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DIETARY PLANT LECTINS MAY BE AN ‘UNKNOWN ETIOLOGY’ IN PARKINSON’S DISEASE AND DIETARY BIOACTIVE COMPOUNDS AFFECT LIFESPAN AND FAT STORAGE ASPECTS OF *CAENORHABDITIS ELEGANS*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Nutrition and Food Sciences

by
Mingming Wang
B.S., Capital Medical University, 2007
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May 2016

To my beautiful wife, Tingting, my beloved son, Jiarui

&

My beloved parents

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ABSTRACT

Dietary bioactive compounds benefit health while some might induce pathological processes. Parkinson's disease (PD) is the second most common neurodegenerative disease. Braak and Hawkes hypothesized that the gastrointestinal tract may be a potential site of neuronal invasion by an "unknown pathogen" leading to some Parkinsonism. Neurotoxin botulinum or ricin can trans-synaptically transport in nervous system. Our hypothesis 1: dietary plant lectins might be responsible for the "unknown pathogen" causing PD. Pomegranate juice (PJ) have antioxidant and anti-obesity effects. Our hypothesis 2: PJ increases lifespan in *C. elegans* and reduces fat storage.

Study 1: Post-feeding rhodamine or TRITC tagged dietary lectins was tracked from gut to dopaminergic (DAergic) neurons in *C. elegans* BZ555 (*egIs1*[*Pdat-1::GFP*]) that has Green Fluorescent Protein (GFP) gene fused to a dopamine transport protein gene labeling DAergic neurons. Although this observation was tested with specific inhibiting sugars (SIS), supplemented with *Escherichia coli*, the high concentrations of monosaccharides necessary may have their own side effects. Results showed that *Amaranthus caudatus agglutinin*, *Euonymus europaeus agglutinin* and *Arachis hypogaea agglutinin* co-localized with DAergic neurons. Lectins affected the number, size or intensity of DAergic neurons, reduced the mobility and affected the lifespan of *C. elegans* to different extents, with the SIS either augmenting or mitigating the effects. Our observations are a tantalizing possible explanation for why dietary plants have been linked to a risk of developing PD.

Study 2: Lifespan of *C. elegans* was increased by PJ treatment in wild type (N2, 56%) and *daf-16* mutant (*daf-16(mgDf50)I*) (GR1307, 18%), by POMx in N2 (28%) and in GR1307 (10%), or by EA (11%). PJ reduced intestinal fat deposition (IFD) in N2 (-68%) or in GR1307 (-33%). The IFD was increased by POMx in N2 (137%) and in GR1307 (26%), by EA in N2 (66%) and in GR1307 (74%), or by UA in N2 (57%) and in GR1307 (43%). Both lectins and PJ are bioactive compounds, playing important roles in

life. These studies may provide useful information for an alternative etiology of PD and offer solutions of using PJ to delay aging and prevent obesity in humans.

CHAPTER I. INTRODUCTION

1.1 General introduction

Dietary bioactive compounds such as phytochemicals, vitamins, minerals and fibers contained in fruits and vegetables benefit humans and animals [1, 2]. Chemical compounds in fruits and vegetables can alter gene expression; have neuroprotective and antiaging effects, and effects in preventing or treating chronic diseases including cancer [3, 4]. Plant lectins were discovered over a century ago [5]. Consumption of lectins contained in seeds or tuber have caused diseases in human beings mainly due to damage on intestinal mucosa [6]. Patients suffered vomiting and diarrhea after consuming a diet high in red kidney beans containing the lectin phytohemagglutinin in a hospital in 1988 [7]. Lectins are also related to disease symptoms in some patients with dysfunction of the immune system [8]. Recent studies report that lectins play important roles in plant defense [9] and legume-rhizobial interactions [10]. Pomegranates (*Punica granatum* L.) have a high content of polyphenols (1.5%), including ellagic acid (EA), gallic acid, anthocynidins, flavan-3-ols, straight chain fatty acids, citric acid, and malic acid. Pomegranates consist of about 80% juice and 20% seeds with water (85%), and 10% sugars consisting primarily of fructose (2.5 g to 17.6 g/100ml) [11, 12]. Dietary polyphenol antioxidants play important roles in health [13-17]. PJ has been shown to extend lifespan in mice [18, 19] and *Drosophila melanogaster* [20].

The increasing age of people in modernized countries creates a greater burden of chronic diseases including cancer, cardiovascular disease, diabetes, and neurodegenerative disorders including PD and Alzheimer's disease [21-25], leading to a high cost of health care and a great financial burden to the public health system and families [26, 27]. The study of dietary bioactive compounds like lectins and PJ and its derivatives in *C. elegans* organism may provide some useful information for an alternative possible etiology of PD and offer some solutions to delay aging and prevent obesity in humans.

1.2 Dietary proteins may be trans-synaptically transported in the nervous system

Protein toxins are able to survive the harsh environment of cooking process, low pH of stomach, and protease digestion of the GI track. Many of them can penetrate intestine epithelial cells and pass into blood or lymphoid circulation [28-30]. Toxins are target specific. Botulinum neurotoxins (BoNTs) or ricin can be trans-synaptically transported in the nervous system causing severe, even lethal damages on humans and other mammals [31, 32]. Cholera toxin exerts toxicities only on the epithelial cells of intestine and causes symptoms like watery diarrhea [33-35]; tetanus toxin (TeNT) affects neuromuscular junctions after being taken up into blood or lymphoid circulation [36].

Glycoproteins and glycolipids are major components of eukaryotic cell membranes along with phospholipids, and are the main binding receptors for toxins, playing a crucial role in the process of binding and internalization [29, 37-39]. Glycoproteins and glycolipids are also essential compounds for intercellular communications and reactions to alterations of the cell environment [40]. Both ricin and some neurotoxins share similar A-B chain structure; in which chain A is responsible for recognizing binding receptors and internalization, and chain B exerts toxicities. Understanding the underlying mechanisms of neurotoxins may help better understand the effects of lectins on neurons.

1.3 Dietary plant lectins as an "unknown pathogen" in Parkinson's disease

Higher prevalence of PD occurs in vegetarians compared to omnivores [41, 42], though controversial. Plant lectins have been used for tracing neurons along nerve fibers in animal studies. Dietary toxin proteins can overcome the barriers of digestion systems and cause severe, even lethal damage on mammals including humans [31, 32]. We hypothesize that dietary plant proteins traverse the GI wall, enter the nerve endings, undergo vesicular transport along nerve fibers, and damage DAergic neurons as one etiology of PD. A recent Danish study showed that patients who had vagal nerves removed 20 years ago had a 40% lower incidence of PD [43], which supported our hypothesis of dietary plant lectins.

PD is the second most common degenerative disorder of the central nervous system that impairs motor skills and cognitive function, the main symptoms of PD are motion disorders, like muscle rigidity, bradykinesia, and tremors [44]. Clinically, PD is characterized by the development of Lewy bodies due to aggregation of a-synuclein (α-SYN) in the brain tissue and the partial loss of DAergic neurons in the substantia nigra [45]. α-SYN also aggregates in microglia and further leads to PD although the detailed mechanism remains unclear [46, 47], while astrocytes convert the PD-causing neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to its active metabolite cation 1-methyl-4-phenylpyridinium (MPP^+) [48]. However, the mechanism of the formation of Lewy bodies is not clear [49]. The etiology of PD is unknown but complex environmental factors play important roles for neurodevelopmental and neurodegenerative disorders including PD [50, 51]. Results of large-scale epidemiological studies using meta-analysis showed that a statistically positive association exists between PD and pesticide or herbicide exposure [52]. Most of these pesticides share common features, such as the ability to induce oxidative stress, mitochondrial dysfunction, α-SYN fibrillization and neuronal cell loss [53]. Insulin dysfunction might be associated as a cause of PD though the detailed mechanism is still unclear [54]. Evidence indicates that in *Caenorhabditis elegans* (*C. elegans*) high glucose concentration (14 mM) increases aggregation of α-SYN while restricted glucose concentration reduces it [55]. Exposure to other environmental factors such as solvents, metals and other pollutants are also associated with the risk of PD in animal models [56, 57]. Despite the long-accepted viewpoint that environment and genetic factors might be most related to the etiology of PD, a recent review in 2015 reported that dietary factors like vitamins, flavonoids, calorie intake, caffeine, alcohol, and metals may also play a role [53, 58].

Plants contain glycoprotein-lectins ("non-immune sugar-binding proteins") in seeds, fruits, and nuts [59], that recognize and reversibly bind specific carbohydrates [60]. They are involved in plant defense [9] and legume-rhizobia [10]. They resist gut enzymes maintaining function under adverse conditions [61, 62]. They can penetrate the GI tract wall by endocytosis [63], probably by first binding a

carbohydrate lectin receptor [64] followed by endocytosis, and, astonishingly, can transfer trans-synaptically in an antegrade and/or retrograde fashion along nerve fibers [60, 65]. Their medical importance is increasingly being recognized by being conjugated with drugs for better drug absorption from the GI tract [64, 66-68]. Particularly relevant to the current studies, lectins have been utilized extensively for neuronal tracing studies [65, 69]. Ricin (*Ricinus communis*) as an extremely cytotoxic lectin has been studied extensively for its function in retrograde transport. It is reportedly via a B-chain mediated endocytosis, following a translocation of the enzymatically catalyzed A-chain, from the endosomes to the Golgi apparatus [70, 71]. This property has been utilized for treatment of malignancies at low doses [72, 73]. Lectins have also been conjugated with DNA for enhanced nervous system gene delivery [74]. Most dietary plant lectins resist gut enzymes and maintain function under usually adverse conditions for proteins [61, 62].

Most lectins are resistant to mild heat and protease [75], lectin *concanavalin A* (Con A) from *Canavalia* seeds can survive high cooking temperatures such as 96 ℃ for up to three hours [76]. These characteristics, theoretically, allow lectins to be 'biologically active' when reaching their "targets" *in vivo*. Controversial reports suggest that a higher prevalence of PD occurs in vegetarians compared to omnivores [41, 77]. The "Vegetarianism in America" study in 2015, published by Vegetarian Times (vegetariantimes.com), shows that 3.2 percent of U.S. adults, or 7.3 million people, follow a vegetarian-based diet and 96.8 percent of U.S. adults or 220.8 million people are omnivores. A 2195 American participants study reported that on average 1/3 of total vegetables people consumed were not cooked, and people who consumed more raw vegetables also preferred raw fruits and grains [78]. Taken together, a subgroup of dietary plant lectins may act as one of the long-term 'pathogens' that slowly cause cellular inclusions of α-SYN.

The Amyotrophic Lateral Sclerosis / Parkinsonism dementia complex (ALS-PDS) in populations from Guam, for example, has been linked to a diet rich in cycad seeds (*Cycas micronesica*) [79, 80]. The cycad

seeds contain ß−methylamino-L-alanine (BMAA) which has excitotoxic properties. Studies on human brain tissue of ALS/PDC, ALS, Alzheimer's disease, PD, Huntington's disease and neurological controls indicated that BMAA is present in non-genetic progressive neurodegenerative disease but not in controls or genetic-based Huntington's disease [81-84]. Animals fed with purified BMAA, however, do not show ALS-PDS traits [85] suggesting that other mechanisms deliver toxin(s) to the target neurons. A newly proposed medical hypothesis suggests that structurally similar glucosides of *helicobacter pylori* might be accountable as neurotoxins [86] though currently unproven. Several lines of evidence suggest a possible role of lectins in neuronal injury. First, lectins have been detected in *Cycas revoluta*, which is in the same family (*Cycadaceae*), and genus (*Cycas*) as *Cycas micronesica* of Guam [87, 88]. Second, horses consuming yellow star thistles (*Centaurea solstitialis*) show liquid necrosis in the substantia nigra and develop nigropallidal encephalomalacia (NPE) symptoms, which are similar to human Parkinsonism. The toxins responsible for NPE have not yet been identified [89]. These studies suggest that lectins might be toxic or chaperone carriers of toxins in PD-like diseases.

1.4 Bioactive compounds of pomegranate juice and extracts improve lifespan in *C. elegans*

Dietary polyphenol antioxidants play important roles in health [13-17]. PJ has been shown to extend lifespan in mice [18, 19] and *Drosophila melanogaster* [20]. PJ extract (POMx) potentiates lifespan extension with dietary restriction, a finding attributed by polyphenols [18, 19]. POMx and PJ can also act as prebiotics, having demonstrated antibacterial properties *in vitro*, and can block DNA repair and inhibit proliferation of breast cancer cells (MCF-7) *in vitro*, as well as modulate the IGF-IGFBP axis [90-94]. POMx and PJ also down-regulate androgen-synthesizing genes to induce apoptosis of human prostate cells (kappaB-dependent) *in vitro* and in mice *in vivo* [95-98]. POMx and PJ decrease prostate specific antigen in humans after surgery or radiation [99, 100], inhibit tumor-associated angiogenesis *in vitro* and *in vivo* [99], suppress inflammatory cell signaling in colon cancer cells [101] (50 mg/L PJ, *in vitro*), improve memory [102] and improve fecundity in humans [20, 103-105].

As the main bioactive component, EA is a measure of the quality of PJ extract products [104, 106, 107]. EA in humans reaches a maximum plasma level in 1 hour (31.9 ng/ml), is eliminated within 4 hours [108] and exerts an antioxidant effect [109]. Urolithin acid (UA) is the main active metabolite of EA and is formed by colonic microflora. UA lasts longer in the human body than EA or other EA metabolites [91, 110], and has better bioavailability [111]. UA also suppresses colorectal, hepatic, and prostate cancers synergistically with EA *in vitro* and in mice *in vivo* [112-116].

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CHAPTER II. DIETARY PLANT LECTINS MAY BE AN 'UNKNOWN ETIOLOGY' IN PARKINSON'S DISEASE

PART A. DIETARY PROTEINS AND TRANS-SYNAPTICAL TRANSPORTATION

1.1 Introduction

Study of mechanisms of protein toxins and lectins has been conducted for decades, aimed at elucidating the interesting phenomenon that a trace amount of botulinum neurotoxins (BoNTs) or ricin could overcome the barriers of digestion systems and cause severe, even lethal damage to mammals including humans [1, 2]. The targets of these toxins in humans vary. For instance, cholera toxin exerts toxicities only on epithelial cells of intestine and causes symptoms like watery diarrhea [3-5]; but tetanus toxin (TeNT) can cause further damages in neuromuscular junctions after being taken up into blood or lymphoid circulation [6]. Several studies have investigated how these protein toxins or ricin survive the harsh environment in the stomach and intestine including low pH and protease, and how the toxin penetrate intestine epithelial cells into blood or lymphoid circulations [7-9]. Though some mechanisms were revealed, the detailed mechanism of this process, especially the process of penetration into the neuronal membrane is still under extensive research [10, 11]. Binding strength to neuronal membranes is a prerequisite of the following translocations and other toxicities [12, 13].

Both ricin and some neurotoxins share similar A-B chain structure; in which chain A is responsible for recognizing binding receptors and internalization, and chain B exerts toxicities. Other lectins can recognize special binding sites on neuron membranes and then build communicating connections with neurons. In this chapter, the travel of neurotoxins from intestine to central nervous system was covered and the stress was placed on how neurotoxins were retrograde transported from axon to central nerve. This part focused on protein toxins because of severe threat to human beings and have been more studied. Understanding the underlying mechanisms would help design new drugs and better understand the effects of lectins on neurons.

1.2 Protein toxins

1.2.1 Botulinum neurotoxins and tetanus neurotoxin

1.2.1.1 Structure components

BoNTs and TeNT have been studied together in most cases because they share similar A-B chain structures, employ similar pathways to exert toxicities; and attack neurons only [6, 14, 15]. BoNTs have seven serotypes, including type A-G. The analysis of protein sequences of seven serotypes of BoNTs and TeNT indicates sequence homology [16, 17]. In addition, BoNTs and TeNT cause similar symptoms of paralyses because they both inhibit the release of neurotransmitter *via* zinc-dependent cleavage of protein components [15]. The main difference between them is that BoNTs target neuromuscular conjunctions while TeNT plays roles at neuron-neuron junctions.

Properties are determined by structures. In the wild with adequate moisture like in the soil, BoNTs exist in the form of binding to a small complex called non-toxic neurotoxin-associated proteins (NAPs, Figure 1), which is composed of Hemagglutinin (HA) and non-toxic non-HA (NTNHA) [18, 19].

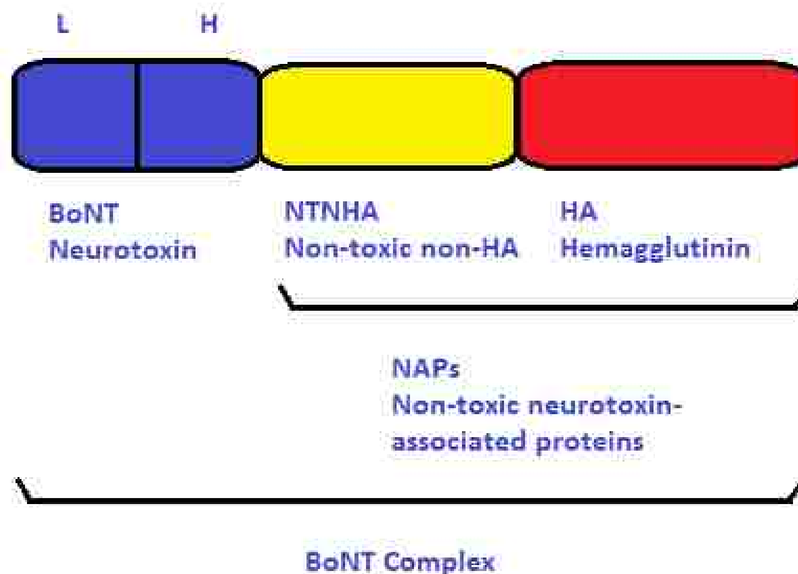

Figure 1. Composition of BoNTs complex.

The structure of NTNHA protein and BoNTs shows mirror symmetry so that they could bind tightly with each other *via* multivalent bonds. This strong binding shield BoNTs from harsh environments like low pH and proteases [20]. After surviving the harsh environment in GI tract and penetrating the GI wall, BoNTs arrive at a moderate environment like blood or lymphoid circulation, where BoNTs are released from the complex and at this time they can travel freely *in vivo* [21].

1.2.1.2 Neurotoxins absorbed from GI tract to circulation and up-taken to nerve endings

After surviving harsh intestinal environments, BoNTs need to overcome the intestinal barrier before entering vascular circulation. Fujinaga *et al.* in 2013 reported that three steps were involved in penetration through the intestinal epithelial barrier, including transcytosis, barrier disruption and absorption from damaged barrier (Figure 2) [19]. In detail, two different pathways are involved in the first step transcytosis. The first pathway is associated with the binding domain of the heavy chain, which facilitates the binding and transcytosis of the whole toxin. BoNT/A mainly takes advantage of this pathway; however, the detailed mechanisms underlying this pathway are still unknown. Couesnon *et al.* proposed that Caco-2 and m-ICc 12 cells can absorb the binding domain of the heavy chain by a Cdc-42-dependent and clathrin-independent pathway. Recently, Couesnon *et al.* further proposed that neuroendocrine intestinal crypt cells play an important role in transcytosis [22, 23]. The second pathway is associated with HA, which could promote the binding of BoNTs with intestinal barrier due to its carbohydrate-binding properties and the ability to transit the barrier. Fujinaga *et al.* reported that HA plays an important role in facilitating the binding of BoNTs complex to the epithelial lining [24]. HA is also involved in internalization of BoNT/C and BoNT/D complex into the intestinal epithelial barrier [25]. The third step is disruption of the epithelial barrier after BoNTs are transferred to the basolateral surface, facilitated by HA. BoNT/A and BoNT/B would not cause some cytotoxic effects in epithelial cells during the disruption [26], however, BoNT/C might do so [27]. The last step is release of BoNTs from the damaged barrier into circulation.

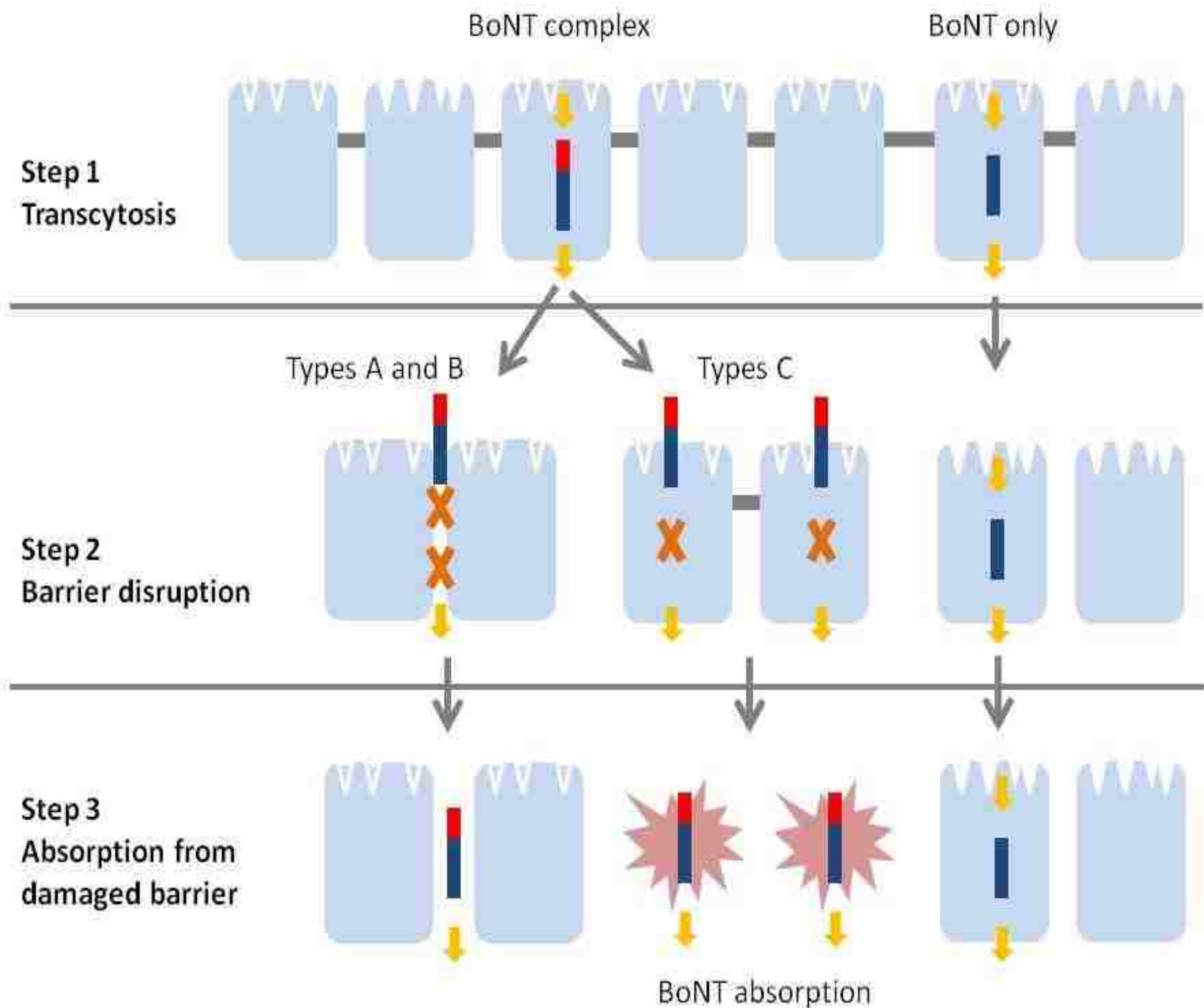

Figure 2. Three steps involved in the process of penetration into the intestinal epithelial barrier, including transcytosis, barrier disruption and absorption from damaged barrier.

1.2.1.3 Internalization into neurons

After entering the blood or lymphatic stream, neurotoxins are transported to neuromuscular junctions. Four steps are required for BoNTs and TeNT to enter neurons and exert toxicities (Figure 3). After entering into neuron terminals, BoNTs' effects are mainly restricted to the neuromuscular conjunction, which prevents release of synaptic vesicles from cell membranes through three different pathways as mentioned above; however, TeNT is retrograde transported to neuron soma in the spinal cord and trans-synaptically transported to the axon of the next neuron [28].

The first step is membrane binding, early research reported that BoNTs and TeNT bound to gangliosides or glycosphingolipids which were found particularly in the outer leaflet of neuronal cell membranes [29, 30]. Later studies suggested that BoNTs and TeNT bind to more than a single receptor, and a dual-receptor theory was then proposed. Strong evidence for this dual-receptor theory is that the binding of TeNT to rat brain membrane was greatly decreased but not abolished after protease pretreatment, implying that other kinds of protein receptors are involved in binding. In detail, the dual-receptor theory proposed that BoNTs and TeNT bound to complex polysialogangliosides first, including GD1b, GT1b and GQ1b, which are abundantly present on the outer leaflet of neuronal membranes; thereafter, they further bind to more sparsely distributed protein receptor(s). Toxins accumulate first on the membrane surfaces, and then further interact with protein receptors to complete the second step of binding. Rummel described the detailed pathway of the first step in 2013 [31].

The second step is known as internalization, after toxins bind to the surface receptors, acid compartments are formed with the endocytic process of toxins, where temperature and available energy could affect this process [16].

The third step is translocation. After BoNTs and TeNT are transported to acidic compartments, the compartments depart the internal cell surface and enter the cytosol. BoNTs and TeNT utilize different pathways for translocation from compartments into cytosol. Due to the low pH in the compartments, the L chain of the BoNTs would be rearranged; leading to higher hydrophobicity, which makes it easier to traverse the lipid bilayer [16, 32]. By this step, the light chains of BoNTs are freely available in the cytosol of the neuron cells.

The fourth step is intracellular action, which mainly inhibits the release of neurotransmitters from neurons into neuromuscular junction. As shown in Figure 3, the L chain of the toxin cleaves essential proteins necessary for the release of neurotransmitters.

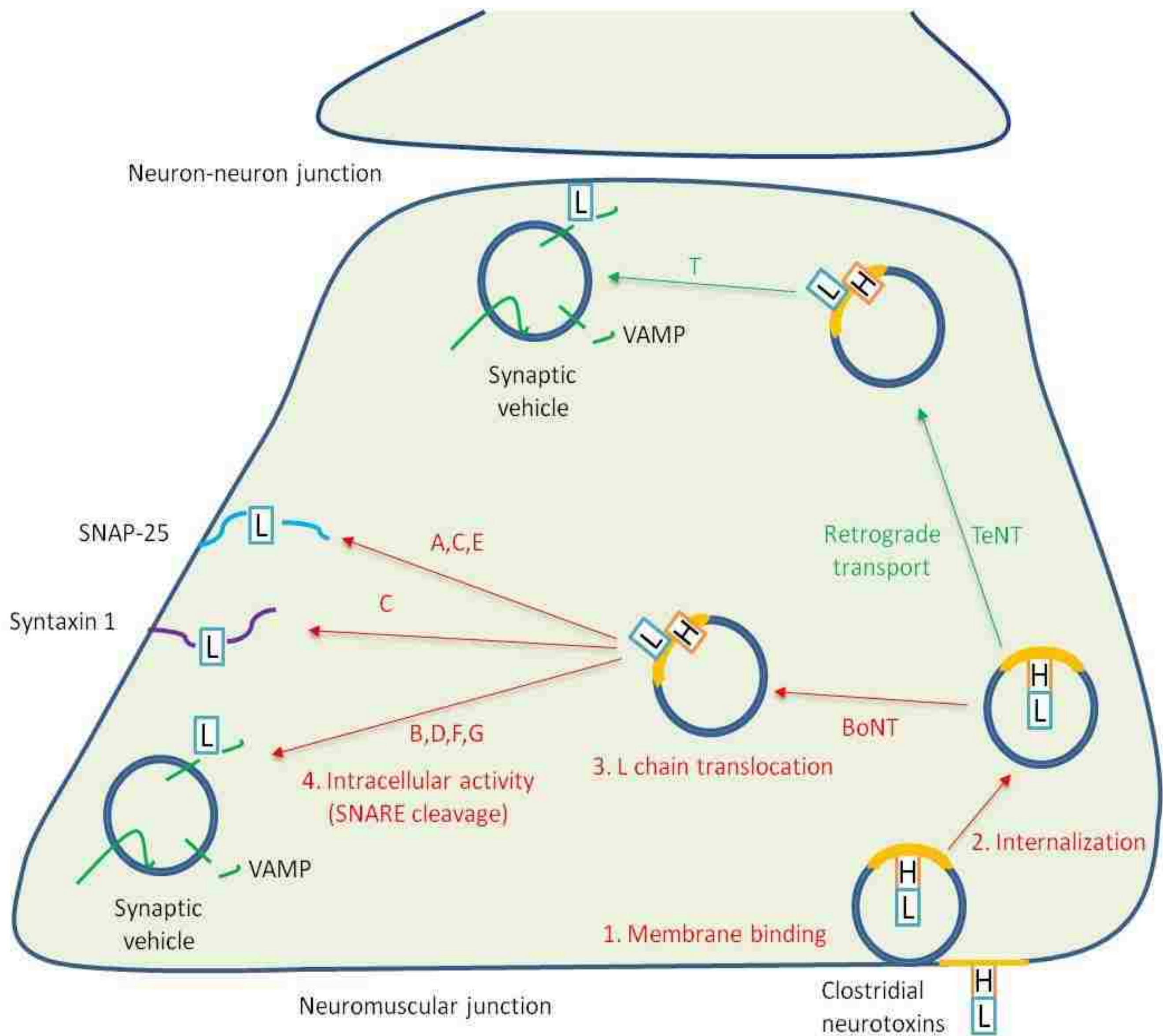

Figure 3. Four steps involved in the internalization and toxic effects.

1.2.2 Shiga toxin

Shiga toxin inhibits protein synthesis in target cells [33]. Globotriaosylceramide (Gb3), the special glycolipid receptor recognized by shiga toxin, was first isolated from both HeLa cells and rabbit jejunal mucosa [34]. Shiga toxin has two sub-types; Stx1 and Stx2, both of which feature A-B_5 structure, where one A-subunit is the active component and five B-subunits are responsible for binding [35]. Stx2 causes more severe damage than Stx1 which only causes some infections [36-38].

Gb3 was first found in the rat sensory neurons and in rabbit brain capillaries by immunohistochemistry [39]. Takahashi, K. first reported that Stx2 might damage neuron cells indirectly using a rabbit model [40]. Other studies reported that Stx2 induced a glial lamellipodia-like process between presynapse and motoneuronal soma, and proposed that neurons were the primary target of Stx2, which could lead to paralysis [35]. Stx2 was further reported to induce expression of Gb3 in neurons and cause dendritic abnormalities in rat brains [41], and to exert direct cytotoxic effect in the thalamus in rats [42].

Shiga toxin employs a clathrin dependent mechanism to enter the cell, binding to glycolipid ligand and endocytosed from coated pits [43, 44]. Binding sites are randomly located on cell membranes, but if the temperature reaches 37 ℃, the binding site Gb3 will concentrate in coated pits. Binding of the subunit B to Gb3 causes induction of narrow tubular membrane invaginations, which drives formation of inward directed membrane tubules for bacterial uptake into the cell. These tubules are essential for the uptake of shiga toxin into host cells [45].

To sum up, neurotoxicity of shiga toxin is mainly due to the subtype Stx2. Gb3 is the primary receptor on neuron cell membranes that bound to Stx2, however, the detailed mechanism of binding and the following internalization is still not clear.

1.3 Lectins

1.3.1 *Ricinus communis agglutinin* (RCA)

Ricin, synthesized and stored in the endosperm cells of maturing *Ricinus communis* seeds (castor beans) is a heterodimeric plant protein that has emerged as the first well-known lectin due to its extremely high toxicity of trace doses and its special A-B chain structure [46, 47]. Similar to BoNTs and TeNT, ricin utilizes the A-B mechanism to penetrate cell membrane. Chain B of ricin attaches to the cell surface first and then the protein undergoes endocytosis into cells. Ricin inhibits the synthesis of protein enzymatically after its chain A enters cytosol and removes a specific adenine residue from the 28S

ribosomal RNA (28SrRNA) of the large subunit of eukaryotic ribosomes [48], other toxins share the similar mechanisms include diphtheria toxin, Shiga toxin, Pseudomonas exotoxin A, abrin and modeccin [49].

Chain B has special binding sites for galactose [50]; galactose exists in many cell membranes because it is widely present in glycoproteins and glycolipids, which are the major components of cell membranes [51], thus ricin is bound to the cell surfaces. Other cell surface receptors include N-acetylgalactosamine, glycoproteins, glycolipids or mannose receptors [52]. After chain B successfully binds to cell surfaces, ricin utilizes both clathrin-dependent and clathrin-independent pathways including caveolae and macropinocytosis to enter cells [53-56]. Ricin mainly utilizes clathrin-coated pits to be internalized, but if this pathway is blocked, ricin could still be endocytosed by clathrin-independent pathways [57]. Catherin-dependent mechanisms are well studied, which is a receptor-mediated uptake of proteins. Special protein motifs like tyrosine residue in the cytosolic tail of the receptors is necessary for the uptake in this mechanism [57, 58]. In detail, chain B of ricin is binding to the galactosyl-residues in cell surface and then internalized [59]. Clathrin-independent mechanism was proposed based on the finding that clathrin-dependent endocytosis was inhibited by transfection of Cos-7 cells with a mutant of the 100~kDa GTPase dynamin, whereas fluid-phase uptake still happened [57, 60], this suggests that clathrin-dependent pathway is not the only pathway that ricin employed. The endocytosis of ricin is reduced to about 50% when the clathrin-dependent pathway is inhibited [61].

Ricin's toxicity largely depends on the exposure route, ingestion mainly caused mass fluid loss by producing mucosal injuries, but injection would cause severe damage by inhibiting the synthesis of proteins in cells [62]. Ricin is able to be transported from peripheral nerves to neurons retrograde, called 'suicide transport' [63]. Rat studies showed that 0.2 microgram of ricin caused heavy fiber degeneration in the sciatic nerve proximal to the injection site, but not in a nearby tributary nerve [64], which suggested that ricin can produce a selective and severe lesion by retrograde "suicide transport". Injection of ricin

bilaterally in the sciatic nerve in rats induces the loss of motor neurons, leading to lower limb paralysis and the deficits that occurs in diseases like amyotrophic lateral sclerosis (ALS) and infantile progressive spinal muscular atrophy (SMA) [65, 66]. Ricin also affects the glial cells in addition to the degeneration of motor neurons in rats. Injection of ricin into rat facial nerve causes the degeneration of facial motor neurons, local microglial cells respond to nerve crush by rapid proliferation and phagocytosis of neuronal debris. After nerve crush, the expression of glial fibrillary acidic protein by fibrous astrocytes is enhanced [67]. Intact ricin is resistant to heat, freezing and proteolysis [68]. These findings suggested a possible route for lectins to transport from peripheral nerves to the central nervous system, which supports the hypothesis that dietary plant lectins might affect DAergic neurons and be one potential cause of PD.

1.3.2 ***Wheat germ agglutinin*** **(WGA)**

WGA is a plant lectin that has been employed as an anterograde tracer research tool for years. WGA has a unique binding affinity for N-Acetylneuraminic acid, a key component of neuronal membranes found in the brain, such as gangliosides which have diverse roles such as cell-to-cell contact, ion conductance, as receptors. WGA also attaches to the protective coating on the nerves known as the myelin sheath [69]. The presence of N-acetylglucosamine inhibits interactions between WGA and cell surface, implying that WGA could bind to N-acetylglucosamine on cell surfaces [70].

WGA is endocytosed by nonfenestrated endothelia throughout the central nervous system, WGA passes through the blood brain barrier (BBB) through a process called “adsorptive endocytosis”, this adsorptive endocytosis was proposed to associate with inclusion of Golgi complex [71, 72]. WGA is uptake via adrenergic nerve terminal and then transported retrograde to the superior cervical ganglion [73], however, the detailed mechanism is not clear and it is suggested that WGA has a single population of binding sites.

Most recently, Damak, *et al.* reported that WGA was transported to the geniculate and petrosal ganglia, and proposed this uptake and transportation was *via* across synapses in vesicles [12]. This

suggested that WGA bind to N-acetylglucosamine or N-Acetylneuraminic acid on cell surfaces first to build some connections, and then by an unknown pathway, WGA is able to form a small channel on the cell membrane, followed by formation of vesicles.

1.3.3 *Phaseolus vulgarwas leucoagglutinin* (PHA-L)

PHA-L has been used as an anterograde tracer of pathways of the central nervous system for a long time, even though it also shows some extent of retrograde transport [74-78]. In mammals, PHA-L is mainly anterograde transported through the nerve system, but some studies reported that PHA-L demonstrated almost the same degree of anterograde and retrograde transport in frogs, which might be due to different distribution of binding sites on cell membranes between frog and mammals [79].

1.3.4 *Concanavalin A* (Con A)

Another interesting lectin is Con A, which can bind to neuronal and synaptic membranes extensively [80], but the uptake of this lectin is very limited [73]. Similar to WGA, Con A has also been widely employed as a tool to study retrograde transport for its special property [81-83]. Con A is taken up by adrenergic nerve terminals and transported retrograde to the superior cervical ganglion [73]. The binding pattern of Con A suggests that the ability of binding to the cell membranes cannot guarantee following internalization; the effective action is a very complicated process requiring several parts to cooperate. The binding site of Con A was confirmed by pre-incubation of Con A with mannose which could greatly decrease Con A activity [84].

In addition to these lectins, other lectins also have shown the ability of recognizing or binding to neurons and their accessories. In recent years, the unique properties of binding to carbohydrate receptors have made lectins powerful research tools for characterizing the distribution of binding sites on cell surfaces.

In 1985, Fabian, R. H. reported the axonal and transneuronal transport of lectins including WGA, *Pisum Sativum Agglutinin* (PSA), *Lens Culinaris Agglutinin* (LCA), *Soybean Agglutinin* (SBA), and Con

A, and proposed that axonal and transneuronal transport of the lectins likely depended upon their respective carbohydrate affinities [83].

Silverman reported utilization of plant lectins to study carbohydrates on sensory ganglion cell surfaces [85]. In rats, the alpha-D-galactose-specific *Griffonia Simplicifolia* I-B4 [86] lectin was used to characterize galactose-terminal glyco-conjugates on a large subpopulation of small neurons, peripheral autonomic, gustatory and visceral sensory, enteric neurons, and the accessory olfactory bulb. L-fucose-binding *Ulex europaeus-I* (UEA) lectin was used to label the substantia gelatinosa in the human spinal cord. In rabbit, a small sensory ganglion cell subset and the spinal cord substantial gelatinosa was co-labelled by both the GSA and UEA lectins [85]. Other research reported use of eight lectins including Con A, *Arachis hypogaea agglutinin* (PNA), *Soybean Agglutinin* (SBA), *Dolichos Biflorus Agglutinin* (DBA), *Phytolacca americana* (PWA), WGA, UEA and PHA from six groups to study the lectin binding sites of olfactory receptor neurons, and all of them demonstrated their special binding properties [84]. The reveal of sugar-binding site of Con A has facilitated the mechanism research [87].

1.4 Discussion

The structure of neurotoxins and lectins play a key role in the binding and interactions with neurons. Special structural characteristics have determined their inherent properties of recognition, binding and all other effects. A-B structure is the most common structure in neurotoxins, in which subunit B is responsible for binding while subunit A for toxic effects. The receptor on cell membranes is another important factor that affects the interactions. For lectins, special receptors on cell membranes determine which lectins they can bind to and the intensity of the interactions, but the detailed mechanism is not all known. Other factors may also affect this binding and internalization, for example, cytofluorometric quantification study demonstrated that regenerated nerve greatly increased the uptake of Con A and WGA in mice [63].

However, due to the potent toxic effects and great value of research, it is of high necessity to illuminate the mechanism as well as the pathways of these neurotoxins and lectins. Many researchers have been working on the mechanisms and applications of these neurotoxins and lectins, but more efforts will be needed. For example, the mechanisms of the action of Shiga toxin and some lectins are still very vague. Research on lectins has focused on the characterization or monitoring the sugar receptors on cell membranes including cancer cells, some studies have started to investigate physiological effects of lectins [88, 89]. In a rat study, after oral administration of ricin, the ricin was absorbed from GI tract into blood and lymphatic circulation and detected in the liver and spleen [90]. This finding together with ricin's ability of 'suicide transport' might suggest that ricin have the ability of traversing the GI tract to enter the central nervous system, which supports our hypothesis that dietary plant lectins may have some effects on neurons. The etiology of PD is still not well defined, but the information presented here provides an alternative possibility of inducing PD.

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PART B. DIETARY PLANT LECTINS APPEAR TO BE TRANSPORTED FROM THE GUT TO GAIN ACCESS TO AND ALTER DOPAMINERGIC NEURONS OF *C. ELEGANS*[1]

1.1 Introduction

Could dietary plant proteins, such as lectins, traverse the gut intact, with vesicular transfer to neurons and be transported intact along axons to affect DAergic neurons as one etiology of PD? A recent Danish study showed that patients who had vagal nerves removed 20 years ago had a 40% lower incidence of PD [1]. Some reports claim that vegetarians have higher rates of PD [2, 3]. This research uses *C. elegans* as a model to investigate dietary lectins transport to DAergic neurons.

PD is the second most common degenerative disorder of the central nervous system that impairs motor skills and cognitive function, the main symptoms of PD are motion disorders, like muscle rigidity, bradykinesia, and tremors [4]. A human study with 490 PD patients and 176 health volunteers showed that the age is a key factor to the severity of the disease on a motor scale, but shows no difference in non-motor symptoms, and in men, the phenotype is characterized by upper-body disease while in women by postural dysfunction [5]. A survey of 210 PD subjects reported that in general men suffer more than women from PD symptoms according to the index of quality of life [6]. Other survey studies reported that men are at higher risk of getting PD than women [7-11]. A study of 1,741 subjects compared motor and non-motor symptoms between male and female subjects according to their age of symptom onset and diagnose, no difference was found between male and female except that women do better than men in non-motor symptoms [12]. The incidence of PD in Italian was estimated at 380/100,100 in year 2011 by

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analyzing drug prescriptions, tax-exemptions as well as hospital discharge records [13]. Longer lifespan has resulted in heavy societal financial burden and emotional burden all over the world [14].

Clinically, PD is characterized by the development of Lewy bodies due to aggregation of a-synuclein (α-SYN) in the brain tissue and the partial loss of DAergic neurons in the substantia nigra [15]. α-SYN also aggregates in microglia and further leads to PD though the detailed mechanism remains unclear [16, 17]. Astrocytes convert neurotoxin MPTP to its active metabolite MPP^+ [18]. However, the mechanism of the formation of Lewy bodies is not clear so far [19]. To date, PD cannot be cured though some treatments may alleviate the symptoms. The etiology of PD is unknown but complex environmental factors play important roles for neurodevelopmental and neurodegenerative disorders including PD [20, 21]. Results of large-scale epidemiological studies using meta-analysis showed that a statistically positive association exists between PD and pesticide or herbicide exposure [22], most of these pesticides share common features, such as the ability to induce oxidative stress, mitochondrial dysfunction, α-SYN fibrillization and neuronal cell loss [23]. Insulin dysfunction might be associated with the cause of PD though the detailed mechanism is still unclear [24]. Evidence indicates that in *C. elegans* high glucose concentration (14 mM) increases the aggregation of α-SYN while restricted glucose concentration reduces it [25]. Exposure to other environmental factors such as solvents, metals and other pollutants are also associated with the risk of PD in animal models [26, 27]. Despite the long-accepted viewpoint that environment and genetic factors might be most related to the etiology of PD, a recent review in 2015 reported that dietary factors like vitamins, flavonoids, calorie intake, caffeine, alcohol, and metals also play an important part in the rise and development of PD [23].

Plant lectins were discovered over a century ago (see review [28]). Toxicity of lectins was first recognized, independently, by Bruylants and Vennemann [29], Warden and Waddell [30] (described by Carl Oppenheimer [31]), and Dixson [32]. Lectin's hemagglutination property was found by Stillmark in 1888 [33], and a general antigenicity of lectins was revealed by Paul Ehrlich in 1890 [34] who won a

"Nobel Prize in Physiology or Medicine 1908" "*in recognition of their work on immunity*" (http://www.nobelprize.org/nobel_prizes/medicine/laureates/1908/). Thereafter, lectins' "immunity" (mainly hemagglutination of red cells for antigen typing) was used for immunological research (see Textbook of Military Medicine [35]). In 1919, Sumner crystallized (*Canavalia ensiformis*, Concanavalin A) [36]. A half century later, investigators began to determine ABO-blood subtypes due to their sugar-binding properties, and the word "lectins" was formally coined [37, 38]. Recent studies report that lectins play important roles in plant defense (see review by [39] and legume-rhizobial interactions [40].

