Amyloid-β-Induced Pathological Behaviors are Suppressed by Ginkgo biloba extract EGb 761 and Ginkgolides in Transgenic Caenorhabditis elegans

Yanjue Wu
*University of Maryland, yj_w2000@yahoo.com*

Zhixin Wu
*University of Southern Mississippi, zhixinwu123@yahoo.com*

Peter Butko
*University of Southern Mississippi, pbutko@chem.nagoya-u.ac.jp*

Yves Christen
*Ipsen*

Mary P. Lambert
*Northwestern University*

See next page for additional authors

Follow this and additional works at: https://aquila.usm.edu/fac_pubs

Part of the Biology Commons

Recommended Citation

Wu, Y., Wu, Z., Butko, P., Christen, Y., Lambert, M. P., Klein, W. L., Link, C. D., Luo, Y. (2006). Amyloid-β-Induced Pathological Behaviors are Suppressed by *Ginkgo biloba* extract EGb 761 and Ginkgolides in Transgenic *Caenorhabditis elegans*. *Journal of Neuroscience, 26*(50), 13102-13113. Available at: https://aquila.usm.edu/fac_pubs/8519

This Article is brought to you for free and open access by The Aquila Digital Community. It has been accepted for inclusion in Faculty Publications by an authorized administrator of The Aquila Digital Community. For more information, please contact aquilastaff@usm.edu.
Authors
Yanjue Wu, Zhixin Wu, Peter Butko, Yves Christen, Mary P. Lambert, William L. Klein, Christopher D. Link, and Yuan Luo

This article is available at The Aquila Digital Community: https://aquila.usm.edu/fac_pubs/8519
Neurobiology of Disease

Amyloid-β-Induced Pathological Behaviors Are Suppressed by Ginkgo biloba Extract EGb 761 and Ginkgolides in Transgenic Caenorhabditis elegans

Yanjue Wu, Zhixin Wu, Peter Butko, Yves Christen, Mary P. Lambert, William L. Klein, Christopher D. Link, and Yuan Luo

1Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Maryland 21201, Departments of 2Biological Sciences and 3Chemistry and Biochemistry, University of Southern Mississippi, Hattiesburg, Mississippi 39406, 4Ipsen, 75016 Paris, France, 5Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois 60208, and 6Institute for Behavioral Genetics, University of Colorado, Boulder, Colorado 80309

Amyloid-β (Aβ) toxicity has been postulated to initiate synaptic loss and subsequent neuronal degeneration seen in Alzheimer’s disease (AD). We previously demonstrated that the standardized Ginkgo biloba extract EGb 761, commonly used to enhance memory and by AD patients for dementia, inhibits Aβ-induced apoptosis in neuroblastoma cells. In this study, we use EGb 761 and its single constituents to associate Aβ species with Aβ-induced pathological behaviors in a model organism, Caenorhabditis elegans. We report that EGb 761 and one of its components, ginkgolide A, alleviates Aβ-induced pathological behaviors, including paralysis, and reduces chemotaxis behavior and 5-HT hypersensitivity in a transgenic C. elegans. We also show that EGb 761 inhibits Aβ oligomerization and Aβ deposits in the worms. Moreover, reducing oxidative stress is not the mechanism by which EGb 761 and ginkgolide A suppress Aβ-induced paralysis because the antioxidant t-ascorbic acid reduced intracellular levels of hydrogen peroxide to the same extent as EGb 761, but was not nearly as effective in suppressing paralysis in the transgenic C. elegans. These findings suggest that (1) EGb 761 suppresses Aβ-related pathological behaviors, (2) the protection against Aβ toxicity by EGb 761 is mediated primarily by modulating Aβ oligomeric species, and (3) ginkgolide A has therapeutic potential for prevention and treatment of AD.

Key words: Aβ peptide; Alzheimer’s disease; behavior; mutant; phenotype; serotonin

Introduction

Despite a widely accepted “amyloid cascade hypothesis” for Alzheimer’s disease (AD) (Hardy and Selkoe, 2002), the current explanation for amyloid-β (Aβ) toxicity in AD still remains controversial in view of the fact that the relationship between Aβ species and Aβ-specific behavior has not been defined in vivo (Lorenzo and Yankner, 1994; Pike et al., 1995; Lambert et al., 1998). A leading theory is that Aβ oligomers are responsible for initiation of synaptic dysfunction, an early event that leads to neurodegeneration observed in AD (Walsh and Selkoe, 2004; Roselli et al., 2005; Lesne et al., 2006; Oddo et al., 2006). The evidence for or against these hypotheses is critical for specific therapeutic strategies. It has previously been demonstrated that small molecules inhibiting Aβ oligomers also reduced its toxicity (Walsh et al., 2005; Yang et al., 2005; Maezawa et al., 2006).

However, most of these studies were conducted in vitro, and the use of a transgenic mice model of AD for pharmacological evaluation and mechanistic studies is time-consuming. Simple invertebrate models of neurodegenerative diseases offer experimental advantages for addressing basic cellular processes that are conserved among all animals (Link, 2005; Wu and Luo, 2005).

The standard Ginkgo biloba leaf extract EGb 761 has been given routinely as a prescription drug in many countries, and as a dietary supplement in the United States, for Alzheimer’s dementia (Christen and Maixent, 2002). Several clinical trials have provided evidence of efficacy (Le Bars et al., 1997; Birks et al., 2002; Mix and Crews, 2002; Le Bars, 2003), comparable with Donepezil (Mazza et al., 2006), as a symptomatic treatment of mild to moderate AD, and suggestive for AD prevention (Andrieu et al., 2003). Currently, a National Institutes of Health-supported GEM (Ginkgo Evaluation of Memory) study in the United States and a GuidAge study in Europe are underway to evaluate EGb 761 as a preventive drug (DeKosky et al., 2006). Substantial experimental data indicate that EGb 761 has neuroprotective and neuromodulatory effects (Lu et al., 2001; Watanabe et al., 2001; Yao et al., 2001; DeFeudis, 2002). Two of its major constituents, flavonoids (24%) and terpenoids (6%), have been actively investigated for their neuroprotective properties (DeFeudis, 1998; Smith and Luo, 2003). The ginkgolides, known as potent antagonists of platelet-
The flavonois are involved in antioxidative properties of
G. biloba (DeFeudis, 1998). The flavonoids are involved in antioxidative properties of
EGb 761 (Chromy et al., 2003) providing evidence that EGb 761 can alter
oligomerization in solution (Luo et al., 2002; Wu et al., 2004). The flavonoids are involved in antioxidative properties of
G. biloba (DeFeudis, 1998).

