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Fluoxetine protects against amyloid-beta toxicity, in part via *daf-16* mediated cell signaling pathway, in *Caenorhabditis elegans*

Roongpetch Keowkase¹, Marwa Aboukhatwa¹, and Yuan Luo^{1,2,*}

¹Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland Baltimore, MD 21201. USA

²Center for Integrative Medicine, School of Medicine, University of Maryland Baltimore, MD 21201. USA

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and is the most common form of dementia in elderly people. The accumulation of amyloid β ($A\beta$) is one of the histopathological hallmarks of AD. $A\beta$ is aggregated to form oligomers which are toxic to neurons and are critical to the onset and progression of AD. In a *Caenorhabditis elegans* (*C. elegans*) model of AD, human $A\beta$ is expressed intracellularly in the body wall muscle. The expression and subsequent aggregation of $A\beta$ in the muscle lead to progressive paralysis. Although the mechanism of action is unknown, antidepressants have been used with FDA approved drugs for dementia in AD and have been shown to enhance cognitive function in human and in animal models of AD. We found that the antidepressant fluoxetine, a selective serotonin reuptake inhibitor, significantly delayed $A\beta$ -induced paralysis in the *C. elegans* model of $A\beta$ toxicity by reducing $A\beta$ oligomers. Our result showed that insulin signaling and DAF-16/FOXO transcription factor were required for fluoxetine-mediated delayed paralysis. We also found that fluoxetine increased thermal stress resistance and extended life span. This finding suggests that fluoxetine may have benefit for the treatment of AD by reduction of proteotoxicity.

Keywords

fluoxetine; Alzheimer's disease; amyloid β ; selective serotonin reuptake inhibitor; *C. elegans*

1 INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and is the most common form of dementia in elderly people. The treatment of patients with AD is only symptomatic treatment with the available FDA approved drugs which are acetylcholinesterase inhibitors (donepezil, rivastigmine, galantamine) and N-methyl-D-aspartate receptor antagonist memantine. These drugs have a limited efficacy and are

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*Correspondence: Yuan Luo, Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, 20 Pine St, PH501, Baltimore, MD 21201, Phone : (410) 706-6639, Fax: (410) 706-4376, yluo@rx.umaryland.edu.

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considered to be effective only in a short period of time ranging from 6 to 12 months (Shah et al., 2006; Raina et al., 2008). The formation of β -amyloid plaques is one of the key causes of the disease (Hardy et al., 2002). A β aggregation is toxic to neurons and eventually leads to clinical symptoms characterized by progressive and irreversible dementia.

AD affects not only cognitive function, but also other behaviors. Increasing evidence suggests that depression is a common psychiatric manifestation in AD patients. There is a high prevalence rate (30-50%) of Alzheimer's disease (AD) and depression comorbidity (Lee et al., 2003). Since depressive symptoms are commonly detected before AD patients manifest cognitive deterioration or are clinically diagnosed (Danion, 1993; Geerlings et al., 2000; Visser et al., 2000), antidepressants may serve as a rational complementary therapy for the treatment of Alzheimer's disease (Aboukhatwa et al., 2010). In the recent years, clinical studies showed positive role of chronic administration of selective serotonin reuptake inhibitor (SSRI) antidepressants in hindering the progression of the AD and improving patient cognitive performance (Altman et al., 1987) either alone or in combination with other FDA approved drugs for treatment of AD (Lai et al., 2002; Finkel et al., 2004; Vythilingam et al., 2004; Schmitt et al., 2006; Mowla et al., 2007; Thompson et al., 2007).

Citalopram, an SSRI, has shown significantly improvement in the score of depressed patients in the Clinical Global Impression Scale (CGIS) and Montgomery Asberg Depression Scale (MADRS), as well as improvement of the emotional and cognitive functions in subgroup of patients who suffer from dementia (Nyth et al., 1992). Drug combination trials reported that patients treated with rivastigmine, a FDA proved drug for AD plus fluoxetine, a SSRI, had better performance in daily life activities and overall function which highlights some of the benefits that may be obtained by adding a serotonin regimen to FDA approved drugs for AD patients (Mowla et al., 2007). Another study also reports the beneficial outcome of combining sertraline (SSRI) with donepezil treatment especially for AD patients with moderate to severe dementia (Finkel et al., 2004). Among currently available antidepressants, selective serotonin reuptake inhibitors (SSRIs) are commonly prescribed to patients with AD to relieve depression, anxiety and agitation (Nyth et al., 1990; Pollock et al., 1999). SSRIs are also frequently used concomitantly with acetylcholinesterase inhibitors (AChEIs) in patients with AD (Pollock et al., 1999). In addition, SSRIs may exert some degree of protection against the negative effects of depression on cognition in AD patients treated with AChEIs (Rozzini et al., 2010). SSRIs increase extracellular serotonin (5-HT) concentration by inhibiting serotonin reuptake transporter and is thought to improve depressive symptom through compensation for a 5-HT deficit in AD. Currently available SSRIs include fluoxetine, sertraline, paroxetine, fluvoxamine, citalopram and escitalopram. Pre-clinic studies also demonstrated restore of cognitive impairments and retards the development of amyloid and tau pathologies in 3xTgAD mice (Duman, 1998). However, the mechanism of action is unknown.

Fluoxetine has emerged as the treatment of choice for depression due to a better safety profile, and fewer side effects compared to tricyclic antidepressants. Moreover, fluoxetine stands out among other SSRIs for its adverse event profile and requirement for dosage titration (Wilde et al., 1998). Fluoxetine may also have therapeutic potential as neuroprotective and disease-modifying agent. There are several studies demonstrating that fluoxetine increased neurogenesis in the hippocampus (Jacobs et al., 2000; Malberg et al., 2000; David et al., 2009). Furthermore, fluoxetine enhanced memory and cognition in the patients with mild cognitive impairment (Mowla et al., 2007). However, the underlying mechanisms of its efficacy remain unclear.