Plants contain glycoprotein-lectins ("non-immune sugar-binding proteins") in seeds, fruits, and nuts [41], and recognize and reversibly bind specific carbohydrates [42]. They are involved in plant defense [39] and legume-rhizobia [40]. They resist gut enzymes maintaining function under adverse conditions [43, 44]. They can penetrate the GI tract wall by endocytosis [45], probably by first binding a carbohydrate lectin receptor [46] followed by endocytosis, and, astonishingly, can transfer trans-synaptically in an antegrade and/or retrograde fashion along nerve fibers [42, 47]. Their medical importance is increasingly being recognized by being conjugated with drugs for better drug absorption from the GI tract [46, 48-50]. Particularly relevant to the current studies, lectins have been utilized extensively for neuronal tracing studies [47, 51].

Most dietary plant lectins resist gut enzymes and maintain function under usually adverse conditions for proteins [43, 44]. Lectins have effects in both pathological and normal processes in living organisms due to their carbohydrate-binding properties [52]. Non-toxic lectins, such as tomato lectin and wheat germ agglutinin, are suggested to show growth factor activity in the GI tract [43]. Bacteria or parasitic protozoa, through their own lectins, attach to carbohydrate receptors on epithelial cells to colonize the GI and urinary tracts. Some lectins are synergistically toxic both locally and systemically to experimental animals [43]. Kidney bean lectin (PHA), for example, damages intestinal epithelial cells, causes bacterial overgrowth, and induces nutritional disorders, effects which are preventable by specific

inhibiting sugars that have competitive binding capacities to lectins by sharing similar terminal structures [43, 53]. Likewise, dietary saccharides or glyco-conjugates, such as probiotic agents and milk oligosaccharides, may act as receptor analogs or decoys to selectively and competitively reduce lectin binding [43, 54, 55]. Soybean lectin has shown potential anticarcinogenic effects [56].

Lectins are widely available in seeds, fruits or nuts of fruits and vegetables. A 2195 American participants study reported that on average 1/3 of total vegetables people consumed were not cooked, and people who consumed more raw vegetables preferred raw fruits and grains [57]. People in different regions of the world have their own preference in consuming certain raw or cooked vegetables, some vegetables like tomatoes, sprout soybeans and carrots are more consumed raw, but other vegetables like peas are more consumed cooked [57]. Many studies have investigated anticarcinogenic effects of fruits and vegetables. For example, high consumption of fruits and vegetables is negatively related to the incidence of cancer in the GI tract [58, 59]. Leafy vegetables have beneficial effects in preventing the development of breast cancer [60]. However, consumption of citrus fruits does not have beneficial effects on stomach cancer [61]. Human cell membranes have different glycoproteins and carbohydrates due to membrane differentiation [62], which may make some people more vulnerable than others. Elder people cannot tolerate raw vegetables as well as cooked vegetables; main symptoms of intolerance include diarrhea and vomiting [63]. Interesting, the prevalence of PD is higher in elder people than in young [64]. Taken together, all this information suggest that people are exposed to lectins daily as they consume varieties of fruits and vegetables, raw or cooked.

Consumption of fruits or vegetables has been reported to cause diseases in human beings mainly due to the damage of lectins on intestinal mucosa [65]. Patients suffered vomiting and diarrhea after consuming a diet high in red kidney beans containing lectin PHA in a hospital in 1988 [66]. Lectins are related to the disease symptoms in some patients with dysfunction of autoimmune system [67]. Overall lectins can be found in about 30% of all the food [67]. The content of lectins in dietary plants vary among

different plants (Table 1). The content of lectin Con A is as high as 15 mg/gram of seeds, the content of lectin UEA I is 0.045 mg/gram. The food content of lectin LcH, EEA, CSA and DBA is not available.

Table 1. Summary of lectins contents from literature search

Lectins	Amount (mg/g)	Comments
UEA I	0.045 [68]	seeds
PSA	0.28 – 0.65 [69]	seeds
PHA	1.07 [70]	seeds
PNA	2 [71]	seeds
GNA	2.5 [72]	bulb tissue
AIA	7.2 [73]	seeds
STA	8 [74]	tuber
ACA	1.7 [75]	seeds
WGA	0.16 [76]	wheat germ
SBA	0.2 [77]	seeds
GSL I	0.4 [78]	seeds
Con A	15 [79]	seeds
CPA	0.5 [80]	Seeds

Direct evidence of neuronal damage caused by daily exposure to dietary plant lectins is not available so far. Consumption of vegetable products, however, is a common factor in the epidemiology of PD-like diseases. The Amyotrophic Lateral Sclerosis / Parkinsonism dementia complex (ALS-PDS) in populations from Guam, for example, has been linked to a diet rich in cycad seeds (*Cycas micronesica*) [81, 82]. The cycad seeds contain ß−methylamino-L-alanine (BMAA) which has excitotoxic properties. Studies on human brain tissue of ALS/PDC, ALS, and Alzheimer’s disease, PD, Huntington's disease and neurological controls indicated that BMAA is present in non-genetic progressive neurodegenerative disease but not in controls or genetic-based Huntington's disease [83-86]. Animals fed with purified BMAA, however, do not show ALS-PDS traits [87] suggesting that other mechanisms deliver the toxin(s) to target neurons.

In equine Parkinsonism, consuming yellow star thistles (*Centaurea solstitialis*) or Russian knapweed (*Acroptilon repens*) causes liquid necrosis in the *substantia nigra pars reticulata* and the *globus pallidus* by destroying DAergic neurons, developing NPE, and creating histopathological features which

resemble human idiopathic PD [88]. These observations suggest transport of toxic substances to neurons. To date, however, epidemiology has not proven dietary lectins to have a significant impact on neuronal degenerative diseases in humans.

Signature pathologies of PD, *e.g.*, Lewey bodies and aggregated α-SYN occur in neurons of the enteric nervous system of the GI wall, in addition to the neurons of the central nervous system (CNS) [89]. The findings reported here support Braak and Hawkes' hypothesis that the GI tract may be a potential site of neuronal invasion by an "unknown etiologic agent", potentially responsible for causing some percentage of PD [89-93]. Braak and Hawkes' hypothesis was based on the finding that α-SYN immunoreactive inclusions were found in neurons of the submucosal Meissner plexus, whose axons project into the gastric mucosa and terminate in direct proximity to fundic glands. These elements provided the first link in an uninterrupted series of susceptible neurons that extend from the enteric to the central nervous system. The existence of such an unbroken neuronal chain lent support to the hypothesis that a putative environmental pathogen capable of passing the gastric epithelial lining might induce α-SYN misfolding and aggregation in specific cell types of the submucosal plexus and reach the brain via a consecutive series of projection neurons. It is suggested herein that one possible etiologic agent could be lectins.

Several animal models have been developed to investigate the etiology and the underlying molecular mechanisms of PD. Neurotoxic models have been most studied and adopted, which are mainly using 6-hydroxydopamine (6-OHDA), MPTP/MPP+ or rotenone to induce PD-like motor symptoms in animals. Neurotoxic models share similar mechanisms of inhibiting the level of complex I of the respiratory chain, which further leads to the apoptosis of DAergic neurons [94]. Genetic models specifically modify certain genes that are related to the etiology of PD. The main limitation of genetic models is that the detailed mechanisms for the effects of genes are still at large though some models have shown effects.

C. elegans as an animal model to study PD was first reported in 2001 [95], and has been widely used [96-101]. *C. elegans* has a high conservation (>65%) of human disease-associated genes [102, 103]. A total of eight DAergic neurons in the hermaphrodite *C. elegans* [104-106], respond to signals from environmental mechano-sensory stimuli, e.g. exhausted food supply, which has offered molecular, genetic, and behavioral tools to aid human disease studies [107-111].

C. elegans modulates locomotion behavior by using dopamine and serotonin to mediate motor circuits in chemical synapses, gap junctions, and neuromuscular junctions [112-114]. Intestinal muscle cells are innervated by pharyngeal motor neurons and DAergic neurons *via* the preanal ganglia. Structures, sensory-motor synapses, gap junction contacts, and activities all resemble those in the mammalian GI tract [115, 116]. A typical phenotype of PD in *C. elegans* caused by the degeneration of DAergic neurons is the slowness of the mobility. In liquid culture, 0.5 mM of MPP+ induces significant degeneration of DAergic neurons and reduces the mobility of *C. elegans* [97, 99]. Due to the automated devices and advanced software, tracking and analyzing the mobility of large number of *C. elegans* at the same time is possible today.

In this study, features of the GFP-dopamine transporter fusion protein *C. elegans* (*egIs1*[*Pdat-1::GFP*]) were evaluated by the numbers, fluorescent intensity, and sizes of GFP-DAergic neurons. Meanwhile, TRITC/rhodamine labeled lectins were also followed post-feeding, to establish the ability of lectins to bind or penetrate the GI wall or nerve cells. The question was whether dietary plant lectins can impair or alter apparently lectin-targeted DAergic neurons. Differences in inherited sugar structures of gut and neuronal cell surface may make some individuals more susceptible in this conceptual disease model. The effects of lectins on the mean lifespan and the movement distance and velocity in liquid culture in *C. elegans* were also evaluated. Our results support Braak and Hawkes' hypothesis suggesting one alternate potential etiology of PD.

1.2 Materials and methods

C. elegans egIs1[Pdat-1::GFP] that express GFP in the 8 DAergic neurons [117, 118] and the standard food *Escherichia coli* (*E. coli*) were obtained from *C. elegans* Genetics Center (CGC, MN). The *C. elegans* model does not require regulation of the Institutional Animal Care and Use Committee (IACUC).

1.2.1 Culture *Escherichia coli* (*E. coli*, OP50)

OP50 were cultured by the standard method described elsewhere [119]. Briefly, approximately 10 μL of stock *E. coli* solution was added to media and incubated at 37 ℃ for 24h. The OP50 were then plated in Petrifilm™ (3M Corporate, St. Paul, MN) at 37 ℃ for 24h until densities of 5×10^{8} to 5×10^{11} colony forming units (cfu/ml) were reached and then were fed to the *C. elegans ad libitum* [119, 120]. The OP50 stock feeding solution was enriched to 2×10^{9}cfu/ml by centrifuging at 2,200 g for 10 minutes and washed with S-complete buffer twice.

1.2.2 *C. elegans* culture

Mature gravid transgenic *C. elegans egIs1[Pdat-1::GFP]* were treated with NaOH (1M) and sodium hypochlorite solution (5.25%, 5:2 ratio) to dissolve the body and release viable eggs [119]. The eggs were hatched overnight fed *ad libitum* with LB broth (200 μL/well) containing OP50 $5x10^{8}$ – $5x10^{11}$ cfu/ml [121], after washing with S-complete solution 3 times. The age-synchronized *C. elegans* were diluted to 100 animals/ml, plated in liquid culture in a 96 well plate (120 μL/well, 10-15 animals) [122]. The plate was tape sealed, bagged, and covered with aluminum foil to avoid contamination. All animals were kept in a 20°C low temperature incubator (Revco Tech., Nashville, NC, USA) throughout the experiments. Thirty microliters of 5-Fluoro-2′-deoxyuridine (FUDR, 0.6 mM) solution was added to each well at larvae 4 stage.

All treatments were applied at day 3 after hatching. Four dose responses of twenty lectins were obtained for each culture condition in a dark room. Control animals were fed with OP50. Experimental

groups were fed rhodamine/TRITC-conjugated lectins. The lectins were incorporated into feeding medium with OP50. Concentrated OP50 were added to each well every other week throughout the experiment. Each group of nematodes was collected and fixed after the first week for the liquid culture as described elsewhere [123]. Briefly, after being collected from wells, animals were washed with S-Basal twice, fixed with paraformaldehyde (4%) over 2h at 4 ℃ and washed with PBS for 5min x 3. Ten microliters of Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) was applied to a glass slide followed by 10 μL of the medium containing *C. elegans*. At last, a cover glass was mounted on the glass slide.

1.2.3 Select lectins

Commercially available plant lectins conjugated with TRITC or rhodamine were purchased from EY labs (San Mateo, USA), Vector Labs (Burlingame, USA), or Sigma-Aldrich (St. Louis, USA). All tested lectins were selected from dietary plants or vegetables. The doses of lectins were determined based on previous studies, literature search or recommendations from product specifications.

1.2.4 Average probability of survival (APS) assay

All average probability of survival (APS) assays were conducted in liquid culture (96-well plate). The animals were synchronized and seeded into each well of a plate (n=10-15) and OP50 was added to each well. Thirty microliters (0.6mM) fluorodeoxyuridine (FUDR) was added to each well and then the plate was shaken evenly to sterilize the animals. Four different treatments (50 μL/treatment, n=6 row) including control or serial of lectins were added. The plate was then covered with aluminum foil. The whole procedure was performed in a dark room to prevent bleaching of fluorophores. After the plate was shaken for 3 minutes, the survival animals were counted every other day until all were dead under an inverted microscope (Nikon, Eclipse T*i* –S, Japan) at 4x or 10x magnification. Animals were exposed to strong lights to stimulate the movements [124] and the movement of pharynx was checked to confirm whether the animals were dead when they were not moving.

1.2.5 Mobility analysis

Mobility analysis was conducted in a 96-well plate. Animals of larvae 1 stage were seeded into the plate with about 20 animals per well. Each well was treated with MPP+ (0.5 mM) [97] or different lectins. All the tested lectins were listed in Table 2. The plate was kept in a low temperature incubator (20 ℃) and analyzed 48 hours later. After about 2 minutes of agitation, the plate was placed on the stage of the microscope (Nikon, Eclipse T*i* –S, Japan). Using a digital camera, a video of the *C. elegans* (n=20-25) with 20 frames (1 frame/second) was acquired from each well and images stored for offline analysis. The video clips were analyzed by tracking the movement of animals using NIS-Elements Advanced Research (version 3.22.11). The distance and the velocity of the movement were used as a function to characterize the effects of lectins on the mobility of *C. elegans*. Quantitative indicators were used to compare the mobility indicators between treatments and the control.

Table 2. List of lectins tested in mobility assay

Lectin	Dose (μM)	Lectin	Dose (μM)	Lectin	Dose (μM)	Lectin	Dose (μM)
ACA	0.032	EEA	0.048	PNA	0.018	PHA-E	0.017
PSA	0.043	UEA I	0.033	WGA	0.046	LcH	0.05
S-WGA	0.046	AIA	0.031	CSA	0.065	DBA	0.018
GNA	0.077	GSL I	0.018	HHA	0.04	PHA-L	0.017
SBA	0.017	Con A	0.019	CPA	0.047	STA	0.02

1.2.6 Fluorescent microscopy

The GFP-DAergic neurons were identified by FTIC filter (480Ex/520Em) and the number of GFP-DAergic neurons counted. Fluorescent intensity of GFP-DAergic neurons and their average sizes (μm^2) were determined by NIS-Elements Advanced Research (version 3.22.11) and compared between the control and lectins group. Briefly, a picture was taken for each animal under the FITC filter, in which the GFP-DAergic neurons were visible. A circle was first drawn around each GFP-DAergic neuron and then the software measured the area and fluorescent intensity automatically. Fluorescent intensity of

rhodamine-lectins was determined by a TRITC filter (580Ex/620Em) to assess co-localization. The magnitude of the effect(s) of the lectin on the DAergic neurons, the number, fluorescent intensity (arbitrary unit), and sizes (μm^2) of GFP-DAergic neurons were determined and compared among each group. Co-localization was initially identified with an inverted microscopy (Nikon, Eclipse T*i* –S, Japan) and then confirmed at a Z-axle with laser scanning microscopy (Leica, TCS SP5, Germany).

1.2.7 Solutions and chemicals

Standard NGM agar plates (g): NaCl 3.0g, Bacto-agar (Becton, MD) 20g, Bacto-peptone 2.5g (Becton, MN), Cholesterol solution 0.1% (0.005/ml 95% ethanol), and dH_2O 975ml were mixed. Additions to the autoclaved solution (M): $CaCl_2$ 1.0 1ml, $MgSO_4$ 1.0 1ml, KPO_4 pH6 1.0 25ml. *LB Broth*: 25.0g, dH_2O 1L (autoclave). *S-basal solution* (M)*:* NaCl 0.1, KPO4 pH6 0.05, Cholesterol 0.1%, was autoclaved. *PBS* (mM)*:* 115 NaCl, 75 $Na_2HPO_4 \cdot 7H_2O$, and 7.5 KH_2PO_4, pH 7.4.

1.2.8 Statistical analyses

All results are presented as mean ±S.E.M. Analyses were carried out using SAS/STAT® software, Version 9.4 of the SAS System for Windows (Cary, NC, USA). Survival curves were displayed by binomial probabilities obtained from logistic regression models as surrogates for survival probabilities and mean lifespan was estimated via Kaplan-Meier (log-rank). ANOVA models were used to analyze neuron data. For each group, 10-15 animals were analyzed for liquid culture. Motion activity analysis was performed by Systat software (version 12.5, SigmaPlot for Windows, San Jose, CA, USA). The normality of the data was evaluated before further analysis, nonparametric analysis Kruskal–Wallis with Dunn's method was used to compare between treatments and the control group if the assumption of normality was not met. An alpha level of 0.5 was considered statistically significant.

1.3 Results

Diets supplemented with varying concentrations of TRITC-conjugated lectins ACA, EEA and PNA in liquid culture were fed to *C. elegans,* and subsequently detected by fluorescence microscopy to be

associated with GFP-DAergic neurons (Table 3). The only explanation for this observation is that these TRITC-labeled lectins traveled in some manner from the gut to the GFP-DAergic neurons. We observed that some lectins had the following effects: a) altering the number of DAergic neurons, b) decreasing fluorescent intensity of GFP-expressing neurons (less GFP-dopamine transporter), or c) altering neuron size. Other seventeen lectins (Table 4) were not observed to be transported to GFP-DAergic neurons, but most of them affected the size, area or intensity of DAergic neurons to different extents, possibly indicating that undetectable amounts of these lectins caused the effects, or that some unexplained secondary effect of the lectins caused those "pathological effects". In addition, the effects of tested lectins on the mean lifespan of *C. elegans* in liquid culture were evaluated. Lectins affected the mean lifespan of *C. elegans* in different manners. PHA-E, PSA, UEA I and WGA increased the mean lifespan ($P<0.05$). ACA, EEA, PNA, AIA, CSA, DBA, GNA, GSL I, HHA, PHA-L and SBA reduced the mean lifespan ($P<0.05$). LcH and S-WGA reduced the mean lifespan at low dose but increased it at high does ($P<0.05$). Con A, CPA and STA did not affect the mean lifespan significantly ($P>0.05$). In mobility assay, larvae 1 stage of *C. elegans* were fed with different lectins. The results of mobility assay demonstrated that all tested lectins reduced both movement distance and velocity of *C. elegans* in liquid culture compared with the blank control group (OP50 only, $P<0.05$).

1.3.1 Lectins co-localized with the GFP-DAergic neurons

There lectins ACA, EEA and PNA were observed to co-localize with GFP-DAergic neurons in *C. elegans*, affect the size, number or fluorescent intensity of GFP-expressing neurons, and affect the mean lifespan (Table 3).

Amaranthus caudatus agglutinin (ACA)-TRITC co-localized with DAergic neurons at the highest dose group (0.32 μM). As shown in the graph, when the green color from GFP fused DAergic neurons (Figure 4a) and the red color from TRITC conjugated ACA (Figure 4b) was overlapped, the co-localized area showed bright yellow color (Figure 4c), which demonstrated that ACA was successfully transported

from gut to DAergic neurons in *C. elegans* organism. ACA did not the affect the number of DAergic neurons (Figure 4d). ACA did not affect the intensity of DAergic neurons (Figure 4e). Area of DAergic neurons was decreased by higher doses of ACA (0.096 μM & 0.32 μM, $P<0.05$, Figure 4f). APS was decreased dose-dependently (Figure 4g). The mean lifespan was reduced by all doses (0.032 μM, 0.096 μM & 0.32 μM) from 18 days to 14, 10 and 7 days dose-dependently (-22%, -44% & -60%, $P<0.05$, Figure 4h).

Table 3. Lectins detected in the neurons by co-localization

Lectins	Dose (μM)	GFP #	GFP intensity	GFP size	Lifespan
ACA	0.032	↔	↔	↔	↓
	0.096	↔	↔	↓	↓
	0.32	↔	↔	↓	↓
PNA	0.018	↑	↔	↔	↔
	0.054	↔	↔	↔	↓
	0.18	↔	↔	↔	↓
EEA	0.048	↔	↔	↔	↓
	0.136	↔	↔	↔	↓
	0.48	↓	↓	↑	↓

↓ Indicates decreasing trend, $P<0.05$
↑ Indicates increasing trend, $P<0.05$
↔ Indicates no significant alternation, $P>0.05$

Arachis hypogaea agglutinin (PNA)-TRITC co-localized with GFP-GAergic neurons after one week of treatment (0.018 μM, 0.054 μM & 0.18 μM, Figure 5). Number of GFP-DAergic neurons was increased at the lowest dose (0.018 μM, $P<0.05$, Figure 5d). The size and intensity of GFP-GAergic neurons was not altered (Figure 5e & Figure 5f). APS was dose-dependently reduced at all doses (Figure 5g). The mean lifespan was reduced by higher doses (0.054 μM and 0.18 μM) from 19 days to 15 days and 14 days (-24% and -27%, $P<0.05$, Figure 5h).

Euonymus europaeus agglutinin (EEA)-TRITC co-localized with DAergic neurons at the lowest dose groups (0.048 μM, Figure 6). As shown in the graph, when the green color from GFP fused DAergic neurons (Figure 6a) and the red color from TRITC conjugated ACA (Figure 6b) overlapped, the co-

localized area showed bright yellow color (Figure 6c), which demonstrated that EEA was successfully transported from gut to DAergic neurons in *C. elegans* organism.

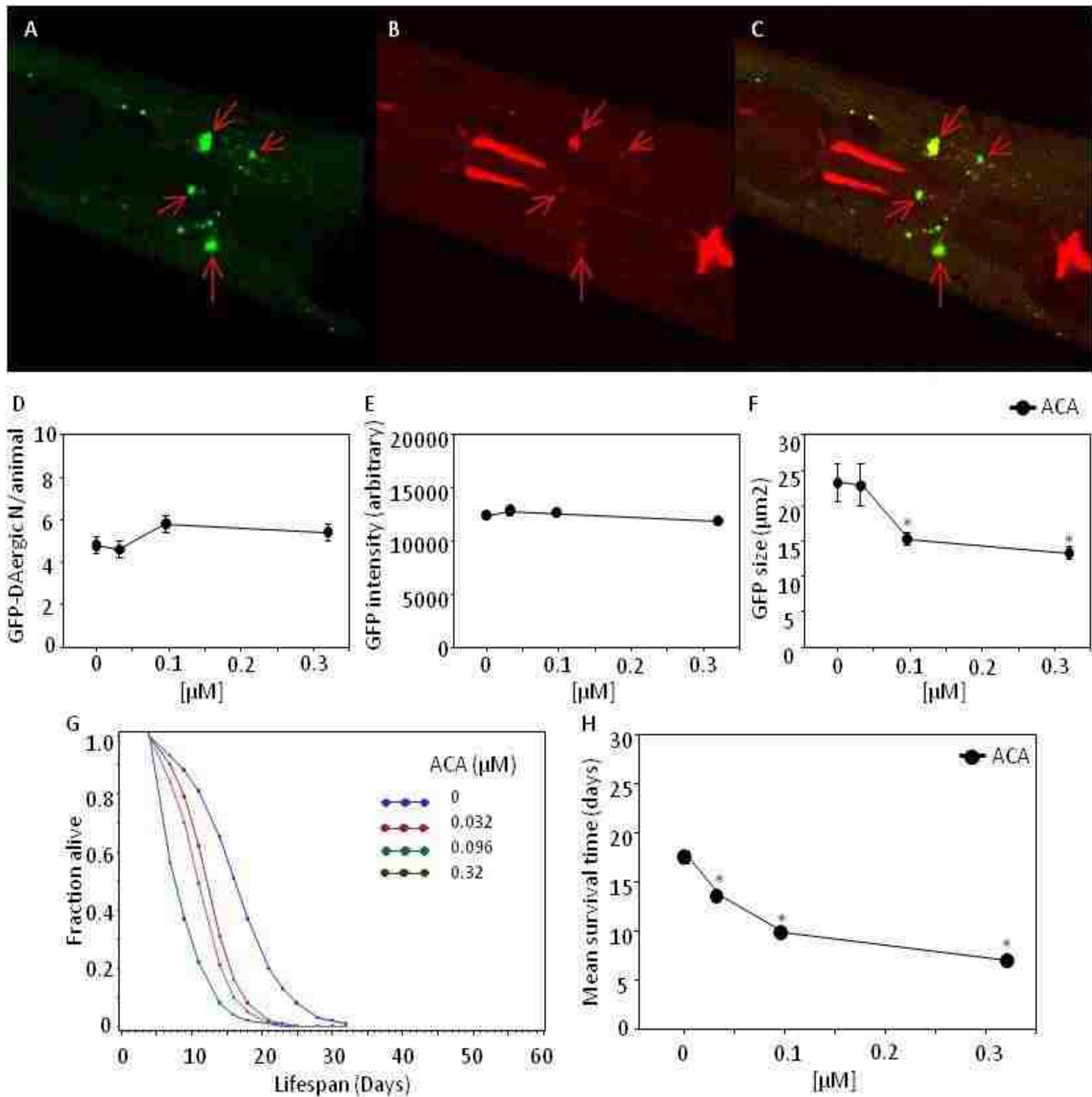

Figure 4. ACA co-localized with GFP-DAergic neurons in *C. elegans* at the highest dose (0.32 μM). A) GFP-DAergic neurons (green), B) PNA-TRITC in the neuron (red), C) Co-localization of the GFP-DAergic neurons in merged A and B (yellow). D) The number of DAergic neurons was not altered. E) The intensity of DAergic neurons was not altered. F) The area of DAergic neurons was reduced by two higher doses (0.096 μM & 0.32 μM, $P<0.05$). G) APS was reduced dose-dependently. H) The mean lifespan was reduced by all doses (0.032 μM, 0.096 μM & 0.32 μM) from 18 days to 14, 10 and 7 days (-22%, -44% & -60%, $P<0.05$). * indicates statistical significance.

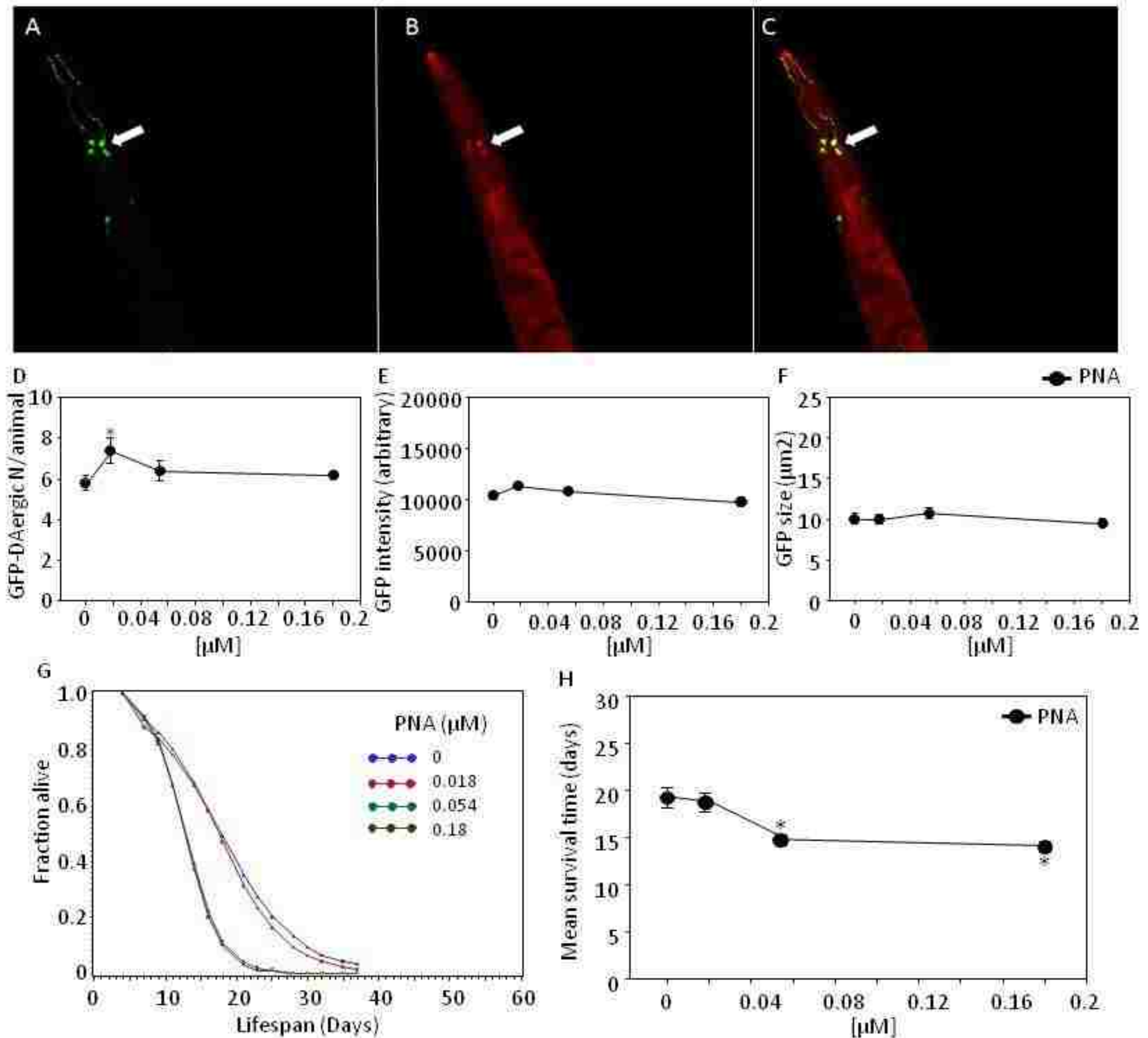

Figure 5. PNA co-localized with GFP-DAergic neurons in *C. elegans* at all doses (0.018 µM, 0.054 µM & 0.18 µM). A) GFP-DAergic neurons (green), B) PNA-TRITC in the neuron (red), C) Co-localization of the GFP-DAergic neurons in merged A and B (yellow). D) Number of GFP-DAergic neurons was increased at the lowest dose (0.018 µM, P<0.05). E) The intensity of GFP-DAergic neurons was not altered. F) The size of GFP-DAergic neurons was not altered. G) APS was dose-dependently reduced at all doses. H) The mean lifespan was reduced by higher doses (0.054 µM and 0.18 µM) from 19 days to 15 days and 14 days (-24% and -27%, P<0.05). * indicates statistical significance

The number of DAergic neurons was reduced by the highest dose of EEA (0.48 µM, P<0.05, Figure 6d). The intensity of DAergic neurons was reduced by the highest dose of EEA (0.48 µM, P<0.05, Figure 6e). Area of DAergic neurons was increased by highest dose of EEA (0.48 µM, P<0.05, Figure

6f). APS was decreased dose-dependently (Figure 6g). The mean lifespan was reduced dose-dependently by all doses (0.048 μM, 0.136 μM & 0.48 μM) from 19 days to 14, 9 and 7 days (-27%, -53% & -62%, P<0.05, Figure 6h).

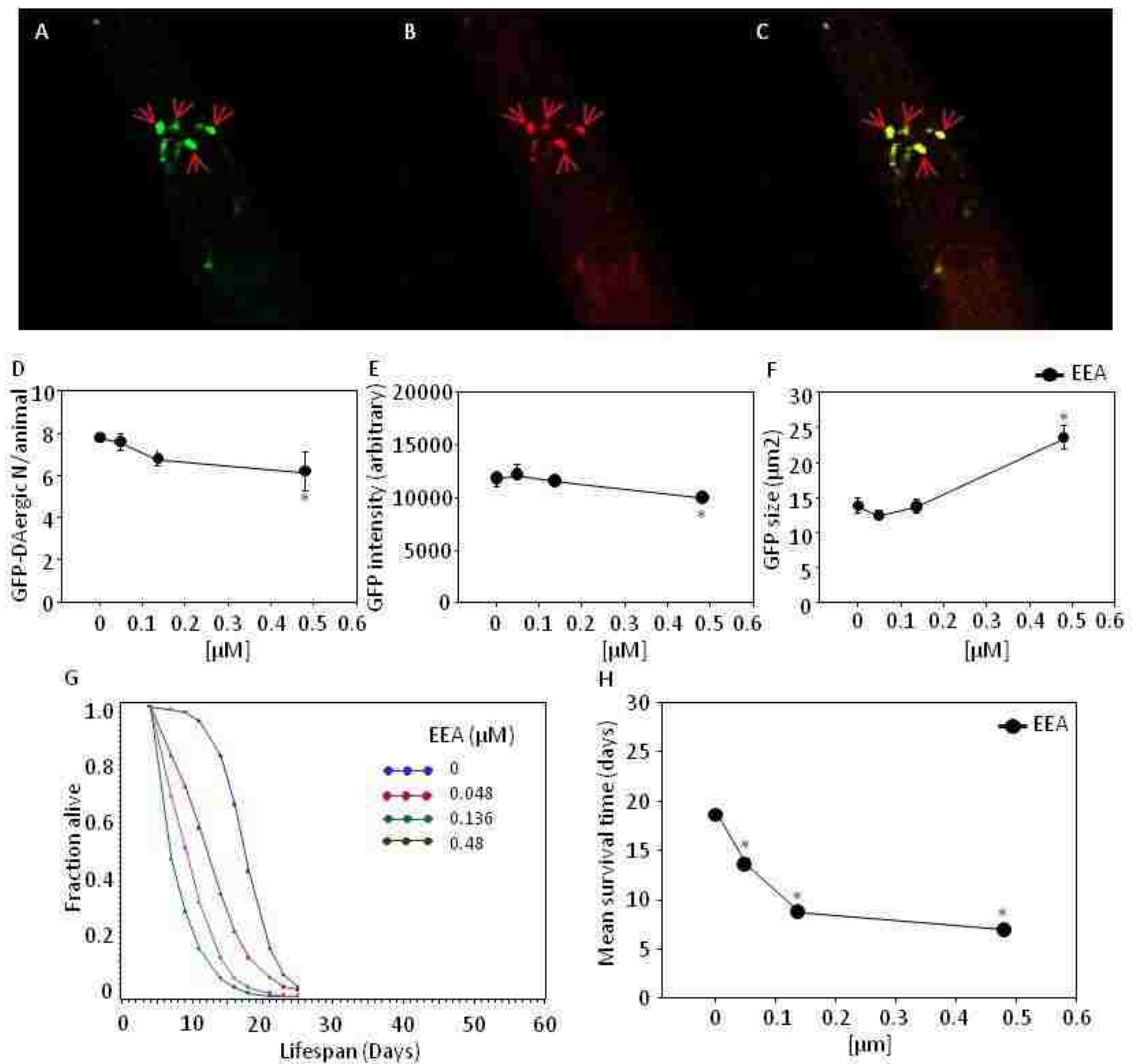

Figure 6. EEA co-localized with GFP-DAergic neurons in *C. elegans* at the lowest dose (0.048 μM). A) GFP-DAergic neurons (green), B) EEA-TRITC in the neuron (red), C) Co-localization of the GFP-DAergic neurons in merged A and B (yellow). D) The number of DAergic neurons was reduced by the highest dose (0.48 μM, P<0.05). E) The intensity of DAergic neurons was reduced by the highest dose of EEA (0.48 μM, P<0.05). F) Area of DAergic neurons was increased by highest dose of EEA (0.48 μM, P<0.05). G) APS was decreased dose-dependently. H) The mean lifespan was reduced dose-dependently by all doses (0.048 μM, 0.136 μM & 0.48 μM) from 19 days to 14, 9 and 7 days (-27%, -53% & -62%, P<0.05). * indicates statistical significance.

1.3.2 Lectins altered the GFP-DAergic neurons without co-localization

Seventeen lectins PHA-E, PSA, UEA I, WGA, LcH, S-WGA, AIA, CSA, DBA, GNA, GSL I, HHA, PHA-L, SBA, Con A or CPA were not observed to co-localize with GFP-DAergic neurons, but most of them affected the number, intensity or size of DAergic neurons or the mean lifespan (Table 4).

Table 4. Lectins which alter number, GFP-intensity, or size of DAergic neurons without observed co-localization

Lectins	Dose (μM)	GFP #	GFP intensity	GFP size	Lifespan
PHA-E	0.017	↔	↑	↔	↔
	0.054	↔	↑	↔	↔
	0.17	↓	↔	↓	↓
PSA	0.043	↔	↔	↔	↑
	0.129	↔	↔	↑	↔
	0.43	↓	↔	↔	↔
UEA I	0.033	↔	↔	↔	↔
	0.099	↔	↔	↔	↔
	0.33	↑	↔	↑	↓
WGA	0.046	↔	↑	↔	↔
	0.138	↔	↑	↔	↔
	0.46	↔	↑	↔	↔
LcH	0.05	↔	↓	↔	↔
	0.15	↑	↓	↔	↓
	0.5	↓	↓	↓	↓
S-WGA	0.046	↔	↑	↔	↔
	0.138	↔	↔	↑	↔
	0.46	↔	↓	↔	↔
AIA	0.031	↔	↔	↔	↔
	0.093	↔	↔	↔	↓
	0.31	↔	↔	↔	↓
CSA	0.065	↔	↔	↔	↔
	0.195	↔	↓	↔	↔
	0.65	↔	↓	↔	↔
DBA	0.018	↔	↑	↔	↔
	0.054	↔	↑	↔	↔
	0.18	↑	↑	↔	↔

↓ Indicates decreasing trend, $P<0.05$
↑ Indicates increasing trend, $P<0.05$
↔ Indicates no significant alternation, $P>0.05$

Table 4 continued. Lectins which alter number, GFP-intensity, or size of DAergic neurons without observed co-localization

Lectins	Dose (μM)	GFP #	GFP intensity	GFP size	Lifespan
GNA	0.077	↔	↔	↔	↔
	0.231	↔	↓	↔	↓
	0.77	↔	↔	↑	↓
GSL I	0.018	↔	↑	↑	↔
	0.054	↔	↑	↑	↔
	0.18	↔	↑	↑	↔
HHA	0.04	↔	↔	↓	↔
	0.12	↔	↔	↔	↔
	0.4	↔	↓	↓	↓
PHA-L	0.017	↔	↓	↑	↑
	0.051	↔	↓	↑	↔
	0.17	↔	↓	↔	↓
SBA	0.017	↔	↑	↔	↔
	0.051	↔	↔	↔	↑
	0.17	↔	↓	↓	↔
Con A	0.019	↔	↔	↔	↔
	0.057	↔	↔	↔	↔
	0.19	↔	↓	↔	↔
CPA	0.047	↔	↔	↔	↔
	0.141	↔	↓	↔	↓
	0.47	↔	↓	↔	↓
STA	0.02	↔	↔	↔	↔
	0.06	↔	↔	↔	↔
	0.2	↔	↔	↔	↔

↓ Indicates decreasing trend, P<0.05
↑ Indicates increasing trend, P<0.05
↔ Indicates no significant alternation, P>0.05

Phaseolus vulgaris (PHA-E)-rhodamine did not show co-localization with but affected DAergic neurons. The number of GFP-DAergic neurons was not altered (Figure 7a). The fluorescence intensity of GFP-DAergic neurons was increased dose-dependently at all doses (0.017 μM, 0.054 μM & 0.17 μM, P<0.05, Figure 7b). The average size of GFP-DAergic neurons was also reduced at the highest dose (0.17 μM, P<0.05, Figure 7c). The APS was increased dose-dependently at lower doses (Figure 7d). The mean lifespan was increased at a medium dose (0.054 μM) from 17 days to 23 days (39%, P<0.05, Figure 7e).

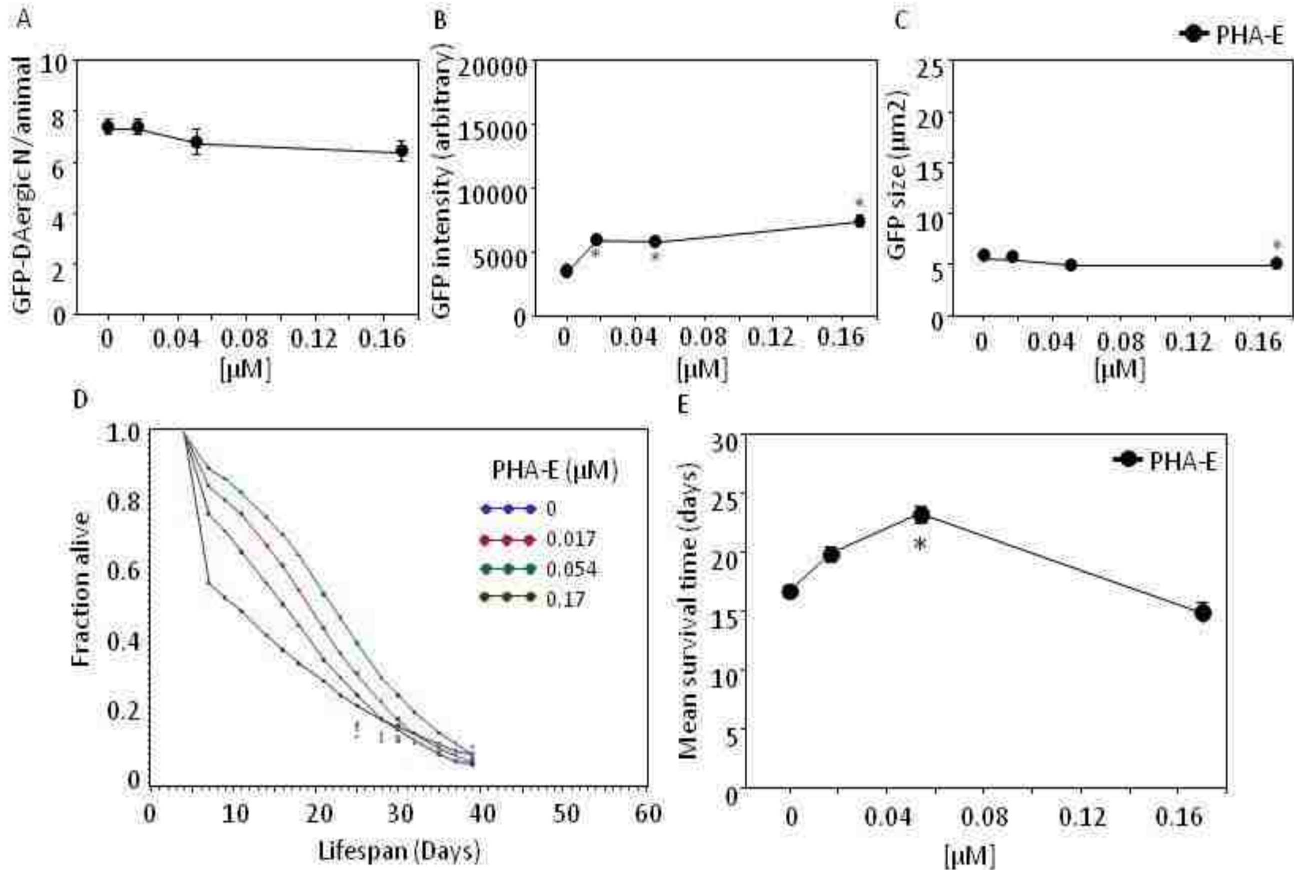

Figure 7. PHA-E-rhodamine did not show co-localization with DAergic neurons in liquid culture but affected the DAergic neurons. A) Number of DAergic neurons was not altered. B) The fluorescence intensity of GFP-DAergic neurons was increased dose-dependently at all doses (0.017 µM, 0.054 µM & 0.17 µM, P<0.05). C) The average size of GFP-DAergic neurons was also reduced at the highest dose (0.17 µM, P<0.05). D) The APS was increased dose-dependently at lower doses. E) The mean lifespan was increased at a medium dose (0.054 µM) from 17 days to 23 days (39%, P<0.05). * indicates statistical significance.

