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EFFICACY AND MECHANISM OF ACTION OF NOVEL SYNTHETIC FATTY ACIDS DERIVATIVES IN A TRANSGENIC *Drosophila melanogaster* MODEL OF ALZHEIMER'S DISEASE.

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That the thesis entitled "EFFICACY AND MECHANISM OF ACTION OF NOVEL SYNTHETIC FATTY ACIDS DERIVATIVES IN A TRANSGENIC *DROSOSPHILA MELANOGASTER* MODEL OF ALZHEIMER DISEASE", presented by RAHEEM JABER MOHAIBES to obtain the Ph.D. DEGREE in the Program of Biotechnology, Genetics and Cellular Biology, has been completed under our supervision in the Department of Biology in the Faculty of Sciences of the University of Balearic Islands. After the revision of the present work, we authorize its presentation to be evaluated by the pertinent committee.

For all intents and purposes, we hereby sign this document.

Palma, July 20th 2015.

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The important thing is not to stop questioning
Curiosity has its own reason for existing
One cannot help but be in awe when he contemplates the mysteries of
Eternity, of life, of the marvelous structure of reality
It is enough if one tries merely to comprehend a little of this mystery every day
Never lose a holy curiosity
Albert Einstein

بسم الله الرحمن الرحيم

In the name of Allah the most Merciful the most Gracious.

All glory belongs to Allah the Almighty, the all Knowing and Seeing He alone can guide. I thank Allah *subhana wa ta'ala* for bestowing his mercy on me, and for guiding me. I send blessings and salutations to His Messenger and his *Ahl*.

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LIST OF ABBREVIATIONS

Aβ: Amyloid β-peptide

Aβ 40: Amyloid β 40

Aβ 42: Amyloid β 42

AD: Alzheimer's Disease

ANOVA: Analysis of variance

ARA: Arachidonic Acid

APOE: Apolipoprotein E

APP: Amyloid Precursor Protein

Appl: Drosophial amyloid precursor protein-like gene or protein

BACE: β-site APP Cleaving Enzyme

C. elegans: Caenorhabditis elegans

DHA: Docosahexaenoic Acid

2OHDHA: 2-hydroxydocosahexaenoic Acid

2OHARA: 2-hydroxyarachidonic Acid

2OHFA: 2-hydroxy Fatty Acid

ED50: Median effective dose

EOAD: Early-onset Alzheimer's disease

ER: Endoplasmic reticulum

EPA: Eicosapentaenoic Acid

FAD: Familial Alzheimer's disease

FAME: Fatty Acyl Methyl Ester

GC: Gas chromatography

LOAD: late-onset Alzheimer's disease

LCFA: long Chain Fatty Acid

IHC: Immunohistochemistry

MAP: Microtubule-associated protein

MUFA: Monounsaturated Fatty Acid

NFT: Neurofibrillar tangles

PI: Performance index

PUFA: Polyunsaturated Fatty Acid

PSEN: Human presenilin gene

SCFA: Short Chain Fatty Acid

SFA: Saturated Fatty Acid

UAS: Upstream activating sequence

UFA: Unsaturated Fatty Acid

WB: Wstern blot

α-secretase: Alpha secretase

β-secretase: Beta secretase

γ-secretase: Gamma secretase

Taken from (Prussing et al., 2013)

To *Drosophila melanogaster*, Thanks for your nice friendship

ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by early synaptic and late neuronal loss, affecting more than 26 million people worldwide.

Among patients affected with dementia, more than half suffer from Alzheimer's disease. The biggest risk factor for developing Alzheimer's disease is age. β-amyloid (Aβ) plaques and neurofibrillary p-Tau tangles accumulates in the brains of elderly patients playing a central role in the pathogenesis of AD. During the last years the fruit fly, *Drosophila melanogaster* has increasingly been used as a model for neurodegenerative disease. Although the adult fly has a simpler nervous system than those of vertebrates, it is capable of higher-order brain functions, including aversive and appetitive learning, and recalling learned information from prior experiences.

The advantages of using the *Drosophila* model are the well-defined genetic characteristics, the quantity, short life span, simplicity in genetic manipulation and the powerful binary UAS-Gal4 transgenic system. The UAS-Gal4 system allows for rapid generation of individual strains in which expression of a specific gene of interest can be directed to different tissues or cell types.

Our transgenic flies expresses human Aβ42 and human Tau fused to a secretion signal for extracellular localization under the control of UAS/GAL4 dual activation system. The overexpression of Aβ42 and Tau in the nervous system of *Drosophila* results in progressive structural and behavioral phenotypes such as locomotor deficits, age-dependent neurodegeneration in the brain and reduced lifespan.

This work has been focused on modeling Alzheimer's diseases in *Drosophila* by expressing two human genes associated with AD (Aβ42 and Tau) in the fly central nervous system**.** This model displays AD-like neuropathological as well as behavioral symptoms. The main goal of developing such a model is to analyse and study the effect of new synthetic fatty acids molecules in the pathogenesis of AD. Additionally, the model organisms established in this study could provide tools that help to understand disease-specific processes resulting in neuronal loss. This study argues that *Drosophila* can be used to study the behavioural basis of human neurodegenerative diseases and may provide a model to identify novel therapeutic avenues for neurodegenerative diseases as Alzheimer's disease.

In this work also was studied the effect of membrane lipid therapy on cognitive decline of a transgenic model of *Drosophila*. This model overexpresses the human amyloid peptide of 42 amino acids (Aβ42), and human Tau protein that play a key role in the development of this

disease. The treatment has been based on the use of DHA and its hydroxylated derivate OHDHA, ARA and its hydroxylated form OHARA and EPA and its hydroxylated form OHEPA at 1, 3, 10, 30, 100 and 250 µg/ml of standard food. After testing the transgenes expression in the F1 generation by PCR analysis and Western blot it was evaluated the toxicity of the compounds, and it was demonstrated that food supplementation with OHDHA, OHARA, OHEPA partially restored the loss of locomotor activity and increased the life-span of the flies expressing the human transgenes whereas the DHA, ARA, EPA, form had not significant effects. It has been observed that the concentrations of 30 and 100 µg/ml of hydroxylated form, including the mixtures of (OHDHA+OHARA), (OHEPA+OHARA), and 30 µg/ml of TGMs, LP183A1, LP183A2, was used, have led to cognitive improvement and have maintained or increased the lifespan with respect to the control group.

No differences were found in the insert expression either in control group or in the treated group as expected since the model was designed to over-express the insert without being influenced by the provided drugs.

In addition it was analyzed the lipid content from *Drosophila* heads by using gas chromatography and it was found that the food supplementation with either hydroxylated or non-hydroxylated compounds induced changes in the fatty acid profile of *Drosophila*. Furthermore it was discovered that the amount of short chain fatty acids (SCFA), from the heads of F1 treated with ARA, EPA and DHA was less than that from untreated F1 flies. Concerning the hydroxylated fatty acids, the reduction in the levels of short chain fatty acid (SCFA) was similar to that of the non-hydroxylated fatty acids. All food supplement tested induced an increase of long chain fatty acids (≥ 18C). ARA, EPA and DHA were present in the fatty acid profile of flies treated with the respective non-hydroxylated food supplements. This fact proves the absorption and incorporation of dietary PUFAs into the *Drosophila* body tissues.

RESUMEN

La enfermedad de Alzheimer (AD, del inglés Alzheimer's disease) es una patología neurodegenerativa caracterizada por una pérdida temprana de conexiones sinápticas y, de manera tardía, de neuronas. Esta enfermedad afecta a unos 40 millones de personas en todo el mundo. Entre las personas con demencia, más de la mitad sufren AD. El mayor riesgo para desarrollar la enfermedad de Alzheimer es la edad. De hecho, las placas β-amiloide (Aβ) y ovillos neurofibrilares de fosfo-Tau se acumulan en los cerebros de pacientes ancianos, jugando un papel central en la patogénesis de AD. Además, se han encontrado reducciones significativas en los niveles de los lípidos fosfatidiletanolamina y ácido docosahexaenoico (DHA) en el cerebro de pacientes con AD. Durante la última década, la mosca de la fruta (*Drosophila melanogaster*) se ha utilizado como modelo para enfermedades neurodegenerativas, debido a que puede ser utilizada para el análisis de conductas como el aprendizaje aversivo y apetitivo, así como su capacidad de utilizar la información aprendida de previas experiencias, aunque la mosca adulta presenta un sistema nervioso mucho más simple que el de vertebrados..

Entre las ventajas de usar el modelo de *Drosophila* están el conocimiento exhaustivo de sus características genéticas, la posibilidad de obtener un número de individuos muy elevado, la corta vida media y la simplicidad en la manipulación genética, incluyendo el sistema utilizado en el presente estudio (Powerful Binary UAS-Gal4 Transgenic System). El sistema UAS-Gal4 permite una rápida generación de cepas en las que se expresan los genes de interés en distintos tejidos o tipos celulares. Mediante este sistema se pueden expresar los péptidos humanos Aβ42 y Tau en el sistema nervioso de *Drosophila melanogaster*, mostrando un fenotipo con deterioro progresivo asociado con el AD tanto estructural como comportamental, con desarrollo de déficit locomotor, neurodegeneración cerebral asociada con la edad y menor esperanza de vida.

La presente investigación se centra en la utilización de *Drosophila* como modelo de AD mediante la sobreexpresión de los genes humanos asociados con AD (Aβ42 y Tau) en el sistema nervioso central de la mosca. El principal objetivo de desarrollar este modelo es analizar y estudiar el efecto de ácidos grasos sintéticos novedosos en la terapia de la AD. Conjuntamente, los organismos modelo establecidos en este trabajo pueden constituir un sistema que permita la comprensión de los procesos específicos de la enfermedad que desencadena la pérdida neuronal. Con todo ello, el presente trabajo demuestra que se puede usar *Drosophila* para estudiar las bases comportamentales de las enfermedades humanas

neurodegenerativas y puede suponer un modelo para identificar nuevas terapias para dichas enfermedades, tales como AD.

Además, se ha estudiado el efecto de la terapia lipídica de membrana en el declive cognitivo del modelo transgénico de AD de *Drosophila*. Este modelo sobreexpresa el péptido amiloide humano de 42 aminoácidos (Aβ42) y la proteína humana Tau, ambas con un papel importante en el desarrollo de esta enfermedad. Los tratamientos empleados se basan en el uso de DHA y su derivado hidroxilado OHDHA, ARA y su forma hidroxilada OHARA y EPA y su forma hidroxilada OHEPA, así como derivados de triacilgliceroles (triacilglicerol miméticos, TGM) a dosis crecientes y añadidos en la comida.

Tras confirmar la expresión de los transgenes en la generación F1 de las moscas por PCR y western blot, se analizó la toxicidad de los distintos compuestos y se demostró que la suplementación de comida con OHDHA, OHARA, OHEPA restauró la pérdida de actividad locomotora, parcialmente, además, aumentó la vida media de las moscas expresando los transgenes humanos, mientras que DHA, ARA, EPA no presentaron efectos significativos. Se observó que las concentraciones de 30 y 100 µg/ml de las formas hidroxiladas, incluyendo las mezclas de (OHDHA+OHARA), (OHEPA+OHARA) y 30 µg/ml de TGMs, LP183A1, LP183A2, mejoraron la capacidad cognitiva y aumentaron la vida media con respecto al grupo control no tratado. No se encontraron diferencias en la expresión del inserto entre los grupos control o tratados, tal y como se esperaba debido a que el modelo se diseñó para sobreexpresar el inserto independientemente de los compuestos administrados.

