by newborn infants. Our goal is to expand our understanding of the *C. elegans* response to anoxia by focusing on the neuropeptide regulation of this stress to address these questions.

Results

Loss of neuropeptide signaling protects against anoxic stress in developing C. elegans

We previously found that loss of function mutations in *egl-3* and *unc-31* protected worms against anoxia (Flibotte et al., 2014). EGL-3 is a proprotein convertase required for maturation of peptide hormones (Thacker and Rose, 2000). It cleaves larger proprotein peptides into smaller peptides with C-terminal basic residue(s). Loss of *egl-3* impedes synthesis of hormones, including neuropeptides. UNC-31 is a calcium-dependent activator protein for secretion (CAPS). It is required for fusion and exocytosis of neuropeptide-containing dense core vesicles at presynaptic membranes (Speese et al., 2007). Wild-type (N2) survival after anoxia is dependent on the duration of the insult: 80-90% of N2 worms survive 24 hours of anoxia while only 30-50% survive 48 hours of anoxia (Van Voorhies and Ward, 2000; Padilla et al., 2002; Flibotte et al., 2014). As a result, we chose 48 hours of anoxic insult to measure changes in susceptibility to this stress.

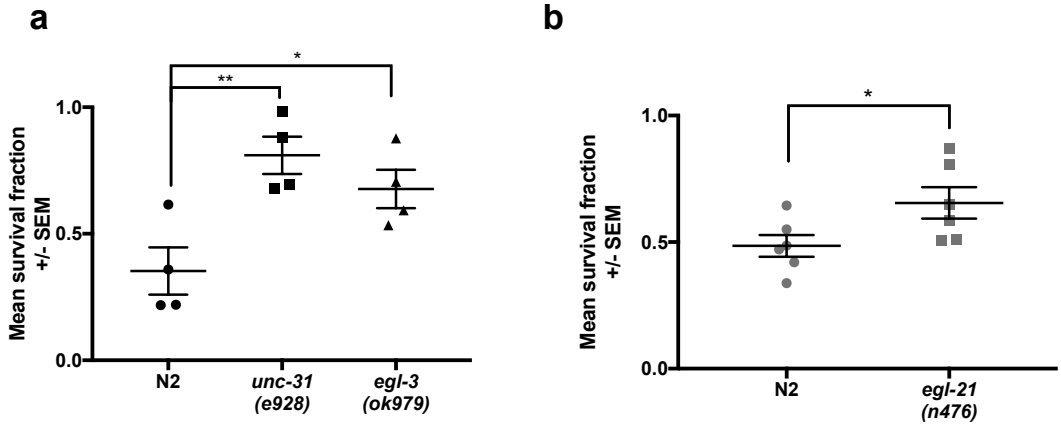


Figure 3.1. Survival of neuropeptide processing and secretion mutants at the L4 stage after 48 hours of anoxia. For a) data were analyzed using one-Way ANOVA with Tukey's test for multiple comparisons (n = 4 independent experiments). For b) data were analyzed using paired t-test (n = 6 independent experiments). *p < 0.05, **p < 0.01

We confirmed these findings in *egl-3* and *unc-31* mutants, and also found that loss of *egl-21* was protective against 48 hours of anoxia (Fig. 3.1a and b). EGL-21 is a carboxypeptidase that is required for maturation of neuropeptides. It removes the C-terminal basic residue(s) from small peptides generated by EGL-3 (Jacob and Kaplan, 2003). Thus, loss of function of multiple genes required for neuropeptide synthesis and secretion protect worms from anoxic stress. Interestingly, all three of these genes have human homologs, and play the same roles in peptide hormone synthesis and secretion (Thacker and Rose, 2000; Jacob and Kaplan, 2003; Speese et al., 2007).

This protective effect was specific to the L4 stage of development, the final larval stage before worms enter adulthood. We found that 90-100% of young adult worms survived 48 hours of anoxia while 30-50% of L4 worms survived the same insult (Fig. 3.2a). The L4 stage of worm development lasts upto 8 hours, during which worms undergo a variety of physiological changes.

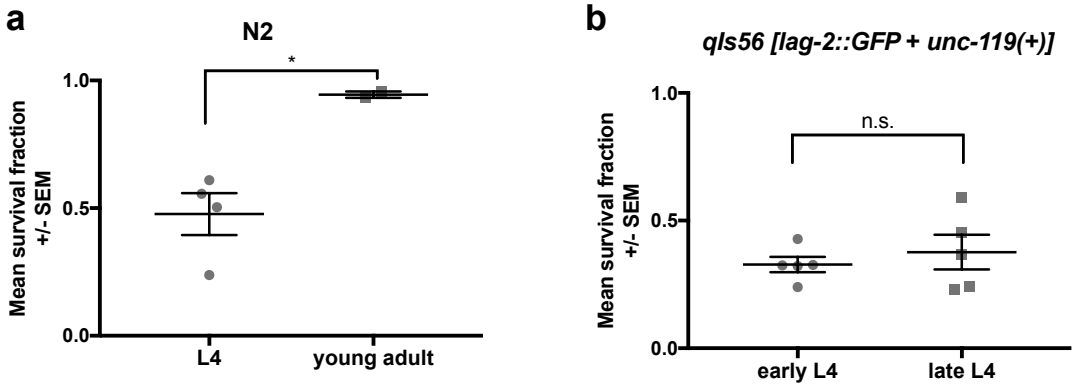


Figure 3.2. Worms are sensitive to 48 hours of anoxia specifically at the L4 stage. a) Survival of L4 vs young adult (L4 + 1day) N2 worms after 48 hours of anoxia (n = 4 independent experiments). b) Survival of early L4 vs late L4 worms after 48 hours of anoxia, using the *qIs56 [lag-2::GFP + unc-119(+)]* reporter worm to define early and late L4 based on tip cell maturation (n = 5 independent experiments). Data were analyzed using paired t-test. *p < 0.05

Given that developing worms respond to 48h anoxia differently than mature worms, we asked if worms in early L4 would be different from those in late L4 in their anoxic

susceptibility. We used a reporter line *qIs56 [(lag-2::GFP) + unc-119(+)]* for distal tip cell maturation (Blelloch et al., 1999) to accurately distinguish early and late L4 worms, and subjected them to 48h anoxia. There was no significant difference in anoxic survival between the two sub-stages (Fig. 3.2b).

In addition to O_2 depletion, 8-12% CO_2 is generated in the biobags used for these studies (per manufacturer). Work by others suggests that worms reduce pharyngeal pumping in response to high CO_2 levels, and this is in part mediated by egl-21 and unc-31 (Sharabi et al., 2014). In order to determine if the worms were responding to hypercarbia instead of anoxia, we placed L4-staged N2, *egl-3* and *unc-31* worms in a chamber with 20% O_2 and 10% CO_2. After 48 hours in this chamber, none of the lines had developmentally arrested ('stunned') or died as they do in the biobags. Rather, they had developed to adulthood, laid eggs and these eggs had hatched (data not shown). We also tested *gcy-9* mutant worms, since GCY-9 is known to be necessary for sensing CO_2, thereby regulating worm avoidance to the gas (Hallem et al., 2011). Loss of *gcy-9*, however, did not alter survival of worms after 48 hours of anoxia compared to N2 animals (data not shown). Therefore, we concluded that the phenotype we studied was specific to anoxia.

We also found that protection conferred by loss of neuropeptide signaling was specific to anoxic stress. Neuropeptide signaling mutants subjected to heat stress, UV stress or tunicamycin-induced ER stress did not survive any better than N2 worms (Fig. 3.3).

Together, these data show that developing (L4) *C.elegans* are sensitive to anoxic stress in a duration-dependent manner, and loss of function mutations in neuropeptide synthesis and secretion specifically confer a survival benefit against this stress. This

indicates that neuropeptide signaling makes worms vulnerable to extreme oxygen deprivation.

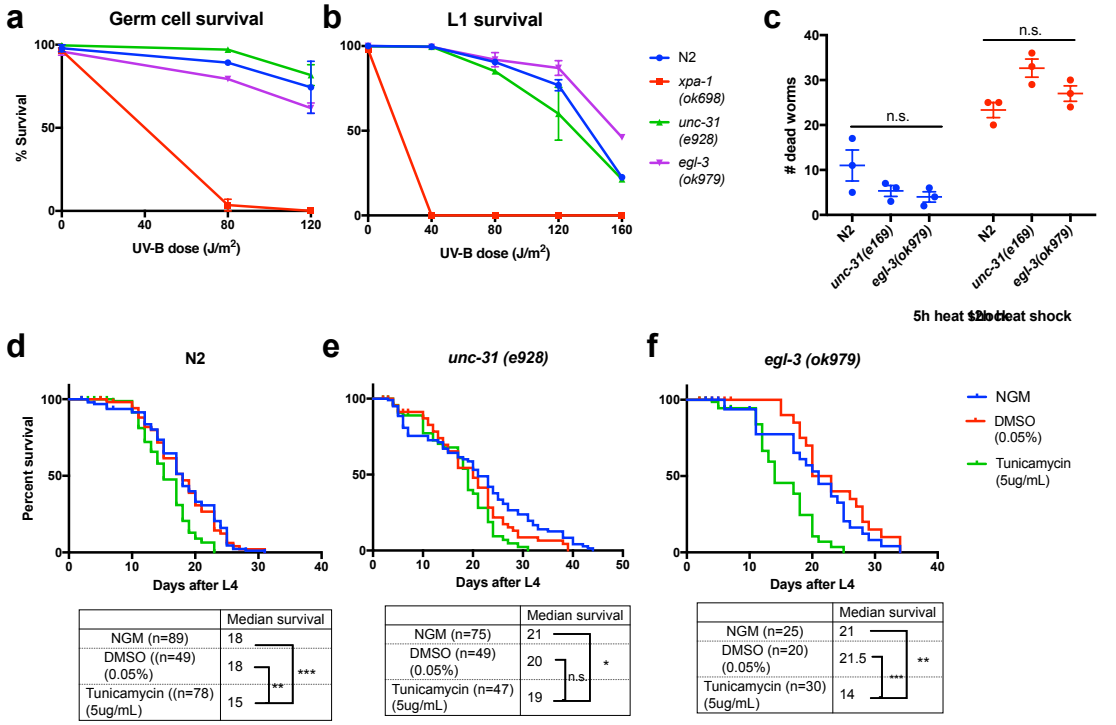


Figure 3.3. Response of neuropeptide signaling mutants to different stresses. a) Survival of embryos after adult parents (n=16-25 animals) are irradiated with different doses of UV-B. b) Survival of L1 worms (n=40-160 animals) irradiated with different doses of UV-B. c) Survival of L4 worms (n=50 animals) after 5h and 12h of 34C heat shock. Data were analyzed by Two-Way ANOVA with Tukey's test for multiple comparisons. d-f) Survival of N2 (d), *unc-31* (e) and *egl-3* (f) worms at L4 placed on NGM, DMSO (Vehicle) or Tunicamycin plates. Data are analyzed using the Mantel-Cox test for survival curve comparison. *p < 0.05, **p < 0.01, ***p < 0.001

Biogenic amines do not underlie unc-31-mediated anoxic survival

In *C.elegans*, dense-core vesicles are known to package biogenic amines in addition to peptides (Berendzen et al., 2016). We wondered if *unc-31*-mediated protection under anoxia was partially due to loss of biogenic amine signaling. There are four biogenic amines in *C.elegans*: serotonin, dopamine, tyramine and octopamine. These are synthesized by tryptophan hydroxylase (*tph-1*), tyrosine hydroxylase (*cat-2*), tyrosine decarboxylase (*tdc-1*) and tyramine β-hydroxylase (*tbh-1*) respectively. In order

to test their contribution to anoxic survival via UNC-31, we measured survival of worms with loss of function mutations in each of these enzymes. We found that none of these mutants had a significant survival benefit compared to N2 (Fig. 3.4), suggesting that loss of *unc-31* confers a survival benefit specifically due to loss of neuropeptide signaling.

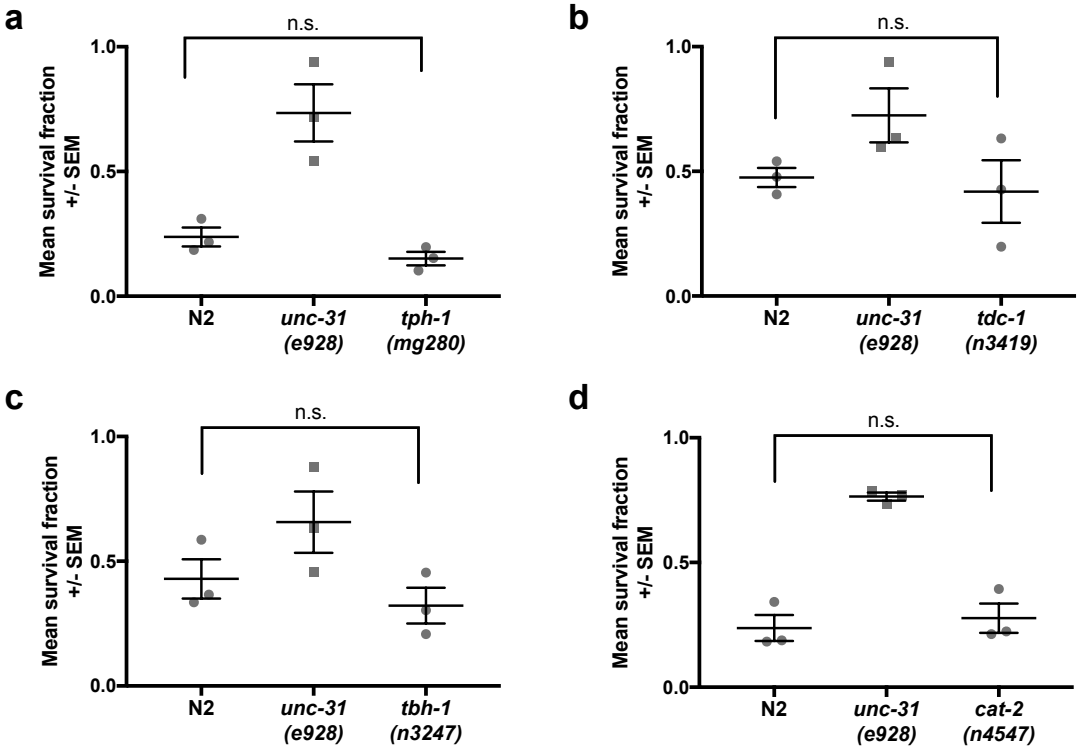


Figure 3.4. Survival of biogenic amine synthesis mutants at L4 after 48 hours of anoxia. N2 and *unc-31* survival was compared against survival of a) tryptophan hydroxylase (*tph-1*), b) tyrosine decarboxylase (*tdc-1*), c) tyramine β-hydroxylase (*tbh-1*) and d) tyrosine hydroxylase (*cat-2*). Data were analyzed using one-way ANOVA with Tukey's test for multiple comparisons (n = 3 independent experiments for all).

Neuropeptide synthesis in the nervous system is necessary for survival under anoxic stress

Reporter studies have shown that UNC-31 and EGL-21 are ubiquitously expressed in the nervous system and have little detectable expression in other tissues

(Jacob and Kaplan, 2003; Speese et al., 2007). Similarly, EGL-3 is broadly expressed in the nervous system, but there is evidence that it is also expressed in non-neuronal cells, such as the intestine (Hung et al., 2014). We wanted to know which tissue *egl-3* loss was required in for its pro-survival effect under anoxic conditions. To this end, we generated worms expressing genomic *egl-3* under either a pan-neuronal, intestinal or muscle-specific promoter into the *egl-3* mutant background. These lines should be null for *egl-3* in the whole animal except neurons, muscles and intestine, respectively. After subjecting these lines to anoxia, we found that intestinal and muscle expression of *egl-3* did not rescue the survival benefit seen in *egl-3* null worms. However, neuronal expression of *egl-3* rescued this survival benefit back to wild-type levels (Fig. 3.5a). We confirmed this finding using pan-neuronal knockdown of *egl-3* using RNAi using a worm strain sensitized for neuronal RNAi (Firnhaber and Hammarlund, 2013). Compared to worms raised on empty vector (EV) bacteria, those raised on pan-neuronal RNAi against *egl-3* survived anoxia significantly better (Fig. 3.5b). This indicates that loss of *egl-3* in neurons is necessary to confer survival under anoxia, and that neuropeptides synthesized in the nervous system make worms susceptible to anoxic stress. These data are also consistent with the expression pattern of *unc-31* and *egl-21*.

In order to determine which neuronal subpopulations were necessary for the *egl-3* loss of function phenotype, we similarly conducted RNAi experiments to knock down *egl-3* in glutamatergic, cholinergic, GABAergic and dopaminergic cells specifically. To do this, we obtained worms that were optimized for dsRNA uptake in the specific neuronal subtypes (Firnhaber and Hammarlund, 2013). We found that glutamatergic and GABAergic neurons do mediate this effect, because we saw significantly higher survival upon *egl-3* knockdown in these tissues compared to the same worms fed EV bacteria after 54h anoxia (Fig 3.5b). In preliminary experiments (n=2) there was no significant

difference between EV and *egl-3* RNAi groups in cholinergic and dopaminergic neurons after 72h anoxia. These experiments were done under higher anoxia durations (54h and 72h) since we found that all these lines survived 48h of anoxia very well, making those data difficult to interpret. Together, these RNAi experiments tell us that loss of *egl-3* function in multiple neuronal subtypes, including glutamatergic and GABAergic neurons, accounts for the survival phenotype of *egl-3* null worms.