Pisum Sativum agglutinin (PSA)-rhodamine did not show co-localization with GFP-DAergic neurons but affected the DAergic neurons. The number of DAergic neurons was not altered (Figure 8a). The intensity was diminished at the highest dose (0.43 µM, P<0.05, Figure 8b). The size was reduced at the lowest dose (0.043 µM, P<0.05, Figure 8c). The APS was increased at all doses (Figure 8d). The mean lifespan was increased at doses (0.043 µM & 0.43 µM) from 22 days to 27 days (22% and 23%, P<0.05, Figure 8e).

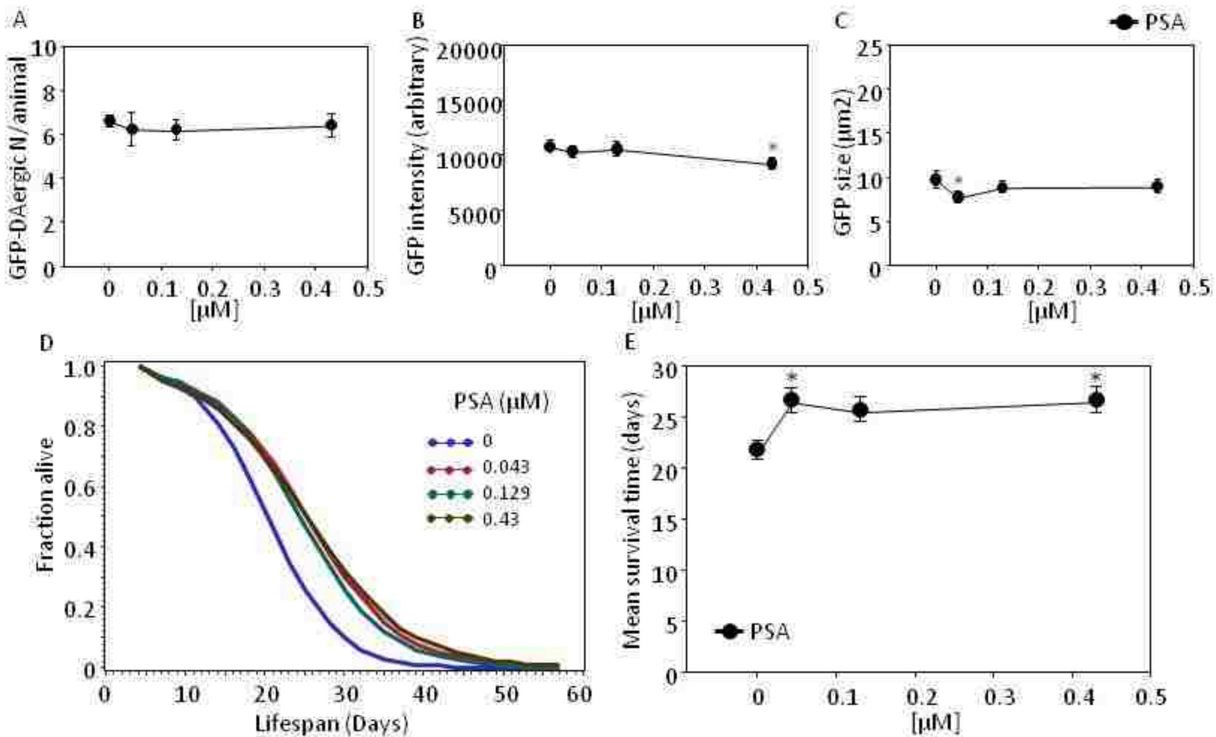

Figure 8. PSA-rhodamine did not show co-localization with GFP-DAergic neurons but affected the GFP-DAergic neurons in *C. elegans*. A) The number of DAergic neurons was not altered. B) The intensity was diminished at the highest dose (0.43 μM, P<0.05). C) The size was reduced at the lowest dose (0.043 μM, P<0.05). D) The APS was increased at all doses. E) The mean lifespan was increased at doses (0.043 μM & 0.43 μM) from 22 days to 27 days (22% & 23%, P<0.05). * indicates statistical significance.

Ulex Europaeus I (UEA I)-TRITC did not show co-localization with GFP-DAergic neurons in *C. elegans* but affected the DAergic neurons. The number of DAergic neurons was not altered (Figure 9a). The intensity was diminished at all doses (0.033, 0.099 & 0.33 μM, P<0.05, Figure 9b). The size was reduced at all doses (0.033, 0.099 & 0.33 μM, P<0.05, Figure 9c). The APS was increased at the low dose (Figure 9d). The mean lifespan was increased by low dose (0.033 μM) from 22 days to 25 days (13%, P<0.05, Figure 9e).

Triticum vulgaris agglutinin (WGA)-rhodamine was not detected as transported to but affected DAergic neurons. The number of DAergic neurons was not altered (Figure 10a). The intensity of the GFP-DAergic neurons was increased at the highest dose (0.46 μM, P<0.05, Figure 10b). The area of the DAergic neurons was reduced at all doses (P<0.05, Figure 10c). The APS was increased at all doses

(Figure 10d). The mean lifespan was increased at the highest dose (0.46 μM) from 20 days to 24 days (22%, P<0.05, Figure 10e).

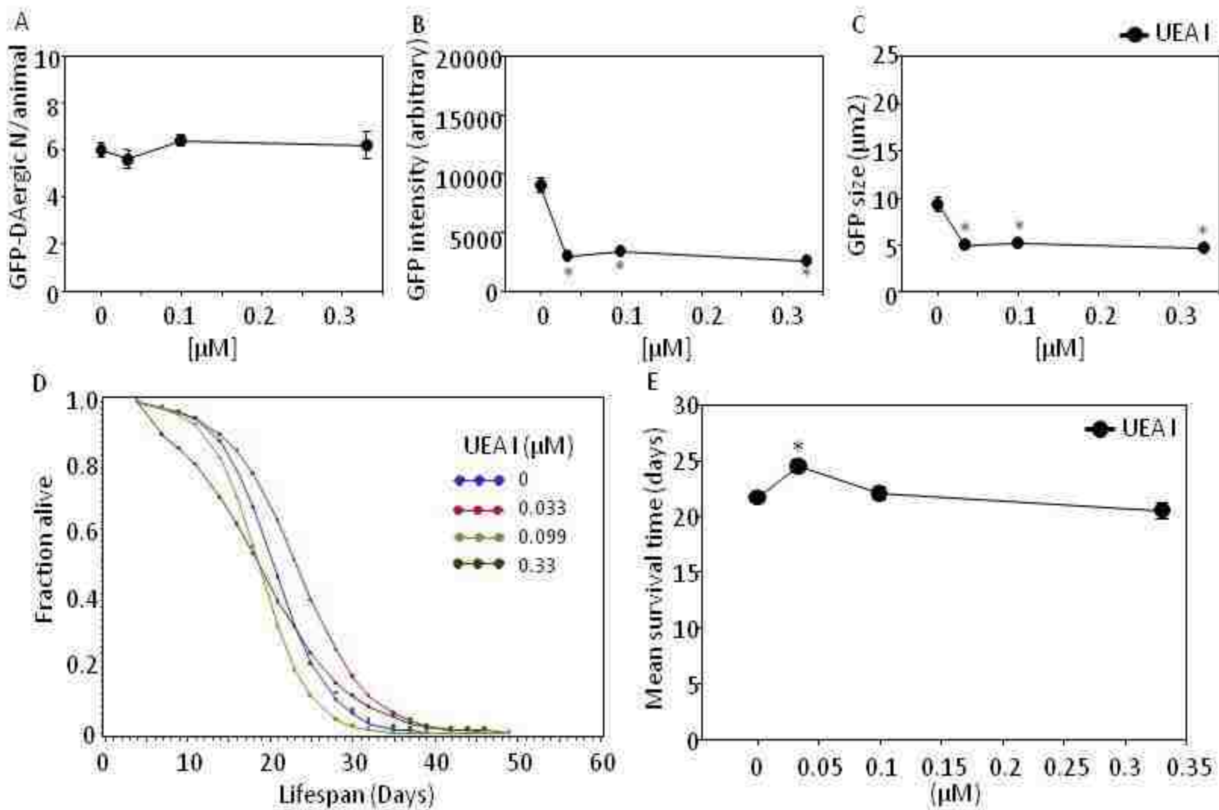

Figure 9. UEA I-TRITC did not show co-localization with GFP-DAergic neurons but affected the DAergic neurons. A) The number of DAergic neurons was not altered. B) The intensity was diminished at all doses (0.033, 0.099 & 0.33 μM, P<0.05). C) The size was reduced at all doses (0.033, 0.099 & 0.33 μM, P<0.05). D) The APS was increased at the low dose. E) The mean lifespan was increased by low dose (0.033 μM) from 22 days to 25 days (13%, P<0.05). * indicates statistical significance.

Lens culinaris (LcH)-TRITC was not detected as transported to but affected DAergic neurons. The number of DAergic neurons was increased at low and high doses (0.05 μM & 0.5 μM, P<0.05, Figure 11a). The intensity of the GFP-DAergic neurons was increased at the lowest dose (0.05 μM, P<0.05, Figure 11b). The area of the DAergic neurons was not altered (Figure 11c). The APS was increased at the lowest dose and reduced at the highest dose (Figure 10d). The mean lifespan was increased at the lowest dose (0.05 μM) from 18 days to 23 days (27%, P<0.05), and decreased at the highest dose (0.5 μM) to 12 days (37%, P<0.05, Figure 11e).

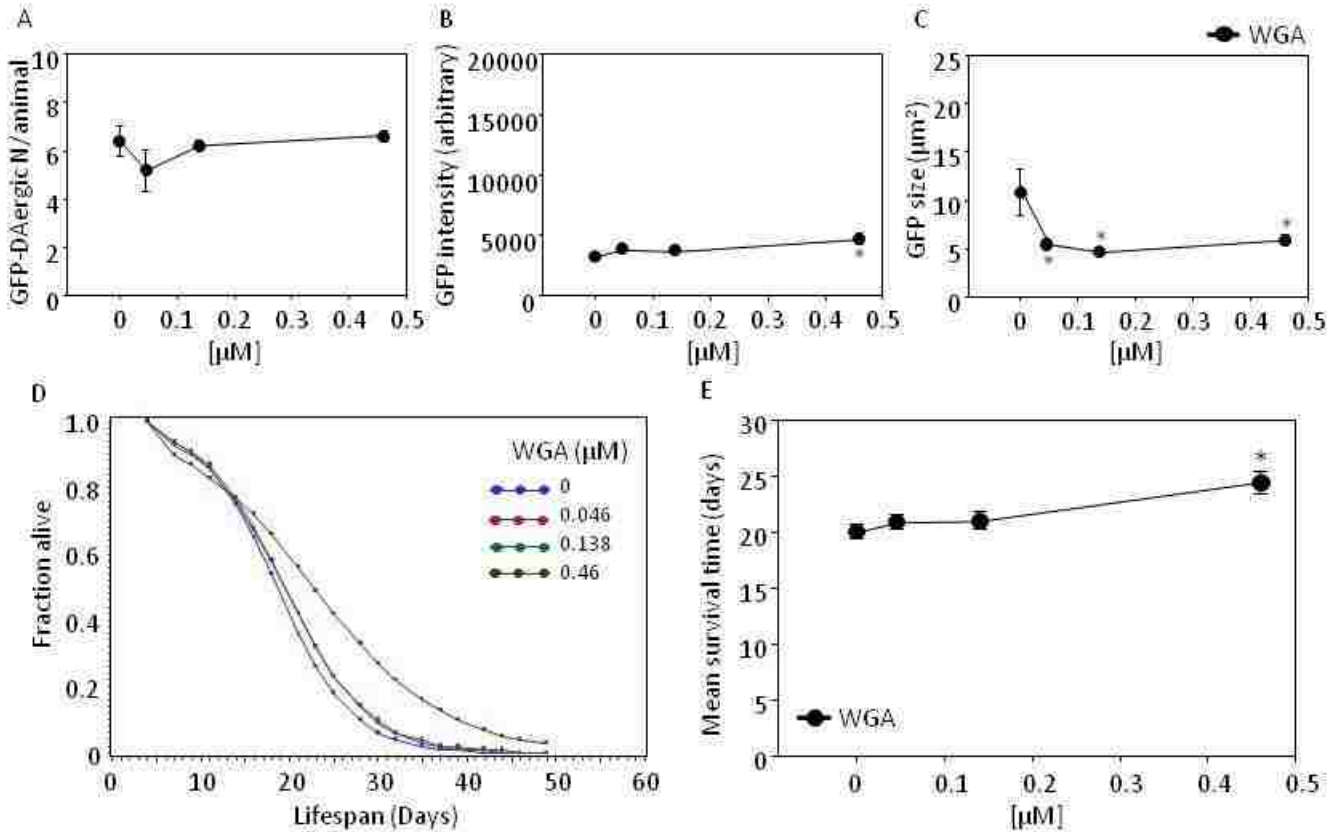

Figure 10. WGA-rhodamine affected the intensity and area of DAergic neurons. A) The number of DAergic neurons was not altered. B) The intensity of the GFP-DAergic neurons was increased at the highest dose (0.46 μM, P<0.05). C) The area of the DAergic neurons was reduced at all doses (P<0.05). D) The APS was increased at all doses. E) The mean lifespan was increased at the highest dose (0.46 μM) from 20 days to 24 days (22%, P<0.05). * indicates statistical significance.

Lens culinaris (LcH)-TRITC was not detected as transported to but affected DAergic neurons. The number of DAergic neurons was increased at low and high doses (0.05 μM & 0.5 μM, P<0.05, Figure 11a). The intensity of the GFP-DAergic neurons was increased at the lowest dose (0.05 μM, P<0.05, Figure 11b). The area of the DAergic neurons was not altered (Figure 11c). The APS was increased at the lowest dose and reduced at the highest dose (Figure 10d). The mean lifespan was increased at the lowest dose (0.05 μM) from 18 days to 23 days (27%, P<0.05), and decreased at the highest dose (0.5 μM) to 12 days (37%, P<0.05, Figure 11e).

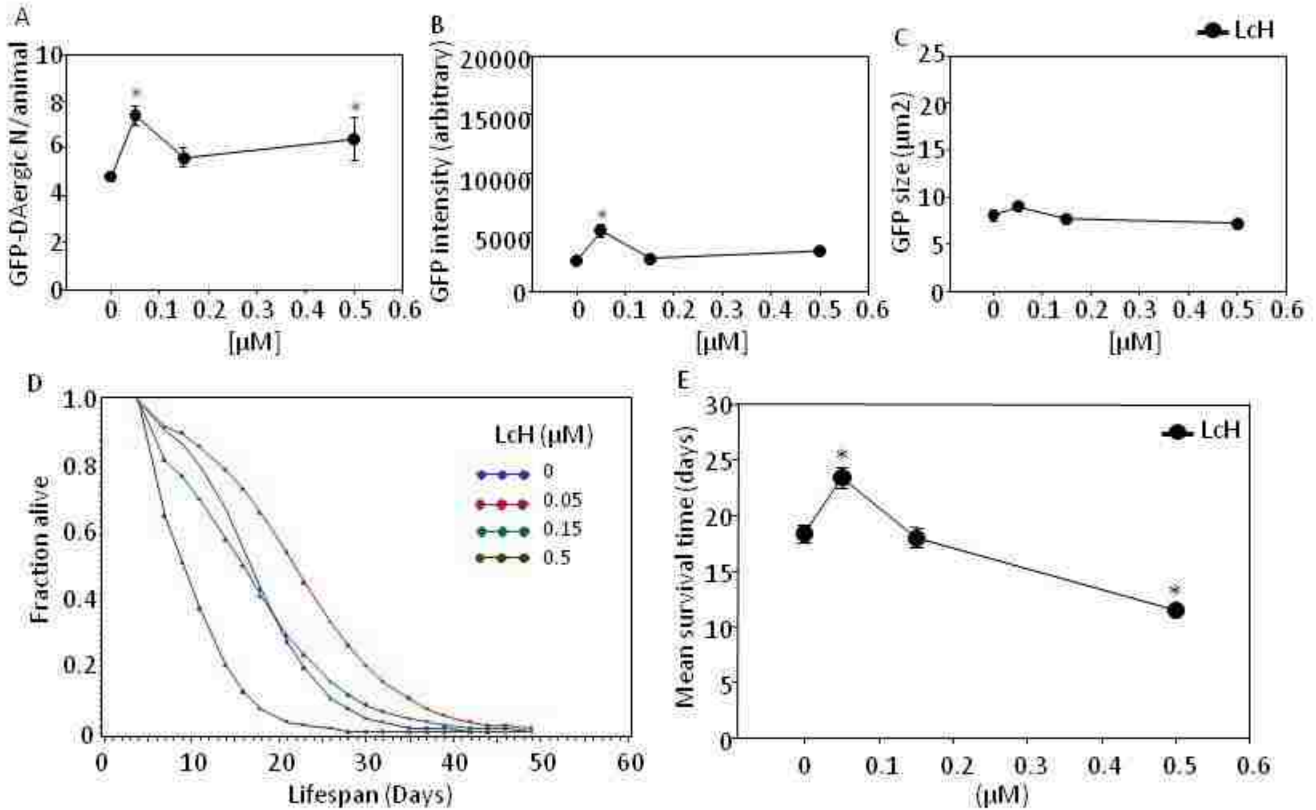

Figure 11. LcH-TRITC was not detected as transported to but affected DAergic neurons. A) The number of DAergic neurons was increased at low and high doses (0.05 μM & 0.5 μM, P<0.05). B) The intensity of the GFP-DAergic neurons was increased at the lowest dose (0.05 μM, P<0.05). C) The area of the DAergic neurons was not altered. D) The APS was increased at the lowest dose and reduced at the highest dose. E) The mean lifespan was increased at the lowest dose (0.05 μM) from 18 days to 23 days (27%, P<0.05), and decreased at the highest dose (0.5 μM) to 12 days (37%, P<0.05). * indicates statistical significance.

Triticum vulgaris (Succinylated) S-WGA-rhodamine did not show co-localization but affected DAergic neurons in *C. elegans*. The number of the GFP-DAergic neurons was decreased at lower doses (0.046 μM & 0.138 μM, P<0.05, Figure 12a). The fluorescent intensity of GFP-DAergic neurons was increased at all doses (0.046 μM, 0.138 μM & 0.46 μM, P<0.05, Figure 12b). The size of GFP-DAergic neurons was decreased at all doses (0.046 μM, 0.138 μM & 0.46 μM, P<0.05, Figure 12c). The APS was increased at a lower dose, and decreased at a higher dose (Figure 12d). The mean lifespan was increased at the lowest dose (0.138 μM) from 21 days to 23 days (9%, P<0.05), and decreased at the highest dose (0.46 μM) to 12 days (-43%, P<0.05, Figure 12e).

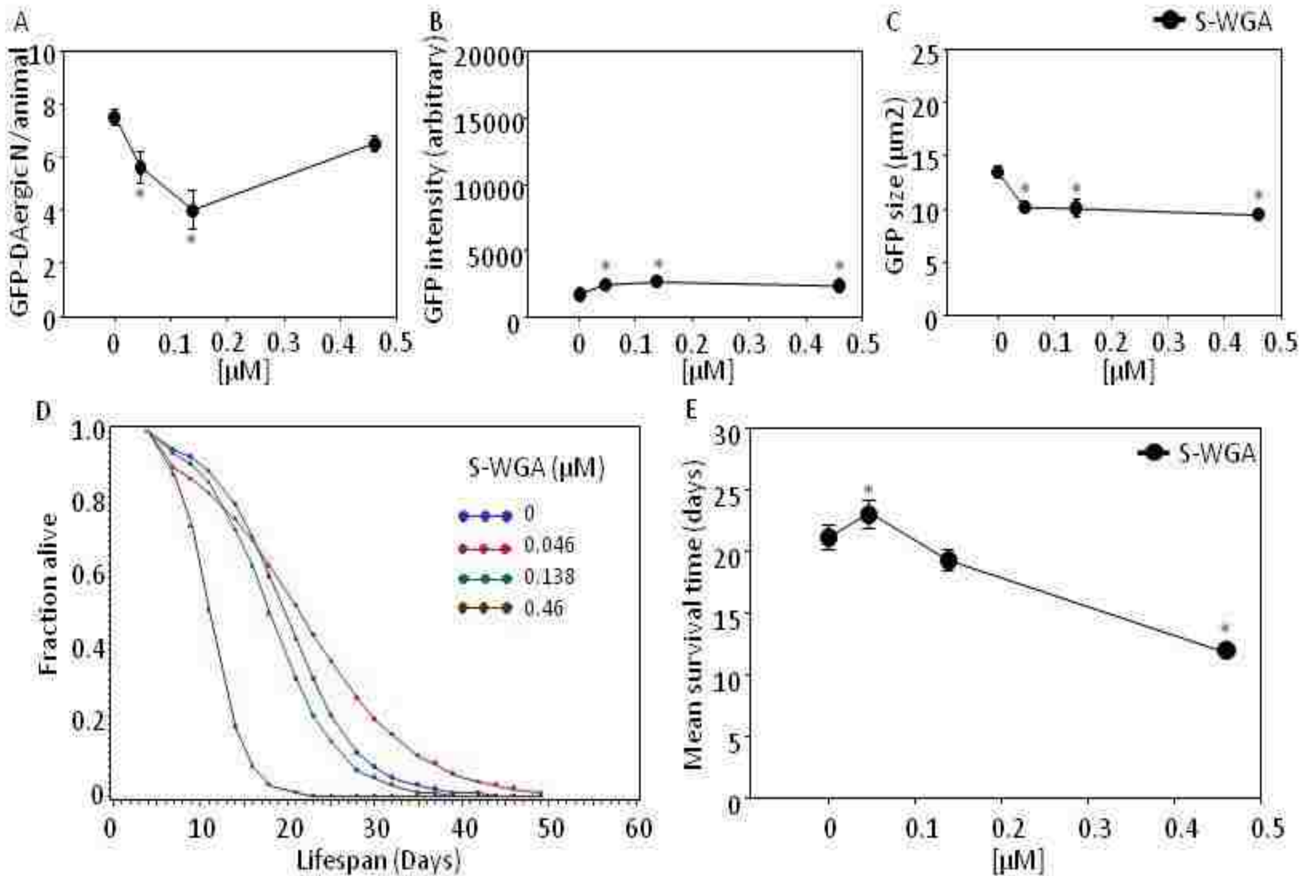

Figure 12. S-WGA-rhodamine affected GFP-DAergic neurons in *C. elegans*. A) The number of the GFP-DAergic neurons was decreased at lower doses (0.046 μM & 0.138 μM, P<0.05). B) The fluorescent intensity of GFP-DAergic neurons was increased at all doses (0.046 μM, 0.138 μM & 0.46 μM, P<0.05). C) The size of GFP-DAergic neurons was decreased at all doses (0.046 μM, 0.138 μM & 0.46 μM, P<0.05). D) The APS was increased at a low dose, and decreased does-dependently at higher doses. E) The mean lifespan was increased at the lowest dose (0.138 μM) from 21 days to 23 days (9%, P<0.05), and decreased at the highest dose (0.46 μM) to 12 days (-43%, P<0.05). * indicates statistical significance.

Artocarpus integrifolia agglutinin (AIA)-TRITC did not show co-localization and did not affect DAergic neurons in *C. elegans*. The number of the GFP-DAergic neurons was not altered (Figure 13a). The fluorescent intensity of GFP-DAergic neurons was not altered by AIA (Figure 13b). The size of GFP-DAergic neurons was not altered by AIA (Figure 13c). The APS was decreased at all doses (Figure 13d). The mean lifespan was decreased at all doses (0.046 μM, 0.138 μM & 0.46 μM) from 24 days to 15 days, 13 days and 11 days (-36%, -47% & -56%, P<0.05, Figure 13e).

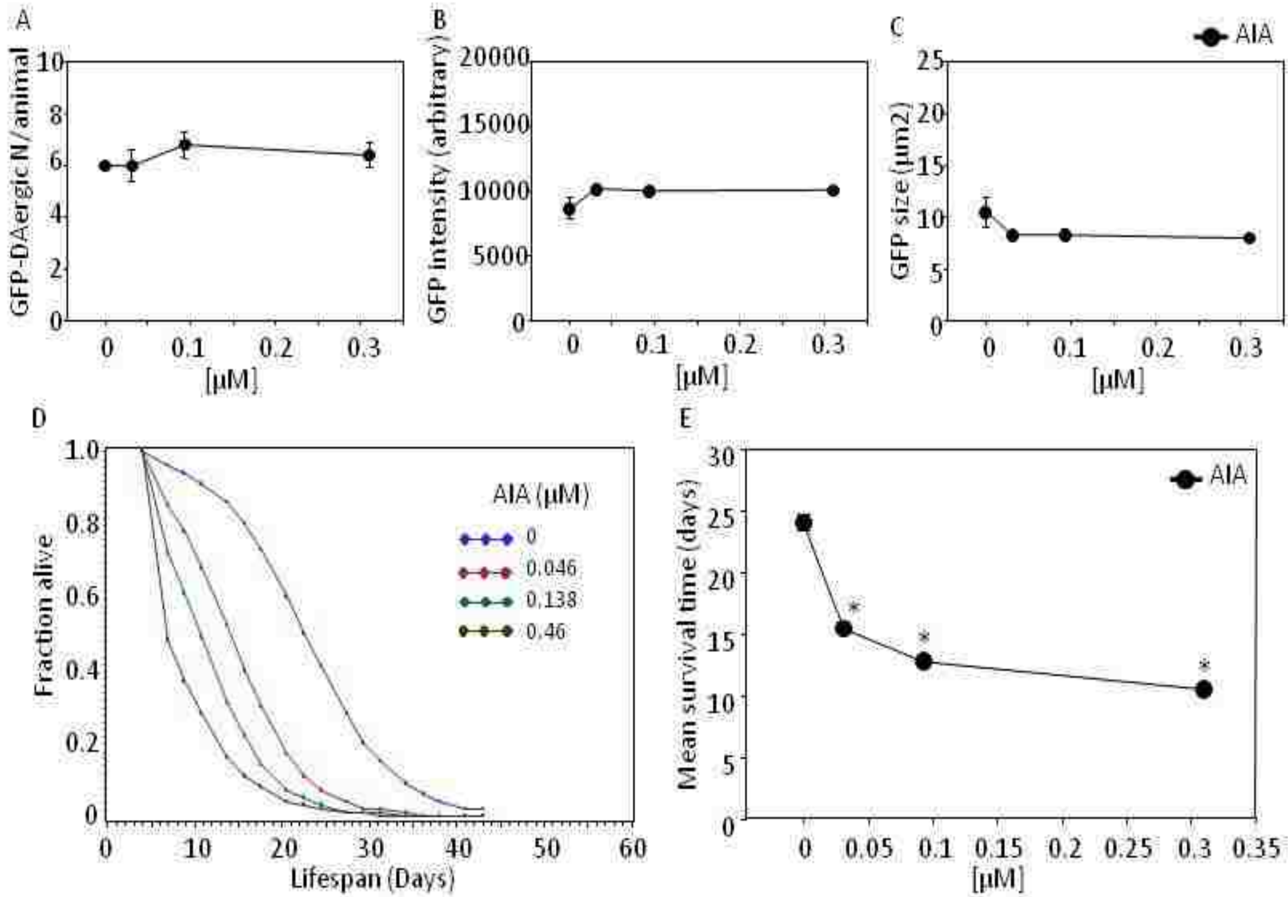

Figure 13. AIA-TRITC did not show co-localization and did not affect DAergic neurons in *C. elegans*. A) The number of the GFP-DAergic neurons was not altered. B) The fluorescent intensity of GFP-DAergic neurons was not altered. C) The size of GFP-DAergic neurons was not altered. D) The APS was decreased at all doses. E) The mean lifespan was decreased at all doses (0.046 μM, 0.138 μM & 0.46 μM) from 24 days to 15 days, 13 days and 11 days (-36%, -47% & -56%, P<0.05). * indicates statistical significance.

Cytisus scoparius agglutinin (CSA)-TRITC did not show co-localization but affected DAergic neurons in *C. elegans*. The number of the GFP-DAergic neurons was reduced at the highest dose (0.65 μM, P<0.05, Figure 14a). The fluorescent intensity of GFP-DAergic neurons was not altered at all doses (Figure 14b). The size of GFP-DAergic neurons was increased at all doses (0.065 μM, 0.195 μM & 0.65 μM, P<0.05, Figure 14c). The APS was decreased at the highest dose (Figure 14d). The mean lifespan was reduced at the highest dose only (0.65 μM) from 18 days to 14 days (-22%, P<0.05, Figure 14e).

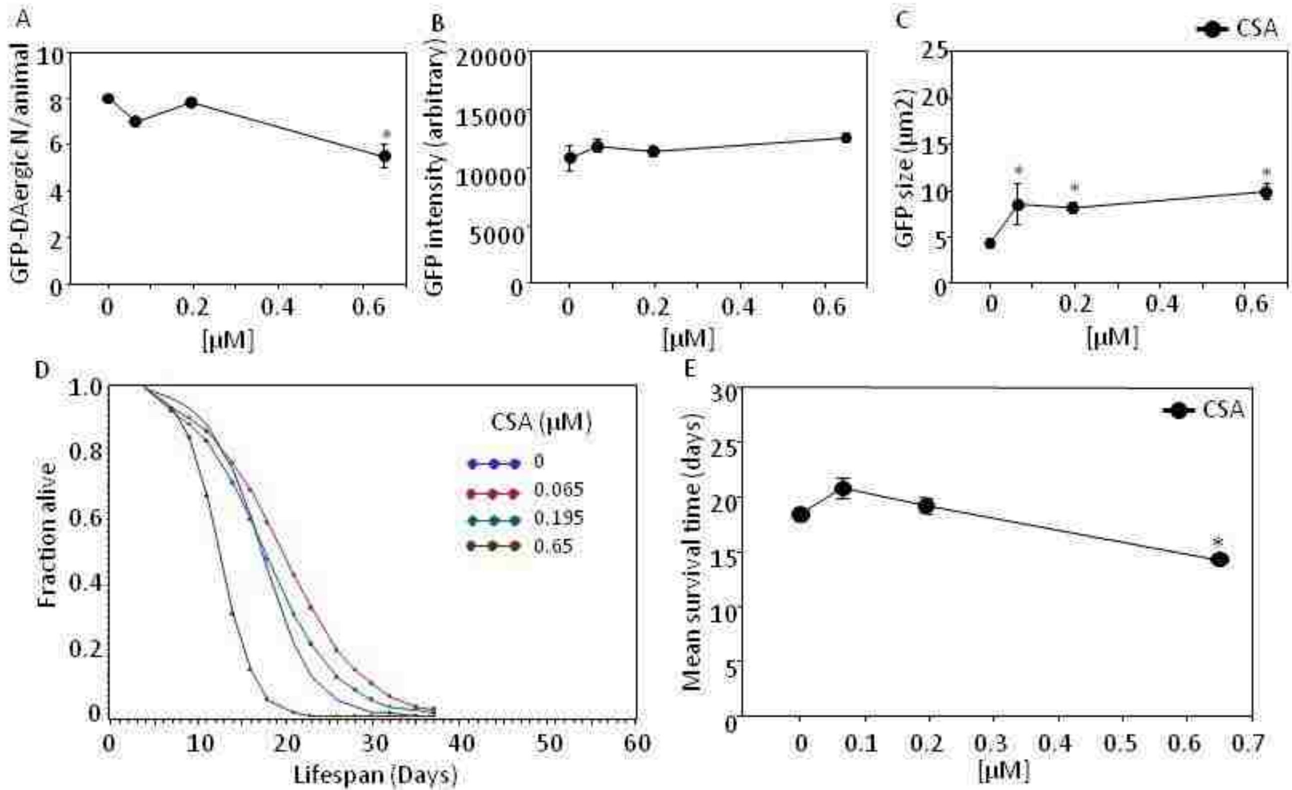

Figure 14. CSA-TRITC did not show co-localization but affected DAergic neurons in *C. elegans*. A) The number of the GFP-DAergic neurons was reduced at the highest dose (0.65 μM, P<0.05). B) The fluorescent intensity of GFP-DAergic neurons was not altered at all doses. C) The size of GFP-DAergic neurons was increased at all doses (0.065 μM, 0.195 μM & 0.65 μM, P<0.05). D) The APS was decreased at the highest dose. E) The mean lifespan was reduced at the highest dose (0.65 μM) from 18 days to 14 days (-22%, P<0.05). * indicates statistical significance.

Dolichos biflorus agglutinin (DBA)-rhodamine did not show co-localization with GFP-DAergic neurons but affected the size and fluorescent intensity of DAergic neurons in *C. elegans*. As shown in the graph, the number of DAergic neurons was not altered (Figure 15a). The fluorescent intensity of GFP-DAergic neurons was increased at the lowest dose (0.018 μM, P<0.05, Figure 15b). The size of GFP-DAergic neurons was increased in a dose-dependent trend at all doses (0.018 μM, 0.054 μM & 0.18 μM, P<0.05, Figure 15c). The APS was increased at a lower dose but decreased at higher doses (Figure 15d). The mean lifespan was reduced at the highest dose only (0.18 μM) from 19 days to 11 days (-43%, P<0.05, Figure 15e).

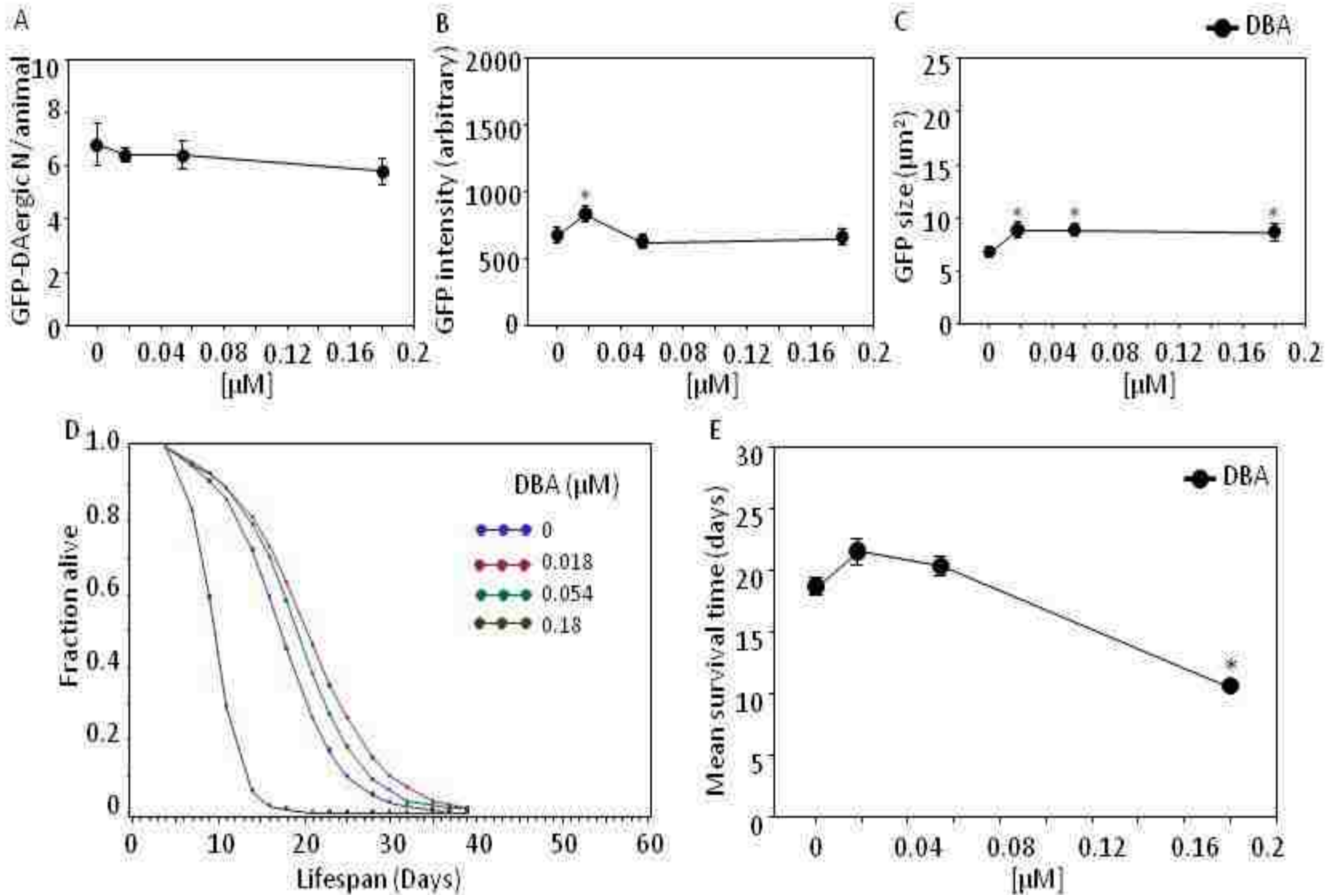

Figure 15. DBA-TRITC did show co-localization but affected the DAergic neurons in *C. elegans.* A) The number of DAergic neurons was not altered. B) The fluorescence intensity of GFP-DAergic neurons was increased at the lowest dose (0.018 μM, P<0.05). C) The size of GFP-DAergic neurons was increased in a dose-dependent trend at all doses (0.018 μM, 0.054 μM & 0.18 μM, P<0.05). D) The APS was increased at lower dose, and decreased at higher doses. E) The mean lifespan was reduced at the highest dose (0.18 μM) from 19 days to 11 days (-43%, P<0.05). * indicates statistical significance.

Galanthus nivalis agglutinin (GNA)-rhodamine did not show co-localization with GFP-DAergic neurons or affect the DAergic neurons. The number of DAergic neurons was not altered (Figure 16a). The fluorescence intensity of GFP-DAergic neurons was not altered (Figure 16b). The size of GFP-DAergic neurons was not altered (Figure 16c). The APS was reduced at all doses (Figure 16d). The mean lifespan was reduced at higher doses dose-dependently (0.077 μM & 0.231 μM) from 24 days to 20 days and 19 days (-16% & -23%, P<0.05, Figure 16e).

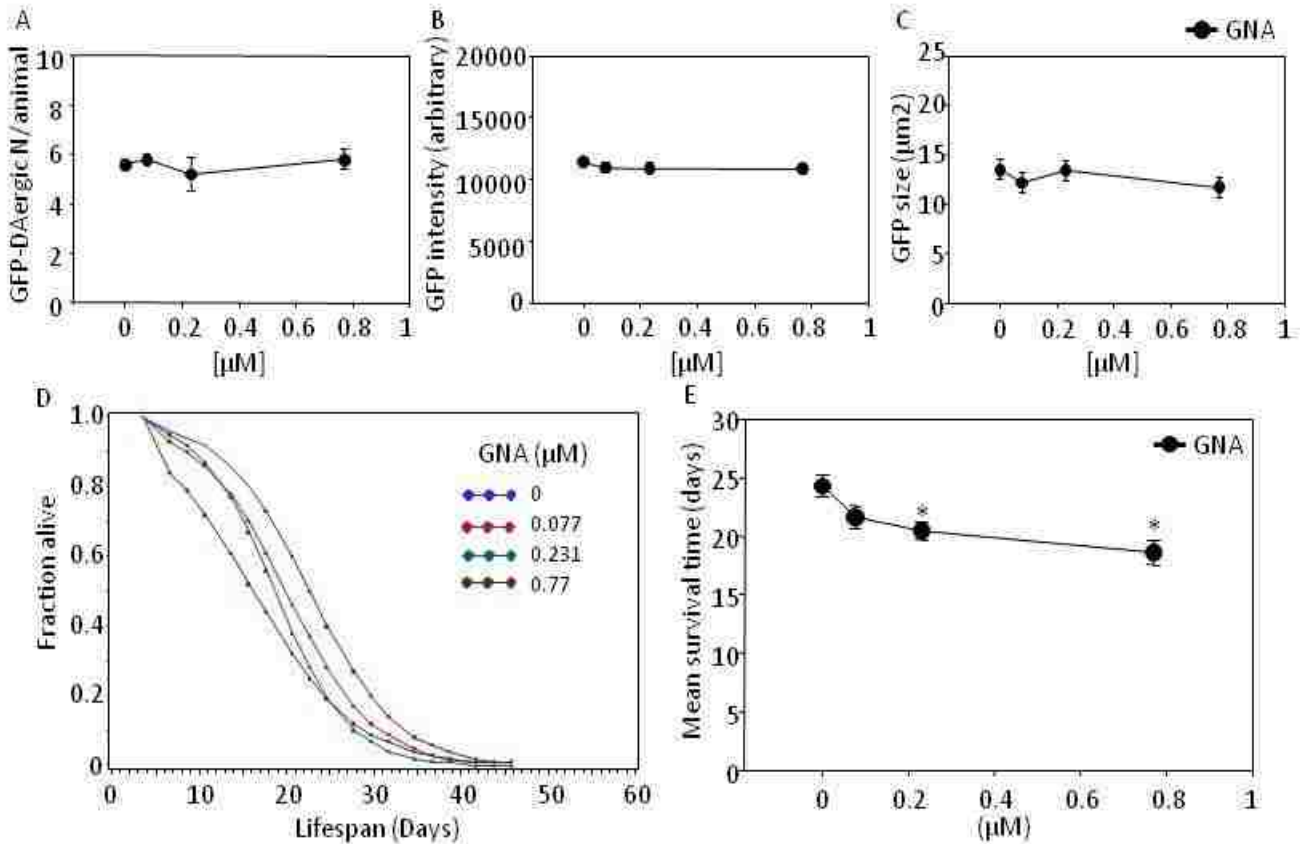

Figure 16. *Galanthus nivalis agglutinin* (GNA)-rhodamine did not show co-localization with GFP-DAergic neurons or affect the DAergic neurons. A) The number of DAergic neurons was not altered. B) The fluorescence intensity of GFP-DAergic neurons was not altered. C) The size of GFP-DAergic neurons was not altered. D) The APS was reduced at all doses. E) The mean lifespan was reduced at higher doses (0.077 μM & 0.231 μM) from 24 days to 20 days and 19 days (-16% & -23%, P<0.05). * indicates statistical significance.

Griffonia Simplicifolia (GSL I)-TRITC did show co-localization but affected the DAergic neurons in *C. elegans*. The number of the GFP-DAergic neurons was not altered in any tested doses (Figure 17a). The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was dose-dependently increased at all doses (0.018 μM, 0.054 μM & 0.18 μM, P<0.05, Figure 17b). The size of GFP-DAergic neurons was not altered (Figure 17c). The APS was dose-dependently decreased at all doses (Figure 17d). The mean lifespan was decreased at the highest dose (0.18 μM) from 17 days to 10 days (-37%, P<0.05, Figure 17e).

