Profiling Membrane Lipids in *C. elegans* Models of Parkinson's Disease

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Abstract

Parkinson's Disease (PD) is a neurodegenerative disease impacting a significant portion of adults worldwide, yet there is no quantitative diagnosis and limited treatments. Several connections have been made between PD and lipids; however, there are limited studies investigating the impacts of PD-linked genes on membrane phospholipids. Here, membrane lipids of *C. elegans* mutants and RNAi knockdowns of putative Parkinson's genes were characterized by mass spectrometry. Altered membrane composition was found in synaptic vesicle recycling gene *unc-26*. Normal membrane composition was rescued with fatty-acid desaturase knockdowns, showing the potential for further studies exploring the disease mechanisms and treatment potential of lipids in Parkinson's Disease.

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Table of Contents

1.0 Introduction

1.1 Parkinson's Disease: A Neurodegenerative Disorder

Parkinson's Disease is the second most common neurodegenerative disorder in humans, affecting 1% of individuals over the age of 60 and 3% of individuals over the age of 80 (Cooper & Van Raamsdonk, 2018; Maulik et al., 2017). PD is a movement disorder clinically characterized by tremor, rigidity, postural instability, and bradykinesia (Cooper & Van Raamsdonk, 2018; Jankovic, 2008; Tysnes & Storstein, 2017). Bradykinesia, or slowness of movement, can manifest as difficulty with fine motor control, in addition to the loss of spontaneous movement. It is one of the most recognizable symptoms of PD, often very obvious before any type of neurological examination. Rest tremor is the most common symptom of PD, with the tremor usually manifesting as a pill-rolling movement in the hands. Although PD is primarily a motor disorder, there are also many non-motor symptoms such as autonomic dysfunction, cognitive disorders, and sensory and sleep abnormalities (Jankovic, 2008). Progressive neuronal degradation, especially of dopaminergic neurons in the substantia nigra, is a contributing factor to these symptoms (Agranoff et al., 1999; Cooper & Van Raamsdonk, 2018).

Currently, there is no definitive diagnostic test to determine if a patient has Parkinson's Disease. Instead, physicians use diagnostic criteria consisting primarily of PD motor symptoms. The National Institute of Neurological Disorders and Stroke (NINDS) categorizes a PD diagnosis as definite, probable, and possible PD, depending on the symptoms present and the patient's response to treatment (Gelb et al., 1999). A definite diagnosis of PD requires histopathological confirmation through a postmortem autopsy (Gelb et al., 1999). By the time a patient exhibits motor symptoms and is clinically diagnosed with PD, neuronal degradation has already taken place (Jankovic, 2008). Developing a diagnostic method, such as a biomarker, is a key goal of PD research. Biomarkers are objective and quantifiable characteristics of a disease that can be used to clinically monitor disease severity (Morgan et al., 2010; Strimbu & Tavel, 2010). An identified biomarker would enable screening and diagnosis of Parkinson's Disease prior to the development of symptoms. Biomarkers would also enable physicians to monitor the efficacy of various treatments (National Institute of Neurological Disorders and Stroke, 2015). Lipids are a promising biomarker for Parkinson's Disease, because of the abundance of lipids present in the

brain, the increasing ease of lipidomic analysis, and the quantifiable nature of lipid abundances (Hussain et al., 2020).

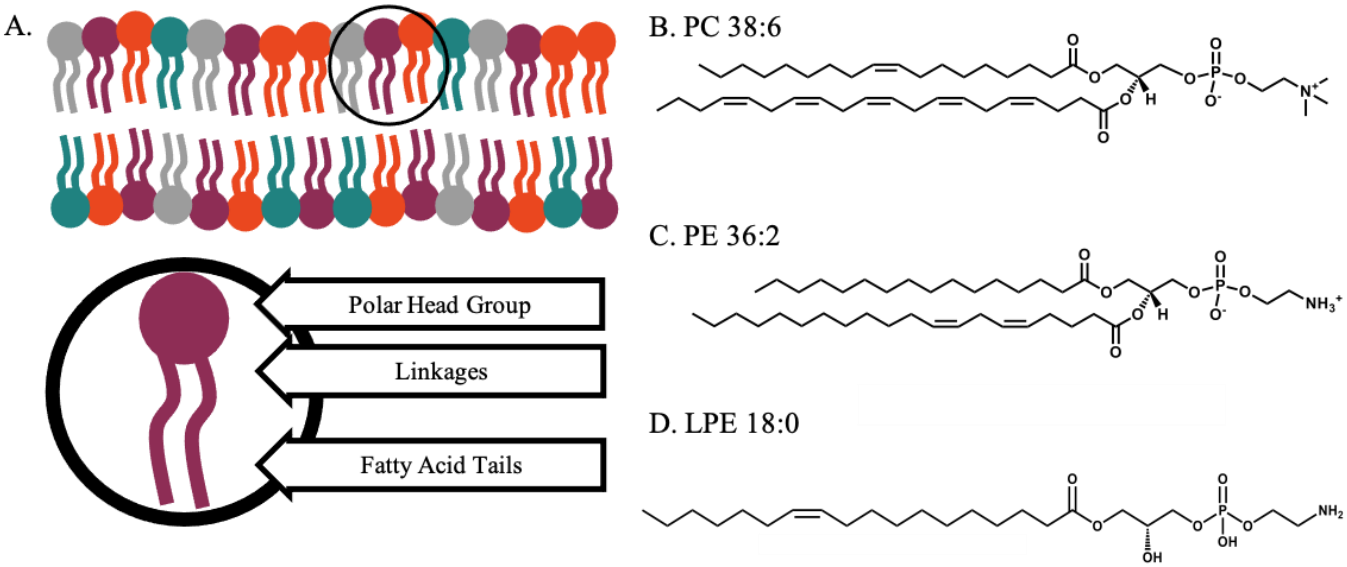
There is no cure for Parkinson's Disease, but some treatment options are available. Currently, a common treatment for PD is dopamine-replacement therapy to counteract the loss of dopaminergic neurons in the substantia nigra (The Parkinson Study Group, 2004). Levodopa is a commonly used treatment, as it is converted to dopamine in the brain. There are limitations to levodopa use, mainly that increasing doses are required to maintain dopamine concentration in the brain. As DA neuron levels decrease with disease progression, the effects of levodopa are shorter lived, creating "on" and "off" periods of motor function in the patient (Poewe et al., 2010). Another concern is that levodopa generates reactive oxygen species, which increases oxidative stress in the brain and contributes to neuronal degradation (Hwang, 2013; The Parkinson Study Group, 2004). A more recent advancement in PD treatments is Deep Brain Stimulation (DBS). DBS can reduce the severity of motor symptoms, and in combination with dopamine replacement therapies, can improve patient quality of life (Vidailhet, 2011). Although it seems like a promising treatment, DBS is recommended only for patients with severe disease progression and poor response to levodopa treatment (Benabid, 2010; Vidailhet, 2011). Other drawbacks of DBS are that it is an invasive surgical procedure, which can make patients ineligible due to preexisting conditions or cost (Benabid, 2010).

Lipids are a promising therapeutic for neurological disorders. Alzheimer's Disease is another age-related neurodegenerative disease, characterized primarily by memory loss. Decreased plasmalogen lipid levels have been identified in Alzheimer's disease, which contributes to oxidative stress and neurodegeneration (Su et al., 2019). A study conducted in patients with Alzheimer's Disease found that supplementing patient diet with plasmalogens isolated from scallops improved patient memory (Fujino et al., 2017). This simple dietary supplementation shows the promise for lipids to be used therapeutically in other neurological diseases, such as Parkinson's Disease.

1.2 The Importance of Lipids in Parkinson's Disease

Lipids are one of the major classes of macromolecules within a cell, characterized by their insolubility in water. Lipids are involved in a wide variety of functions that are essential for life and have diverse structures that facilitate their functions. Major functions include energy

storage, signaling, and membrane formation (van Meer et al., 2008). Changes to lipid homeostasis can result in or indicate a neurological disease because the brain is the organ with the second highest lipid content, and 50-60% of the brain's composition is lipids (Agranoff et al., 1999; Hussain et al., 2020). Many alterations in lipid metabolism and composition have been observed in several neurological diseases, including Parkinson's Disease (Hussain et al., 2020). Key functions of lipids in the brain include signaling through synaptic vesicles, which are



formed from cell membrane components (Hussain et al., 2020; Mundigl & De Camilli, 1994).

Figure 1. Membranes and Membrane Lipids: A. Membranes are highly diverse, with a variety of phospholipid species. Phospholipid diversity comes from the different possible combinations of polar head groups, fatty acid tails, and linkages between the head groups and tails. The nomenclature used with phospholipids follows the following format: C X:YnZ, where C is the headgroup, X is the number of carbons, Y is the number of double bonds, and Z is the position of the first double bond. **B.** Phosphatidylcholine 38:6, a phospholipid composed of a PC head group, and fatty acid tails C18:1 and C20:5. **C.** Phosphatidylethanolamine 36:2, a phospholipid composed of a PE head group, and fatty acid tails C16:0 and C20:2. **D.** Lysophosphatidylethanolamine 18:0, a phospholipid composed of a PE head group, and single C18:0 fatty acid tail.

Membranes are selectively permeable barriers that separate cells and organelles from their surroundings. Cell membranes are composed primarily of phospholipids, consisting of a hydrophilic head group attached to two hydrophobic fatty acid tails. The variety of head groups and fatty acid tails allows for the formation of hundreds of species of phospholipids, each with their own properties (Figure 1A).

Two of the most abundant head groups are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) lipids, with PC lipids accounting for approximately 50% of a eukaryotic membrane (van Meer et al., 2008). PC lipids self-assemble into bilayers and promote formation of a membrane with little to no curvature (Figure 1B). PE lipids are the second most abundant lipid species and promote formation of a membrane with negative curvature (Figure 1C) (Holthuis & Menon, 2014; van Meer et al., 2008). PE lipids are also involved in regulating interactions between membrane lipids and membrane proteins (van Meer et al., 2008). Lyso-phospholipids, such as lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE), are phospholipids with a single fatty acid tail (Figure 1D), which promotes the formation of a membrane with positive curvature (Holthuis & Menon, 2014). Other common head groups include phosphatidylinositol (PI), which make up around 2% of brain lipids, phosphatidylserine (PS), and phosphatidylglycerol (PG) (Agranoff et al., 1999; van Meer et al., 2008).

Maintaining head group diversity is important for overall cell health and homeostasis. A characteristic of Parkinson's Disease is the aggregation of misfolded α-synuclein in the brains of PD patients. The neuronal protein α-synuclein is localized in presynaptic clefts, and controls neurotransmitter release through association with membrane and vesicle lipids (Bosco et al., 2006; Stefanis, 2012). α-synuclein folding is mediated by phospholipids, and optimal protein folding requires abundant phospholipid availability. A mixture of 30% phosphatidylserine (PS) and 70% phosphatidylcholine (PC) lipids was needed for proper protein folding (Chandra, et al., 2003). Decreases in several PC lipid species have been observed in the substantia nigra in a rat model of Parkinson's Disease, indicating that PD can trigger altered membrane composition within the brain (Farmer et al., 2015). Initial clinical studies show the potential for the use of altered phospholipids as biomarkers in PD diagnosis. Increased PC/LPC ratios have been found in the blood plasma of PD patients (Vukajlović et al., 2020). This study demonstrates that altered lipids are not limited to specific tissues in the brain and can be detected easily throughout the body in non-neuronal tissues (Morgan et al., 2010; Strimbu & Tavel, 2010; Vukajlović et al., 2020).