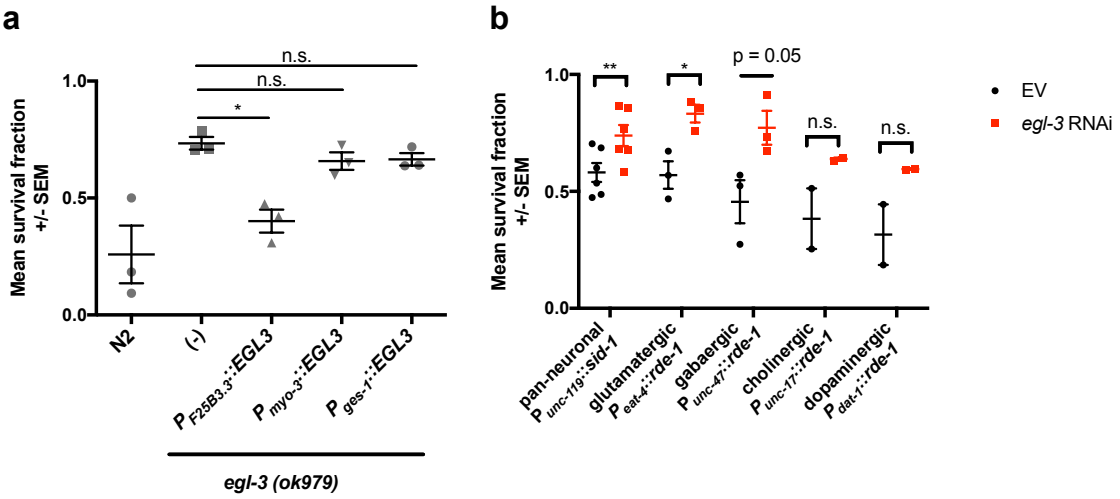


Figure 3.5. Neuron-specific regulation of *egl-3* mediated survival after anoxia. a) Survival of worm lines expressing genomic EGL-3 in neurons ($P_{F2B3.3}$), body wall muscle (P_{myo-3}) and intestine (P_{ges-1}) in an *egl-3* null background (n = 3 independent experiments). Data were analyzed using one-way ANOVA with Tukey's test for multiple comparisons. b) Survival of lines fed EV (TU3311) or *egl-3* RNAi bacteria for at least 2 generations. Each line expresses either SID-1 or RDE-1 to allow RNAi mediated *egl-3* knockdown in neuronal subtypes. Pan-neuronal = TU3311 (*Punc-119::sid-1*) (n=6), glutamatergic = XE1582 (*Peat-4::rde-1*) (n=3), GABAergic = XE1375 (*Punc-47::rde-1;Punc-47::mCherry)* (n=3), cholinergic = XE1581 (*Punc-17::rde-1*) (n=2), dopaminergic = XE1474 (*Pdat-1::rde-1*) (n=2). Data were analyzed using paired t-test between EV and *egl-3* RNAi for each line. *p < 0.05, **p < 0.01

Known stress-resistance/longevity pathways do not regulate neuropeptide-mediated survival under anoxia

We wanted to understand the mechanism by which neuropeptide signaling affects survival under anoxia. Do genes and pathways previously shown to regulate stress responses also underlie neuropeptide-mediated anoxic susceptibility?

To test this, we interrogated loss of function mutations in a variety of such genes in the *unc-31* and/or *egl-3* background. We tested the following genes due to their described role in anoxia, hypoxia or general stress resistance/longevity: (i) *daf-16* (Scott et al., 2002; Mendenhall et al., 2006a), (ii) *hif-1* (Zhang et al., 2009), (iii) *hsf-1* (Hsu et al., 2003), (iv) *skn-1* (Ghose et al., 2013), (v) *nsy-1* (Hayakawa et al., 2011). Briefly, we made double mutants between the mutant gene of interest (i-v above) and *unc-31/egl-3* mutants, and subjected them to anoxia. Our reasoning was that if increased anoxic survival of *unc-31/egl-3* mutants was due to the activity of any one of the genes i-v, then loss of that gene in the *unc-31/egl-3* mutant background would reverse the survival benefit.

We found no significant difference in survival between *unc-31* or *egl-3* mutant strains and the respective double mutant strains (Fig. 3.6). Thus, none of these genes were unable to rescue survival benefit conferred by loss of *unc-31* or *egl-3*. These data indicate that either these genes act in parallel to neuropeptide signaling, or that neuropeptide-mediated regulation of anoxia requires the activity of an untested or novel cellular factor and does not involve most known stress resistance or longevity promoting pathways.

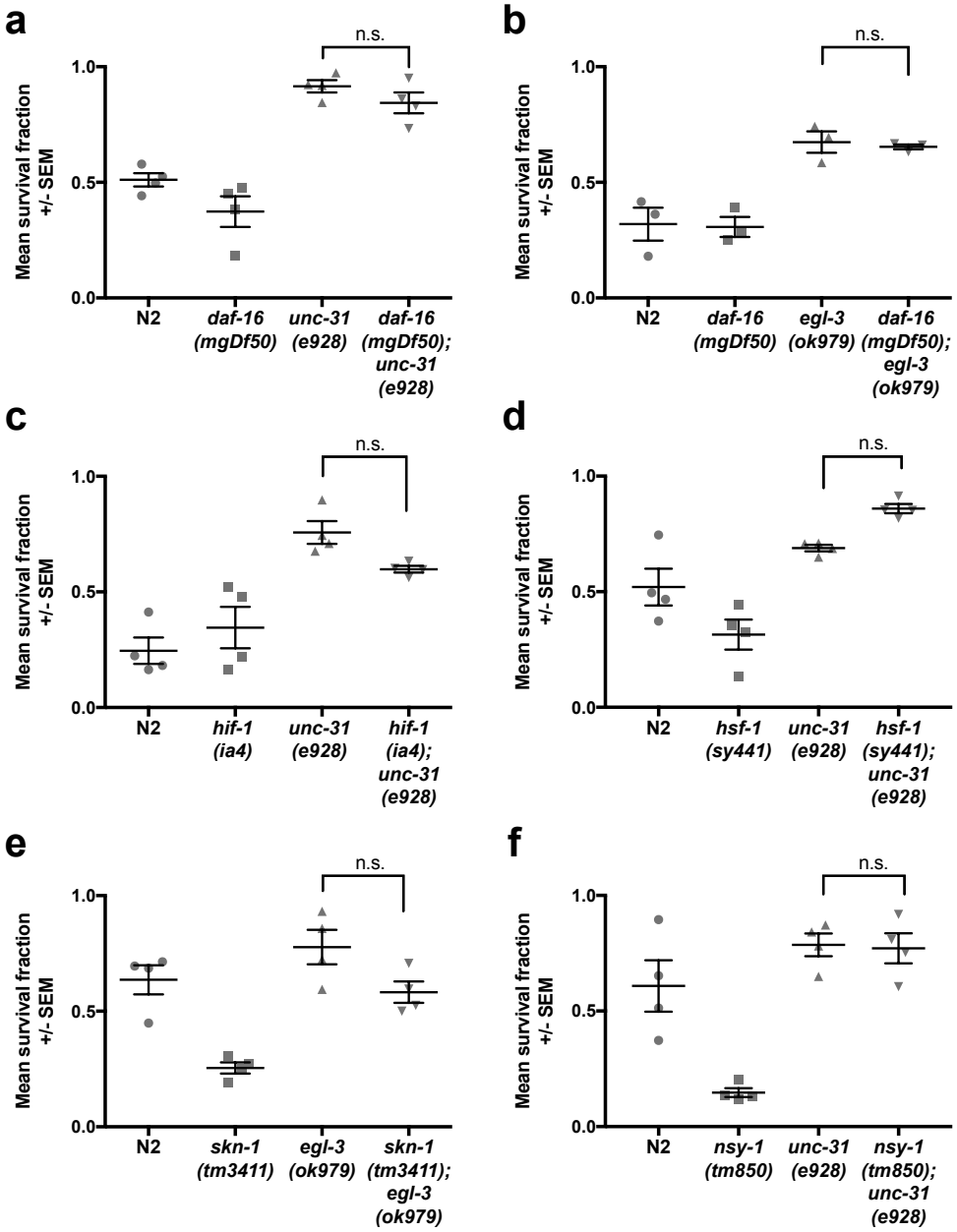


Figure 3.6. Survey of canonical stress-resistance and longevity promoting genes in regulating neuropeptde-mediated anoxia sensitivity. Survival of the following double mutants containing *egl-3* or *unc-31* was compared with *egl-3* or *unc-31* alone. a) *daf-16;unc-31* b) *daf-16;egl-3* c) *hif-1;unc-31* d) *hsf-1;unc-31* e) *skn-1;egl-3* f) *nsy-1;unc-31.* (n = 3 independent experiments for b), n = 4 for rest). Data were analyzed using one-way ANOVA with Tukey's test for multiple comparisons.

Genes involved in cellular metabolism suppress survival benefit induced by loss of neuropeptide signaling

Since worms enter a hypometabolic, suspended animation state in order to survive long term anoxia, we wondered if disrupting genes involved in regulating cellular metabolism would affect neuropeptide-mediated sensitivity to anoxic stress. AMP-associated kinase (AMPK) is the master cellular sensor of a decrease in the ATP/AMP ratio. When cellular ATP levels decline, AMPK is activated by phosporylation, and in turn it promotes anabolic pathways and restricts catabolic pathways in the cell. Studies have shown that ATP levels decrease during worm anoxia (Van Voorhies and Ward, 2000; Padilla et al., 2002), and that loss of the worm AMPK, *aak-2*, also makes worms vulnerable to anoxia (LaRue and Padilla, 2011). Previous studies have also implicated the short chain (C20-22) ceramide syntase HYL-2 in regulating *C. elegans* response to anoxia (Menuz et al., 2009). Ceramides are essential components of the lipid bilayer, and are involved in regulating metabolic homeostasis (Bikman and Summers, 2011). Loss of function mutations in *hyl-2* made worms extremely sensitive to anoxia, and lack of *hyl-2* signficantly decreased the survival benefit conferred by mutant *daf-2* (Menuz et al., 2009). We decided to test these two genes to assess their involvement in the neuropeptide-mediated survival phenotype we observe in developing worms.

First, we confirmed that loss of *hyl-2* and *aak-2* renders worms hypersensitive to our anoxic conditions. Next, we studied the genetic doubles *hyl-2;unc-31*, *hyl-2;egl-3*, and *aak-2;unc-31*. In contrast to other stress-resistance genes discussed in the previous section, loss of *hyl-2* and *aak-2* completely suppressed the survival benefit in the *egl-3/unc-31* mutant background (Figure 3.7a-c and 3.7e). We confirmed the effect of *hyl-2* in two different ways: (i) we tested two different loss of function mutations and obtained the same result (Fig. 3.7a and 3.7b), and (ii) we outcrossed *hyl-2* from the *unc-31;hyl-2*

double mutant to directly test *hyl-2* involvement in survival suppression (Fig. 7d). Outcrossing *hyl-2* reversed the sensitivity to anoxia seen in the double mutant. In fact, the outcrossed strain had similar survival as the *unc-31* mutant alone, confirming that *hyl-2* was necessary for suppression of the *unc-31* phenotype. These data indicate that cellular pathways regulating energy metabolism in the cell are critical to surviving anoxic stress, and that neuropeptide signaling likely acts downstream of these metabolic factors in regulating anoxia response.

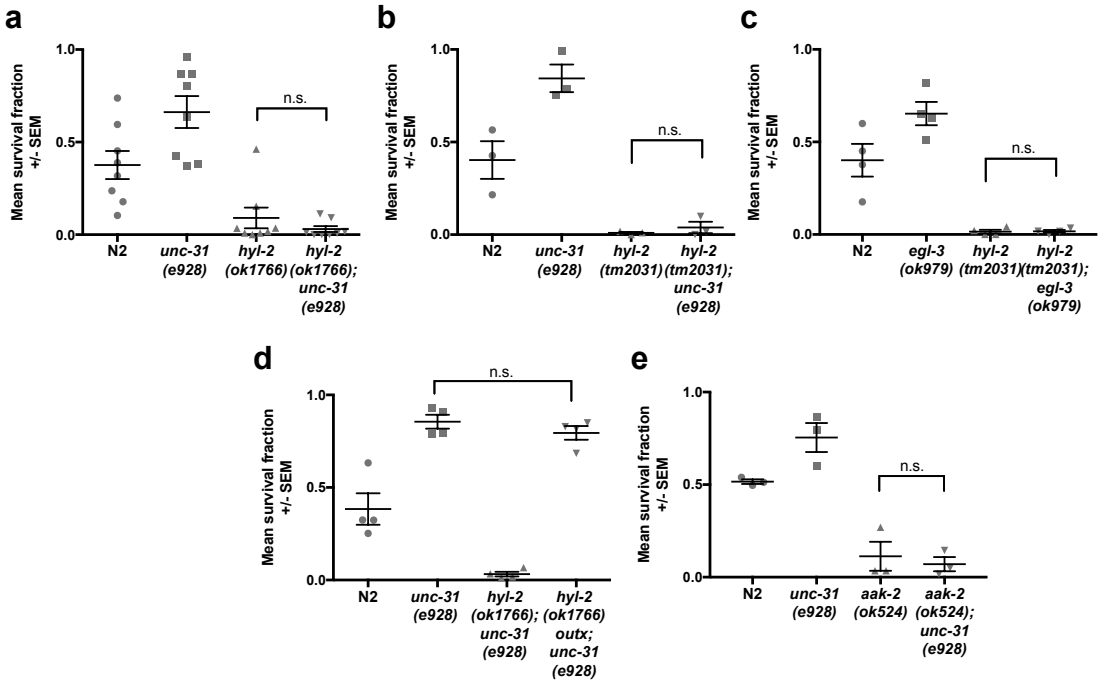


Figure 3.7. Anoxia resistance of *egl-3 and unc-31* mutants is suppressed by loss of the ceramide synthase *hyl-2* and the ATP/AMP sensor *aak-2*. All experiments were done with L4 animals under 48 hours anoxia. a) and b) Survival of two different mutant alleles of *hyl-2* in the *unc-31* mutant background (n = 8 and 3 independent experiments respectively). c) Survival of mutant *hyl-2* in mutant *egl-3* background (n=4). d) Survival of mutant *unc-31* after outcrossing *hyl-2* from the *hyl-2;unc-31* strain tested in (a) (n=4). e) Survival of *aak-2* mutant in the *unc-31* mutant background (n=3). Data were analyzed using one-way ANOVA with Tukey's test for multiple comparisons.

The neuropeptide nlp-40 partly regulates C. elegans response to anoxia

Loss of *egl-3*, *egl-21* and *unc-31* should lead to an inhibition of all neuropeptide synthesis in the worm. We predicted that the anoxic survival benefit in these mutant worms is mediated by either a single neuropeptide or a group of neuropeptides. There is a rich body of literature that implicates single neuropeptides in regulating complex behaviors and pathways in worms (Nelson et al., 2013; Cheong et al., 2015; Chen et al., 2016; Shao et al., 2016; Turek et al., 2016). Therefore, we set out to find putative neuropeptide(s) that may underlie the effect of *egl-3/egl-21/unc-31* loss of function under anoxic conditions. There are 113 genes encoding over 250 distinct neuropeptides in *C.elegans*, most of which do not have clearly defined functions (www.wormbook.org). These neuropeptide genes are broadly divided into three groups: insulins (*ins*), FMRF-like peptides (*flp*) and non-insulin/non-FMRF like peptides (*nlp*).

The first class of neuropeptides we addressed was the insulins, because (i) loss of function in the insulin receptor *daf-2* is also protective against anoxia and this protection is dependent on its downstream transcription factor *daf-16* (Scott et al., 2002), and (ii) EGL-3 is known to process ins propeptides to generate their mature, active forms (Hung et al., 2014). However, the *unc-31;daf-16* and *egl-3;daf-16* double mutants have similar survival compared to *unc-31* and *egl-3* mutants alone (Fig. 3.6a and 3.6b) suggesting that the *unc31/egl-3* effect on anoxia is independent of the DAF-2/DAF-16 pathway. This allowed us to rule out the *ins* class of neuropeptides, and focus our search to *flp*'s and *nlp*'s. We tested survival of almost all available deletion mutants of *flp*'s (29 of 31 total *flp*'s) and *nlp*'s (36 of 42 total *nlp*'s) after 48 hours anoxia (Fig. 3.8a and 3.8b) in order to find mutants that mimicked the survival benefit seen in *unc-31* loss of function worms.

For our initial screen, we tested each mutant 2 independent times, with 3 technical replicates each time. We had to reconcile the number of independent experiments we could carry out here because of the need to balance two competing issues – getting through the large screen of available mutants, and testing them thoroughly to avoid the substantial biological variability that is a constant feature of this anoxia assay. As expected, we did encounter a large amount of biologic variability in these experiments, making candidate identification challenging. Ultimately, we decided to narrow down an initial list of mutants that had an average survival greater than 50% across these 6 observations. The only exception was *nlp-15*, which was included in the candidate list because it had very high survival in one of the two experiments. This left us with 8 *flp*'s and 9 *nlp*'s for the secondary screen. One of the strains we tested had a very large deletion that removed many hundreds of genes (VC30122, which spanned *nlp-25*, *nlp-26* and *nlp-42*). Because of this, we had 15 strains encompassing the 17 total mutations after our initial screen.

To narrow down these candidates further, we employed three different tests – (i) repeat the experiment for each candidate >3-4 more times, (ii) backcross each line to N2 at least 2X to remove mutations in the background genotype and (iii) rescue the deleted region in each mutant by injecting a wild-type copy of the gene. We were able to repeat the experiment in 11 of 14 strains, backcross 11 of 14 strains and rescue 7 of 14 strains. For most lines, repeating the experiment many times dropped the average survival to well below 50%, suggesting a high false-positive rate in our initial screen. Additionally, we noticed that backcrossing the lines led to high variability in the data. One line that had high survival (*flp-3*), had to be excluded because backcrossing it reduced its survival to N2 levels, and fosmid rescue did not reduce its high survival. This indicated that, at least for this line, background genotype led to a false positive hit in the initial screen.