También se analizó el contenido lipídico en membranas de la cabeza de moscas mediante cromatografía de gases y se observó que la suplementación de la comida, tanto con los compuestos hidroxilados como los no-hidroxilados estudiados, indujo cambios en el perfil de ácidos grasos de *Drosophila melanogaster*. Entre ellos, se observó una menor cantidad de ácidos grasos de cadena corta en cabezas de moscas F1 tratadas con ARA, EPA and DHA en comparación con moscas no tratadas. En cuanto a los ácidos grasos hidroxilaqdos, presentaron un nivel similar en la reducción de los niveles de ácidos grasos de cadena corta. Además, todos los suplementos añadidos a la comida indujeron un aumento de los ácidos grasos de cadena larga (≥ 18C). Finalmente, se observó la presencia de ARA, EPA y DHA en el perfil de ácidos grasos de las moscas tratadas con el correspondiente ácido graso no-hidroxilado. Este hecho prueba la absorción e incorporación de los ácidos grasos poliinsaturados presentes en la dieta en los tejidos de la *Drosophila.*

INTRODUCTION

Alzheimer's Disease

Neurodegenerative diseases (NDDs) are a family of disorders characterized by progressive loss of neuronal structure and its function, resulting in neuronal death in the nervous system (Maciotta et al., 2013). At least there are more than 50 different diseases that can lead to dementia (Tomlinson, 1977). There were an estimated 44.4 million people with age related dementia worldwide as in the 2013. This number will increase to an estimated 75.6 million in 2030, and 135.5 million in 2050 (Acerra et al., 2014). The most common cause of senile dementia, is Alzheimer's disease (AD), with advanced age as the most significant risk factor for this disease (Bertram and Tanzi, 2012; Jiang et al., 2012; Mhatre et al., 2014; Mosher and Wyss-Coray, 2014). AD accounts for two thirds of these dementia cases, and turns it to the leading causes of dementia (Mhatre et al., 2014). The estimated number of AD is ~30 million patients worldwide (Nicolas and Hassan, 2014).

The disease was first described in 1901 by Dr. Alois Alzheimer, a german psychiatrist and neuropathologist. The patient was a 51 year old woman named Auguste D (Figure 1), who was presented to him by her family after they found out changes in her personality and behavior. Her family reported her problems with memory, difficulty speaking, and impaired comprehension. Dr. Alzheimer described Auguste D patient as having an aggressive form of dementia, manifested in her memory, language and behavioral deficit (Radebaugh et al., 1996). Dr. Alzheimer has noted many abnormal symptoms, including difficulty with speech, agitation and confusion. He followed her progressive mental decline till she died in 1906, then he did his findings public in November 1906, he had made a presentation to the meeting of South West Germany describing his observation of tangles and plaques during the post examination of Auguste's brain (Hardy, 1996). At autopsy, Dr. Alzheimer noticed that the patient's cerebral cortex looked atrophied, with widening of the sulci. He also identified histopathologic changes, which are known as the pathologic hallmarks of the degenerative condition of AD: neurofibrillary tangles and neuritic plaques. Finally, the name of the disease was given in 1910 by Kreapelin, Dr Alzheimer senior colleague.

The knowledge of AD reaches behind the scientific communities and medical professionals. In particular condition, AD became a digressing and is sometimes used synonymously or being disquieted with dementia by nonprofessionals and public media. However the effect of the disease on aging societies becomes more and more subject of public discussion (Wadman, 2012).

AD is a neurodegenerative procedure of the central nervous system (CNS) which is clinically characterized by the development of early amnestic and executive dysfunction, which leads to

the eventually spreads across cognitive domains, that leads to the complete incapacity and development of end-stage dementia (Jicha and Markesbery, 2010). It is recognized as a multifactorial illness with both genetic and nongenetic causes. Four major genes have been identified related with patrimonial risk for AD: presenilin-1, presenilin-2, amyloid precursor protein (APP), and apolipoprotein E. Mutations in these genes cause dysregulation of amyloid precursor protein processing, and in particular of the handling of a proteolytic derivative termed beta-amyloid (Aβ) that eventually causes neuronal dysfunction and death (Cifuentes and Murillo-Rojas, 2014).

As the disease progress the clinical profile include delusions, focal symptoms, hallucinations and executive dysfunction leading to advanced helplessness in the more advanced stages (Goedert and Spillantini, 2006; Van Dam and De Deyn, 2011). The disease duration could range from 1 to 25 years and the cause of death is associated with malnutrition or pneumonia (Bird, 2008).

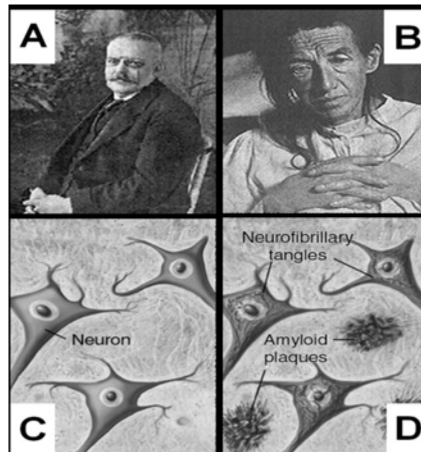


Figure 1. The beginning of Alzheimer's disease. Dr. Alois Alzheimer (A) and his patient Auguste Deter (B) in whom he first described the pathological characteristics of Alzheimer's disease (D) compared to the normal situation (C). Reproduced courtesy of Alzheimer's Disease Research, a program of the American Health Assistance Foundation. © 2012.

The high incidence of AD in older individuals together with the progressive increase in the current life expectancy, an aging population in developed countries and high emotional costs and health from the disease, make AD one of the most important social and health issues of the day and near-future.

The etiology of this disease is still unknown and there is currently no effective drug treatment for alleviating neurological deficits that occur during the course of the same or at least to delay the onset of their symptoms. In this regard, the most common treatment is symptomatic using acetylcholine esterase inhibitors. However, the use of these inhibitors only partially slows the cognitive deficits observed in patients. Furthermore, only 50% of these patients respond to the treatment, which means that the cholinergic system is not an optimal

pharmacological target in combating the symptoms and/or progression of this disease. Thus, an effective treatment of this pathology, or even of the cognitive deficits of normal aging, should prevent or delay the progressive neuronal death that occurs in this process.

Neuropathological features of Alzheimer's disease

In Alzheimer's disease (AD), the amyloid hypothesis postulates that a short peptide, amyloid-β (Aβ), is a prime causative agent in the pathological sequence of the disease. Of the two predominant Aβ isoforms—Aβ40 and Aβ42— the latter is more often involved in AD (Saad et al., 2014). The expressing of either Aβ42 or its precursor protein (AβPP) in transgenic strains of the fruit fly, *Drosophila melanogaster*, leads to AD-like phenotypes, including Aβ aggregation, neuronal dysfunction, shorter lifespan, and age-dependent behavioral deficits (Saad et al., 2014).

In the AD brain, the degeneration observed is a result of the loss of both neurons and synapses engaged in a diversity of neurotransmitter systems containing the cholinergic, glutamatergic, noradrenergic and serotonergic (Francis, 2005; Wenk, 1988). The neuropathological changes of AD are manifested in certain areas, brain vulnerable to this disease, initially in the entorhinal cortex and progresses to the hippocampus, the neocortex finally engaging with the progress of the disease. According to the changes observed in the structure of the brain, the lateral ventricles containing cerebrospinal fluid are fond extremely enlarged (Bakkour et al., 2009), (Figure 2).

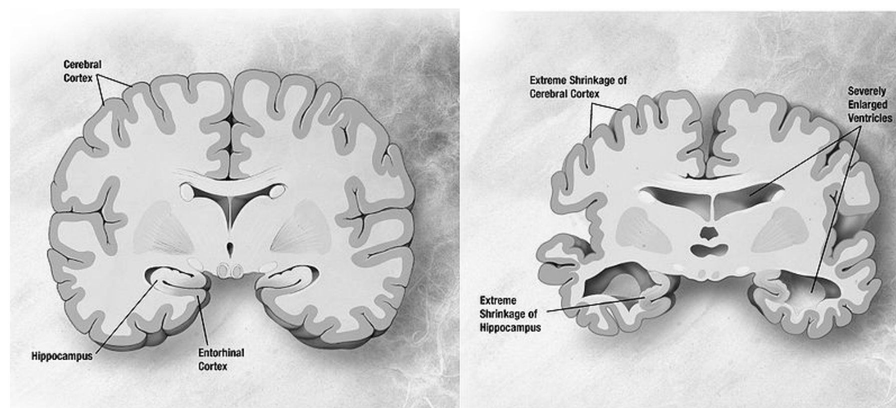


Figure 2. Atrophy of the brain during Alzheimer's disease. The image on the left represents a normal, healthy brain whereas the image on the right represents typical gross changes to the brain in severe AD (Image taken from www.nia.nih.gov/Alzheimers/Resources/HighRes.htm).

AD can be characterized by two major neuropathological features: extracellular accumulations of amyloid-β peptides in the form of plaques and intracellular tangles, consisting of hyperphosphorylated Tau proteins (Liebscher and Meyer-Luehmann, 2012). The

prevailing hypothesis for how AD develops is deceptively simple (Niedowicz et al., 2011), (Figure 3), it is generated by sequential proteolytic processing of transmembrane amyloid precursor protein (APP) by two enzymes, β-secretase (β-site APP cleaving enzyme or BACE-1) and γ-secretase, in the reference amyloidogenic processing pathways. The main sources of Aβ and phosphorylated Tau in the cerebrospinal fluid of patients suffering of Alzheimer's disease are supposed to get together with neuronal injury or neurodegeneration. Aβ and phosphorylated Tau in cerebrospinal fluid are, thus, important biomarkers for the diagnosis of Alzheimer's disease.

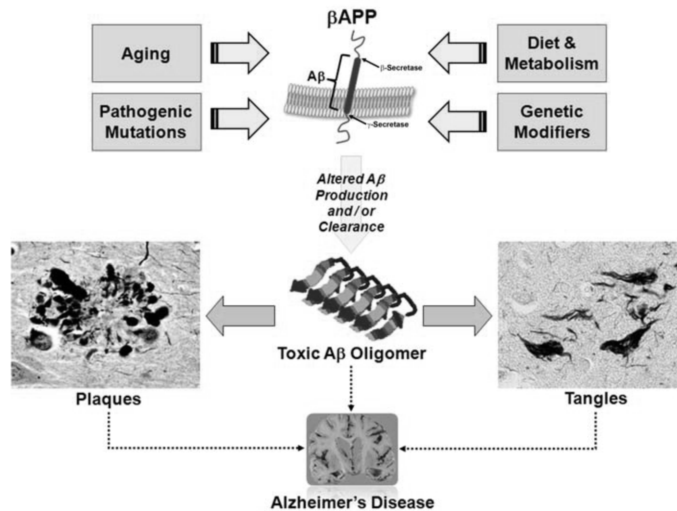


Figure 3. The affecting factors of amyloid accumulation and AD pathology. Adapted from (Niedowicz et al., 2011)

Increased concentration of Aβ and phosphorylated Tau with disease progression appear to correlate with the conversion from cognitive normalcy or mild cognitive impairment to dementia (Perrin et al., 2009), (Figure 4). As specific anatomical pattern of pathological changes initiated, it propagates between neighboring neuronal cells, possibly spreading along the axonal network (Santa-Maria et al., 2012). In this regard, it has been demonstrated that axonopathy, mainly manifesting as impairment of axonal transport and swelling of the axon and varicosity may play a very important role in the neuropathological mechanisms (Zhang et al., 2012b).

Brain with AD exhibit gross cerebral cortex atrophy and also microscopic extracellular amyloid-β (Aβ) neuritic plaques and intraneuronal neurofibrillary tangles (NFT) (Bird, 2008). Although in the brain there are mainly two variants of peptide 40 and 42 amino acids (named Aβ40 and Aβ42 respectively) (Naslund et al., 1994), in normal conditions, the Aβ40 is the most abundant variant, whereas the Aβ42 peptide is the main component of the amyloid plaques. Aβ42 is the most insoluble form and aggregates (Jarrett et al., 1993; Younkin, 1995). In fact, the increase in the Aβ42/Aβ40 relationship is one of the most useful parameters to

confirm the diagnosis of probable AD. The neuritic plaques Aβ42 plays a central role in the pathogenesis of AD, and Tau acts downstream of Aβ42 as modulator of disease progression (Iijima-Ando and Iijima, 2010).

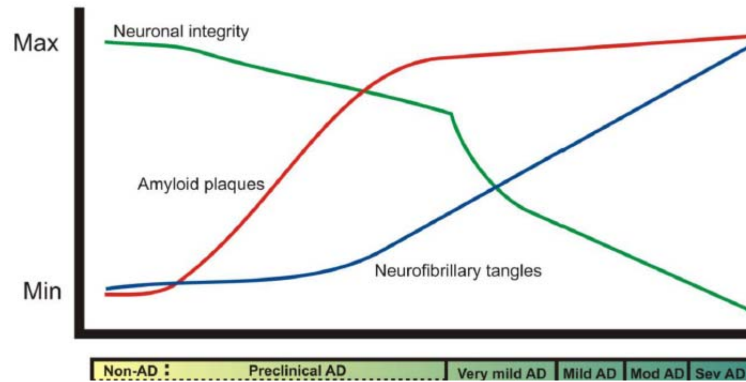


Figure 4. The quantities of senile plaques (red), neurofibrillary tangles (blue) and the neuronal integrity (green). In a relationship with the time route of pathological and clinical stages of Alzheimer's disease (AD). Adapted from (Perrin et al., 2009).