Additional diversity in the phospholipid population comes from the linkages between the head groups and fatty acid tails which can also alter membrane properties. Canonical PC and PE lipids have two ester linkages between the head group and both fatty acid tails. Plasmalogens are lipids with an ether linkage at one of the linkage positions. An alkenyl-ether group at the linkage

will result in a plasmenyl lipid, indicated by a P- in the lipid nomenclature. An alkyl-ether bond results in a plasmanyl lipid, indicated by a O- in the nomenclature. Ether-linked lipids have shown to play a role in healthy reproduction, lifespan, and oxidative stress resistance (Dancy et al., 2015; van Meer et al., 2008).

Finally, the fatty acid tails of phospholipids also determine various properties of the membrane such as fluidity. Saturated fatty acids have no double bonds, and therefore can pack compactly (Holthuis & Menon, 2014). Unsaturated fatty acids have one or more double bonds, which cause kinks and bends within the chain and therefore cannot pack together as tightly as the saturated fatty acids (Holthuis & Menon, 2014). Increasing the abundance of unsaturated fatty acid species will increase the fluidity of the membrane.

In relationship to Parkinson's disease, monounsaturated fatty acids contribute to α-synuclein aggregation and toxicity, and metabolites from oxidized lipids trigger α-synuclein misfolding and aggregation (Bosco et al., 2006; Chandra et al., 2003; Fanning et al., 2019). This protein aggregation in cells is toxic, and can cause cell death, especially of dopaminergic neurons. A study in yeast expressing human α-synuclein found that α-synuclein toxicity in cells is rescued with knockdowns of fatty acid desaturase enzymes (Fanning et al., 2019). These connections between Parkinson's Disease and phospholipids indicate that further studies are needed since limited studies have been conducted on whole-animal membrane composition, especially in connection to specific PD genes.

1.3 Modeling Parkinson's Disease in *C. elegans*

C. elegans is a small nematode commonly used as a model organism in a variety of research fields including human diseases and lipidomics (Figure 2) (Corsi et al., 2015). Wild-type *C. elegans* reach adulthood in 2-3 days and have a lifespan of 2-3 weeks (Corsi et al., 2015). This short lifespan is useful for modeling an age-related disease like Parkinson's, as phenotypes of the disease can be observed throughout the life of the animal (Cooper & Van Raamsdonk, 2018; Corsi et al., 2015). The rapid generation time allows for easy characterization of reproductive phenotypes, which can be an indicator of Parkinson's Disease as altered dopamine signaling can reduce brood size and fertility in *C. elegans* (Maulik et al., 2017). In short, *C. elegans* is a useful model for Parkinson's Disease due to many advantageous features including a

short lifespan, genetic parallels to humans, and the ability to conduct various molecular and behavioral analyses (Cooper & Van Raamsdonk, 2018; Corsi et al., 2015).

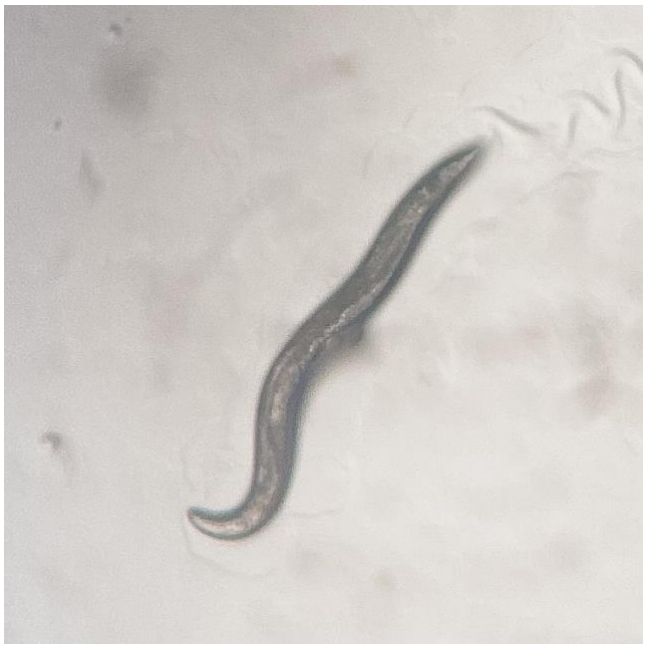


Figure 2. Adult wild-type *C. elegans.*

C. elegans have many human gene orthologs, which allows for the modeling of several human diseases, including Parkinson's Disease (Cooper & Van Raamsdonk, 2018). Although *C. elegans* is a less complex organism compared to humans, these genetic parallels allow for modeling of genetic pathways and for the identification of disease biomarkers with the advantage of a short generation time and lifespan. Mutants with deletions of genes can be used to model the lifelong and generational impacts of the lack of a gene (Cooper & Van Raamsdonk, 2018). Additionally, RNAi can be used to knockdown genes at specific times, which is beneficial to avoid impacts on worm development to adulthood (Cooper & Van Raamsdonk, 2018; Fanning et al., 2019; Maulik et al., 2019).

Various PD phenotypes such as altered movement, altered chemotaxis, and altered reproduction can easily be quantified in *C. elegans* (Cooper & Van Raamsdonk, 2018; Maulik et al., 2017). One chemotaxis assay that can be conducted in *C. elegans* is ethanol preference. An avoidance response to ethanol is dependent on functional dopaminergic neuron pathways. With dopaminergic neuron degradation in PD mutants, the animals will lack an avoidance response to ethanol (Cooper & Van Raamsdonk, 2018; Maulik et al., 2019).

Several studies conducted in *C. elegans* models of Parkinson's Disease have made important conclusions related to the disease. For example, a study of major Parkinson's Disease gene PTEN-induced kinase 1 (*pink-1)* in *C. elegans* found that these genes have antagonistic functions in oxidative stress response. In this study, *pink-1* mutant worms had a reduced survival when exposed to oxidative stress through paraquat. This reduced survival was rescued in *pink-1* and leucine-rich repeat serine/threonine-protein kinase 1 (*lrk-1*) double mutants. This study also found that altered mitochondrial morphology in *pink-1* mutant *C. elegans* was rescued in the *pink-1* and *lrk-1* double mutant. These findings provide more insight into the cellular mechanisms that lead to the development of Parkinson's Disease (Sa¨mann et al., 2009). Other studies provide insight into potential therapeutic targets of Parkinson's Disease. *C. elegan*s mutants expressing human α-synuclein have several PD phenotypes analogous to PD symptoms in humans, such as dopaminergic neuron degeneration and reduced movement. These phenotypes were rescued with desaturase enzyme knockdowns, further highlighting the potential for lipids to serve as a therapeutic target for Parkinson's Disease (Maulik et al., 2019).

1.4 Selection of Parkinson's Disease Genes

Both genetic and environmental factors have been identified in disease development (Maulik et al., 2017). Around 15% of all Parkinson's Disease diagnoses have been linked solely to a genetic component (Cooper & Van Raamsdonk, 2018). Genes chosen for this study were selected due to their connection to familial Parkinson's Disease. The work here focused on 6 genes: uncoordinated 26 (*unc-26*), PTEN-induced kinase 1 (*pink-1*), cation transporting ATPase (*catp-6*), leucine-rich repeat serine/threonine-protein kinase 1 (*lrk-1*), glutathione-independent glyoxalase 1.1 (*djr-1.1*), and sorbitol dehydrogenase 2 (*sodh-2)*.

Studies have shown that mutations in these genes are involved in disease pathology and causing disease symptoms (Anand et al., 2020; Bae et al., 2018). Some genes have been definitively linked to PD, such as mutations in *lrk-1* causing autosomal-dominant PD, and mutations in *pink-1, djr-1.1,* and *catp-6* causing autosomal-recessive forms of PD (Anand et al., 2020; Bae et al., 2018; Cooper & Van Raamsdonk, 2018; Valente, 2004). Other genes have been identified to play a role in PD through large scale genome wide association studies and familial genotyping. For example, homozygous mutations in *unc-26* have been traced in three families with early-onset Parkinson's Disease (Drouet & Lesage, 2014). Mutations in alcohol

dehydrogenase genes, such as *sodh-2*, have been identified to be prevalent in populations with Parkinson's Disease (Buervenich et al., 2000; Murphy et al., 2003). Because these genes have been implicated to play a role in PD development, they are commonly studied in many model organisms, including *C. elegans*, indicating that Parkinson's can be successfully modeled in the nematodes (Cooper & Van Raamsdonk, 2018). All of the chosen genes have a human ortholog implicated in familial Parkinson's Disease, which can allow for comparisons between the impacts of the genes in *C. elegans* and humans.

Table 1. A summary of Parkinson's Disease genes studied, with information on the human orthologs, currently known gene functions, and manifestations of phenotypes in *C. elegans*.

***C. elegans* Gene**	**Human Ortholog**	**Known Gene Functions**	**Phenotypes in loss of function mutants or gene knockdowns in *C. elegans***
unc-26	SYNJ1/PARK20	synaptic vesicle recycling	reduced movement, reduced brood size, small size, jerky motion (Harris et al, 2000)
pink-1	PINK1/PARK6	kinase, mitophagy, autophagosome formation	decreased basal slowing, increased oxidative stress sensitivity, mitochondrial accumulation (Cooper & Van Raamsdonk, 2018; Valente, 2004; Wu et al., 2018; Xiong et al., 2009)
catp-6	ATPase12A3/ PARK9	ATPase, regulates autophagy and lysosomal function	loss of DA neurons, decreased basal slowing, decreased movement, increased stress sensitivity (Cooper & Van Raamsdonk, 2018; Gitler et al., 2009)
lrk-1	LRRK1/PARK8	kinase, phosphorylates endophilin, regulates α-synuclein aggregation	decreased oxidative stress sensitivity, reduced lifespan, reduced brood size (Bae et al., 2018; Cooper et al. 2015; Cooper & Van Raamsdonk, 2018)
djr-1.1	DJ1/PARK7	glyoxylase	mitochondrial fragmentation, decreased sensitivity to stress (Cooper & Van Raamsdonk, 2018; Sa¨mann et al., 2009)
sodh-2	ADH4	alcohol dehydrogenase	increased lifespan (Murphy et al., 2003)

In addition to having strong connections to Parkinson's Disease, the targeted genes were selected because it was believed they would influence membrane dynamics based on their previously studied functions within the cell. For example, *unc-26* has direct interactions with membrane lipids as it is involved in the synaptic vesicle recycling pathway (Drouet & Lesage, 2014). Similarly, *catp-6* is a lysosomal transport protein, and has direct interactions with membranes (Cooper & Van Raamsdonk, 2018). A summary of information on the genes studied can be found in Table 1.