Since we did not backcross all the lines before initially testing them, it is possible that by the same rationale, we may have missed potential hits due to background genetic contamination. Despite this limited approach, one of the candidates, *nlp-40*, was able to pass all three tests of the secondary screen (Fig. 3.8c and 3.8d). However, *nlp-40* mutant survival hovers around 50-60% despite backcrossing, suggesting that it is only partially responsible for the anoxia survival phenotype of *unc-31*. It is likely that more than one neuropeptide regulates anoxic survival in *C. elegans*.

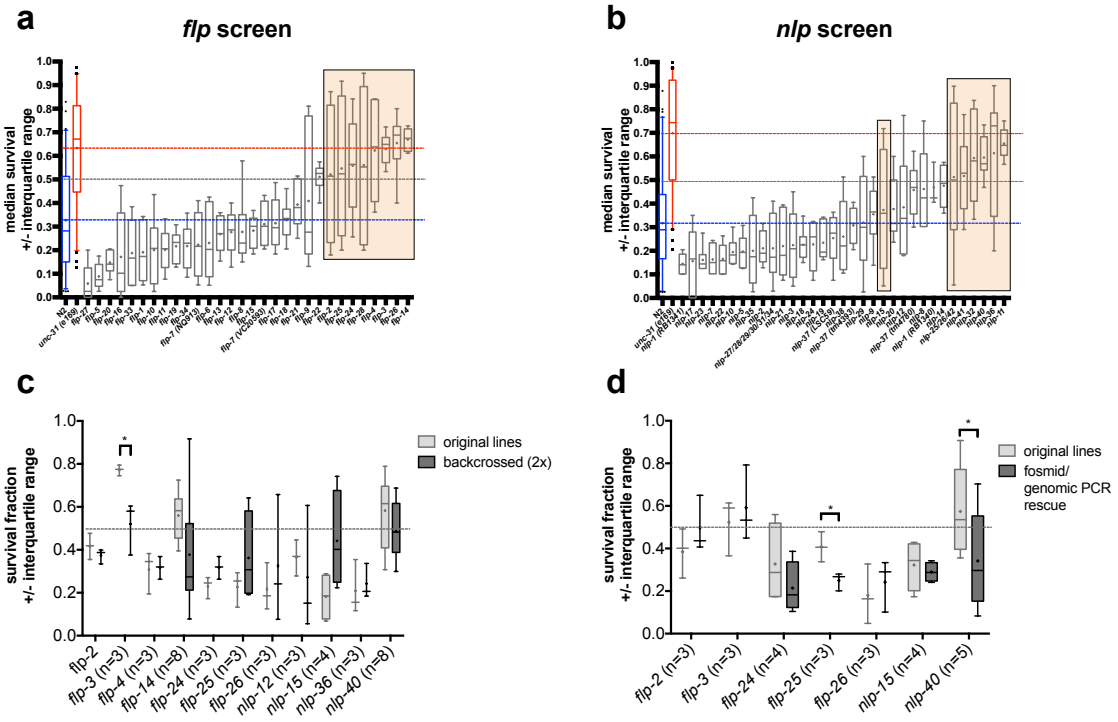


Figure 3.8. Screen for *flp* and *nlp* neuropeptides regulating survival under anoxia. a) Survival of 31 strains containing deletions of individual *flp*'s after 48h anoxia (n = 2 independent experiments). b) Survival of 33 strains comprising 36 deletions of individual *nlp*'s after 48h anoxia (n=2). Strains highlighted in the orange box in (a) and (b) were selected as candidates for further screening. c) Survival of candidate *flp*'s and *nlp*'s before and after 2X backcrossing to N2 (n=3-9). d) Survival of candidate *flp*'s and *nlp*'s before and after rescue with a fosmid or a PCR fragment containing the genomic locus (n=3-5). Data were analyzed by a two-tailed paired t-test for each *flp/nlp*. Data appear as 'boxes' instead of 'lines' if the experiment was conducted more than 3 times. *p <0.05.

Discussion

C. elegans employs neuropeptides to regulate a wide range of physiological functions. They are involved in many behaviors such as, among others, locomotion (Hums et al., 2016), sleep (Nelson et al., 2013), feeding (Cheong et al., 2015), egg-laying (Ringstad and Horvitz, 2008), mechanosensation (Hu et al., 2011) and chemosensation (Cohen et al., 2009). Genes encoding neuropeptides are expressed widely throughout the nervous system but are also found in non-neuronal tissue such as intestine, the vulval hypodermis and the gonads. They act both locally at synapses and over long distances as hormones to coordinate these behaviors within and between tissues (Rabinowitch et al., 2016).

Here, we establish a novel role for neuropeptides in regulating a specific stress – extreme oxygen deprivation – in developing *C. elegans*. We confirm and expand on previous work (Flibotte et al., 2014) to demonstrate that loss of neuropeptide synthesis by *egl-3* and *egl-21*, as well as loss of neuropeptide secretion by *unc-31*, lead to increased survival when exposed to anoxia. This protective effect of neuropeptide loss is specific to anoxic stress (Fig. 3.3). Hence, neuropeptide signaling makes worms vulnerable to anoxic insult in particular. We found that neuropeptide synthesis is necessary in the nervous system for this effect (Fig.3.5), is independent of insulin signaling and does not rely on established stress-resistance and longevity-promoting genes (Fig. 3.6).

Cell autonomous vs cell non-autonomous actions of neuropeptides

The insulin signaling pathway is known to regulate stress-resistance and longevity in worms, flies and even mammals (Holzenberger et al., 2002; Broughton et al., 2005; Henis-Korenblit et al., 2010). DAF-2 is the insulin/insulin-like growth factor

receptor homolog in worms. Insulin-like peptides can bind and activate DAF-2, which initiates an intracellular phosphorylation signaling cascade ultimately leading to the sequestration of the transcription factor DAF-16 (worm homolog of FOXO) to the cytosol, thus preventing transcription of DAF-16 targets. DAF-2 loss of function leads to increased lifespan and increased tolerance to many stressors including heat, UV radiation, oxidative stress, hypoxia and anoxia (G J Lithgow, 1995; Murakami and Johnson, 1996; Honda and Honda, 1999; Scott et al., 2002; Mendenhall et al., 2006b). We show here that neuropeptide-mediated regulation of survival against anoxia is independent of the DAF-2/DAF-16 pathway. DAF-2 expression in neurons and muscles, but not intestines, rescues the extreme hypoxic (<0.3%) survival benefit of *daf-2* mutant worms (Scott et al., 2002). Our data shows that neuropeptide signaling is necessary in neurons and acts in a parallel pathway to DAF-2/DAF-16, which suggests that the *daf-2* mediated survival benefit must be due to its expression in muscles. If the DAF-2/DAF-16 pathway acts in non-neuronal tissues for regulating response to anoxia, it would argue for two separate, cell autonomous regulatory pathways influencing survival under anoxic stress. Future studies need to address tissue-specific regulation of anoxia by DAF-2/DAF-16.

Neuropeptide-mediated regulation of behaviors can also occur in a cell non-autonomous fashion (Shao et al., 2016). Here, we find that for inducing sensitivity to anoxia, neuropeptide synthesis is necessary in neurons. The BAG sensory neurons in worms are responsible for detecting a decrease in environmental oxygen. However, surviving anoxic stress during development does not require neuropeptide signaling from BAG neurons (Flibotte et al., 2014), since *egl-3* null worms with BAG-specific rescue of *egl-3* continue to survive anoxia similar to *egl-3* null worms. The neuropeptide *flp-17* is primarily expressed in BAG neurons (Ringstad and Horvitz, 2008). In this study, we find

that loss of *flp-17* does not protect L4 worms from anoxia (Fig.3.8a), further suggesting that neuropeptide-mediated regulation of survival under anoxic stress acts in non-BAG neurons. However, where the neuropeptides act remains to be determined.

We describe *nlp-40* as a partial regulator of the worm response to anoxia. The only published paper about *nlp-40* describes its expression to be limited to the intestine (Wang et al., 2013), which does not agree with our results of neuropeptides in anoxic regulation. There are two ways to reconcile the differences in our findings: first, it could be that the *nlp-40* mediated regulation of anoxic survival is independent of *egl-3* and *unc-31*. Perhaps *nlp-40* is processed by a proprotein convertase other than *egl-3*. There are 5 different proprotein convertases in *C. elegans*, and their expression and function remain to be fully understood. Second, it is likely that the reporter construct used in the study by Sieburth *et al* did not fully capture *nlp-40* expression. Finally, the putative receptor for *nlp-40* is *aex-2* (Wang et al., 2013), which is thought to be expressed in GABAergic neurons, and loss of function mutants of *aex-2* are smaller in size and have a pale appearance similar to *nlp-40* mutants. Studying *aex-2* mutants under anoxia may also provide insight into the mechanism of action and cell-type specificity of *nlp-40* function. Understanding which cells neuropeptides act on will provide insight into the mechanism by which peptides induce anoxic sensitivity in worms, and elucidate their local vs. long range actions for modulating physiological responses.

Metabolism and anoxia

Worms enter a hypometabolic, 'suspended animation' state when exposed to anoxia, wherein they dramatically reduce their metabolic rate (Van Voorhies and Ward, 2000; Padilla et al., 2002). In the absence of oxygen, energy consumption occurs via breakdown of carbohydrates instead of fats. In fact, carbohydrate stores are depleted by

two-thirds in adult animals exposed to 24 hours of anoxia (Föll et al., 1999), and worms mutant for the glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase (*gpd-2* and *gpd-3*), are sensitive to anoxia (Mendenhall et al., 2006a). ATP/AMP levels are reduced in embryos exposed to anoxia (Padilla et al., 2002). Consistent with this, worms mutant for AMPK (*aak-2*), the master sensor of ATP/AMP, are also sensitive to anoxia, as shown previously in adult worms (LaRue and Padilla, 2011) and here in L4 worms. Further, a lipid synthesis promoting transcription factor, SREBP1 is required for fatty acid accumulation (which requires oxygen) *after* anoxia (Taghibiglou et al., 2009b). Ceramides are important components of the lipid bilayer in cells, and HYL-2, a short chain ceramide synthase, is essential for surviving anoxia (Menuz et al., 2009), suggesting a role for lipids *before* anoxia as well. Additionally, diet and nutrition also contribute to anoxic survival, as worms fed a higher carbohydrate food source survive long-term anoxia better (LaRue and Padilla, 2011). Mutants for *nlp-40* are shown to deficient in their defacation cycle (Wang et al., 2013) and have a pale appearance, presumably due to intestinal swelling and altered fat deposits. Together, these observations suggest an important, but incompletely understood contribution of metabolism in regulating survival from long-term anoxia exposure.

We find that survival promoted by loss of neuropeptide signaling is completely suppressed by loss of the ATP/AMP sensor AMPK as well as the short-chain ceramide synthase HYL-2. AMPK and ceramide synthases are important modulators of cellular metabolism and cell membrane integrity. What does the lethality of *neuropeptide mutant;aak-2* and *neuropeptide mutant;hyl-2* tell us about the role of cellular metabolism in anoxia? It is interesting to note that *hyl-2* loss of function also suppresses the survival benefit induced by *daf-2* in L4 and young adult worms (Menuz et al., 2009; Garcia et al., 2015). Similarly, loss of *aak-2* significantly reduces the survival benefit of *daf-2* animals

(LaRue and Padilla, 2011). Our work here shows that EGL-3/UNC-31 mediated neuropeptide signaling and DAF-2/DAF-16 mediated insulin signaling are independent pathways in regulating anoxia survival. AMPK and HYL-2 can thus suppress the effects of two independent pathways involved in anoxia. These two genes may thus act as master regulators of energy metabolism in the cell acting upstream of these two pathways, further implying that cellular energy is critical to surviving anoxia.

Enhancing understanding of neuropeptides and anoxia

While this work reveals a new role for neuropeptide signaling, it also opens up many potential directions for future study. First, we don't know why neuropeptide signaling would make worms sensitive to anoxia. It would be beneficial to understand how much energy neuropeptide synthesis and secretion requires. Given the important role of hypometabolism to surviving anoxia, perhaps the cell cannot afford the energy consumed by neuropeptide signaling. Second, we don't know *when* neuropeptide signaling is necessary for inducing anoxic sensitivity in worms. Is neuropeptide signaling needed before worms are exposed to anoxia or during the recovery from anoxia? Given that many N2 worms already appear dead immediately after re-exposure to oxygen, we would hypothesize that neuropeptide signaling is at least in part necessary before and/or during exposure to anoxia. The *egl-3, egl-21* and *unc-31* mutants we use have loss of function in these genes throughout their life. In order to address this timing issue, constitutive mutants that lose gene function at different stages of life, and at different time points before, during and after anoxia will need to be generated. Third, while we rule out many canonical stress resistance pathways in contributing to anoxia, we do not know how neuropeptides lead to anoxic sensitivity. One pathway that remains unexplored in this context is the unfolded protein response (UPR) pathway, which is

implicated in different stress responses, including anoxia (Kaufman and Crowder, 2015). Neuropeptide signaling may play a role in regulating the UPR, thus regulating the response to anoxia.

Conclusion

This work establishes a novel role for neuropeptide signaling in *C. elegans* response to extreme oxygen deprivation, thereby expanding on genetic and molecular regulation of anoxic stress in worms. It suggests that cell non-autonomous regulation may play a key role of the response to anoxia that is independent of known stress resistance pathways in worms.

Methods

Worm husbandry

C. elegans were cultivated at 20 degrees Celsius on nematode growth medium (NGM) agar surface, unless otherwise stated. NGM plates were seeded with a lawn of the *E. coli* strain OP50 as the food source, except in RNAi experiments.

Worm Strains

Strains used in this study are listed in Table 3.1. Double mutants were made using standard genetic techniques. Mutations were confirmed by PCR genotyping, and the primers used are also listed in Table 3.1.

Anoxia assay

Worms were synchronized by hypochlorite treatment and eggs were placed on fresh plates. For each experiment, 30-50 synchronized L4 animals from each genotype were placed on three fresh plates. Each plate was placed in a separate anerobic biobag (Becton Dickson, catalog # 261216) along with appropriate co-bagged controls. The bags were sealed and an anoxic environment (<0.1% O_2) was generated by palladium catalyst mediated consumption of ambient oxygen in the bag (per manufacturer instructions). A resazurin indicator was included in each bag to confirm oxygen deprivation. Unless otherwise stated, worms were placed under anoxia for 48h, after which the bags were opened, and the resazurin indicator confirmed reintroduction of ambient oxygen in the bag. Anoxia exposure was also confirmed by 'stunned' phenotype of worms once the bags were opened ((Padilla et al., 2002; Flibotte et al., 2014)). Any bags that failed to achieve anoxia according to the indicator and/or due to the absence

of ‘stunning’ were excluded from the experiment. Animals were allowed to recover for 24h after anoxia, after which dead and alive animals were manually scored. Animals were considered alive if they were moving, pumping their pharynx and/or responded to light touch. Each experiment thus had three technical replicates (3 bags), and experiments were generally repeated 3-6 times. The average survival fraction across experiments was reported.

Heat shock assay

Synchronized L4 worms were picked onto fresh plates, sealed with parafilm and placed in a plastic bag submerged in a 34 degree Celsius water bath. Individual plates were removed after 5 hours and 12 hours to manually score living/dead animals and promptly returned to the water bath. Animals were scored alive if they were moving, pumping their pharynx and/or responded to light touch.

ER stress assay

NGM plates were supplemented with 5mg/mL tunicamycin or equivalent DMSO vehicle (0.05%) and seeded with OP50 bacteria. Synchronized L4 worms raised on regular OP50 plates were placed on vehicle or tunicamycin plates and lifespan of worms on either plate was determined by scoring live/dead animals daily.

UV stress assay

To determine ‘germ cell’ survival, young adult animals were irradiated with 0, 80 and 120 kJ/m^2 UV-B and allowed to recover for 24h. Survival of the progeny (eggs laid for ~3 hrs) of the irradiated adults was then determined by counting dead and alive eggs. Average results across two independent experiments are presented. In each

experiment, survival on four replicate plates was determined (the offspring of 16-25 irradiated animals). The *xpa-1* mutant, necessary for nucleotide excision repair, was included as a positive control to demonstrate UV toxicity.

Larval survival was determined by irradiating L1 larvae and scoring the percentage of animals that arrested/died or survived (developed into L4/adult) 48 hours later. Average results across two independent experiments are presented. In each experiment, survival of three replicate plates, each containing between 40 – 160 L1 larvae, was determined. The *xpa-1 (ok698)* mutant was included as a positive control (Lans et al., 2010).

Hypercarbia assay

Carbon dioxide was introduced into a sealed chamber fitted with a CO_2 controller ($ProCO_2$ from BioSpherix, Inc) until its concentration was 10% of room air, as measured by a CO_2 sensor in the chamber. A separate sensor measured O_2 concentration, which was 18% of room air. Synchronized L4 worms were placed in this chamber for 48 hours, after which they were removed and assessed for a 'stunned' phenotype and survival.

RNAi experiments

NGM plates were seeded with *E. coli* expressing empty vector (L4440) or *egl-3* RNAi-encoding (C51E3.7) plasmids in order to perform feeding RNAi experiments. We tested worm strains sensitized to import dsRNA from the bacterial source, and induce RNAi against the target gene in either all neurons or specific subsets of neurons to achieve cell-type specific knockdown of *egl-3*. The strains used were: TU3311 (pan-neuronal knockdown), XE1581 (cholingergic neuron knockdown), XE1582 (glutamatergic neuron knockdown), XE1375 (GABAergic neuron knockdown) and

XE1474 (dopaminergic neuron knockdown). Worms from each of these strains were placed on either empty vector or *egl-3* RNAi plates for 3-4 generations to ensure successful knockdown of *egl-3*. For anoxia experiments, worms were placed in anerobic biobags for 54h and allowed to recover for 24h before scoring survival.