Materials and Methods

G. biloba extract EGb 761 was provided by Schwabe Pharmaceuticals
(Karlsruhe, Germany); the extract is well characterized and is the one
used in the ongoing clinic trials. The flavonoid fraction (Fig. 1C) was
from E. coli (OP50) for food. To prepare age-
synchronized animals, nematodes were transferred to fresh NGM plates
on reaching reproductive maturity at 3 d of age and allowed to lay eggs for
4–6 h (overnight for Western blotting of CL1476 and CL2355). Isolated
hatchlings from the synchronized eggs (day 1) were cultured on fresh
NGM plates in either a 20°C or a 16°C (for CL4176, CL2355, and their
control strains) temperature-controlled incubator (model 2005; Sheldon
Manufacturing, Cornelius, OR). The worms were fed with the drugs
either from stage L1 (1 d of age) or starting from the egg.

Paralysis assays. The strain CL1476 (Drake et al., 2003; Link et al., 2003)
maintained at 16°C was egg-synchronized onto the 35 × 10 mm
culture plates containing either a vehicle or drug. Transgene expression
was induced by upshifting the temperature from 16 to 23°C, started at the
start of the assay plate, 1 μl of control odorant (100% ethanol) were added. Assay
plates were incubated at 23°C for 1 h and chemotaxis index (CI) was
scored. Chemotaxis index is defined as follows: (number of worms at the
attractant location – number of worms at the control location)/total
number of worms on the plate.

Table 1. Description of the well characterized transgenic C. elegans used in the
study

| Strains | Transgene | Expression | Phenotype |
|---------|-----------|------------|-----------|
| N2      |           | Wild type  |           |
| CL1476  | myo-3/Δβ1,42 | Inducible muscle Δβ1,42 | Rapid paralysis |
| CL1175  | myo-3     | Control for CL1476 | Normal |
| CL2006  | unc-54/Δβ1,42 | Constitutive muscle Δβ1,42 | Progressive paralysis |
| CL2355  | snb-1/Δβ1,42 | Inducible neuronal Δβ1,42 | Reduced chemotaxis |
| CL2122  | mtl-2/GFP | Control for CL2355 |           |
| CL2179  | myo-3/GFP | GFP control for CL1476 |           |

N2 (Bristol) was from Caenorhabditis Genetics Center (University of
Minnesota, Minneapolis, MN). The construction and characterization of
the transgenic nematode strains CL2006 (unc-54/Δβ1,42) and CL1476
(smg-1/ts [myo-3/Δβ1,42 long 3′-untranslated region (UTR)]) and its
control strain CL1175 (smg-1) have been described previously (Link,
1995; Link et al., 2001). The CL2006 strain constitutively produces a
body-wall muscle-specific Δβ1,42 whereas the expression of muscle-
specific Δβ1,42 in CL1476 depends on upshifting temperature from 16 to
23°C. The transgenic arrays in the CL2006, CL1175, and CL1476 strains
all contain the dominant rol-6(su1066) morphological marker. The
CL2355 strain [smg-1 in (snb-1/Δβ1,42 long 3′-UTR)] is used as a control for CL1476. This strain has
a GFP reporter tagged to the same promoter as the transgenic strains and has detectable GFP at 16°C, which is significantly stronger at higher
temperatures.

C. elegans maintenance and treatment. The wild-type (N2) and the
transgenic CL2006 were propagated at 20°C, CL1476 and CL2355 and
their controls at 16°C, on solid nematode growth medium (NGM)
seeded with 100 μl of control odorant (100% ethanol) along with 1
μl of odorant (0.1% benzaldehyde in 100% ethanol) (Sigma) along with 1 μl of 1 mM sodium azide were added to the
original spot. On the opposite side of the attractant, 1 μl drop of sodium
azide and 1 μl of control odorant (100% ethanol) were added. Assay
plates were incubated at 23°C for 1 h and chemotaxis index (CI) was
scored. Chemotaxis index is defined as follows: (number of worms at the
attractant location – number of worms at the control location)/total
number of worms on the plate.
5-HT sensitivity assay. Synchronized transgenic worms (CL2355) and the control strain (CL2122) fed with or without the drugs were collected at 36 h after temperature upshift. Serotonin (creatine sulfate salt; Sigma) was dissolved in M9 buffer to 1 mM. Twenty worms in each group were washed with M9 buffer for three times and transferred into 200 μl serotonin solution in a 96-well assay plate. Worms were scored after 5 min as active or paralyzed (nonmotile for 5 s).

Western blotting of Aβ species. The Aβ species in the transgenic C. elegans strains was identified by immunoblotting using a Tris-Tricine gel and the standard Western blotting protocol except that the polyvinylidene difluoride membranes were boiled for 5 min after the transfer. After the experimental treatments, the worms were collected by washing with distilled water, quickly frozen in liquid nitrogen, sonicated in the cell lysis buffer (50 mM HEPES, pH 7.5, 6 mM MgCl₂, 1 mM EDTA, 75 mM sucrose, 25 mM benzamidine, 1 mM DTT, 1% Triton X-100), and heated with sample buffer containing 5% β-mercaptoethanol (1:1; Bio-Rad, Hercules, CA). To detect Aβ oligomers, samples were extracted in PBS containing proteinase inhibitor mixture (Sigma) with (for antibody A11) or without 2% SDS (for antibody NU4). After mixing with the sample buffer, proteins were unheated and loaded on the gel. Equal amounts of the total protein (80 μg) were loaded in each lane. Antibody to Aβ₁₋₁₇ (6E10; at 1:500 dilutions) was from Signet (Dedham, MA). Antibody selective against oligomers (A-11; at 1:1000 dilution) was from Biosource (Camarillo, CA) (Kayed et al., 2003). Antibody specific to Aβ oligomers (NU4; 1:3000) was generated in W. L. Klein’s laboratory (Lambert, 2006). Anti-mouse IgG or anti-rabbit IgG (1:5000; Signet) was used as the secondary antibody. The mean densities of the Aβ bands were analyzed by a gel documentation system (Alpha Innotech 801054; Imgen Technologies, Alexandria, VA).