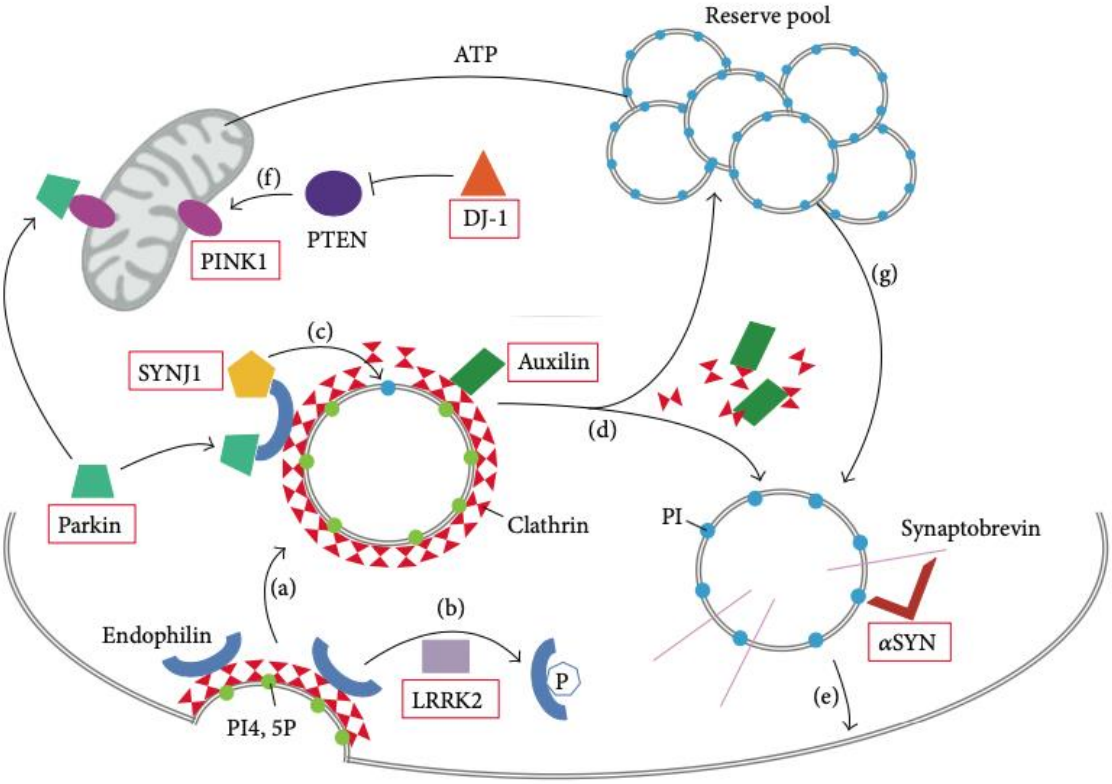


Figure 3. Parkinson's Disease Genes in the Synaptic Vesicle Recycling Pathway (Drouet & Lesange, 2014).

Many of PD genes are involved in the same pathways and can regulate other PD genes, suggesting that a defect in a single gene can be detrimental to entire pathways. Several PD genes are involved in synaptic vesicle recycling pathways (Figure 3). *lrk-1* (LRRK2) causes endophilin to dissociate from vesicles (b), which will then allow *unc-26* (SYNJ1) to dephosphorylate synaptic vesicles (c). Synaptic vesicles fuse to the membrane with the assistance of a multiprotein complex that includes α-synuclein (αSYN), the hallmark PD protein (e). In a

NMDA receptor signalling pathway, *djr-1.1* (DJ-1) can inhibit proteins that increase *pink-1* (PINK1) levels in the mitochondria (f). Proper mitochondrial function, as regulated by *pink-1*, will generate ATP required for vesicle mobilization (g) (Drouet & Lesage, 2014). Other studies suggest that *pink-1* forms a complex with *djr-1.1* to promote protein ubiquitination (Thomas et al., 2011; Xiong et al., 2009; Zhang et al., 2005).

Previous studies in yeast expressing α-synuclein found that aggregating α-synuclein disrupted lipid homeostasis by inhibiting the storage of triglyceride neutral lipids. α-synuclein aggregation also caused increases of C18:1 fatty acid abundances, which proved to be toxic to cells because it promoted further α-synuclein aggregation, leading to toxic accumulation of triglycerides within the cell. Knockdowns of a stearoyl-CoA desaturase rescued the toxicity seen with the increases of C18:1 (Fanning et al., 2019). In *C. elegans* models expressing α-synuclein, stearoyl-CoA desaturase knockdowns rescued several PD phenotypes, including normal levels of DA neurons, ethanol avoidance, and movement (Fanning et al., 2019; Maulik et al., 2019). One stearoyl-CoA desaturase that rescued PD phenotypes is *fat-7*, a desaturase that acts on C18:0 and produces C18:1n9 (Maulik et al., 2019; Watts & Ristow, 2017). Due to known impacts on lipid metabolism and the connection to PD, *fat-7* was selected for use as the desaturase used to rescue altered lipid species and as a positive control (Dancy et al., 2015; Maulik et al., 2019).

Homologous loss of function mutants for *unc-26*, *pink-1*, *catp-6*, and *fat-7* and RNAi knockdowns of *unc-26*, *pink-1*, *catp-6*, *lrk-1*, *djr-1.1*, *sodh-2* and *fat-7* were used in this study. The goal was to characterize membrane lipids of these mutants and gene knockdowns in *C. elegans*, and ultimately identify a lipid biomarker and lipid treatment target.

2.0 Methods and Materials

2.1 *C. elegans* Mutant Strains

N2, *fat-7* (wa36), *catp-6* (ok3473), *unc-26* (e1196), and *pink-1* (ok3538) strains were provided by the Caenorhabditis Genetics Center (CGC), which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). The *catp-6* and *pink-1* strains were given to the CGC by the *C. elegans* Gene Knockout Project at the Oklahoma Medical Research Foundation, which was part of the International *C. elegans* Gene Knockout Consortium.

2.2 *C. elegans* Maintenance

Unless otherwise noted, *C. elegans* strains were maintained on HG plates seeded with *E. coli* strain OP50 at 20°C.

2.2 Mutant Characterization & Lifespan

100-150 synchronized L1s were plated on a 6cm NGM plate seeded with OP50 *E. coli*. After 24 hours, worms were transferred onto a 3cm plate with 1 worm on each plate. Worms were checked every 24 hours for the development of L4 characteristics. Once egg laying started, worms were transferred every 24 hours to a fresh plate. The number of eggs and L1s on each plate were recorded. The plates with eggs were incubated for 24 hours in 20°C, and the number of L1 worms on the plate was recorded (Cooper et al., 2017). After reproduction ended, the animals were checked every 24 hours and transferred every 48 hours to fresh plates, and the deaths, bagging, movement, and other phenotypes were recorded.

2.3 RNAi Gene Knockdowns

2.3.1 RNAi Bacteria

Cultures of HT115 *E. coli* transformed with an empty vector (L4440) or the RNAi of interest were obtained from the Ahringer RNAi library. RNAi strains were streaked on LB + Carbenicillin (100 mg/L) and Tetracycline (15 mg/L) plates and grown overnight at 37°C. RNAi strains were cultured in LB + Carbenicillin (100 mg/L) and Tetracycline (15 mg/L) liquid media for 18-24 hours at 37°C. The bacteria were pelleted and resuspended in M9 (100 uL/plate) to

seed NGM + Carbenicillin (100 mg/L) and isopropyl β-D-thiogalactoside (IPTG) (1 g/L) plates. NGM + CI plates were seeded 48-72 hours prior to plating worms.

2.3.2 Adult RNAi Protocol

The worm strain was bleached to create a synchronized population. 2000-2500 L1 worms were plated on NGM + CI plates seeded with L4440 (empty vector) bacteria. After 48 hours of incubation at 20°C, L4 / young adult worms were transferred to NGM + CI plates seeded with RNAi bacteria for the genes of interest. Additional food was added to the plates as needed. After 48 hours on RNAi bacteria, the worms were collected and the larvae were removed through gravity separation. The worm pellets were stored at -80°C until extraction and separation.

2.4 Lipid Extraction, Separation, and Analysis

2.4.1 Mutant Lipid Characterization

The *C. elegans* strain was bleached to create a synchronized population. 2000-2500 L1 worms were plated on NGM + CI plates seeded with L4440 (empty vector) bacteria. After 48 hours of incubation at 20 °C, L4 / young adult worms were transferred to fresh NGM + CI plates seeded with L4440 bacteria. Additional food was added to the plates as needed. After 48 hours, the worms were collected and the larvae were removed through gravity separation. The worm pellets were stored at -80 °C until extraction and separation.

2.4.2 Lipid Extraction for HPLC-MS/MS

4 ml of chloroform/methanol (2:1) was added to a glass vial with a PTFE-cap to limit exposure to plastics which interfere with lipid analysis. The worm pellet (8,000-10,000 worms) and 10 µL of lipid standards (PC 11:0 and TAG 13:0) were added to the glass vial. The vials were rotated for 1.5 hours at room temperature. 600 µL of 0.9% NaCl were added to the vials, and the vials were centrifuged for 2 minutes at 2000 rpm. The bottom phase in the vials was transferred to a fresh tube and dried down under nitrogen. The extracted lipids were resuspended in 200 µL of dilution buffer (acetonitrile/ 2-propanol/water (65:30:5) for HPLC-MS/MS analysis. Samples were stored at -20°C until separated for GC-MS analysis (Dancy et al., 2015).

2.4.3 Lipid Analysis using HPLC-MS/MS

Abundances of intact phospholipids were identified using HPLC-MS/MS (Dancy et al., 2015). Data from the HPLC-MS/MS was analyzed using the Lipid Data Analyzer (Hartler et al., 2011).

2.4.4 Lipid Separation for GC-MS

The previously extracted lipids analyzed with HPLC-MS/MS were purified for phospholipid and neutral lipid fatty acid analysis with GC/MS. PLs and TAGs were purified by solid phase exchange (SPE) chromatography. Extracted lipid was resuspended in chloroform and loaded onto SPE columns (100 mg capacity, Fisher Scientific) pre-equilibrated with 3 ml of chloroform. TAGs were eluted first with 3 ml of chloroform. Glycosphingolipids were eluted next with 5 ml of a 9:1 acetone: methanol mixture, and phospholipids were eluted last with 3 ml of methanol. Purified lipids were dried, resuspended in methanol/2.5% H_2SO_4, and incubated for 1 hour at 80°C to create FAMEs. FAMEs were analyzed by gas chromatography/mass spectrometry (GC/MS) (Dancy et al., 2015).

2.5 Ethanol Avoidance: Quadrant Assay

Two quadrants of a 6cm plate were seeded with 45 uL of ethanol, applied 15 uL at a time. The quadrants were allowed to dry between applications. Ten day 3 adult worms were picked onto the center of the plate. The plates rested for 30 minutes, and the worms in each quadrant at the end were counted. The ethanol avoidance index was calculated as follows: [(number of worms in control quadrants) − (number of worms in ethanol seeded quadrants)]/total number of worms (Cooper et al., 2017; Maulik et al., 2017).

3.0 Results and Discussion

3.1 Altered Development and Developmental Phenotypes in Parkinson's Disease Mutants

Some Parkinson mutants, including *catp-6* and *pink-1*, have quantifiable phenotypes including altered development and lifespan (Cooper et al., 2017; Zielich et al., 2018). Selected mutations were characterized to identify any similar changes as these are disease indicators. In humans, reproduction seems to impact the age of onset of PD, and PD can cause a decrease in life expectancy (Frentzel et al., 2017; Savica, Turcano, Bower, Ahlskog, & Mielke, 2019). Identifying PD phenotypes in *C. elegans* analogous to PD symptoms in humans allows for the ability to monitor and test for disease manifestation and progression. Therefore, it is important to use reproducing animals in these experiments, and note altered developmental, reproductive, and motor phenotypes as indicators of PD in *C. elegans*.