Neuropeptide screen

We assembled a list of all *nlp*'s and *flp*'s (wormbook.org) and interrogated Wormbase (wormbase.org) to identify mutants that were putative null. This list of neuropeptide mutants was obtained from either the CGC or the Japanese Consortium. We posited that deletion mutants would have the most severe loss of function phenotype. Any flp or nlp that did not have a deletion mutant available was excluded from the screen. Using this criterion, we were able to test 29 of 31 total flps (all but flp-23 and flp-32) and 36 of 42 total nlps (all but *nlp-4*, *nlp-6*, *nlp-13*, *nlp-16*, *nlp-33* and *nlp-39*). For the initial screen, each strain was tested two independent times, each time with three bags and with co-bagged N2 and *unc-31* worms. The average survival of each strain across these 6 bags was used to narrow candidates for the secondary screen. Any stain with >50% average survival was selected for secondary screening. In the secondary screen, strains were (i) tested in 3 additional independent experiments, (ii) backcrossed 2x to N2 and retested, and (iii) subjected to rescue experiments using available fosmid DNA for each mutant (Source Bioscience, Nottingham UK). Fosmids were injected at 160ng/uL along with pmyo-2::mCherry as a coinjection marker. For *nlp-40* rescue experiments, the genomic rescue line used (OJ949) was generously donated by Dr. Derek Sieburth (Wang et al., 2013).

Statistics

Data were analyzed using Prism (GraphPad Software, La Jolla, CA). Significant differences between two groups were determined using paired Student's t-test (two-tailed). Significant differences within groups greater than two were determined using one-way ANOVA followed by Tukey's test for multiple comparisons. Survival curves were analyzed using the log-rank (Mantel-Cox) test for significance. For all tests, the significance threshold was set to $p < 0.05$.

Table 3.2: A list of worm strains used in this study, along with their genetic description and primers used for genotyping (where applicable)

Strain	*gene(allele)/genotype*	PCR primers
N2	*wild-type, Bristol*	
CB928	*unc-31(e928)*	
VC671	*egl-3(ok979)*	Fwd Primer: agtccaactccattcatttgc Rev Primer (outer): aattccagaactaaggacacg Rev Primer (inner): atctctacggaagatgcacc
VC461	*egl-3(gk238)*	Fwd Primer: ccattggagaaagtggaagc Rev Primer (outer): aagccagtttgacttttcagc Rev Primer (inner): ttcccacggaactcgatgc
KP2018	*egl-21(n476)*	
JK2868	*qIs56[(lag-2::GFP) + unc-119(+)]*	
MT14525	*gcy-9(n4470)*	
RB864	*xpa-1(ok698)*	
MT15434	*tph-1(mg280)*	
MT15620	*cat-2(n4547)*	
MT13113	*tdc-1(n3419)*	
MT9455	*tbh-1(n3247)*	
RK132	*egl-3(ok979);$P_{F2B23.3}$::egl-3*	
RK133	*egl-3(ok979);P_{ges-1}::egl-3*	
RK134	*egl-3(ok979);P_{myo-3}::egl-3*	
TU3311	*uIs60[unc-119p::YFP + unc-119p::sid-1]*	
XE1582	*wpSi11[eat-4p::rde-1::SL2::sid-1 + Cbr-unc-119(+)]; eri-1(mg366); rde-1(ne219); lin15(n744)*	

XE1375	*wpIs36[unc-47p::mCherry]; wpSi1[unc-47p::rde-1::SL2::sid-1 + Cbr-unc-119(+)]; eri-1(mg366); rde-1(ne219); lin15(n744)*	
XE1581	*wpSi10[unc-17p::rde-1::SL2::sid-1 + Cbr-unc-119(+)]; eri-1(mg366); rde-1(ne219); lin15(n744)*	
XE1474	*wpSi6[dat-1p::rde-1::SL2::sid-1 + Cbr-unc-119(+)]; eri-1(mg366); rde-1(ne219); lin15(n744)*	
GR1307	*daf-16(mgDf50)*	Fwd Primer: ctctctctgtttctccccgc Rev Primer (outer): acggacactgttcaactcgt Rev Primer (inner): gcgagagtagcgatgttgga
ZG31	*hif-1(ia4)*	Fwd Primer: gaatgccgcatgttccgatc Rev Primer (outer): cggagcagcaatacaagatg Rev Primer (inner): atggtgtcttcagtccatacc
PS3551	*hsf-1(sy441)*	
FX03411	*skn-1(tm3411)/nT1[qIs51]*	Fwd Primer (outer): ctccgaaatctggaacgcc Fwd Primer (inner): gagattccgaagagaggcg Rev Primer: caggacgtcaacagcagac
FX0850	*nsy-1(tm850)*	Fwd Primer: gcgattccaggaaatgcacg Rev Primer (outer): ctccgtatcacactgcttatgg Rev Primer (inner): tcaacaagtgccacgtcagc
FX02031	*hyl-2(tm2031)*	Fwd Primer (outer): cgtactaccatttgtataccg Fwd Primer (inner): tctcacttctggtctccgg Rev Primer: cgtcgtcgcaacatctcct
RB1498	*hyl-2(ok979)*	Fwd Primer (outer): cgtactaccatttgtataccg Fwd Primer (inner): tctcacttctggtctccgg Rev Primer: cgtcgtcgcaacatctcct
RB754	*aak-2(ok524)*	Fwd Primer: cccaatctgccaaatactgac Rev Primer (outer): cacgaccatacatcaacttcg Rev Primer (inner): cattgttctgctcatcgagc
RK135	*unc-31(e928); daf-16(mgDf50)*	
RK136	*egl-3(e928); daf-16(mgDf50)*	
RK137	*unc-31(e928); hif-1(ia4)*	
RK138	*unc-31(e928); hsf-1 (sy441)*	
RK139	*egl-3 (ok979); skn-1(tm3411)/nT1*	
RK140	*unc-31(e928); nsy-1(tm850)*	
RK141	*unc-31(e928); hyl-2(tm2031)*	
RK142	*unc-31(e928); hyl-2(ok979)*	
RK143	*egl-3(ok979); hyl-2(tm2031)*	
RK144	*unc-31(e928); aak-2(ok524)*	
NY16	*flp-1(yn4)*	

VC2591	*flp-2(ok3351)*	Fwd Primer: gcggattgaatcgttttccg Rev Primer (outer): ttctcgatgcggaaagttgc Rev Primer (inner): tttcctcgtccttctcgcc
RK145	*flp-2(ok3351)* *2X backcrossed*	Fwd Primer: gcggattgaatcgttttccg Rev Primer (outer): ttctcgatgcggaaagttgc Rev Primer (inner): tttcctcgtccttctcgcc
VC2497	*flp-3(ok3265)*	Fwd Primer: gccaaatggagcactgtcg Rev Primer (outer): tttggtagcgtcgggtagg Rev Primer (inner): aatgagatttggaaagctgc
RK146	*flp-3(ok3265)* *2X backcrossed*	Fwd Primer: gccaaatggagcactgtcg Rev Primer (outer): tttggtagcgtcgggtagg Rev Primer (inner): aatgagatttggaaagctgc
NY226	*flp-4(yn35)*	Fwd Primer: cacatgccagtctgcctaca Rev Primer (outer): ttgttcggtcaagactcggc Rev Primer (inner): ttgagacagagacgtgacgc
RK147	*flp-4(yn35)* *2X backcrossed*	Fwd Primer: cacatgccagtctgcctaca Rev Primer (outer): ttgttcggtcaagactcggc Rev Primer (inner): ttgagacagagacgtgacgc
VC20382	*flp-5(gk320541)*	
VC2324	*flp-6(ok3056)*	
VC20593	*flp-7(gk951274)*	
NQ913	*flp-7*	
PT501	*flp-8(pk360)*	
RB2067	*flp-9(ok2730)*	
RB1989	*flp-10(ok2624)*	
FX02706	*flp-11(tm2706)*	
RB1863	*flp-12(ok2409)*	
NQ602	*flp-13(tm2427)*	
VC1957	*flp-14(gk1055)*	Fwd Primer: aggaaaaccggcaagcctag Rev Primer (outer): cggcgccataataacatctgc Rev Primer (inner): cgcccctgtttcctacttct
RK148	*flp-14(gk1055)* *2X backcrossed*	Fwd Primer: aggaaaaccggcaagcctag Rev Primer (outer): cggcgccataataacatctgc Rev Primer (inner): cgcccctgtttcctacttct
VC30176	*flp-15(gk960606)*	
FX04829	*flp-16(tm4829)*	
MT15933	*flp-17(n4894)*	
AX1410	*flp-18(db99)*	
RB1902	*flp-19(ok2460)*	
PT505	*flp-20(pk1596)*	
RB982	*flp-21(ok889)*	
VC3465	*flp-22(gk1201)/hT2*	
VC1971	*flp-24(gk3109)*	Fwd Primer (outer): ctaagcaggcatactacagg Fwd Primer (inner): aaccacgcaaaatattattcg Rev Primer: ttggcatgccgaaaaaaggg

RK149	*flp-24(gk3109)* *2X backcrossed*	Fwd Primer (outer): ctaagcaggcatactacagg Fwd Primer (inner): aaccacgcaaaatattattcg Rev Primer: ttggcatgccgaaaaaaggg
VC1982	*flp-25(gk1016)*	Fwd Primer: tttgacccaattcacttgacg Rev Primer (outer): cttgattggacattgcgcga Rev Primer (inner): gcactccttttttgcttcgg
RK150	*flp-25(gk1016)* *2X backcrossed*	Fwd Primer: tttgacccaattcacttgacg Rev Primer (outer): cttgattggacattgcgcga Rev Primer (inner): gcactccttttttgcttcgg
VC3017	*flp-26(gk3015)*	Fwd Primer (outer): ggtttgtcatgatcggttgg Fwd Primer (inner): tcaagtttccctaatccccc Rev Primer: ttgaagtcttcattagctccg
RK151	*flp-26(gk3015)* *2X backcrossed*	Fwd Primer (outer): ggtttgtcatgatcggttgg Fwd Primer (inner): tcaagtttccctaatccccc Rev Primer: ttgaagtcttcattagctccg
VC2012	*flp-27(gk3331)*	
VC2502	*flp-28(gk1075)*	
VC2423	*flp-33(gk1038)*	
RB2269	*flp-34(ok3071)*	
RB1340	*nlp-1(ok1469)*	
RB1341	*nlp-1(ok1470)*	
FX01908	*nlp-2(tm1908)*	
FX03023	*nlp-3(tm3023)*	
RB1609	*nlp-5(ok1981)*	
FX02984	*nlp-7(tm2984)*	
VC1309	*nlp-8(ok1799)*	
FX03572	*nlp-9(tm3572)*	
FX06232	*nlp-10(tm6232)*	
VC40940	*nlp-11(gk891596)*	
RB607	*nlp-12(ok335)*	Fwd Primer: tttttgcaacacagtcgccg Rev Primer (outer): tgatgttcagtacggtctgc Rev Primer (inner): tcgattggtggtttggatgg
RK152	*nlp-12(ok335)* *2X backcrossed*	Fwd Primer: tttttgcaacacagtcgccg Rev Primer (outer): tgatgttcagtacggtctgc Rev Primer (inner): tcgattggtggtttggatgg
VC1108	*nlp-14(ok1517)*	
VC1063	*nlp-15(ok1512)*	Fwd Primer: tttatgccgtgtcttatgtcc Rev Primer (outer): gccaggctgtcctattacg Rev Primer (inner): tcgtttgacgcacttcttcg
RK153	*nlp-15(ok1512)* *2X backcrossed*	Fwd Primer: tttatgccgtgtcttatgtcc Rev Primer (outer): gccaggctgtcctattacg Rev Primer (inner): tcgtttgacgcacttcttcg
VC30240	*nlp-17(gk960682)*	
RB1372	*nlp-18(ok1557)*	
VC40619	*nlp-19(gk951891)*	

RB1396	*nlp-20(ok1591)*	
FX02569	*nlp-21(tm2569)*	
FX06351	*nlp-22(tm6351)*	
FX05531	*nlp-23(tm5531)*	
FX02105	*nlp-24(tm2105)*	
VC30122	*nlp-25; nlp-26; nlp-42(gk963304)*	
VC40271	*nlp-27; nlp-28; nlp-29; nlp-30; nlp-31; nlp-34(gk963785)*	
FX01931	*nlp-29(tm1931)*	
VC40420	*nlp-32(gk628779)*	
FX05434	*nlp-35(tm5434)*	
FX05156	*nlp-36(tm5156)*	Fwd Primer: tgagagacccaccagattgc Rev Primer (outer): gggaccaagcttcatgtcga Rev Primer (inner): tgttgcttcaagtcgacgga
RK154	*nlp-36(tm5156) 2X backcrossed*	Fwd Primer: tgagagacccaccagattgc Rev Primer (outer): gggaccaagcttcatgtcga Rev Primer (inner): tgttgcttcaagtcgacgga
LSC59	*nlp-37(tm4393); lstEx24*	
FX04393	*nlp-37(tm4393)*	
FX04780	*nlp-37(tm4780)*	
VC2357	*nlp-38(ok2330)*	
FX04085	*nlp-40(tm4085)*	Fwd Primer: caagtcgccacatatcccg Rev Primer (outer): ccacgcgaccattctcttc Rev Primer (inner): gccgctgaaagttgtgttgt
RK155	*nlp-40(tm4085) 2X backcrossed*	Fwd Primer: caagtcgccacatatcccg Rev Primer (outer): ccacgcgaccattctcttc Rev Primer (inner): gccgctgaaagttgtgttgt
VC20740	*nlp-41(gk963053)*	
RK156	*flp-2(ok3351); WRM061B_H10; pmyo2::mCherry*	
RK157	*flp-3(ok3265); WRM061B_H10; pmyo2::mCherry*	
RK158	*flp-24(gk3109); WRM0636B_B10; pmyo2::mCherry*	
RK159	*flp-25(gk1016); WRM0627A_A09; pmyo2::mCherry*	
RK160	*flp-26(gk3015); WRM0634D_E12; pmyo2::mCherry*	
RK161	*nlp-15(ok1512);*	

	WRM066C_H12; pmyo2::mCherry	
OJ949	*nlp-40(tm4085); vjEx330[Pttx-3::RFP, nlp-40 genomic DNA(~8.8kb)]*	

CHAPTER 4

General Discussion

This body of work highlights the important role of metabolism in two different scenarios: (i) in **Chapter 2**, I use a mouse model to study the contribution of a change in metabolism at the organism-level to ALS progression and (ii) in **Chapter 3**, I use *C. elegans* to study how neuropeptides may contribute to surviving an environmental stress (severe oxygen deprivation) during which worms enter a hypometabolic state. Each of these projects generated some key insights into disease and stress and raised interesting questions for further investigation, some of which I highlight below.

Bridging cellular energy homeostasis and organism-wide metabolic defects in ALS

The findings presented here focus on organism wide metabolic perturbations in ALS. However, a key feature of all neurodegenerative diseases is the presence of mitochondrial dysfunction in the brain and spinal cord (Lin and Beal, 2006). It remains to be understood how nervous system mitochondrial dysfunction at the cellular level and organism level metabolic abnormalities are related. Does energy imbalance at the cellular level, caused by dysfunctional mitochondria, contribute to organism level changes in weight and glucose homeostasis? Alternatively, is organism level metabolic dysfunction uncoupled from neuronal mitochondrial damage, and can it be targeted independently to alter the course of disease progression?

In familial ALS, as well as the G93A SOD1 mouse model used in the studies here, mutations in ubiquitously expressed genes such as C9orf72 and SOD1 lead to specific degeneration of motor neurons. It is not known why motor neurons are selectively vulnerable to damage. Mitochondrial damage is observed in neurons but also in non-neuronal tissues, such as liver and skeletal muscle (Nakano and Hirayama, 1987; Siklós et al., 1996; Sasaki and Iwata, 2007). There are two potential mechanisms by which cellular mitochondrial dysfunction and organism-wide hypermetabolism in ALS might be related. (i) Mutant SOD1 leads to peripheral tissue metabolic dysfunction, which, in aggregate, results in organism-wide metabolic phenotypes. (ii) The nervous system undergoes pathologic changes due to mutant SOD1, and through cell non-autonomous processes such as hormone signaling, leads to altered metabolism in peripheral tissue. In either scenario, the trigger is cellular-level abnormality due to mutant SOD1, which causes a cascade of events in both cell autonomous and non-autonomous ways. The overall, compounded effect of these abnormalities is whole body metabolic derangement. In addition to changes in ATP production, features of dysfunctional mitochondria in neurodegenerative diseases include generation of reactive oxygen species and alterations in the redox status of cells (Lin and Beal, 2006). It is possible that aberrant mitochondria influence cellular metabolism through any of these different ways. Regardless, these make mitochondrial dysfunction an attractive candidate for this trigger.

Figuring out the mechanistic link between mitochondrial defects at the cellular level and organism-wide hypermetabolic profile in ALS will be a key development in understanding disease progression.

Targeting peripheral tissue for hypometabolism

In these studies, MC4R was knocked out in the hypothalamus to induce hypometabolism in the G93A SOD1 background. Loss of MC4R, in addition to hypometabolism, also induces hyperphagia and increased fat deposits in these mice (Balthasar et al., 2005). The increased fat deposition makes mice lethargic and sedentary, and complicates interpretation of motor function in the ALS context. We were unable to dissociate its effects on feeding from its effects on metabolic rate. Our goal was to first generate mice that lacked MC4R globally in the G93A SOD1 background (G93A;$MC4R^{-/-}$), and then cross them to a Sim1-Cre mouse line to re-express MC4R in the paraventricular hypothalamus. As reported in work by Balthasar *et al*, this partial restoration of MC4R was able to reduce obesity in $MC4R^{-/-}$ mice by 60%, while maintaining their hypometabolic phenotype (Balthasar et al., 2005). We attempted to make this triple mutant cross in our mice, but did not achieve large enough brood sizes in both sexes to do the entire range of our experiments. Therefore, we studied the double mutant mouse containing a global knockout of MC4R in the G93A SOD1 background. Our grip strength data suggest that MC4R mutant animals, while obese and sedentary, can exert similar if not higher muscle force when compared to WT mice. Physiologically, however, it is difficult to tease apart these dual actions of MC4R, and practically, drugs targeting MC4R for obesity have met limited success due to a lack of *in vivo* efficacy, side effects and blood brain barrier penetrance (Fani et al., 2014).