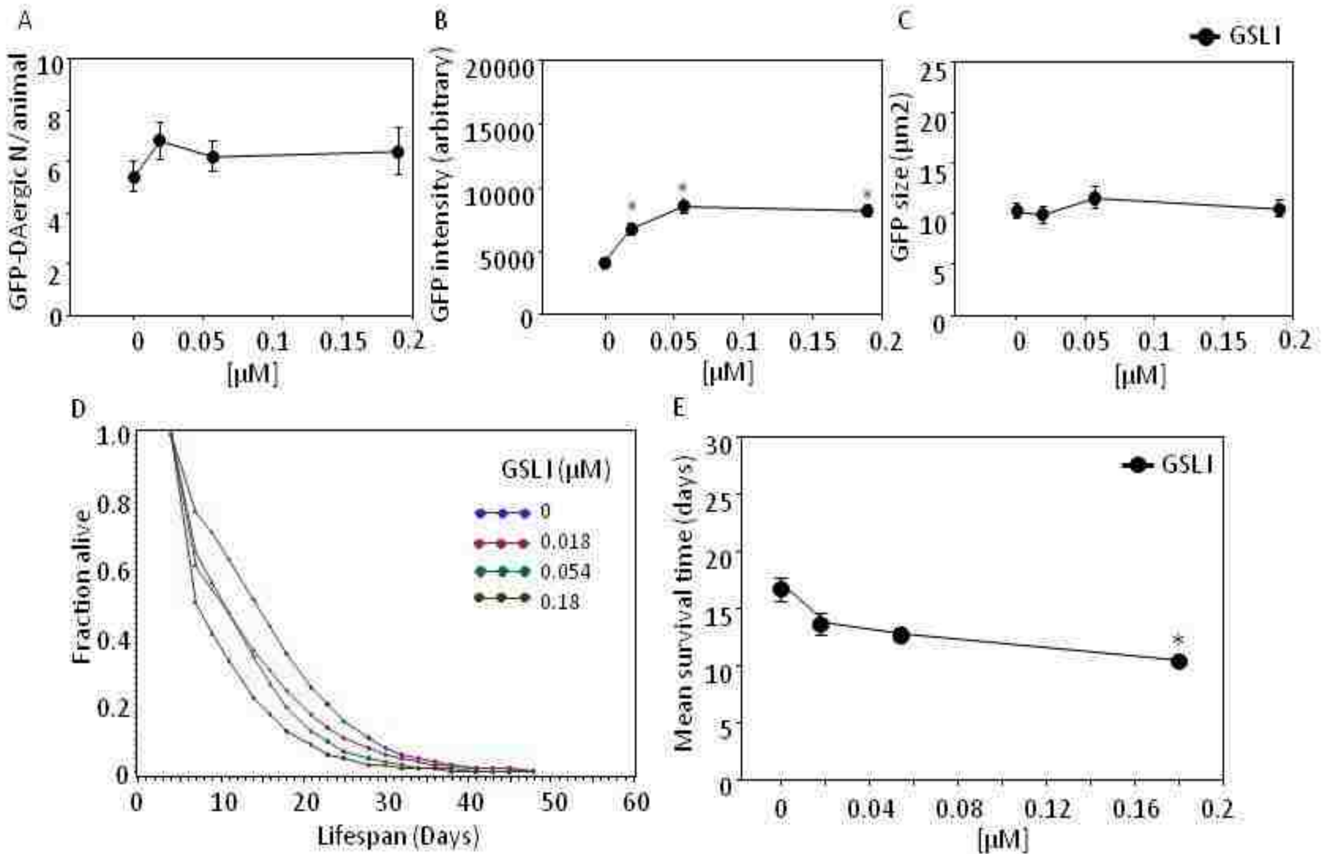

Figure 17. GSL I-TRITC did show co-localization but affected the DAergic neurons in *C. elegans.* A) The number of the GFP-DAergic neurons was not altered. B) The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was dose-dependently increased at all doses (0.018 µM, 0.054 µM & 0.18 µM, P<0.05). C) The size of GFP-DAergic neurons was not altered. D) The APS was dose-dependently decreased at all doses. E) The mean lifespan was decreased at the highest dose (0.18 µM) from 17 days to 10 days (-37%, P<0.05). * indicates statistical significance.

Hippeastrum hybrid agglutinin (HHA)-TRITC did show co-localization but affected the DAergic neurons in *C. elegans*. The number of the GFP-DAergic neurons was not altered (Figure 18a). The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was increased at all doses (0.04 µM, 0.12 µM & 0.4 µM, P<0.05, Figure 18b). The size of GFP-DAergic neurons was increased at the lowest dose (0.04 µM, P<0.05, Figure 18c). The APS was dose-dependently decreased at all doses (Figure 18d). The mean lifespan was decreased at higher doses (0.12 µM & 0.4 µM) from 21 days to 15 days and 11 days (-29% & -47%, P<0.05, Figure 18e).

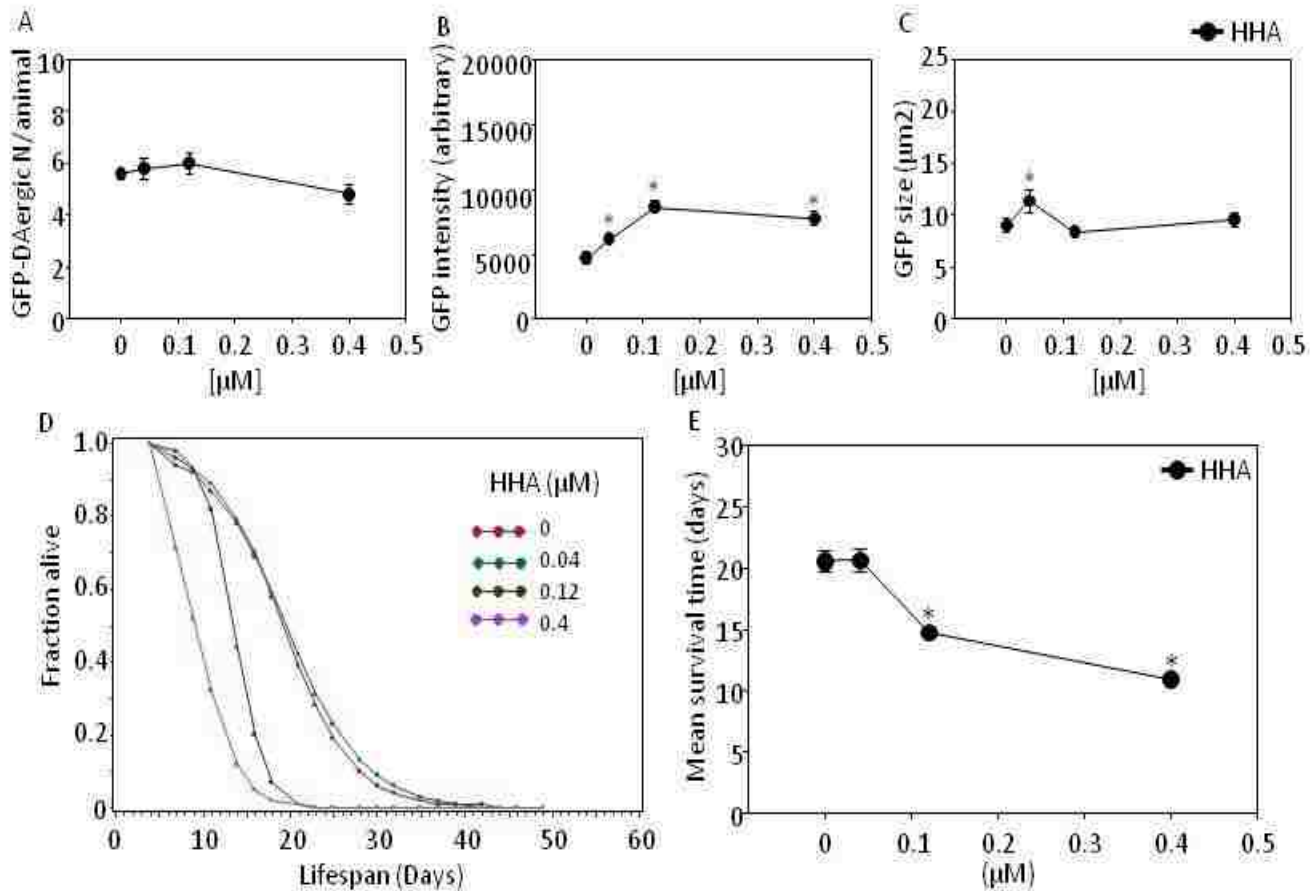

Figure 18. HHA-TRITC did show co-localization but affected the DAergic neurons in *C. elegans*. A) The number of the GFP-DAergic neurons was not altered. B) The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was increased at all doses (0.04 μM, 0.12 μM & 0.4 μM, P<0.05). C) The size of GFP-DAergic neurons was increased at the lowest dose (0.04 μM, P<0.05). D) The APS was dose-dependently decreased at all doses. E) The mean lifespan was decreased at higher doses (0.12 μM& 0.4 μM) from 21 days to 15 days and 11 days (-29% & -47%, P<0.05). * indicates statistical significance.

Phaseolus vulgaris agglutinin-L (PHA-L)-TRITC did show co-localization but affected the DAergic neurons in *C. elegans*. The number of the GFP-DAergic neurons was not altered (Figure 19a). The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was reduced at lower doses (0.017 μM & 0.051 μM, P<0.05, Figure 19b). The size of GFP-DAergic neurons was increased at all doses (0.017 μM, 0.051 μM & 0.17 μM, P<0.05, Figure 19c). The APS was increased at a low dose and reduced at higher doses (Figure 19d). The mean lifespan was reduced at the highest dose (0.17 μM) from 20 days to 13 days (-34%, P<0.05, Figure 19e).

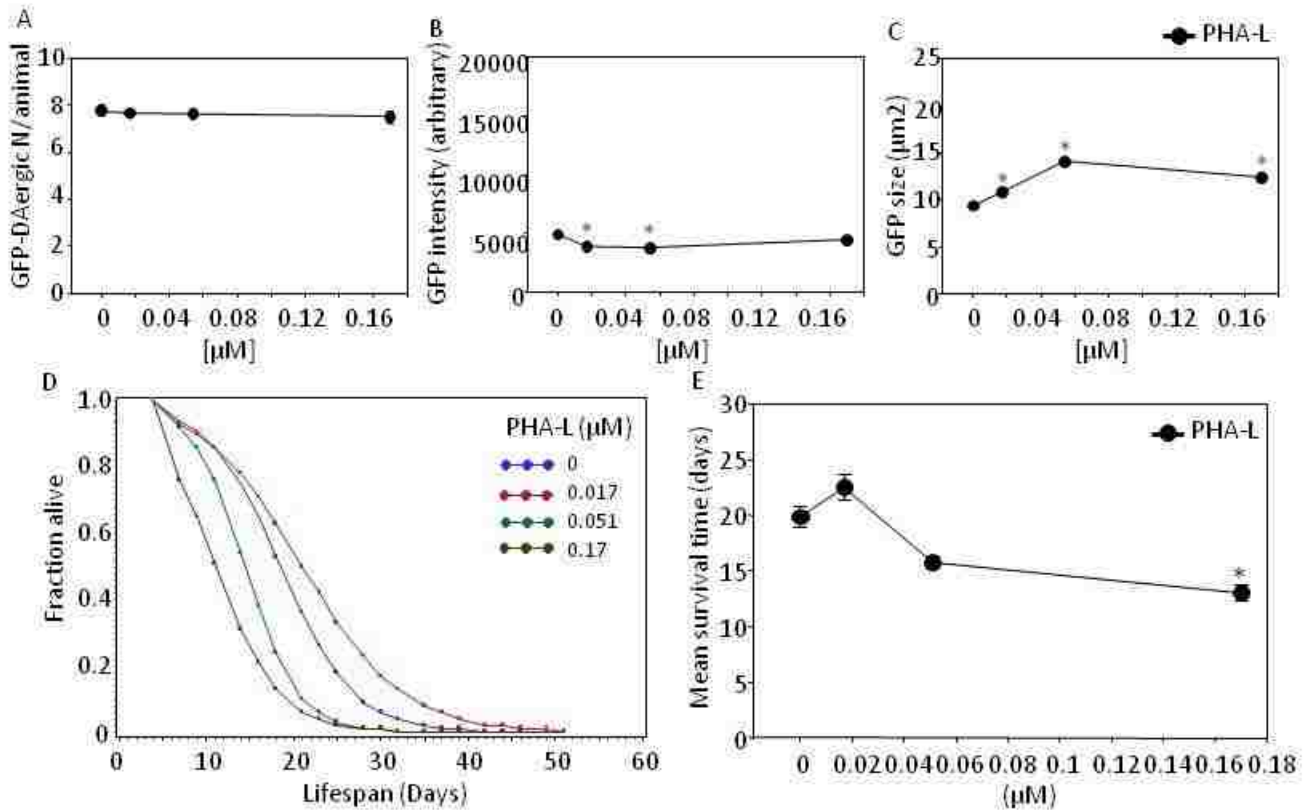

Figure 19. PHA-L-TRITC did show co-localization but affected the DAergic neurons in *C. elegans*. A) The number of the GFP-DAergic neurons was not altered. B) The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was reduced at lower doses (0.017 μM & 0.051 μM, P<0.05). C) The size of GFP-DAergic neurons was increased at all doses (0.017 μM, 0.051 μM & 0.17 μM, P<0.05). D) The APS was increased at a low dose and reduced at higher doses. E) The mean lifespan was reduced at the highest dose (0.17 μM) from 20 days to 13 days (-34%, P<0.05). * indicates statistical significance.

Soybean agglutinin (SBA)-TRITC did show co-localization but affected the DAergic neurons in *C. elegans*. The number of the GFP-DAergic neurons was decreased at the lowest dose (0.017 μM, P<0.05, Figure 20a). The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was not altered (Figure 20b). The size of GFP-DAergic neurons was reduced at the lowest dose (0.017 μM, P<0.05, Figure 20c). The APS was increased at low doses and reduced at a higher dose (Figure 20d). The mean lifespan was reduced at the highest dose (0.17 μM) from 20 days to 14 days (-31%, P<0.05, Figure 20e).

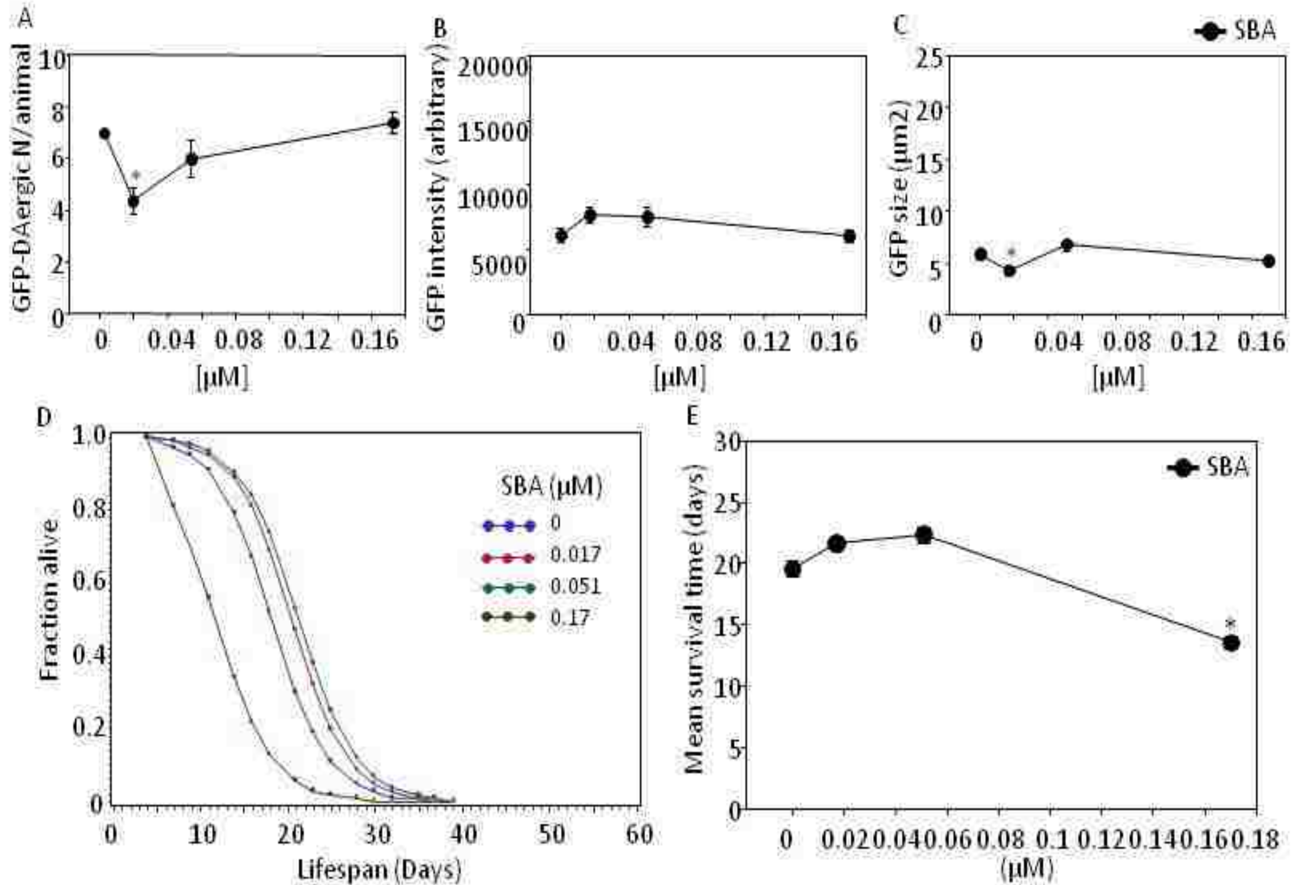

Figure 20. Soybean *agglutinin* (SBA)-TRITC did show co-localization but affected the DAergic neurons in *C. elegans*. A) The number of the GFP-DAergic neurons was decreased at the lowest dose (0.017 μM, P<0.05). B) The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was not altered. C) The size of GFP-DAergic neurons was reduced at the lowest dose (0.017 μM, P<0.05). D) The APS was slightly increased at low doses and reduced at a higher dose. E) The mean lifespan was reduced at the highest dose (0.17 μM) from 20 days to 14 days (-31%, P<0.05). * indicates statistical significance.

Concanavalin A (Con A)-TRITC did not show co-localization with but affected GFP-DAergic neurons in *C. elegans*. The number of DAergic neurons was not altered (Figure 21a). The fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons was increased at the highest dose (0.19 μM, P<0.05, Figure 21b). The size was reduced at medium dose (0.057 μM, P<0.05, Figure 21c). The APS was increased at all doses (Figure 21d). The mean lifespan was not altered by Con A (Figure 21e).

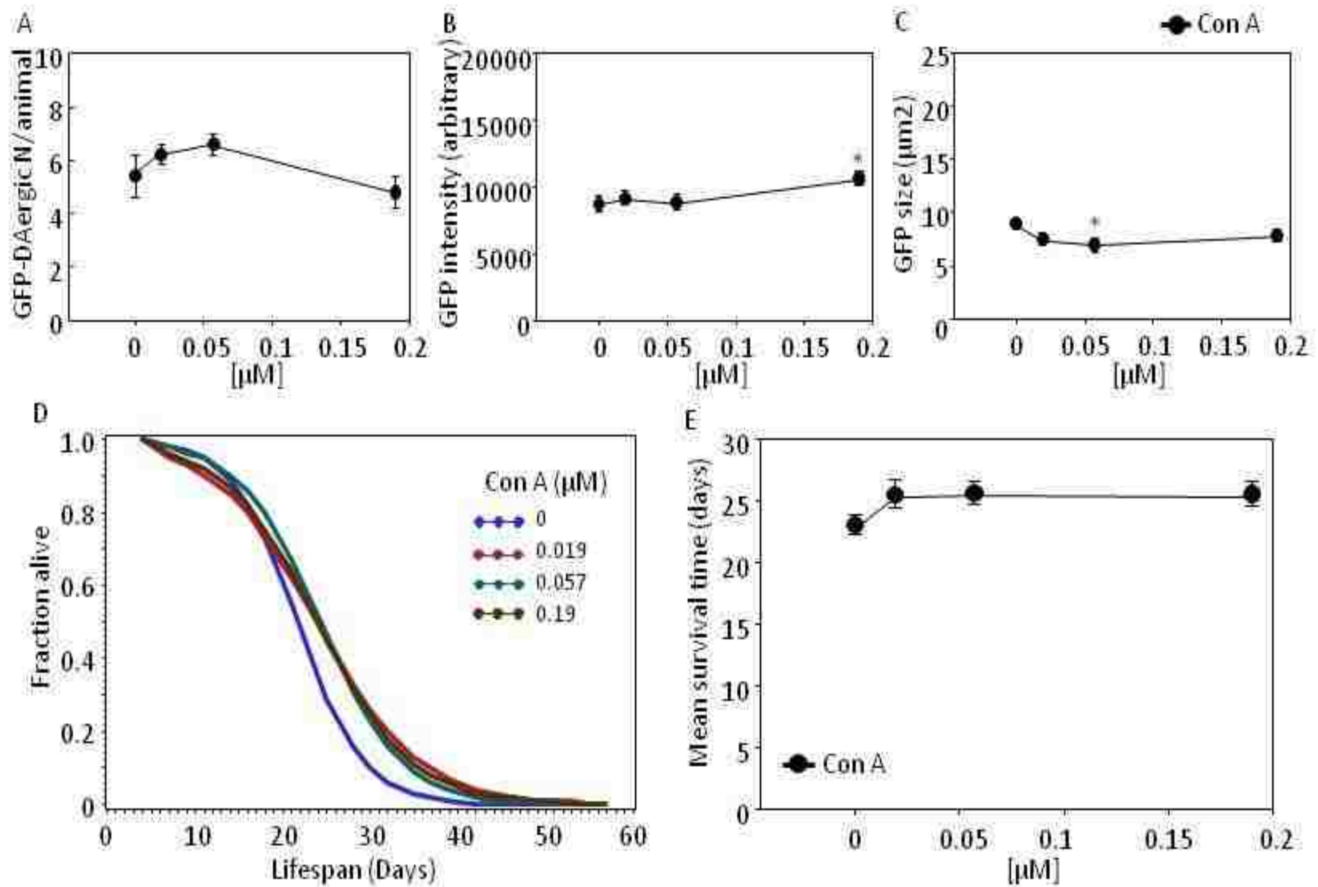

Figure 21. Con A-rhodamine affected the GFP-DAergic neurons in *C. elegans*. A) The number of DAergic neurons was not altered. B) The fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons was increased at the highest dose (0.19 μM, P<0.05). C) The size was reduced at medium dose (0.057 μM, P<0.05). D) The APS was increased at all doses. E) The mean lifespan was not altered. * indicates statistical significance.

Cicer arietinum agglutinin (CPA)-TRITC did not show co-localization with but affected GFP-DAergic neurons in *C. elegans*. The number of DAergic neurons was not altered (Figure 21a). The fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons was reduced at the lowest and the highest doses (0.047 μM & 0.47 μM, P<0.05, Figure 22b). The size of GFP-DAergic neurons was reduced at the doses (0.047 μM & 0.47 μM, P<0.05, Figure 22c). The APS was increased at all doses (Figure 21d). The mean lifespan was not altered by any tested doses significantly (P>0.05, Figure 22e).

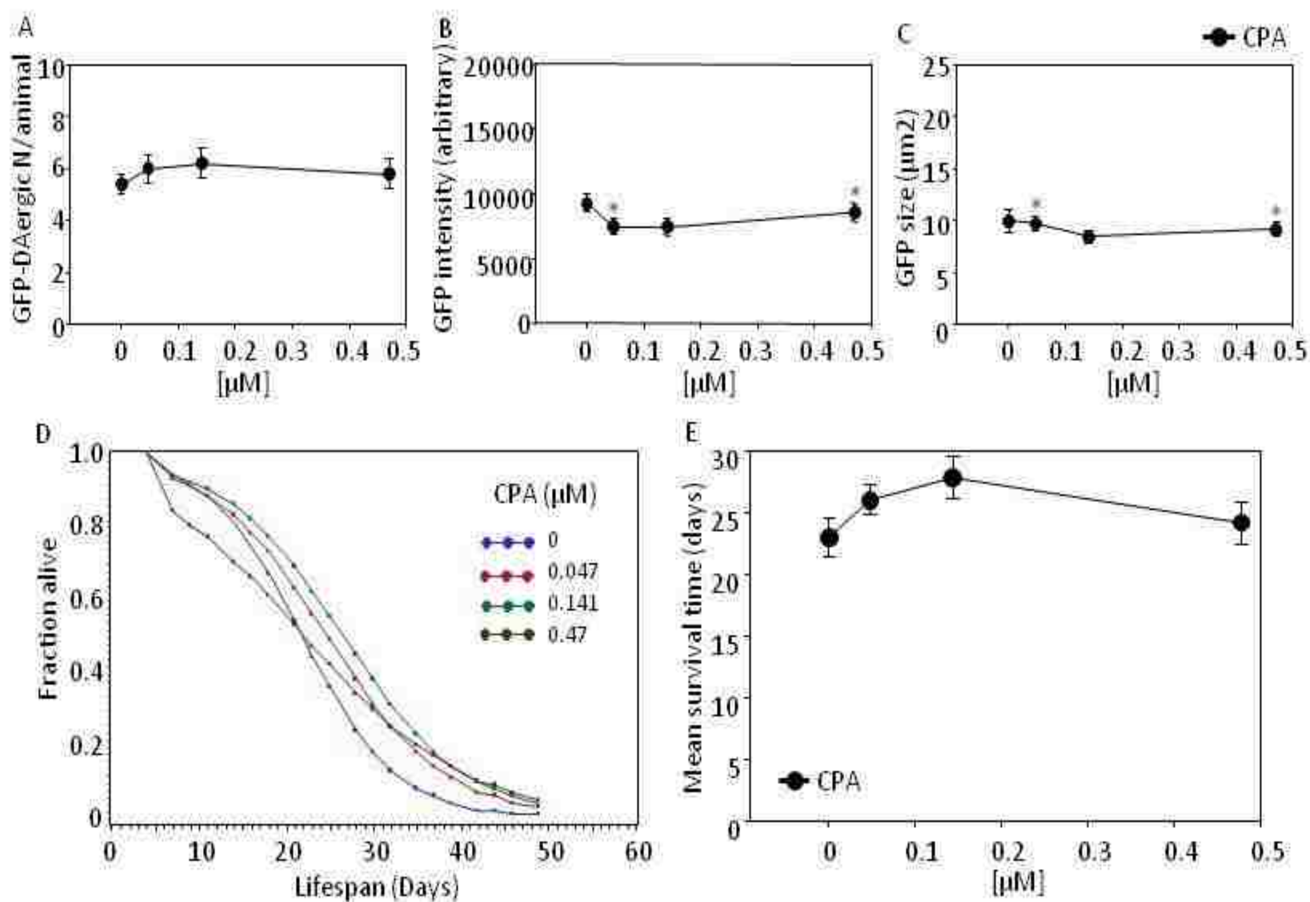

Figure 22. CPA-TRITC did not show co-localization with but affected GFP-DAergic neurons in *C. elegans*. A) The number of DAergic neurons was not altered. B) The fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons was reduced at the doses (0.047 μM & 0.47 μM, P<0.05). C) The size was reduced at the doses (0.047 μM & 0.47 μM, P<0.05). D) The APS was increased at all doses. E) The mean lifespan was not altered. * indicates statistical significance.

Solanum tuberosum agglutinin (STA)-TRITC did not show co-localization with but affected GFP-DAergic neurons in *C. elegans*. The number of DAergic neurons was not altered in all tested doses (Figure 23a). The fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons was not altered (Figure 23). The size was reduced at all doses (0.02 μM, 0.06 μM & 0.2 μM, P<0.05, Figure 23c). The APS was slightly increased at all doses (Figure 23d). The mean lifespan was not altered in all tested doses significantly (P<0.05, Figure 23e).

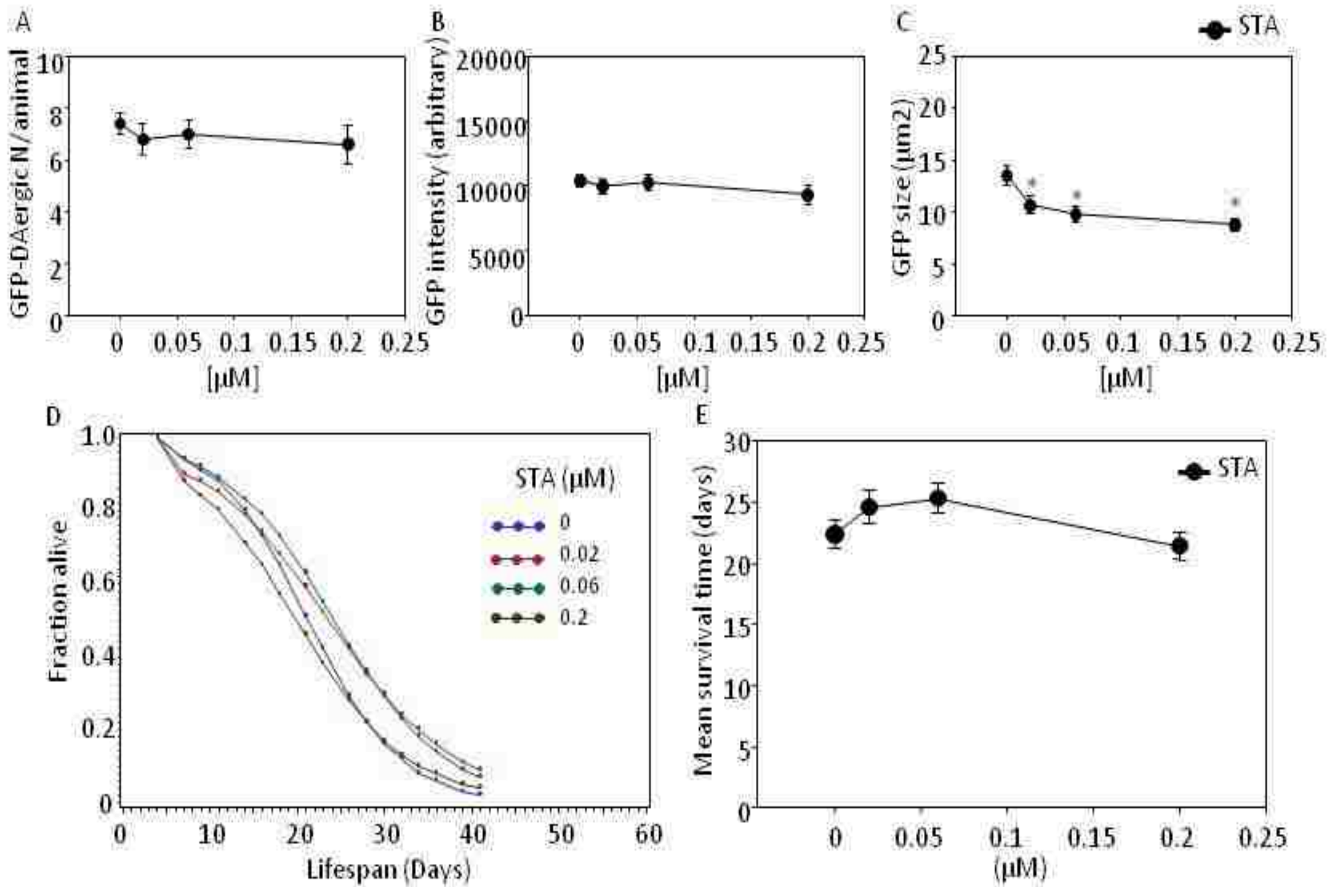

Figure 23. STA-TRITC did not show co-localization with but affected GFP-DAergic neurons in *C. elegans*. A) The number of DAergic neurons was not altered. B) The fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons was not altered. C) The size was reduced at all doses (0.02 μM, 0.06 μM & 0.2 μM, P<0.05). D) The APS was slightly increased. E) The mean lifespan was not altered. * indicates statistical significance.

1.3.3 Lectins affect mobility of *C. elegans*

Larvae 1 stage of *C.elegans* were treated with lectins or MPP+ (0.5 mM) as a positive control. The blank control was supplemented with OP50 only. Travel distance and velocity were evaluated by measuring the moving pixels of *C. elegans* that were displayed in the 20 frames of a video clip. All evaluated twenty lectins significantly reduced the travel distance and velocity of *C. elegans* showing similar effects as the positive control MPP+ compared with the blank control OP50 only (Figure 24, Figure 25).

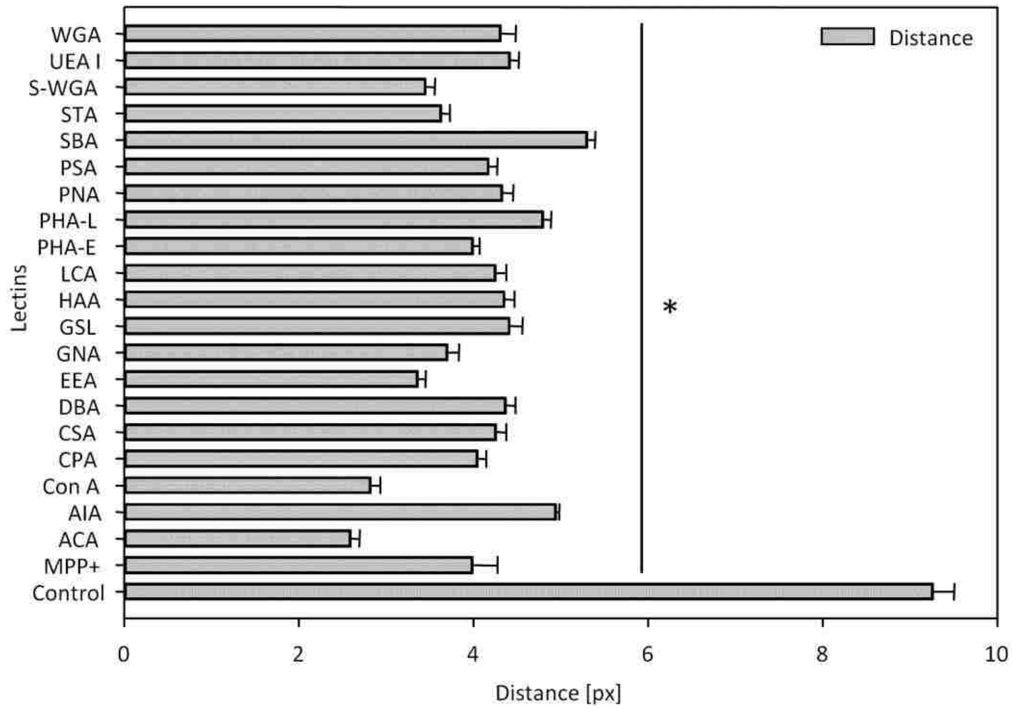

Figure 24. Lectins and MPP+ decreased the travel distance of *C.elegans* compared with the blank control group. * indicates statistical significance compared with the blank control.

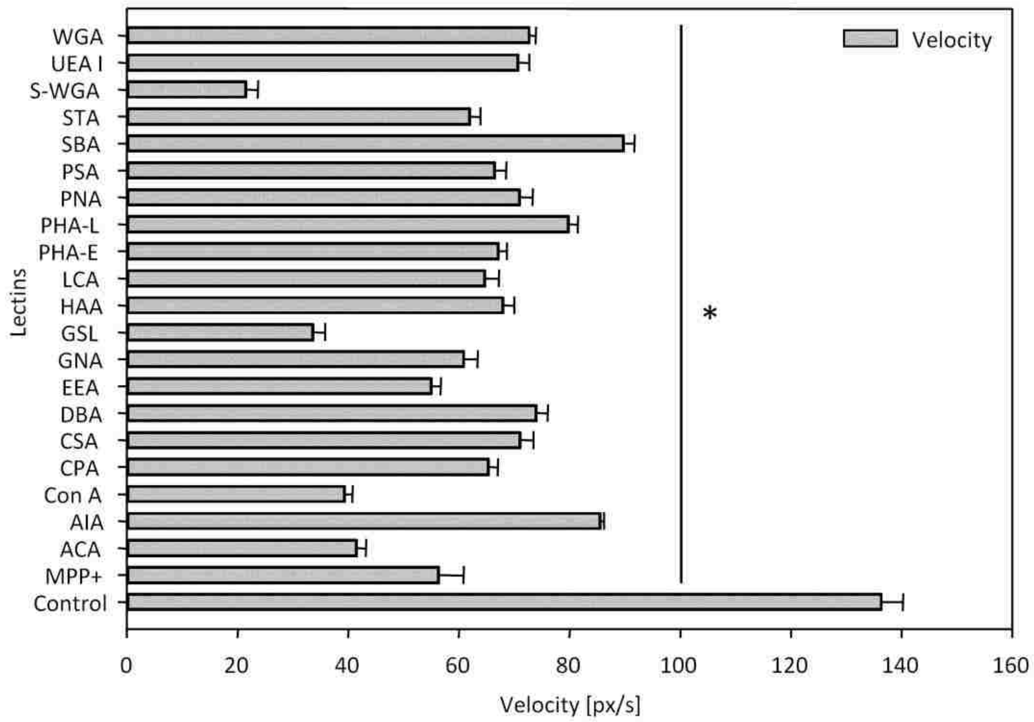

Figure 25. Lectins and MPP+ decreased the travel velocity of *C.elegans* compared with the blank control group. * indicates statistical significance compared with the blank control.

1.4 Discussion

Twenty examined dietary plant lectins conjugated to TRITC or rhodamine were tested in the *in vivo C. elegans* (*egIs1*[*Pdat-1::GFP*]) model. An elevated GFP-dopamine transporter that is expressed under control of the dopamine transporter (DAT) gene promoter shows enhanced DAT expression and trafficking by the promoter, transcription factor, and nuclear receptor [125]. All three lectins ACA, EEA and PNA that co-localized with DAergic neurons decreased the mean lifespan significantly in a dose-dependent manner.

Carbohydrate-binding protein toxins are known to survive and traverse the gut intact, as an acutely toxic substance and can induce serious life-threatening illness in humans and animals. Distance pathogenicity of botulinum toxin, as well as cholera toxin, impairs the central nervous system [126]. The present study was aimed at a new, surprising property of lectins based upon the hypothesis that lectins may be transported directly by gut absorption to local neurons and transported axonally to distal neurons where they have an anatomical and potentially a physiological pathophysiological effect. Fluorescent intensity and co-localization of lectins was observed to suggest transport to GFP-DAergic neurons. Number and area changes of GFP-dopamine receptor fluorescence in DAergic neurons were observed to be an effect of the lectins. Involvement of other pathways like indirect effects by interaction of lectins with glial cells that affected DAergic neurons may be possible, as it was reported that in patients with PD or Parkinsonism had α-SYN deposited not only in DAergic neurons but also in different kinds of glial cells [127-129]. Lectins have been used for histochemistry and neuronal tracing only, but were not previously associated with neuronal toxicity [51, 130, 131].

1.4.1 The occurrence and intensity of individual fluorescently labeled lectins in GFP-DAergic neurons detected by co-localization

Three lectins (ACA, EEA or PNA) appeared to co-localize with a subgroup of GFP-DAergic neurons while other lectins had effects on GFP-DAergic neurons where co-localization was not observed.

Because some effects were seen in neurons where lectins were not detected, this may be due to undetectable amount of transported lectin, or some unexplained indirect effect. Thus, the number and GFP-dopamine transporter image of neurons were also evaluated, even when the fed lectin was not detected. The lack of observation of fluorescence in the neurons using other fed lectins (which, however, seemed to affect these neurons) may be due to a variety of characteristics. For instance, a critical window for the lectin to be detectable may have been missed, the lectin may have been partially degraded, losing the fluorophore, but still retaining neuron-effective activity, or, more likely, undetectable levels of the lectin have activities in the cytoplasm or nucleus. Future studies with ELISA, other immunocytochemical studies or radiolabeling may confirm the transport of small amounts of these specific lectins where an effect is observed without fluorescence co-localization being observed. In addition to the DAergic neurons, lectins were found mainly contained in the intestine of *C. elegans*. Few amount of lectins were also found scattered in the whole body of the animals under the fluorescent microscopy. Observation of lectins in the whole body may suggest that lectins have access to other organs in *C. elegans*. ACA was first isolated from the seeds of *Amaranthus caudatus* in 1989 [132], and it has been mainly used to describe the recognition and migration of cell surface receptors of cancer cells [133] [134]. EEA was mainly known to bind to blood group-related carbohydrates (mainly B and H determinants) and as a new category of lectins [135]. Structural analysis of EEA indicated that EEA had a β-trefoil fold similar to ricin B-like (R-type) lectins [136], which is known for its neurotoxicity. PNA was reported to facilitate to isolate PNA-binding glycoprotein in central nervous system in humans [137], which implied that further interactions between PNA and these proteins was possible.

1.4.2 Lectin-caused differences in the number of GFP-DAergic neurons

Four lectins (CSA, EEA, SBA and S-WGA) reduced the number of DAergic neurons, it seemed that these effects were related to the dose used. For CSA and EEA, only the highest dose reduced the number of DAergic neurons. For SBA and S-WGA, only low doses reduced the number. In contrast to

EEA, low doses of S-WGA reduced the number of DAergic neurons while the highest dose did not. CSA and SBA reduced the number of DAergic neurons. The reduction of neuron numbers indicated the toxicity of these lectins, which was consistent with lifespan results, that both EEA and S-WGA reduced the mean lifespan significantly. The lectin LcH increased the number of DAergic neurons at both the lowest dose and the highest dose.

1.4.3 Lectin feeding effects on the fluorescent intensity of GFP-DAergic neurons

Four lectins (CPA, EEA, PHA-L, PSA or UEA I) reduced GFP-dopamine transporter fluorescence in D-Aergic neurons suggesting damage to DAT, while Con A, CSA, DBA, GSL I, HHA, LcH, PHA-E, S-WGA or WGA induced an increase indicating a promotion of DAT in the DAergic neurons. PSA, in some studies, has been shown to be essentially nontoxic in mice both *in vivo* and *in vitro* [138]. However, toxicity measurements in these studies may not include more subtle long term effects of neuronal damage. Although WGA did not affect the number of GFP-DAergic neurons in the present study, and in other laboratories, in the *in vivo* rat gut lumen, reduced expression of heat shock proteins resulting in lowered protection and greater permeability of epithelial cells. WGA also increases thrombin in human platelets, and escalates adipogenesis in mesenchymal cells of the mouse limb bud *in vitro* [50, 139-141], by unknown mechanisms.

1.4.4 Sizes of GFP-DAergic neurons

ACA, CPA, Con A, PHA-E, PSA, SBA, S-WGA, STA UEA I or WGA significantly reduced the size of GFP-DAergic neurons. Whether these effects are due to damage to the neurons is not known. Increased neuron size of a subgroup of GFP-DAergic neurons, however, was also observed with DBA, EEA, HHA or PHA-L which may have promoted DAT expression, however, whether decrease or increase in the apparent size of neurons has a physiological effect, or indicates lectin-mediated damage is not yet known. Toxicity of some lectins and newly discovered side effects of ingestion of PHA and WGA lectins in human and animals have been observed, mitigated by sucrose feeding [141-143]. In addition, the size

of the GFP-DAergic neurons was significantly reduced suggesting a possible toxic effect of cytoplasmic PHA-E. This observation is in agreement with other studies showing that PHA can damage intestinal epithelial cells [53, 142], which was prevented or reversed by a PHA-E SIS sucrose [142]. Interestingly, PHA-E did not show significant change in the number and size of GFP-neurons in *C. elegans* but demonstrated enhanced expression of GFP-dopamine transporter fluorescence intensity. Con A strengthens extracellular matrixes by promoting production of proteoglycan in mouse chondrocytes *in vitro* [140]. In our study, Con A altered GFP-DAergic neurons by reducing the area of GFP-dopamine transporter fluorescence.

Some specific beneficial activities of a variety of lectins have been reported [140, 144]. In our studies, DBA was observed in the GFP-DAergic neurons which had the effect of increasing the observed area of the GFP-labelled DA transporter. This increase may suggest enhanced DAT expression and trafficking, where GFP is expressed under the dopamine transporter (DAT) promoter [125, 145]. A major adverse effect of DBA lectin has not been reported elsewhere. In fact, DBA significantly facilitates cartilagenesis and osteogenesis in mouse limb bud mesenchymal cells *in vitro* [140].

1.4.5 Elevated fluorescent intensity and size of DAergic neurons

These alterations may reflect a relationship with the insulin receptor and DAT. Glucose provides a vital energy source for brain and clearly modulates neuronal function [146, 147]. In *C. elegans*, hyperglycemia reduces APS, related to human diabetes. These relationships in our study, however, may represent some signaling interaction of glycemia/insulinemia and DAT. As with other catecholamine neurotransmitters, inhibitory neurotransmitters are inversely proportional to glycemia, and DA kinetics is sensitive to hypoglycemia in a complex manner [148]. In rodents, insulin receptors and DAT are densely present in *substantia nigra*, insulin may increase DAT mRNA expression, and glycemic index is inversely associated with the risk of PD [149, 150].

1.4.6 Lectins affect the lifespan of *C. elegans*

Lectins can be categorized into four groups based on their effects on the mean lifespan of *C. elegans*. 1) Lectins PHA-E, PSA, UEA I or WGA increased the mean lifespan. 2) Lectins ACA, AIA, CSA, DBA, EEA, GNA, GSL I, HHA, PHA-L, PNA or SBA reduced the mean lifespan. 3) Lectins LcH or S-WGA showed dual effects in affecting the mean lifespan, with low dose increasing and high dose decreasing it. 4) Lectins Con A, CPA or STA did not alter the mean lifespan significantly.

1.4.7 Lectins affect the mobility of *C. elegans*

PD includes four cardinal features, tremor, rigidity, bradykinesia (slowness of movement) and postural instability, with bradykinesia as the most characteristic and easily recognizable symptom [151]. In addition, among the four symptoms, bradykinesia correlates best with the degree of dopamine deficiency, rigidity and postural instability correlates less and tremor does not show any correlation [152]. In this study, two important surrogate markers of movement, distance and velocity were evaluated in *C. elegans*. *C. elegans* is a practical and affordable animal model to study symptoms of neurodegenerative diseases, as they respond to a wide range of stimuli and exhibit characteristic movement patterns [153].