THE AMYLOID-B (AB) PEPTIDE

Amyloid plaques are spherical and complex protein accumulations with β-sheet structure. They are divided into neuritic plaques and diffuse plaques (Duyckaerts et al., 2009; Holtzman et al., 2011), (Figure 5). β-amyloid forms insoluble protein aggregates that deposited extracellularly and mainly formed by the Aβ peptide (Glenner and Wong, 1984; Masters et al., 1985). The amyloid plaques are mainly composed of Aβ42, derived from the cleavage of APP by both β-secretase (N-terminus) and γ-secretase (C-terminus) enzymes (Chow et al., 2010), and result in the formation of Aβ(1-40) and/or Aβ(1-42) amyloid precursor protein (APP) by sequential proteolysis, catalyzed by the aspartyl protease BACE, and followed by presenilin-dependent gamma-secretase cleavage (Phiel et al., 2003). The peptides Aβ40 and Aβ42, particularly Aβ42, are secreted by neurons, oligomerize, can disrupt synaptic function, and eventually cause cognitive deficits and neurodegeneration (Bertram et al., 2010). Aβ42 peptide expressed in the fly central nervous system leads to reduce life span (Finelli et al., 2004).

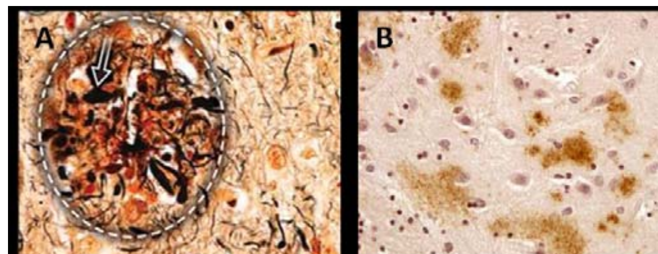


Figure5. Amyloid plaques visualized by immunohistochemistry. (A) Neuritic plaque with dystrophic neurites (small arrow). Adapted from (Holtzman et al., 2011). (B) Diffuse plaques. Adapted from (Duyckaerts et al., 2009).

It has been developed an adult-onset *Drosophila* model of AD by using the gene-switch system (an inducible gene expression system to express the Aβ42 Arctic mutant specifically in adult neurons), thus avoiding the complications associated with expression of this protein during development (Sofola et al., 2010). The Aβ display a disposition to aggregate as β-sheet pleated protein and exists at least intermediately as soluble oligomers before it forms highly insoluble fibrils that eventually deposit as plaques in the brain parenchyma (Niedowicz et al., 2011). There is evidence that the soluble forms are the main neurotoxins (Niedowicz et al., 2011), that damage the neurons and stimulate the formation of NFT, in that way linking the two major AD pathologies (Kawabata et al., 1991; Niedowicz et al., 2011). Accumulation of aggregated Aβ, as the key pathological case driving neurodegeneration in AD and Down's syndrome was introduced by George Glenner (Glenner and Wong, 1984). Consecutive cleavage of APP, by its cleaving enzyme termed γ-secretase, generates 38-43-amino-acid Aβ peptides (Haass and De Strooper, 1999). Aβ42, the longer peptide that represents 10% of all Aβ-species in the brain, is sound to become the more amyloidogenic form of the peptide since it is prone to aggregate. From here Aβ42 peptides have an increased propensity to accumulate as extracellular amyloid deposits in senile plaques and cerebral blood vessels, simultaneously with shorter fragments of the peptide in amorphous form they comprise senile plaques in AD brains (Selkoe, 1999). Aβ increases phosphorylation of Tau protein and concomitantly activates glycogen synthase kinase, GSK-3 (Iijima-Ando and Iijima, 2010). The oligomers present a variety of lengths: a width of approximately 1-3 nm, and a various lengths from between 5-10 nm. Larger oligomers with a length of 15-25 nm and a width ranging between 2-8 nm, are common at high concentrations of Aβ (Mastrangelo et al., 2006).
On the other hand, diffuse plaques vary in size from 50 µm to several hundred µm and consist of amorphous, non-fibrillar plaques of Aβ with little or no detectable neuritic dystrophy (Yamaguchi et al., 1988). Four kDa Aβ peptides becomes neurotoxic in excess quantities or when it fails to become degraded and the polymerization will occur (Koechling et al., 2010). The small protein of Aβ is designated as β-amyloid protein (Aβ), after the β-pleated sheet structure. Beyreuther and Masters have extended this work and they showed that the same protein (Aβ) is the major constituent of amyloid cores in the parenchyma of AD brains (Masters et al., 1985). Most of the "Familial AD" mutations in APP and presenilin (α-secretase catalytic component) genes result in overproduction of Aβ42 (Saido, 2013).

NEUROFIBRILLARY TANGLES.

Neurofibrillary tangles (NFTs) consist of large non-membranous bundles of abnormal fibers present in the perikarya or in the apical dendrites (Holtzman et al., 2011), (Figure 6).

Intraneuronal neurofibrillary tangles (iNFTs) are found in the brains of Alzheimer's disease patients (Lasagna-Reeves et al., 2012). The iNFTs are caused by hyperphosphorylation of the Tau protein, leading to inhibition of Tau's ability to bind and stabilize microtubules (Lin et al., 2007). In AD brain neurofibrillary degeneration is seen as intra-neuronal neurofibrillary tangles, neuropil threads, and as dystrophic neurites surrounding the β-amyloid core in neuritic (senile) plaques. Neurofibrillary tangle (NFTs) formation is a late event and is observed in brains affected by various neurodegenerative diseases as well as AD (Morishima-Kawashima and Ihara, 2002). The number of neurofibrillary tangles directly correlate**s** with the presence and the degree of dementia in AD (Iqbal et al., 2009).

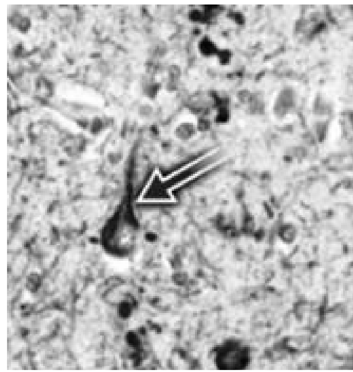


Figure 6. Neurofibrillary tangles in AD. Anti–phospho-Tau antibody reveals hyperphosphorylated Tau accumulation in neuronal cell bodies (large arrow). Adapted from (Holtzman et al., 2011).

TAU

Tau protein tangles are found in the brains of Alzheimer's disease patients (Umahara et al., 2002). The major component of the neurofibrillary (NFTs) is the protein Tau, a microtubule binding protein responsible for axonal transportation and cellular communication (Price et al., 1998). The gene coding for Tau, *mapt*, is located on chromosome 17 and consists of 16 exons, even though only 11 are constitutive for the isoforms found in the central nervous system (CNS) (Goedert et al., 1988; Neve et al., 1986). Tau in non-diseased mature neurons phosphorylates at a nanomolar degree and binds to tubulin that way promoting microtubuli assembly. The higher degree of phophorylation results in Tau detachment from microtubuli (Iqbal et al., 2010). The normal Tau is hydrophilic and therefore soluble and virtually all Tau is bound to microtubules (Iqbal et al., 2010)

Tau is abnormally hyperphosphorylated in AD brain and, in this form, is the major protein subunit of the paired helical filaments (PHF) and straight filaments (SF) forming neurofibrillary tangles, neuropil threads, and plaque dystrophic neurites in AD (Iqbal et al., 2009). Tau hyperphosphorylated do not bind to tubulin or promote microtubule assembly, but instead inhibits assembly and disrupts microtubuli (Goedert et al., 1992).

Cyclin -dependent kinase 5 (CDK5) and glycogen synthase kinase 3 (GSK3) are two main protein kinases that have an important role in the abnormal hyperphosphorylation of microtubule associated protein Tau (MAPT) which leads to Alzheimer's disease (Jayapalan and Natarajan, 2013). GSK3 and CDK5 are also called Tau kinase I and Tau kinase II (Ishiguro et al., 1993). Glycogen synthase kinase 3β (GSK3β) is the major kinase that phosphorylates Tau (Cho and Johnson, 2004).

These kinases may be activated by elements of AD pathology like inflammation, oxidative stress, Aβ and cell cycle re-entry (Arnaud et al., 2006), and play an important role in the phosphorylation of Tau. GSK-3 is located in the regions adjacent to the domain of Tau implicated in microtubule binding residues. Furthermore, GSK-3 interacts with other proteins, such as the presenilin 1 (PS1), which have an important role in the initiation of the pathology (Anderton, 1999; Ballatore et al., 2007). CDK5 phosphorylates Tau protein also, may facilitate subsequent modification of Tau by GSK3. CDK5 phosphorylation appears to be regulated by the p35 protein in AD and the former can undergo proteolysis processing and thereby achieving aberrantly phosphorylated Tau (Patrick et al., 1999). Importantly, Tau mutations do not result in AD but to frontotemporal dementia, probably linked to other neurodegenerative dementias such as corticobasal degeneration and progressive supranuclear palsy (Kar et al., 2005).

Types of Alzheimer's disease.

There are two forms of AD based on the age that AD is diagnosed. Early onset familial AD (EOFAD) which is diagnosed before the age of 65, usually in the 40s or 50s, and comprises about 5-10% of all AD patients and late onset AD (LOAD), also called sporadic AD which is diagnosed after the age of 65 and comprises approximately 90-95% of all AD patients (Tanzi and Bertram, 2005).

Familial Alzheimer's disease (FAD).

Although the majority of AD cases are sporadic, 10% are familial (AD) and are inherited in autosomal dominant fashion. FAD represents a small percentage of AD cases and it is considered that most non-hereditary or sporadic forms of the disease have a complex etiology involving a variety of environmental and genetic factors. A large number of genes are believed to be involved in AD pathogenesis based on either their chromosomal location or their function. Moreover, more than 1,000 polymorphisms in over 350 genes have been involved in increasing AD susceptibility (Bertram et al., 2007; Rocchi et al., 2003). FAD is

clinically and pathologically indistinguishable from AD with the exception that the onset of symptoms typically occurs earlier in AD (Bird, 2008). FAD has been linked to three genetic loci, amyloid precursor protein (APP) (Goate et al., 1991), presenilin-1 (PSEN1) (Sherrington et al., 1995), presenilin -2 (PSEN2) (Rogaev et al., 1995), however all these mutations affect APP processing and increase the total amount of Aβ, increase the Aβ42/Aβ40 ratio or to increase the propensity for Aβ to aggregate (Table 1). Biochemically, FAD-associated mutations in *PS* gene increase the relative concentration of the aggregation-prone Aβ42 (Brunholz et al., 2012; Iwatsubo, 2004; Sisodia and St George-Hyslop, 2002).

Table 1. Overview of the diversified FAD. The mutations causing and their effects on APP processing and the production of Aβ. Adapted from (Brouwers et al., 2008).

Gene	Type of mutations	Location of mutations	Effect of mutations
APP	missense	N-terminal of Aβ peptide (β-secretase site)	enhanced β-secretase cleavage → increased Aβ production
APP	missense	Aβ peptide encoding region	alteration of the Aβ sequence and its properties → increased aggregation propensity → increased protofibril and/or fibril formation reduced α-secretase cleavage → increase in β-secretase substrate
APP	missense	C-terminal of Aβ peptide (γ-secretase sites)	decreased cleavage at $Aβ_{40}$ and/or increased cleavage at $Aβ_{42}$ → relative increased production of $Aβ_{42}$ compared to $Aβ_{40}$
APP	gene/locus duplication	whole gene	increased levels of APP as substrate for Aβ production; relatively increased production of $Aβ_{42}$ compared to $Aβ_{40}$
APP	promoter mutations	5′ regulatory region	increased levels of APP as substrate for Aβ production
PSENs	missense mutations—insertions/deletions—genomic deletions	scattered over the protein	decreased γ-secretase activity alterations in the position of the cleavage site → relative increased production of $Aβ_{42}$ compared to $Aβ_{40}$

MUTATIONS IN THE GENE FOR AMYLOID PRECURSOR PROTEIN.