It may, therefore, be beneficial to target peripheral sources of metabolic rate control. One interesting candidate for modulating disease is the gut microbiota, which harbor as many as 100 trillion diverse bacteria (Ley et al., 2006). Recently, the relationship between the microbiome and the CNS has begun to emerge (Sharon et al., 2016). For example, mice lacking the gut microbiome (termed germ-free mice) have

impaired spatial and object recognition, altered expression of critical neuronal molecules such as subunits of the dopamine receptor, as well as the brain derived neurotrophic factor (Bercik et al., 2011; Heijtz et al., 2011; Möhle et al., 2016). They also show impairments in the blood brain barrier (Braniste et al., 2014). The role of the gut microbiota is much less understood in the context of neurodegenerative diseases. One study showed that the G93A SOD1 mouse has a presymptomatic increase in intestinal permeability and an altered gut microbiome compared to wild type mice (Wu et al., 2015). The same group did a follow up study and showed that administration of 2% butyrate, a by product of bacterial fermentation in the gut, reduced intestinal 'leakiness', decreased mutant SOD1 aggregation and increased lifespan by an average of 38 days in the G93A SOD1 mouse (Zhang et al., 2017). However, there is no report of how the altered microbiome of G93A SOD1 mice might alter whole body metabolism. It might be worthwhile to do fecal transplantation studies in these mice, where healthy microbiota are transplanted into the ALS-like mice, and study changes in metabolism and disease progression.

In PD, there is a study of fecal transplantation that demonstrated the role the microbiome might play in motor dysfunction (Sampson et al., 2016). PD patients have an altered gut microbiome compared to healthy controls (Scheperjans et al., 2015). Sampson and colleagues showed that the presence of the microbiome led to manifestation of motor deficits as well as microglial activation in the caudate/putamen of mice overexpressing α-synuclein. This was dependent on short chain fatty acids produced by the gut bacteria. Antibiotic administration diminished the microbiome and led to improved motor pathology. Strikingly, compared to control mice transplanted with fecal microbiomes from healthy human donors, those transplanted with fecal microbiomes from PD patients developed significantly altered gut microbiomes of their

own. They also developed motor dysfunction compared to the control mice. Together, these studies demonstrate the important role of the microbiome in altering disease progression, and may act as a peripheral target to manipulate organismal metabolism as well.

Neuropeptide signaling and metabolism in C. elegans anoxia

Worms have the remarkable ability to survive near-complete oxygen deprivation. A hallmark of this phenotype is that worms enter a state of suspended animation where they cease most physiological functions including feeding, developing, reproducing and locomotion for the duration of the anoxic stress. In this state, they deplete a large amount of their carbohydrate reserves and decrease ATP production, indicating a hypometabolic phenotype. Our data also shows that *aak-2* (AMPK) mutants are hypersensitive to this insult, demonstrating a vital role for AMPK in regulating and directing appropriate energy consumption to survive the stress. The absence of AMPK continued to be fatal to worms in anoxia even in *unc-31* mutant worms, which normally are anoxia resistant. Work by others shows that loss of AMPK has a similar effect in the absence of *daf-2*, which too is anoxia resistant (Mendenhall et al., 2006a). This indicates that functional AMPK is essential to worm survival in anoxia, and acts as a 'bottleneck', effectively masking the contribution of other pathways such as neuropeptide and insulin signaling. Similarly, we confirm here that *hyl-2* (ceramide synthase) mutants are hypersensitive to anoxia and that this effect too masks that of anoxia tolerance in both *egl-3* and *unc-31* mutants. Ceramides are a class of lipid molecules that are essential for proper maintenance of cell membranes. Here, too, *hyl-2* loss was detrimental to worm survival even in the *daf-2* mutant background (Menuz et al., 2009), suggesting a broad suppression of anoxia tolerance-promoting pathways.

There is additional data suggesting a critical role for metabolic enzymes/transcription factors in anoxia tolerance. A key transcription factor essential for glucose metabolism and lipid/fatty acid synthesis is SREBP1. Levels of SBP-1, the worm homolog for SREBP1 (sterol regulatory element binding protein), increase with increasing duration of anoxia in developing (L3) worms (Taghibiglou et al., 2009b). Although the authors used different tools to generate anoxia than us, they found that 5 hours of anoxia induced lipid accumulation and increased body size in these worms, both of which were reversed by knocking down *sbp-1*. Further, loss of *gpd-2* and *gpd-3*, worm homologs of isoforms of the glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase, led to impairments in worm behavior after anoxia, and they act downstream of the *daf-2*/*daf-16* pathway (Mendenhall et al., 2006a). Otherwise protected *daf-2* mutants lost their survival benefit when *gpd-2* was also mutated.

How are all these genes, essential for various aspects of cellular metabolism, coordinating their activity under anoxia? Ceramides negatively regulate transcription of SREBP1 in mammalian cells (Worgall et al., 2002). If this is also true in worms, it might provide a potential explanation for the mechanism of *hyl-2* mutant sensitivity to anoxia. Perhaps, in the absence of *hyl-2,* there is an increase in SREBP1 expression and an increase in lipid accumulation that is toxic to worms. Further experiments are needed to explore these possibilities and clearly define these relationships with regard to their regulation of anoxic stress.

Understanding how oxygen deprivation alters metabolism and induces metabolic changes has important consequences for human health. In an elegant study, it was demonstrated that SREBP1 inhibition is protective in an *in vitro* model of focal ischemia (Taghibiglou et al., 2009a). *C. elegans* is an excellent tool to study genetic and

molecular interactions and is ideally suited to study these metabolic gene interactions in the context of oxygen deprivation.

Multifactorial response to anoxia in worms

Many genes and pathways are implicated in regulating the response to anoxic stress in worms, such as *daf-2* (insulin signaling), *nsy-1* (MAP kinase signaling), *rars-1* (protein translation), *skn-1* (oxidative stress response) and *hyl-2* (ceramide synthesis). To add to this growing list are genes described here – *egl-3*, *unc-31* and *egl-21* (neuropeptide signaling). We tested many of the above genes to see if neuropeptide signaling was dependent on their function for its effects in anoxia. We were unable to find any interaction, suggesting that most of these pathways acted in parallel to the neuropeptide signaling pathway. Hence, multiple pathways can potentially regulate the response to this stress in worms.

Previous work also suggests that there may be more than one mechanism by which neuropeptide signaling mediates survival in anoxia. Anderson and colleagues (Anderson et al., 2009) identified that suppressing protein translation (*rars-1* loss of function) makes worms resistant to extreme oxygen deprivation. This was mediated in part by induction of the unfolded protein response pathway, suggesting that other mechanisms are also recruited by protein translation to regulate survival after anoxia. Similarly, there may be small changes in multiple pathways as a result of disrupted neuropeptide signaling, which in sum lead to improved survival in anoxia.

This idea is also bolstered by the fact that we were unable to find a strong effect of a single neuropeptide in regulating anoxia susceptibility. Based on results from our screen, our best candidate is *nlp-40*, however loss of *nlp-40* only provides a partial increase in survival compared to N2 worms. This suggests the involvement of additional

neuropeptides in *egl-3* mediated anoxia tolerance. Further, our RNAi experiments show that knocking down *egl-3* in multiple neuronal subtypes continues to protect worms from anoxia, suggesting that many neuronal subtypes may be involved in this regulation. Overall, our data are consistent with the idea that multiple neuropeptides are released from multiple neuron-types to make worms sensitive to anoxia. Perhaps small downstream effects of these neuropeptides summate and make worms broadly vulnerable to anoxic insult. It may be essential to knock down/out *egl-3* in many neurons to induce an appreciable survival benefit.

Timing of neuropeptide signaling in anoxia

An important question that was not addressed in this dissertation is *when* inhibiting neuropeptide signaling is most protective to worms. In other words, when does neuropeptide activity make wild type worms sensitive to anoxia? We use genetic knockouts or RNAi to diminish neuropeptide signaling via *egl-3, unc-31* and *egl-21*, so these worms lack neuropeptide activity throughout their life. Is neuropeptide signaling requires before, during or after the anoxic exposure (or some combination of these) to sensitize worms to the stress? In future experiments, conditional knockouts or RNAi experiments could help to answer this question. Having this information may give us clues about the role of neuropeptides in modulating this phenotype, and more appropriately target therapeutic interventions if this were to be translational.

Another important observation in our work, which confirms observations by others, is that there is a developmental regulation of worm susceptibility to anoxia (Van Voorhies and Ward, 2000; Padilla et al., 2002). In our assay, L4 staged developing worms were sensitive to 48h of anoxia, while young adults were resistant. The major physiological events between these two stages of worm development are vulval

development, a period of lethargus and the final molt/cuticle development (Raizen et al., 2008; Schindler et al., 2014; Mok et al., 2015). Studying mutants in these pathways may clarify if disruption of these events contributes to the sensitivity of worms to anoxia during L4.

Protein translation, neuropepitde synthesis and anoxia

As mentioned earlier, Anderson *et al* showed that mutating aminoacyl-tRNA synthetases had a strong positive effect on worm survival in anoxia (Anderson et al., 2009). This suggests that decreasing protein translation is beneficial in worms. From an energetic perspective, observation is consistent with preserving ATP loads during anoxia and redirecting cell biology away from energy-expensive catabolic processes like protein translation (one of the primary roles of AMPK). In their study, the authors found that the effect of reducing protein translation was protective partially due to upregulation of the unfolded protein response (UPR) machinery.

One idea that we did not explore in our work, is if there is decreased neuropeptide translation and decreased neuropeptide signaling in the aminoacyl-tRNA synthetase mutants such as *rars-1*. In other words, if we tested double mutants bearing a loss of function in *egl-3*/*unc-31* as well as *rars-1*, would there be an additive survival benefit in anoxia, or would the double mutant be similar to either individual mutant? If there is no additive effect of the two mutations, they likely occur in the same genetic pathway, suggesting that a *rars-1* mutation may act to increase anoxia survival by decreasing neuropeptide signaling.

Additionally, is the UPR induced in neuropeptide signaling-deficient worms? We show that *egl-3* and *unc-31* mutant worms are sensitive to tunicamycin (a UPR inducer) and have reduced lifespan when raised on tunicamycin. This is a preliminary indicator

that neuropeptide mutant worms are not resistant to sustained tunicamycin-induced toxicity, but it is not known if the UPR is induced in *unc-31* and *egl-3* mutant worms at baseline or after anoxia. If neuropeptide signaling does not rely on the UPR for its effects on anoxia, it may help to partially explain the protection conferred by *rars-1* deletion.

Data variability

On a technical note, one of the major challenges in the anoxia experiments was the significant amount of biological variability in the data. N2 survival ranged from 10-80% and *unc-31* survival varied from 20-95% over 40-50 independent experiments of 48h anoxia in L4 worms. This made some data difficult to interpret, affected the results of our neuropeptide screen and necessitated many independent experiments to reliably conclude a biological effect. By and large, there was very little variation between the 3 technical replicates within an experiment, but the variability was substantial between different experiments. We tried to address the source of this variability by accurately staging animals at the same stage of L4 and positioning plates differently in the bags, but we were unable to find a systematic factor that affected the range of survival. However, there are some additional factors that may contribute to the variability that we did not explore. Some of these are: precise volume of agar in plates, gentleness of handling worms while placing them on plates, lot-to-lot variability in culture reagents, small changes in the incubator environment and age of food on the plate.

We know that preconditioning worms to a stressor like a different diet, a different growth temperature, or a short bout of anoxia enhances their survival under long term anoxia (Dasgupta et al., 2007; LaRue and Padilla, 2011). Could it be that the source of the variability comes from previous experience? Factors such as plate crowding, freshly seeded food plates (‘younger’ OP50 vs ‘older’ OP50) and contamination in an earlier

generation are some factors that can be readily controlled for in a systematic way. Minimizing variability will be a huge benefit to ease of conducting and interpreting future experiments.

Conclusion

A central theme bridging these two projects is that cell autonomous and non-autonomous factors regulate how an organism responds environmental stress and disease. In the case of ALS, we demonstrate that decreasing metabolic rate by loss of MC4R in the hypothalamus does not alter progression or outcome of ALS. However, we do not know if there are cellular level changes to mitochondrial health, and if this is different in the nervous system compared to muscles. In fALS, a mutation in a single gene in all cells in the body affects mitochondrial health in many cells, whole body metabolism but preferentially causes death of motor neurons. This points to cell autonomous cellular damage. However, it remains to be determined what the source of metabolic derangement in ALS is and how it relates to preferential death of motor neurons. As suggested earlier, one potential mechanism is that a cell non-autonomous factor, potentially released by energy starved neurons, signals to the rest of the body and causes whole body hypermetabolism.

In the case of anoxic stress, we establish that loss of neuropeptide signaling is beneficial to worms undergoing anoxia in a manner that is independent of many established stress-resistance pathways. There is likely a group of neuropeptides, of which *nlp-40* is one, that regulates this response, and the neuropeptides are secreted from the nervous system. This work demonstrates that soluble factors such as neuropeptides can act cell non-autonomously to regulate organism level response to an external stress.

This work sheds light on the bidirectional role of metabolism in disease and stress. Changes in metabolic rate can be an adaptive response to external stress, and can be modulated to impact disease in sophisticated and specific ways.

APPENDIX

Some additional observations of anoxia-related *C. elegans* physiology in wild type and neuropeptide signaling mutants

Shachee Doshi[1,2], Emma Price[1], Robert G. Kalb[1,2,3]

[1]Division of Neurology, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA 19104,
[2]Neuroscience Graduate Group and [3]Department of Neurology, University of Pennsylvania, Philadelphia, PA 19104

Lifespan of neuropeptide signaling mutants

In Chapter 3, the readout for all our experiments was survival of worms 24h after recover from a 48h anoxic insult. However, this did not tell us anything about the biology of the worms after this 24h post-anoxia window. We were curious to see if the various neuropeptide signaling mutants we used in Chapter 3 had any differences in lifespan pre- and post- anoxia. We placed L4 worms on plates supplemented with 5-Fluoro-2′-deoxyuridine (FUdR) at a final concentration of 200uM, and measured their survival on these plates either at baseline, or that of surviving worms after 48h of anoxia. FUdR is a potent inhibitor of DNA synthesis, and was used here to prevent eggs laid by test animals from hatching. In this manner, we avoided 'contamination' by progeny and limited repeated handling and moving the test worms.

We found that all the neuropeptide mutants – *egl-3* (*ok979*), *egl-3* (*gk238*), *egl-21* (*n476*), *unc-31* (*e928*) – were significantly longer lived than the wild type line (Fig A1a). Additionally, we measured the lifespan of surviving worms after the 48h anoxic insult, and found that they continued to be longer-lived post anoxia (Fig. A1b). Interestingly the lifespan of all lines, including N2, was higher post anoxia than that at baseline. Hence, L4 staged neuropeptide mutant worms have significantly greater survival compared to N2 after 48h anoxia (see Chapter 3), and the worms that survive anoxia have a greater lifespan compared to N2 worms. Additionally, anoxia may act as a stressor that allows worms that can survive the stress to have increased lifespan than those that do not experience this stress.