MPP+ has been widely used in various animal models to induce symptom of PD, which can be selectively taken up by DAergic neurons via DAT[99]. MPP+ (0.5 mM) has been used to induce motor symptoms that mimic PD's in *C. elegans*, which cause apparent mobility reduction after 48 hours of treatment [96, 97, 154]. Present study showed that all twenty lectins evaluated reduced travel distance and velocity ($P<0.05$), mimicking bradykinesia in humans, as the positive control MPP+ (0.5 mM) in *C. elegans* organism. The decrease of the distance and velocity of animals implies that motor neurons were affected, which might be due to neurodegeneration caused by lectins. This observed reduction in the travel distance and velocity was consistent with the reduction of the number, area or intensity of DAergic neurons for most tested lectins. Some lectins like PNA, LcH, AIA, DBA, GNA, GSL I or HAA, were not observed to have any detrimental effects on DAergic neurons in *C. elegans* in this study, but still caused

PD like symptoms of slow movement in *C. elegans*. This phenomenon might indicate that lectins might have other mechanisms in inducing the PD besides affecting DAergic neurons. α-SYN deposit in both DAergic neurons and glial cells, lectins might also affect the glial cells in addition to directly or indirectly participate in the development of the PD [128]. The detailed mechanism of bradykinesia remains unknown, but a disruption in normal motor cortex activity caused by reduced DAergic function might be the main cause of bradykinesia [151]. Functional imaging studies suggest that bradykinesia correlates with impairment in the recruitment of cortical and subcortical systems that regulate kinematic parameters of movement like velocity [155]. Anatomically, bradykinesia and tremor scores significantly correlate with cerebral metabolic rate of glucose inversely in bilateral putamen, the metabolic rate of glucose is higher when the bradykinesia is worse [156], which leads to reduction in the muscle force produced at the initiation of movement [151]. In a human study, patients with PD were asked to make ballistic elbow flexion movements, electromyographic signals were recorded and showed that patients with bradykinesia are unable to energize the appropriate muscles to perform long time movements compared with the control [157]. Taken together, the results of current study might lend some support to the hypothesis of reduced DAergic function caused bradykinesia, and the reduced DAergic neuron number, area or intensity caused by lectins correlate with the reduced mobility in *C. elegans* to some extent.

Neurotoxins like botulinum toxin or ricin bind to specific receptors on cell membrane, to be internalized, and exert toxic effects [158, 159]. Similarly, lectins bind to carbohydrate ligands of targeted cells to be functional, producing effects. Specific sugars may competitively bind and inhibit lectins uptake [160]. Structurally similar lectins might share similar binding receptors and compete for each other's binding sites. The complex GI environment including nutrients and cellular environment might alter the absorption of certain lectins and their potential biological consequences [161].

In a recent Danish report, patients who had vagal nerves removed 20 years earlier had 40% lower incidence of PD than control populations. If dietary proteins were one potential etiology for PD, by

transport to neurons from the gut, as hypothesized here, removal of the vagal nerve would have prevented or reduced this etiology pathway. Symptoms of motor impairment are typical in PD patients, and dysfunction of aspects of the autonomic nervous system are often underrated, such as GI motility [162], rapid eye movement [163], *etc*. The current study indicates potential transport of some dietary plant lectins from the GI tract to the DAergic neurons in *C. elegans*, with direct or indirect effects on these neurons and diverse effects on APS. This observation may be related to the Braak and Hawkes' hypothesized unknown etiologic agent for PD or related, for example, to damaged DAergic neurons those have been found in PD [89, 93]. If related, the process may be gradual, may be additive, related to the frequency of consumption of certain lectins, and may be determined by the association of lectins with other factors. Certainly, there is potential for inputs from individual genetic susceptibility, varying sugar structures profiles in different cell membranes, the receptivity to endocytosis, a disorder or leakage of the GI lining, and dietary content. Our observations are a tantalizing possible explanation for why dietary plants have been linked to a risk of developing PD.

1.5 References

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CHAPTER III. SPECIFIC INHIBITING SUGARS AFFECT THE EFFECTS OF LECTINS ON LIFESPAN AND DOPAMINERGIC NEURONS IN *C. ELEGANS*

1.1 Disclaimer

There were anticipated problems with feeding high amounts of sugars to *C. elegans* as inhibitors of lectin binding. For example, D-galactosamine perfusion in the liver of rats led to D-galactosamine accumulate as sugar nucleotides, and reduced the level of uridine diphosphate (UDP), which was needed for RNA synthesis [1]. Other problems can arise, for example, feeding xylose to rats and mice cause cataracts due to protein cross linking by "non enzymatic glycosylation" [2, 3]. Sucrose has effects on the levels of dopamine and opioid mRNA in rat brain similar to the effects of opiate [4]. Other sugars may cause similar side effects because they spend considerably more time in the open chain aldehyde or ketone form than glucose, which is the evolutionarily chosen circulating sugar in animals for that reason. However, we conducted some experiments with simple sugars as inhibitors, which experiments and results are described here.

1.2 Materials and methods

C. elegans egIs1[Pdat-1::GFP] that express GFP in the 8 DAergic neurons [5, 6] and the standard food *Escherichia coli* (*E. coli*) were obtained from *C. elegans* Genetics Center (CGC, MN). The *C. elegans* model does not require regulation of the Institutional Animal Care and Use Committee.

1.2.1 Culture *Escherichia coli* (*E. coli*, OP50)

OP50 were cultured by the standard method described elsewhere [7]. Briefly, approximately 10 μL of stock *E. coli* solution was added to media and incubated at 37 ℃ for 24h. The OP50 were then plated in Petrifilm™ (3M Corporate, St. Paul, MN) at 37 ℃ for 24h until densities of 5×10^{8} to 5×10^{11} colony forming units (cfu/ml) were reached and then were fed to the *C. elegans ad libitum* [7, 8]. The OP50 stock feeding solution was enriched to 2×10^{9}cfu/ml by centrifuging at 2,200 g for 10 minutes and washed with S-complete buffer twice.

1.2.2 *C. elegans* culture

Mature gravid transgenic *C. elegans egIs1[Pdat-1::GFP]* were treated with NaOH (1M) and sodium hypochlorite solution (5.25%, 5:2 ratio) to dissolve the body and release viable eggs [7]. The eggs were hatched overnight fed *ad libitum* with LB broth (200 μL/well) containing OP50 $5 \times 10^8 - 5 \times 10^{11}$ cfu/ml [9], after washing with S-complete solution 3 times. The age-synchronized *C. elegans* were diluted to 100 animals/ml, plated in liquid culture in a 96 well plate (120μl/well, 10-15 animals) [10]. The plate was tape sealed, bagged, and covered with aluminum foil. All animals were kept in a 20°C low temperature incubator (Revco Tech., Nashville, NC, USA) throughout the experiments. Thirty microliters of 5-Fluoro-2′-deoxyuridine (FUDR, 0.6 mM) stock solution was added to each well at larvae 4 stage.

All treatments were applied at day 3 after hatching. Four dose responses of twenty lectins were obtained for each culture condition in a dark room. Control animals were fed with OP50. Experimental groups were fed rhodamine/TRITC-conjugated lectins with or without additional SIS(s). The SIS, or lectin plus SIS were incorporated into feeding medium with OP50. Each group of nematodes were collected and fixed after the first week for the liquid culture as described elsewhere [11]. Briefly, after being collected from wells, animals were washed with S-Basal twice, fixed with paraformaldehyde (4%) over 2h at 4℃ and washed with PBS for 5min x 3. Ten microliters of Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) was applied to a glass slide followed by 10 μL of the medium containing *C. elegans*. At last, a cover glass was mounted on the glass slide.

1.2.3 Select lectins and specific inhibiting sugars (SIS)

Commercially available plant lectins conjugated with TRITC or rhodamine were purchased from EY labs (San Mateo, USA), Vector Labs (Burlingame, USA), or Sigma-Aldrich (St. Louis, USA). Each lectin-SIS-inhibition experiment had a SIS-only control group. The dose of SIS for each fluorescence-conjugated lectin was determined by searching histology literature or product manuals. Doses of lectins and their SISs were used comparable to published work in histochemistry or neuronal tracing [12-14].

1.2.4 Average probability of survival (APS) assay

All average probability of survival (APS) assays were conducted in liquid culture (96-well plate). The animals were synchronized and seeded into each well of a plate (n=10-15) and OP50 was added to each well. Thirty microliters (0.6mM) of fluorodeoxyuridine (FUDR) was added to each well to sterilize the animals. Four different treatments (50 μL/treatment, n=6 row) including control, SIS only, or lectin plus SIS were added. The plate was then covered with aluminum foil. The whole procedure was performed in a dark room to prevent bleaching of fluorophores. After the plate was shaken for 3 minutes, the survival animals were counted every other day until all were dead under an inverted microscope (Nikon, Eclipse T*i* –S, Japan) at 4x or 10x magnification. Animals were exposed to strong lights to stimulate the movements [15] and the movement of pharynx was checked to confirm whether the animals were dead when they were not moving.

1.2.5 Fluorescent microscopy

The GFP-DAergic neurons were identified by FTIC filter (480Ex/520Em) and the number of GFP-DAergic neurons counted. Fluorescent intensity of GFP-DAergic neurons and their average sizes (μm^2) were determined by NIS-Elements Advanced Research (version 3.22.11) and compared among the following groups: control, SISs, and lectins plus SIS. Fluorescent intensity of rhodamine-lectins was determined by a TRITC filter (580Ex/620Em) to assess co-localization. The magnitude of the effect(s) of the lectin on the DAergic neurons, the number, fluorescent intensity (arbitrary unit), and sizes (μm^2) of GFP-DAergic neurons were determined and compared among each group. Co-localization was initially identified with an inverted microscopy (Nikon, Eclipse T*i* –S, Japan) and then confirmed at a Z-axle with laser scanning microscopy (Leica, TCS SP5, Germany).

1.2.6 Solutions and chemicals

Standard NGM agar plates (g): NaCl 3.0g, Bacto-agar (Becton, MD) 20g, Bacto-peptone 2.5g (Becton, MN), Cholesterol solution 0.1% (0.005/ml 95% ethanol), and dH_2O 975ml were mixed.

Additions to the autoclaved solution (M): $CaCl_2$ 1.0 1ml, $MgSO_4$ 1.0 1ml, KPO_4 pH6 1.0 25ml. *LB Broth*: 25.0g, dH_2O 1L (autoclave). *S-basal solution* (M)*:* NaCl 0.1, KPO4 pH6 0.05, Cholesterol 0.1%, was autoclaved. *PBS* (mM)*:* 115 NaCl, 75 $Na_2HPO_4{\cdot}7H_2O$, and 7.5 KH_2PO_4, pH 7.4.

1.2.7 Statistical analyses

All results are presented as mean ±S.E.M. Analyses were carried out using SAS/STAT® software, Version 9.4 of the SAS System for Windows (Cary, NC, USA). Survival curves were displayed by binomial probabilities obtained from logistic regression models as surrogates for survival probabilities and mean lifespan was estimated via Kaplan-Meier (log-rank). ANOVA models were used to analyze neuron data. For each group, 10-15 animals were analyzed for liquid culture. An alpha level of 0.5 was considered statistically significant.

1.3 Results

Diets supplemented with varying concentrations of TRITC-conjugated lectins and their SIS in liquid culture were fed to *C. elegans* (**Error! Reference source not found.**). We observed that some SIS had the following effects: a) enhancing the effects of lectins on the DAergic neurons or mean lifespan, b) mitigating the effects of lectins on the DAergic neurons or mean lifespan, or c) reversing the effects of lectins on the DAergic neurons or mean lifespan.

Amaranthus caudatus agglutinin (ACA)-TRITC co-localized with DAergic neurons at the highest dose (0.32 μM), with or without the SIS GalNAc. As shown in the graph, when the green color from GFP fused DAergic neurons (Figure 26a) and the red color from TRITC conjugated ACA (Figure 26b) was overlapped, the co-localized area showed bright yellow color (Figure 26c), which demonstrated that ACA was successfully transported from gut to DAergic neurons in *C. elegans* organism. ACA did not the affect the number of DAergic neurons (Figure 26d), the intensity of DAergic neurons was reduced by the highest dose of ACA with the presence of the SIS (0.32 μM, $P<0.05$, Figure 26e).

Table 5. Effects of lectins combined with specific inhibiting sugars on lifespan and DAergic neurons in *C. elegans*

Lectins	Dose (μM)	GFP #	GFP intensity	GFP size	Lifespan
ACA	0.032	↔	↔	↔	↔
	0.096	↔	↔	↔	↓
	0.32	↔	↓	↓	↓
EEA	0.048	↔	↑	↔	↔
	0.136	↔	↔	↓	↓
	0.48	↔	↓	↑	↓
PNA	0.018	↔	↔	↔	↔
	0.054	↔	↔	↓	↔
	0.18	↔	↔	↓	↑
PHA-E	0.017	↔	↑	↔	↔
	0.054	↔	↑	↔	↔
	0.17	↓	↔	↓	↓
PSA	0.043	↔	↔	↔	↑
	0.129	↔	↔	↑	↔
	0.43	↓	↔	↔	↔
UEA I	0.033	↔	↔	↔	↔
	0.099	↔	↔	↔	↔
	0.33	↑	↔	↑	↓
WGA	0.046	↔	↑	↔	↔
	0.138	↔	↑	↔	↔
	0.46	↔	↑	↔	↔
LcH	0.05	↔	↓	↔	↔
	0.15	↑	↓	↔	↓
	0.5	↓	↓	↓	↓
S-WGA	0.046	↔	↑	↔	↔
	0.138	↔	↔	↑	↔
	0.46	↔	↓	↔	↔
AIA	0.031	↔	↔	↔	↔
	0.093	↔	↔	↔	↓
	0.31	↔	↔	↔	↓
CSA	0.065	↔	↔	↔	↔
	0.195	↔	↓	↔	↔
	0.65	↔	↓	↔	↔

↓ Indicates decreasing trend, $P<0.05$
↑ Indicates increasing trend, $P<0.05$
↔ Indicates no significant alternation, $P>0.05$

Table 5 continued. Effects of lectins combined with specific inhibiting sugars on lifespan and DAergic neurons in *C. elegans*

Lectins	Dose (μM)	GFP #	GFP intensity	GFP size	Lifespan
DBA	0.018	↔	↑	↔	↔
	0.054	↔	↑	↔	↔
	0.18	↑	↑	↔	↔
GNA	0.077	↔	↔	↔	↔
	0.231	↔	↓	↔	↓
	0.77	↔	↔	↑	↓
GSL I	0.018	↔	↑	↑	↔
	0.054	↔	↑	↑	↔
	0.18	↔	↑	↑	↔
HHA	0.04	↔	↔	↓	↔
	0.12	↔	↔	↔	↔
	0.4	↔	↓	↓	↓
PHA-L	0.017	↔	↓	↑	↑
	0.051	↔	↓	↑	↔
	0.17	↔	↓	↔	↓
SBA	0.017	↔	↑	↔	↔
	0.051	↔	↔	↔	↑
	0.17	↔	↓	↓	↔
Con A	0.019	↔	↔	↔	↔
	0.057	↔	↔	↔	↔
	0.19	↔	↓	↔	↔
CPA	0.047	↔	↔	↔	↔
	0.141	↔	↓	↔	↓
	0.47	↔	↓	↔	↓
STA	0.02	↔	↔	↔	↔
	0.06	↔	↔	↔	↔
	0.2	↔	↔	↔	↔

↓ Indicates decreasing trend, $P<0.05$
↑ Indicates increasing trend, $P<0.05$
↔ Indicates no significant alternation, $P>0.05$

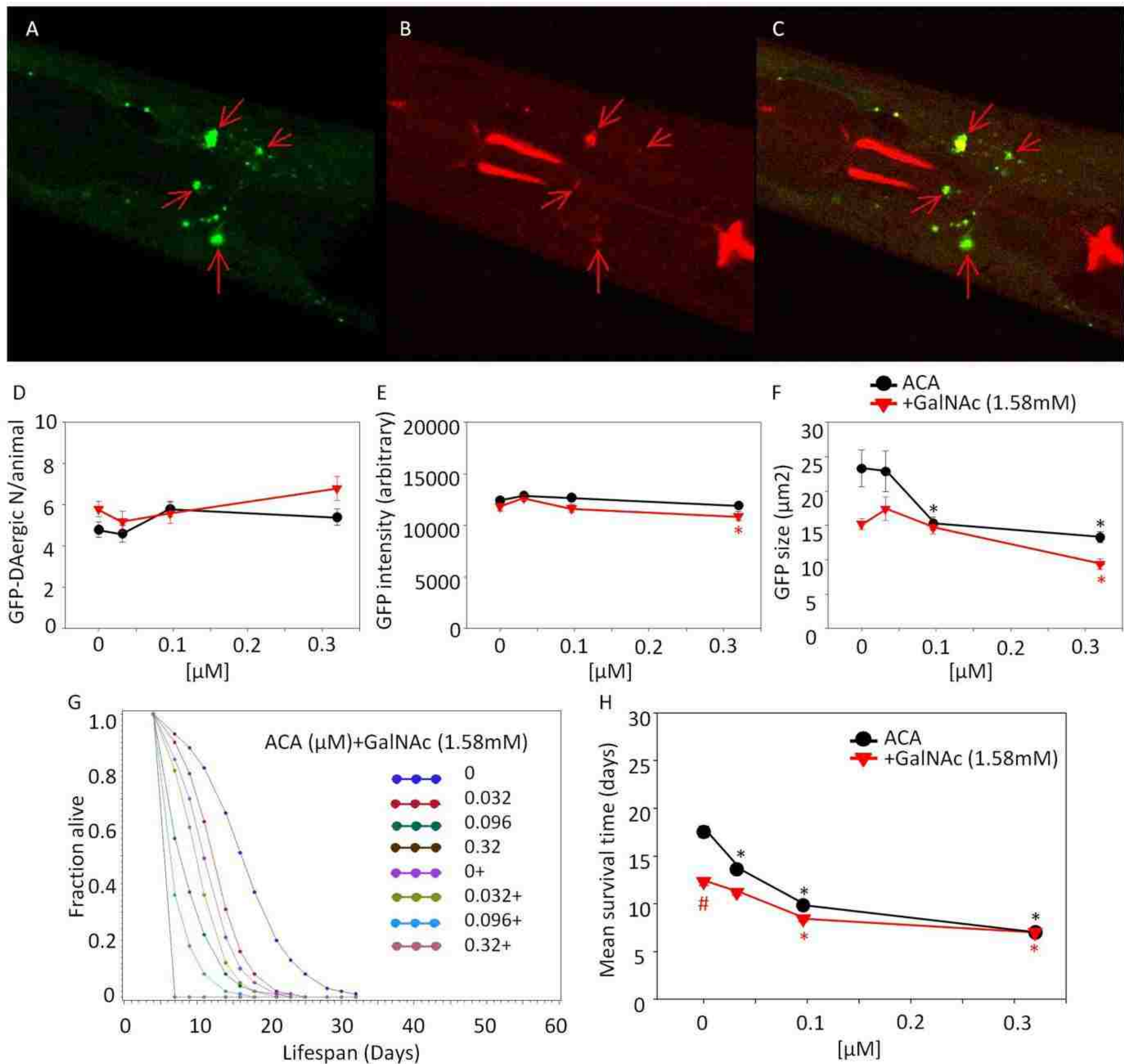

Figure 26. ACA co-localized with GFP-DAergic neurons in *C. elegans* at the highest dose (0.32 µM). A) GFP-DAergic neurons (green), B) PNA-TRITC in the neuron (red), C) Co-localization of the GFP-DAergic neurons in merged A and B (yellow). D) The number of DAergic neurons was not altered. E) The intensity of DAergic neurons was decreased by the highest dose (0.32 µM) plus the presence of the SIS (GalNAc 1.58 mM). F) The area of DAergic neurons was reduced by two higher doses (0.096 µM & 0.32 µM, P<0.05) and the highest dose (0.32 µM, P<0.05) when the SIS was present. G) The APS was reduced with or without the SIS. H) The mean lifespan was reduced by all doses (0.032 µM, 0.096 µM & 0.32 µM) from 18 days to 14, 10 and 7 days (-22%, -44% & -60%, P<0.05), or by the SIS only from 18 days to 12 days (-30%, P<0.05). With the presence of the SIS, the mean lifespan was reduced by higher doses (0.096 µM & 0.32 µM) from 12 days to 8 days and 7 days (-32% & -43%, P<0.05). * indicates statistical significance, # indicates SIS plus OP50 vs. OP50 only.

Area of DAergic neurons was decreased by higher doses of ACA (0.096 µM & 0.32 µM, P<0.05), and decreased by the highest dose of ACA plus GalNAc (0.32 µM, P<0.05, Figure 26f). APS was decreased dose-dependently with or without the presence of the SIS (Figure 26g). The mean lifespan was reduced by all doses (0.032 µM, 0.096 µM & 0.32 µM) from 18 days to 14, 10 and 7 days (-22%, -44% & -60%, P<0.05), or by the SIS only from 18 days to 12 days (-30%, P<0.05). With the presence of the SIS, the mean lifespan was reduced by higher doses (0.096 µM & 0.32 µM) from 12 days to 8 days and 7 days (-32% & -43%, P<0.05, Figure 26h).

Euonymus europaeus agglutinin (EEA)-TRITC co-localized with DAergic neurons at the lowest dose (0.048 µM), with or without the SIS lactose. As shown in the graph, when the green color from GFP fused DAergic neurons (Figure 27a) and the red color from TRITC conjugated EEA (Figure 27b) was overlapped, the co-localized area showed bright yellow color (Figure 27c), which demonstrated that EEA was successfully transported from gut to DAergic neurons in C. elegans organism. The number of DAergic neurons was reduced by the highest dose (0.48 µM, P<0.05), which was blocked by the SIS lactose (10 mM, Figure 27d). The intensity of DAergic neurons was reduced by the highest dose of EEA (0.48 µM, P<0.05) which was reversed by the SIS at the lowest dose (0.048 µM, P<0.05). The SIS did not block the effect of the highest dose of EEA in reducing the intensity of DAergic neurons (Figure 27e). Area of DAergic neurons was increased by highest dose (0.48 µM, P<0.05), the presence of the SIS reduced the magnitude of this effect. Medium dose of EEA plus the SIS reduced the size of DAergic neurons (0.136 µM, P<0.05, Figure 27f). APS was decreased dose-dependently with or without the SIS (Figure 27g). The mean lifespan was reduced dose-dependently by all doses (0.048 µM, 0.136 µM & 0.48 µM) from 19 days to 14, 9 and 7 days (-27%, -53% & -62%, P<0.05), or by the SIS only from 19 days to 11 days (-43%, P<0.05). With the presence of the SIS, the mean lifespan was reduced by higher doses (0.136 µM & 0.48 µM) from 11 days to 7 days and 7 days (-32% & -35%, P<0.05, Figure 27h).

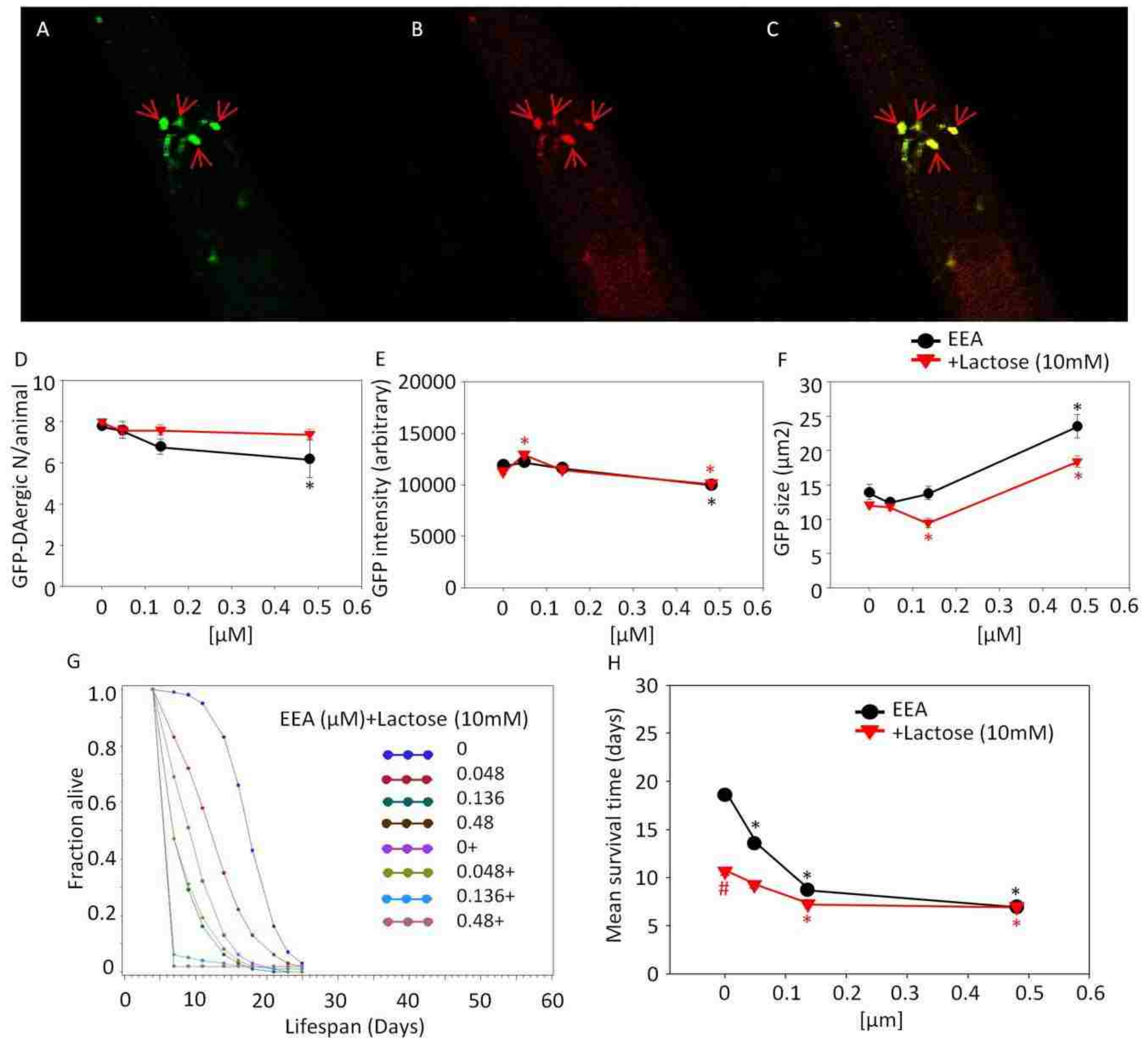

Figure 27. EEA co-localized with GFP-DAergic neurons in *C. elegans* at the lowest dose (0.048 µM). A) GFP-DAergic neurons (green), B) PNA-TRITC in the neuron (red), C) Co-localization of the GFP-DAergic neurons in merged A and B (yellow). D) The number of DAergic neurons was reduced by the highest dose (0.48 µM, P<0.05), which was blocked by the SIS lactose 10 mM. E) The intensity of DAergic neurons was reduced by the highest dose of EEA (0.48 µM, P<0.05) which was reversed by the SIS at the lowest dose (0.048 µM, P<0.05). F) Area of DAergic neurons was increased by highest dose of EEA (0.48 µM, P<0.05), the presence of the SIS reduced the magnitude of this effect. Middle dose of EEA plus the SIS reduced the size of DAergic neurons (0.136 µM, P<0.05). G) APS was decreased dose-dependently with or without the presence of the SIS. H) The mean lifespan was reduced dose-dependently by all doses (0.048 µM, 0.136 µM & 0.48 µM) from 19 days to 14, 9 and 7 days (-27%, -53% & -62%, P<0.05), or by the SIS only from 19 days to 11 days (-43%, P<0.05). With the presence of the SIS, the mean lifespan was reduced by higher doses (0.136 µM & 0.48 µM) from 11 days to 7 days and 7 days (-32% & -35%, P<0.05). * indicates statistical significance, # indicates SIS plus OP50 vs. OP50 only.

Arachis hypogaea agglutinin (PNA)-TRITC co-localized with GFP-GAergic neurons after one week of treatment (0.018 μM, 0.054 μM & 0.18 μM). As shown in the graph, when the green color from GFP fused DAergic neurons (Figure 28a) and the red color from TRITC conjugated EEA (Figure 28b) was overlapped, the co-localized area showed bright yellow color (Figure 28c), which demonstrated that PNA was successfully transported from gut to DAergic neurons in C. elegans organism. Number of GFP-DAergic neurons was increased at the lowest dose (0.018 μM, $P<0.05$), which was blocked by the SIS galactose 200 mM (Figure 28d). The size and intensity of GFP-GAergic neurons was not altered (Figure 28e & Figure 28f). SIS galactose (200 mM) treatment decreased the size of GFP-DAergic neurons (0.054 μM & 0.18 μM, $P<0.05$) without altering the number and intensity. APS was dose-dependently reduced at all doses, reversed by SIS, and was reduced by SIS galactose (200 mM) only (Figure 28g). The mean lifespan was reduced by higher doses (0.054 μM and 0.18 μM) from 19 days to 15 days and 14 days (-24% and -27%, $P<0.05$), which was reversed in presence of SIS galactose with an increase at the highest dose (0.18 μM) from 12 days to 16 days (33%, $P<0.05$). The mean lifespan was reduced by SIS only from 19 days to 12 days (-36%, $P<0.05$, Figure 28h).

Phaseolus vulgaris (PHA-E)-rhodamine did not show co-localization with DAergic neurons in liquid culture but affected the DAergic neurons. The number of GFP-DAergic neurons was not altered, but reduced at the highest dose with the SIS present (0.17 μM, $P<0.05$, Figure 29a). The fluorescence intensity of GFP-DAergic neurons was increased dose-dependently at all doses (0.017 μM, 0.054 μM & 0.17 μM, $P<0.05$), which was blocked by the SIS at the highest dose (0.17 μM, Figure 29b). The average size of GFP-DAergic neurons was also reduced at the highest dose (0.17 μM, $P<0.05$), which was augmented by the presence of the SIS (Figure 29c). The APS was increased dose-dependently at lower doses, which was blocked by SIS GalNAc (1.58 mM), and was reduced by GalNAc only (Figure 29d), possibly due to Uridine diphosphate (UDP) depletion [1].

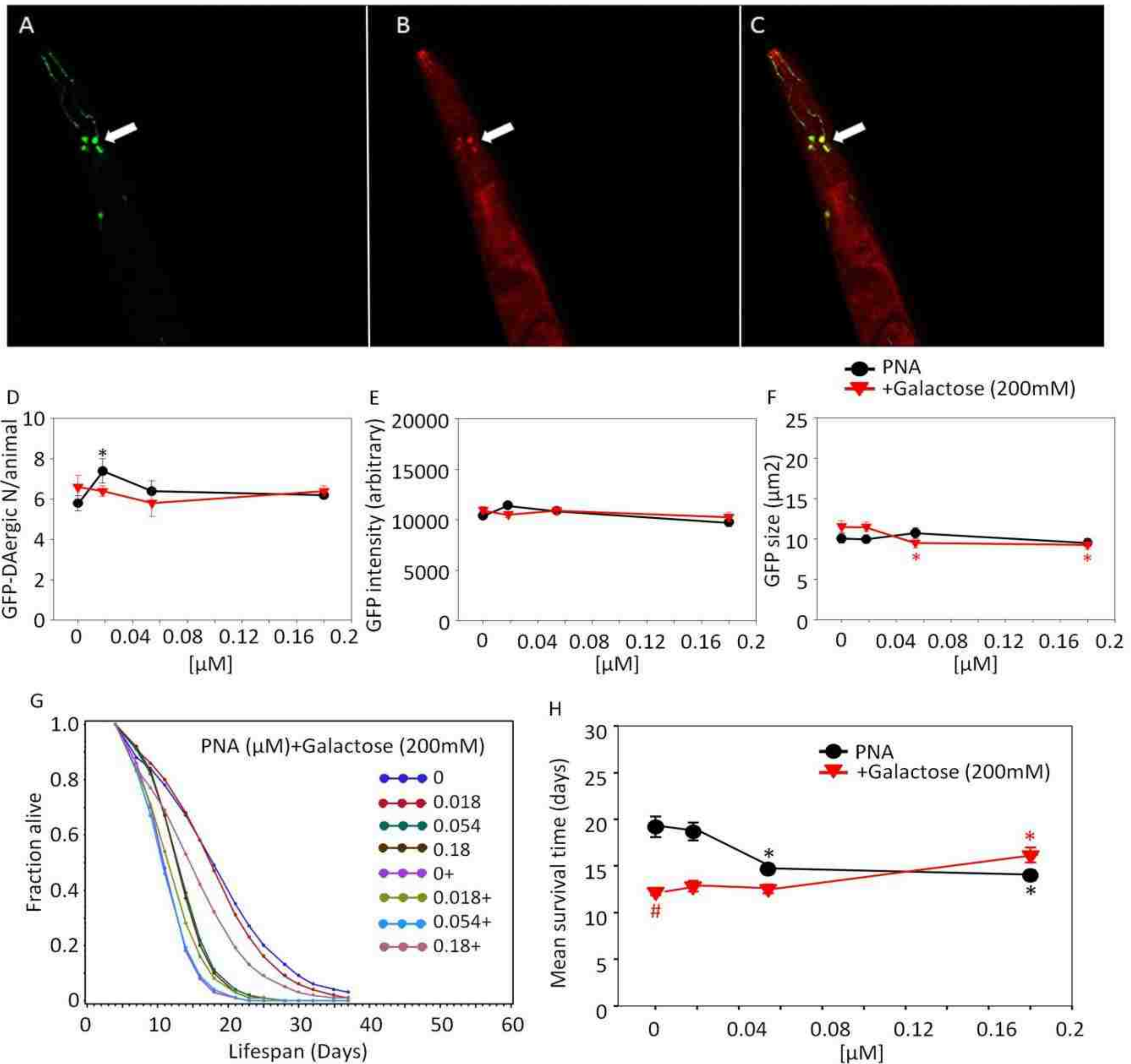

Figure 28. PNA co-localized with GFP-DAergic neurons in *C. elegans* at all doses (0.018 μM, 0.054 μM & 0.18 μM). A) GFP-DAergic neurons (green), B) PNA-TRITC in the neuron (red), C) Co-localization of the GFP-DAergic neurons in merged A and B (yellow). D) Number of GFP-DAergic neurons was increased at the lowest dose (0.018 μM, P<0.05), which was blocked by the SIS galactose 200 mM. E) The intensity of GFP-DAergic neurons was not altered. F) The size of GFP-DAergic neurons was not altered, but was reduced with the presence of the SIS at higher doses (0.054 μM & 0.18 μM, P<0.05). G) APS was dose-dependently reduced at all doses, reversed by SIS, and was reduced by SIS galactose (200 mM) only. H) The mean lifespan was reduced by higher doses (0.054 μM and 0.18 μM) from 19 days to 15 days and 14 days (-24% and -27%, P<0.05), which was reversed in presence of SIS galactose with an increase at the highest dose (0.18 μM) from 12 days to 16 days (33%, P<0.05). The mean lifespan was reduced by SIS galactose (200 mM) only from 19 days to 12 days (-36%, P<0.05). * indicates statistical significance, # indicates SIS plus OP50 vs. OP50 only.

The mean lifespan was increased at a middle dose of PHA-E (0.054 μM) from 17 days to 23 days (39%, P<0.05), which was blocked by GalNAc, and was reduced at a higher dose (0.17 μM) from 11 days to 8 days (-28%, P<0.05) in presence of SIS. GalNAc (1.58 mM) only reduced the mean lifespan from 17 days to 11 days (-34%, P<0.05, Figure 29e).

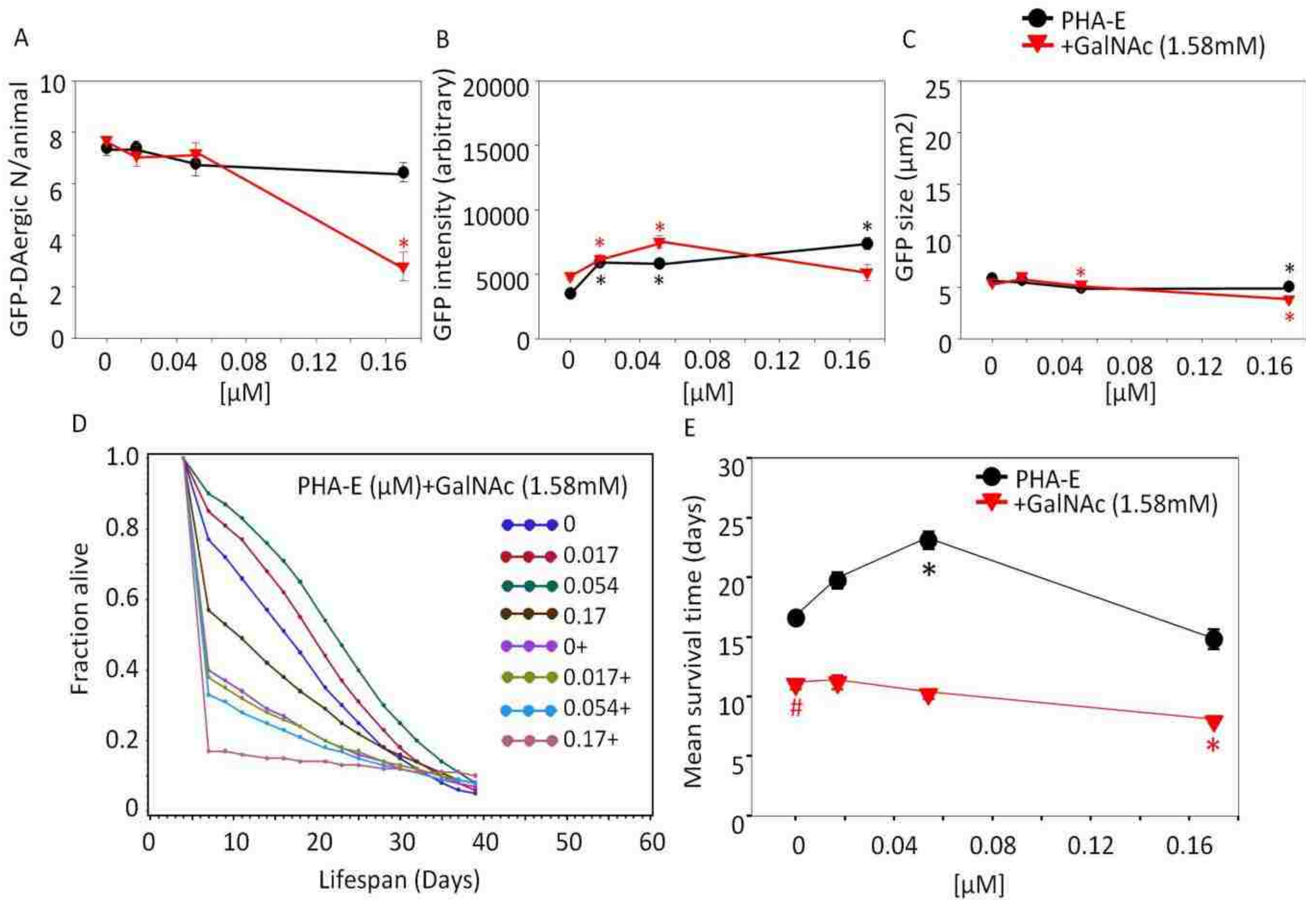

Figure 29. PHA-E-rhodamine did not show co-localization with DAergic neurons in liquid culture but affected the DAergic neurons. A) Number of DAergic neurons was reduced at the highest dose (0.17 μM) with the SIS present. B) The fluorescence intensity of GFP-DAergic neurons was increased dose-dependently at all doses (0.017 μM, 0.054 μM & 0.17 μM, P<0.05), which was blocked by the SIS at the highest dose (0.17 μM). C) The average size of GFP-DAergic neurons was also reduced at the highest dose (0.17 μM, P<0.05), which was augmented by the presence of the SIS. D) The APS was increased dose-dependently at lower doses, which was blocked by SIS GalNAc (1.58 mM), and was reduced by GalNAc only. E) The mean lifespan was increased at a middle dose (0.054 μM) from 17 days to 23 days (39%, P<0.05), which was blocked by GalNAc, and was reduced at a higher dose (0.17 μM) from 11 days to 8 days (-28%, P<0.05) in presence of SIS. GalNAc (1.58 mM) only reduced the mean lifespan from 17 days to 11 days (-34%, P<0.05). * indicates statistical significance, # indicates SIS plus OP50 vs. OP50 only.

Pisum Sativum agglutinin (PSA)-rhodamine did not show co-localization with GFP-DAergic neurons but affected the DAergic neurons. The number of DAergic neurons was not altered, but was reduced at the highest dose (0.43 μM, P<0.05) in presence of the SIS α-methylmannoside (200 mM) plus α-methylglucoside (200 mM, Figure 30a). The intensity was diminished at the highest dose of PSA (0.43 μM, P<0.05), which was eliminated in presence of the SIS (Figure 30b).

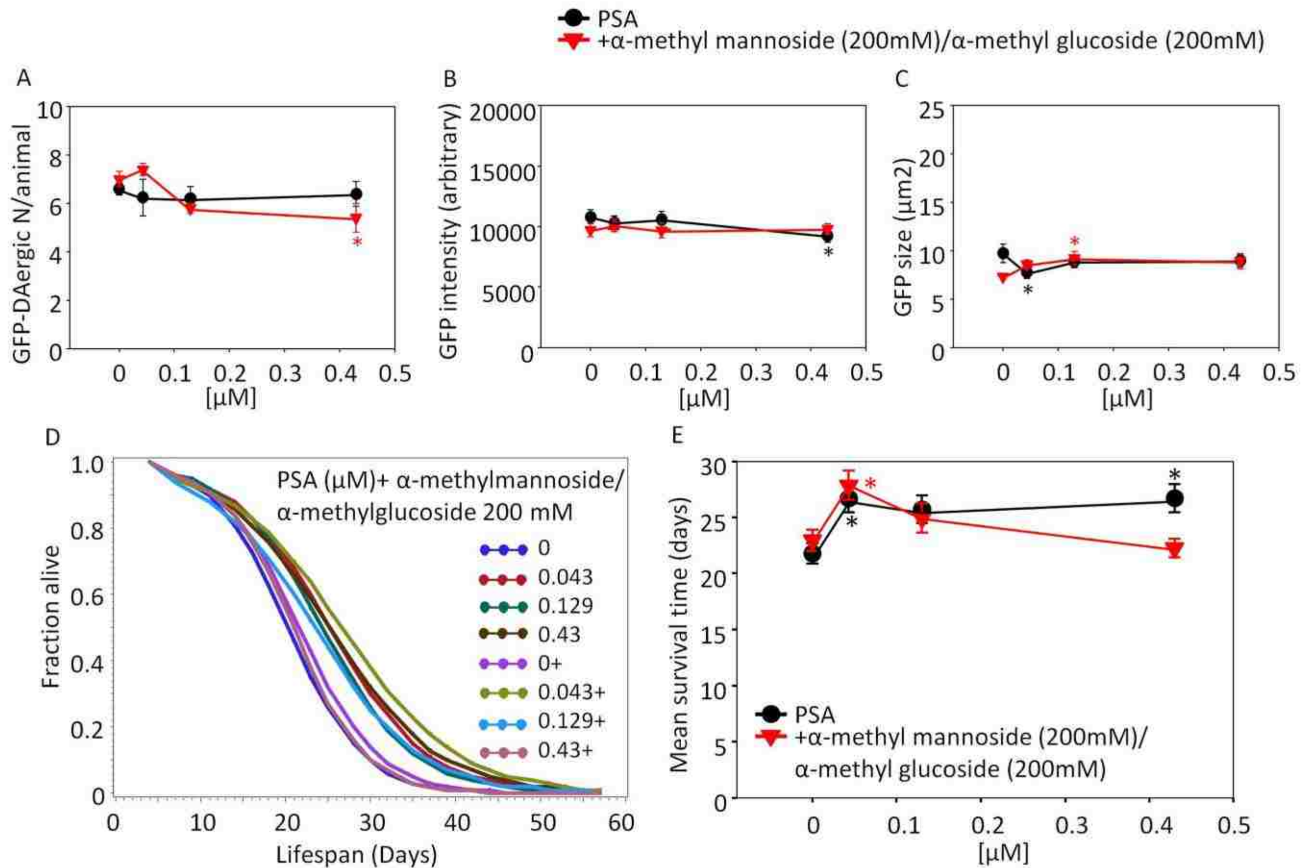

Figure 30. PSA-rhodamine did not show co-localization with GFP-DAergic neurons but affected the GFP-DAergic neurons in *C. elegans*. A) The number of DAergic neurons was not altered, but was reduced at the highest dose (0.43 μM, P<0.05) in presence of the SIS α-methylmannoside (200 mM) plus α-methylglucoside (200 mM). B) The intensity was diminished at the highest dose (0.43 μM, P<0.05), which was eliminated in presence of the SIS. C) The size was reduced at the lowest dose (0.043 μM, P<0.05), which was blocked by the SIS, and was increased it at middle dose (0.129 μM, P<0.05) with the SIS. D) The APS was increased at all doses, which was blocked at the highest dose in presence of the SIS. E) The mean lifespan was increased by lower doses (0.043 μM & 0.43 μM) from 22 days to 27 days (22% & 23%, P<0.05), which was blocked by SIS at the highest dose (0.43 μM), and was increased at a lower dose (0.043 μM) from 23 days to 28 days in presence of the SIS (21%, P<0.05). * indicates statistical significance.