Mutations in the *APP* gene have been found in a small number of families with disease onset before 65 years of age (Guo et al., 2000), and these mutations, localized on chromosome 21 have been found in some rare forms of familial early-onset AD (FAD**)** (Alafuzoff et al., 1987; Chartier-Harlin et al., 1991; Goate et al., 1991; Murrell et al., 1991). Most identified mutations are located in close vicinity to the cleavage sites of α-, β- and γ-secretases resulting in increased production of Aβ peptides (Suzuki et al., 1994). The APP gene sequencing and testing for mutations in individuals with the disease, confirmed that the APP gene was associated with this pathology. Most of APP missense mutations alter APP processing in a pathological manner by increasing either overall production of Aβ-peptide or generating highly fibrillogenic Aβ variants, like Aβ42 (Citron et al., 1992; Haass et al., 1994; Price and Sisodia, 1998; Price et al., 1998; Suzuki et al., 1994). All the mutations in APP result in higher Aβ42 generation, leading to accumulation of Aβ42. Aggregation properties of Aβ42

lead to rapid assembly of peptides into oligomers and fibrils inducing neuronal dysfunction and neuronal loss (Reddy and Beal, 2008).

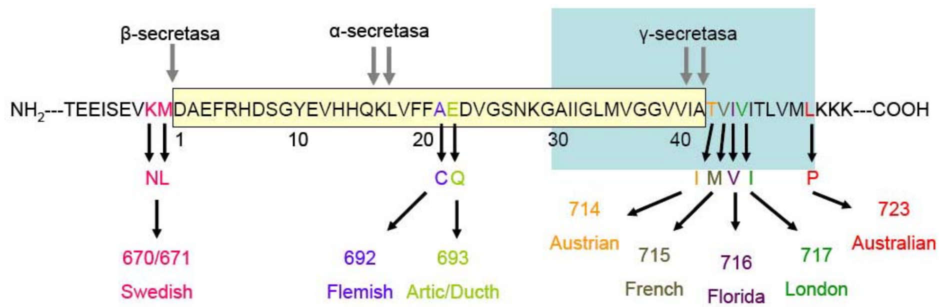


Figure 7. Amino acid sequence of the APP protein. The mutations described in familial AD and processing sites, the sequence of Aβ is framed in the light yellow box. The blue rectangle delimits the transmembrane domain. The arrows above indicate the breakpoints secretase, while the dotted arrow indicates the cleavage site generated by the amyloid peptide insoluble. The mutations described in cases of familial AD are highlighted in color. Adapted from (Evin and Weidemann, 2002).

Interestingly, AD related APP mutations were found within the region encoding Aβ or immediately adjacent to β and γ-secretase cleavage sites (Hardy, 1996), that strengthen the amyloid cascade hypothesis. The mutations found in the APP gene (currently 27 known mutations, www.uia.ac.be/ADMutations) account for a small proportion, around 5% of cases of familial Alzheimer’s. Sequencing of exon 17 of the APP gene led to the discovery of mutations in families with early development of AD (Figure 7).

MUTATIONS IN PRESENILIN GENE PRESENILIN 1 AND 2.

Mutations in the presenilins PSEN1 and PSEN2 are the most common cause for FAD. PSEN1/2 encode the catalytic subunits of the γ-secretase complex (Lai et al., 2003).

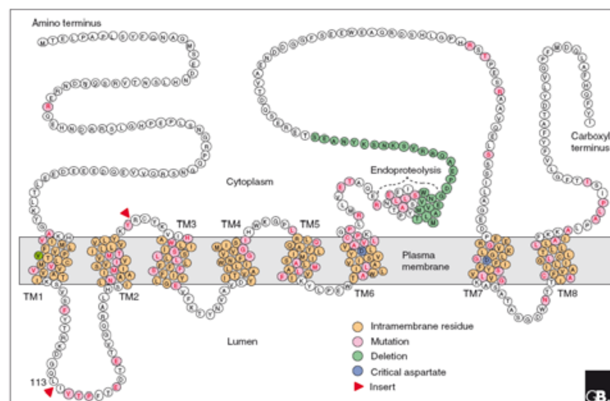


Figure 8. Structure of PS1. Amino acid sequence of presenilin-1 not processed (full-length), and mutations described familiar AD. The protein consists of 8 transmembrane domains. Color code indicated the mutations present in the familial Alzheimer's. Adapted from (Tandon and Fraser, 2002).

Presenilins mutations account for up to 50% of FAD cases (Guo et al., 2000), and are also leading to increase production of Aβ peptides; in particular mutations in PSEN2 are consistently raising production of Aβ42 (Bentahir et al., 2006). In the year 1995, independent

groups identified genetic linkage and mutations within PSEN*1* (chromosome14) and PSEN*2* (chromosome 1) genes in several early onset familial FAD (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995).

The protein is thought to have eight transmembrane domains (Tandon and Fraser, 2002), (Figure 8). However, mutations that alter the remaining structure of the protein were also found. Currently more than 150 mutations are known. Presenilins in vertebrates, are broadly expressed (Lee et al., 1996), and primarily localized to the endoplasmic reticulum and Golgi apparatus (Cook et al., 1996; De Strooper et al., 1997; Walter et al., 1996). At a cellular level presenilins primarily localize to the ER and Golgi apparatus and to a lesser extent to the plasma membrane (Berezovska et al., 2003). Even though the function of presenilins is not fully understood, a role in protein processing was first proposed based, in part, on their subcellular distribution and their ability to affect processing of APP and Notch, a protein that regulates cell-fate decisions in virtually all species (Borchelt et al., 1996; Citron et al., 1997; Song et al., 1999; Struhl and Greenwald, 1999; Tomita et al., 1997). Although PS1 is expressed relatively at higher levels than PS2*,* both proteins ubiquitously expressed in the brain and peripheral tissues in adult human and rodent (Vetrivel et al., 2006).

The proteolysis and stability of presenilin depends on its combination with the three other molecules in the γ-secretase complex core, nicastrin, Aph-1 and Pen-2 (Takasugi et al., 2003). Although little is known about its regulation and activity, γ-secretase recognizes and cleaves a growing list of transmembrane proteins with very short extracellular domains generated by prior processing (De Strooper, 2003; Struhl and Adachi, 2000; Wolfe and Kopan, 2004).

Sporadic Alzheimer’s disease.

Sporadic Alzheimer’s disease (senile or late onset), has a low incidence to 60-65 years of age, affecting 3 or 4 % of the population. From this age, the incidence rate doubles every 5 years, which means that more than half the population over 80 may suffer from this disease. In the sporadic form of the disease, the main risk factor is genetic or environmental factors, which by themselves are not sufficient to trigger the disease. Therefore, its appearance can be determined by the existing polymorphism between individuals, which may predispose to a greater or lesser degree to the neurodegenerative process. Therefore, the genes involved in the development of Alzheimer's disease -causing agents can be the same and be involved in the initial stages as in cases of familial, can be a predisposing factor for developing it, where in more associated with later stages and the sporadic form (Rocchi et al., 2003).

Gene ApoE.

The apolipoprotein E (ApoE) is the only gene has been definitively associated with the risk for AD (Brouwers et al., 2008), through molecular genetic analysis of a variant that is a confirmed risk factor for both late-onset AD as the earliest forms of this degenerative disease (Mahley et al., 2006; Rocchi et al., 2003). APOE is involved in lipid metabolism, and neuronal maintenance remodeling, toxin removal and repair of tissues. Furthermore, it plays a specific role in the central nervous system, including neuronal development, regeneration and certain neurodegenerative processes. In the brain, astrocytes and microglial cells are the main producers of APOE (Poirier, 2000). It is postulated that APOE modulates the intracellular cytoskeleton and alters extension and branching of neurites in the presence of cholesterol (Handelmann et al., 1992). The isoform of apolipoprotein E, ApoE4, has been shown to confer dramatically increased risk for late onset AD (LOAD) (Roses et al., 1995).

The polymorphism of the ApoE gene determines three polymorphic isoforms of APOE protein called ApoE2, ApoE3 and ApoE4, or variants encoded by alleles of ApoE gene called (ε2, ε3 and ε4) with different conformation and lipid binding properties (Cedazo-Minguez and Cowburn, 2001). ApoE levels are elevated in AD (Mahley et al., 2006) . This protein appears to be involved in the formation of amyloid plaques and neurofibrillary aggregates by interacting with both the peptide Aβ as with Tau protein. In fact, it has been shown that ApoE4 can modulate the processing of APP protein and increase production of Aβ (Ye et al., 2005). In addition, ApoE4 can bind to Aβ to regulate its clearance and degradation, and plays critical roles in regulating brain Aβ peptide levels, as well as their deposition and clearance (Jiang et al., 2008). It has been shown that APOE ε4 increase the rate of Aβ deposition in animal models (Myers and Goate, 2001).

The increase is due to a decrease in clearance of Aβ (Holtzman et al., 2012). This protein may be processed by acid proteases in the lysosome, generating toxic fragments that affect mitochondrial function (Mahley et al., 2006). Finally, it should also be noted that the ApoE4 is able to stimulate the production of prostaglandin E2, produced by inflammatory microglia factor, which also contributes to the pathogenesis of AD (Chen et al., 2005).

Other non-genetic factors.

In addition to age, there is a variety of risk factors that are associated with LOAD. Factors that are associated with increased risk of acquiring AD include a family history of the disease, cardiovascular disease, diabetes, hypertension, heart disease, prior head injury, high alcohol intake, and stroke. Regular exercise, higher education, and proper diet have been reported to be protective, as has smoking (Lee, 1994), despite its other negative health effects.

Amyloid cascade hypothesis

Even though the exact etiology of AD remains elusive, the amyloid cascade hypothesis remains the best-defined and most studied conceptual framework for AD. The amyloid cascade hypothesis proposes that increased production or decreased clearance of Aβ42 peptides is the primary influence driving all forms of AD (Hardy and Selkoe, 2002). The hypothesis starting with an increase in Aβ production and accumulation and the formation of soluble Aβ oligomers. While aberrant Aβ production is generally considered a cause of neurodegeneration, it may be merely a symptom of an underlying cause, namely, altered γ-secretase activity (Pimplikar, 2009), (Figure 9).

This culminates in a series of downstream pathological events including synaptic injury, inflammation, oxidative damage and Tau dysfunction that in turn result in widespread neuronal dysfunction and cell death. However, in the last decade there have been described the oligomeric soluble form of Aβ, able to diffuse into the brain parenchyma, leading to synaptic loss and severe cognitive failures (Haass and Selkoe, 2007).

Consequently, the cascade hypothesis of Aβ has been modified in recent years, so that certain changes in Aβ metabolism provoke the beginning of the cascade, generating elevated levels of Aβ and Aβ42 proportion / Aβ40, either by overproduction or lack of clearance. Because its shape is more hydrophobic Aβ42 than Aβ40 tends to oligomerize more easily, which would enhance the formation of diffusible oligomers.

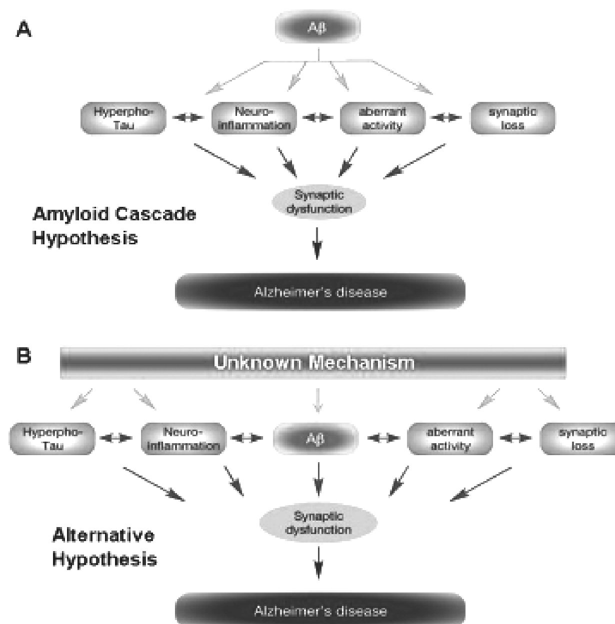


Figure 9. Hypotheses for the role of β amyloid (Aβ) plaques in Alzheimer's disease. (A) Amyloid cascade hypothesis. This well supported hypothesis suggests that Aβ plaques are the root cause of the neurodegeneration

responsible for AD; (B) Alternative hypothesis. This hypothesis proposes that Aβ is a symptomatic product of an unknown mechanism which is also responsible for the other AD characteristics. Adapted from (Pimplikar, 2009).