Reproductive phenotypes of Parkinson's Disease mutants *unc-26* (e1196), *catp-6* (ok3473), and *pink-1* (ok3538) were characterized by determining reproductive period and brood size. Wild-type worms began laying eggs around 3 days after hatching. The *unc-26* mutant strain had significantly delayed development, with egg-laying starting around 24 hours after wild-type worms (Figure 4A). The length of the reproductive period was increased in *unc-26* as well, with animals laying eggs for an average of 10.3 ± 0.78 days compared to control animals laying eggs for an average of 4.63 ± 0.38 days ($p = 0.00002$). The brood size for *unc-26* animals was 159.40 ± 18.63 offspring, similar to the brood size of 196.00 ± 26.80 offspring for control animals (Figure 4B). The *catp-6* mutant animals had a significantly decreased brood size of 51.00 ± 10.94 offspring ($p = 0.0006$) (Figure 4B), although the onset and duration of reproduction was similar (Figure 4A). The *pink-1* worms did not have significantly altered reproductive periods or brood sizes compared to wild-type worms (Figure 4A, 4B).

A small-scale lifespan was conducted with the mutant strains. The lifespan of *pink-1* and *catp-6* strains was decreased considerably compared to wild-type worms (Figure 4C). The reduction in *catp-6* lifespan was in part due to a high frequency of bagging, with 70% of the animals bagged between 4 to 5 days of plating. In *pink-1* worms, an internal organ expulsion phenotype was observed in several of the worms, potentially contributing to the decreased lifespan (Figure 4D). Published studies indicated that there is no significant difference in the

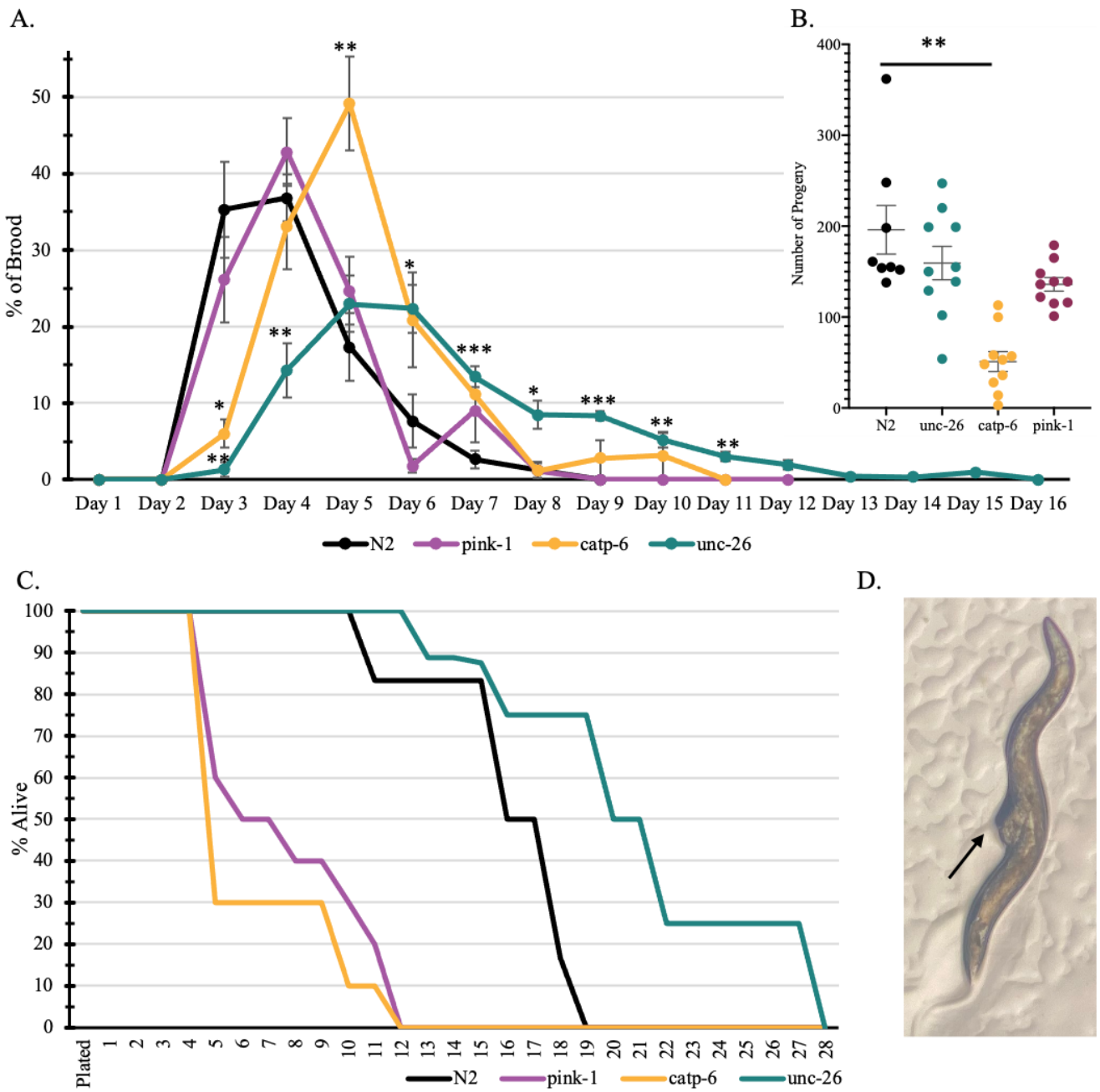


Figure 4. Parkinson's Disease mutants had altered development, reproduction, and lifespans. A. *unc-26* (e1196) reproduction was delayed from wild-type by approximately 24 hours and lasted for a greater duration than wild-type animals. *catp-6* (ok3473) reproduction was delayed from wild type slightly. *pink-1* (ok3538) reproduction was consistent with wild-type. (N2: n=8, unc-26: n=10, catp-6: n=10, pink-1: n=10) **B.** *catp-6* brood size was significantly reduced compared to wild type worms. (N2: n=8, unc-26: n=10, catp-6: n=10, pink-1: n=10) **C.** *catp-6* and *pink-1* strains had a decreased lifespan compared to wild-type, partially due to bagging seen in 70% of the *catp-6* animals, and internal organ expulsion seen in 50% of *pink-1* animals. (N2: n=9, unc-26: n=10, catp-6: n=10, pink-1: n=10) **D.** A D5 adult *pink-1* mutant animal with the internal organ expulsion phenotype. (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$).

lifespan of *pink-1* mutants and wild type worms (Cooper et al., 2017). In this study, worms with bagging phenotypes and internal organ expulsion events were excluded, and the animals were rendered sterile with the use of FUdR (Cooper et al., 2017). The differences in these results and the previously published results can likely be as a result of the differences in experimental protocol and data interpretation. A lifespan with a larger sample size should be considered in the future to verify the frequency of the bagging and internal organ expulsion phenotypes.

It was also observed that *unc-26* animals have lifelong impaired motor phenotypes, with circular crawling and limited movement. Interestingly, after *pink-1* reproduction stopped at day 8 after plating, the *pink-1* mutants had progressively reduced movement, with similar circular and jerky crawling that was seen in the *unc-26* worms. It would be valuable to attempt to quantify movement, either by determining thrashing rate or distance traveled over time.

Altered development in the *unc-26* mutants, reduced fertility in the *catp-6* worms, reduced lifespan in the *catp-6* and *pink-1* worms, and impaired movement observed in *unc-26* and *pink-1* animals are indicators that the animals are unhealthy and exhibiting Parkinsonian movement defects. These phenotypes observed in *C. elegans* have also been observed in humans with Parkinson's Disease, which makes these observations valuable for future experiments (Frentzel et al., 2017; Gelb et al., 1999; Savica et al., 2019). Identification of changes in biochemical pathways could explain these altered phenotypes, and a rescue of these phenotypes can be used as indicators of health in a treatment-based experiment.

3.2 *C. elegans unc-26* Mutants Demonstrate Altered Phospholipid Composition

Membrane lipids of Parkinson's Disease mutants were characterized in an effort to identify changes that could be contributing to the manifestation of disease phenotype. Mutant lipids were analyzed with high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS). Extracted lipids were suspended in a liquid phase and ejected into a solid phase silica column to separate the sample based on lipid size and affinity to the column. After separation, the samples are analyzed with tandem mass spectrometry to first analyze the total mass of the molecular, then identify the molecule based on its ion fragmentation pattern. Lipid species were characterized by head groups and linkages. As the most abundant species, the

phospholipids with PC and PE head groups were analyzed in further detail to determine abundances based on the fatty acid tails.

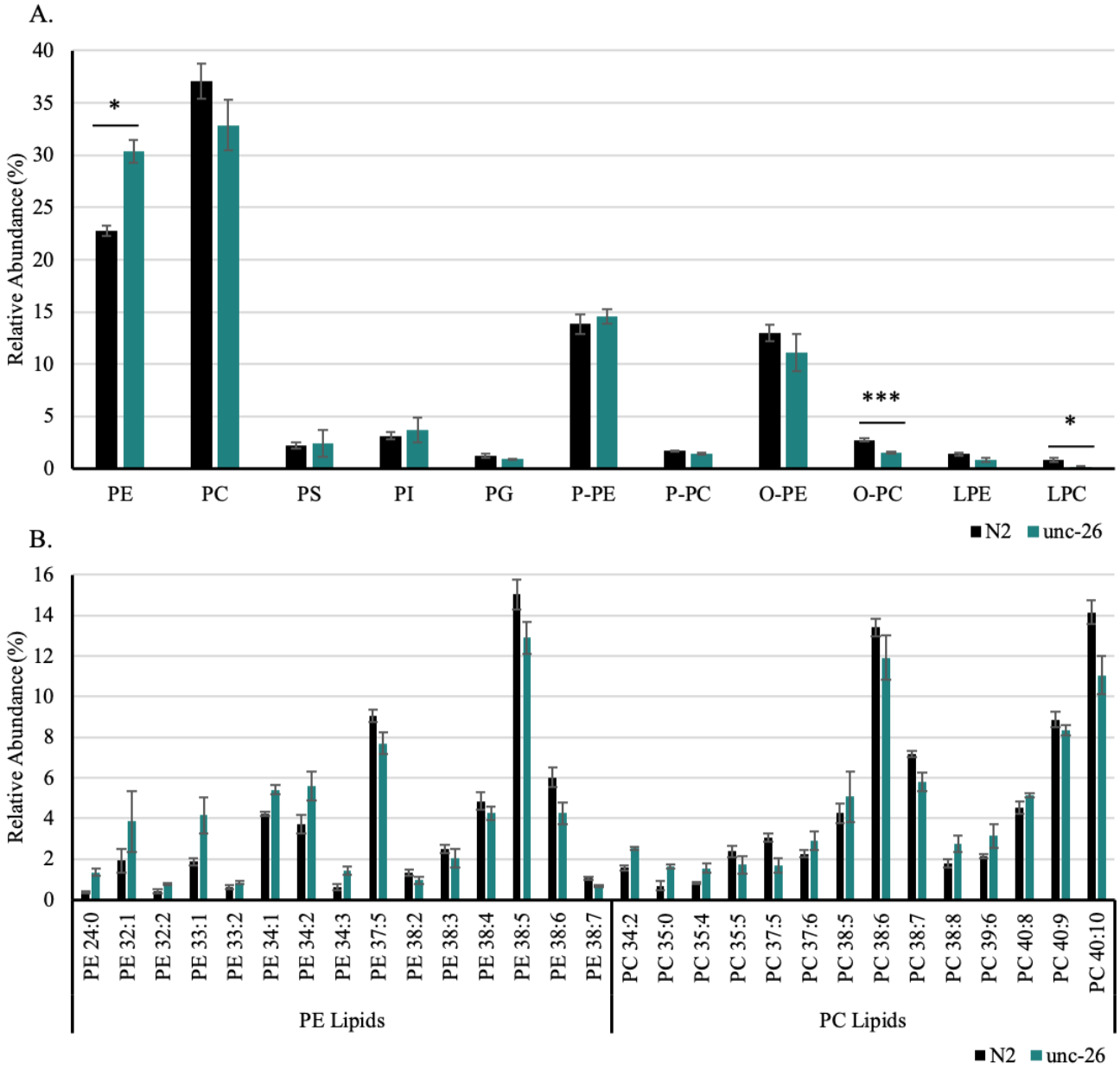


Figure 5. Membrane Lipids in *unc-26* Mutant Animals. A. The abundance of phospholipid head groups in *unc-26* mutants. B. The abundance of PE and PC lipid Species in *unc-26* mutants. (N2: n=3, *unc-26*: n=3). (* p< 0.05, ** p<0.005, *** p< 0.001).