Fluorescence staining of Aβ deposits. Individual CL2006 transgenic nematodes were fixed in 4% paraformaldehyde/PBS, pH 7.4, for 24 h at 4°C, and permeabilized in 5% fresh β-mercaptoethanol, 1% Triton X-100, 125 mM Tris, pH 7.4, in a 37°C incubator for 24 h. The nematodes were stained with 0.125% thioflavin S (Sigma) in 50% ethanol for 2 min, destained, at 4°C, and permeabilized in 5% fresh β-mercaptoethanol, 1% Triton X-100, 125 mM Tris, pH 7.4, in a 37°C incubator for 24 h. The nematodes were stained with 0.125% thioflavin S (Sigma) in 50% ethanol for 2 min, destained, and mounted on slides for microscopy. Fluorescence images were acquired at the same exposure parameters using a 40× objective of the microscope (BX 60; Olympus, Tokyo, Japan) equipped with a digital camera (Micropublisher 5.0; Qimaging, Burnaby, British Columbia, Canada). The number of thioflavin S-reactive deposits in the area anterior of the pharyngeal bulb in individual animals was scored.

H₂O₂ assay in C. elegans. Intracellular ROS were measured in C. elegans using 2,7-dichlorofluorescein diacetate (DCF-DA) (Invitrogen, Eugene, OR) as described previously (Smith and Luo, 2003). Age-synchronized C. elegans were collected at 36 h after temperature upshift (CL4176/1175) into 100 μl of PBS with 1% Tween 20 (PBST) in Eppendorf tubes in groups of 40 worms. The worms were then subjected to equally timed homogenization (Pellet Pestle Motor; MG Scientific, Pleasant Prairie, WI) and sonication (Branson Sonifier 250; VWR Scientific, Suwanee, GA) to break up the outer cuticle. Samples were collected into wells of 96-well plates. DCF-DA (final concentration, 50 μM in PBS) was added to each well at 37°C for quantification of fluorescence in an FLx800 Microplate Fluorescence Reader (Bio-Tek Instruments, Winooski, VT) with the excitation at 485 nm and emission at 530 nm. Samples were read every 20 min for 2.5 h.

Statistical analyses. Differences between untreated and drug-treated groups were analyzed for statistical significance by independent Student’s t test of two groups using Origin 6.0 software (Microcal Software, Northampton, MA). A value of p < 0.05 is considered statistically significant. Correlation analysis was performed with the GraphPad (San Diego, CA) Prism 4.0a, using a one-tailed Pearson test.
Results
EGB 761, ginkgolide A and J alleviate Aβ-induced paralysis in the transgenic C. elegans
To determine whether EGB 761 specifically protects against Aβ-induced toxicity \textit{in vivo}, we first fed EGB 761 to a transgenic C. elegans line, in which expression of human Aβ peptide in the muscle cells induces an Aβ-dependent paralysis phenotype in the worms (muscle Aβ strain, CL4176) (Link et al., 2003). Synchronized eggs from transgenic worms, or control worms were placed on OP50 food containing vehicle or EGB 761 (100 μg/ml) for 36 h at 16°C, followed by temperature upshift from 16 to 23°C to induce transgene expression. Figure 2A is a set of photographs representing the following: the transgenic muscle Aβ expression strain (CL4176) without the temperature upshift and untreated with EGB 761 [control (Ctrl), no Aβ] (Fig. 2A, left panel), the temperature upshifted (for 36 h) CL4176, untreated with EGB 761 (Ctrl, muscle Aβ) (Fig. 2A, middle panel), or treated with EGB 761 (EGB, muscle Aβ) (Fig. 2A, right panel). Worms that did not move or only moved the head, under a gentle touch with a platinum loop were scored as paralyzed. In Figure 2A, the shape of movement and tracks left on the food (indicated with black lines) in nonparalyzed worms are obvious. The arrowheads indicate paralyzed worms (straight line shape), and the arrows indicate the moving worms (the “C” or “S” shape). At 56 h, ~100% worms without the transgene expression were nonparalyzed (Fig. 2A, left). At this time, only ~20% Aβ transgene expressing worms without EGB feeding were nonparalyzed (Fig. 2A, middle), and ~60% transgene expressing worms fed with EGB were nonparalyzed (Fig. 2A, right). Note the tracks left behind on the plate in the EGB 761-fed C. elegans (Fig. 2A, right panel).

Figure 2B is a time course of paralysis in the C. elegans CL4176 fed with a vehicle (0.01% ethanol; open circles), EGB 761 (EGB, 100 μg/ml; filled circles), or CR (139 μg/ml; filled squares) from day 1 till after the temperature upshift for 36 h. CL1175 (filled triangles) is a transgenic control strain that does not express Aβ. A notable delay of paralysis was observed in the transgenic worms fed with EGB 761 compared with the untreated controls. Congo red also moderately delayed paralysis. Interestingly, the worms fed with 100 μg/ml l-ascorbic acid, a known antioxidant, did not show any delay in paralysis (for clarity of the graph, data are presented only in Table 2). To further determine which constituent(s) of EGB 761 contributed to reducing Aβ-induced paralysis, transgenic C. elegans CL4176 were fed with GA, GB, GC, GJ, BB, and a flavonoid (Flav) fraction (10 μg/ml each). Among individual components tested, only GA and GJ exhibited significant delay of Aβ-induced paralysis (for clarity, the paralysis time courses for other components are not shown in Fig. 2C) (for statistical analysis, see Table 1). Reproducibility of paralysis at a given temperature in individual trials was consistent. Because the transgene product is temperature sensitive, the onset of paralysis is related to the specific value of room temperature, at which the paralysis assays were conducted. Thus, the variation in the onset of paralysis between Figure 2, B and C, is attributed to the slight difference in room temperature on the days when the assays were performed (22 vs 23°C). Nevertheless, the difference between the worms fed with and without EGB 761 was consistently significant at both 22 and 23°C.

For quantitative analysis, we define PT50 as the time interval, from the onset of paralysis, at which 50% of the worms were paralyzed (i.e., PT50 of 5.7 h for control was obtained by subtracting the onset time of paralysis 27 h from the time when 50% of the worms were paralyzed at 32.7 h) (Table 1). A statistically significant delay of Aβ-induced paralysis was observed in the worms fed with EGB 761 (Table 2) (control vs EGB, p = 0.03; n = 9 assays; 40 worms in each assay group), but not in Congo red-treated animals (control vs CR, p = 0.15; n = 9 assays; 30 worms in each assay group). Among six single components of EGB 761, only ginkgolides A and J exhibited a statistically significant delay of paralysis (control vs GA, p = 0.001; control vs GJ, p = 0.01; n = 3 assays; 30 worms in each assay group). Moreover, EGB 761 feeding was found to delay paralysis through a wide range of concentrations (10–500 μg/ml) (data not shown). Paralysis was not delayed in the worms fed with EGB 761 before Aβ induction, at the time of Aβ induction, or after Aβ induction by temperature upshift (data not shown) (see diagram in Fig. 2D), suggesting that short duration of feeding may not be sufficient to alleviate the Aβ toxicity.