The nematode model organism *Caenorhabditis elegans* (*C. elegans*) is an important model to study the molecular mechanisms of drug action and disease pathogenesis. For example, some behaviors of *C. elegans* such as egg laying have been used as a simple genetic system for identification of the action of fluoxetine and imipramine (Dempsey et al., 2005). *C. elegans* has been used as a model for various age-associated neurodegenerative diseases, including Alzheimer's (Link, 1995; Link, 2001), Parkinson's (Nass et al., 2002; Lakso et al., 2003) and Huntington's diseases (Faber et al., 1999). There is strong genetic conservation between *C. elegans* and mammals as demonstrated by the fact that 60-80% of human gene have been identified in *C. elegans* (Kaletta et al., 2006). The transgenic *C. elegans* model of AD has been developed by expressing human A β in the muscle. The expression and subsequent aggregation of A β in the muscle lead to progressive paralysis (Link, 2006).

In *C. elegans*, the insulin/IGF-1 signaling pathway controls many biological processes such as lifespan, metabolism and stress response. This pathway is comprised of many proteins including insulin/IGF-1 receptor (DAF-2), PI 3-kinase (AGE-1) and FOXO transcription factor (DAF-16). In mammals, DAF-16 homologues are forkhead (FOXO) transcription factors, of which specific functions have been identified for multiple FOXO proteins such as FOXO1a and FOXO3a (Kuningas et al., 2007). Some FOXO proteins initiate stress-induced apoptosis, which eliminate damaged dysfunctional cells. FOXO proteins also up-regulate antioxidant defense and DNA repair-facilitating gene (Vijg et al., 2008). Recently it was reported that modulation of the insulin/IGF-1 signaling pathway increases thermotolerance and delays the onset of A β toxicity in *C. elegans* expressing human A β (Cohen et al., 2006). In this study, we aim to determine if fluoxetine protects against A β toxicity in transgenic *C. elegans* expressing human A β . We found that fluoxetine significantly delayed A β -induced paralysis in the *C. elegans* model of A β toxicity by reducing A β oligomers. In addition, DAF-16/FOXO transcription factor was required for fluoxetine protection against A β toxicity in *C. elegans*. These findings suggested that some antidepressants used in AD patients might have additional therapeutic benefits beyond their efficacy to ameliorate depressive symptoms.

2. MATERIALS AND METHODS

2.1 Strain

The wild type *C. elegans* strain N2, the transgenic strain CL2006, TJ356 and *daf-16* were obtained from the Caenorhabditis Genetic Center (University of Minnesota).

Maintenance of all strains was routinely performed at 20°C on Nematode Growth Medium (NGM) plates with *Escherichia coli* strain OP50 as a food source as previously described (Brenner, 1974).

2.2 Drug treatment

Fluoxetine (fluoxetine hydrochloride; Sigma-Aldrich, USA), citalopram (citalopram hydrobromide; Sigma-Aldrich, USA) and paroxetine (paroxetine hydrochloride hemihydrates; Sigma-Aldrich, USA) were added to the OP50 bacteria to a desired final concentration. The treatment was given to the transgenic worm from egg stage onward.

2.3 Paralysis assay

Transgenic *C. elegans* strain CL2006 maintained at 20°C was egg-synchronized onto the 35×10 mm culture plates containing either a vehicle or drug. The worms were tested everyday for paralysis. To identify the paralysis, each worm was gently touched with a platinum loop. The worm was considered paralyzed if it did not move or moved head only after touching.

2.4 Thermotolerance assay

Wild-type (N2) and *daf-16* *C. elegans* at 3 days of age was incubated at 35 °C. At 7 hr plates were removed and worms were scored as alive or dead. Worms failing to respond to the gentle touch with platinum loop were scored as dead.

2.5 Life span assay

Synchronized eggs of N2 *C. elegans* were maintained at 20 °C on the 35×10 mm culture plates (~35 eggs/plate) containing vehicle or fluoxetine. Once the worms reached to young adult stage, they were shifted everyday to fresh plates until they stop laying egg. Then they were shifted to fresh plates every 2 days. The life span was counted from adult day 1 onward. The worm was considered death if it did not move at all after gently touch with the platinum loop.

2.6 Subcellular DAF-16 localization

Subsequent to the treatment, living worms were placed on microscope slides, capped with cover slips and the subcellular DAF-16 distribution was analyzed by fluorescence microscopy. TJ356 worms were classified into three categories (cytosolic, intermediate and nuclear) with respect to the major localization of the DAF-16::GFP fusion protein.

2.7 RNA Interference (RNAi)

RNAi was performed in *C. elegans* by feeding the worms with dsRNA-containing bacteria. *C. elegans* was fed with *E.coli* HT115 strains expressing dsRNA specific to *daf-16* or *hsf-1* or *tph-1* gene. After 3-4 h, worms were removed and eggs were permitted to mature to L4 young larvae. The L4 larvae were transferred to another plate containing dsRNA and allowed to lay eggs. The resultant adult worms were used for the paralysis assay.

2.8 Dot blot of A β species

After the experimental treatments, the worms were collected by washing with M9 and distilled water, quickly frozen in liquid nitrogen, homogenized in the cell lysis buffer (25 mM Tris 7.5, 5 mM NaCl, 1 mM DTT, 5 mM EDTA) with protease inhibitor cocktail (sigma, Saint Louis, MO). Equal protein samples (2 μ g) were applied to nitrocellulose membrane (Bio-rad, Hercules, CA). The membranes were probed with a primary anti-A β oligomer antibody NU-4 (1:1,000) (Lambert et al., 2007) or anti-A β 42 antibody 6E10 (1:1,000) followed by corresponding secondary antibodies.