Of the three mutants, *unc-26* mutants had the most significant changes to membrane composition. *unc-26* is responsible for the dephosphorylation of phosphatidylinositol 4,5-bisphosphate (PI4,5P) into phosphatidylinositol (PI), so it was hypothesized that there would be

altered abundances of PI lipids; however, this was not observed (Figure 5A) (Holthuis & Menon, 2014). PI lipids are critical in membrane trafficking and vesicle formation, so it is possible that compensatory mechanisms are involved to maintain PI populations within the membrane (Li et al., 2020).

A significant increase was seen in *unc-26* mutant PE lipids (Figure 5A). There is also a trend towards a decrease in PC species, and significant decreases in O-PC and LPC lipids (Figure 5A). Within the PE lipids, there are some trends towards increases in phospholipids with saturated and monounsaturated fatty acids, especially in PE 32:1, PE 33:1, PE 34:1, and PE 34:2 (Figure 5B). There are also trends towards decreases in PE phospholipids with unsaturated fatty acids, especially PE 37:5, PE 38:5, and PE 38:7 (Figure 5B). There are interesting trends in PC phospholipids, especially a decrease in PC 40:10 (Figure 5B) which may indicate reduced synthesis of unsaturated fatty acids.

With a biological model like *C. elegans*, there will be natural variability between replicates, which can lead to data presenting as not statistically significant. Additional replicates can improve the precision of results, and be used to identify any outliers, and therefore would be beneficial to verify these trends.

3.3 *C. elegans unc-26* Knockdowns Trend Towards Altered Phospholipid Composition

Mutant animals are constantly impacted by the lack of the gene for generations which, as observed through the characterization of reproductive phenotypes, can impact the development of the animal. In addition, long-term absences of the gene may enable the activation of alternative compensatory pathways that regulate lipid homeostasis in an effort to maintain cellular health. Therefore, changes seen may not be directly because of the loss of the gene, but instead due to adaptations to the lack of the gene. With RNAi, the gene knockdown can be targeted towards certain time periods in the animal lifespan. For this experiment, adult *C. elegans* were targeted with the RNAi knockdown to avoid impacting development of the worm, and to model Parkinson's Disease progression more accurately because manifestation of Parkinson's Disease in humans happens in older populations. This RNAi knockdown period allowed for the effects of the gene knockdown on daily membrane maintenance to be observed.

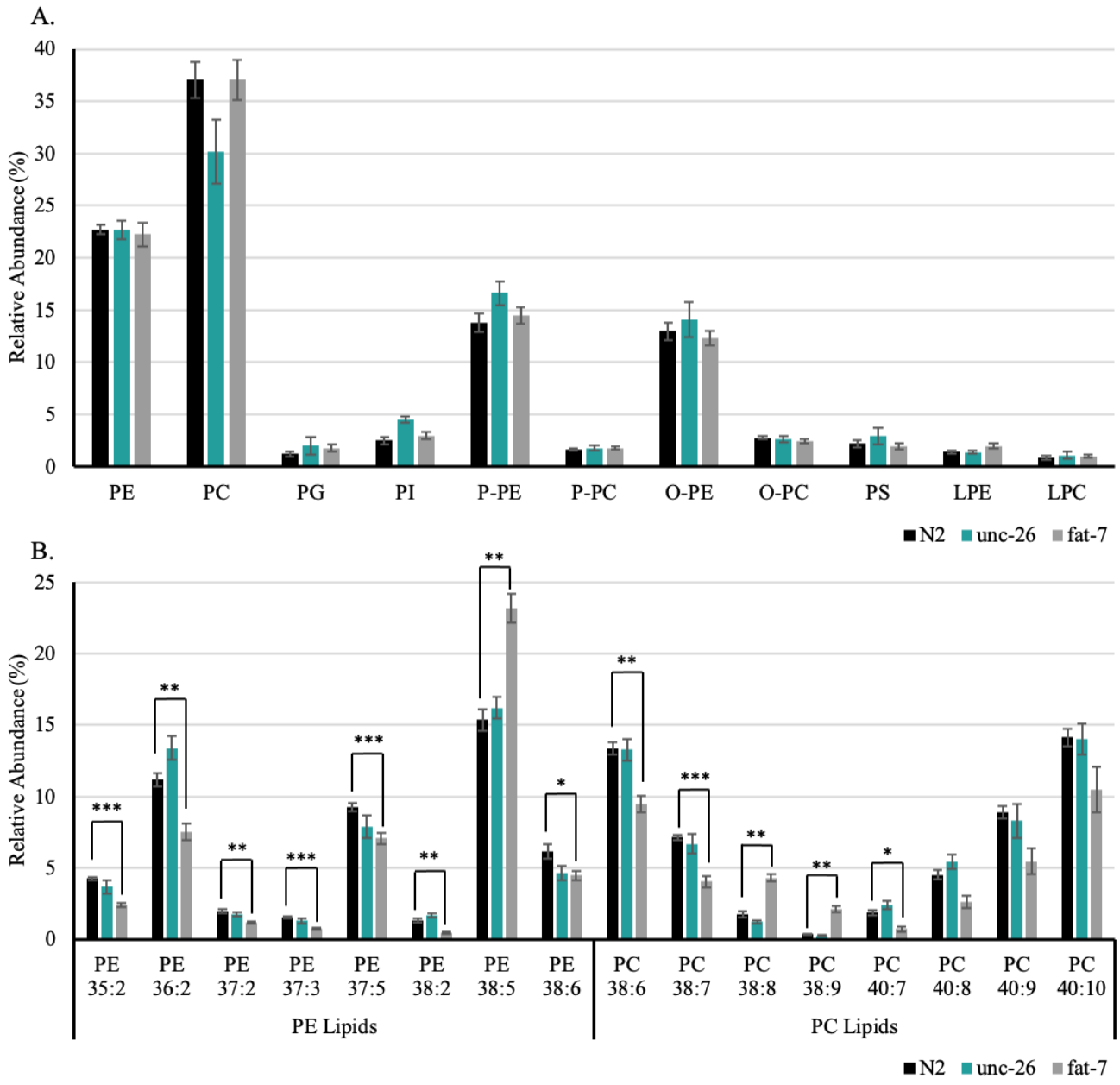


Figure 6. Membrane Lipids in *unc-26* RNAi Knockdowns. **A.** The abundance of phospholipid head groups in *unc-26* knockdowns. **B.** The abundance of PE and PC lipid Species in *unc-26* knockdowns. (N2: n=12, *unc-26*: n=5, *fat-7*: n=8). (* p< 0.05, ** p<0.005, *** p< 0.001).

In addition to the chosen PD genes, the RNAi knockdown protocol was completed with *fat-7*, a fatty acid desaturase enzyme that produces C18:1n9 from C18:0. For this experiment, *fat-7* served as a positive control, as the impacts of *fat-7* knockdowns on membrane lipids has been previously characterized (Dancy et al., 2015). Altered lipid species in the *fat-7* knockdowns will indicate that the RNAi protocol is working and that any changes, or lack thereof, seen in the previously unstudied genes are valid. Knockdowns of *fat-7* have previously been shown to

rescue PD phenotypes in *C. elegans* as well, so comparing the membrane composition of the PD genes and *fat-7* can provide an insight into how the desaturase might be rescuing these phenotypes (Fanning et al., 2019; Maulik et al., 2019).

Significant changes consistent with previously published data were seen in the PC and PE lipid species in the *fat-7* knockdown animals, indicating that the protocol was working (Dancy et al., 2015). qRT-PCR was not completed to determine the extent of the knockdown, so it is possible that the length of knockdown was not enough time to impact lipids, or that the lack of impact on development resulted in a reduced impact on lipids.

With significant changes seen in *unc*-26 mutant lipids, similar changes to membrane composition were expected in the *unc-26* RNAi knockdown animals. Similar to the trend seen in *unc-26* mutants, the *unc-26* RNAi knockdowns had a trend towards a decrease in PC species (Figure 6A). However, the increase in PE species seen in the *unc-26* mutant worms was not seen with the *unc-26* RNAi knockdowns. There were no significant changes in the lipid species within either the PE or PC classes (Figure 6B).

The differences between lipids in the *unc-26* mutants and the *unc-26* knockdowns could be because the development of the animals was not impacted with the RNAi knockdowns. It is also possible that the RNAi knockdown was not conducted for enough time to cause a significant impact on the lipids, and a longer RNAi knockdown period would result in more significant alterations in lipid species. The trend of decreased PC lipids seen in both *unc-26* mutants and *unc-26* RNAi knockdowns may indicate that PC species are the first impacted by the lack of *unc-26*. The increase in PE lipids observed in *unc-26* mutants may be a consequence of the decreased PC lipids and may be observed with a longer period of RNAi knockdown.

3.4 Knockdowns of *fat-7* Rescues Altered Lipids in *unc-26* Knockdowns

As mentioned, previous studies have shown that knockdowns of fatty-acid desaturases in *C. elegans* models of Parkinson's Disease were able to partially rescue wild-type phenotypes, including normal dopaminergic neuron levels (Maulik et al., 2019). In addition, the previous experiments demonstrated that different components of phospholipids are impacted by *unc-26* and *fat-7*. The abundance of head groups is impacted by *unc-26*, while the abundance of fatty-acid tails is impacted by *fat-7*. Therefore, it was hypothesized that normal membrane composition could be rescued with a knockdown of *fat-7* and *unc-26*.