EGB 761 suppresses neuronal Aβ expression-induced defect in chemotaxis behavior and 5-HT sensitivity
The paralysis phenotype in muscle of the Aβ expression strain CL4176, used above, has been established and used to illustrate several important molecular processes related to Aβ toxicity (Drake et al., 2003; Link et al., 2003). To relate Aβ toxicity with neurological functions, we characterized a behavior phenotype of the transgenic strain in which Aβ was expressed in the neuronal cells (CL2355). Two characteristic neuronal controlled behaviors, chemotaxis and 5-HT sensitivity, were assayed in these worms. The chemotaxis response in C. elegans is mediated by activation of several sensory neurons and interneurons to stimulate the motor neurons (Hobert, 2003). The CI is a measure of the fraction of worms that are able to arrive at the location of the attractant (Matsuura et al., 2005). To investigate the effect of EGB 761 on the performance of chemotaxis behavior, we applied the chemical benzaldehyde as an attractant and ethanol as a control, both containing sodium azide, which paralyzes the worms on contact (Fig. 3A). The chemotaxis index was scored in the transgenic strain and the control strain at day 4 of age. Figure 3B shows that the transgenic mutant CL2355 exhibits a significant reduction of CI compared with the control strain CL2122, or no Aβ strain untreated (Ctrl CI\textsubscript{low Aβ}, 0.36 ± 0.02 vs Ctrl CI\textsubscript{high Aβ}, 0.27 ± 0.02; n = 4; p = 0.01). EGB 761 feeding of the control strain had no effect on their CI (Ctrl CI\textsubscript{low Aβ}, 0.36 ± 0.02, vs EGB CI\textsubscript{low Aβ}, 0.35 ± 0.04; n = 4; p = 0.31), but it significantly normalizes the reduced CI in the neuronal Aβ transgenic strain (Ctrl CI\textsubscript{Aβ}, 0.27 ± 0.02, vs EGB CI\textsubscript{Aβ}, 0.37 ± 0.03; n = 4; p = 0.01; a total of 240 worms was used in each assay). A similar effect was observed

| Table 2. Quantitative analysis of paralysis |
| Experiments | PT50 | p value |
|---|---|---|
| Control | 5.7 ± 0.8 | 0.03 |
| EGB 761 | 9.4 ± 1.3* | 0.17 |
| Ascorbic acid | 3.4 ± 0.3 | 0.15 |
| Congo red | 7.5 ± 0.8 | 0.18 |
| Control | 3.5 ± 0.3 | 0.24 |
| Ginkgolide A | 7.2 ± 0.3** | 0.001 |
| Ginkgolide B | 2.8 ± 0.4 | 0.01 |
| Ginkgolide C | 3.5 ± 0.3 | 1.00 |
| Ginkgolide J | 6.8 ± 0.6* | 0.01 |
| Bilobalide B | 2.8 ± 0.4 | 0.18 |
| Flavonoids | 2.7 ± 0.6 | 0.24 |

The paralysis assays were quantified for mean time duration at which 50% worms were paralyzed (PT50) from the transgenic worms fed with or without the drugs. Values of p were obtained from nine independent assays for the worms fed with EGB 761 or Congo red, each paired with untreated controls, and from three assays for l-ascorbic acid, and single components paired with the control.

Wu et al. • EGB Reduces Aβ-Specific Pathological Behaviors
J. Neurosci., December 13, 2006 • 26(50):13102–13113 • 13105
in the transgenic worms fed with GA (Ctrl CI_{AP} 0.27 ± 0.02, vs GA CI_{AP} 0.34 ± 0.01; n = 4; p = 0.03).

Taking advantage of the ability of C. elegans to take up exogenous 5-HT (Sawin et al., 2000), we conducted a serotonin (5-HT) hypersensitivity assay to further determine whether 5-HT-mediated neurotransmission is affected by expressing Aβ transgene in the neurons. 5-HT is a key neurotransmitter that modulates several behaviors of C. elegans, including egg laying, locomotion, and olfactory learning (Schafer and Kenyon, 1995; Sawin et al., 2000; Nuttley et al., 2002; Zhang et al., 2005). When exogenous 5-HT is applied to the nematodes, they become paralyzed as a result of the sensitivity to excessive 5-HT. This 5-HT sensitivity assay has previously been used to identify 5-HT hyper-sensitive mutants, which revealed the functional relationship of the genes involved in 5-HT signaling (Schafer et al., 1996; Ranganathan et al., 2001). We used this assay to test the response to 5-HT in the transgenic mutant CL2355 in comparison with the control strain CL2122. Twenty animals from each strain were placed in 200 μl of 5-HT solution (1 mM) in 96-well microtiter wells. Paralysis was scored at 5 min after exposure to 5-HT. Figure 3C demonstrates that the transgenic neuronal line CL2355 untreated (Ctrl) was particularly sensitive to 5-HT, compared with the transgenic control CL2122 (no Aβ strain) untreated (percentage of Ctrl_{CL2355} 35.9 ± 7.8%, vs percentage of Ctrl_{CL2122} 73.2 ± 3.3; n = 3; p < 0.001). The neuronal Aβ worms (CL2355) then were fed with EGB 761 or its single components. Only feeding with EGB 761 (EGB vs Ctrl, 69.5 ± 4.9%, vs 35.9 ± 7.8%; n = 3; p = 0.003) and ginkgolide A (GA vs Ctrl, 65.5 ± 4.0, vs 35.9 ± 7.8%; n = 3; p = 0.005) significantly normalized the defective response to 5-HT in the transgenic strain. Neither the antioxidant l-ascorbic acid (VC), kaempferol, the flavonoid fraction of EGB 761 (KA), or CR normalized the paralysis in the neuronal Aβ strain CL2355.

Among behavior assays available to examine neuronal toxicity (including egg laying, body bend, pharyngeal pumping, locomotion/swimming, and paralysis), the serotonin sensitivity assay (swimming) was performed to determine a behavior phenotype in neuronal Aβ-expressing strain for the following reasons: (1) early work by Horvitz et al. (1982) demonstrated that exogenous 5-HT affected the wild-type C. elegans locomotion, or swimming behavior. Subsequently, it was reported that the effect was blocked in the MOD-1 mutant (a 5-HT-gated chloride channel) (Ranganathan et al., 2000), suggesting that this assay measures a 5-HT-mediated response; (2) in C. elegans, the experience-dependent behavior (an enhanced slowing of the response) is modulated by 5-HT (Sawin et al., 2000), which provides a link between the behavior plasticity and the neuronal plasticity associated with the impairment of cognitive function observed in AD; (3) among others, this phenotype exhibited the most significant difference between the control strain (CL2122) and the transgenic strain (CL2355). Thus, the Aβ-dependent behavior may provide the opportunity to elucidate 5-HT-mediated neuroprotection against Aβ neurotoxicity.