2.9 Western blotting of A β species

The A β species in the transgenic *C. elegans* strains was identified by immunoblotting using a Tris-Tricine gel and the standard Western blotting protocol. After the experimental treatments, the worms were collected by washing with distilled water, quickly frozen in liquid nitrogen, sonicated in cell lysis buffer (50 mM HEPES, pH 7.5, 6 mM MgCl₂, 1 mM EDTA, 75 mM sucrose, 25 mM benzamide, 1 mM DTT, 1% Triton X-100) with protease inhibitor cocktail (sigma, Saint Louis, MO). The samples were then heated with loading buffer containing 5% β -mercaptoethanol (2:1; Bio-Rad, Hercules, CA). After mixing with the loading buffer, proteins were unheated and loaded on the gel. Equal amounts of the total protein (50 μ g) were loaded in each lane. The membranes were probed with a primary anti-A β oligomer antibody NU-4 (1:1,000) (Lambert et al., 2007) or anti-A β 42 antibody 6E10 (1:1,000) followed by corresponding secondary antibodies.

2.10 Cell culture

2.10.1 N2a cells (A β expressing mutated cell line)—This cell line expresses a Swedish mutation in APP 695 and another mutation in PS1 where exon-9 is deleted. These double mutations are similar to the one seen in early stage familial Alzheimer's disease. N2a cells produce high levels of amyloid peptide. This cell line is a gift from Dr H Xu at University of California in San Diego, CA, USA. This cell line is used to test the effect of fluoxetine on the levels of amyloid species intracellularly and extracellularly (in the medium).

2.10.1 Western blotting—N2a cells were maintained in non stimulating medium (47% Dulbecco's modified eagle medium, 5% fetal bovine serum, 47% opti-MEM, 0.2 mg/ml G418 and 1% antibiotics) at 37°C and 5% CO₂/95% O₂. When the cells reach 80% confluency, they were shifted to stimulating medium (50% Dulbecco's modified eagle medium, 0.5% fetal bovine serum, 50% opti-MEM, 0.2 mg/ml G418, 1% antibiotics and 10 mM sodium butyric acid). N2a cells were treated with increasing concentration of fluoxetine for 24 hr. After 24 hr, the cells were washed twice with cold phosphate buffer saline (PBS) and then collected from the plates. The cells were lysed by sonication in lysis buffer (75 mM Tris-HCl, 1 mM DTT, 50 mM NaCl, 1 mM EGTA and 5 mM EDTA) with added protease inhibitor cocktail. The undisturbed cells were removed by centrifugation (12,000 g, 15 min, at 4 °C). Equal amount of protein (50 μ g) from each sample were loaded in each lane. The membranes were probed with a primary anti-A β ₄₂ antibody 6E10 (1:1,000) followed by a secondary anti-mouse IgG antibody (1:2,000).

2.11 APP and PS1 double transgenic mouse model of AD

These mice are heterozygous double transgenic mice (APP^{swe}/PS1 Δ 9) that have been purchased from Jackson laboratory. The mice are conserved by cross breeding the transgenic mice with the wild type (B6C3) and then the offspring is genotyped. This mouse model is used in western blotting.

2.12 Statistic analysis

Data are represented as mean \pm SEM. Significant differences between groups were assessed by unpaired t test. A *p* value < 0.05 was considered statistically significant. GraphPad Prism 5 software was used for the paralysis analysis. *P* value calculations were made between treated and untreated animals using the log-rank (Mantel-Cox) test.

3. RESULTS

3.1 Fluoxetine and paroxetine but not citalopram, delays A β -induced paralysis in *C. elegans* strain CL2006

According to the amyloid hypothesis, AD is thought to be caused by the production and deposition of neurotoxic A β -peptide in the brain (Blennow et al., 2006). The deposition of A β in the brain leads to many consequences such as the formation of neurofibrillary tangles, oxidative stress, glutamatergic excitotoxicity, inflammation, neuronal cell death and eventually the clinical symptoms of AD (Cummings, 2004; van Marum, 2008). In transgenic *C. elegans* model of AD, human A β ₄₂ protein has been expressed intracellularly in the body wall muscle and the expression and subsequent aggregation of A β in the muscle lead to progressive paralysis (Link, 1995; Link, 2006). To investigate the protective effect of fluoxetine, the worm strain CL2006 which produces A β constitutively in the muscle was used. The worms were treated with fluoxetine at concentration of 1, 10, 100 and 1,000 μ M (fig 1A). Fluoxetine at 10 and 1,000 μ M significantly delayed A β -induced paralysis in this transgenic worm (*p* = 0.02 and 0.0001 respectively). As a comparison, paroxetine and citalopram, other selective serotonin reuptake inhibitors, were also tested. Paroxetine at dose

100 nM significantly delayed A β -induced paralysis ($p = 0.05$) (fig 1C). However, citalopram at dose up to 2,000 μ M did not delay A β -induced paralysis (fig 1B) ($p = 0.29$).

3.2 Fluoxetine reduces A β oligomer in transgenic *C. elegans* and N2a neuroblastoma cells

Growing evidence suggests that amyloid plaques do not always correlate with neurodegeneration and cognitive decline (Resende et al., 2008). In stead, the concentration of soluble A β (oligomers) is well correlated with cognitive deficits (McLean et al., 1999). To determine whether fluoxetine treatment affected the toxic species A β oligomer in the worm we measured A β oligomer using an oligomer specific antibody NU4. Fluoxetine 1 mM showed a trend of decreased levels of A β oligomer in comparison with the untreated control, suggesting that fluoxetine may alleviated A β toxicity by reducing A β oligomers (fig 2A; $p = 0.09$, $n = 3$). However, fluoxetine 1 mM did not decrease total A β as shown in figure 2B ($p = 1.2$, $n = 3$), supporting a role of fluoxetine in specifically inhibition of toxic species of A β . We also analyzed A β oligomer in *C.elegans* fed with 1 mM fluoxetine by Western blotting using antibody against A β oligomers (NU-4). Fig 2C shows A β -immunoreactive band (MW ~6-53 kDa) detected in the tissues from transgenic worms (CL2006) fed with 1mM fluoxetine or 100nM paroxetine, another SSRI antidepressant drug as a control. The treatment of CL2006 *C.elegans* strain with two selective serotonin reuptake inhibitors (fluoxetine and paroxetine) results in reduction in A β oligomers species with molecular weight ~ 12 and ~53 kDa (Fig 2C).