The size was reduced at the lowest dose (0.043 μM, P<0.05), which was blocked by the SIS, and was increased it at medium dose (0.129 μM, P<0.05) with the SIS (Figure 30c). The APS was increased at all doses of PSA, which was blocked at the highest dose in presence of the SIS (Figure 30d). The mean lifespan was increased by lower doses (0.043 μM & 0.43 μM) from 22 days to 27 days (22% and 23%, P<0.05), which was blocked by SIS at the highest dose (0.43 μM), and was increased at a lower dose (0.043 μM) from 23 days to 28 days in presence of the SIS (21%, P<0.05, Figure 30e).

Ulex Europaeus I (UEA I)-TRITC did not show co-localization with GFP-DAergic neurons but affected the DAergic neurons. The number of DAergic neurons was not altered, but was increased at the highest dose (0.33 μM, P<0.05) in presence of the SIS L-fucose (50 mM, Figure 31a). The intensity was diminished at all doses (0.033, 0.099 & 0.33 μM, P<0.05), which was eliminated in presence of the SIS (Figure 31b). The size was reduced at all doses (0.033, 0.099 & 0.33 μM, P<0.05), which was blocked by the SIS, and was increased at the highest dose (0.33 μM, P<0.05) in presence of the SIS (Figure 31c). The APS was increased at the lowest dose, which was blocked by the SIS (Figure 31d). The mean lifespan was increased by the lowest dose (0.033 μM) from 22 days to 25 days (13%, P<0.05), which was blocked by SIS, and was reduced at the highest dose (0.99 μM) from 21 days to 16 days in presence of the SIS (26%, P<0.05, Figure 31e).

Triticum vulgaris agglutinin (WGA)-rhodamine was not detected as being transported to the DAergic neurons but affected the DAergic neurons. The number of DAergic neurons was not altered (Figure 32a). The intensity of the GFP-DAergic neurons was increased at the highest dose (0.46 μM, P<0.05), which was augmented in presence of the SIS (Figure 32b). The area of the DAergic neurons was reduced at all doses (P<0.05), which was blocked by the SIS (Figure 32c). The APS was increased at all doses, which was blocked by SIS (Figure 32d). The mean lifespan was increased at the highest dose (0.46 μM) from 20 days to 24 days (22%, P<0.05), which was blocked by the SIS (Figure 32e).

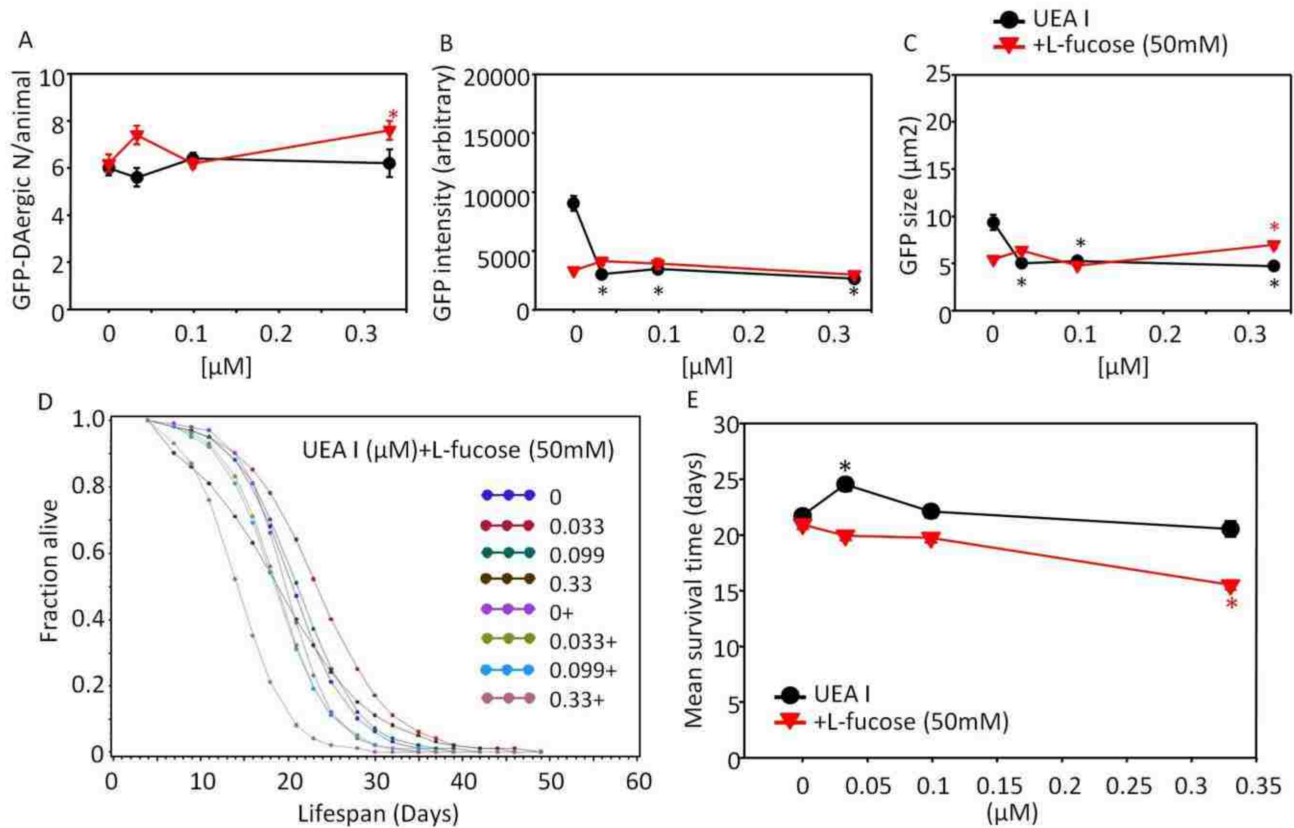

Figure 31. UEA I-TRITC did not show co-localization with GFP-DAergic neurons but affected the DAergic neurons. A) The number of DAergic neurons was not altered, but was increased at the highest dose (0.33 μM, P<0.05) in presence of the SIS L-fucose (50 mM). B) The intensity was diminished at all doses (0.033, 0.099 & 0.33 μM, P<0.05), which was eliminated in presence of the SIS. C) The size was reduced at all doses (0.033, 0.099 & 0.33 μM, P<0.05), which was blocked by the SIS, and was increased at the highest dose (0.33 μM, P<0.05) in presence of the SIS. D) The APS was increased at the low dose, which was blocked in presence of the SIS. E) The mean lifespan was increased by low dose (0.033 μM) from 22 days to 25 days (13%, P<0.05), which was blocked by SIS, and was reduced at the highest dose (0.99 μM) from 21 days to 16 days in presence of the SIS (26%, P<0.05). * indicates statistical significance.

Lens culinaris (LcH)-TRITC was not detected as transported to but affected DAergic neurons. The number of DAergic neurons was increased at low and high doses (0.05 μM & 0.5 μM, P<0.05), which was blocked at low dose and reversed at high does (0.5 μM, P<0.05, Figure 33a). The intensity of the GFP-DAergic neurons was increased at the lowest dose (0.05 μM, P<0.05), which was reversed in the presence of the SIS. Besides, the intensity was reduced at higher doses (0.05 μM & 0.15 μM, P<0.05) in presence of the SIS (Figure 33b). The area of the DAergic neurons was not altered (P>0.05), which was

reduced by the highest dose (0.5 μM, P<0.05) in presence of the SIS (Figure 33c). The APS was increased at the lowest dose and reduced at the highest dose, which was enhanced by the SIS (Figure 33d). The mean lifespan was increased at the lowest dose (0.05 μM) from 18 days to 23 days (27%, P<0.05) which was blocked by the SIS, and decreased at the highest dose (0.5 μM) to 12 days (-37%, P<0.05) which was enhanced in presence of the SIS. The mean lifespan was increased by the SIS only from 18 days to 27 days (45%, P<0.05, Figure 33e).

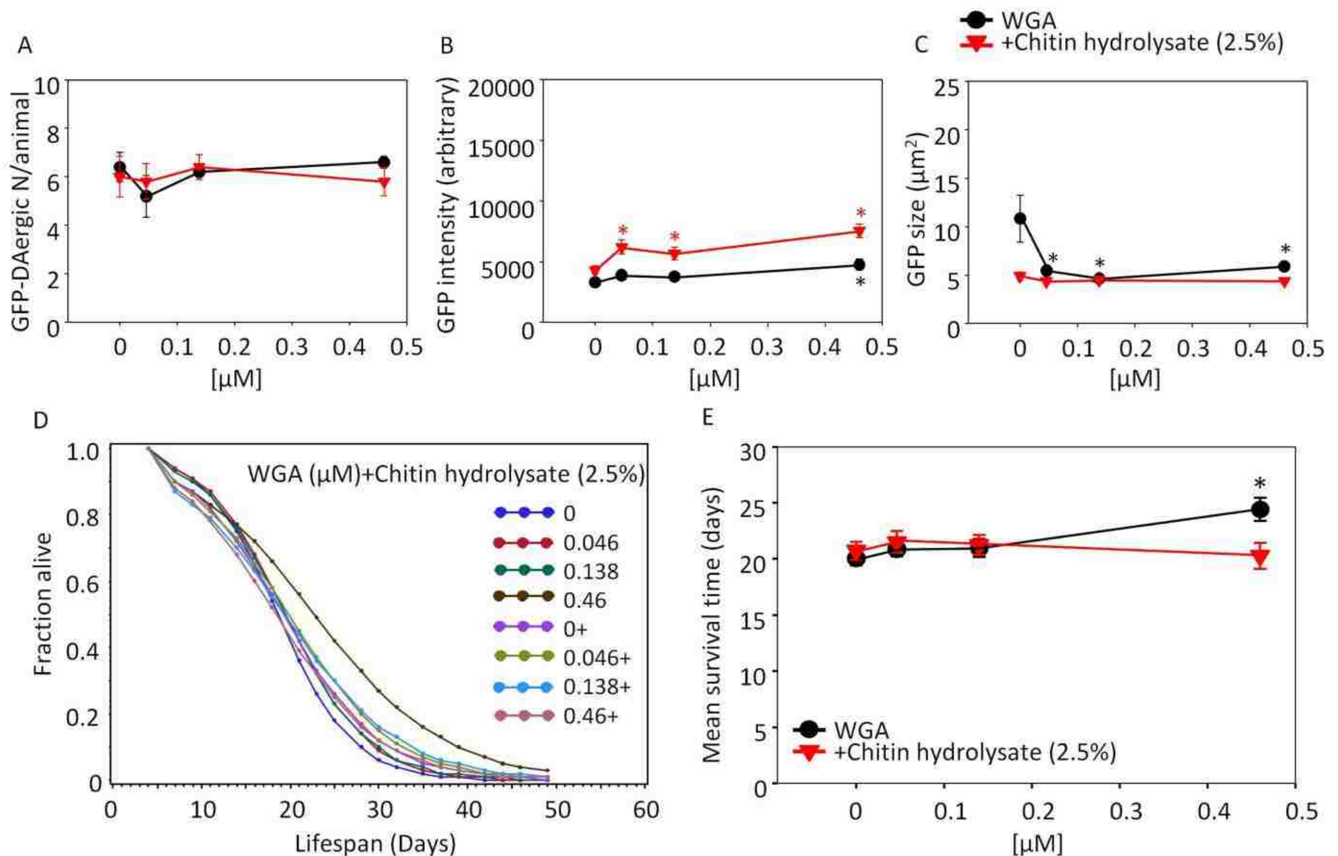

Figure 32. WGA-rhodamine affected the intensity and area of DAergic neurons. A) The number of DAergic neurons was not altered by WGA with or without the SIS chitin hydrolysate (2.5%). B) The intensity of the GFP-DAergic neurons was increased at the highest dose (0.46 μM, P<0.05), which was augmented in the presence of the SIS. C) The area of the DAergic neurons was reduced at all doses (P<0.05), which was blocked by the SIS. D) The APS was increased at all doses, which was blocked by the SIS. E) The mean lifespan was increased at the highest dose (0.46 μM) from 20 days to 24 days (22%, P<0.05), which was blocked by the SIS. * indicates statistical significance.

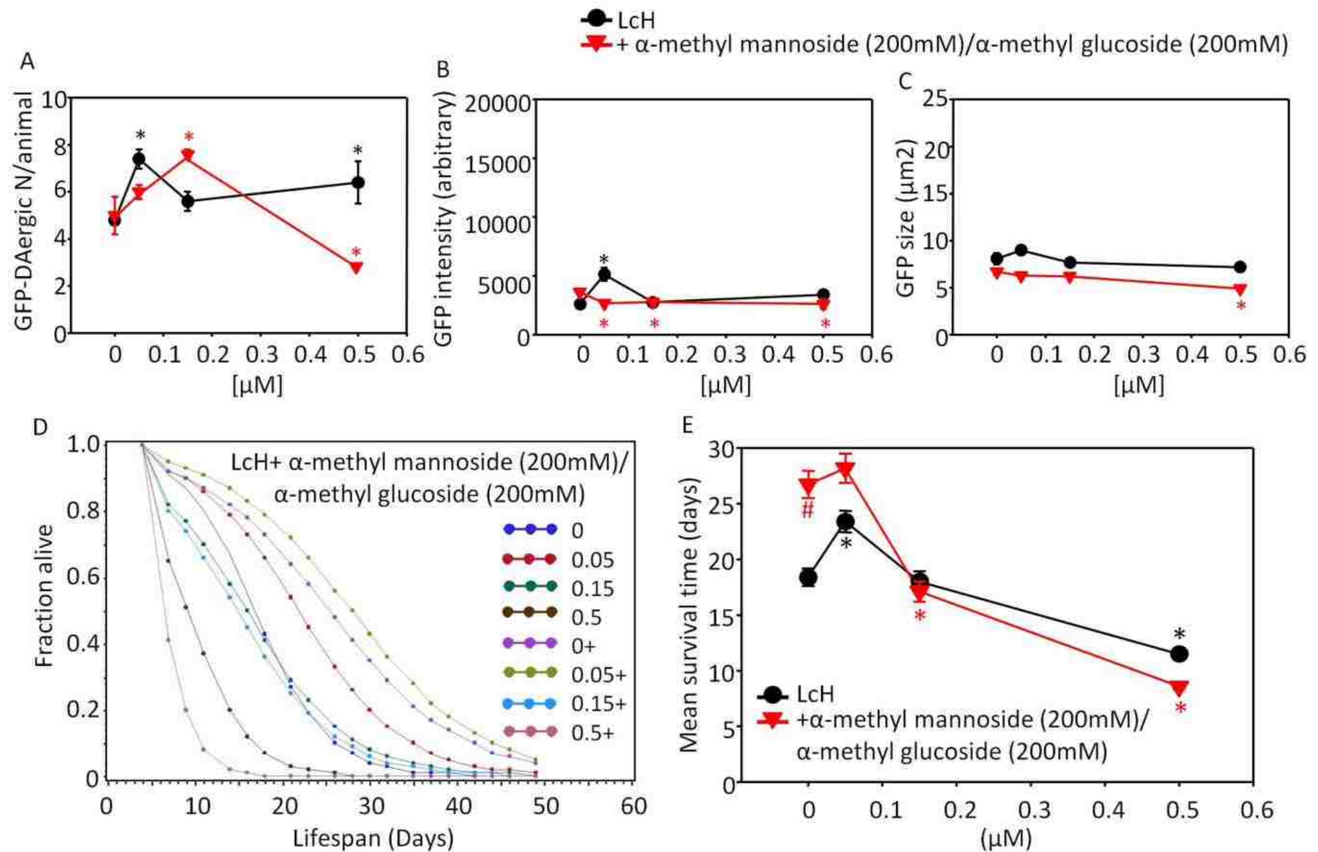

Figure 33. LcH-TRITC was not detected as transported to but affected DAergic neurons. A) The number of DAergic neurons was increased at low and high doses (0.05 μM & 0.5 μM, P<0.05), which was blocked at low dose and reversed at high does (0.5 μM, P<0.05). B) The intensity of the GFP-DAergic neurons was increased at the lowest dose (0.05 μM, P<0.05), which was reversed in the presence of the SIS. Besides, the intensity was reduced at higher doses (0.05 μM & 0.15 μM, P<0.05) in presence of the SIS. C) The area of the DAergic neurons was not altered (P>0.05), which was reduced by the highest dose (0.5 μM, P<0.05) in presence of the SIS. D) The APS was increased at the lowest dose and reduced at the highest dose, which was enhanced by the SIS. E) The mean lifespan was increased at the lowest dose (0.05 μM) from 18 days to 23 days (27%, P<0.05) which was blocked by the SIS, and decreased at the highest dose (0.5 μM) to 12 days (37%, P<0.05) which was enhanced in presence of the SIS. The mean lifespan was increased by the SIS only from 18 days to 27 days (45%, P<0.05). * indicates statistical significance, # indicates SIS plus OP50 vs. OP50 only.

Triticum vulgaris (Succinylated) S-WGA-rhodamine did not show co-localization but affected DAergic neurons in *C. elegans*. The number of the GFP-DAergic neurons was decreased at lower doses (0.046 μM & 0.138 μM, P<0.05), which was blocked by the SIS chitin hydrolysate (2.5%, Figure 34a). The fluorescent intensity of GFP-DAergic neurons was increased at all doses (0.046 μM, 0.138 μM & 0.46 μM, P<0.05), which was blocked by the SIS at medium dose (0.138 μM, P>0.05) and reversed by

the highest dose (0.46 μM, P<0.05, Figure 34b). The size of GFP-DAergic neurons was decreased at all doses (0.046 μM, 0.138 μM & 0.46 μM, P<0.05), which was blocked by the SIS (Figure 34c).

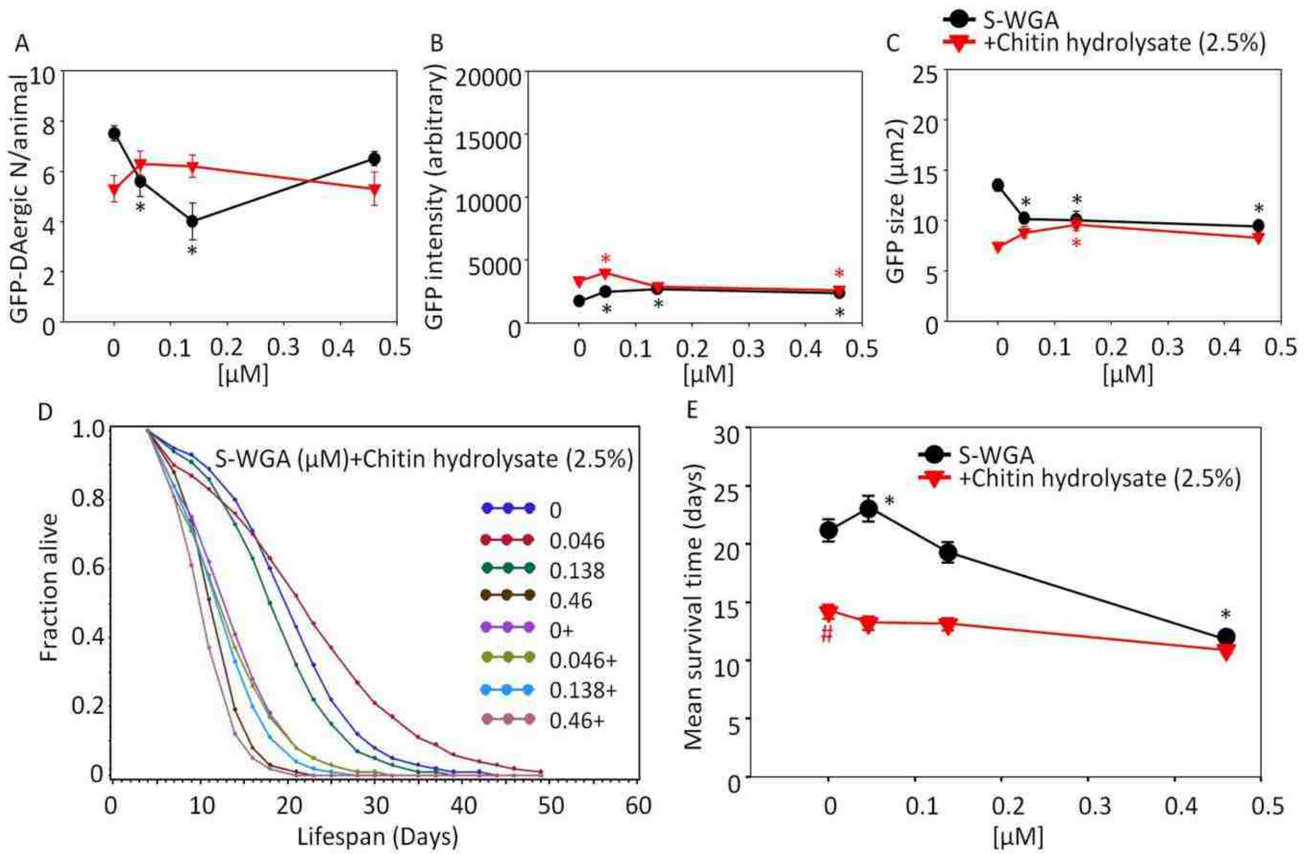

Figure 34. S-WGA-rhodamine affected GFP-DAergic neurons in *C. elegans*. A) The number of the GFP-DAergic neurons was decreased at lower doses (0.046 μM & 0.138 μM, P<0.05), which was blocked by the SIS chitin hydrolysate (2.5%). B) The fluorescent intensity of GFP-DAergic neurons was increased at all doses (0.046 μM, 0.138 μM & 0.46 μM, P<0.05), which was blocked by the SIS at medium dose (0.138 μM, P>0.05) and reversed by the highest dose (0.46 μM, P<0.05). C) The size of GFP-DAergic neurons was decreased at all doses (0.046 μM, 0.138 μM & 0.46 μM, P<0.05), which was blocked by the SIS. D) The APS was increased at a low dose, and decreased does-dependently at higher doses, which was blocked by SIS. E) The mean lifespan was increased at the lowest dose (0.138 μM) from 21 days to 23 days (9%, P<0.05), and decreased at the highest dose (0.46 μM) to 12 days (-43%, P<0.05), which was blocked by the SIS at all doses. The mean lifespan was reduced by the SIS only from 21 days to 14 days (-33%, P<0.05). * indicates statistical significance, # indicates SIS plus OP50 vs. OP50 only.

The APS was increased at a low dose, and decreased at higher doses, which was blocked by SIS (Figure 34d). The mean lifespan was increased at the lowest dose (0.138 μM) from 21 days to 23 days (9%, P<0.05), and decreased at the highest dose (0.46 μM) to 12 days (-43%, P<0.05), which was blocked

by the SIS at all doses. The mean lifespan was reduced by the SIS only from 21 days to 14 days (-33%, P<0.05, Figure 34e).

Artocarpus integrifolia agglutinin (AIA)-TRITC did not show co-localization and did not affect DAergic neurons in *C. elegans*. The number of the GFP-DAergic neurons was not altered by AIA, with or without the SIS galactose (32 mM, P>0.05, Figure 35a).

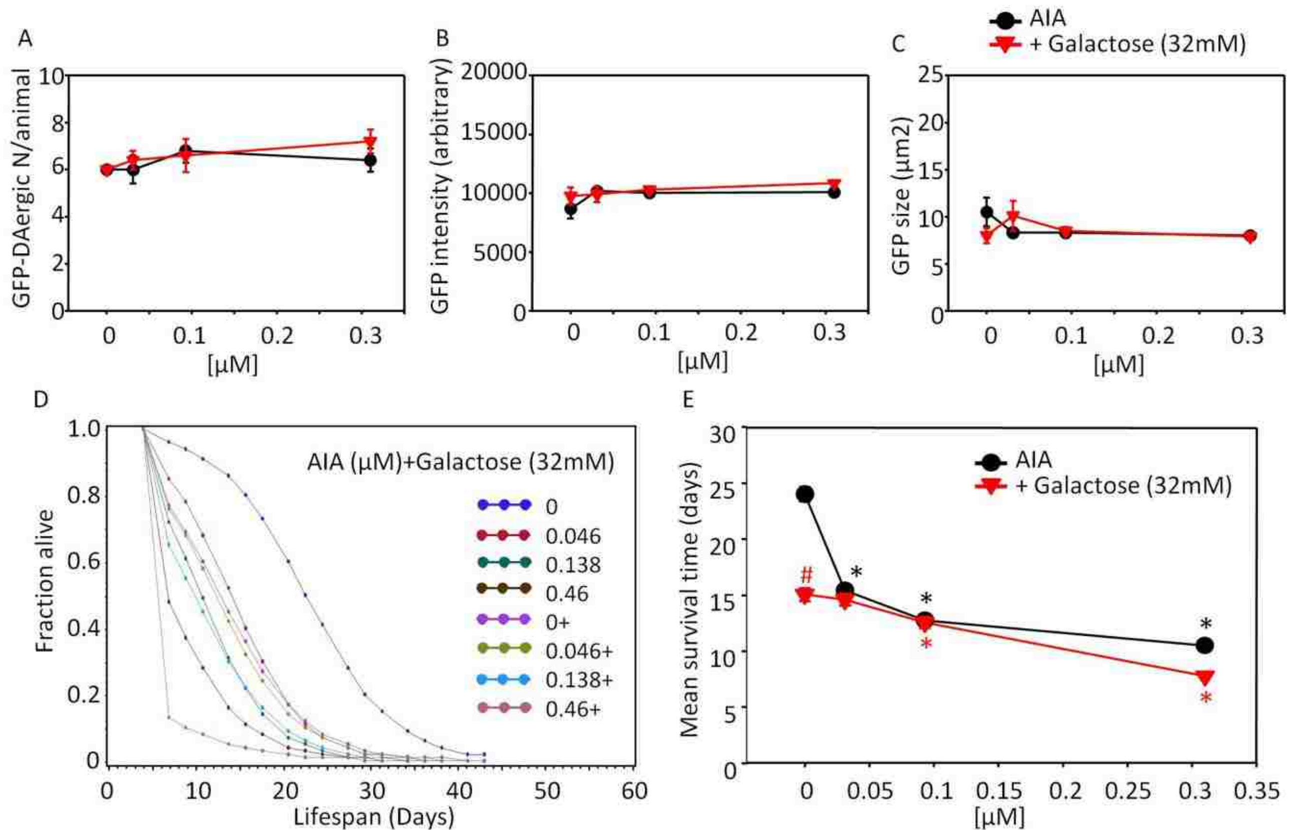

Figure 35. AIA-TRITC did not show co-localization and did not affect DAergic neurons in *C. elegans*. A) The number of the GFP-DAergic neurons was not altered by AIA, with or without the SIS galactose (32 mM, P>0.05). B) The fluorescent intensity of GFP-DAergic neurons was not altered by AIA (P>0.05). C) The size of GFP-DAergic neurons was not altered by AIA (P>0.05). D) The APS was decreased at all doses, which was enhanced by the SIS. E) The mean lifespan was increased at all doses (0.046 μM, 0.138 μM & 0.46 μM) from 24 days to 15 days, 13 days and 11 days (-36%, -47% & -56%, P<0.05), which was blocked at the lowest dose (0.046 μM) in presence of the SIS. The mean lifespan was reduced by the SIS only from 24 days to 15 days (-37%, P<0.05). * indicates statistical significance, # indicates SIS plus OP50 vs. OP50 only.

The fluorescent intensity of GFP-DAergic neurons was not altered by AIA (P>0.05, Figure 35b). The size of GFP-DAergic neurons was not altered by AIA (P>0.05, Figure 35c). The APS was decreased

at all doses, which was enhanced by the SIS (Figure 35d). The mean lifespan was increased at all doses (0.046 μM, 0.138 μM & 0.46 μM) from 24 days to 15 days, 13 days and 11 days (-36%, -47% & -56%, P<0.05), which was blocked at the lowest dose (0.046 μM) in presence of the SIS. The mean lifespan was reduced by the SIS only from 24 days to 15 days (-37%, P<0.05, Figure 35e).

Cytisus scoparius agglutinin (CSA)-TRITC did not show co-localization but affected DAergic neurons in *C. elegans*. The number of the GFP-DAergic neurons was reduced at the highest dose (0.65 μM, P<0.05), which was blocked by the SIS GalNAc 20 mM (Figure 36a).

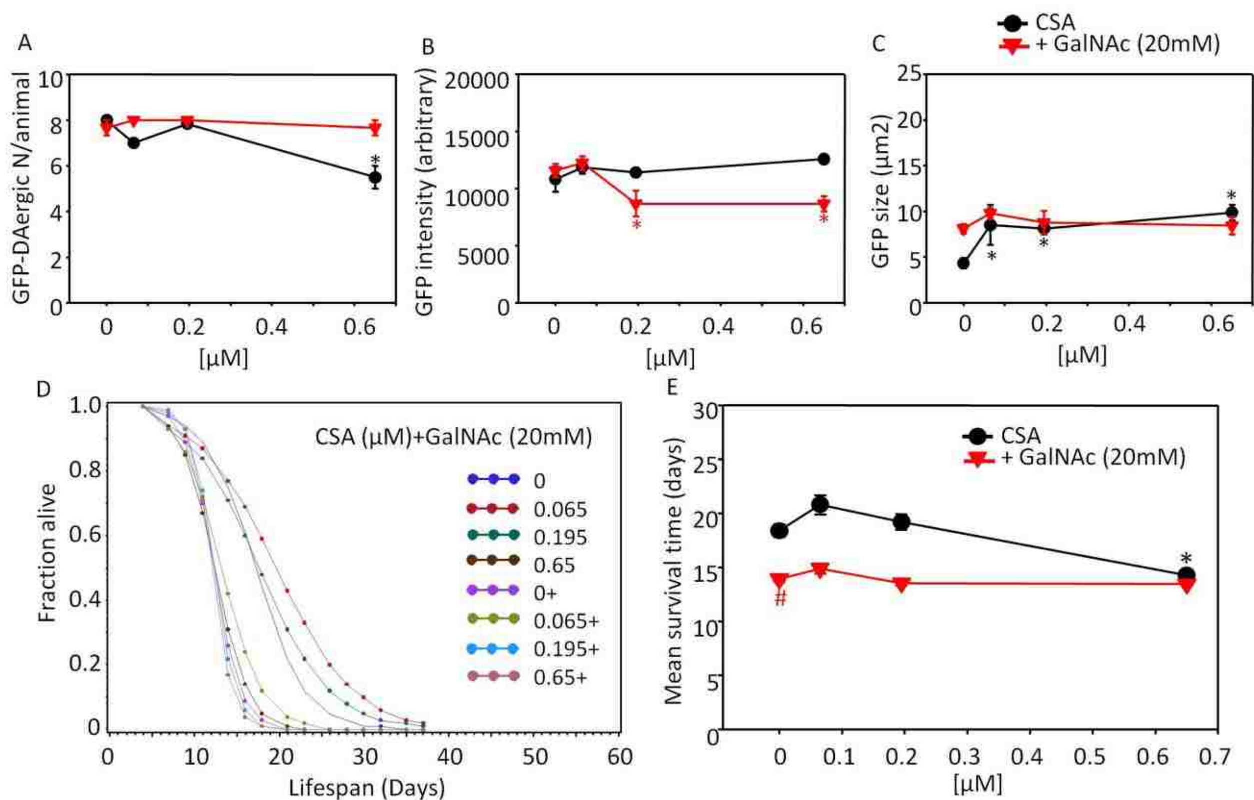

Figure 36. CSA-TRITC did not show co-localization but affected DAergic neurons in *C. elegans*. A) The number of the GFP-DAergic neurons was reduced at the highest dose (0.65 μM, P<0.05), which was blocked by the SIS GalNAc 20 mM. B) The fluorescent intensity of GFP-DAergic neurons was not altered at all doses (P>0.05), which was reduced at high doses (0.195 μM & 0.65 μM, P<0.05) in presence of the SIS. C) The size of GFP-DAergic neurons was increased at all doses (0.065 μM, 0.195 μM & 0.65 μM, P<0.05), which was blocked in presence of the SIS. D) The APS was decreased at the highest dose, which was blocked by the SIS. E) The mean lifespan was reduced at the highest dose (0.65 μM) from 18 days to 14 days (-22%, P<0.05), which was blocked in presence of the SIS. The mean lifespan was reduced by the SIS only from 18 days to 14 days (-24%, P<0.05). * indicates statistical significance, # indicates SIS plus OP50 vs. OP50 only.

The fluorescent intensity of GFP-DAergic neurons was not altered at all doses ($P>0.05$), which was reduced at high doses (0.195 μM & 0.65 μM, $P<0.05$) in presence of the SIS (Figure 36b). The size of GFP-DAergic neurons was increased at all doses (0.065 μM, 0.195 μM & 0.65 μM, $P<0.05$), which was blocked in presence of the SIS (Figure 36c). The APS was decreased at the highest dose, which was blocked by the SIS (Figure 36d). The mean lifespan was reduced at the highest dose (0.65 μM) from 18 days to 14 days (-22%, $P<0.05$), which was blocked in presence of the SIS. The mean lifespan was reduced by the SIS only from 18 days to 14 days (-24%, $P<0.05$, Figure 36e).

Dolichos biflorus agglutinin (DBA)-rhodamine did not show co-localization with GFP-DAergic neurons but affected the DAergic neurons. The number of DAergic neurons was not altered, but was increased at the highest dose (0.18 μM, $P<0.05$) in presence of the SIS GalNAc (50 mM, Figure 37a). The fluorescence intensity of GFP-DAergic neurons was increased at the lowest dose (0.018 μM, $P<0.05$), which was reversed by the SIS, and was increased at higher doses (0.054 μM & 0.18 μM, $P<0.05$) in presence of the SIS (Figure 37b). The size of GFP-DAergic neurons was increased in a dose-dependent trend at all doses (0.018 μM, 0.054 μM & 0.18 μM, $P<0.05$), which was blocked by the SIS (Figure 37c). The APS was increased by lower doses of DBA, and decreased by higher doses (Figure 37d). The mean lifespan was reduced at the highest dose (0.18 μM) from 19 days to 11 days (-43%, $P<0.05$). The animals did not survive in presence of GalNAc (50 mM) within two days, possibly because of UDP depletion (Figure 37e).

Galanthus nivalis agglutinin (GNA)-rhodamine did not show co-localization with GFP-DAergic neurons or affect the DAergic neurons. The number of DAergic neurons was not altered with or without the SIS mannose (34 mM, Figure 38a). The fluorescence intensity of GFP-DAergic neurons was not altered at all doses, which was reduced at the medium dose (0.231 μM, $P<0.05$) in presence of the SIS (Figure 38b).

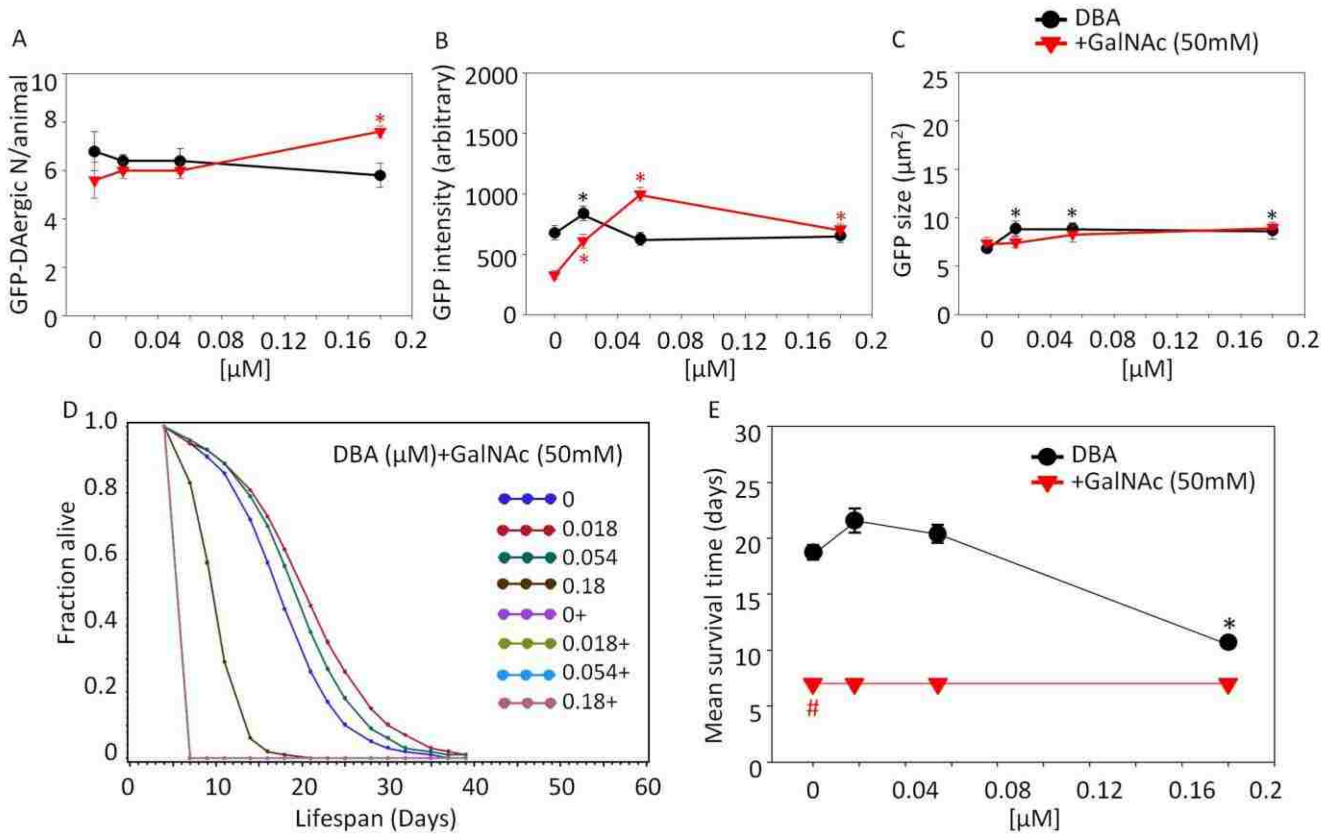

Figure 37. DBA-TRITC did show co-localization but affected the DAergic neurons in *C. elegans.* A) The number of DAergic neurons was not altered, but was increased at the highest dose (0.18 μM, P<0.05) in presence of the SIS GalNAc (50 mM). B) The fluorescence intensity of GFP-DAergic neurons was increased at the lowest dose (0.018 μM, P<0.05), which was reversed by the SIS, and was increased at higher doses (0.054 μM & 0.18 μM, P<0.05) in presence of the SIS. C) The size of GFP-DAergic neurons was increased in a dose-dependent trend at all doses (0.018 μM, 0.054 μM & 0.18 μM, P<0.05), which was blocked by the SIS. D) The APS was increased at lower dose, and decreased at higher doses. E) The mean lifespan was reduced at the highest dose (0.18 μM) from 19 days to 11 days (-43%, P<0.05). The animals did not survive in presence of GalNAc (50 mM) within two days. * indicates statistical significance, # indicates SIS plus OP50 vs. OP50 only.

The size of GFP-DAergic neurons was increased at all doses (0.077 μM, 0.231 μM & 0.77 μM, P<0.05), which was blocked by the SIS (Figure 38c). The APS was reduced at all doses, which was enhanced by the SIS (Figure 38d). The mean lifespan was reduced at higher doses (0.077 μM & 0.231 μM) from 24 days to 20 days and 19 days (-16% & -23%, P<0.05), which was enhanced in presence of the SIS (Figure 38e).

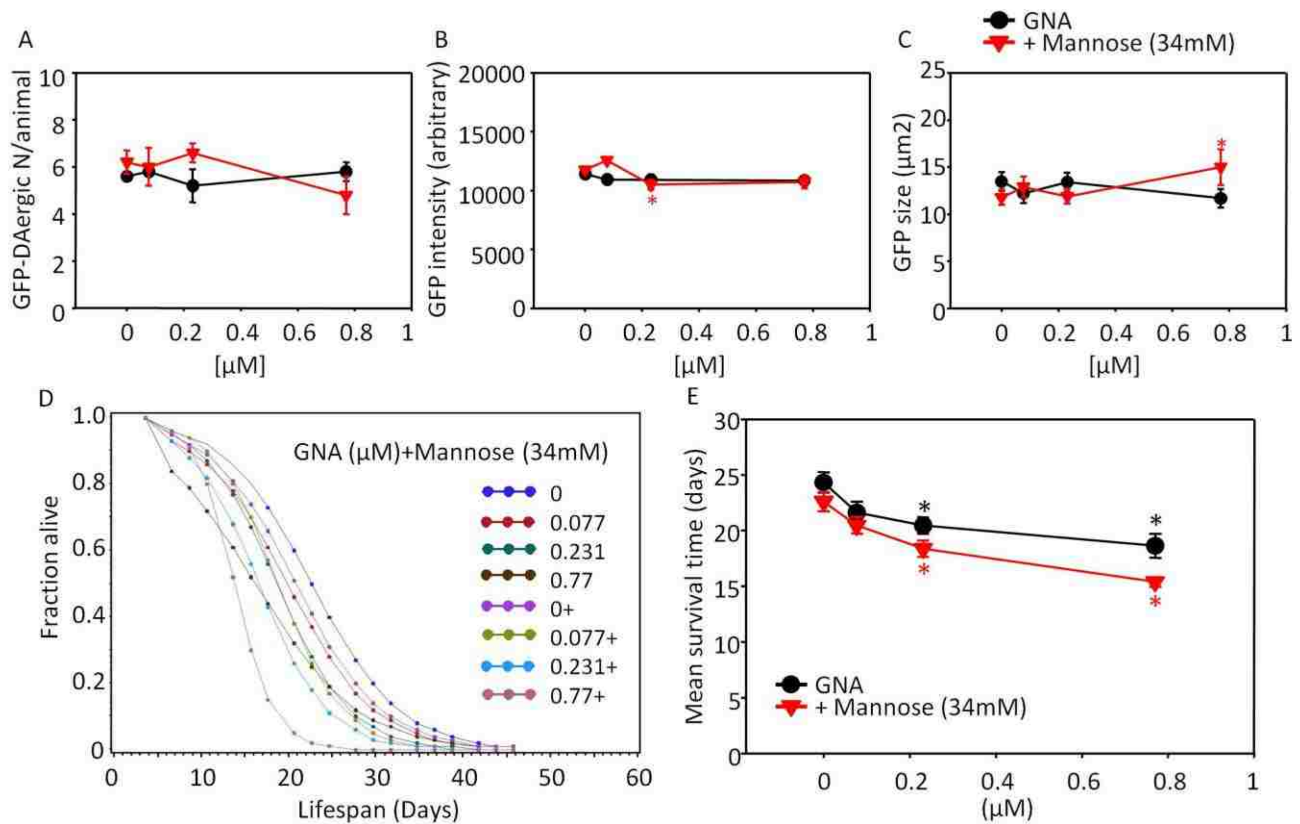

Figure 38. *Galanthus nivalis agglutinin* (GNA)-rhodamine did not show co-localization with GFP-DAergic neurons or affect the DAergic neurons. A) The number of DAergic neurons was not altered with or without the SIS mannose (34 Mm). B) The fluorescence intensity of GFP-DAergic neurons was not altered at all doses, which was reduced at the medium dose (0.231 µM, P<0.05) in presence of the SIS. C) The size of GFP-DAergic neurons was increased at all doses (0.077 µM, 0.231 µM & 0.77 µM, P<0.05), which was blocked by the SIS. D) The APS was reduced at all doses, which was enhanced by the SIS. E) The mean lifespan was reduced at higher doses (0.077 µM & 0.231 µM) from 24 days to 20 days and 19 days (-16% & -23%, P<0.05), which was enhanced in presence of the SIS. * indicates statistical significance. * indicates statistical significance.