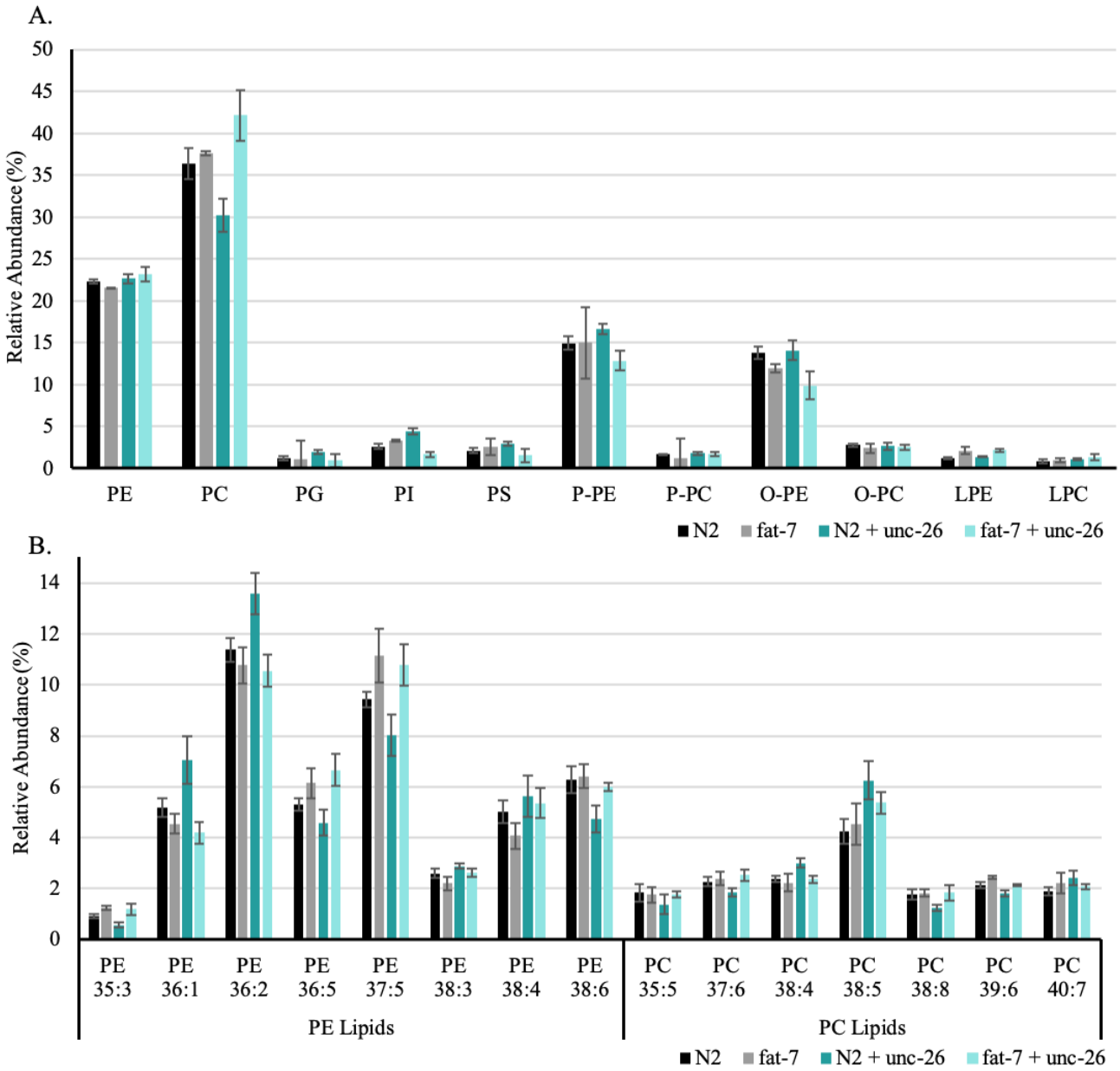


Figure 7. Membrane Lipids in *unc-26* RNAi Knockdowns in *fat-7* mutants. A. The abundance of phospholipid head groups of *unc-26* knockdowns in *fat-7* mutants**. B.** The abundance of PE and PC lipid species of *unc-26* knockdowns in *fat-7* mutants. (N2: n=12, N2 + unc-26: n=5, *fat-7*: n=4, fat-7 + unc-26: n=4). (* $p < 0.05$, ** $p<0.005$, *** $p < 0.001$).

The decreased trend of PC lipids in *unc-26* knockdown animals seen in the abundances of phospholipid head groups seems to be rescued in the *unc-26* knockdown of *fat-7* worms (Figure 7A). The abundance of PC lipids in the *unc-26* knockdown of *fat-7* worms has increased, even above wild-type PC levels. Similarly, many altered species within the PE and PC lipids in *unc-26*

knockdowns in wild-type animals seem to be rescued with the *unc-26* knockdown in *fat-7* mutants (Figure 7B). For example, increases seen in PE 36:1 and PE 36:2 abundances in *unc-26* knockdowns are not present in the *unc-26* knockdowns in *fat-7* mutants. *fat-7* mutants did not have the same impacts on lipids as RNAi, probably because the animals have adapted to the lack of this desaturase through generational and developmental lack of the gene.

An increased abundance of PE head groups with unsaturated fatty acid chains destabilizes membranes and impacts the binding of membrane proteins (Holthuis & Menon, 2014). Within the synaptic vesicle recycling pathway involving *unc-26*, a protein complex which includes alpha-synuclein is responsible for docking and fusing the synaptic vesicle to the cell membrane (Drouet & Lesage, 2014). The increase in PE lipids in *unc-26* worms may be contributing to the altered phenotypes seen, such as delayed reproduction and impaired movement. The altered lipid species, specifically increases in unsaturated PE lipids, may result in the inability of proteins like α-synuclein to assist in the binding of synaptic vesicles to the cell membrane and release neurotransmitters. When a desaturase, such as *fat-7,* is knocked down, the increase in saturated fatty acids and the decrease in unsaturated fatty acids may alleviate some of the stress caused by the altered head groups and rescue normal membrane composition and function.

3.5 Phospholipid Composition of *C. elegans pink-1* and *catp-6* Mutants

Membrane composition of *catp-6* and *pink-1* worms was characterized. Analysis of *catp-6* and *pink-1* mutant membranes showed no significant alterations or trends in the phospholipid head groups (Supplementary Figure 1A). There may be some interesting trends within PE and PC lipids for *catp-6*, specifically with increases in PE 37:5 and decreases in PE 38:5, PC 40:8, PC 40:9, and PC 40:10 (Supplementary Figure 1B and 1C).

3.6 Phospholipid Composition of RNAi Knockdowns of Selected Parkinson's Disease Genes

Parkinson's Disease genes *pink-1*, *catp-6*, *lrk-1*, *djr-1.1*, and *sodh-2* were knocked down in wild-type *C. elegans* following the adult-only RNAi Protocol. In RNAi knockdowns of *pink-1*, *catp-6*, *lrk-1*, *djr-1.1*, and *sodh-2*, there were minimal alterations in the membrane (Supplementary Figure 2). Unlike the *catp-6* mutant, there seems to be a decrease in the PC

headgroups in the *catp-6* RNAi knockdowns. No significant changes were seen within the PC and PE lipids for any of the genes.

In addition, RNAi knockdowns of *pink-1* and *sodh-2* were done in *fat-7* mutant *C. elegans*, and there were no significant changes to head group abundances or to lipid species for either of the genes studied (Supplementary Figures 3 & 4). The lack of impact could be due to the relatively short period of RNAi knockdown, as seen in the lack of altered lipids in the wild-type knockdowns of *pink-1* and *sodh-2*.

3.7 Fatty Acid Extraction of RNAi knockdowns in Wild Type *C. elegans*

To further characterize the impacts of RNAi knockdowns on lipids, the fatty acids were analyzed with GC-MS. The HPLC-MS/MS analyzes intact phospholipids and provides information on fatty acids associated with the head groups and linkages. GC-MS is a more targeted analysis of only the fatty acid species present in the phospholipids. With the GC-MS, information on the head groups is lost, but more information can be gained regarding abundances of fatty acid tails present in the membrane. In combination with the HPLC-MS/MS data on intact phospholipids, GC-MS data can be extremely valuable in tracking the saturated and unsaturated fatty acid species in the membrane.

The lipid samples that were run on the HPLC-MS/MS were used in the lipid separation protocol and fatty acids were isolated through FAME creation. There were limited samples with data that was able to be analyzed, and there were several species with lower abundances that did not meet the minimum threshold in the GC-MS. These initial results show promise for further GC-MS analysis, as there are some trends towards changes in fatty acid abundances in *unc-26* (Supplementary Figure 5).

Fatty acid separation after HPLC-MS/MS analysis was useful for the purposes of this project, but not recommended for consistent future use. It was difficult to get the abundances needed, and it is easier to get significant thresholds of lipids abundances by growing more *C. elegans* and separating the pellet during extraction.

3.8 Ethanol Assays

An ethanol assay was conducted on mutant strains and RNAi knockdowns of wild-type animals as a way to test for dopaminergic neuron function. Ethanol avoidance is reduced with the

loss of dopaminergic neurons. Results between trials were variable and did not match previous literature (Cooper et al., 2017; Maulik et al., 2019). Although not significant, there was a trend towards decreased ethanol avoidance in *unc-26* and *pink-1* mutants compared to wild-type worms (Supplementary Figure 6). Interestingly, the *fat-7* RNAi knockdowns also seemed to have a trend towards decreased ethanol avoidance. The protocol used may need adjustment. It is possible that there may not have been enough ethanol on the plates, and increased concentrations of ethanol may give more consistent results. Additional steps may need to be taken to ensure that light, heat, or other factors did not influence the worm response to ethanol.

4.0 Conclusions

The goal of this project was to characterize the impacts of Parkinson's Disease genes on membrane lipids in order to close diagnostic and treatment gaps of the disease. This study shows that membrane lipids were impacted by *unc-26*, a gene linked to familial Parkinson's Disease. In *unc-26* mutants, the abundance of PE and PC head groups was impacted, and trends towards decreased unsaturated fatty acids and increased saturated fatty acids were observed. The altered fatty acids may indicate activation of regulatory mechanisms in the cell that prevent destabilization of the membrane, which can happen with increased abundances of PE head groups with unsaturated fatty acid chains (Holthuis & Menon, 2014). Therefore, a desaturase *fat-7* was knocked down in combination with *unc-26*, which showed trends towards a rescue of normal membrane composition. The increased saturated fatty acids due to the loss of desaturase function may be alleviating stress placed on the membranes due to the loss of *unc-26.*

This study shows promise for further investigation of *unc-26*. Future experiments will be valuable to increase understanding of the full impacts and mechanisms of *unc-26* on membrane lipids. One future direction would be to do RNAi knockdowns of *fat-7* in *unc-26* mutant worms. This experiment would be a more realistic model of Parkinson's Disease, as the mutation in *unc-26* is present in PD patients, and the *fat-7* knockdown would be the treatment. This experiment will determine if the significant alterations in PE lipids in the *unc-26* mutants can be rescued with the *fat-7* knockdown.

A treatment-based experiment would be to supplement saturated fat to *unc-26* mutants to see if altered phenotypes and altered lipids can be rescued. With rescues in lipid alterations being seen with the loss of function of *fat-7* in *unc-26* knockdowns, it is hypothesized that the increase in saturated fat is causing these rescues. With a saturated fat supplementation, it can be determined if the rescue is due to increased abundances of saturated fat and can investigate the possibility of saturated fat treatments for Parkinson's Disease.

Another direction would be to conduct longer RNAi knockdowns. The RNAi treatment was for 48 hours, which may not be enough time for the effects of the gene knockdown to impact the lipids. By doing the RNAi knockdowns for a longer period of time, it will allow more time for the knockdown to affect the membranes. As PD is age-linked, the longer knockdown period will also allow for any age related changes to be observed. However, because worm feeding

decreases with age, a different RNAi knockdown protocol may need to be used to maintain the effectiveness (Corsi et al., 2015).

5.0 Abbreviations

C. elegans: *Caenorhabditis elegans*; DA neurons: dopaminergic neurons; DBS: Deep Brain Stimulation; GC-MS: gas chromatography-mass spectrometry; HG: High Growth media; HPLC-MS/MS: high performance liquid chromatography-mass spectrometry/mass spectrometry; LPC: lysophosphatidylcholine; LPE: lysophosphatidylethanolamine; NGM: Nematode Growth Media; NGM + CI: Nematode Growth Media + Carbenicillin and isopropyl β-D-thiogalactoside (IPTG); PC: phosphatidylcholine; PD: Parkinson's Disease; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PS: phosphatidylserine; RNAi: RNA interference.