These produce changes in synaptic function, subtle first and subsequently tightened. Along with the increase in extracellular levels of Aβ level, the first extracellular plaques appear and the subsequent activation of the inflammatory response (astroglia and microglia), causing the loss of synaptic spines and generate dystrophic neurites. These changes would cause neuronal phosphatase kinase activity were altered resulting in hyperphosphorylation of Tau and therefore, damage and axonal transport in the formation of neurofibrillary tangles. Finally, the cascades terminate by the massive neuronal death and a state of progressive dementia (Haass and Selkoe, 2007). The disturbance in calcium homeostasis can promote many neurodegenerative disorders, among other mechanisms, processes mediated by the formation of free radicals (Yatin et al., 1998), and the phosphorylation of Tau protein (Takashima et al., 1993).

APP cleavage

APP is a membrane protein with a large N-terminal glycosylated domain and a short C-terminal cytoplasmic, that belongs to type I membrane glycoprotein, which contains a large amino terminal extracellular/cytosolic domain and a small intracellular C-terminal domain (De Strooper and Annaert, 2000; Nunan and Small, 2000; Schenk et al., 1995). APP presents isoforms that differ in size from 695-770 amino acid residues (Kang et al., 1987; Mattson, 1997; Selkoe, 2001). APP gene is located in chromosome 21, and is a 110-120 kDa protein.

This ubiquitously expressed protein is modified in the secretory pathway by N-glycosylation and O-glycosylation in the endoplasmic reticulum (ER) and the Golgi apparatus (Sinha and Lieberburg, 1999; Weidemann et al., 1989). APP fraction can bypass the endosomal compartment post re-internalization and directly get trafficked to lysosomes (Brunholz et al., 2012). The APP expression is found to be restrictive in neurons for some organisms, indicating its importance in neuronal functions (Brunholz et al., 2012). It has been shown that APP regulates various cellular processes like kinase-based signaling mechanisms, calcium regulation, cell adhesion and others (Thinakaran and Koo, 2008). Aβ-domain of APP leads to targeting of APP to the axonal compartment (Tienari et al., 1996).

The region of Aβ is located at the cell surface with a part of the peptide embedded in the membrane (Kang et al., 1987). However only 10-20% of APP localizes to the plasma membrane, the rest is found within the intracellular compartments of the cell (Bignante et al., 2013). APP is involved in a variety of important roles like cell adhesion, cell migration, apoptosis and also synaptogenesis and insulin and glucose homeostasis (Zheng and Koo, 2006). APP is known to play a central role in the pathogenesis of AD but it’s physiological

function is still not fully understood. APP is enriched at the growth cones of the developing neurites during synaptogenesis, suggesting a functional role of APP in neuronal network formation (Nalivaeva and Turner, 2013). APP plays a role in cell proliferation, cell survival, neuroprotection, memory enhancement, neuronal excitability and regulation of synaptic plasticity (Ma et al., 2008). Mammalian APP belongs to a protein family with two other members: the amyloid precursor like protein 1 and 2 (APLP1 and APLP2).

The three members of APP family are expressed in the brain; APP and APLP2 are also expressed ubiquitously in other tissues (Bignante et al., 2013). Two homologues have been identified in invertebrates: the amyloid protein-like protein 1 (APL-1) in *Caenorhabditis elegan,* (Daigle and Li, 1993), and the amyloid precursor protein-like protein (APPL) in *Drosophila melanogaster,* (Rosen et al., 1989).

APP might play an important role in the neurite outgrowth, axonal transport and apoptotic cell death (Koo, 2002). Furthermore the APP protein is extensively modified by glycosylation (N- or O-glycosylation) that occur just after the APP protein synthesized in the endoplasmic reticulum. On the other hand, phosphorylation and sulfation have place during transit to the plasma membrane in the secretory pathway (Walter et al., 1997). The APP proteolytic processing is a membrane related event, which occurs by sequential cleavage of APP by proteases termed α-, β- and γ-secretase (Sastre et al., 2008), (Figure 10).

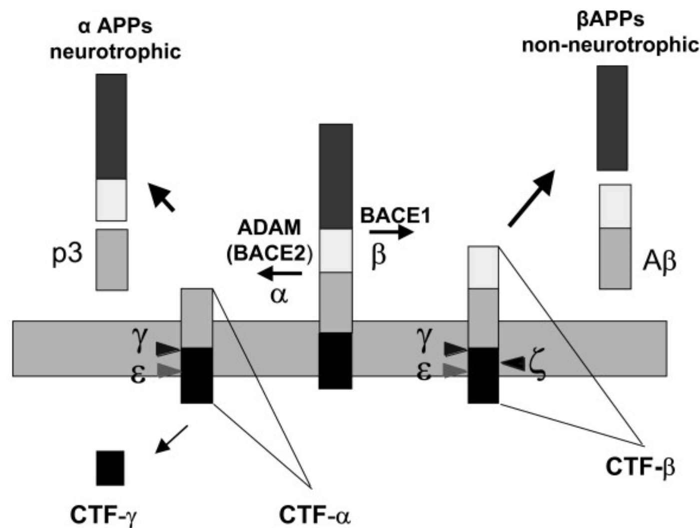


Figure 10. Proteolytic processing of APP. Proteolysis of APP by α-secretase or β-secretase leads to the secretion of αAPPs or βAPPs. Both secretases generate C-terminal fragments (CTF) of 10 kDa and 12 kDa respectively, which are inserted in the membrane (grey). These fragments can be cut by γ-secretase to release the peptides P3 and Aβ. Two further cleavage sites, termed ε and ζ, have been identified in the CTF. Adapted from (Sastre et al., 2008).

The proteolysis of APP can occur in two ways: through the amyloidogenic and non-amyloidogenic pathway (Figure 11). The non-amyloidogenic pathway, α-secretase cleaves APP in the ectodomain within the Aβ region of APP protein that prevents the generation of

Aβ peptide (Chow et al., 2010; Zhang et al., 2012a). Around 90 % of the AβPP cleavage derives from the nonamyloidogenic pathway (Cohen and Kelly, 2003).

Whereas around 10 % of the AβPP cleavage derives from the amyloidogenic pathway, from which the Aβ peptide is released as one of its cleavage products (Cohen and Kelly, 2003). The proteolysis of APP by α-secretase or β-secretase leads to the secretion of soluble α-APPs or β-APPs that generates C-terminal fragments of 10 kDa and 12 kDa respectively, which are integrated in the membrane. These fragments can be cut by γ-secretase to release the peptides P3 and Aβ (Walter et al., 2001), and a cytoplasmic fragment identified as AICD (APP intracellular domain) (Sastre et al., 2001).

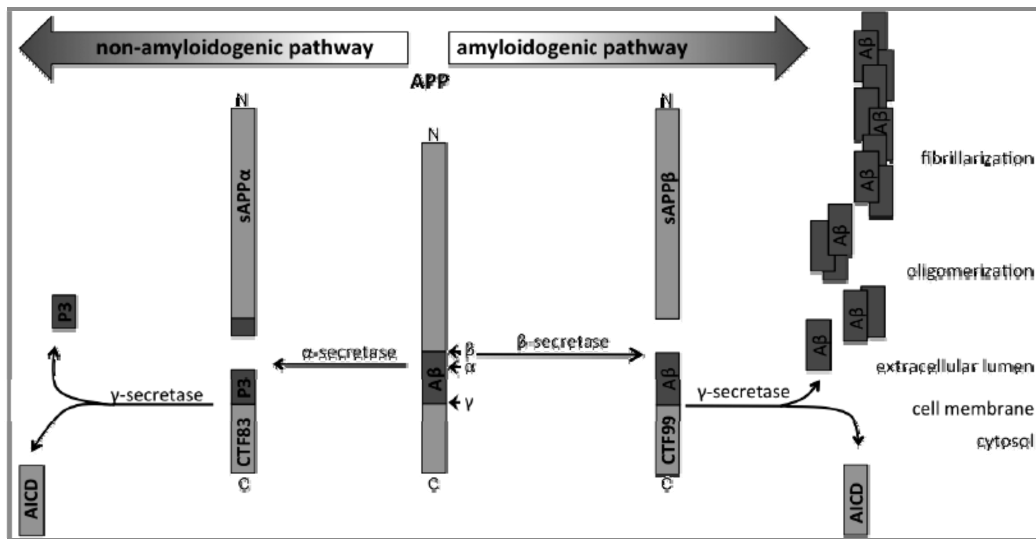


Figure 11. Amyloidogenic and non-amyloidogenic pathway. On the left side the non-amyloidogenic pathway pictured involves APP cleavages by α- and γ-secretases resulting in the generation of a sAPPα and C-terminal fragments CTF83/AICD and P3. On the right side, the amyloidogenic pathway depicted involves APP cleavage by β- and γ-secretases resulting in the generation of sAPPβ, a C-terminal fragment (CTF99/AICD) and Aβ peptides. Aβ peptides oligomerise and fibrillise resulting in amyloid plaques characteristic for AD. Adapted from (Chow et al., 2010; Thomas and Fenech, 2007).

Lately, p3 was shown to induce neuronal toxicity by altering cell calcium homeostasis (Jang et al., 2010). Nevertheless, the amyloidogenic pathway results in formation of Aβ peptides varying in length ranging from 38 to 42 amino acids. Cleavage of APP by β-site APP-cleaving enzyme (BACE) as well termed β-secretase (Howlett et al., 2000), releasing sAPPβ into the extracellular space, leaving a 99-amino-acid C- terminal fragment (C99). Otherwise sAPPα and sAPPβ display a neuroprotective function. The sAPPβ is shown to cause and increase neurite outgrowth, but with 10 times less efficiency than sAPPα (Chasseigneaux et al., 2011), and sAPPβ induces a rapid neural differentiation of human embryonic stem cells more activity than sAPPα (Freude et al., 2011).

Following β-secretase cleavage, C99 is cleaved within the membrane by γ-secretase complex cleaves the remaining fragment at the γ-site, resulting in the release of Aβ peptides into

extracellular lumen and release of CTF99/AICD (De Strooper, 2003). Depending on the exact point of cleavage by γ-secretase, three principal forms of Aβ, comprising 38, 40 or 42 amino acids, are produced. The relative amount of Aβ42 formed is particularly noteworthy, because this longer form of Aβ is more hydrophobic and more prone to oligomerize and form amyloid fibrils (Jarrett et al., 1993), than the produce of Aβ40. Aβ42 is the predominant isoform found in both amyloid and diffuse plaques (Iwatsubo et al., 1994). Many studies have indicate that Aβ42 forms the core of the amyloid plaque found in AD patients, implicating Aβ42 as the initiating factor for plaque formation (Balin and Hudson, 2014).

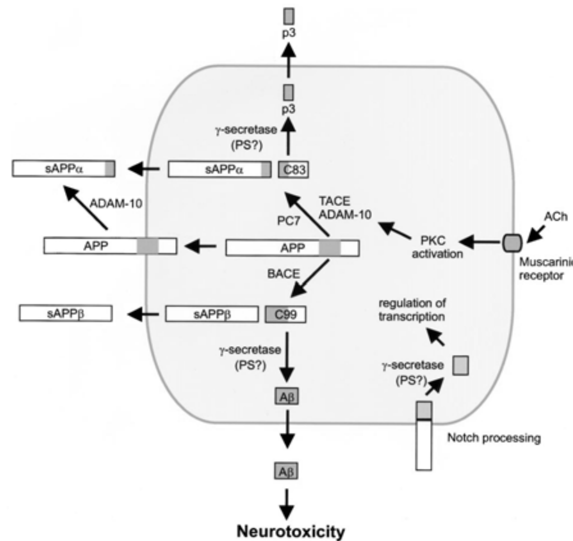


Figure 12. Pathways of APP processing by α-, β- and γ-secretases. Cleavage by K-secretase (PC7, TACE or ADAM-10) produces sAPPK and a C terminal fragment C83. Both TACE and ADAM-10 can be activated by protein kinase C (PKC) which is regulated by the muscarinic acetylcholine (ACh) receptor. C83 is cleaved by γ-secretase to produce p3. Cleavage of APP by β-secretase (BACE) produces sAPPβ and C99. γ-secretase, play a role in the proteolysis of Notch, cleaves C99 to release Aβ which has neurotoxic properties. Presenilins (PS) 1 and 2 have been proposed as γ-secretases. Adapted from (Nunan and Small, 2000).