Griffonia Simplicifolia (GSL I)-TRITC did show co-localization but affected the DAergic neurons in *C. elegans*. The number of the GFP-DAergic neurons was not altered (Figure 39a). The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was dose-dependently increased at all doses (0.018 µM, 0.054 µM & 0.18 µM, P<0.05), which was augmented at lower doses (0.018 µM & 0.054 µM, P<0.05) and mitigated at the highest dose (0.18 µM, P<0.05, Figure 39b) by the SIS galactose (40 mM). The size of GFP-DAergic neurons was not altered, but was dose-dependently increased at all doses (0.018 µM, 0.054 µM & 0.18 µM, P<0.05) in presence of the SIS (Figure 39c). The APS was dose-

dependently decreased at all doses, which was reversed by SIS (Figure 39d). The mean lifespan was decreased at the highest dose (0.18 μM) from 17 days to 10 days (-37%, P<0.05), which was blocked by the SIS (Figure 39e).

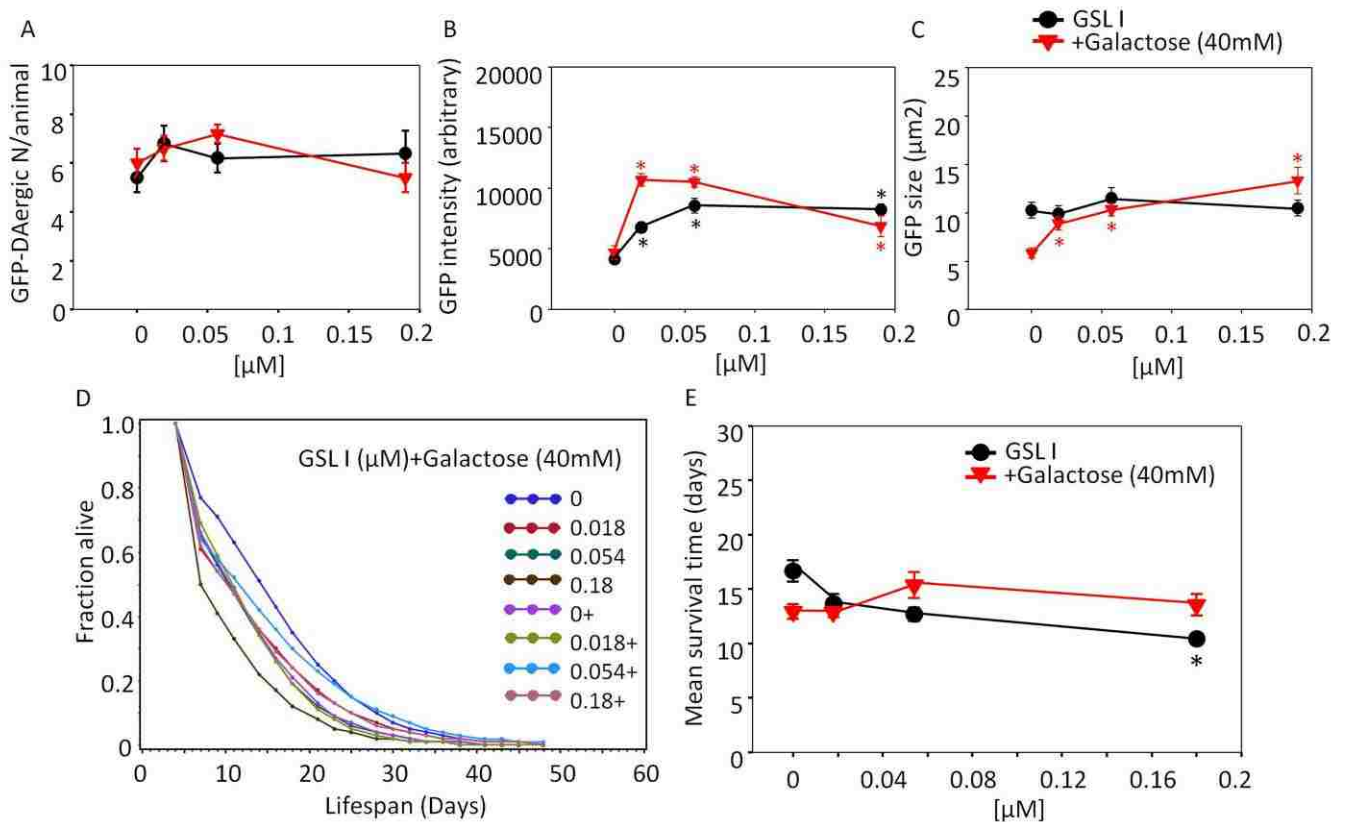

Figure 39. GSL I-TRITC did show co-localization but affected the DAergic neurons in *C. elegans.* A) The number of the GFP-DAergic neurons was not altered. B) The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was dose-dependently increased at all doses (0.018 μM, 0.054 μM & 0.18 μM, P<0.05), which was augmented at lower doses (0.018 μM & 0.054 μM, P<0.05) and mitigated at the highest dose (0.18 μM, P<0.05) by the SIS galactose (40 mM). C) The size of GFP-DAergic neurons was not altered, but was dose-dependently increased at all doses (0.018 μM, 0.054 μM & 0.18 μM, P<0.05) in presence of the SIS. D) The APS was dose-dependently decreased at all doses, which was blocked by SIS. E) The mean lifespan was decreased at the highest dose (0.18 μM) from 17 days to 10 days (-37%, P<0.05), which was blocked by the SIS. *indicates statistical significance.

Hippeastrum hybrid agglutinin (HHA)-TRITC did show co-localization but affected the DAergic neurons in *C. elegans*. The number of the GFP-DAergic neurons was not altered with or without the SIS mannose (10 mM, Figure 40a). The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was increased at all doses (0.04 μM, 0.12 μM & 0.4 μM, P<0.05), which was blocked

at lower doses (0.04 μM & 0.12 μM, P>0.05) and reversed at the highest dose (0.2 μM, P<0.05) in presence of the SIS (Figure 40b).

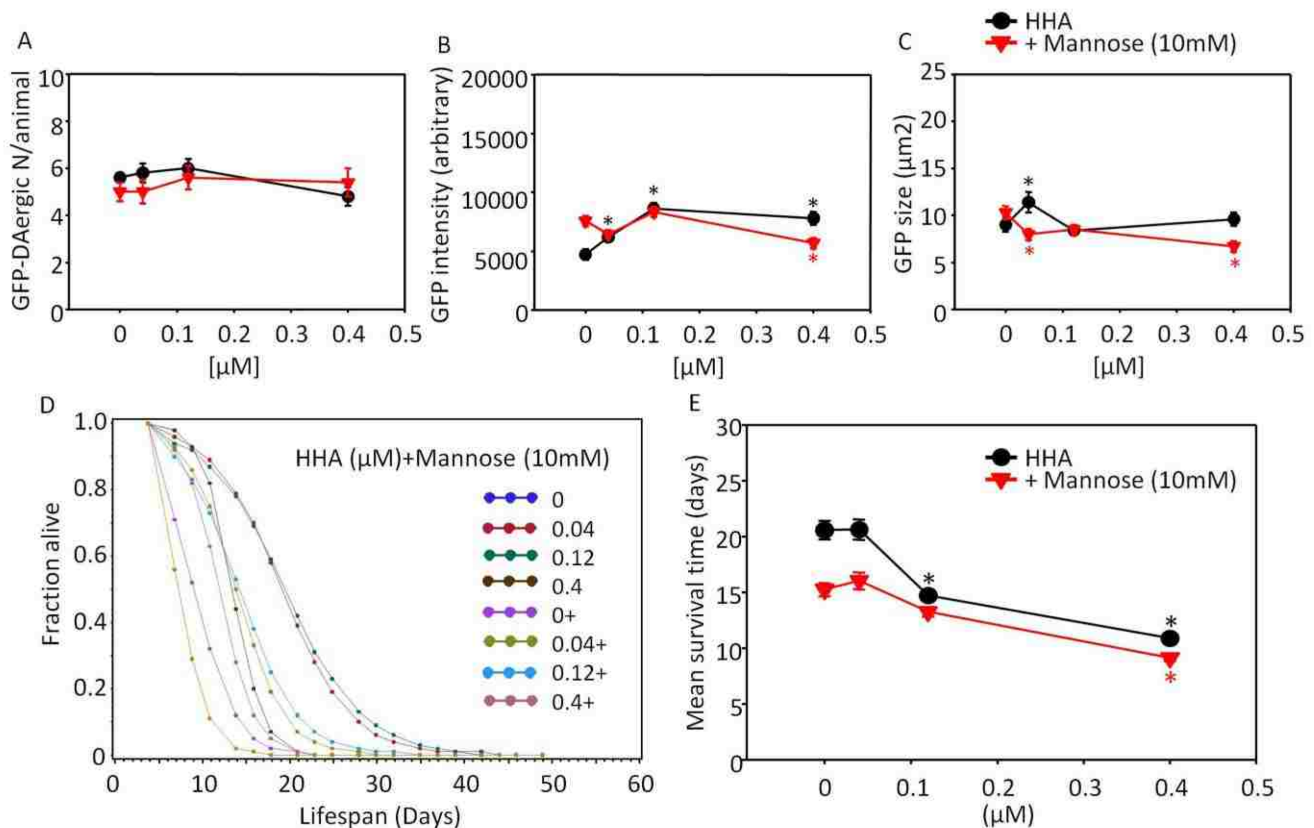

Figure 40. HHA-TRITC did show co-localization but affected the DAergic neurons in *C. elegans*. A) The number of the GFP-DAergic neurons was not altered with or without the SIS mannose (10 mM). B) The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was increased at all doses (0.04 μM, 0.12 μM & 0.4 μM, P<0.05), which was blocked at lower doses (0.04 μM & 0.12 μM, P>0.05) and reversed at the highest dose (0.2 μM, P<0.05) in presence of the SIS. C) The size of GFP-DAergic neurons was increased at the lowest dose (0.04 μM, P<0.05), which was reversed in presence of the SIS and reduced at the highest dose (0.4 μM, P<0.05) in presence of the SIS. D) The APS was dose-dependently decreased at all doses. E) The mean lifespan was decreased at higher doses (0.12 μM& 0.4 μM) from 21 days to 15 days and 11 days (-29% & -47%, P<0.05), which was blocked at the medium dose (0.12 μM) in presence of the SIS. * indicates statistical significance.

The size of GFP-DAergic neurons was increased at the lowest dose of HHA (0.04 μM, P<0.05), which was reversed in presence of the SIS and reduced at the highest dose (0.4 μM, P<0.05) in presence of the SIS (Figure 40c). The APS was dose-dependently decreased at all doses (Figure 40d). The mean lifespan was decreased at higher doses (0.12 μM& 0.4 μM) from 21 days to 15 days and 11 days (-29%

& -47%, P<0.05), which was blocked at the medium dose (0.12 μM) in presence of the SIS (Figure 40e).

Phaseolus vulgaris agglutinin-L (PHA-L)-TRITC did show co-localization but affected the DAergic neurons in *C. elegans*. The number of the GFP-DAergic neurons was not altered with or without the SIS GalNAc (1.58 mM, Figure 40a). The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was reduced at lower doses (0.017 μM & 0.051 μM, P<0.05), which was reduced at the highest dose (0.17 μM, P<0.05) in presence of the SIS (Figure 40b).

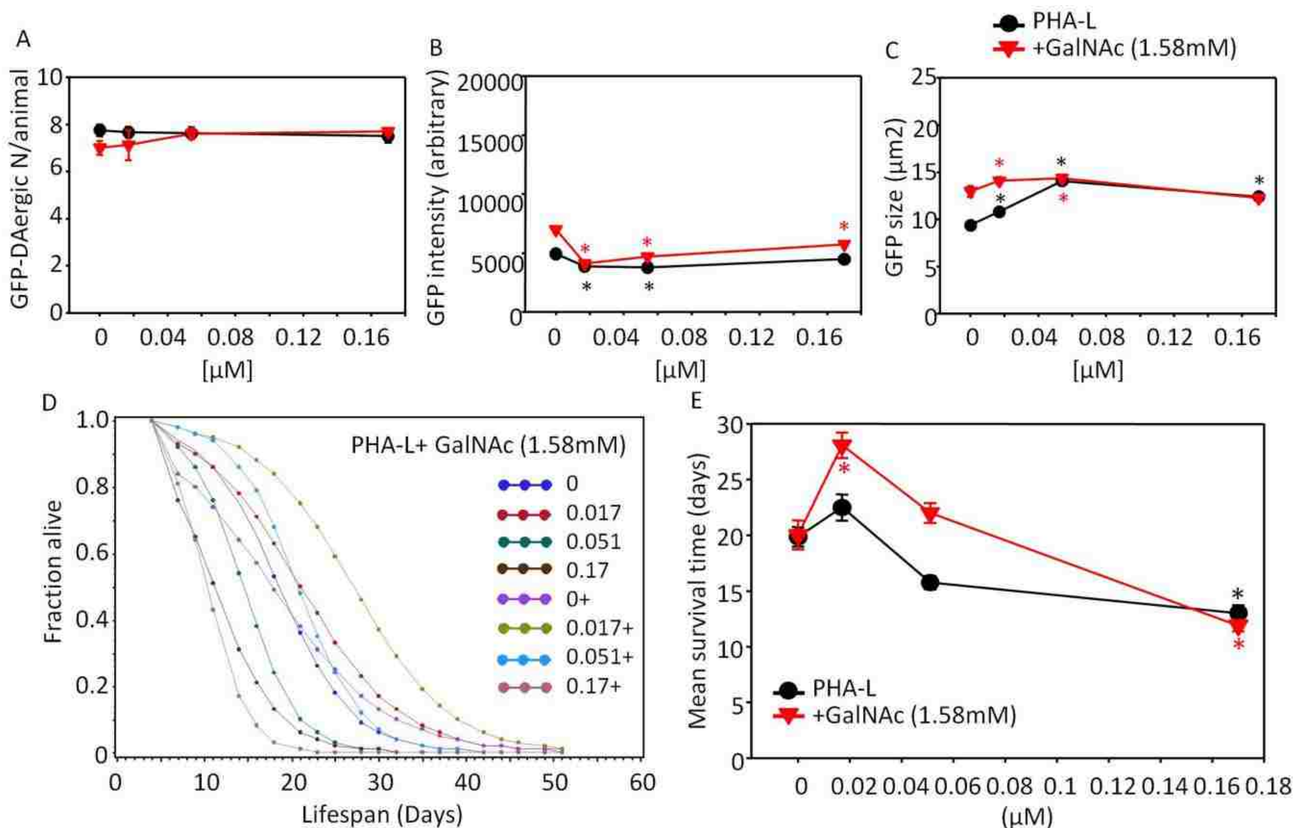

Figure 41. PHA-L-TRITC did show co-localization but affected the DAergic neurons in *C. elegans*. A) The number of the GFP-DAergic neurons was not altered with or without the SIS GalNAc (1.58 mM). B) The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was reduced at lower doses (0.017 μM & 0.051 μM, P<0.05), which was reduced at the highest dose (0.17 μM, P<0.05) in presence of the SIS. C) The size of GFP-DAergic neurons was increased at all doses (0.017 μM, 0.051 μM & 0.17 μM, P<0.05), which was blocked at the highest dose (0.17 μM) in presence of the SIS. D) The APS was increased at low dose and reduced at higher doses. E) The mean lifespan was reduced at the highest dose (0.17 μM) from 20 days to 13 days (-34%, P<0.05), which was increased at the lowest dose (0.017 μM) in presence of the SIS. * indicates statistical significance.

The size of GFP-DAergic neurons was increased at all doses (0.017 μM, 0.051 μM & 0.17 μM,

P<0.05), which was blocked at the highest dose (0.17 μM) in presence of the SIS (Figure 40c). The APS was increased at low dose and reduced at higher doses (Figure 40d). The mean lifespan was reduced at the highest dose (0.17 μM) from 20 days to 13 days (-34%, P<0.05), which was increased at the lowest dose (0.017 μM) in presence of the SIS (Figure 40e).

Soybean agglutinin (SBA)-TRITC did show co-localization but affected the DAergic neurons in *C. elegans* (Figure 42).

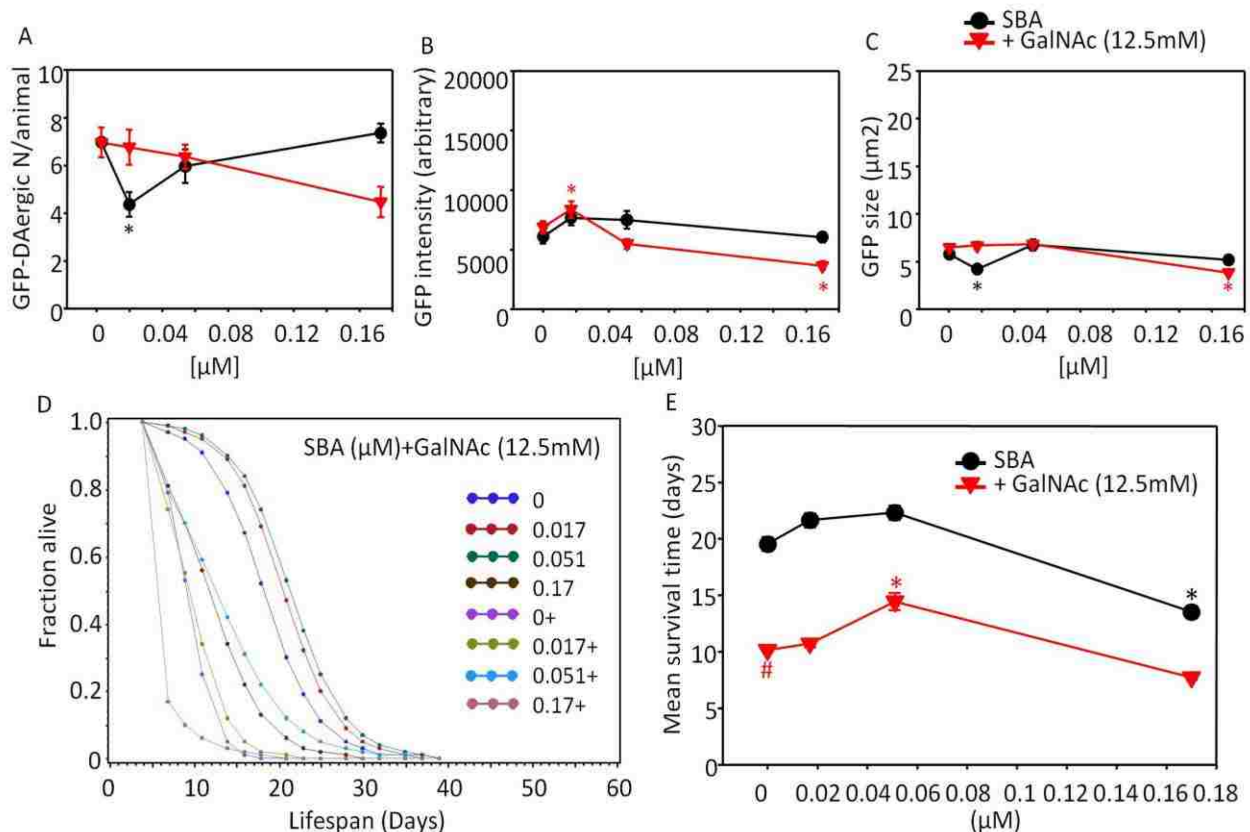

Figure 42. Soybean *agglutinin* (SBA)-TRITC did show co-localization but affected the DAergic neurons in *C. elegans*. A) The number of the GFP-DAergic neurons was decreased at the lowest dose (0.017 μM, P<0.05), which was blocked by the SIS GalNAc (12.5 mM). B) The intensity of DAergic neurons was not altered at all doses (P>0.05), which was increased at the lowest dose (0.017 μM, P<0.05) and reduced at the highest dose (0.17 μM, P<0.05) in presence of the SIS. C) The size of GFP-DAergic neurons was reduced at the lowest dose (0.017 μM, P<0.05), which was blocked in the presence of the SIS. D) The APS was slightly increased at low doses and reduced at higher dose. E) The mean lifespan was reduced at the highest dose (0.17 μM) from 20 days to 14 days (-31%, P<0.05), which was blocked by the SIS. The mean lifespan was reduced by the SIS only from 20 days to 11 days (-48%, P<0.05). * indicates statistical significance, # indicates SIS plus OP50 vs. OP50 only.

The number of the GFP-DAergic neurons was decreased at the lowest dose (0.017 μM, $P<0.05$), which was blocked by the SIS GalNAc (12.5 mM, Figure 42a). The fluorescence intensity of GFP-dopamine transporter protein in DAergic neurons was not altered at all doses ($P>0.05$), which was increased at the lowest dose (0.017 μM, $P<0.05$) and reduced at the highest dose (0.17 μM, $P<0.05$) in presence of the SIS (Figure 42b). The size of GFP-DAergic neurons was reduced at the lowest dose (0.017 μM, $P<0.05$), which was blocked in the presence of the SIS (Figure 42c). The APS was slightly increased at low doses and reduced at higher dose (Figure 42d). The mean lifespan was reduced at the highest dose (0.17 μM) from 20 days to 14 days (-31%, $P<0.05$), which was blocked by the SIS. The mean lifespan was reduced by the SIS only from 20 days to 11 days (-48%, $P<0.05$, Figure 42e).

Concanavalin A (Con A)-TRITC did not show co-localization with but affected GFP-DAergic neurons in *C. elegans*. The number of DAergic neurons was not altered with or without the SIS α-methylmannoside (200 mM) plus α-methylglucoside (200 Mm, Figure 43a). The fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons was increased at the highest dose (0.19 μM, $P<0.05$) and reversed by the SIS ($P<0.05$, Figure 43b). The size was reduced at middle dose (0.057 μM, $P<0.05$), which was blocked by the SIS (Figure 43c). The APS was increased at all doses, which was blocked by the SIS (Figure 43d). The mean lifespan was not altered with or without SIS (Figure 43e).

Cicer arietinum agglutinin (CPA)-TRITC did not show co-localization with but affected GFP-DAergic neurons in *C. elegans*. The number of DAergic neurons was not altered with or without the SIS bovine fetuin (0.77 μM, Figure 44a). The fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons was reduced at the doses (0.047 μM & 0.47 μM, $P<0.05$), which was blocked at the lowest dose (0.047 μM) by the SIS (Figure 44b). The size was reduced at middle at the doses (0.047 μM & 0.47 μM, $P<0.05$), which was blocked by the SIS (Figure 44c). The APS was increased at all doses, which was reversed by the SIS (Figure 44d). The mean lifespan was not altered by CPA, which was decreased by CPA from 29 days to 22 days and 11 days (-25% & -64%, $P<0.05$) in presence of the SIS.

The mean lifespan was increased by the SIS only from 23 days to 29 days (28%, P<0.05, Figure 44e).

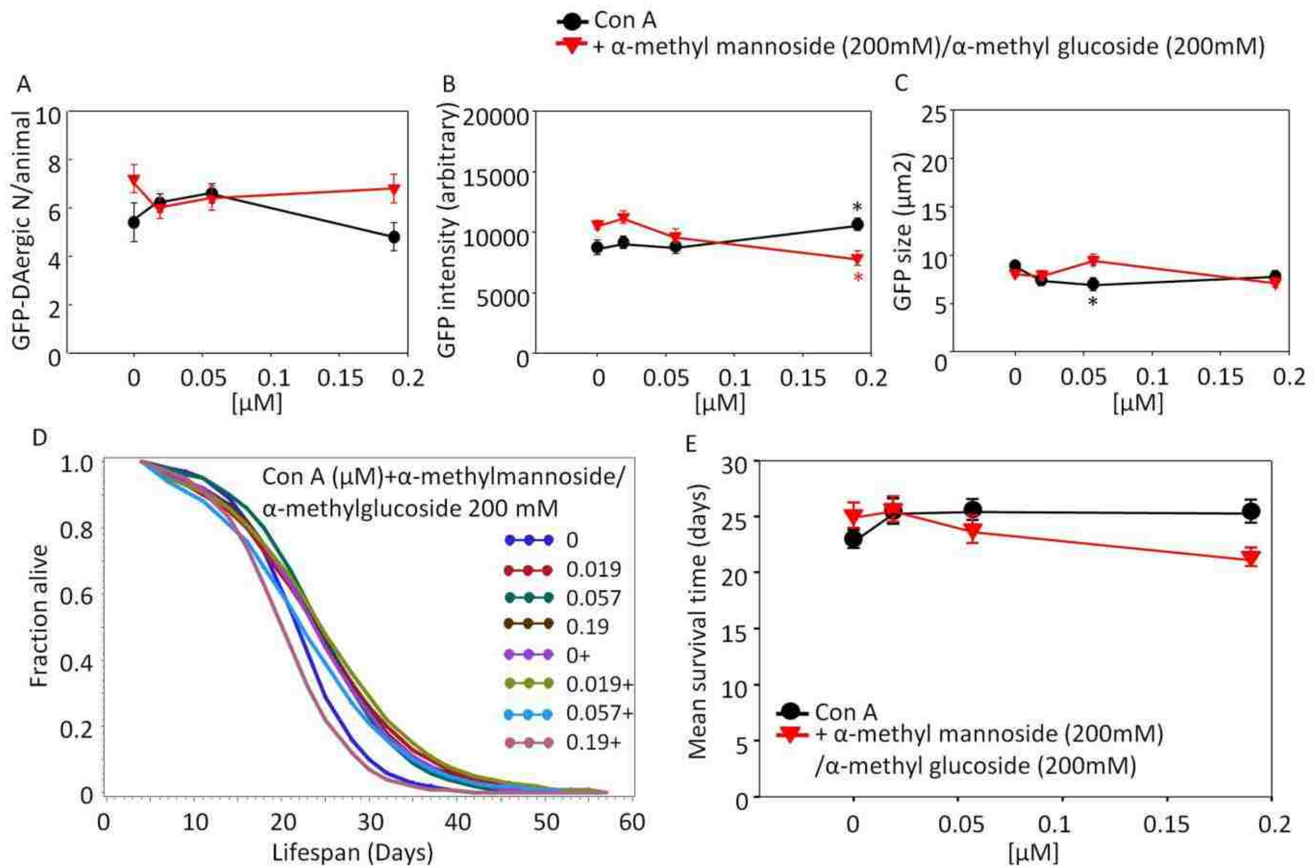

Figure 43. Con A-rhodamine affected the GFP-DAergic neurons in *C. elegans*. A) The number of DAergic neurons was not altered with or without the SIS α-methylmannoside (200 mM) plus α-methylglucoside (200 Mm). B) The fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons was increased at the highest dose (0.19 μM, P<0.05) and reversed by the SIS (P<0.05). C) The size was reduced at middle dose (0.057 μM, P<0.05), which was blocked by the SIS. D) The APS was increased at all doses, which was blocked by the SIS. E) The mean lifespan was not altered with or without SIS. * indicates statistical significance.

Solanum tuberosum agglutinin (STA)-TRITC did not show co-localization with but affected GFP-DAergic neurons in *C. elegans*. The number of DAergic neurons was not altered with or without the SIS chitin hydrolystate (2.5%, Figure 45a). The fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons was not altered (Figure 45b).

The size was reduced at all doses of STA (0.02 μM, 0.06 μM & 0.2 μM, P<0.05), which was blocked by the SIS (Figure 45c). The APS was slightly increased without the SIS (Figure 45d). The mean

lifespan was not altered by STA. The animals died within one week of adding treatments. The mean lifespan was decreased by the SIS only from 22 days to 10 days (-54%, P<0.05, Figure 45e).

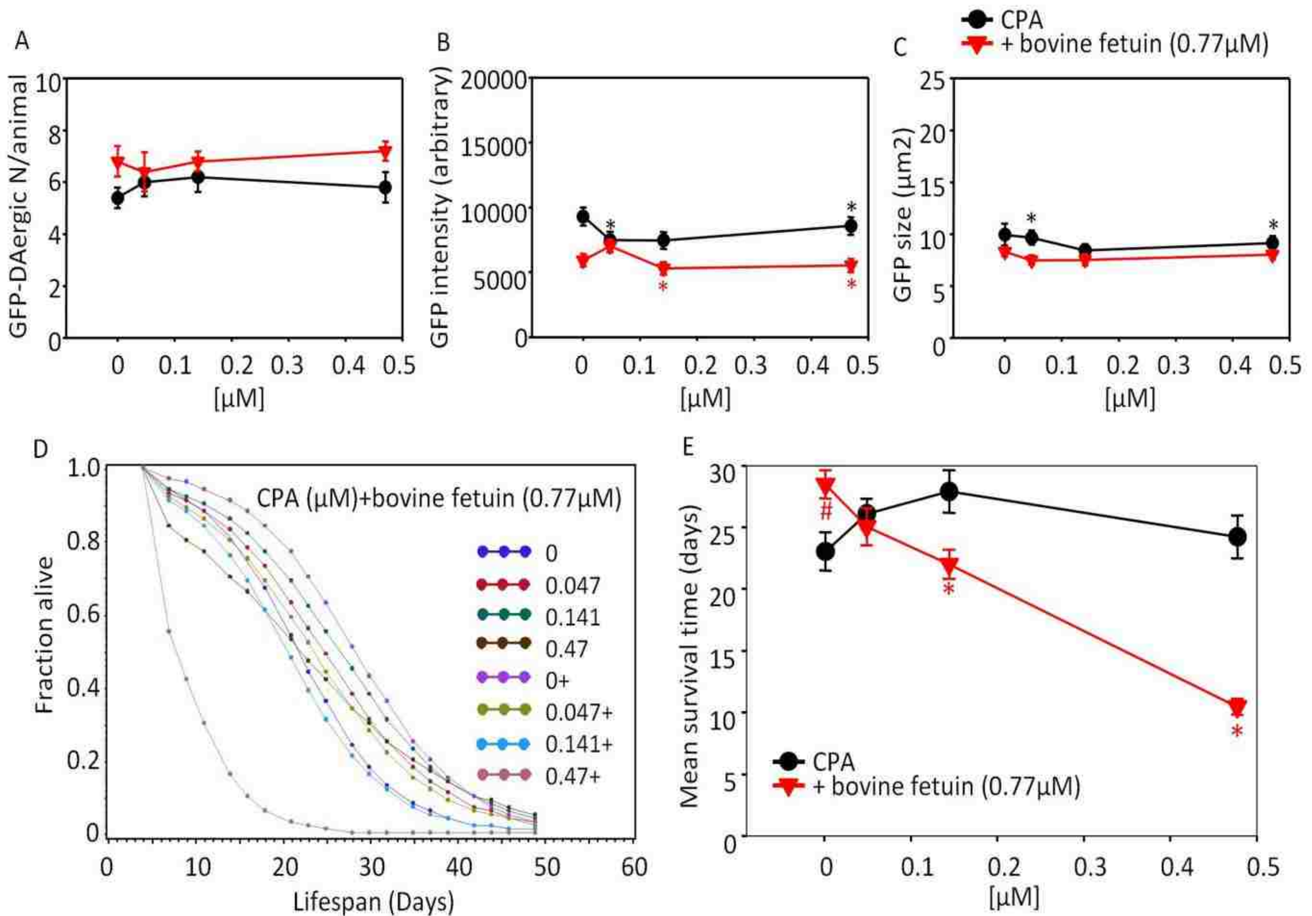

Figure 44. CPA-TRITC did not show co-localization with but affected GFP-DAergic neurons in *C. elegans*. A) The number of DAergic neurons was not altered with or without the SIS bovine fetuin (0.77 μM). B) The fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons was reduced at the doses (0.047 μM & 0.47 μM, P<0.05), which was blocked at the lowest dose (0.047 μM) by the SIS. C) The size was reduced at middle at the doses (0.047 μM & 0.47 μM, P<0.05), which was blocked by the SIS. D) The APS was increased at all doses, which was reversed by the SIS. E) The mean lifespan was not altered by CPA, which was decreased by CPA from 29 days to 22 days and 11 days (-25% & -64%, P<0.05) in presence of the SIS. The mean lifespan was increased by the SIS only from 23 days to 29 days (28%, P<0.05). * indicates statistical significance, # indicates SIS plus OP50 vs. OP50 only.

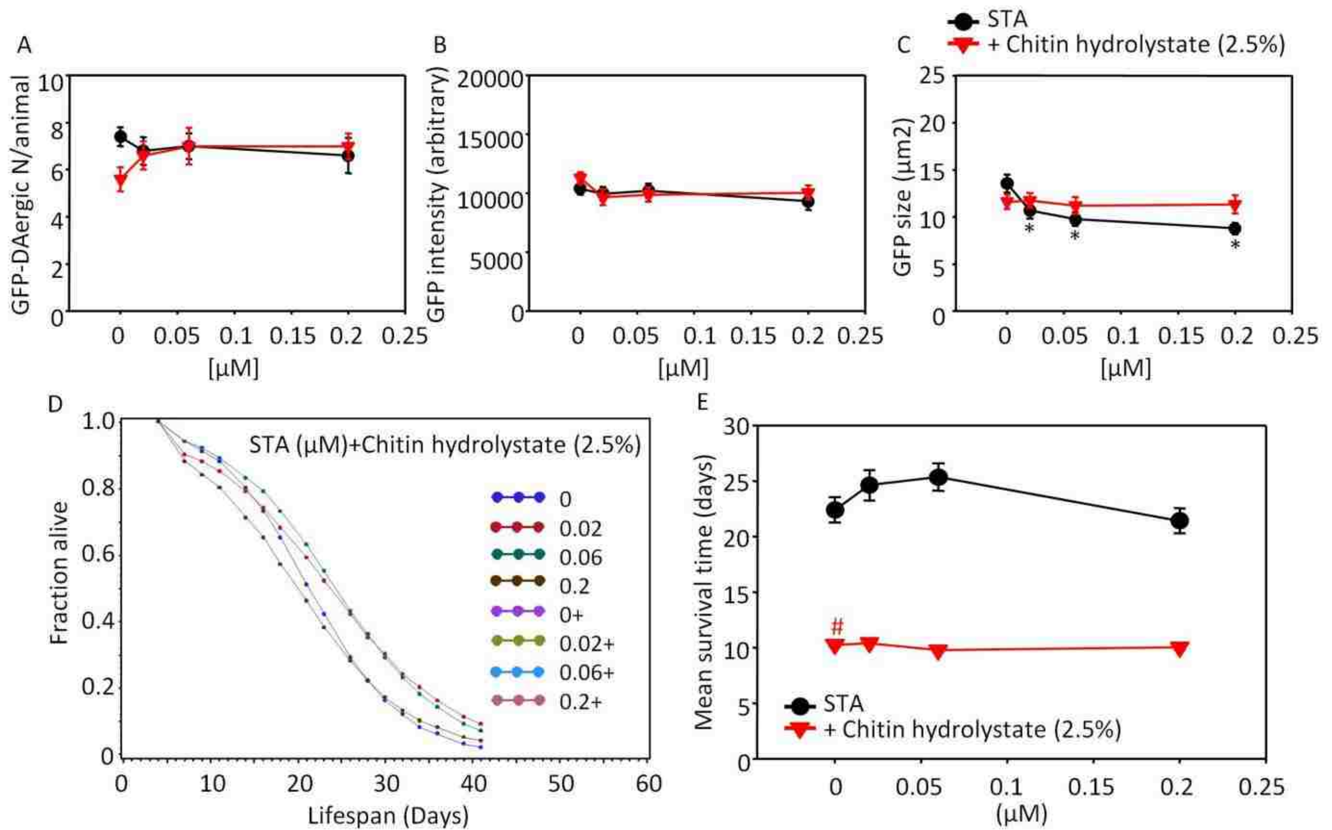

Figure 45. STA-TRITC did not show co-localization with but affected GFP-DAergic neurons in *C. elegans*. A) The number of DAergic neurons was not altered with or without the SIS chitin hydrolystate (2.5%). B) The fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons was not altered. C) The size was reduced at all doses (0.02 µM, 0.06 µM & 0.2 µM, P<0.05), which was blocked by the SIS. D) The APS was slightly increased without the SIS. E) The mean lifespan was not altered by STA. The animals died within one week of adding treatments. The mean lifespan was decreased by the SIS only from 22 days to 10 days (-54%, P<0.05). * indicates statistical significance, # indicates SIS plus OP50 vs. OP50 only.

1.4 Discussion

The purpose of the specific inhibiting sugar was to bind with, and then block the corresponding lectins. As stated in the beginning of this chapter, using high concentration of sugars would cause various other effects other than binding with lectins in the *C. elegans* organism, like UDP depletion causing the ceasing of RNA synthesis. In the current study, SIS was observed to have various effects when supplemented with lectins in liquid culture.

The interaction between lectins and specific sugars is complicated, depending on properties of the sugar-binding sites in lectins and the moieties in the sugars. Lectins have several sugar-binding sites available due to the repeat of sequences, some lectins like EEA and PNA bind to monosaccharide, other lectins like ACA and CSA bind to polysaccharides. The sequence of amino acids in the sugar-binding sites of the lectins has an important role in selecting the specific sugars [16]. The binding between lectins and simple sugars is not as strong as between lectins and polysaccharides, but the multiple binding sites in simple sugars produce multivalent bindings with lectins [17], which increases the affinity of simple sugars and lectins to some extent. The dissociation constant of lectin Con A from glycoprotein asialofetuin increases as the concentration of inhibiting sugars increases [18], which suggest that the binding between the sugars and lectins is reversible; the existence of the third compound may compete with sugars for the binding with lectins.

1.4.1 SIS inhibited the effects of lectins on GFP-DAergic neurons or mean lifespan

SIS were observed to block the effects caused by lectins on the GFP-DAergic neurons or mean lifespan. The possible explanation is that the sugar binding sites of lectins were covered by the SIS first, which prevented the binding of lectins with other receptors on cell surface in *C. elegans* organism. The effect of ACA (0.096 μM) on reducing the size of GFP-DAergic neurons was blocked by the SIS GalNAc, which might be due to the binding of SIS with ACA. The observation that the SIS GalNAc did not block the effects of the highest dose of ACA (0.32 μM) might suggest the concentration of the SIS was not high enough to bind to all sugar binding sites of ACA. Similarly, the SIS GalNAc only blocked the effects of lowest dose of ACA (0.032 μM) on reducing the mean lifespan but not on higher doses.

1.4.2 SIS mitigated the effects of lectins on GFP-DAergic neurons or mean lifespan

Some SIS were observed to alleviate the effects of lectins in *C. elegans*. The reason that the SIS did not block the effect but only mitigated the effects might be due to the partial binding of the SIS with sugar binding sites of lectins. The highest dose of EEA (0.48 μM) increased the size of GFP-DAergic neurons;

the adding of the SIS lactose did not block these effects but only reduced the magnitude. The effect of medium dose of EEA (0.136 μM) on reducing the mean lifespan was mitigated by the SIS.

1.4.3 SIS affected the effects of lectins on GFP-DAergic neurons or mean lifespan

SIS were also observed to enhance or the effects of SIS on lectins in *C. elegans*. Lower doses of GSL I (0.018 & 0.054 μM) increased the fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons, with the SIS galactose present, these effects of increasing was strengthened. In addition, the SIS reversed the effect of the highest dose of Con A on increasing the fluorescence intensity of GFP-dopamine transporter protein image in DAergic neurons. These findings were not caused by the effects of lectins only; the possible explanation might be due to some unknown effects caused by the SIS or the interactions between the SIS and lectins.

The fact that only certain doses reduced the number of DAergic neurons but other doses did not suggests that this effect is dose-related. The presence of lactose (10 mM) blocked this effect probably because lactose bound with the binding sites of EEA, thus reduced the activity of EEA. In contrast to EEA, low doses of S-WGA reduced the number of DAergic neurons while the highest dose did not. Similarly, this effect was blocked by the presence of the SIS chitin hydrolysate (2.5%), probably due to the same mechanism as EEA. The effects of CSA and SBA on reducing the number of DAergic neurons were also blocked by their SISs. The reduction of neuron numbers indicated the toxicity of these lectins, which was consistent with the lifespan results, that both EEA and S-WGA reduced the mean lifespan significantly. The lectin LcH increased the number of DAergic neurons at both the lowest dose and the highest dose. These effects were either blocked at low dose or reversed at high dose by the SIS, which implied that high dose of the SIS had detrimental effects.

The toxic effects of high concentration of sugars in *C. elegans* were expected, and lower concentration of sugars would not be enough to counteract the effects of lectins. The present study showed

that the SIS blocked the effects caused by lectins on GFP-DAergic neurons or mean lifespan, whereas, other effects might be due to the toxic effects of the SIS.

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CHAPTER IV. DIETARY BIOACTIVE COMPOUNDS MAY AFFECT LIFESPAN AND FAT STORAGE ASPECTS IN *C. ELEGANS*

1.1 Introduction

Health determines human lifespan as well as optimal quality of life [1, 2]. Aging at the cellular level involves complex interacting mechanisms that lead to functional declines which become manifest following birth and proceed through life. A U.S. population based study demonstrated that mortality risk was directly correlated with body mass index (BMI). Thus, above 25 kg/m^2, BMI inversely correlated with lifespan [3]. The increasing age of western societies creates a greater burden of chronic diseases including cancer, cardiovascular disease, diabetes, and neurodegenerative disorders including PD and Alzheimer's disease [4-8], leading to a high cost of health care and a great financial burden to the public health system and families [9, 10]. Among complex factors, both obesity and aging decrease insulin sensitivity, impair the immune response, increase inflammation, impair the gut-bloodstream barrier, and decrease physical mobility [11]. Many dietary interventions provide noninvasive approaches to reinforce optimal nutrition, fight metabolic dysfunction, enhance physiological function, and promote a healthy lifespan [12-14].

Dietary polyphenol antioxidants play important roles in health [15-19]. Pomegranates (*Punica granatum* L.) have a high content of polyphenols (1.5%), including ellagic acid (EA), gallic acid, anthocynidins, flavan-3-ols, straight chain fatty acids, citric acid, and malic acid. Pomegranates consist of about 80% of juice and 20% of seeds with water (85%), and 10% sugars consisting primarily of fructose (2.5 g to 17.6 g/100ml) [20, 21] (Table 6). PJ has been shown to extend lifespan in mice [22, 23] and *Drosophila melanogaster* [24]. PJ extract (POMx) potentiates lifespan extension with dietary restriction, a finding attributed by polyphenols [22, 23]. POMx and PJ can also act as prebiotics, having demonstrated antibacterial properties *in vitro*, and can block DNA repair and inhibit proliferation of breast cancer cells (MCF-7) *in vitro*, as well as modulate the IGF-IGFBP axis [25-29]. POMx and PJ also down-regulate

androgen-synthesizing genes to induce apoptosis of human prostate cells (kappaB-dependent) *in vitro* and in mice *in vivo* [30-33]. POMx and PJ decrease prostate specific antigen in humans after surgery or radiation [34, 35], inhibit tumor-associated angiogenesis *in vitro* and *in vivo* [34], suppress inflammatory cell signaling in colon cancer cells [36] (50 mg/L PJ, *in vitro*), improve memory [37] and improve fecundity in humans [24, 38-40].

As the main bioactive component, EA is a measure of the quality of PJ extract products [39, 41, 42]. EA in humans reaches a maximum plasma level in 1 hour (31.9 ng/ml), is eliminated within 4 hours [43] and exerts an antioxidant effect [44]. Urolithin acid (UA) is the main active metabolite of EA and is formed by the colonic microflora. UA lasts longer in the human body than EA or other EA metabolites [26, 45], and has better bioavailability [46]. UA also suppresses colorectal, hepatic, and prostate cancers synergistically with EA *in vitro* and in mice *in vivo* [47-51].

Table 6. Nutrition facts of pomegranate juice

Serving size 8 fl. oz. (236 mL)	
Amounts per serving	
Calories	150
Calories from Fat	0
% Daily Value	
Total Fat 0 g	0%
Saturated Fat 0 g	0%
Trans Fat 0 g	
Cholesterol l0 mg	
Sodium 0 mg	0%
Potassium 600 mg	17%
Total Carbohydrate 36 g	12%
Fiber 0 g	0%
Sugars 31 g	
Protein 1 g	
Vitamin A	<2%
Vitamin C	<2%
Calcium	<2%
Iron	0%
Percent Daily Values are based on a 2,000 calorie diet	

C. elegans model organism is the first animal model to have its genome completely sequenced. *C. elegans* conserves 65% of the genes associated with human diseases, and has been increasingly used for functional biomedical research with more than 300 transgenic and mutant strains available [52-62]. The *C. elegans daf-16* gene, a homologue of the human gene FOXO, regulates *C. elegans* lifespan and mediates both lipid metabolism and insulin signaling pathways [63, 64].