**EGB 761 modulates Aβ oligomers in the transgenic C. elegans**

To determine whether inhibition of Aβ oligomerization by EGB 761 underpins its mechanism for suppressing pathological behaviors in C. elegans, we analyzed Aβ species from the transgenic C. elegans fed with or without EGB 761 by Western blotting using antibodies against Aβ (6E10) or specific against Aβ oligomers (A-11 and NU-4). Figure 4A shows Aβ-immunoreactive (6E10) bands (Mr, ~7.2–28 kDa) detected in the tissues from the transgenic worms (CL4176) fed with or without EGB 761 and the
comparison chemicals. Two immunoreactive Aβ species with molecular weights at ~20 and 28 kDa were significantly decreased in the worms fed with EGB 761 (100 µg/ml for 72 h) (Fig. 4A, EGB, lane 3). Interestingly, a simultaneous increase of the Aβ monomers was observed in those worms. Consistent with the observation in CL4176, a similar inhibitory effect by EGB 761 was observed in the transgenic strain CL2006. In this strain, EGB 761 inhibited the same Aβ species and, at the same time, increased Aβ monomers in the same tissue extract (data not shown). In contrast, L-ascorbic acid (VC) (100 µg/ml for 72 h) did not inhibit the Aβ oligomerization or increase the monomer content as EGB 761 did, in the transgenic worm CL4176 (Fig. 4A, lane 4). CR (139 µg/ml) inhibited the oligomerization to a lesser degree than EGB 761 but also failed to increase the Aβ monomer content in the same samples in the transgenic worms (Fig. 4A, CR, lane 5). The mean densities of the Aβ oligomer band at ~20 kDa as well as the Aβ monomer bands were analyzed. Statistically (Fig. 4B), EGB 761 and Congo red significantly reduced the oligomers (Fig. 4B, solid bars) (n = 3; control vs EGB, p = 0.04; Ctrl vs CR, p = 0.02). Most importantly, only EGB 761 significantly increased the Aβ monomers from the same C. elegans samples (Fig. 4B, dashed bars) (n = 3; control vs EGB, p = 0.01).

Neuroprotection by EGB 761 is known as a “multipotent” action (i.e., it is achieved by modulating a variety of biological pathways simultaneously because of its complex nature) (DeFeudis, 2002; Smith and Luo, 2003). We next asked whether the inhibitory effect on oligomerization by an individual constituent is sufficient to correlate with its alleviative effect on Aβ-induced paralysis. The transgenic C. elegans was fed with different single constituents and Aβ species were analyzed by Western blotting using antibody 6E10. Figure 4C represents a noticeable shift of Aβ oligomer species to monomers by EGB 761 and ginkgolide A. Blots from three independent experiments were quantified in Figure 4D. It shows that EGB 761 (EGB), GA, and GJ remarkably reduced the Aβ oligomers band (filled bars) (Ctrl vs EGB, * p = 0.006; Ctrl vs GA, ** p = 0.006; Ctrl vs GJ, *** p = 0.007). GB, GC, and GJ also significantly reduced this Aβ species, to a somehow lesser degree (Ctrl vs GB, * p = 0.04; Ctrl vs GC, ** p = 0.04), compared with untreated transgenic worms (Ctrl). Only EGB 761 and GA significantly enhanced the Aβ monomers (dashed bars) (Ctrl vs EGB, # p = 0.02; Ctrl vs GA, p = 0.03).

To confirm the properties of the Aβ species modulated by EGB 761, we used two independent antibodies A11 (Fig. 4E) and NU4 (Fig. 4F). Antibody A11 is selective for high molecular weight Aβ oligomers as well as other oligomeric proteins (Kayed et al., 2003). This antibody was used to reveal a temporal profile of Aβ oligomerization in mice model of
AD (Oddo et al., 2006). Antibody NU-4 is a mice monoclonal antibody generated in the W. L. Klein laboratory (Lambert, 2006), which is an improved version of the previously generated low molecular weight oligomer [Aβ-derived diffusible ligand (ADDL)]-selective polyclonal rabbit antibody (M93/M94) (Lambert et al., 2001). Levels of oligomers reactive to this antibody have been reported to be 70-fold higher in the AD patient’s brain compared with the control brains (Gong et al., 2003). Prevention by EGb 761 of the oligomer formation in vitro was demonstrated using this antibody (Chromy et al., 2003).

Multiple immunoreactive bands were recognized by A11 in the transgenic C. elegans CL4176 (Fig. 4E, lane 2). Among these, a major band at ~50 kDa disappeared in the CL4176 strain fed with EGb 761 (Fig. 4E, lane 3). The blot is representative of three independent experiments. Because A11 also recognizes oligomeric structures other than Aβ, we further performed the immunoblotting using an antibody specific to the toxic Aβ oligomers referred to as ADDL (NU-4). Figure 4F is representative of two independent blots using antibody NU-4. An immunoreactive band at 50 kDa, presumably a 12-mer species previously found to be abundant in AD brain (Gong et al., 2003), was detected only in the transgenic strain CL4176, but not in the transgenic control strain. EGb 761 inhibited formation of this species completely (Fig. 4F, lane 3). EGb 761 also inhibited formation of higher-order Aβ species (~70–80 kDa, probably 16- to 18-mers) in the neuronal strain CL2355. In addition, the Aβ species detected by 6E10 and A11/NU4 do not overlap, in terms of the size, which suggests that multiple Aβ oligomers are attenuated by EGb 761. The shift from larger Aβ oligomers to monomers caused by EGb 761 is striking, but it only can be detected by 6E10, because A11 and NU-4 do not recognize monomeric Aβ (Chromy et al., 2003; Lambert, 2006).

To determine whether EGb 761 affects the expression levels of the Aβ transgene, rather than interacting with the Aβ peptide species, an integrated myo-3/GFP strain (CL2179) with the same promoter tagged with a GFP reporter was used as a control for CL4176. To determine whether EGb 761 affects transcription of the transgenes, the strain was handled the same way as CL4176, in terms of temperature upshift regimen and EGb 761 feeding. Figure 4G shows that comparing the worms (CL2179) fed with or without EGb, there is no visible difference in GFP fluorescence density and intensity in the two groups, suggesting that EGb 761 feeding does not affect the levels of Aβ transgene expression in the worms. Data from a microarray of the transgenic worms fed with or without EGb 761 further supported this observation (data not shown).