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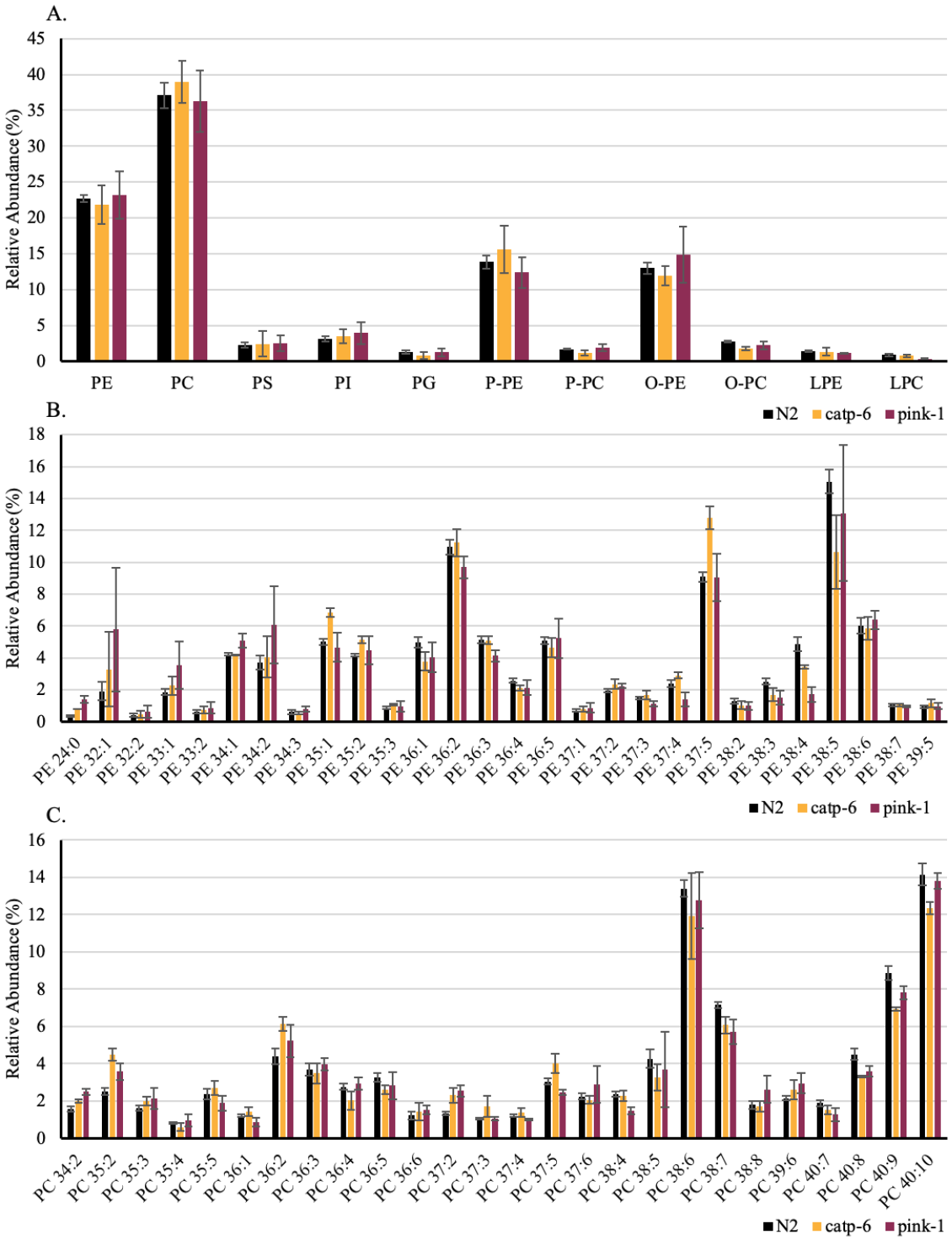
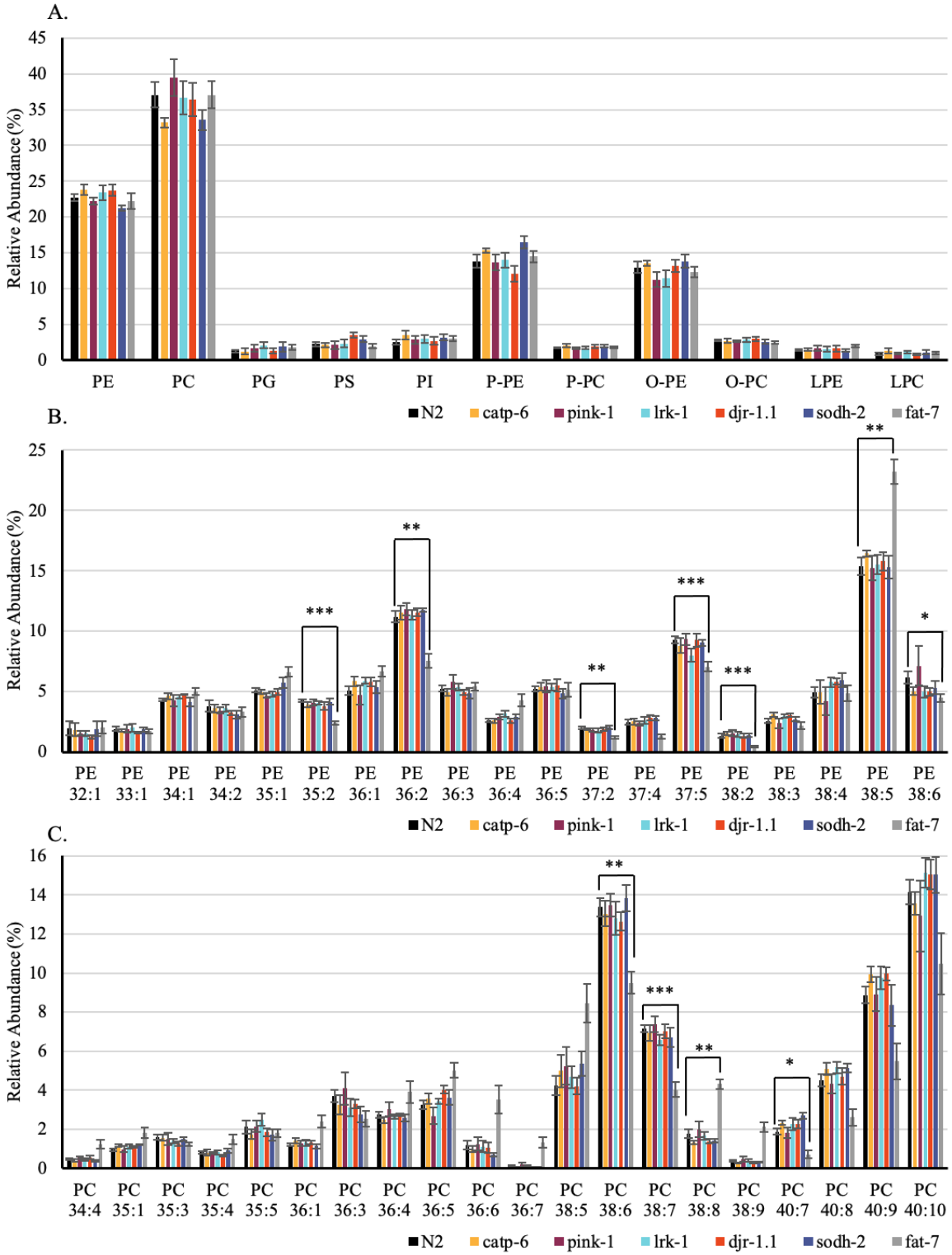
Supplementary Figure 1



Supplementary Figure 1. Membrane Lipids in Parkinson's Disease Mutant Strains. A. The abundance of phospholipid head groups in *pink-1* and *catp-6* mutants. **B.** The abundance of PE Lipids in *pink-1* and *catp-6* mutants. *catp-6* had some interesting trends, specifically with increases in PE 37:5 and decreases in PE 38:5 **C.** The

abundance of PC Lipids in *pink-1* and *catp-6* mutants. *catp-6* has trends towards decreases in PC 40:8, PC 40:9, and PC 40:10. (N2: n=3, *catp-6*: n=3, *pink-1*: n=3). (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$).

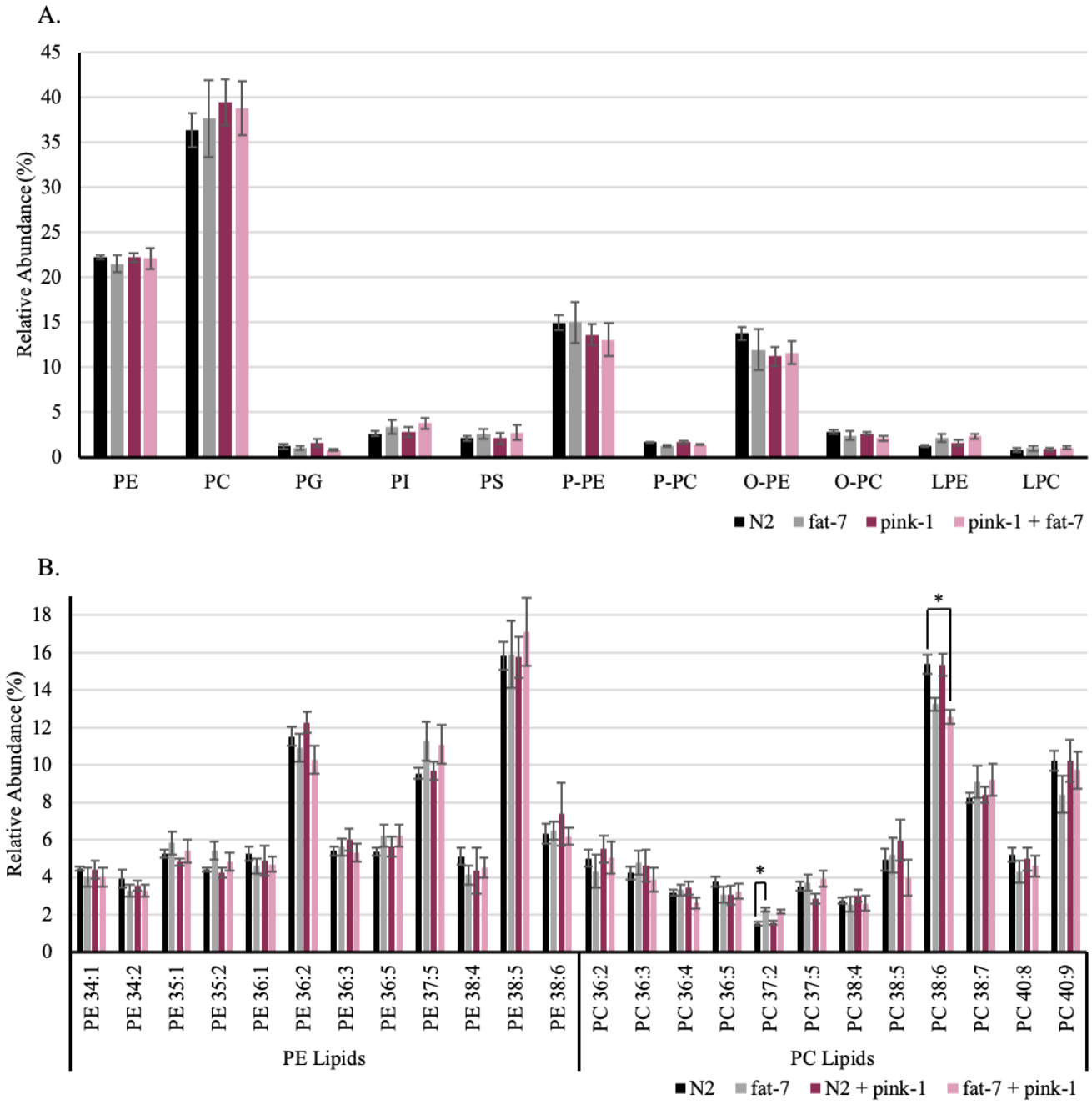
Supplementary Figure 2



Supplementary Figure 2. Membrane Lipids in Parkinson's Disease RNAi knockdowns. A. The abundance of phospholipid head groups in in RNAi Knockdowns. B. The abundance of PE Lipids in in RNAi Knockdowns. C.