We hypothesized that pomegranate would increase lifespan while reducing intestinal fat deposition (IFD). We evaluated four PJ products using the *C. elegans* wild type (N2) to assess their effect on lifespan and IFD, and to predict their potential effect on aging and obesity in humans. In addition, we also used a *daf-16* deficient mutant to assess the role of the FOXO signaling pathway in mediating the effects of botanicals evaluated.

1.2 Materials and methods

C. elegans strains wild type (N2) and *daf-16* mutant (*daf-16(mgDf50)I*) GR1307, and their standard lab food, *Escherichia coli* (*E. coli*, OP50, *Uracil auxotroph),* were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN). PJ (POM Wonderful® LLC, Los Angeles, CA, USA) was purchased from a local grocery store. POMx (extract of pomegranate) was a gift from POM Wonderful® LLC (Los Angeles, CA, USA). EA was purchased from Sigma (St. Louis, MO, USA). UA was synthesized by Dr. David Heber's laboratory [65].

1.2.1 Culture of *C. elegans*

Mature gravid wild type *C. elegans* (N2, Bristol) and *daf-16* deficient mutant *[daf-16(mgDf50)I]* were treated with NaOH (1M) and sodium hypochlorite solution (5.25%, 5:2 ratio) to dissolve the body and release viable eggs [61]. The eggs were hatched overnight after washing with S-complete solution three times. The age-synchronized *C. elegans* were diluted to 100 animals/ml, plated in liquid culture in a 96-well plate (120 µl/well, 10-15 animals) [66] with OP50 (10^9 cfu/ml), and incubated in 20°C (N2) or

15°C (*daf-16* mutant) low temperature incubators (Revco Tech., Nashville, NC, USA). Thirty microliters of 5-Fluoro-2′-deoxyuridine (FUDR, 0.6mM) stock solution was added to each well at Larvae 4 stage.

1.2.2 Culture of *Escherichia coli* (*E. coli*, OP50)

OP50 were cultured by the standard method described elsewhere [61]. Briefly, approximately 10 μL of stock *E. coli* solution was added to media and incubated at 37 ℃ for 24h. The OP50 were then plated in Petrifilm™ (3M Corporate, St. Paul, MN) at 37 ℃ for 24 h until densities of 5×10^{8} to 5×10^{11} colony forming units (cfu/ml) were reached and then were fed to the *C. elegans ad libitum* [61, 67]. The OP50 stock feeding solution was enriched to 2×10^{9} cfu/ml by centrifuging at 2,200 g for 10 minutes and washed with S-complete buffer twice.

1.2.3 Lifespan assays

Fifty microliters of the treatments was added to each well three days after egg-synchronization. The control group received OP50 only. The experimental groups received additional PJ (0.01%, 0.1%, 1%, 3%, 5%, 10% or 25%, v/v), POMx (5, 10, 20, 40, 80, 160, and 320 μg/ml), EA (1, 2, 5, 10, 25, and 50 μM in dimethyl sulfoxide (DMSO 0.05%), or UA (1, 2, 5, 10, 25, and 50 μM in DMSO) (n=10-15/well/6well). A second control (DMSO, 0.05%) was used in the EA and UA groups. Additional OP50 was added to each well every other week until all animals were dead. The numbers of live animals were manually recorded every other day under a microscope (Nikon, Eclipse T*i* –S, Japan).

1.2.4 Fluorescence microscopy

Same treatments as in lifespan assay were added to each well at larvae 1 stage of animals. Lipophilic dye, Nile red, was used to stain for IFD, and fluorescent intensity was evaluated [61]. *C. elegans* in each group were collected after 3 days of treatments, washed with S-Basal twice, fixed with paraformaldehyde (4%) over 2h at 4 ℃ and washed with PBS for 5 min x 3. Nile red (50 μL) was applied to the specimens for 10 min. Ten microliters of Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) was applied to a glass slide followed by 20 μL of the medium containing Nile red stained *C. elegans*. A

cover glass was mounted on the glass slide, and the slides were viewed with an epifluorescence microscope (Nikon Eclipse, *Ti*) equipped with a Texas Red filter. Fluorescent micrographs were taken with a digital camera (Andor, DU-885k) and analyzed using Nikon-Elements (version 3.22.11). Optical densities (arbitrary units) of Nile red stained IFD were determined for *C. elegans* (larvae 4).

1.2.5 Statistical Analysis

Analyses were carried out using SAS/STAT® software, Version 9.4 of the SAS System for Windows (Cary, NC, USA). All results were expressed as mean ±S.E.M. Survival curves were displayed by binomial probabilities obtained from logistic regression models as surrogates for survival probabilities and mean lifespan was estimated via Kaplan-Meier (log-rank). ANOVA models with post hoc Tukey adjustment were used to analyze fluorescence intensity data. Statistical significance was defined as $P<0.05$.

1.3 Results

PJ treatment dose-dependently extended the mean lifespan of N2 up to 56% or *daf-16* mutant up to 30% in an A-shape curve, the magnitude of the lifespan extension was decreased at higher doses. A similar trend with half the magnitude was observed in POMx treated animals in N2 (28%). EA or UA did not significantly affect lifespan overall, however, lifespan extension was observed in several days of the experiment in each treatment (Supplement Table 8c & d). The fluorescent intensity of IFD in *C. elegans* was reduced by PJ treatment in N2 (-68%) or in *daf-16* (-33%). In contrast, IFD was increased in N2 more than in the *daf-16* mutant by POMx (137% at 320 μg/ml vs. 26% at 20 μg/ml), or UA (57% at 10 μM vs. 43% at 50 μM). IFD was increased by EA in N2 (66% at 5 μM) and in *daf-16* mutant (74% at 25 μM).

1.3.1 PJ dose-dependently extended then reduced lifespan in N2 and the *daf-16* mutant

In a dose-dependent manner, PJ added to the cultures significantly increased mean lifespan in N2 up to the 1% dose but produced reduced lifespans at higher doses, an A-shape curve relationship (Figure 46a & b).

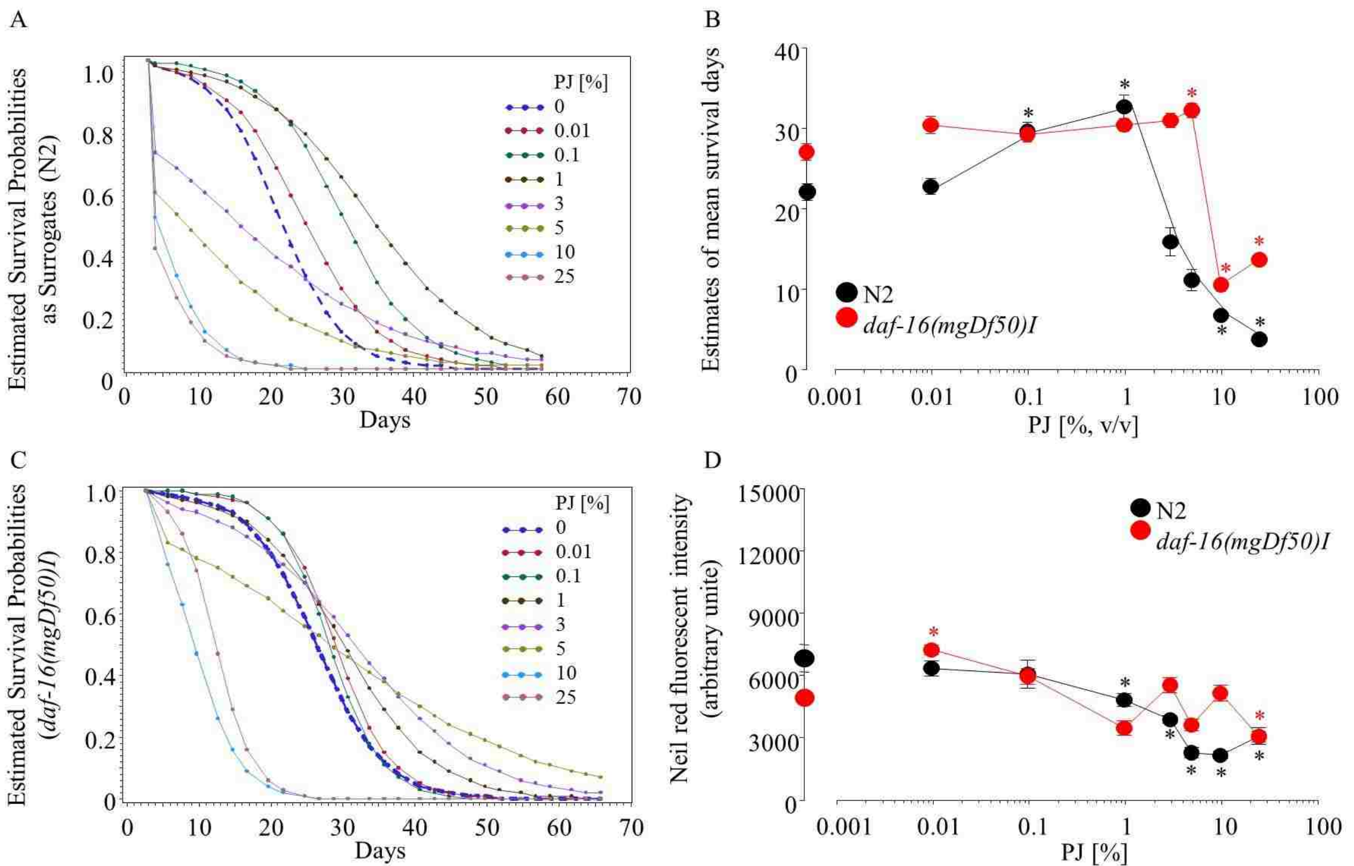

Figure 46. In an A-shape, PJ dose-dependently increased lifespan in both N2 (a & b) and *daf-16* mutant (c & b) at lower doses, and dose-dependently reduced it at higher doses. PJ dose-dependently reduced the fluorescent intensity of IFD (arbitrary unit) in N2 and *daf-16* mutant (d). *P<0.05

The average probability of survival across the lifespan was increased by lower doses of PJ (0.01%, 0.1%, & 1%, P<0.05), while lifespan was decreased at higher doses (10% & 25%, P<0.05, Figure 46a). The mean lifespan was increased at lower doses (0.1% - 1%) from 21 to 33 days (42% - 56%, P<0.05) while it was decreased at higher doses (10% & 25%) from 33 to 4 days (-67% & -81%, P<0.05) (Figure 46b). Similarly, in the *daf-16* mutant, PJ also significantly increased mean lifespan in a dose-dependent

manner up to 5 % after which survival was significantly reduced. The dose-response curve shifted to the right. The APS across the lifespan of the *daf-16* mutant was increased at lower doses (1%, 3%, & 5%, P<0.05) and reduced at higher doses (10% & 25%, P<0.05, Figure 46c). The mean lifespan of the *daf-16* mutant was increased from 27 to 32 days at a dose (5%) that reduced lifespan in N2 (18%, P<0.05) and decreased at the higher doses (-61% at 10% & -49% at 25%, P<0.05, Figure 46b). Additionally, specific days during the experiment at which significant differences in survival probabilities were observed are provided (Supplement Table 8a).

PJ dose-dependently reduced the fluorescent intensity of IFD in N2 and the *daf-16* mutant (L4). In N2, the IFD was reduced from 6971±607 to 2372±245 (-30% at 1%, -44% at 3%, -66% at 5%, -68% at 10% & -55% at 25%, n=10, P<0.05). The IFD was increased at lower doses in the *daf-16* mutant from 4677±136 to 7222±240 (54% at 0.01%, 28% at 0.1%, n=10, P<0.05), followed by reduction at higher doses to 3134±252 (-24% at 1%, -21% at 5% & -33% at 25%, n=10, P<0.05) (Figure 46d).

1.3.2 POMx dose-dependently increased lifespan in N2 and in the *daf-16* mutant

A dose-response curve for lifespan extension was also present in the N2 group treated with POMx, but was absent in the daf-16 mutant. In N2, the APS across the lifespan was increased at all doses (5, 10, 20, 40, 80, 160, & 320 μg/ml, P<0.05, Figure 47a). The mean lifespan was elevated from 18 to 22 days (18% at 10 μg/ml & 28% at 20 μg/ml, P<0.05, Figure 47b). The APS across the lifespan of the *daf-16* mutant was increased at lower doses (5, 10, & 40 μg/ml, P<0.05) and reduced at the highest dose (320 μg/ml, P<0.05, Figure 47c). The mean lifespan of the *daf-16* mutant was not altered (Figure 47b). Additionally, specific days during the experiment at which significant differences in survival probabilities were observed are provided (Supplement Table 8b).

In N2, lower doses of POMx slightly reduced the IFD (P>0.05) followed by an increase from 3323±63 to 7515±125 at the highest dose (137% at 320 μg/ml, n=10, P<0.05). Similarly in the *daf-16*

mutant, POMx increased the IFD from 5012±1032 to 6327±716 at mid-dose (26% at 20 μg/ml, n=10, P<0.05) followed by a reduction to 3815±736 at a higher dose (23.9% at 80 μg/ml, n=10, P<0.05) (Figure 47d).

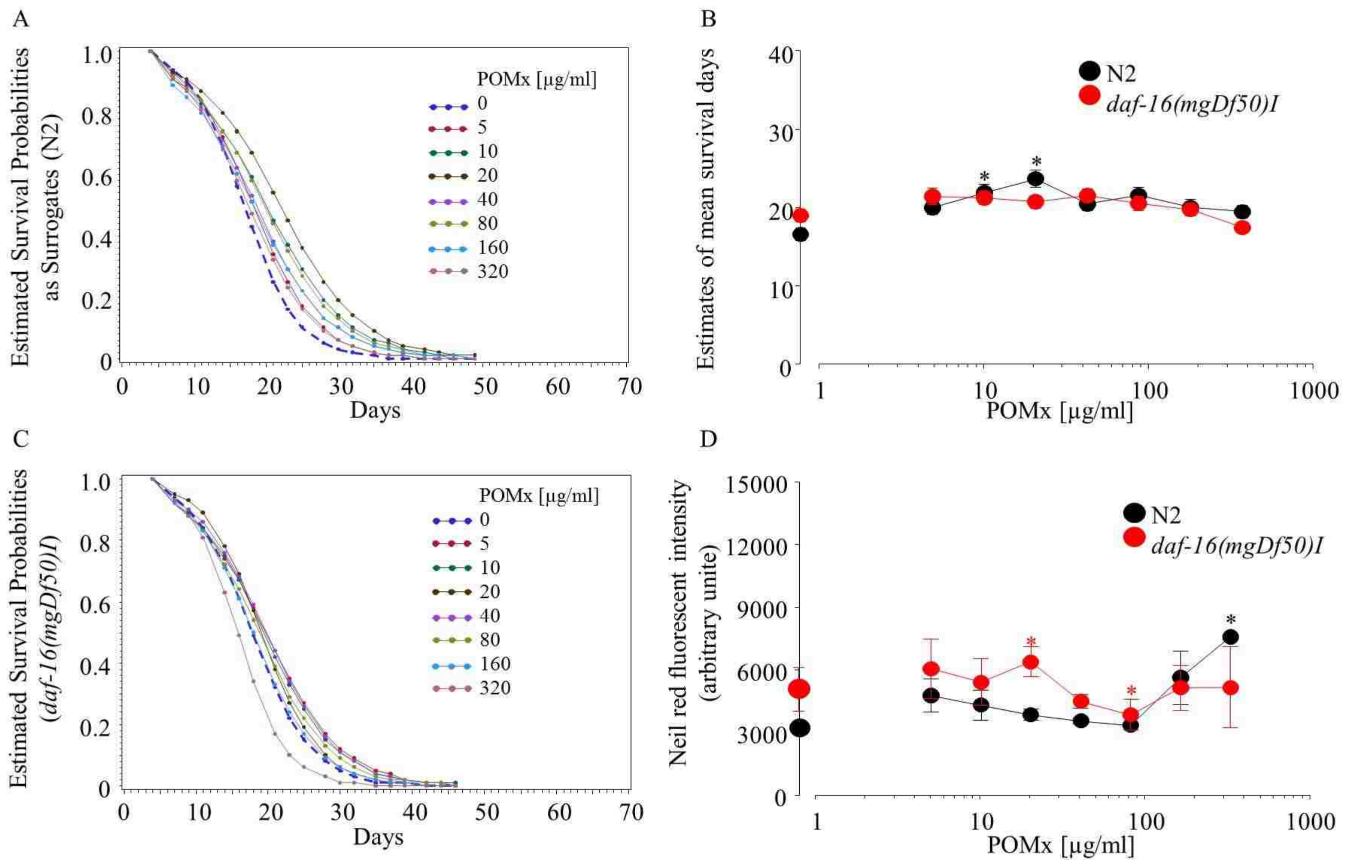

Figure 47. An A-shape dose-responses of lifespan extension was present in POMx treated group in both N2 (a & b) and the *daf-16* mutant (c & b), however, POMx was less potent than PJ. In N2, had an initial elevation of life span in the presence of lower doses of POMx which also reduced the IFD (d). IFD was increase at the highest dose. In the *daf-16* mutant, POMx increased IFD at mid-dose followed by a reduction at a higher dose. *P<0.05

1.3.3 EA did not alter lifespan in N2 or in the *daf-16* mutant

Unlike PJ and POMx that increased lifespan dose-dependently, EA did not alter lifespan in N2 or the *daf-16* mutant. There was a mild increase of lifespan in N2 at lower doses and a mild decrease at higher doses (P>0.05, Figure 48a) with the mean lifespan also being unchanged (P>0.05, Figure 48b).

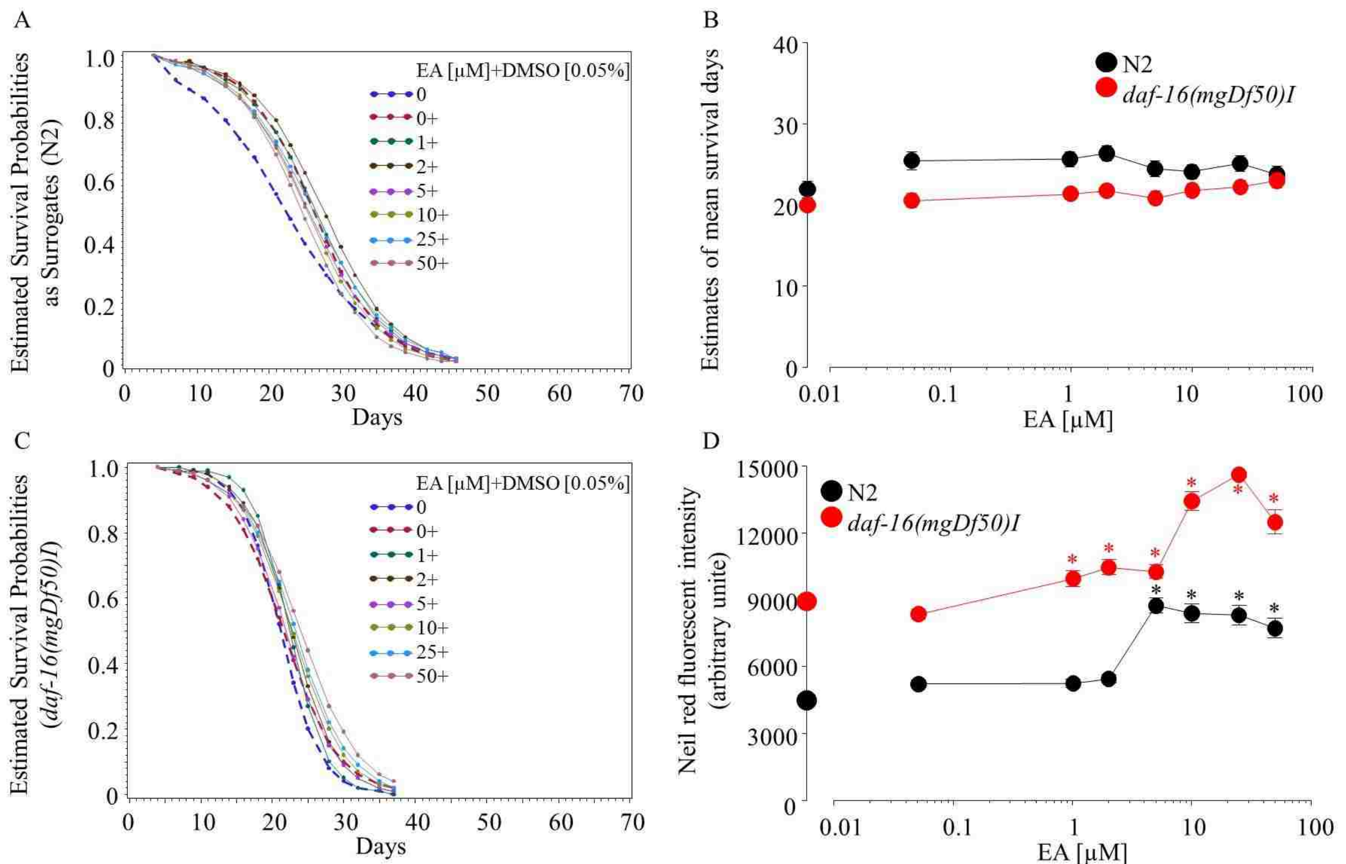

Figure 48. EA did not alter lifespan in N2 (a & b) but increased the lifespan in the *daf-16* mutant (c & b) at the highest dose. The IFD (d) was dose-dependently elevated by EA in both N2 and *daf-16* mutant, and the responses were in parallel with a greater elevation (by 2-fold) in the *daf-16* mutant. *P<0.05

The APS of the *daf-16* mutant was elevated at the highest dose (50μM, P<0.05, Figure 48c), but the increase in mean lifespan was not significant (P>0.05, Figure 48b). The specific days during the experiment at which significant differences in survival probabilities were observed are provided (Supplement Table 8c). The IFD elevation by EA was dose-dependent, similar in both N2 and the *daf-16* mutant, and in parallel with a 2-fold elevation in the *daf-16* mutant. In N2, EA increased IFD from 5190±158 to 8608±323 at higher doses (66% at 5 μM, 59% at 10 μM, 58% at 25 μM, & 47% at 50 μM, n=10, P<0.05). Similarly in the *daf-16* mutant, EA increased IFD at the higher doses from 8237±227 to 14324±113 (60% at 10 μM, 74% at 25 μM, & 49% at 50 μM, n=10, P<0.05) (Figure 48d).

1.3.4 UA did not alter lifespan in N2 or in the *daf-16* mutant

As with EA, UA did not alter the lifespan in N2 or the *daf-16* mutant. In N2, the APS across the lifespan was increased at the highest dose (50 μM, P<0.05, Figure 49a).

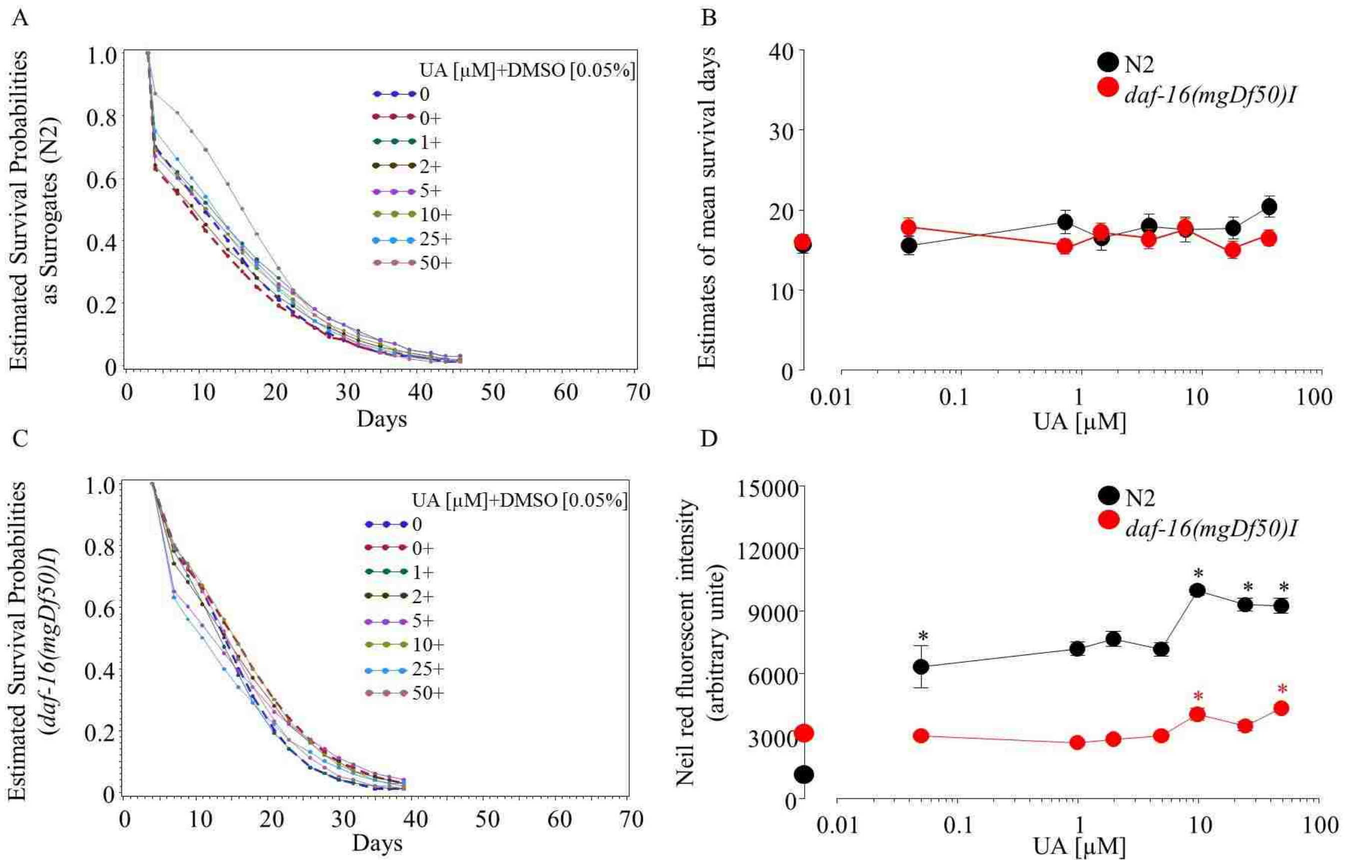

Figure 49. The mean lifespan was increased in N2 (a & b) but decreased in *daf-16* mutant (c & b) in UA treated group. Animals treated with UA had an increased IFD (d) in both N2 and the *daf-16* mutant with a parallel pattern of response with N2 showing a greater increase than the *daf-16* mutant. *P<0.05

The mean lifespan was not changed (P>0.05, Figure 49b). The APS across the lifespan of the *daf-16* mutant was reduced at doses (1, 25, & 50 μM, P<0.05, Figure 49c), while the mean lifespan was not changed (P>0.05, Figure 49b). Additionally, specific days during the experiment at which significant differences in survival probabilities were observed are provided (Supplement Table 8d). The IFD was increased in N2, from 1362±61 to 6278±991 in the DMSO-control group (361% at 0.05% DMSO, n=10, P<0.05) and greater elevation to 9860±216 was observed at higher doses (57% at 10 μM, 47% at 25 μM,

& 46% at 50 μM, n=10, $P<0.05$). The IFD in the *daf-16* mutant was increased from 3021±127 to 4324±177 dose-dependently (34% at 10 μM & 43% at 50 μM, n=10, $P<0.05$) (Figure 49d).

1.4 Discussion

Our findings reveal that the lifespan extension has the same trend, either on a larger or on a smaller scale, in N2 and the *daf-16* mutant by treatment with PJ or POMx, and with minimal effects on lifespan in cultures treated with EA or UA. These results indicated that the *daf-16* pathway was partially required for lifespan extension in the present study. The survival curves displayed are approximated by estimated probabilities of survival across the lifespan (Binomial). Although some treatment groups show statistically significant differences, comparisons based on these probabilities cannot be used to draw conclusions regarding lifespan.

As energy sources, appropriate amount of sugar should have beneficial effects on lifespan extension. However, high sugar (>2%) in the diet is detrimental to the *C. elegans* and reduces lifespan [62, 68-70]. The dose-response curves with an A-shape were seen in PJ and POMx treated animals. We were puzzled by the fact that the lower doses of PJ increased lifespan, while the higher doses decreased the lifespan. We wondered if this might be related to PJ's high sugar content. We found that the reversal of the lifespan extension by PJ was at a dose that contained 0.4% sugar (Table 7). PJ has a high content of sugars in which the fructose is 1.25-fold greater than that of glucose which, in turn, is 4.7-fold higher than that of mannitol [71]. In agar dish culture, extra glucose (>0.001% or 2%) reduces lifespan mainly due to reducing signals of the DAF-16/FOXO and heat shock transcription factor (HSF-1) gene signaling [72-76]. The effect of fructose, however, is controversial. Supplemental fructose reduces the plasma glucose level, glycohemoglobin, serum cholesterol, triglycerides, lactate, and body weight in type 2 diabetes [75, 77]. Many epidemiological studies link the consumption of high dietary fructose usage to an increased prevalence of obesity, which has been reported with lack of sufficient evidence. Since associations do not show cause and effect, clinical trials are needed to support this hypothesized cause for

obesity [78-80]. The A-shape dose-response relationship with the lifespan in the present studies appears to be related to the increasing sugar content. On the other hand, the level of sugar tolerance may be specie-dependent, since a diet supplemented with PJ (10%) increase lifespan of flies [24]. The data also implies involvement of the *daf-16* pathway, since the reduced lifespan was observed only at the highest dose in the *daf-16* mutant group. The A-shape curve of PJ showed the longest lifespan to be at a sugar content of 0.13% in N2 and 0.66% in the *daf-16* mutant. The fact that PJ increased lifespan of the *daf-16* mutant in our study may indicate alternative mechanism. The lifespan curve shifting rightward might indicate an "independent or compensatory effect of the *daf-16* pathway".

Table 7. Sugar content of the PJ feeding media

Doses [%]	0	0.01	0.1	1	3	5	10	25
Sugar [%]	0	0.001	0.013	0.13	0.39	0.66	1.31	3.28

Fat storage is one of the outcomes of energy consumption and energy expenditure in living organisms. In *C. elegans*, the fluorescent intensity of IFD in the PJ group was reduced in both N2 and the *daf-16* mutant. This inverse relationship of lifespan and fat content (PJ 1%) is in agreement with studies that PJ is effective in reducing cardiovascular risk factors in overweight humans [81].

POMx, extracted from PJ, which is characterized by rich polyphenols extracted from pomegranate, extended the lifespan in a similar manner as PJ with an A-shape curve in N2. POMx in the *daf-16* mutant extended lifespan by several days (Supplement Table 8b). Unlike PJ, POMx did not reduce lifespan at higher doses. These results may be attributed to either an absent or reduced sugar content and/or other unknown factors. The effect of POMx on lifespan extension was only half the magnitude of the lifespan extension induced by PJ, which suggests that multiple factors in the PJ products extend lifespan as was suggested by the Heber's studies [82]. Also unlike PJ, a V-shape dose-response curve was detected for the IFD in POMx treated animals at the doses that were used in this study, and only a minimal reduction of IFD was observed ($P>0.05$). The reversal of the pattern of increase in lifespan extension and decrease

in IFD occurred at 80 μg/ml, which seems to relate to the sugar content as it did in PJ treated group. Elevating IFD in the *daf-16* mutant suggests the involvement of this pathway controlling body fat accumulation.

Unlike either PJ or POMx, EA did not alter lifespan in N2 or in the *daf-16* mutant at the doses that was used. Lifespan extension was observed on several days of the experiment, which is similar with a study in N2 in agar culture that the EA (50 μM at 0.3% DMSO) prolonged mean lifespan [83].

DMSO is a solvent that extends lifespan dose-dependently in N2 with an "A-shape " curve, in agar culture (24% at 0.05% to 5%, v/v) [84] and in a liquid culture (20% at 0.9%, v/v) [85]. In the present study, DMSO doses were limited to the minimum. The lower DMSO dose (0.05%) extended lifespan up to 12.7%, while the higher amount (0.1%) decreased the lifespan which is different from the observations of the Wang group [84], which observed a 30% reduction in lifespan in the *daf-16* mutant (9.4%). The effect of EA on extending lifespan suggested complex mechanisms involving *daf-16* that is known as the gene for lifespan in *C. elegans*. EA also dose-dependently increased IFD at higher doses in N2 with parallel findings in the *daf-16* mutant. The enhanced IFD in the *daf-16* mutant uncovered the important role of the *daf-16* on lipid/energy metabolism, which was not seen in the PJ treated groups.

Like all polyphenols, EA's large molecular weight and hydrophilic properties could have limited the absorption in the intestine as well as the bioavailability to the hosts. Since the main metabolites of EA have many beneficial effects, the effects of UA which is a metabolite of the colonic microbiota that has a higher absorption index has attracted extensive research in recent years [86]. UA has anti-inflammatory, anti-carcinogenic, anti-glycative, anti-oxidant, and anti-microbial effects [86-88]. In our study, some of the treatment groups showed a statistically significant increase in mean survival probabilities in the presence of UA. Although one cannot draw conclusions from these observations, it is possible that higher doses of UA may alter lifespan, and the *daf-16* mutant pathway is predicted to mediate the effect of UA on lifespan. UA also dose-dependently increased IFD at higher doses in both N2 and in the *daf-16* mutant,

in parallel, with the latter having half the increase in IFD.

The antioxidant potency of PJ is higher (by at least 20%) compared to other polyphenol-rich products including red wine, Concord grape juice, blueberry juice, black cherry juice, cranberry juice, orange juice, iced tea beverages, and apple juice *in vitro* [89]. Thus, PJ has superior antioxidant bioactivity compared to its purified polyphenols, EA, punicalagin, or total pomegranate tannin. These conclusions are consistent with Seeram *et al.* who cited multifactorial effects and chemical synergy of the action of multiple compounds in PJ compared to single purified active ingredients [82].

EA content varies by variety of the fruit, and many commercial pomegranate extracts were found not to contain the same amount of EA [42]. We used a commercially available PJ product [90-93], described in a full analysis of 477 commercial PJ in the market across North America and Asia [94]. Although studies in humans the pharmacokinetics of EA and UA that are equivalent [65], our study showed, as previously reported, that PJ was the most potent in extending lifespan and reducing IFD with POMx being only half as potent. The total phenolic content, by organic phenolic acid gallic acid equivalent (GAE), are 4.7-fold higher in PJ (2,825 μg/ml GAE) than in that of POMx (606 μg/mg GAE) [25]. In the present study, the estimated GAE (μg/ml) by calculation was also directly related to the lifespan extension at lower doses by PJ or POMx, which was reduced by higher doses (supplement Table 9, Table 10 & Figure 50). Thus, the fact that PJ was more potent than POMx may be beyond different amount of polyphenols but the synergy of various components rather than, for example, EA only [95]. The lower or lack of effect of other tested substances compared to PJ suggest that some effective compounds may be reduced or absent, and multifactorial effects were present.

Our data from the *C. elegans* model indicate that the extension of lifespan by pomegranate is partially dependent on the *daf-16* pathway, but mechanisms other than those involving polyphenols such as EA and its metabolites including UA may play a role as well. The unique effects of PJ in reducing IFD suggest that the effects are not simply attributed to one component. The results of the *C. elegans* studies

provide useful information and suggest that, at the proper dilution, PJ may have optimal health benefits including control of fat storage, prevent obesity and offer a solution to delay aging in humans.

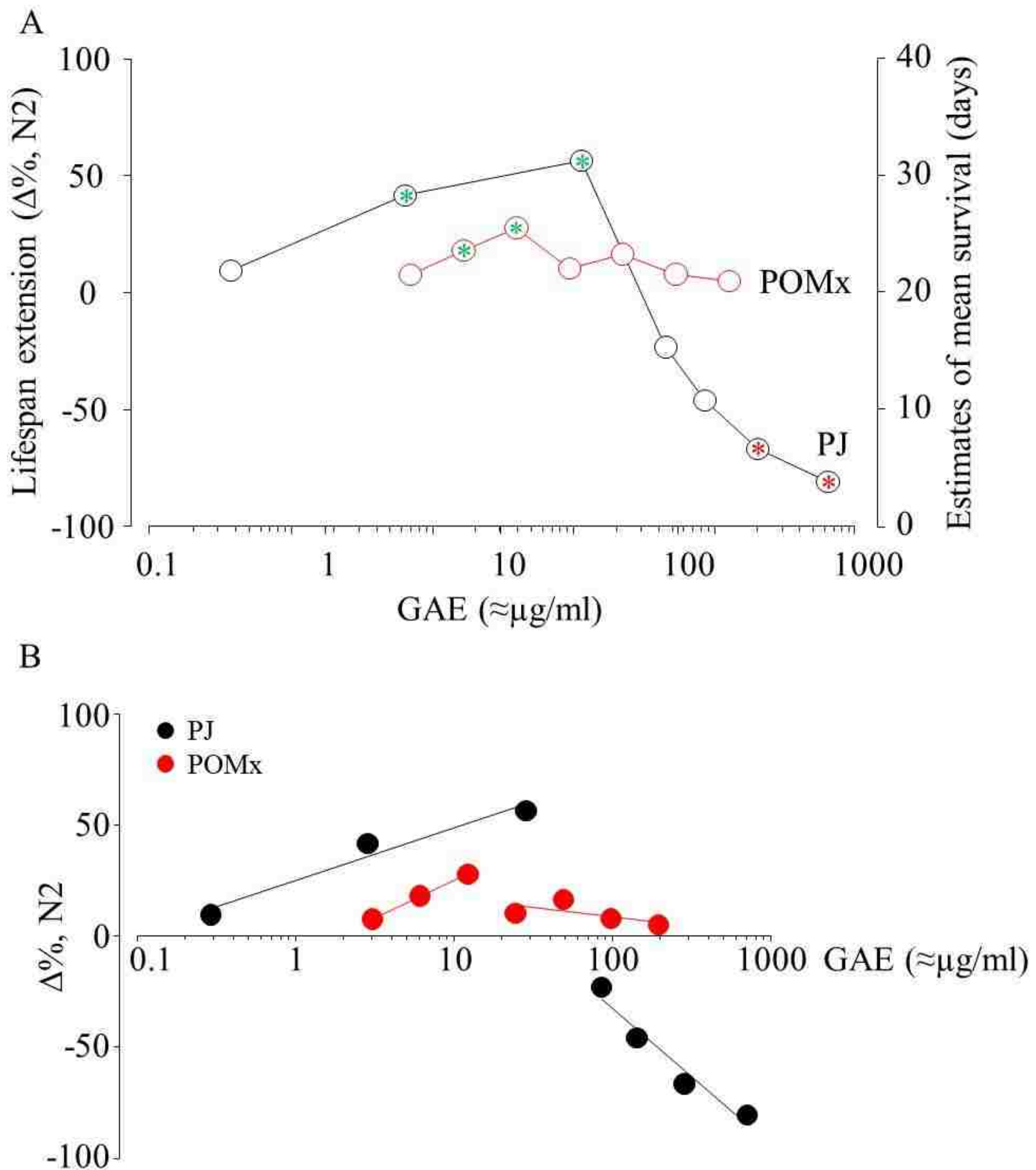

Figure 50. The estimated GAE (μg/ml) by calculation was also directly related to the lifespan extension at lower doses by PJ (black) or POMx (red) (a). The correlation of estimated GAE with lower doses of PJ (black) and POMx (red) (b). The correlation with higher doses of PJ that reduced lifespan and POMx that did not (b).

1.5 Supplementary materials

Table 8. Additional statistics for lifespan ($P<0.05$)

a. PJ treated group

Days/Dose (%)	0.01	0.1	1	3	5	10	25
N2				16	16		
				30			
	44			44	44		
daf-16	20	20			20	20	20
			36	36	36	36	36
			52	52	52	52	52
			67	67	67	67	67

b. POMx treated group

Days/Dose (μg/ml)	5	10	20	40	80	160	320
N2			15				
	27	27	27	27	27	27	27
	38	38	38	38	38	38	38
		49	49	49	49	49	49
daf-16	25	25		25			25
	37	37		37			37
	46	46		46			46

c. EA treated group

Days/Dose (μM)	DMSO	1	2	5	10	25	50
N2							
daf-16		11					
							21

d. UA treated group

Days/Dose (μM)	DMSO	1	2	5	10	25	50
N2						15	15
		26		26			
daf-16				13		13	
		22				22	
		31					
		39					

Table 9. Calculated GAE content in the doses of the PJ and POMx used in this study

PJ [%]	Calculated GAE [μg/ml][*]	POMx [μg/ml]	Calculated GAE [μg/ml][*]
0.01	0.2825	5	3.03
0.1	2.825[**]	10	6.06[**]
1	28.25[**]	20	12.12[**]
3	84.75	40	24.24
5	141.25	80	48.48
10	282.5[***]	160	96.96
25	706.25[***]	320	193.92

[*]Calculation was based on [25]
[**]Increased lifespan ($P<0.05$)
[***]Reduce lifespan ($P<0.05$)

Table 10. Correlation of estimated GAE with the dose-responses (A-shape)

	Low doses		High doses	
PJ	$y = 0.1388\ln(x) - 0.0502$	$R^2 = 0.9999$	$y = 0.1452\ln(x) - 0.1856$	$R^2 = 0.9995$
POMx	$y = -0.267\ln(x) + 0.8962$	$R^2 = 0.9485$	$y = -0.069\ln(x) + 0.4027$	$R^2 = 0.7102$

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CHAPTER V. CONCLUSIONS

In a recent Danish report, patients who had vagal nerves removed 20 years earlier had 40% lower incidence of PD than control populations. If dietary proteins were one potential etiology for PD, by transport to neurons from the gut, as hypothesized here, removal of the vagal nerve would have prevented or reduced this etiology pathway. Symptoms of motor impairment are typical in PD patients, and dysfunction of aspects of the autonomic nervous system are often underrated, such as GI motility, rapid eye movement, *etc*. The current study indicates potential transport of some dietary plant lectins from the GI tract to the DAergic neurons in *C. elegans*, with direct or indirect effects on these neurons and diverse effects on APS. This observation may be related to the Braak and Hawkes' hypothesized unknown etiologic agent for PD or related, for example, to damaged DAergic neurons those have been found in PD. If related, the process may be gradual, may be additive, related to the frequency of consumption of certain lectins, and may be determined by the association of lectins with other factors. Certainly, there is potential for inputs from individual genetic susceptibility, varying sugar structures profiles in different cell membranes, the receptivity to endocytosis, a disorder or leakage of the GI lining, and dietary content. Our observations are a tantalizing possible explanation for why dietary plants have been linked to a risk of developing PD.

Our data from the *C. elegans* model indicate that the extension of lifespan by pomegranate is partially dependent on the *daf-16* pathway, but mechanisms other than those involving polyphenols such as EA and its metabolites including UA may play a role as well. The unique effects of PJ in reducing IFD suggest that the effects are not simply attributed to one component. The results of the *C. elegans* studies provide useful information and suggest that, at the proper dilution, PJ may have optimal health benefits including control of fat storage, preventing obesity and offering solutions to delay aging in humans.

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Dear editor,

Our manuscript titled "**Dietary plant lectins appear to be transported from the gut to gain access to and alter dopaminergic neurons of *Caenorhabditis elegans*, a potential etiology of Parkinson's disease**" is in the stage of author's proof.

I am a graduate student in Louisiana State University and I want to include some data from the manuscript into my dissertation, would you please guide me what procedure do I need to get a permission for this?

Thank you and best regards,
Mingming

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VITA

Mingming Wang was born in Anqing, Anhui Province in China, in 1985. He graduated from Capital Medical University (Beijing, China) in 2007 and received a Bachelor of Science degree, majoring in pharmacy, and a Master of Science degree majoring in pharmaceutics in 2010. He started his first job as a clinical research associate in Venturepharm, a local contract research organization from 2010-2011, then in George Clinical from 2011-2014 before joining Louisiana State University to pursue a doctoral degree majoring in Food Science. He anticipates graduating with his Ph.D. degree in May 2016.