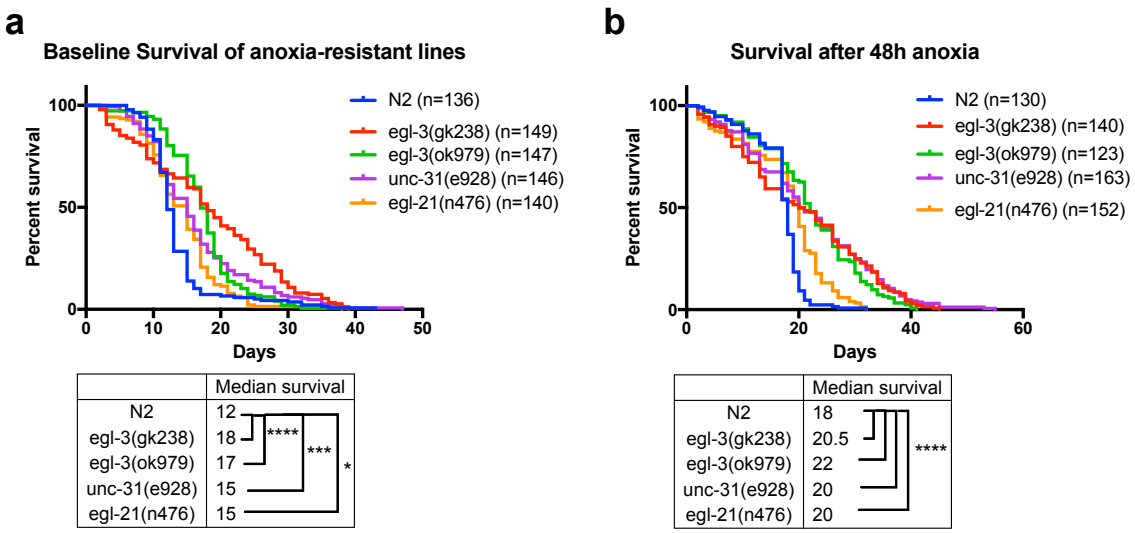


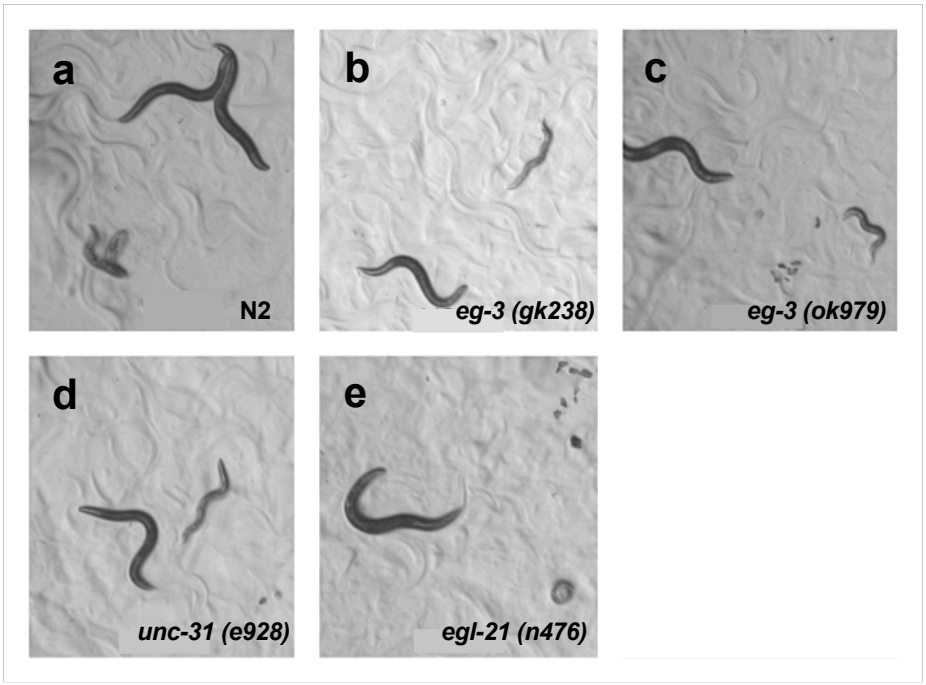
Fig. A1. Survival of anoxia tolerant neuropeptide mutants. Lifespan of mutants in *egl-3*, *egl-21* and *unc-31* are plotted against N2 lifespan a) at baseline and b) after 48h anoxia. Data are analyzed using the Mantel-Cox test for survival curve comparisons. *p < 0.05, ***p < 0.001, ****p < 0.0001

'Healthspan' of neuropeptide signaling mutants

The lifespan of different worm lines tells us about how long they live, but does not tell us about how *well* they live. We attempted to get a measure of worm 'health' post anoxia. To do this, we scored anoxia-surviving worms 6 days after they were removed from the anoxic biobags. We looked to see if worms had developed to adulthood, had sexually matured and had normal locomotion on the plates. If they did, we scored these as 'healthy'. The worms that did not achieve these benchmarks were scored as 'unhealthy'. These 'unhealthy' worms generally had one or more of the following phenotypes, that did not change throughout their life: (i) they were much smaller than normal adult worms, (ii) they had aberrant locomotion and stayed in the same spot on the plate, (iii) they did not bear eggs, (iv) they appeared very pale, seemingly lacking the characteristic dark color of adult worms due to fat deposits, (v) they had a 'sheath'

surrounding the entire body, presumably the cuticle that they were unable to shed during molting.

If the worms met any of these phenotypic criteria, we scored them as ‘unhealthy’. Images of smaller, paler worms without eggs alongside ‘healthy’ worms are presented in Fig. 2a-e. We also noticed that these worms tended to have shorter lifespan compared to the ‘healthy’ worms on the same plate. These ‘unhealthy’ animals were present in all the strains, including N2. However, there were strikingly fewer ‘unhealthy’ N2 worms post-anoxia than all the other strains (Fig. A2f). This indicates that although a smaller fraction of N2 worms survive the initial anoxic insult, those that do have a ‘healthier’ lifespan. Conversely, a higher proportion of neuropeptide signaling mutant worms survive the initial anoxic insult, but many of them are ‘unhealthy’ after anoxia. Additionally, the lifespan data in Fig A1b contains neuropeptide signaling mutants that are both ‘healthy’ and ‘unhealthy’. This suggests that the anoxic insult generates two populations of worms in these mutants, ones that are shorter-lived and sicker, and ones that are very long-lived and healthier.



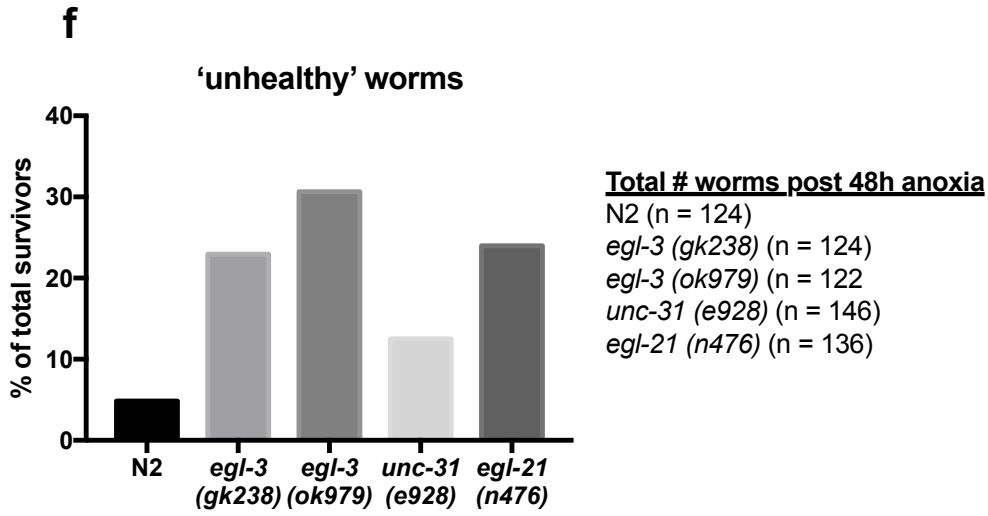


Fig. A2. 'Healthspan' of anoxia-tolerant neuropeptide signaling mutants after anoxia. a-e) Representative pictures of 'healthy' and 'unhealthy' surviving worms in N2 and anoxia-tolerant neuropeptide signaling mutants 6 days after 48h anoxia. f) Quantification of percent 'unhellthy' worms in every genotype, alongside total number of worms.

Survival of egl-3 and unc-31 mutants after anoxia is additive

Since *egl-3* is required for neuropeptide maturation and *unc-31* is required for neuropeptide secretion, we hypothesized that they are in the same genetic pathway, where *egl-3* acts upstream of *unc-31*. Compared to wild type worms, loss of function mutants in each of these genes leads to increased survival after 48h anoxia in L4 worms (Chapter 3). If they are in the same pathway, survival of *egl-3;unc-31* double mutants should not be any higher than either of the single mutants. On the other hand, if they were in unrelated, parallel pathways, the combined survival of the double mutant would be the sum of their individual survival phenotypes. In order to test this hypothesis, we generated the double mutants and tested their survival after 54h anoxia. We chose a higher anoxia duration because there was a ceiling effect of survival in these mutants at 48h, making it difficult to ascertain if they were truly in the same or parallel pathways.

To our surprise, we found that *egl-3;unc-31* double mutants had significantly greater survival compared to either of the single mutants (Fig. A3). This suggests that *egl-3* and *unc-31* may act via different mechanisms, or in different cells, to confer a survival benefit under anoxia. However, this requires further study. Further, it is worth noting that survival of *egl-3* and *unc-31* mutants is much lower at 54h (30-40%) than at 48h (70-90%), and is not significantly different from N2. This suggests that there is a limited window of anoxic exposure where loss of *egl-3* and *unc-31* activity is protective in worms.

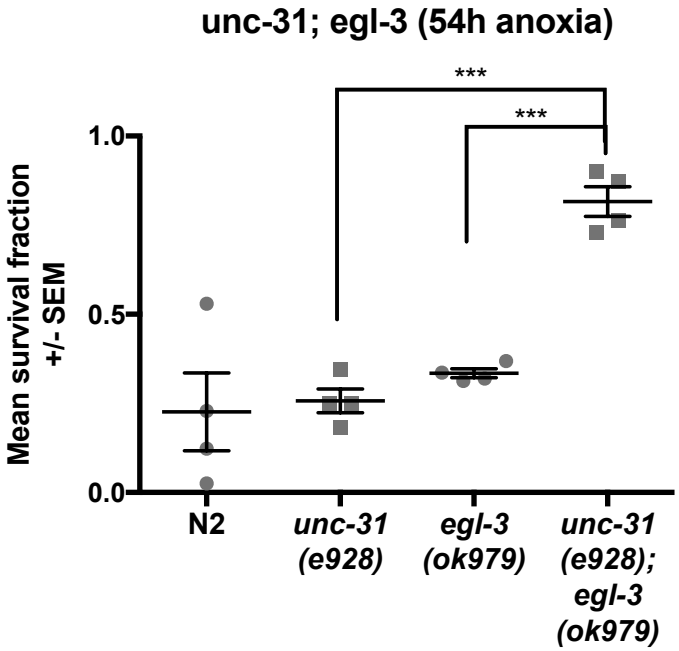


Fig. A3. Survival of *egl-3;unc-31* double mutants after anoxia. Worms were exposed to 54h anoxia, and allowed to recover for 24h before scoring live/dead worms (n = 4 independent experiments). Data were analyzed by one-way ANOVA with Tukey's test for multiple comparisons. ***p < 0.001.

BIBLIOGRAPHY

Ahima RS, Bjorbaek C, Osei S, Flier JS (1999) Regulation of Neuronal and Glial Proteins by Leptin: Implications for Brain Development 1. Endocrinology 140:2755–2762.

Ahmad Aziz N, Pijl H, Frölich M, Maurits van der Graaf AW, Roelfsema F, Roos RAC (2009) Leptin secretion rate increases with higher CAG repeat number in Huntington's disease patients. Clinical Endocrinology 73:206–211.

Ahmed RM, Mioshi E, Caga J, Shibata M, Zoing M, Bartley L, Piguet O, Hodges JR, Kiernan MC (2014) Body mass index delineates ALS from FTD: implications for metabolic health. J Neurol 261:1774–1780.

Albanese E, Launer LJ, Egger M, Prince MJ, Giannakopoulos P, Wolters FJ, Egan K (2017) Body mass index in midlife and dementia: Systematic review and meta-regression analysis of 589,649 men and women followed in longitudinal studies. Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring 8:165–178.

Allen SP, Rajan S, Duffy L, Mortiboys H, Higginbottom A, Grierson AJ, Shaw PJ (2014) Neurobiology of Aging. Neurobiology of Aging:1–11.

Anderson LL, Mao X, Scott BA, Crowder CM (2009) Survival from Hypoxia in C. elegans by Inactivation of Aminoacyl-tRNA Synthetases. Science 323:630–633.

Ari C, Poff AM, Held HE, Landon CS, Goldhagen CR, Mavromates N, D'Agostino DP (2014) Metabolic therapy with Deanna Protocol supplementation delays disease progression and extends survival in amyotrophic lateral sclerosis (ALS) mouse model. PLoS ONE 9:e103526.

Arthur KC, Calvo A, Price TR, Geiger JT, Chiò A, Traynor BJ (2016) Projected increase in amyotrophic lateral sclerosis from 2015 to 2040. Nature Communications 7:12408.

Aziz NA, Pijl H, Frolich M, Snel M, Streefland TCM, Roelfsema F, Roos RAC (2010) Systemic energy homeostasis in Huntington's disease patients. J Neurol Neurosurg Psychiatry 81:1233–1237.

Balthasar N et al. (2005) Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell 123:493–505.

Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, Deng Y, Blennerhassett P, Macri J, McCoy KD, Verdu EF, Collins SM (2011) The Intestinal Microbiota Affect Central Levels of Brain-DerivedNeurotropic Factor and Behavior in Mice. Gastroenterology 141:599–609.e3.

Berendzen KM, Durieux J, Shao L-W, Tian Y, Kim H-E, Wolff S, Liu Y, Dillin A (2016) Neuroendocrine Coordination of Mitochondrial Stress Signaling and Proteostasis. Cell 166:1553–1563.e10.

Bikman BT, Summers SA (2011) Ceramides as modulators of cellular and whole-body metabolism. J Clin Invest 121:4222–4230.

Blelloch R, Anna-Arriola SS, Gao D, Li Y, Hodgkin J, Kimble J (1999) The gon-1 gene is required for gonadal morphogenesis in Caenorhabditis elegans. Developmental Biology 216:382–393.

Bouteloup C, Desport JC, Clavelou P, Guy N, Derumeaux-Burel H, Ferrier A, Couratier P (2009) Hypermetabolism in ALS patients: an early and persistent phenomenon. J Neurol 256:1236–1242 Available at: http://www.springerlink.com/index/10.1007/s00415-009-5100-z.

Braniste V, Al-Asmakh M, Kowal C, Anuar F, Abbaspour A, Toth M, Korecka A, Bakocevic N, Ng LG, Kundu P, Gulyas B, Halldin C, Hultenby K, Nilsson H, Hebert H, Volpe BT, Diamond B, Pettersson S (2014) The gut microbiota influences blood-brain barrier permeability in mice. Science Translational Medicine 6:263ra158–263ra158.

Brenner S (1974) THE GENETICS OF CAENORHABDITIS ELEGANS. Genetics 77:71–94.

Broughton BRS, Reutens DC, Sobey CG (2009) Apoptotic Mechanisms After Cerebral Ischemia. Stroke 40:e331–e339.

Broughton SJ, Piper MDW, Ikeya T, Bass TM, Jacobson J, Driege Y, Martinez P, Hafen E, Withers DJ, Leevers SJ, Partridge L (2005) Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands. Proc Natl Acad Sci USA 102:3105–3110.

Bruijn LI, Becher MW, Lee MK, Anderson KL, Jenkins NA, Copeland NG, Sisodia SS, Rothstein JD, Borchelt DR, Price DL, Cleveland DW (1997) ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. Neuron 18:327–338.

Brzoska T, Luger TA, Maaser C, Abels C, Böhm M (2008) α-Melanocyte-Stimulating Hormone and Related Tripeptides: Biochemistry, Antiinflammatory and Protective Effects in Vitroand in Vivo, and Future Perspectives for the Treatment of Immune-Mediated Inflammatory Diseases. Endocrine Reviews 29:581–602.

Cacabelos D, Ayala V, Ramírez-Nunez O, Granado-Serrano AB, Boada J, Serrano JCE, Cabré R, Nadal-Rey G, Bellmunt MJ, Ferrer I, Pamplona R, Portero-Otin M (2014) Dietary Lipid Unsaturation Influences Survival and Oxidative Modifications of an Amyotrophic Lateral Sclerosis Model in a Gender-Specific Manner. Neuromol Med 16:669–685.

Cai H, Cong W-N, Ji S, Rothman S, Maudsley S, Martin B (2012) Metabolic Dysfunction in Alzheimers Disease and Related Neurodegenerative Disorders. Current Alzheimer Research 9:5–17.

Caruso C, Durand D, Schiöth HB, Rey R, Seilicovich A, Lasaga M (2007) Activation of Melanocortin 4 Receptors Reduces the Inflammatory Response and Prevents Apoptosis Induced by Lipopolysaccharide and Interferon-γ in Astrocytes. Endocrinology 148:4918–4926.

Chen H, Zhang SM, Hernán MA, Willett WC, Ascherio A (2003) Weight loss in Parkinson's disease. Annals of Neurology 53:676–679.

Chen Y-C, Chen H-J, Tseng W-C, Hsu J-M, Huang T-T, Chen C-H, Pan C-L (2016) A C. elegans Thermosensory Circuit Regulates Longevity through crh-1/CREB-Dependent flp-6 Neuropeptide Signaling. Dev Cell 39:209–223.

Chen Z, Zhong C (2013) Decoding Alzheimer's disease from perturbed cerebral glucose metabolism: implications for diagnostic and therapeutic strategies. Progress in neurobiology 108:21–43.

Cheong MC, Artyukhin AB, You Y-J, Avery L (2015) An opioid-like system regulating feeding behavior in C. elegans. eLife Sciences 4:e06683.

Chiang P-M, Ling J, Jeong YH, Price DL, Aja SM, Wong PC (2010) Deletion of TDP-43 down-regulates Tbc1d1, a gene linked to obesity, and alters body fat metabolism. Proc Natl Acad Sci U S A 107:16320–16324.

Choi DW, Rothman SM (1990) The Role of Glutamate Neurotoxicity in Hypoxic-Ischemic Neuronal Death. Annu Rev Neurosci 13:171–182.

Cistaro A, Pagani M, Montuschi A, Calvo A, Moglia C, Canosa A, Restagno G, Brunetti M, Traynor BJ, Nobili F, Carrara G, Fania P, Lopiano L, Valentini MC, Chiò A (2014) The metabolic signature of C9ORF72-related ALS: FDG PET comparison with nonmutated patients. Eur J Nucl Med Mol Imaging 41:844–852.

Cohen M, Reale V, Olofsson B, Knights A, Evans P, de Bono M (2009) Coordinated regulation of foraging and metabolism in C. elegans by RFamide neuropeptide signaling. Cell Metabolism 9:375–385.

Cohen P, Yang G, Yu X, Soukas AA, wolfish CS, Friedman JM, Li C (2005) Induction of Leptin Receptor Expression in the Liver by Leptin and Food Deprivation. Journal of Biological Chemistry 280:10034–10039.

Cone RD (2005) Anatomy and regulation of the central melanocortin system. Nature Neuroscience 8:571–578.

Cranford JA (1983) Body temperature, heart rate and oxygen consumption of normothermic and heterothermic western jumping mice (Zapus princeps). Comparative Biochemistry and Physiology Part A: Physiology 74:595–599.

Dark J (2005) ANNUAL LIPID CYCLES IN HIBERNATORS: Integration of Physiology and Behavior. Annu Rev Nutr 25:469–497.

Dasgupta N, Patel AM, Scott BA, Crowder CM (2007) Hypoxic Preconditioning Requires the Apoptosis Protein CED-4 in C. elegans. Current Biology 17:1954–1959.

Dausmann KH, Glos J, Ganzhorn JU, Heldmaier G (2004) Physiology: Hibernation in a tropical primate. Nature 429:825–826.