**EGb 761 inhibits amyloid deposits in transgenic C. elegans**

To decide whether the inhibitory effect of EGb 761 on Aβ oligomerization would affect amyloid formation, the number of amyloid deposits was scored in the worm head region, which is separated from the rest of the body by the pharyngeal bulb (black arrows). Figure 5A shows Aβ deposits (black arrowheads) detected in the transgenic C. elegans (CL2006) (Fig. 5Ac) but not the wild type (N2) (Fig. 5Ab), as previously observed (Link, 1995). The number of Aβ deposits per nematode was reduced in the transgenic C. elegans CL2006 fed with EGb 761 (Fig. 5Ad). The worms exhibit background fluorescence without thioflavin S staining (Fig. 5Aa), which provides a guide to define the head area in the animals. Figure 5B shows that the mean number of Aβ deposits, from 24 worms in each group, was significantly reduced in worms fed with 100 µg/ml EGb 761 (Ctrl, 7.7 ± 0.5, vs EGb, 4.9 ± 0.6; n = 24; p = 0.002) or with 200 µM Congo red (Ctrl, 6.2 ± 0.8, vs CR, 5.5 ± 1.1; n = 24; p = 0.025), but not with the flavinon fraction of EGb 761 (Ctrl, 7.1 ± 0.9, vs Flav, 6.9 ± 1.0; n = 24; p > 0.05). These results suggest that EGb 761 inhibits Aβ oligomerization, which leads to an increase in the nontoxic Aβ monomers and the reduced amyloid deposits. Congo red, which binds to Aβ fibrils, also reduced the Aβ deposits in C. elegans.

**Levels of H2O2 in the transgenic C. elegans fed with EGb 761 and other chemicals**

Given that numerous lines of evidence have associated oxidative stress with AD and Aβ toxicity, we hypothesized that the antioxidative properties also contribute to the protective effects of EGb 761 against Aβ toxicity. We first tested the levels of H2O2 in the transgenic control strain (CL1175) and the transgenic muscle Aβ strain (CL4176). Figure 6A demonstrates that the untreated transgenic strain CL4176 (Ctrl) exhibits increased levels of H2O2.
Three concentrations of EGB 761 (25, 50, and 100 μg/ml) were added to the transgenic *C. elegans* diet from day 1 of age until the end of the temperature upshift. Feeding EGB 761 attenuated the intracellular levels of H$_2$O$_2$ in a dose-dependent manner (Fig. 6A). Feeding 100 μg/ml EGB 761 exhibited most significant reduction (Ctrl, 100 ± 23%; EGB, 42 ± 7%; n = 6; p = 0.04). Interestingly, no significant reduction was observed in the control worms fed with EGB 761 (Fig. 6A), suggesting that the effect of EGB 761 on attenuating H$_2$O$_2$ may be specific to Aβ expression. In comparison, 50 μg/ml L-ascorbic acid (VC) (Ctrl, 100 ± 3%; VC, 31 ± 3%; n = 3, p = 0.04), but not CR (139 μg/ml) (Ctrl, 100 ± 3%; CR, 101 ± 18%; n = 3; p = 0.11; total of 300 worms in each group), also showed significant attenuation of the Aβ-induced elevation of H$_2$O$_2$. Among single components of EGB 761 tested (10 μg/ml each), only ginkgolide A significantly attenuated the levels of H$_2$O$_2$ (p = 0.047) (Fig. 6C).

Paralysis is associated with Aβ oligomers but not the level of H$_2$O$_2$

To determine whether there is a correlative association between the behavioral rescue and the decrease in Aβ oligomers by EGB 761 and its constituents, correlation analysis was performed. Figure 7A shows a clear correlation between the amount of Aβ oligomers (mean density) and paralysis (PT$_{50}$), with a value of the Pearson correlation coefficient r of 0.566 (p = 0.044). Among all of the compounds, GJ, GA, and EGB 761 appear to contribute the most to the correlation, in contrast with GC, GB, and BB. The latter three compounds decrease the Aβ oligomers density without improving the worms’ paralysis. Figure 7B is a plot of paralysis (PT$_{50}$) versus levels of H$_2$O$_2$ measured by DCF fluorescence (see Materials and Methods). Paralysis is dissociated from the level of reactive oxygen species (ROS), because no correlation of the paralysis with the levels of H$_2$O$_2$ was found. The value of the Pearson correlation coefficient r was 0.27 (p = 0.07).

As summarized in Table 3, chemotaxis behavior does not correlate with the mean density of the Aβ oligomers. It is possible that the chemosensory neural circuits are less sensitive to the Aβ toxicity, and thus the chemotaxis behavior may not be the best assay for the latter. However, the serotonin hypersensitivity and paralysis correlate well with the Aβ oligomers density, supporting our hypothesis that EGB 761 and its constituents alleviate the behavioral abnormalities by decreasing the levels of the toxic Aβ oligomers.

Discussion

In the present experiments, we sought to associate Aβ species with Aβ-specific pathological behaviors using transgenic *C. elegans* as a model and EGB 761 as a pharmacological modulator. We found that EGB 761 alleviated Aβ-induced paralysis, chemotaxis dysfunction, and 5-HT hypersensitivity in the transgenic *C. elegans* expressing Aβ. EGB 761 also modulated Aβ oligomers and attenuated levels of H$_2$O$_2$ in the transgenic *C. elegans* (CL4176). Interestingly, suppression of paralysis is associated with inhibition of Aβ oligomerization but is disassociated with the antioxidative effect (Fig. 7A,B), suggesting that protective effects of EGB 761 against Aβ toxicity is mediated primarily by inhibition of Aβ oligomerization. If a similar mechanism is shared with other species, it may represent a rationale for the beneficial effects of EGB 761 in humans with AD-related dementia (Le Bars et al., 2000; Mazza et al., 2006) and for the enhanced

![Figure 6.](image-url)
A Correlation analysis between paralysis and Aβ oligomers

![Graph showing correlation between paralysis and Aβ oligomers](image)

B Correlation analysis between paralysis and levels of H₂O₂

![Graph showing correlation between paralysis and H₂O₂ levels](image)

Table 3. Summary of correlation analysis

| Behavior | Pearson r value | p value | Correlation with Aβ oligomers |
|----------|----------------|---------|-------------------------------|
| Paralysis | -0.566 | 0.044 | Yes |
| 5-HT sensitivity | -0.644 | 0.043 | Yes |
| Chemotaxis | -0.451 | 0.131 | No |

The data are from 10 different drugs tested in three behavior assays. Correlation of paralysis (PT₅₀), chemotaxis (CI), and 5-HT sensitivity with the 20 kDa Aβ oligomers mean density was tested by Pearson's test (see Materials and Methods).