The brain extract from wild and double transgenic APP/PS1 mice was used as a positive control for the endogenous A β oligomers. *C.elegans* was treated with 1mM fluoxetine or 100nM paroxetine from the egg stage of the worm to day one of the adulthood (3-4 days). The doses of drugs used in this experiment are determined based on the effective concentration of the drugs in paralysis assay (Fig 1). In this experiment, Nu4 antibody which is specific for A β oligomers was used.

The effect of Fluoxetine on A β oligomers was further confirmed by using N2a cells that express A β transgene. The effect of increasing concentrations of fluoxetine (0.1-10 μ M) on intracellular A β oligomers in N2a cells was tested using Western blotting with 6E10 antibody to determine the effect on different A β species (Fig 2D). The results showed that 10 μ M fluoxetine treatment resulted in significant reduction of A β oligomers in N2a cells. One-way ANOVA showed that there was a significant difference for the fluoxetine treatment ($p=0.0230$). *posthoc* Dunnett test shows that 10 μ M fluoxetine treatment caused a significant reduction in A β oligomers in comparison to the control ($p < 0.01$).

3.3 DAF-16 and TPH-1 are required for the protective effect of fluoxetine on delaying paralysis

It has been revealed recently that DAF-16, HSF-1 and insulin signaling pathway play a role in the protection against A β toxicity (Cohen et al., 2006; Cohen et al., 2008). It is also well known that *C. elegans* FOXO transcription factor DAF-16 is a key mediator for regulating longevity and stress resistance (Mukhopadhyay et al., 2006; Murphy, 2006; Schaffitzel et al., 2006). To test whether DAF-16 and HSF-1 are required for the protective effect of fluoxetine against A β toxicity, we performed the experiment by using RNAi knockdown of DAF-16 or HSF-1 expression. We found that fluoxetine at 1 mM significantly delayed A β -induced paralysis in worms grown on bacteria containing empty vector (fig 3A, $p = 0.01$) but not on *daf-16* RNAi bacteria (fig 3B, $p = 0.1$). This result indicated that reducing the activity of DAF-16 abolished the protective effect of fluoxetine, suggesting requirement of DAF-16 for protective effect of fluoxetine. On the other hand, fluoxetine at 1 mM still significantly delayed A β -induced paralysis in worms fed with *hsf-1* RNAi bacteria (fig 3C, $p = 0.001$), indicating that HSF-1 is not required for the protective effect of fluoxetine. In

addition, we knocked down *tph-1* gene which is responsible for transcription of tryptophan hydroxylase. This enzyme is a rate-limiting step enzyme for the synthesis of serotonin. Because fluoxetine blocks the reuptake of serotonin into the cell and increases serotonin in synaptic cleft, so we test if serotonin is required for the protective effect of fluoxetine. We found that fluoxetine did not delay paralysis in worms fed with *tph-1* RNAi bacteria (fig 3D, $p = 0.17$) when compared to control, indicating that serotonin is required for the protective effect of fluoxetine.

3.4 Fluoxetine, but not citalopram, induces the translocation of DAF-16 from cytosol to nucleus

The localization of DAF-16 in the nucleus is an essential prerequisite for its ability to initiate the transcription of target genes. To further confirm the involvement of DAF-16 in fluoxetine-reduced A β toxicity, we examined the effect of fluoxetine on subcellular distribution of DAF-16 using the transgenic *C. elegans* strain TJ356. This strain of worm carries a fusion construct of DAF-16 coupled to GFP as a reporter gene allowing the determination of the subcellular localization of this transcription factor. The worms can be scored in three stages; 1) for cytosolic localization, fluorescences are evenly distributed throughout *C. elegans* body (Fig. 4A), 2) for nuclear localization, the distribution of the fluorescence is enclosed in the nucleus as arrow shown in fig 4B, 3) for intermediate localization, the fluorescences are distributed in both nuclear and cytosole. Eighty percent of the control group showed a cytosolic localization of DAF-16 and only small fraction showed an intermediate localization (fig 4B). Fluoxetine 1 mM induced the translocation of DAF-16 from cytosol to the nucleus by increasing the percentage of worms with intermediate DAF-16 localization by 92.38%. This result indicated that fluoxetine has partial effect on the translocation of DAF-16 from cytosol to nucleus. On the other hand, citalopram 2 mM did not alter the subcellular distribution of DAF-16 (fig 4C). The observation that fluoxetine caused a translocation of DAF-16 suggested that fluoxetine might affect insulin signaling by regulating the activity of DAF-16.

3.5 Fluoxetine increases thermal stress resistance and extends life span in wild type *C. elegans*

Insulin signaling pathway regulates aging, stress resistance and neurodegeneration (Cohen et al., 2008). To determine if fluoxetine specifically delay A β -induced paralysis, but not physiological wellness, we further evaluated whether fluoxetine could protect against heat stress and prolong survival. We tested the effect of fluoxetine on thermal stress resistance by exposing the worm to a higher temperature (35°C). Worm normally live at 20-25°C, when exposed to 35°C, they will die gradually within 7-8 hr. We found that worms fed with fluoxetine (1 mM) showed 38% increase in thermal stress resistance and the effect was lost in *daf-16* mutation worms (Fig 5A). These results suggest that thermal protection by fluoxetine required DAF-16. Survival assays was also conducted in worms fed with or without increasing concentrations of fluoxetine. In worms fed with 100 μ M fluoxetine, there was a significant prolongation of mean life span of the *C. elegans* ($p=0.04$, $n=70$).