Davis JF, Choi DL, Schurdak JD, Fitzgerald MF, Clegg DJ, Lipton JW, Figlewicz DP, Benoit SC (2011) Leptin Regulates Energy Balance and Motivation Through Action at Distinct Neural Circuits. Biological Psychiatry 69:668–674.

Desport JC, Preux PM, Magy L, Boirie Y, Vallat JM, Beaufrère B, Couratier P (2001) Factors correlated with hypermetabolism in patients with amyotrophic lateral sclerosis. Am J Clin Nutr 74:328–334.

Dupuis L, Corcia P, Fergani A, Gonzalez De Aguilar JL, Bonnefont-Rousselot D, Bittar R, Seilhean D, Hauw JJ, Lacomblez L, Loeffler JP, Meininger V (2008) Dyslipidemia is a protective factor in amyotrophic lateral sclerosis. Neurology 70:1004–1009 Available at: http://proxy.library.upenn.edu:2079/ovftpdfs/FPDDNCFBLCBGBP00/fs047/ovft/live/gv024/00006114/00006114-200803250-00006.pdf.

Dupuis L, Oudart H, Rene F, de Aguilar JLG, Loeffler JP (2004) Evidence for defective energy homeostasis in amyotrophic lateral sclerosis: Benefit of a high-energy diet in a transgenic mouse model. Proc Natl Acad Sci U S A 101:11159–11164.

Dupuis L, Pradat P-F, Ludolph AC, Loeffler J-P (2011) Energy metabolism in amyotrophic lateral sclerosis. The Lancet Neurology 10:75–82.

Duran-Aniotz C, Hetz C (2016) Glucose Metabolism: A Sweet Relief of Alzheimer's Disease. Current Biology 26:R806–R809.

Erecińska M, Silver IA (2001) Tissue oxygen tension and brain sensitivity to hypoxia - ScienceDirect. Respiration physiology 128:263–276 Available at: https://proxy.library.upenn.edu/login?url=http://www.library.upenn.edu/biomed/ [Accessed October 3, 2017].

Eschbach J, Schwalenstöcker B, Soyal SM, Bayer H, Wiesner D, Akimoto C, Nilsson A-C, Birve A, Meyer T, Dupuis L, Danzer KM, Andersen PM, Witting A, Ludolph AC, Patsch W, Weydt P (2013) PGC-1α is a male-specific disease modifier of human and experimental amyotrophic lateral sclerosis. Human Molecular Genetics 22:3477–3484.

Evidente VGH, Caviness JN, Adler CH, Gwinn-Hardy KA, Pratley RE (2001) Serum leptin concentrations and satiety in Parkinson's disease patients with and without weight loss. Mov Disord 16:924–927.

Fani L, Bak S, Delhanty P, van Rossum EFC, van den Akker ELT (2014) The melanocortin-4 receptor as target for obesity treatment: a systematic review of emerging pharmacological therapeutic options. International Journal of Obesity 38:163–169.

Feeney SJ, McKelvie PA, Austin L, Jean-Francois MJ, Kapsa R, Tombs SM, Byrne E (2001) Presymptomatic motor neuron loss and reactive astrocytosis in the SOD1 mouse model of amyotrophic lateral sclerosis. Muscle Nerve 24:1510–1519.

Ferraiuolo L, Kirby J, Grierson AJ, Sendtner M, Shaw PJ (2011) Molecular pathways of motor neuron injury in amyotrophic lateral sclerosis. Nature Reviews Neurology 7:616–630.

Figlewicz DP, Bennett JL, Naleid AM, Davis C, Grimm JW (2006) Intraventricular insulin and leptin decrease sucrose self-administration in rats. Physiology & Behavior 89:611–616.

Firnhaber C, Hammarlund M (2013) Neuron-specific feeding RNAi in C. elegans and its use in a screen for essential genes required for GABA neuron function. PLoS Genet 9:e1003921.

Flibotte JJ, Jablonski AM, Kalb RG (2014) Oxygen Sensing Neurons and Neuropeptides Regulate Survival after Anoxia in Developing C. elegans. PLoS ONE 9:e101102.

Forero-Vivas ME, Hernández-Cruz A (2014) Increased firing frequency of spontaneous action potentials in cerebellar Purkinje neurons of db/db mice results from altered auto-rhythmicity and diminished GABAergic tonic inhibition. Gen Physiol Biophys 33:29–41.

Föll RL, Pleyers A, Lewandovski GJ, Wermter C, Hegemann V, Paul RJ (1999) Anaerobiosis in the nematode Caenorhabditis elegans. Comp Biochem Physiol B, Biochem Mol Biol 124:269–280.

Fried SK, Lee M-J, Karastergiou K (2015) Shaping fat distribution: New insights into the

molecular determinants of depot- and sex-dependent adipose biology. Obesity 23:1345–1352.

Frölich L, Blum-Degen D, Bernstein HG, Engelsberger S, Humrich J, Laufer S, Muschner D, Thalheimer A, Türk A, Hoyer S, Zöchling R, Boissl KW, Jellinger K, Riederer P (1998) Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease. J Neural Transm 105:423–438.

Funalot B, Desport J-C, Sturtz F, Camu W, Couratier P (2009a) High metabolic level in patients with familial amyotrophic lateral sclerosis. Amyotroph Lateral Scler 10:113–117.

Funalot B, Desport J-C, Sturtz F, Camu W, Couratier P (2009b) High metabolic level in patients with familial amyotrophic lateral sclerosis. Amyotroph Lateral Scler 10:113–117.

G J Lithgow TMWSMTEJ (1995) Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. Proc Natl Acad Sci USA 92:7540–7544.

Gahete MD, Rubio A, Córdoba-Chacón J, Gracia-Navarro F, Kineman RD, Avila J, Luque RM, Castaño JP (2010) Expression of the Ghrelin and Neurotensin Systems is Altered in the Temporal Lobe of Alzheimer's Disease Patients. JAD 22:819–828.

Garcia AM, Ladage ML, Dumesnil DR, Zaman K, Shulaev V, Azad RK, Padilla PA (2015) Glucose Induces Sensitivity to Oxygen Deprivation and Modulates Insulin/IGF-1 Signaling and Lipid Biosynthesis in Caenorhabditis elegans. Genetics 200:167–184.

Ghose P, Park EC, Tabakin A, Salazar-Vasquez N, Rongo C (2013) Anoxia-Reoxygenation Regulates Mitochondrial Dynamics through the Hypoxia Response Pathway, SKN-1/Nrf, and Stomatin-Like Protein STL-1/SLP-2. PLoS Genet 9:e1004063.

Godau J, Herfurth M, Kattner B, Gasser T, Berg D (2010) Increased serum insulin-like growth factor 1 in early idiopathic Parkinson's disease. J Neurol Neurosurg Psychiatry 81:536–538.

Gorges M, Vercruysse P, Müller H-P, Huppertz H-J, Rosenbohm A, Nagel G, Weydt P, Petersén Å, Ludolph AC, Kassubek J, Dupuis L (2017) Hypothalamic atrophy is related to body mass index and age at onset in amyotrophic lateral sclerosis. J Neurol Neurosurg Psychiatry:jnnp–2017–315795.

Gray JM, Karow DS, Lu H, Chang AJ, Chang JS, Ellis RE, Marletta MA, Bargmann CI (2004) Oxygen sensation and social feeding mediated by a C. elegans guanylate cyclase homologue. Nature 430:317–322.

Gurney M, Pu H, Chiu A, Dal Canto M, Polchow C, Alexander D, Caliendo J, Hentati A, Kwon Y, Deng H, et A (1994) Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science 264:1772–1775.

Hallam J, Boswell RG, DeVito EE, Kober H (2016) Gender-related Differences in Food Craving and Obesity. The Yale Journal of Biology and Medicine 89:161.

Hallem EA, Spencer WC, McWhirter RD, Zeller G, Henz SR, Rätsch G, Miller DM, Horvitz HR, Sternberg PW, Ringstad N (2011) Receptor-type guanylate cyclase is required for carbon dioxide sensation by Caenorhabditis elegans. Proc Natl Acad Sci U S A 108:254–259.

Han Z, Liu X, Luo Y, Ji X (2015) Therapeutic hypothermia for stroke: Where to go? Experimental

neurology 272:67–77.

Hardiman O, van den Berg LH (2017) Edaravone: a new treatment for ALS on the horizon? The Lancet Neurology 16:490–491.

Hardiman O, van den Berg LH, Kiernan MC (2011) Clinical diagnosis and management of amyotrophic lateral sclerosis. Nature Reviews Neurology 7:639–649.

Hargrove JL (2005) Adipose energy stores, physical work, and the metabolic syndrome: lessons from hummingbirds. Nutrition Journal 2005 4:1 4:36.

Hayakawa T, Kato K, Hayakawa R, Hisamoto N, Matsumoto K, Takeda K, Ichijo H (2011) Regulation of Anoxic Death in Caenorhabditis elegans by Mammalian Apoptosis Signal-Regulating Kinase (ASK) Family Proteins. Genetics 187:785–792.

Heijtz RD, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, Hibberd ML, Forssberg H, Pettersson S (2011) Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A 108:3047–3052.

Heiman-Patterson TD, Deitch JS, Blankenhorn EP, Erwin KL, Perreault MJ, Alexander BK, Byers N, Toman I, Alexander GM (2005) Background and gender effects on survival in the TgN(SOD1-G93A)1Gur mouse model of ALS. J Neurol Sci 236:1–7.

Henis-Korenblit S, Zhang P, Hansen M, McCormick M, Lee S-J, Cary M, Kenyon C (2010) Insulin/IGF-1 signaling mutants reprogram ER stress response regulators to promote longevity. Proc Natl Acad Sci U S A 107:9730–9735.

Hoelzl F, Bieber C, Cornils JS, Gerritsmann H, Stalder GL, Walzer C, Ruf T (2015) How to spend the summer? Free-living dormice (Glis glis) can hibernate for 11 months in non-reproductive years. J Comp Physiol B 185:931–939.

Holden KF, Lindquist K, Tylavsky FA, Rosano C, Harris TB, Yaffe K (2009) Serum leptin level and cognition in the elderly: Findings from the Health ABC Study. Neurobiology of Aging 30:1483–1489.

Holzenberger M, Dupont J, Ducos B, Leneuve P, Géloën A, Even PC, Cervera P, Le Bouc Y (2002) IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature 421:182–187.

Honda Y, Honda S (1999) The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. FASEB J 13:1385–1393.

Hopkins RO, Haaland KY (2004) Neuropsychological and neuropathological effects of anoxic or ischemic induced brain injury. J Int Neuropsychol Soc 10:957–961.

Hsu A-L, Murphy CT, Kenyon C (2003) Regulation of Aging and Age-Related Disease by DAF-16 and Heat-Shock Factor. Science 300:1142–1145.

Hu Z, Pym ECG, Babu K, Vashlishan Murray AB, Kaplan JM (2011) A neuropeptide-mediated stretch response links muscle contraction to changes in neurotransmitter release. Neuron 71:92–102.

Hums I, Riedl J, Mende F, Kato S, Kaplan HS, Latham R, Sonntag M, Traunmüller L, Zimmer M (2016) Regulation of two motor patterns enables the gradual adjustment of locomotion strategy in Caenorhabditis elegans. eLife Sciences 5:e14116.

Hung WL, Wang Y, Chitturi J, Zhen M (2014) A Caenorhabditis elegans developmental decision requires insulin signaling-mediated neuron-intestine communication. Development 141:1767–1779.

Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F (1997) Targeted Disruption of the Melanocortin-4 Receptor Results in Obesity in Mice. Cell 88:131–141.

Jacob TC, Kaplan JM (2003) The EGL-21 Carboxypeptidase E Facilitates Acetylcholine Release at Caenorhabditis elegans Neuromuscular Junctions. Journal of Neuroscience 23:2122–2130.

Jawaid A, Murthy SB, Wilson AM, Qureshi SU, Amro MJ, Wheaton M, Simpson E, Harati Y, Strutt AM, York MK, Schulz PE (2010a) A decrease in body mass index is associated with faster progression of motor symptoms and shorter survival in ALS. Amyotroph Lateral Scler 11:542–548.

Jawaid A, Salamone AR, Strutt AM, Murthy SB, Wheaton M, McDowell EJ, Simpson E, Appel SH, York MK, Schulz PE (2010b) ALS disease onset may occur later in patients with pre-morbid diabetes mellitus. European Journal of Neurology 17:733–739.

Jiang H, Guo R, Powell-Coffman JA (2001) The Caenorhabditis elegans hif-1 gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. Proc Natl Acad Sci USA 98:7916–7921.

Kaneb HM, Sharp PS, Rahmani-Kondori N, Wells DJ (2011) Metformin Treatment Has No Beneficial Effect in a Dose-Response Survival Study in the SOD1G93A Mouse Model of ALS and Is Harmful in Female Mice. PLoS ONE 6:e24189.

Kass J, Jacob TC, Kim P, Kaplan JM (2001) The EGL-3 Proprotein Convertase Regulates Mechanosensory Responses of Caenorhabditis elegans. Journal of Neuroscience 21:9265–9272.

Kaufman DM, Crowder CM (2015) Mitochondrial Proteostatic Collapse Leads to Hypoxic Injury. Current Biology 25:2171–2176.

Kiernan MC, Vucic S, Cheah BC, Turner MR, Eisen A, Hardiman O, Burrell JR, Zoing MC (2011) Amyotrophic lateral sclerosis. The Lancet 377:942–955.

Kim HJ, Magrane J, Starkov AA, Manfredi G (2012) The mitochondrial calcium regulator cyclophilin D is an essential component of oestrogen-mediated neuroprotection in amyotrophic lateral sclerosis. Brain 135:2865–2874.

Kimura KD, Tissenbaum HA, Y L, Ruvkun G (1997) daf-2, an Insulin Receptor-Like Gene That Regulates Longevity and Diapause in Caenorhabditis elegans. Science 277:942–946.

Kirkinezos IG, Bacman SR, Hernandez D, Oca-Cossio J, Arias LJ, Perez-Pinzon MA, Bradley WG, Moraes CT (2005) Cytochrome c Association with the Inner Mitochondrial Membrane Is

Impaired in the CNS of G93A-SOD1 Mice. Journal of Neuroscience 25:164–172.

Krashes MJ, Lowell BB, Garfield AS (2016) Melanocortin-4 receptor–regulated energy homeostasis. Nature Neuroscience 19:206–219.

Krishnan SN, Sun Y-A, Mohensin A, Wyman RJ, Haddad GG (1997) Behavioral and electrophysiologic responses of Drosophila melanogaster to prolonged periods of anoxia. Journal of insect ….

Lans H, Marteijn JA, Schumacher B, Hoeijmakers JHJ, Jansen G, Vermeulen W (2010) Involvement of Global Genome Repair, Transcription Coupled Repair, and Chromatin Remodeling in UV DNA Damage Response Changes during Development Maizels N, ed. PLoS Genet 6:e1000941.

LaRue BL, Padilla PA (2011) Environmental and Genetic Preconditioning for Long-Term Anoxia Responses Requires AMPK in Caenorhabditis elegans. PLoS ONE 6:e16790.

Ley RE, Peterson DA, Gordon JI (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124:837–848.

Lieb W, Beiser AS, Vasan RS, Tan ZS, Au R, Harris TB, Roubenoff R, Auerbach S, decarli C, Wolf PA, Seshadri S (2009) Association of Plasma Leptin Levels With Incident Alzheimer Disease and MRI Measures of Brain Aging. JAMA 302:2565–2572.

Lim MA, Bence KK, Sandesara I, Andreux P, Auwerx J, Ishibashi J, Seale P, Kalb RG (2014) Genetically altering organismal metabolism by leptin-deficiency benefits a mouse model of amyotrophic lateral sclerosis. Human Molecular Genetics 23:4995–5008.

Lin MT, Beal MF (2006) Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 443:787–795.

Ling S-C, Polymenidou M, Cleveland DW (2013) Converging Mechanisms in ALS and FTD: Disrupted RNA and Protein Homeostasis. Neuron 79:416–438.

Lipton P (1999) Ischemic Cell Death in Brain Neurons. Physiological Reviews 79:1431–1568.

Lo EH (2008) A new penumbra: transitioning from injury into repair after stroke. Nat Med 14:497–500.

Lyden PD, Krieger D, Yenari M, Dietrich WD (2006) Therapeutic Hypothermia for Acute Stroke. International Journal of Stroke 1:9–19.

Ma K, McLaurin J (2014) α-Melanocyte Stimulating Hormone Prevents GABAergic Neuronal Loss and Improves Cognitive Function in Alzheimer's Disease. Journal of Neuroscience 34:6736–6745.

Magrané J, Cortez C, Gan W-B, Manfredi G (2014) Abnormal mitochondrial transport and morphology are common pathological denominators in SOD1 and TDP43 ALS mouse models. Human Molecular Genetics 23:1413–1424.

Mao XR, Crowder CM (2010) Protein misfolding induces hypoxic preconditioning via a subset of the unfolded protein response machinery. Mol Cell Biol 30:5033–5042.

Mattiazzi M (2002) Mutated Human SOD1 Causes Dysfunction of Oxidative Phosphorylation in Mitochondria of Transgenic Mice. Journal of Biological Chemistry 277:29626–29633.

Mauvais-Jarvis F, Clegg DJ, Hevener AL (2013) The role of estrogens in control of energy balance and glucose homeostasis. Endocrine Reviews.

McCombe PA, Henderson RD (2010) Effects of Gender in Amyotrophic Lateral Sclerosis. Gender Medicine 7:557–570.

Mendenhall AR, LaRue B, Padilla PA (2006a) Glyceraldehyde-3-Phosphate Dehydrogenase Mediates Anoxia Response and Survival in Caenorhabditis elegans. Genetics 174:1173–1187.

Mendenhall AR, LaRue B, Padilla PA (2006b) Glyceraldehyde-3-Phosphate Dehydrogenase Mediates Anoxia Response and Survival in Caenorhabditis elegans. Genetics 174:1173–1187.