α-SECRETASE

The major route of APP processing is through the α-secretase pathway, which cleaves on the C-terminal side of residue 16 of the AL sequence, generating an 83-residue C-terminal fragment (C83) (Esch et al., 1990). APP forms containing 751 and 770 amino acids are widely expressed in non- neural cells, while the higher levels correspond to neuronal isoform APP 695 (Haass et al., 1991). The APP cleavage by α-secretase may occur in the *trans*-Golgi compartment (Sinha and Lieberburg, 1999), at the cell surface or within caveolae (Kosik, 1999; Lammich et al., 1999; Nunan and Small, 2000), however α-secretase is present predominantly at the plasma membrane (Haass et al., 2012). The cleavage of sAPPα is secreted and shown to be neuroprotective and has memory enhancement effects (Vincent and Govitrapong, 2011). APP is cleaved by α-secretase in the center of the Aβ domain (Nunan

and Small, 2000), (Figure 12). Three related metalloproteases of the ADAM (a disinterring and metalloprotease) family, ADAM-9, ADAM-10 and ADAM-17, also termed TACE (tumor necrosis factor converting enzyme), (Figure 12) appear to exert α-secretase activity (Goate and Hardy, 2012; Walter et al., 2001). ADAM10 mediated proteolysis is essential for substrate subsequent cleavage by γ-secretase complex (Vingtdeux and Marambaud, 2012).

β- SECRETASE

β-Secretase (BACE1 for β-site APP cleaving enzyme) was cloned and identified as a type I transmembrane aspartyl protease (Vassar et al., 1999). They are two β-secretases identified in humans: the β-site AβPP-cleaving enzymes 1 and 2 (BACE-1 and BACE-2), both belonging to a type-1 transmembrane aspartyl proteases family (Yan et al., 2001). Whilst BACE2 cleaves in the Aβ sequence, it displayed to act similar to α-secretase (Fluhrer et al., 2002). Consequently, BACE1 acts as a sole β-secretase controlling Aβ production (Roberds et al., 2001), and has two active site motifs (DTGS and DSGT) and these motifs are present within the luminal domain (Kwak et al., 2011). Both β-secretases are widely expressed in the human brain (Jacobsen and Iverfeldt, 2009).

β-secretase is involved in amyloidogenic processing of APP and is the first prerequisite for the generation of Aβ peptide. This enzyme cleaves APP at the Asp+1 residue of Aβ sequence, and generates two products: a secreted soluble fragment (sAPPβ) and the membrane bound APP C-terminal fragment, composed of 99 amino acids (βCTF or C99).

Subsequently γ-secretase cleaves C99 producing a spectrum of intact β-amyloid peptide plus the APP intracellular domain (AICD). A β-site APP cleaving enzyme, BACE (beta-site APP-cleaving enzyme; also called Asp-2 and memapsin-2) has been identified by several groups by genetic screening, and by direct enzyme purification and sequencing (Hussain et al., 1999; Yan et al., 1999). BACE is the unusual member of the pepsin family of aspartyl proteases, that has an N-terminal catalytic domain, which containing two important aspartate residues, linked to a 17-residue transmembrane domain and a short C-terminal cytoplasmic tail (Vassar et al., 1999). BACE have four potential N-linked glycosylation sites and a propeptide sequence at the N-terminus. Within the cell, BACE is expressed first as a pre-protein, and thereafter efficiently processed to its mature form in the Golgi (Haniu et al., 2000). Anywise, BACE possesses many of the features of β-secretase. BACE can cleave full-length APP at Asp1 of the Aβ sequence and also at Glu11, residues of the Aβ sequence. These cleavage sites are known as β- and β'-site, respectively (Vassar et al., 2009). The activity of β-secretase is highest at acidic pH of 5.5 (Nawrot, 2004). Therefore, BACE activity is highest in the acidic

subcellular compartments of the secretase pathways including the trans-Golgi network and endosomes (Haass et al., 1995).

γ-SECRETASE

The γ-Secretase protein Consists of four essential membrane proteins called aph-1, pen-2, nicastrin and presenilin (PS) (Schellenberg and Montine, 2012), (Figure 13). γ-secretase plays a vital role in both non-amyloidogenic as well as amyloidogenic APP proteolytic pathways (Gandy, 2005).

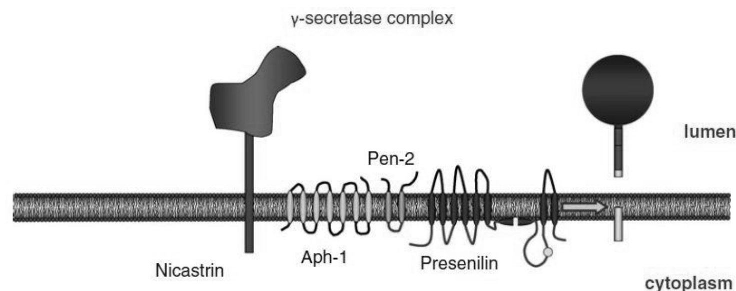


Figure 13. Topology of γ-secretase complex. γ-Secretase consists of four components: presenilin, nicastrin, PEN-2 and APH-1. Adapted from (Schellenberg and Montine, 2012)

Presenilins located at most at the plasma membrane and in the endosomal/lysosomal system (Haass et al., 2012). The activity of γ-secretase is located dramatically in the lipid raft membrane (Matsumura et al., 2014). It has been shown that more than 150 AD-causing mutations were found in *PSEN* gene that had an effect on Aβ peptide production (Hirth, 2010). The γ - secretase APP processing within the transmembrane region, involves the participation of four different subunits: presenilin, nicastrin, Aph -1 and P 2 (De Strooper, 2003), whereas aph-1, pen-2 and nicastrin function in the assembly and subcellular transport of γ-secretase and in the recognition of protein substrates (Shah et al., 2005; Shahani and Brandt, 2002; Takasugi et al., 2003; Yu et al., 2000). γ-Secretase inactivation causes accumulation of APP carboxy-terminal fragments (APP-CTF) to levels which are two- to threefold higher than what is observed in wildtype cells, and Ab peptide is no longer produced. inactivation of γ-Secretase causes accumulation of APP carboxy-terminal fragments (APP-CTF) to levels which are two- to threefold higher than what is observed in wildtype cells, and Aβ peptide is no longer produced (Hirth, 2010). The assembly of the γ-secretase complex might occur through several cycles of vesicular exchange between the endoplasmic reticulum and Golgi apparatus.

Once the mature complex is assembled and presenilin endoproteolysis occurs, the complex passes the quality control and is basically in the plasma membrane and other cellular

compartments, where catalytic activity on APP and other substrates takes place. Besides the role in the amyloidogenic pathway, γ-secretase is involved in the processing of other substrates such as Notch, cadherins, LDR (LDL receptor), CD44, nectin -1α , among others, involved in various cellular functions such as stabilization of β-catenin, calcium homeostasis, cell adhesion, all of which are essential for cell survival (Parent and Thinakaran, 2010; Wakabayashi and De Strooper, 2008). Currently, they are few γ-secretase modulators compounds in early to mid-stage of clinical trials (Lanz et al., 2010).

Drosophila as a model organism for neurodegenerative diseases

Animal model systems are used to study specific functional aspects of human diseases in general and neurodegenerative diseases in particular. These models range from yeast (Winderickx et al., 2008), and *Caenorhabditis elegans* (Teschendorf and Link, 2009), to mammals and human cell culture systems. They combine essential criteria like cognitive and behavioural dysfunction caused by cell type-specific neurodegeneration, cellular pathophysiology including aggregate formation, clear pattern of inheritance and genetic homogeneity. Using comparable short living models allows getting detailed information about disease formation and progression from conception to age-related death. As described above vertebrate model organisms reflect pathologic hallmarks of human diseases very well. However, large-scale experiments demand organisms that can be handled in large numbers and simultaneously mimic disease progression in an acceptable time frame.

Drosophila melanogaster has been used as a model organism since the beginning of the last century when it first was used for genetic studies by Thomas H. Morgan. Research using *Drosophila* has led to important insights into the mechanisms of human developmental and physiological processes and has resulted in many Nobel Prizes with the first to Thomas H. Morgan in 1933 (Fields and Johnston, 2005). *Drosophila*, has been used as a model for neurodegenerative diseases, such as Alzheimer's disease (Chakraborty et al., 2011; Gama Sosa et al., 2012; Mhatre et al., 2013), Huntington's disease (Jackson et al., 1998), and familial amyloidotic polyneuropathy (Berg et al., 2009; Pokrzywa et al., 2007). Biologically, *Drosophila* is easier than the human with only 4 chromosomes and ~14 000 genes and a volume of the brain of ~0.002 mm3 compared to the human brain of ~1200000 mm3, therefore making the fly an interesting model in neuroscience. In spite of its small size, it has been shown that the adult fly is capable of higher-order brain functions including aversive and appetitive learning (Kim et al., 2007; Schwaerzel et al., 2003). A drug-screen in *Drosophila* can be performed by mixing the drug in the food, by exposing the flies to vaporized

chemicals, or by drug delivery to individual headless (Manev et al., 2003). By mixing the drug into the flies' food is so far the most used drug delivery route.

Comparative analysis of whole genomes revealed striking similarities between structural composition of human and *Drosophila* genes (Rubin et al., 2000). The genetic mutants of *Drosophila* are readily available (http://flybase.org). Because of sufficient high reproduction rate of flies, progress genetic screens using chemical mutagenesis, insertional mutagenesis or RNA-interference (RNAi) can be easily performed to identify the mutations associated with neurodegeneration (Sapiro et al., 2013), and nearly 70 % of human disease-causing genes have homologs in the fly (Fortini et al., 2000). Presented this, it is not surprising that also homologs associated to known AD genes exist in *Drosophila* exhibiting functional conservation. The fly´s behavior ranges from simple avoidance to learning and memory (McGuire et al., 2005).

Furthermore because of its size and low care requirements it is cheap and easy to breed in big numbers. Another characteristic related to its usefulness in biomedical research especially in the field of neurodegenerative diseases is its short lifespan. Depending on diet and stress it ranges between 50 up to 120 days (Piper et al., 2005). Taken together this organism is the perfect choice for high throughput approaches as it can also be easily handled in high numbers.

***Drosophila* models of Alzheimer's Disease**

The fruit fly *Drosophila melanogaster* has been an extremely useful animal model in the history of developmental biology, which allowed the identification of a great deal of developmental control genes by means of classical genetics in combination with molecular biology methods. In this context, *Drosophila* has become also a useful animal model with which to uncover and characterize molecular pathways that are perturbed in human diseases (Marsh and Thompson, 2006), including neurodegenerative diseases (Crowther et al., 2006; Iijima and Iijima-Ando, 2008; Marsh and Thompson, 2006), (Figure 14).

Information about many human diseases has benefited from *Drosophila* research from cancer (Polesello et al., 2011), to sleep problems (Huber et al., 2004). The fruit flies have been used to answer important questions about the onset of Alzheimer's disease (Chakraborty et al., 2011; McBride et al., 2010).

From the data of molecular cloning and analysis of genes in *Drosophila* with functional genetic from mammalian systems has shown that amazingly large number of *Drosophila* genes have homologs with similar function in mammalian systems. Including transcription factors and their regulatory targets, structural proteins, chromosomal proteins, ion channels,

and signaling proteins. Moreover the evolutional maintenance extends to higher-level processes, like development (Riddle and Tabin, 1999), behavior, sleep (Hendricks et al., 2000), and responses to drugs at the physiological level (Moore et al., 1998).