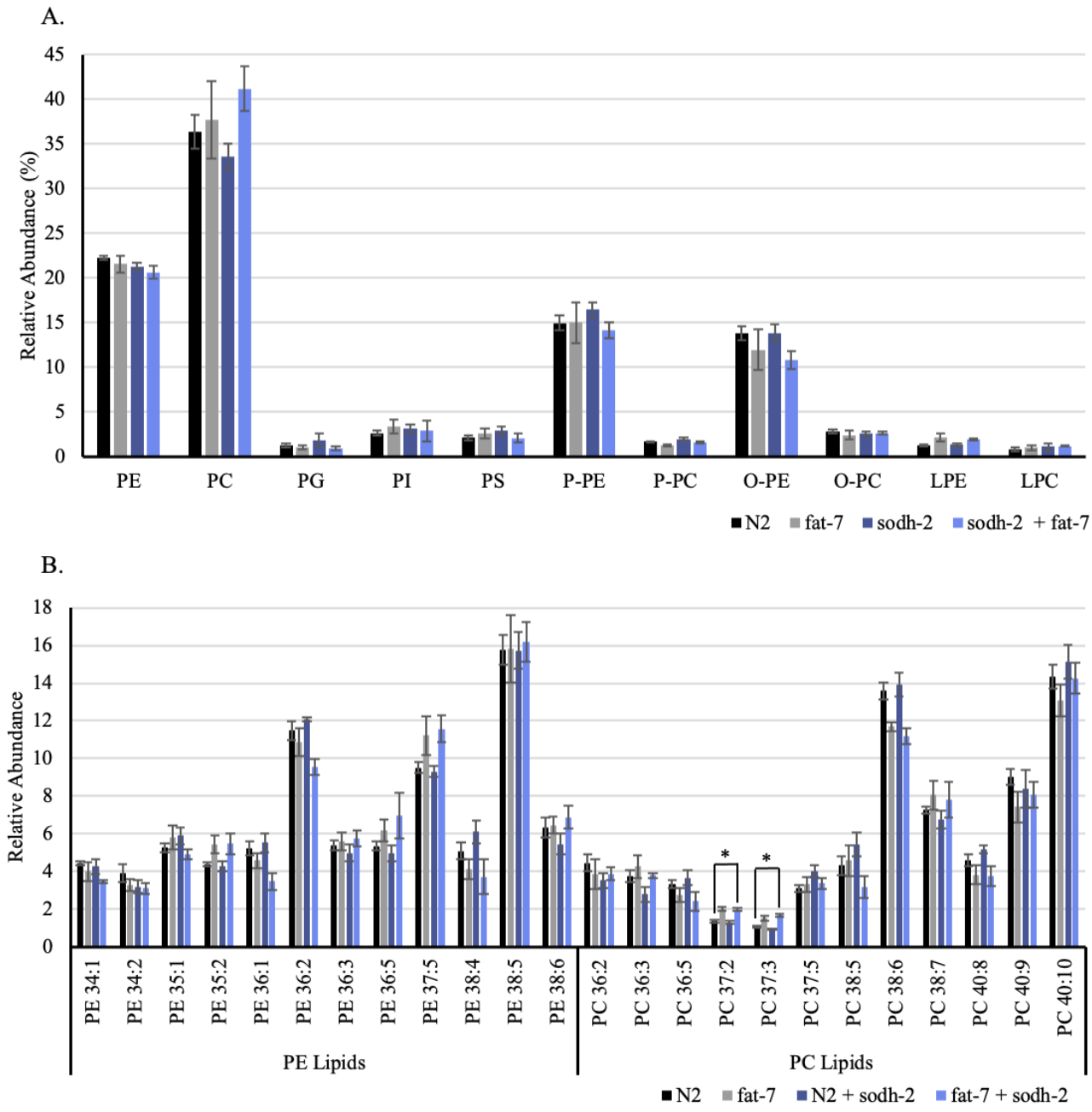
The abundance of PC Lipids in RNAi Knockdowns. (N2: n=12, *catp-6*: n=5, *pink-1*: n=5, *lrk-1*: n=5, *djr-1.1*: n=5, *sodh-2*: n=5, f*at-7*: n=8). (* p< 0.05, ** p<0.005, *** p< 0.001).

Supplementary Figure 3



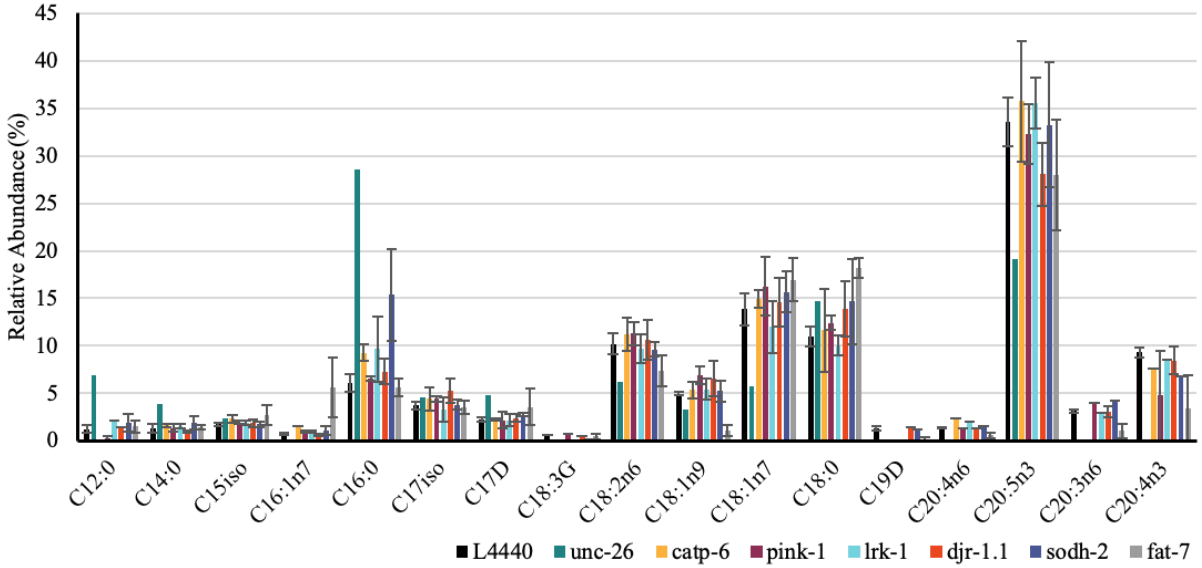
Supplementary Figure 3. Membrane Lipids in *pink-1* RNAi Knockdowns in *fat-7* mutants. A. The abundance of phospholipid headgroups in *pink-1* knockdowns of *fat-7* mutants **B.** The abundance of PE and PC lipid species in *pink-1* knockdowns. (N2: n=12, *fat-7*: n=4, N2 + *pink-1*: n=5, fat-7 + *pink-1*: n=4). (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$).

Supplementary Figure 4



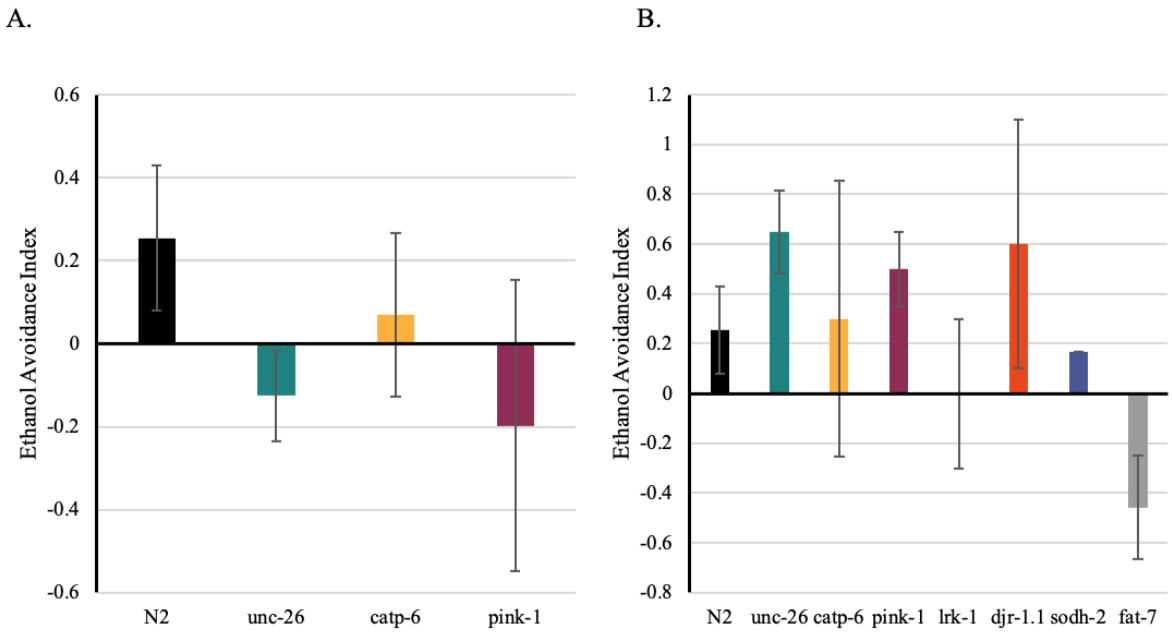
Supplementary Figure 4. Membrane Lipids *sodh-2* RNAi Knockdowns in *fat-7* mutants. A. The abundance of phospholipid headgroups in *sodh-2* knockdowns of *fat-7* mutants. **B.** The abundance of PE and PC lipid Species in *sodh-2* knockdowns. (N2: n=12, *fat-7*: n=4, N2 + *sodh-2*: n=5, fat-7 + *sodh-2*: n=4). (* p< 0.05, ** p<0.005, *** p< 0.001).

Supplementary Figure 5



Supplementary Figure 5. Fatty Acids in Parkinson's Disease RNAi knockdowns (N2: n=5, *unc-26*: n=1, *catp-6*: n=3, *pink-1*: n=4, *lrk-1*: n=2, *djr-1.1*: n=3, *sodh-2*: n=4, *fat-7*: n=3).

Supplementary Figure 6



Supplementary Figure 6. Ethanol Avoidance Index. A. The Ethanol Avoidance Index of *C. elegans* Mutants. (N2: n=6, *unc-26*: n=4, *catp-6*: n=4, *pink-1*: n=4) **B.** Ethanol Avoidance Index of *C. elegans* RNAi Knockdowns. (N2: n=6, *unc-26*: n=2, *catp-6*: n=2, *pink-1*: n=2, *lrk-1*: n=2, *djr-1.1*: n=2, *sodh-2*: n=2, *fat-7*: n=2).