Menuz V, Howell KS, Gentina S, Epstein S, Riezman I, Fornallaz-Mulhauser M, Hengartner MO, Gomez M, Riezman H, Martinou JC (2009) Protection of C. elegans from Anoxia by HYL-2 Ceramide Synthase. Science 324:381–384.

Miller DL, Roth MB (2009) C. Elegans Are Protected from Lethal Hypoxia by an Embryonic Diapause. Current Biology 19:1233–1237.

Miyazaki K, Masamoto K, Morimoto N, Kurata T, Mimoto T, Obata T, Kanno I, Abe K (2012) Early and Progressive Impairment of Spinal Blood Flow—Glucose Metabolism Coupling in Motor Neuron Degeneration of ALS Model Mice. Journal of Cerebral Blood Flow & Metabolism 32:456–467.

Mok DZL, Sternberg PW, Inoue T (2015) Morphologically defined sub-stages of C. elegans vulval development in the fourth larval stage. BMC Developmental Biology 2015 15:1 15:26.

Mosconi L (2005) Brain glucose metabolism in the early and specific diagnosis of Alzheimer's disease. Eur J Nucl Med Mol Imaging 32:486–510.

Möhle L, Mattei D, Heimesaat MM, Bereswill S, Fischer A, Alutis M, French T, Hambardzumyan D, Matzinger P, Dunay IR, Wolf SA (2016) Monocytes Provide a Link between Antibiotic-Induced Changes in Gut Microbiota and Adult Hippocampal Neurogenesis. CellReports 15:1945–1956.

Murakami S, Johnson TE (1996) A Genetic Pathway Conferring Life Extension and Resistance to UV Stress in Caenorhabditis elegans. Genetics 143:1207–1218.

Nagel S, Papadakis M, Hoyte L, Buchan AM (2014) Therapeutic hypothermia in experimental models of focal and global cerebral ischemia and intracerebral hemorrhage. Expert Review of Neurotherapeutics 8:1255–1268.

Nakano Y, Hirayama K (1987) Hepatic Ultrastructural Changes and Liver Dysfunction in Amyotrophic Lateral Sclerosis. Archives of Neurology 44:103–106.

Nelson MD, Trojanowski NF, George-Raizen JB, Smith CJ, Yu CC, Fang-Yen C, Raizen DM (2013) The neuropeptide NLP-22 regulates a sleep-like state in *Caenorhabditis elegans*. Nature Communications 4:ncomms3846.

Ngo ST, Steyn FJ, McCombe PA (2014) Body mass index and dietary intervention: implications for prognosis of amyotrophic lateral sclerosis. J Neurol Sci 340:5–12.

Nystul TG, Goldmark JP, Padilla PA, Roth MB (2003) Suspended Animation in C. elegans Requires the Spindle Checkpoint. Science 302:1038–1041.

O'Reilly ÉJ, Wang H, Weisskopf MG, Fitzgerald KC, Falcone G, McCullough ML, Thun M, Park Y, Kolonel LN, Ascherio A (2013) Premorbid body mass index and risk of amyotrophic lateral sclerosis. Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration 14:205–211.

O'Rourke JG, Bogdanik L, Yáñez A, Lall D, Wolf AJ, Muhammad AKMG, Ho R, Carmona S, Vit JP, Zarrow J, Kim KJ, Bell S, Harms MB, Miller TM, Dangler CA, Underhill DM, Goodridge HS, Lutz CM, Baloh RH (2016) C9orf72 is required for proper macrophage and microglial function in mice. Science 351:1324–1329.

Oldreive CE, Harvey J, Doherty GH (2008) Neurotrophic effects of leptin on cerebellar Purkinje but not granule neurons in vitro. Neuroscience Letters 438:17–21.

Padilla PA, Roth MB (2001) Oxygen deprivation causes suspended animation in the zebrafish embryo. Proc Natl Acad Sci USA 98:7331–7335.

Padilla PA, Zager RA, Nystul TG, Johnson ACM, Roth MB (2002) Dephosphorylation of Cell Cycle–regulated Proteins Correlates with Anoxia-induced Suspended Animation in Caenorhabditis elegans. Molecular Biology of the Cell 13:1473–1483.

Paganoni S, Ma JD, Jaffa M, Cudkowicz ME, Wills A-M (2011) Body mass index, not dyslipidemia, is an independent predictor of survival in amyotrophic lateral sclerosis. Muscle Nerve 44:20–24.

Park TJ et al. (2017) Fructose-driven glycolysis supports anoxia resistance in the naked mole-rat. Science 356:307–311.

Parone PA, Da Cruz S, Han JS, McAlonis-Downes M, Vetto AP, Lee SK, Tseng E, Cleveland DW (2013) Enhancing Mitochondrial Calcium Buffering Capacity Reduces Aggregation of Misfolded SOD1 and Motor Neuron Cell Death without Extending Survival in Mouse Models of Inherited Amyotrophic Lateral Sclerosis. Journal of Neuroscience 33:4657–4671.

Peña S, Sherman T, Brookes PS, Nehrke K (2016) The Mitochondrial Unfolded Protein Response Protects against Anoxia in Caenorhabditis elegans. PLoS ONE 11:e0159989.

Perera ND, Turner BJ (2015) AMPK Signalling and Defective Energy Metabolism in Amyotrophic Lateral Sclerosis. Neurochem Res 41:544–553.

Phukan J, Pender NP, Hardiman O (2007) Cognitive impairment in amyotrophic lateral sclerosis. The Lancet Neurology 6:994–1003.

Picher-Martel V, Valdmanis PN, Gould PV, Julien J-P, Dupré N (2016) From animal models to human disease: a genetic approach for personalized medicine in ALS. Acta Neuropathologica Communications 2016 4:1 4:70.

Pradat P-F, Bruneteau G, Gordon PH, Dupuis L, Bonnefont-Rousselot D, Simon D, Salachas F, Corcia P, Frochot V, Lacorte J-M, Jardel C, Coussieu C, Le Forestier N, Lacomblez L, Loeffler J-P, Meininger V (2010) Impaired glucose tolerance in patients with amyotrophic

lateral sclerosis. Amyotroph Lateral Scler 11:166–171.

Pratley RE, Salbe AD, Ravussin E, Caviness JN (2000) Higher sedentary energy expenditure in patients with Huntington's disease. Annals of Neurology 47:64–70.

Prudencio M et al. (2015) Distinct brain transcriptome profiles in C9orf72-associated and sporadic ALS. Nature Neuroscience 18:1175–1182.

Rabinowitch I, Laurent P, Zhao B, Walker D, Beets I, Schoofs L, Bai J, Schafer WR, Treinin M (2016) Neuropeptide-Driven Cross-Modal Plasticity following Sensory Loss in Caenorhabditis elegans. PLoS Biol 14:e1002348.

Raizen DM, Zimmerman JE, Maycock MH, Ta UD, You Y-J, Sundaram MV, Pack AI (2008) Lethargus is a Caenorhabditis elegans sleep-like state. Nature 451:569–572.

Renton AE, Chiò A, Traynor BJ (2014) State of play in amyotrophic lateral sclerosis genetics. Nature Neuroscience 17:17–23.

Riar AK, Burstein SR, Palomo GM, Arreguin A, Manfredi G, Germain D (2017) Sex specific activation of the ERα axis of the mitochondrial UPR (UPRmt) in the G93A-SOD1 mouse model of familial ALS. Human Molecular Genetics 26:1318–1327.

Rich PR (2003) The molecular machinery of Keilin's respiratory chain. Biochm Soc Trans 31:1095–1105.

Ringstad N, Horvitz HR (2008) FMRFamide neuropeptides and acetylcholine synergistically inhibit egg-laying by C. elegans. Nature Neuroscience 11:1168–1176.

Robberecht W, Philips T (2013) The changing scene of amyotrophic lateral sclerosis. :1–17.

Salehzadeh F, Rune A, Osler M, Al-Khalili L (2011) Testosterone or 17{beta}-estradiol exposure reveals sex-specific effects on glucose and lipid metabolism in human myotubes. Journal of Endocrinology 210:219–229.

Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, Challis C, Schretter CE, Rocha S, Gradinaru V, Chesselet M-F, Keshavarzian A, Shannon KM, Krajmalnik-Brown R, Wittung-Stafshede P, Knight R, Mazmanian SK (2016) Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell 167:1469–1480.e12.

Sasaki S, Iwata M (2007) Mitochondrial Alterations in the Spinal Cord of Patients With Sporadic Amyotrophic Lateral Sclerosis. J Neuropathol Exp Neurol 66:10–16.

Saxena S, Cabuy E, Caroni P (2009) A role for motoneuron subtype–selective ER stress in disease manifestations of FALS mice. Nature Neuroscience 12:627–636.

Scheperjans F, Aho V, Pereira PAB, Koskinen K, Paulin L, Pekkonen E, Haapaniemi E, Kaakkola S, Eerola-Rautio J, Pohja M, Kinnunen E, Murros K, Auvinen P (2015) Gut microbiota are related to Parkinson's disease and clinical phenotype. Mov Disord 30:350–358.

Schindler AJ, Baugh LR, Sherwood DR (2014) Identification of Late Larval Stage Developmental Checkpoints in Caenorhabditis elegans Regulated by Insulin/IGF and Steroid Hormone Signaling Pathways Ashrafi K, ed. PLoS Genet 10:e1004426.

Scott BA, Avidan MS, Crowder CM (2002) Regulation of Hypoxic Death in C. elegans by the Insulin/IGF Receptor Homolog DAF-2. Science 296:2388–2391.

Shaffer HB et al. (2013) The western painted turtle genome, a model for the evolution of extreme physiological adaptations in a slowly evolving lineage. Genome Biology 2013 14:3 14:R28.

Shao L-W, Niu R, Liu Y (2016) Neuropeptide signals cell non-autonomous mitochondrial unfolded protein response. Cell Research 26:1182–1196.

Sharabi K, Charar C, Friedman N, Mizrahi I, Zaslaver A, Sznajder JI, Gruenbaum Y (2014) The Response to High CO2 Levels Requires the Neuropeptide Secretion Component HID-1 to Promote Pumping Inhibition. PLoS Genet 10:e1004529.

Sharon G, Sampson TR, Geschwind DH, Mazmanian SK (2016) The Central Nervous System and the Gut Microbiome. Cell 167:915–932.

Siklós L, Engelhardt J, Harati Y, Smith RG, Joó F, Appel SH (1996) Ultrastructural evidence for altered calcium in motor nerve terminals in amyotrophc lateral sclerosis. Annals of Neurology 39:203–216.

Simonson TS, Wei G, Wagner HE, Wuren T, Qin G, Yan M, Wagner PD, Ge RL (2015) Low haemoglobin concentration in Tibetan males is associated with greater high-altitude exercise capacity. The Journal of Physiology 593:3207–3218.

Simonson TS, Yang Y, Huff CD, Yun H, Qin G, Witherspoon DJ, Bai Z, Lorenzo FR, Xing J, Jorde LB, Prchal JT, Ge R (2010) Genetic Evidence for High-Altitude Adaptation in Tibet. Science 329:72–75.

Speese S, Petrie M, Schuske K, Ailion M, Ann K, Iwasaki K, Jorgensen EM, Martin TFJ (2007) UNC-31 (CAPS) Is Required for Dense-Core Vesicle But Not Synaptic Vesicle Exocytosis in Caenorhabditis elegans. Journal of Neuroscience 27:6150–6162.

Stanley M, Macauley SL, Holtzman DM (2016) Changes in insulin and insulin signaling in Alzheimer's disease: cause or consequence? Journal of Experimental Medicine 213:1375–1385.

Ste Marie L, Miura GI, Marsh DJ, Yagaloff K, Palmiter RD (2000) A metabolic defect promotes obesity in mice lacking melanocortin-4 receptors. Proc Natl Acad Sci USA 97:12339–12344.

Stribl C et al. (2014) Mitochondrial Dysfunction and Decrease in Body Weight of a Transgenic Knock-in Mouse Model for TDP-43. Journal of Biological Chemistry 289:10769–10784.

Sun CL, Kim E, Crowder CM (2014) Delayed innocent bystander cell death following hypoxia in Caenorhabditis elegans. Cell Death & Differentiation 21:557–567.

Taghibiglou C, Martin HGS, Lai TW, Cho T, Prasad S, Kojic L, Lu J, Liu Y, Lo E, Zhang S, Wu JZZ, Li YP, Wen YH, Imm J-H, Cynader MS, Wang YT (2009a) Role of NMDA receptor–dependent activation of SREBP1 in excitotoxic and ischemic neuronal injuries. Nat Med 15:1399–1406.

Taghibiglou C, Martin HGS, Rose JK, Ivanova N, Lin CHC, Lau HL, Rai S, Wang YT, Rankin CH (2009b) Essential role of SBP-1 activation in oxygen deprivation induced lipid accumulation and increase in body width/length ratio in Caenorhabditis elegans. FEBS Letters 583:831–

834.

Tan W, Pasinelli P, Trotti D (2014) Role of mitochondria in mutant SOD1 linked amyotrophic lateral sclerosis. Biochimica et Biophysica Acta 1842:1295–1301.

Taylor JP, Brown RH Jr, Cleveland DW (2016) Decoding ALS: from genes to mechanism. Nature 539:197–206 Available at: https://proxy.library.upenn.edu/login?url=http://www.library.upenn.edu/biomed/.

Tefera TW, Borges K (2017) Metabolic Dysfunctions in Amyotrophic Lateral Sclerosis Pathogenesis and Potential Metabolic Treatments. Front Neurosci 10.

Thacker C, Rose AM (2000) A look at the Caenorhabditis elegans Kex2/Subtilisin-like proprotein convertase family. Bioessays 22:545–553.

Turek M, Besseling J, Spies J-P, König S, Bringmann H (2016) Sleep-active neuron specification and sleep induction require FLP-11 neuropeptides to systemically induce sleep. eLife Sciences 5:e12499.

Ultsch GR, Jackson DC (1982) Long-term submergence at 3 degrees C of the turtle Chrysemys picta bellii in normoxic and severely hypoxic water. III. Effects of changes in ambient PO2 and subsequent air breathing. Journal of Experimental Biology 97:87–99.

Van Doorn C, Macht VA, Grillo CA, Reagan LP (2017) Physiology & Behavior. Physiology & Behavior:1–7.

Van Voorhies WA, Ward S (2000) Broad oxygen tolerance in the nematode Caenorhabditis elegans. Journal of Experimental Biology 203:2467–2478.

Vercruysse P et al. (2016) Alterations in the hypothalamic melanocortin pathway in amyotrophic lateral sclerosis. Brain 139:1106–1122.

Volpe JJ (2001) Perinatal brain injury: From pathogenesis to neuroprotection. Ment Retard Dev Disabil Res Rev 7:56–64.

Wang H, Girskis K, Janssen T, Chan JP, Dasgupta K, Knowles JA, Schoofs L, Sieburth D (2013) Neuropeptide Secreted from a Pacemaker Activates Neurons to Control a Rhythmic Behavior. Current Biology 23:746–754.

Wang L, Gutmann DH, Roos RP (2010) Astrocyte loss of mutant SOD1 delays ALS disease onset and progression in G85R transgenic mice. Human Molecular Genetics 20:286–293.

Wang R, Ross CA, Cai H, Cong W-N, Daimon CM, Carlson OD, Egan JM, Siddiqui S, Maudsley S, Martin B (2014) Metabolic and hormonal signatures in pre-manifest and manifest Huntington's disease patients. Front Physiol 5.

Wiedemann FR, Manfredi G, Mawrin C, Beal MF, Schon EA (2002) Mitochondrial DNA and respiratory chain function in spinal cords of ALS patients. J Neurochem 80:616–625.

Wills A-M et al. (2014a) Hypercaloric enteral nutrition in patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled phase 2 trial. The Lancet 383:2065–2072.

Wills A-M et al. (2014b) Hypercaloric enteral nutrition in patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled phase 2 trial. Lancet 383:2065–2072.

Worgall TS, Johnson RA, Seo T, Gierens H, Deckelbaum RJ (2002) Unsaturated fatty acid-mediated decreases in sterol regulatory element-mediated gene transcription are linked to cellular sphingolipid metabolism. J Biol Chem 277:3878–3885.

Wu S, Yi J, Zhang Y-G, Zhou J, Sun J (2015) Leaky intestine and impaired microbiome in an amyotrophic lateral sclerosis mouse model. Physiological Reports 3:e12356.

Yogaratnam J, Jacob R, Naik S, Magadi H, Sim K (2013) Prolonged Delirium Secondary to Hypoxic-ischemic Encephalopathy Following Cardiac Arrest. Clin Psychopharmacol Neurosci 11:39–42.

Zhang K et al. (2015) The C9orf72 repeat expansion disrupts nucleocytoplasmic transport. Nature 525:56–61.

Zhang Y, Shao Z, Zhai Z, Shen C, Powell-Coffman JA (2009) The HIF-1 Hypoxia-Inducible Factor Modulates Lifespan in C. elegans Zhang Y, Shao Z, Zhai Z, Shen C, Powell-Coffman JA, eds. PLoS ONE 4:e6348.

Zhang Y-G, Wu S, Yi J, Xia Y, Jin D, Zhou J, Sun J (2017) Target Intestinal Microbiota to Alleviate Disease Progression in Amyotrophic Lateral Sclerosis. Clinical Therapeutics 39:322–336.

Zhao Z, Lange DJ, Voustianiouk A (2006) A ketogenic diet as a potential novel therapeutic intervention in amyotrophic lateral sclerosis. BMC ….

Zimmer M, Gray JM, Pokala N, Chang AJ, Karow DS, Marletta MA, Hudson ML, Morton DB, Chronis N, Bargmann CI (2009) Neurons Detect Increases and Decreases in Oxygen Levels Using Distinct Guanylate Cyclases. Neuron 61:865–879.