The effects of known antioxidants. We believe that the effect of paralysis in a model organism, in a manner that does not parallel AD (Tg2576) (Stackman et al., 2003).

Cognitive function by EGb 761 and ginkgolide A suppress both Aβ oligomerization and Aβ-induced paralysis in a model organism, in a manner that does not parallel the effects of known antioxidants. We believe that the effect of EGb 761 and ginkgolide A on Aβ toxicity is specific for the following reasons.

**EGb 761 modulates Aβ specific pathological behaviors**

(Figs. 2, 3)

*Caenorhabditis elegans* is an ideal model organism for functional analysis of the age-associated neurodegeneration because of its available genetic information as well as the simple structure of its nervous system, which consist of only 302 neurons in an adult nematode. We took advantage of an established relationship between onset of Aβ expression and paralysis phenotype in a transgenic *C. elegans* model (Link, 1995). The absence of endogenous Aβ production in the worms offers an opportunity to find a direct role of the Aβ involvement in pathological behaviors (Wu and Luo, 2005). In addition, predominantly intracellular expression of Aβ provides another tool to address specific role of intracellular Aβ in relation to its toxicity (Gutierrez-Zepeda and Luo, 2004). Substantial evidence implicates intracellular Aβ oligomers in early events related to AD (Kienlen-Campard et al., 2002).

For the first time, two neuronal behavior phenotypes were characterized in a neuronal Aβ-expressing strain CL2355. Both chemotaxis and 5-HT signaling phenotypes are biologically relevant to Aβ-induced toxicity. *C. elegans* uses six primary sensory neurons to respond to >40 different attractants and repellents (Bargmann et al., 1993). The reinforcement is mediated by 5-HT signaling in the worms (Zhang et al., 2005). Several possible mechanisms could explain the 5-HT hypersensitive phenotype of the Aβ transgene, including the following: (1) response to 5-HT, which is modulated by calcium channel-dependent calcium influx (Schafer and Kenyon, 1995), was affected by the transgene Aβ expression (Ingram, 2005); (2) reduced acetylcholine (ACh) by Aβ accumulation in the worms; ACh is a negative regulator of 5-HT sensitivity in *C. elegans* (Schafer et al., 1996); and (3) Aβ may directly or indirectly block 5-HT reuptake in the worms. The pathological behavior assays used here are simple and relatively reproducible compared with other behavior assays in the worms. Whether this form of pathological behavior uses molecular mechanisms common to higher animals remains to be determined. A similar approach has recently revealed polyglutamin threshold toxicity in a transgenic *C. elegans* model of Huntington’s disease (Brignull et al., 2006).

**EGb 761 and ginkgolide A inhibit Aβ oligomerization**

(Fig. 4A–D)

Accumulation of Aβ oligomers seems to be one of the earliest events in the transgenic mice of AD (Oddo et al., 2006), which impairs long-term potentiation (LTP) and memory (Walsh et al., 2005; Lesne et al., 2006) and which correlates better with severity of dementia in AD patients than the density of amyloid plaques (Gong et al., 2003). Some oligomers (~20 kDa) inhibited by EGb 761 and ginkgolide A, as observed in this study (Fig. 4A–D), might be similar to, or identical with the neurotoxic ADDLs, or Aβ-derived diffusible ligands (Lambert et al., 1998). Previous studies showed that EGb 761 could inhibit formation of these species in solution (Luo et al., 2002; Chromy et al., 2003). The higher-order Aβ oligomers (~50 kDa) inhibited by EGb 761 in the worms (Fig. 4E,F) might be relevant to the species previously reported to be abundant in the AD brain (Gong et al., 2003). Our results suggest, but do not prove, that all of the oligomeric Aβ species inhibited by EGb 761 and ginkgolide A are toxic.

Based on *in vitro* studies, a linear pathway leading from Aβ monomers via paranuclei, oligomers, to protofibrils, and then
to fibrils was proposed (Bitan et al., 2003; Urbanc et al., 2004). This pathway may provide an explanation for our observation that Congo red, although it reduced Aβ deposits (Fig. 5B), did not significantly delay Aβ-induced paralysis (Fig. 2B). We speculate that EGB 761 and Congo red may bind to Aβ oligomers differently. For example, EGB 761 may have a higher affinity for certain oligomeric species, whereas Congo red favors the fibril form of Aβ. Thus, Congo red probably enters the linear process of fibrillogenesis at later stages than EGB 761, which would still lead to reduced overall Aβ oligomers (Fig. 4A) and decreased Aβ deposits (Fig. 5B), but not the appearance of Aβ monomers or significant suppression of paralysis (Fig. 2B) in the transgenic C. elegans. This theory agrees with the previous observation that paralysis occurs before detectable β-amyloid deposition in C. elegans (Drake et al., 2003). Therefore, the paralysis suppression by EGB 761 and ginkgolide A might be a consequence of the shift from the toxic Aβ oligomer to the nontoxic Aβ monomers (Fig. 4A). Given its “multipotent” nature, it is also possible that EGB 761 differentially modulates different processes of oligomerization.

The unique structure of ginkgolide A provides a rationale for its specific effect

It is believed that the unique biological properties of ginkgolides arise from their unique “cage skeleton” structure. This structure may share a common motif with Congo red and/or curcumin (Yang et al., 2005), which all display affinity for amyloidogenic conformations. It is not surprising that, among all ginkgolides, only GA exhibited a correlation between reducing β-amyloid oligomers (Fig. 4A) and decreased Aβ deposits (Fig. 5B), but not the appearance of Aβ monomers or significant suppression of paralysis (Fig. 2B) in the transgenic C. elegans. This theory agrees with the previous observation that paralysis occurs before detectable β-amyloid deposition in C. elegans (Drake et al., 2003). Therefore, the paralysis suppression by EGB 761 and ginkgolide A might be a consequence of the shift from the toxic Aβ oligomer to the nontoxic Aβ monomers (Fig. 4A). Given its “multipotent” nature, it is also possible that EGB 761 differentially modulates different processes of oligomerization.

References

Andrieu S, Gillette S, Amouyal K, Nourhashemi F, Reynish E, Ousset PJ, Albareda JL, Vellas B, Grandjean H (2003) Association of Alzheimer’s disease onset with Ginkgo biloba and other symptomatic cognitive treatments in a population of women aged 75 years and older from the EPI-DOS study. J Gerontol A Biol Sci Med Sci 58:372–377.

Bargmann CI, Hartwig E, Horvitz HR (1993) Odorant-selective genes and neurons mediate olfaction in C. elegans. Cell 74:515–527.