4. DISCUSSION

In the present study, we found that fluoxetine delayed A β -induced paralysis in transgenic *C. elegans* model of A β toxicity. We also found that paroxetine, another SSRIs, which has been reported to improve cognitive performance in 3xTgAD mouse model of AD and reduce the concentrations of A β in the hippocampus (Nelson et al., 2007), delayed A β -induced paralysis. However, this benefit does not apply to all SSRIs because citalopram did not delay A β -induced paralysis. Furthermore, A β oligomers were reduced in the transgenic worm treated with fluoxetine. It is well recognized that A β oligomers are toxic to neurons

and are critical to the onset and progression of AD and that A β oligomers are particularly damaging to the synapse (Lambert et al., 1998; Lacor et al., 2004; Lacor et al., 2007). Therefore, our result suggested that fluoxetine ameliorated A β toxicity in transgenic *C. elegans* by reducing the toxic form of A β . Fluoxetine only affected on A β oligomers but not on total concentration of A β . The potential reason is that A β species are at a dynamic balance between monomer, oligomers and fibrils. Our previous results shown that when A β oligomers were reduced by EGb 761, the levels of A β monomers were enhanced (Wu et al., 2006). This could be one of the explanations why the total concentrations were unchanged.

We further investigated the possible mechanisms underlying the reduction of A β oligomers in *C. elegans* by fluoxetine. There is evidence from human and different model organisms indicating a role of insulin receptor/IGF-1 receptor signaling in A β metabolism and clearance (Townsend et al., 2007; Zhao et al., 2008; Zhao et al., 2009). It has been recently shown that knocking down DAF-2 in *C. elegans*, the homolog of the mammalian insulin receptor/IGF-1 receptor, reduces A β ₁₋₄₂ toxicity (Cohen et al., 2008). The authors hypothesized that DAF-16 regulates the process that convert the toxic form (oligomers) to less-toxic form (high-molecular-mass aggregates). Our results indicated that DAF-16 is required for the protective effect of fluoxetine, which is further supported by our observation that fluoxetine induced, at least partially, the translocation of DAF-16 from cytosol to the nucleus. In contrast, citalopram did not affect translocation of DAF-16 which might explain why citalopram could not delay A β -induced paralysis. However, how DAF-16 mediates the protection by fluoxetine against A β toxicity remains to be elucidated.

Translation of results from extending life span in *C.elegans* to mammals is not straight forward. However, mammalian homologues of *C.elegans* daf-16, FOXO1 and FOXO3 genes variants were segregated with survival to age 85 and older, and in female, gene variants that reduce insulin/IGF-1 signaling are associated with longevity. Recently, heterozygous mutations in the IGF-IR were found overrepresented in centenarians (Vijg and Campisi, 2008), further validity that indeed *C.elegans* would be a model of importance for modulating this signaling and potentially predictive for other drugs.

Fluoxetine is a potent antidepressant that increases serotonin at the synapse via inhibition of the serotonin reuptake transporter. However, fluoxetine can act on additional targets other than the serotonin transporter (Kaletta et al., 2006). Because citalopram did not show the effect on delaying paralysis, this support the idea that fluoxetine might act on alternative target which is the insulin signaling pathway. In addition, although the SSRIs share a common primary pharmacology by inhibition of the serotonin reuptake transporter, their secondary pharmacology is remarkably heterogeneous (Carrasco et al., 2005), which may account for the different outcomes between fluoxetine and citalopram found in this study. For example, fluoxetine is a more potent inhibitor of 5-HT_{2C} receptor while citalopram has the highest affinity for histamine H₁ receptors compared to other SSRIs (Palvimaki et al., 1999; Owens et al., 2001). This might be an explanation why fluoxetine showed the effect on delaying paralysis whereas citalopram did not.

Fluoxetine might act directly or indirectly through the insulin signaling pathway, since Murakami et al. (2007) revealed an interaction between the serotonin pathway and the insulin/IGF-1 pathway (Murakami et al., 2007). Their study showed that a mutation of certain serotonin receptor genes increased longevity and was likely mediated through the insulin/IGF-1 pathway. Our results are in agreement with this finding because we found that serotonin was also required for the protective effect of fluoxetine. Nevertheless, additional investigation is needed to explore the mechanisms involved in the relationship between serotonin pathway and insulin/IGF-1 signaling pathway.

There are controversies about the interaction of insulin signaling and pathophysiology of Alzheimer's disease. It has been shown that insulin signaling has a protective role in prevention or slowing pathogenesis of Alzheimer's disease (Cole et al., 2007; De Felice et al., 2009) while insulin resistance increases the risk of Alzheimer's disease (Craft, 2007). In addition, insulin and insulin sensitizers improve cognitive and memory functions in animal models of Alzheimer's disease (Craft et al., 2003; Watson et al., 2005). In contrast, metformin, an insulin-sensitizer, significantly increases the generation of both intracellular and extracellular A β species (Chen et al., 2009). Impaired insulin signaling reduces A β accumulation and protects against premature death in a model of Alzheimer's disease (Freude et al., 2009). Our results are in agreement with this finding since fluoxetine reduced A β oligomer in part by working through DAF-16. The increase of the activity of DAF-16 was revealed by the impairment of insulin signaling by knocking down the insulin receptor (Cohen et al., 2006).

Other antidepressants have also been reported for their efficacy in reduction of A β toxicity. Most recently, imipramine, a tricyclic antidepressant, has been reported to prevent cognitive decline and A β accumulation in a mouse model of Alzheimer's disease (Chavant et al., 2009). Taken together, these findings and our results provide insight that some SSRIs and tricyclic antidepressant might have disease-modifying properties in addition to providing depressive symptoms relief.

In conclusion, this study is the first to demonstrate that fluoxetine can delay A β -induced paralysis in a model organism. The mechanism underlying its protective efficacy was shown in part through DAF-16 and insulin signaling pathway. However, an additional investigation is needed to better understand the mechanisms underlying the protective effect of fluoxetine and the possibility of the interaction between serotonin and insulin signaling pathway.