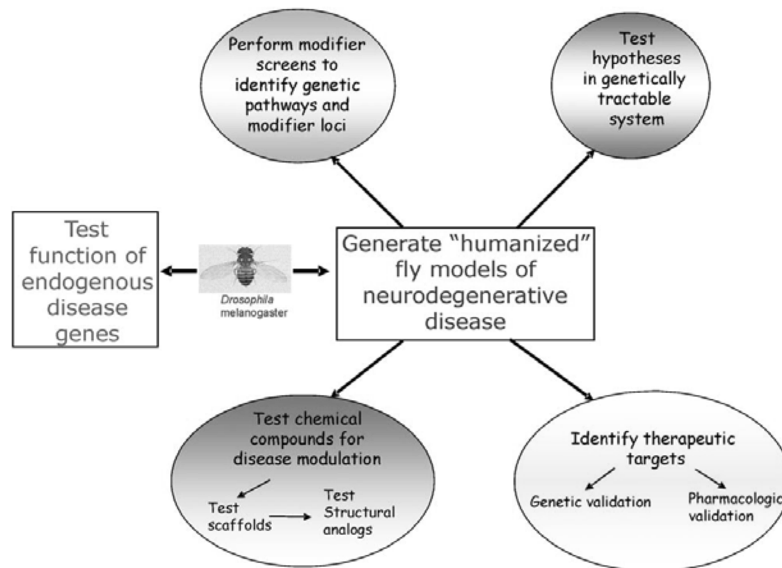


Figure 14. The schema of usefulness of *Drosophila* models of neurodegenerative disease. Adapted from (Marsh and Thompson, 2006).

There are many features of the fruit fly make it a perfect model organism for the study of human neurodegenerative diseases. For example, flies have a short generation time and short life span. This is important so there is no need to spend a long time (months/year) to determine the aging- neurodegenerative processes induced by AD as is in the case of mice models. The nervous system of *Drosophila* composed of some 200,000 neurons and supporting glia relative to the millions of neurons found in the mammalian brain.

The neurophysiology of the fly is very similar to its mammalian counterpart. For instance, fly neurons exhibit synaptic plasticity and neurotransmission mediated by many of the same neurotransmitters, synaptic proteins, receptors and ion channels found in the mammalian brain (Yoshihara et al., 2001). Over 70% of human disease genes have homologs in *Drosophila* and these genes often share greater than 90% nucleotide sequence identity (Bier, 2005; Lloyd and Taylor, 2010). Also the flies exhibit complex behaviors and like humans, many of these behaviors deteriorate with age including learning, memory and motor ability (Mockett et al., 2003; Simon et al., 2006).

The expression of amyloidogenic peptides into *Drosophila* have linked the phenotype of the flies' lifespan and behavior to the predicted aggregation propensity of the expressed peptides (Luheshi et al., 2007).

Drosophila life cycle

Drosophila develops over different stages they are called embryo, larval, pupal and adult stage (Figure 15). Every stage is extensively characterized, and many genetic manipulating tools are obtainable to determine the effect of mutation on specific cell/tissue type, including neurons. The life cycle of a fly is between 10-12 days (at 25 °C). Newly laid eggs take 24 hours to submit embryogenesis before hatching into first instar larvae, which continue to develop for another 48 hours into second, and then third instar larvae. Two days later, larvae transform into immobile pupa, undergo metamorphosis and eclose as adult 5-7 days later.

Importantly, adult females are fertile 12 hours post-eclosion and a single fly can produce hundreds of offspring within days making it relatively easy to perform analysis on hundreds of flies within a matter of days. The evolutionary conservation extends to higher-level processes, like development (Bello et al., 2006), behavior, sleep (Hirth et al., 1998), and responses to drugs at the physiological level (Moore et al., 1998).

The genome of the fly has been completely sequenced and annotated, and encodes for a little more than 14,000 genes on four chromosomes, only three of carry the bulk of the genome. *Drosophila melanogaster* have four pairs of chromosomes: a pair of sexual chromosomes (I): XX in females and XY in males; and three pairs of autosomes: II, III and IV (punctual). In *Drosophila* sex is determined by the ratio of X to autosomes.

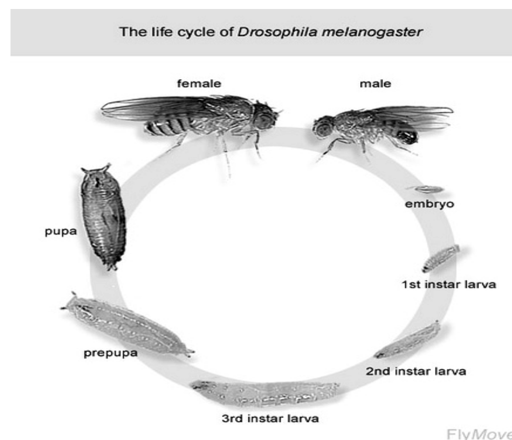


Figure 15. The life cycle of *Drosophila melanogaster*. The development of a fertile egg to an adult fly over a 10-day period at 25ºC. Following hatching (eclosion), larvae go through three instars before reaching pupariation at which time metamorphosis takes place resulting in the emergence of an adult fly. Figure taken from FlyMove (http://flymove.uni-muenster.de).

UAS/GAL4 system

The system of UAS/GAL4 is a tissue-specific gene expression system based on the yeast GAL4 transcription factor that activates the transcription of genes that contains the GAL4

DNA binding site, known as (UAS) (upstream activating sequence) (Brand and Perrimon, 1993).

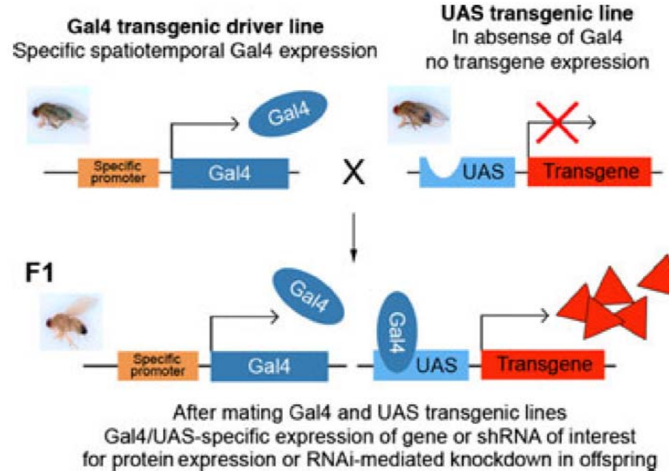


Figure 16. The UAS/Gal4 system. Crossing files bearing Gal4 with files carrying UAS produce flies (F1) expressing the model genes for AD study. The GAL4/UAS system is composed of two parts. The first part is the yeast GAL4 transcriptional activator which is under the control of a native promoter of interest. The second is a short upstream activating sequence (UAS) to which GAL4 binds and initiates transcription. Adapted from (Lenz et al., 2013).

UAS/GAL4 system in *Drosophila* is a bipartite. In this system, the yeast transcription activator GAL4 binds to the enhancer UAS and initiates transcription. This produces F1 individuals that carry both constructs and express the transgene in a spatially restricted manner as determined by the GAL4 promoter. In the case of modelling AD, the pan-neuronal elav-GAL4 or eye specific GMR-GAL4 drivers have been used to drive either UAS-APP/UAS-BACE or UAS-Aβ transgenes.

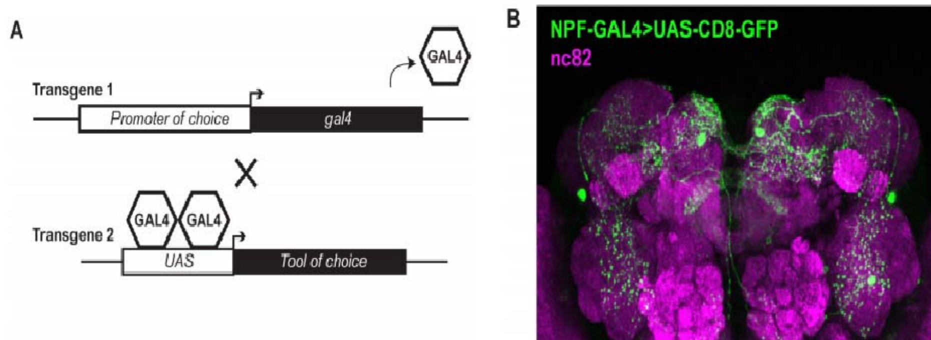


Figure 17. Genetically labeling small groups of neurons. (A) The bilateral GAL4/UAS genetic expression system. (B) Genetic labeling of neuropeptide in the adult Drosophila brain. NPF-GAL4 transgene drives expression of membrane- tethered CD8-GFP in NPF-positive neurons (green). Adapted from (Jones, 2009).

An example of the use of the GAL4/UAS system is to model AD (Figure 16). The progeny will express the gene of interest in a conformable to the GAL4 expression modality, when the UAS and GAL4 strains are crossed. The UAS/GAL4 system allows for spatial-temporal

expression of a targeted gene like human Aβ gene in flies of AD model (Duffy, 2002). A gene of interest can be introduced into a UAS strain and expressed in a variety of spatial patterns by mating with various GAL4 drivers.

The GAL4/UAS system is exceedingly used to overexpress or knockdown (by RNAi) the gene of interest and the promoter of a gene of interest (e.g. neuropeptide) under this system (Figure 17) is cloned and inserted upstream of the yeast transcription factor gene GAL4. Other manipulating tools are hormone inducible GAL4 system (Jones, 2009), split-Gal4 (Pfeiffer et al., 2010).

The brain of *Drosophila melanogaster*

The central nervous system of *Drosophila melanogaster* constitutes two parts, the brain and the ventral nerve cord (Sanchez-Soriano et al., 2007). The nervous system of *Drosophila* is composed of some 200,000 neurons and supporting glia relative to the millions of neurons found in the mammalian brain. Although simpler, fly neurophysiology is very similar to mammalian counterpart. For instance, fly neurons show synaptic plasticity and neurotransmission mediated by many of the same neurotransmitters, synaptic proteins, receptors and ion channels found in the mammalian brain (Yoshihara et al., 2001). Also the flies' exhibit complex behaviors and many of these behaviors, like humans, deteriorate with age including learning, memory and motor ability.

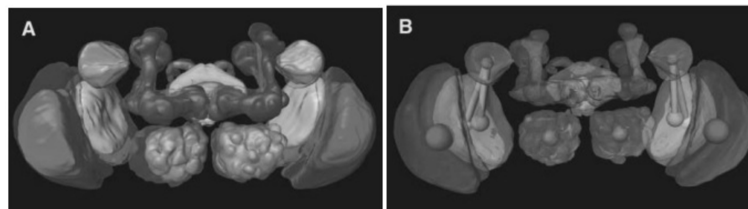


Figure18. The standard brain of *Drosophila melanogaster*. (A) Superimposed polygonal models of the representative CS brain (transparent) and arbitrarily chosen rol image data set. (B) show centers of gravity of labeled structures. (Rein et al., 2002).

They are three phases of neurogenesis in the development of *Drosophila* brain, the first phase takes place in the embryo including neuroectodermal proliferation of neuroblasts, patterning of the neuraxis and differentiation into neurons and glia that constitute the brain of the 1st instar larvae. The optic lobe and central brain neuroblasts become quiescent towards the end of embryogenesis (Figure 18). There is exception in this regard which is the neuroblasts building the mushroom bodies, as they continue to proliferate through all developmental stages. A second wave of neurogenesis during the larval stages begins to build the structures for the adult brain. The second phase is based on re-activation of quiescent embryonic

neuroblasts. The third phase of neurogenesis happens during metamorphosis in the pupal stages where the proliferation of neuroblasts stops and the neurons terminally differentiate into mature adult cells (Truman and Bate, 1988).

Along the ventral nerve cord is the midline marked with a dashed line and inside, on either side of the midline are the neuropils (synaptically dense regions) containing dopamine and serotonin terminals (Blennow et al., 2006; Borue et al., 2009; Vickrey et al., 2009).

On the outside of the ventral nerve cord are the motorneuron connection that protrude out to the rest of the larval body to the neuromuscular junctions, where the action potential causes the muscles to contract. For the adult fly, the CNS is distinctly different from that of the larva. The brain consists of two parts, the central brain, and the two optic lobes that receive inputs from the eyes. An interesting neuropil area in the adult fly is the mushroom body, which consists of two pared structures. In the larva the mushroom body is a part of the supraesophageal ganglion.

The ~2500 Kenyon cells (Waddell and Quinn, 2001), around each calyx project through the calyx and the pedunculus and bifurcate into the vertical (V) lobes or the horizontal (H) lobes. The vertical lobes contains the two subunits α and α' and the horizontal lobes the three β, β', and γ. There are three distinct clusters around the mushroom body that contain dopamine neurons, the protocerebral anterior median (PAM), protocerebral posterior lateral 1 (PPL1) and 2ab (PPL2ab) clusters and they terminate in the mushroom bodies.

The PAM neurons project into the medial portion of the horizontal lob in the mushroom body, PPL1 neurons project to the vertical lobes, the junction area, the heel and distal peduncle, and PPL2ab neurons project to the calyx (Mao and Davis, 2009).