Bastianetto S, Ramassamy C, Dore S, Christen Y, Poirier J, Quirion R (2000) The Ginkgo biloba extract (EGB 761) protects hippocampal neurons against cell death induced by beta-amyloid. Eur J Neurosci 12:1882–1890.

Birks J, Grimes EV, Van Dongen M (2002) Ginkgo biloba for cognitive impairment and dementia. Cochrane Database Syst Rev 2:CD003120.

Bitan G, Kiriakidze MD, Lomakin A, Vollers SS, Benedek GB, Teplow DB (2003) Amyloid beta-protein (Abeta) assembly: Abeta 40 and Abeta 42 oligomerize through distinct pathways. Proc Natl Acad Sci USA 100:330–335.

Brignull HR, Moore FE, Tang SJ, Morimoto RI (2006) Polyglutamyl proteins at the pathogenic threshold display neuron-specific aggregation in a pan-neuronal Caenorhabditis elegans model. J Neurosci 26:7597–7606.

Christen Y, Maixent JM (2002) What is Ginkgo biloba extract EGB 761? An overview—from molecular biology to clinical medicine. Cell and Mol Biol 48:601–611.

Chromy BA, Nowak RJ, Lambert MP, Viola KL, Chang L, Velasco PT, Jones BW, Fernandez SJ, Lacor PN, Horowitz P, Finch CE, Kraft GA, Klein WL (2003) Self-assembly of Abeta(1–42) into globular neurotoxins. Biochemistry 42:12749–12760.

Cohen E, Bleschke J, Pericavalle RM, Kelly JW, Dillin A (2006) Opposing activities protect against age-onset proteotoxicity. Science 313:1604–1610.

DeFeudis FV (1998) Ginkgo biloba extract (EGB 761): from chemistry to clinical use. Cell and Mol Biology 44:257–267.

DeFeudis FV (2002) Effects of Ginkgo biloba extract (EGB761) on gene expression: possible relevance to neurological disorders and age-associated cognitive impairment. Drug Dev Res 57:214–235.

DeKosky ST, Fitzpatrick A, Ives DG, Saxton J, Williamson J, Lopez OL, Burke G, Fried L, Kuller LH, Robbins J, Tracy R, Woolard N, Dunn L, Kolmac KE, Nahin R, Farberg C (2006) The Ginkgo Evaluation of Memory (GEM) study: design and baseline data of a randomized trial of Ginkgo biloba extract in prevention of dementia. Contemp Clin Trials 27:238–253.

Drake J, Link CD, Butterfield DA (2003) Oxidative stress precedes fibrillar deposition of Alzheimer’s disease amyloid beta-peptide (1–42) in a transgenic Caenorhabditis elegans model. Neurobiol Aging 24:415–420.

Gong Y, Chang L, Viola KL, Lacor PN, Lambert MP, Finch CE, Kraft GA, Klein WL (2003) Alzheimer’s disease-aﬀected brain: presence of oligomeric Aβ beta ligands (ADDLs) suggests a molecular basis for reversible memory loss. Proc Natl Acad Sci USA 100:10417–10422.

Gutierrez-Zepeda A, Lue Y (2004) Testing the amyloid toxicity hypothesis of Alzheimer’s disease in transgenic Caenorhabditis elegans model. Front Biosci 9:3333–3338.
Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer’s disease: progress and problems on the road to therapeutics. Science 297:353–356.

Holm AL (2003) Behavioral plasticity in C. elegans: paradigms, circuits, genes. J Neurobiol 54:203–223.

Horvitz HR, Chalfie M, Trent C, Sulston JE, Evans PD (1982) Serotonin and octopamine in the nematode Caenorhabditis elegans. Science 216:1012–1014.

Ingram VM (2005) The role of Alzheimer Abeta peptides in ion transport across cell membranes. Subcell Biochem 38:339–349.

Ivic L, Sands TT, Fishkin N, Nakanishi K, Kriegstein AR, Stromgaard K (2006) Temporal profile of amyloid-beta (Abeta) oligomerization in an in vivo model of Alzheimer disease. J. Neurochem 99:12449–12454.

Oddo S, Caccamo A, Tran L, Lambert MP, Glabe CG, Klein WL, LaFerla FM (2006) Temporal profile of amyloid-beta (Abeta) oligomerization in an in vivo model of Alzheimer disease. J. Neurochem 99:12449–12454.

Netzer WJ, Xu H, Butko P (2002) Inhibition of amyloid-beta aggregation and caspase-3 activation by the Ginkgo biloba extract EGb 761. Prog Natl Acad Sci USA 99:12449–12454.
Walsh DM, Selkoe DJ (2004) Oligomers on the brain: the emerging role of soluble protein aggregates in neurodegeneration. Protein Pept Lett 11:213–228.

Walsh DM, Townsend M, Podlisny MB, Shankar GM, Fadeeva JV, Agnaf OE, Hartley DM, Selkoe DJ (2005) Certain inhibitors of synthetic amyloid β-peptide (Ab) fibrillogenesis block oligomerization of natural Ab and thereby rescue long-term potentiation. J Neurosci 25:2455–2462.

Watanabe CM, Wolffram S, Ader P, Rimbach G, Packer L, Maguire JJ, Schultz PG, Gohil K (2001) The in vivo neuromodulatory effects of the herbal medicine Ginkgo biloba. Proc Natl Acad Sci USA 98:6577–6580.

Wood W (1988) The nematode Caenorhabditis elegans. Plainview, NY: Cold Spring Harbor Laboratory.

Wu Y, Luo Y (2005) Transgenic C. elegans as a model in Alzheimer’s research. Curr Alzheimer Res 2:37–45.

Yang F, Lim GP, Begum AN, Ubeda OJ, Simmons MR, Ambegaokar SS, Chen PP, Kayed R, Glabe CG, Frautschy SA, Cole GM (2005) Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid in vivo. J Biol Chem 280:5892–5901.

Yao Z, Drieu K, Papadopoulos V (2001) The Ginkgo biloba extract Egb 761 rescues the PC12 neuronal cells from beta-amyloid-induced cell death by inhibiting the formation of beta-amyloid-derived diffusible neurotoxic ligands. Brain Res 889:181–190.

Zandi PP, Anthony JC, Khachaturian AS, Stone SV, Gustafson D, Tschanz JT, Norton MC, Welsh-Bohmer KA, Breitner JC. (2004) Reduced risk of Alzheimer disease in users of antioxidant vitamin supplements: the Cache County Study. Arch Neurol 61:82–88.

Zhang Y, Lu H, Bursmann CI (2005) Pathogenic bacteria induce aversive olfactory learning in Caenorhabditis elegans. Nature 438:179–184.