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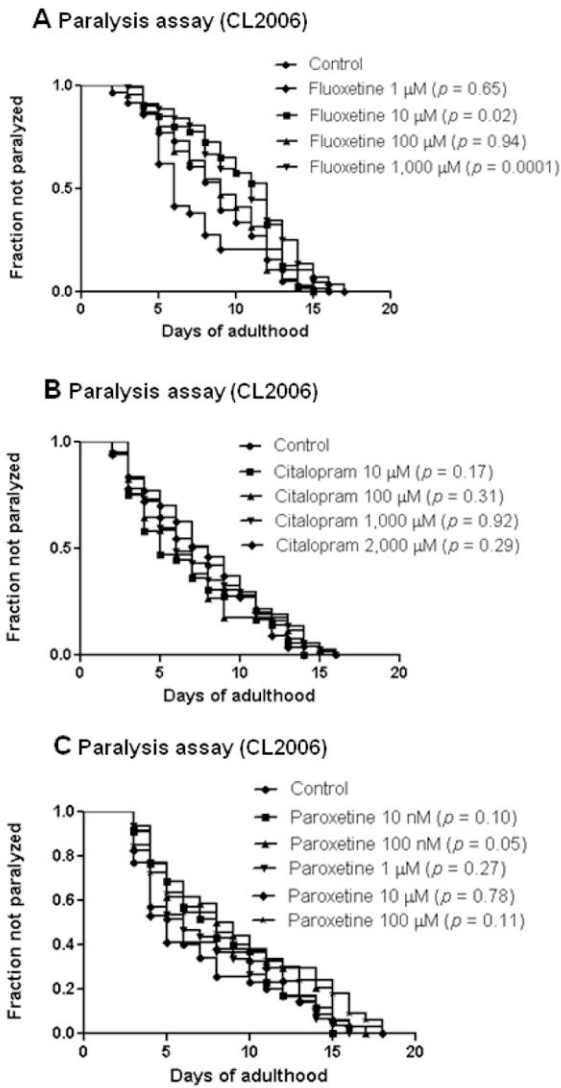
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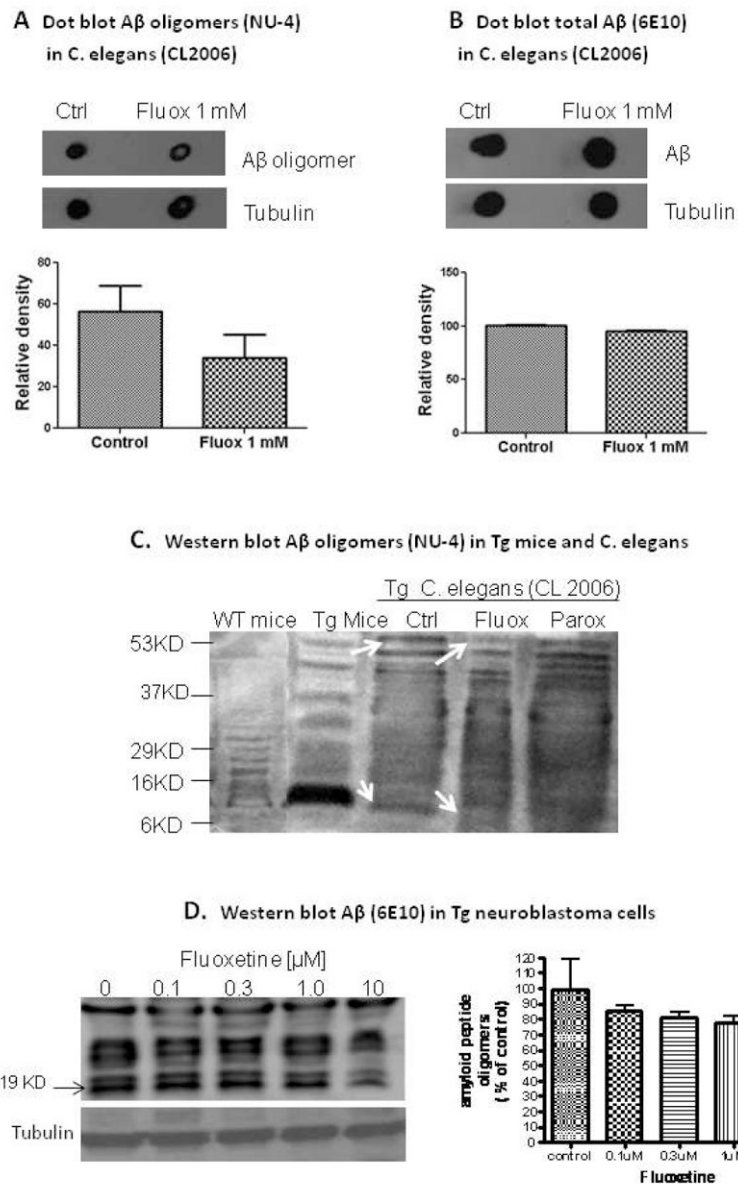
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**Fig. 1.**

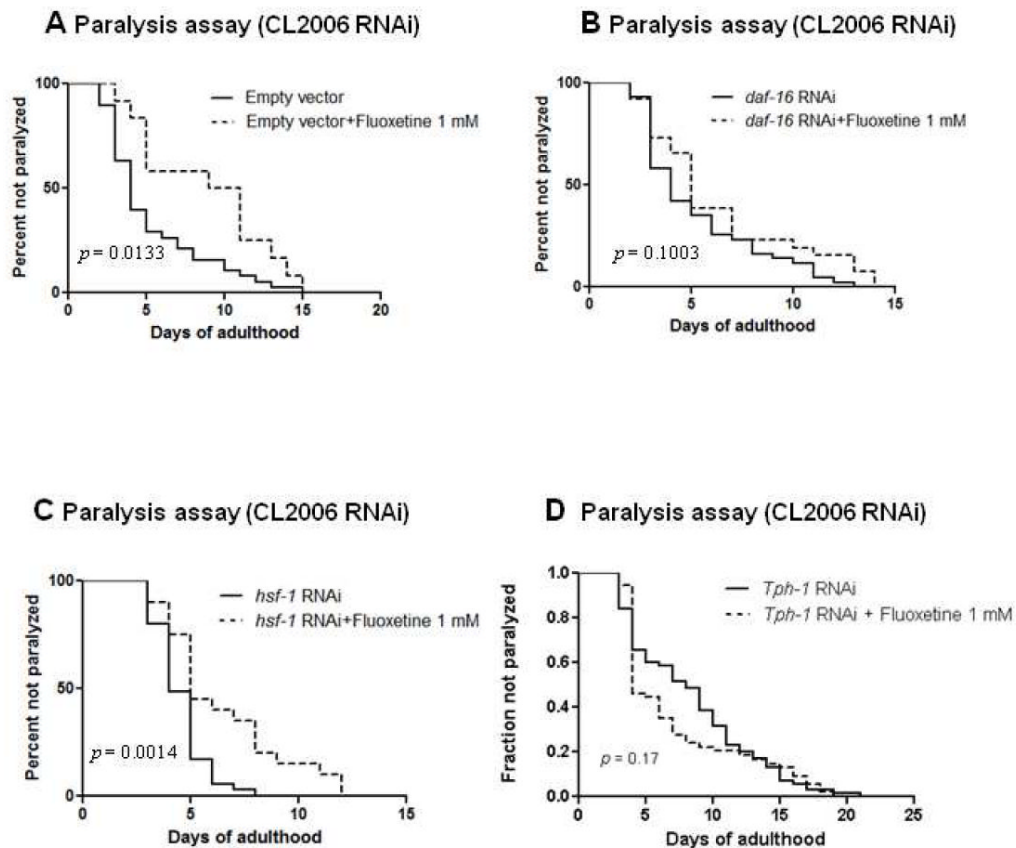
$\text{A}\beta$ -induced paralysis in *C. elegans* strain CL2006 fed with antidepressants. (A) Effect of fluoxetine at concentration of 1, 10, 100, 1000 μM on $\text{A}\beta$ -induced paralysis. (B) Effect of citalopram at concentration of 10, 100, 1,000 and 2,000 μM on $\text{A}\beta$ -induced paralysis. (C) Effect of paroxetine at concentration of 10 nM, 100 nM, 1 μM , 10 μM and 100 μM .

Synchronized eggs of CL2006 *C. elegans* were maintained at 20 $^{\circ}\text{C}$, on the 35 \times 10 mm culture plates (~35 eggs/plate) containing vehicle (control) or drugs. The treatment was given to the worm from egg stage onward until the worm completely paralyzed. (35 worms in each experiment, 3 independent experiments).

**Fig 2.**

Dot blot assay of A β oligomers in transgenic *C. elegans* treated with or without fluoxetine (Fluox at 1 mM). (A) Relative density of A β oligomer immunoreactivity (NU-4) in CL2006 worms untreated (control) or treated with fluoxetine (n = 3). Inset, representative dot blot of A β oligomers in untreated worms and treated worm. (B) Relative density of total A β immunoreactivity (6E10) in worms untreated (control) or treated with fluoxetine (n = 3). Inset, representative dot blot of total A β in untreated worms and treated worm. Equal amount of protein (2 μ g) were loaded on the membrane. Tubulin was used to normalize protein loading. Error bars indicate SEM. C: The treatment of *C. elegans* with 1mM fluoxetine and 100nM paroxetine result in reduction in A β oligomers in comparison to the control. D: Effect of fluoxetine on amyloid peptide species is determined by western blotting. Fluoxetine significantly decreased the levels of intracellular A β oligomers (ANOVA, P=0.0230). *posthoc* Dunnett test shows that 10 μ M fluoxetine treatment causes

significant reduction in A β oligomers in comparison to the control ($P < 0.01$). Data is expressed as mean \pm SEM (N=3). ** represents $P < 0.01$.

**Fig. 3.**

Paralysis in CL2006 with or without DAF-16, HSF-1 and TPH-1 knock down by RNAi. (A) Paralysis in CL2006 treated with or without fluoxetine in *C. elegans* fed with vector control RNAi. Solid line, worms grown on bacteria containing empty vector alone; dotted line, worms grown on bacteria containing empty vector and treated with fluoxetine. (B) Paralysis in CL2006 treated with or without fluoxetine in *C. elegans* fed with RNAi to down regulate DAF-16. Solid line, worms grown on *daf-16* RNAi bacteria alone; dotted line, worms grown on *daf-16* RNAi bacteria and treated with fluoxetine. (C) Paralysis in CL2006 treated with or without fluoxetine in *C. elegans* fed with RNAi to down regulate HSF-1. Solid line, worms grown on *hsf-1* RNAi bacteria alone; dotted line, worms grown on *hsf-1* RNAi bacteria and treated with fluoxetine. (D) Paralysis in CL2006 treated with or without fluoxetine in *C. elegans* fed with RNAi to down regulate TPH-1. Solid line, worms grown on *tph-1* RNAi bacteria alone; dotted line, worms grown on *tph-1* RNAi bacteria and treated with fluoxetine. (70 worms in each experiment, 2 independent experiments)

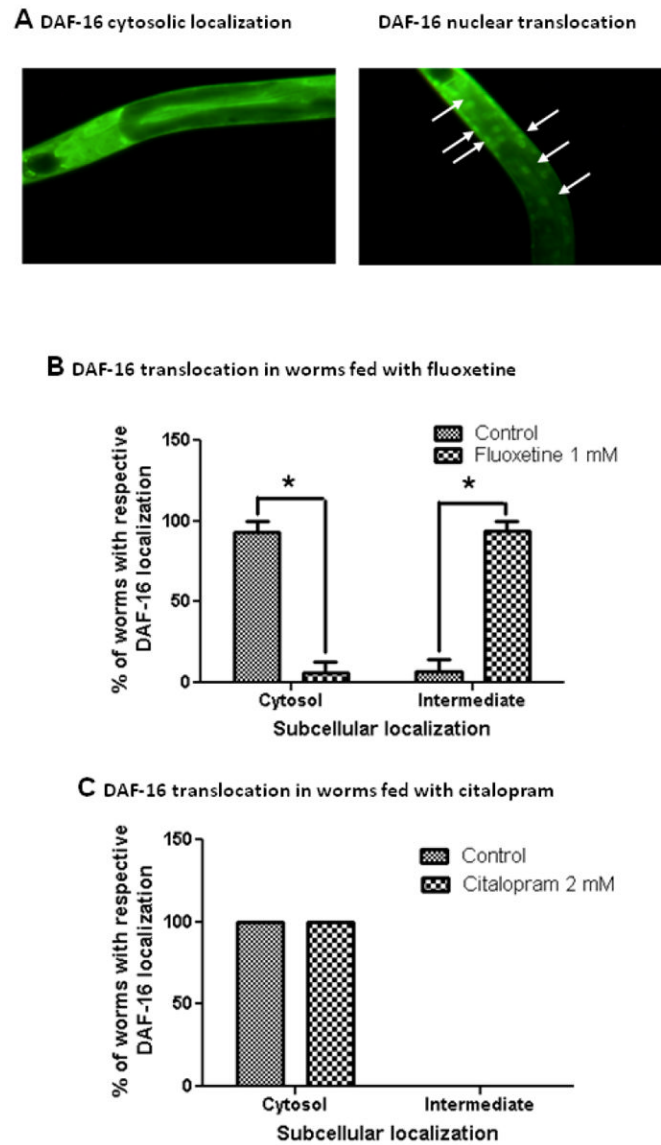


Fig. 4. The effect of fluoxetine and citalopram on subcellular DAF-16 localization. (A) Representative fluorescence images of transgenic strain TJ356 showing cytosolic (left) and nucleus (right, arrows) localization of DAF-16. Subcellular distribution of DAF-16 in worms treated with fluoxetine (B) or citalopram (C) was analyzed by fluorescence microscopy. The worms were classified into 2 categories: cytosol, translocate according to their localization phenotype. (* $p < 0.05$ vs control, $n = 24$)

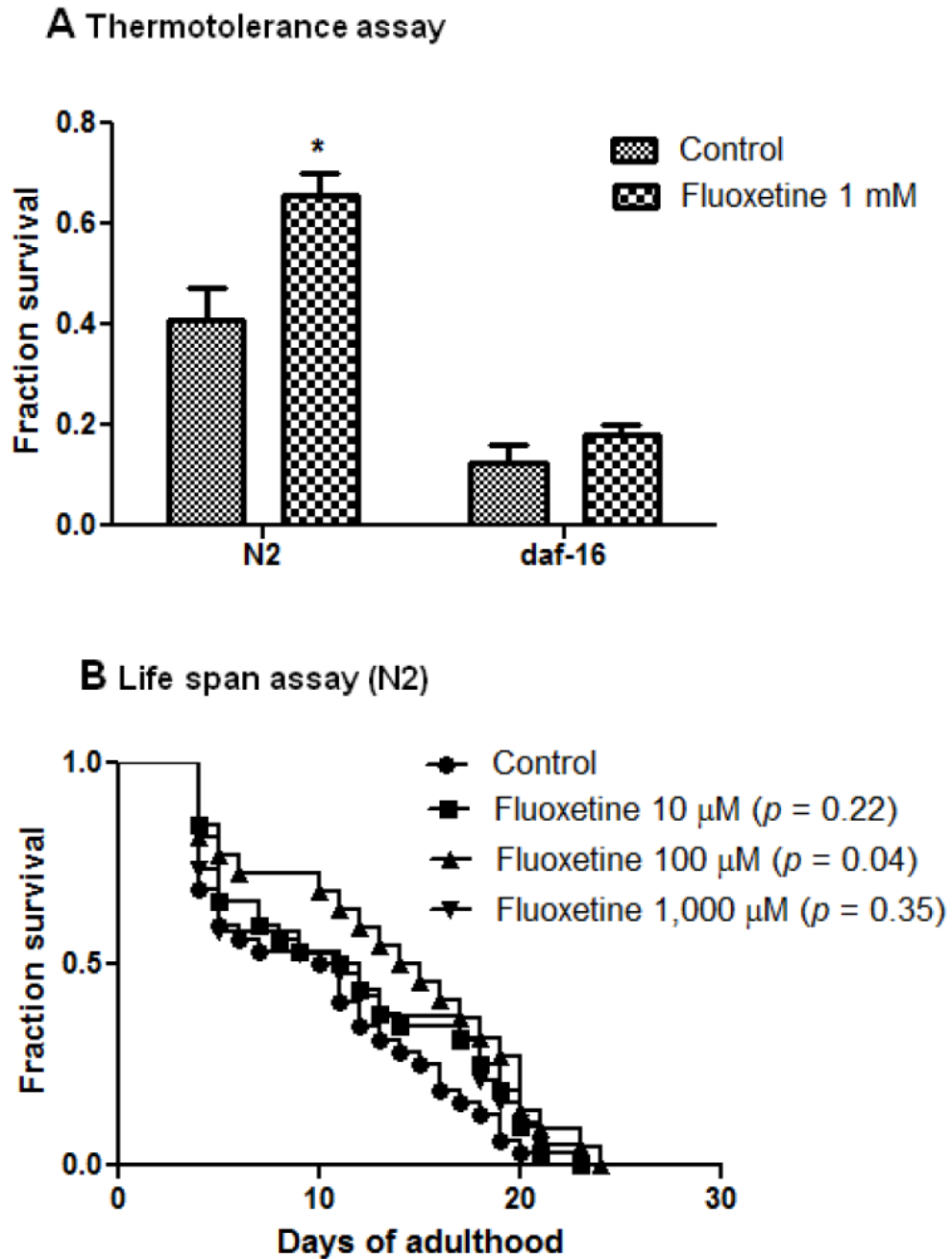


Fig. 5. Effect of fluoxetine on thermal stress resistance and life span in *C. elegans*. **(A)** Thermotolerance in wild type (N2) and *daf-16* *C. elegans* treated with or without Fluoxetine 1 mM (* $p = 0.01$ vs control) and **(B)** Life span assay in wild type (N2) *C. elegans* treated with vehicle (control) or with increasing dose of fluoxetine (10, 100 and 1,000 μ M). Synchronized eggs of N2 and *daf-16* *C. elegans* were maintained at 20 °C, on the 35×10 mm culture plates (~35 eggs/plate) containing vehicle or drug. The treatment was given to the worm from egg stage onward.