APP processing it is conserved in *Drosophila*.

Drosophila has a clear homolog of APP, APP-like protein (dAPPL) (Figure 19) (Iijima-Ando and Iijima, 2010). Studies in *Drosophila* have been useful to understand the physiological function of AD relevant genes. For instance, a neuronal-specific APP homolog, APPL, has been identified and is believed to be involved in neural development and axonal transport (Borue et al., 2009; Torroja et al., 1996; Watson et al., 2008).

APP is known to become a member of a larger gene family, the amyloid precursor-like proteins (APLPs), that includes APLP1 and APLP2 in humans, the fly homolog Appl (Rosen et al., 1989), and like mammalian APP, it is expressed specifically in neuronal tissue (Luo et al., 2001). All genes in this family encode type 1 membrane proteins with a large extracellular

domain and short cytoplasmic region and undergo processing similar to APP. The dAPPL shares about 30% overall sequence identity with human APP.

In addition, all known components involved in the APP processing have their structural and functional homologues in *Drosophila* (Figure 19) (Iijima-Ando and Iijima, 2010). The region in dAPPL that corresponds to the Aβ-peptide lacks significant homology with the human peptide which has led to the assumption that amyloidogenic peptides cannot be produced from fly APPL (Carmine-Simmen et al., 2009). The appl transcripts in *Drosophila* are at most localized in the cortical region of the fly nervous system (Martin-Morris and White, 1990). Knock down of Appl protein in flies, particularly in adult brains, leads to defective long term associative memory (Watson et al., 2008).

Over-expression of Appl in the fly brain leads to increased post developmental axonal arborization, suggesting a potential role in synaptic plasticity (Leyssen et al., 2005). Mammalian γ-secretase complex: Presenilin1/2, Nicastrin, Aph-1, and Pen-2 are conserved in flies and worms (Chakraborty et al., 2011; Chung and Struhl, 2001; Francis et al., 2002; Goutte et al., 2000). The γ-secretase complex of *Drosophila* is able to cleave both endogenous Appl as well as expressed human AβPP (Chakraborty et al., 2011; Greeve et al., 2004).

Drosophila melanogaster harbors all components of γ-secretase complex (Periz and Fortini, 2004), (Figure 19), while *Drosophila* has very low β-secretase activity (Bolkan et al., 2012; Yagi et al., 2000). Latterly, β-secretase-like enzyme (dBACE) was specified (Figure 19) that has 25% identity to human BACE1 and 28% identity to human BACE2. dBACE does cleave human APP but not at *β*-site (Carmine-Simmen et al., 2009). *Drosophila* model for Aβ-induced plaque formation was generated by expressing human APP in conjunction with human β- secretase and *Drosophila* presenilin (Greeve et al., 2004; Watson et al., 2008).

The APP homolog of *Drosophila* (dAPPL) shares the characteristic domains with vertebrate APP family members (Luo et al., 1992; Soldano et al., 2013). The region in APPL that corresponding to the Aβ peptide lacks significant homology with the human peptide which has led to the assumption that amyloidogenic peptides cannot be produced from fly APPL (Luo et al., 1992; Prussing et al., 2013), therefore, there is no endogenous Aβ production in the fly and only the overexpression of dBACE resulted in cleavage of dAPPL, producing a fragment corresponding to the human Aβ peptide (Iijima-Ando and Iijima, 2010).

However, this fragment is also able to aggregate and cause age-dependent behavioral deficits and neurodegeneration (Carmine-Simmen et al., 2009). *Drosophila* α-cleaved ectodomains were shown to have neuroprotective effects in multiple fly neurodegeneration mutants (Wentzell et al., 2012). In the non-amyloidogenic pathway, APP is cleaved within the Aβ

domain by α-secretase precluding deposition of intact Aβ peptide. The workable orthologue of α-secretase in the fly is the *kuzbanian* gene (Kuz), (Bourdet et al., 2015).

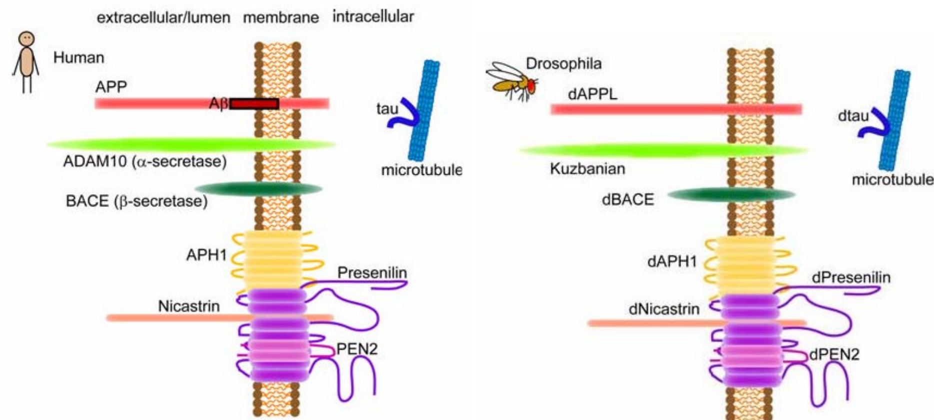


Figure 19. Schematic representation of the human APP and dAPPL. In the figure represent the α-secretase, the β-secretase and the components complex of y-secretase and Tau in humans and *Drosophila* Adapted from (Iijima-Ando and Iijima, 2010).

The final step in the production of Aβ is the cleavage of APP C99 fragment by γ-secretase. The position of cleavage by γ-secretase is critical for the development of AD (Artavanis-Tsakonas et al., 1999; Funamoto et al., 2013) .

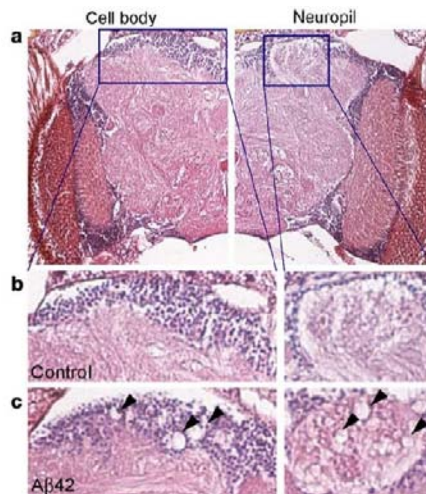


Figure 20. The cell body and neuropil degeneration in Aβ42 flies. a–c: Neurodegeneration in Aβ42 flies at 25 days old. The cell body and neuropil region in the mushroom body are enlarged. Arrowheads in **c** indicate neurodegeneration. Adapted from (Iijima-Ando and Iijima, 2010).

Expressing of Aβ in *Drosophila.*

The study of human Aβ peptide-induced amyloid formation and neurodegeneration in transgenic flies has been generated by several approaches using the GAL4/UAS system (Figure 19), (Lenz et al., 2013). The genes UAS were inserted corresponding to the peptides Aβ1-40, Aβ1-42, a double inserted Aβ1-42, and Aβ1-42 E22G (the Arctic mutation), which

also contained a secretion signal peptide at the N-terminus causing the peptides to be secreted towards the extracellular space (Crowther et al., 2005; Iijima et al., 2004).

Both the Aβ40 and the Aβ42 peptides accumulate during aging in the fly brain (Prussing et al., 2013), but only the Aβ42 formed amyloid deposits (Crowther et al., 2005; Iijima et al., 2004; Kopan and Goate, 2002). This result is coordinated with prior studies *in vivo* amyloid deposits in AD, or down syndrome patient brains that have shown that Aβ42 first accumulates in amyloid plaques in the brain parenchyma (Serrano-Pozo et al., 2011).

The Aβ42 and not Aβ40 caused reduced lifespan, locomotor defects and age-dependent neurodegeneration in the brain (Figure 20). The genotype of these flies that has short-term memory is affected, locomotor deficits are obvious in later stages and survival is reduced (Figure 21), (Iijima et al., 2008; Moloney et al., 2010).

Flies do possess an APP homologue, Appl (Soldano et al., 2013). The lacking of APPL or overexpressing human APP in the flies and *Drosophila* APPL constructs produce axonal transport defects that are enhanced by reductions in kinesin 1 expression. However, over-expression of the Aβ-domain-containing APP, but not APPL, induced neuronal apoptosis (Gunawardena and Goldstein, 2001; Torroja et al., 1999). Likewise, APPL has been implicated in promoting synaptic formation at the neuromuscular junction (NMJ) in flies (Tian and Wu, 2015).

Greeve and co-workers generated a triple transgenic animal expressing human APP, human β-secretase and *Drosophila* γ-secretase presenilin (dPsn) with point mutations corresponding FAD mutants (Greeve et al., 2004; Ye and Fortini, 1999). These flies developed amyloid plaques age-dependent neurodegeneration dependent on dPsn expression and mutation.

The fly Aβ peptide shows neurotoxic properties similar to human Aβ peptides (Carmine-Simmen et al., 2009). The models share key features of human disease and are consequently well suited to gain more insight into pathomechanisms of AD.

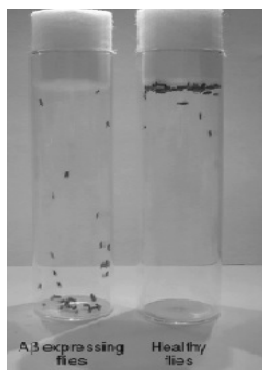


Figure 21. Quantifiable locomotor defects in flies expressing Aβ. Flies expressing Aβ peptides in their brains exhibit locomotor abnormalities, while healthy flies are typically straight and oriented vertically. Adapted from (Moloney et al., 2010).

Expressing of Tau in *Drosophila*

Flies and worms contain an ortholog of Tau as well, *C. elegans* protein PTL-1 (protein with Tau-like repeats) (Mhatre et al., 2013). *Drosophila* expressing human wild type Tau, the ortholog of human Tau (h-Tau) is known as *Drosophila* Tau, several studies indicate that d-Tau and h-Tau share functional roles. For instance, d-Tau shows significant cytotoxic effects when over-expressed in fly tissues. The Tau ortholog of *Drosophila*, dTau, expressed throughout the nervous system (Gama Sosa et al., 2012; Heidary and Fortini, 2001), and could be phosphorylated by doubled kinases (Chatterjee et al., 2009; Feuillette et al., 2010). The expressing of d-Tau in adult mushroom body neurons leads to behavioral deficits in associative learning and memory (Link, 2005; Mershin et al., 2004).

The *Drosophila* homologue of MAPtau exhibits 46% identity and 66% similarity with the human protein. However, the fly protein does not contain the N-terminal repeats found in several human isoforms of tau (Hirth, 2010).

The expression of human wild type or mutant Tau in *Drosophila* affects the development of neurons correlating with learning and memory, connected with these neurons, reduced lifespan and an age dependent progressive neurodegeneration (Link, 2005). However, the over-expression of d-Tau in motor neurons leads to similar morphological abnormalities in the fly larval neuromuscular junction as the expression of h-Tau (Ubhi et al., 2007).

Characteristic of *Drosophila* toxicity model

Drosophila melanogaster has been used moderately as a tool to assay toxicity. Fly has found some limited use in the study of herbicide toxicity and to research the mechanisms of drug toxicity (McClung and Hirsh, 1998; Mhatre et al., 2013).

The fly life cycle has four discrete parts: embryonic, larval, pupa, and adult. Each of these life cycle stages poses unique opportunities to assess toxicity. Compare with mouse or zebra fish toxicity models, *Drosophila melanogaster* has been a powerful *in vivo* model because it has less increase in the genome, facilitating the rapid analysis of gene functions (Chakraborty et al., 2011; Hsu and Schulz, 2000). Also the fly can be manipulated experimentally much more easily than vertebrate models, according to both ethical and technical issues.

Fatty acid analysis of lipids from *Drosophila melanogaster*

The available genetic toolkit in *Drosophila melanogaster* and the accessibility of multiple genome sequences for the Drosophilids have made this tiny fruit fly a model organism for animal genetics, development and cell biology, as well as for comparative genomics,

transcriptomic and proteomics (Graveley et al., 2011). The metabolomics is a valuable tool for *Drosophila melanogaster* functional genomics (Chintapalli et al., 2013). *Drosophila melanogaster* offers the best balance between genetic tractability, availability of well-characterized genetic mutant stocks, and organismal complexity (Carvalho et al., 2012). For nearly a century, some mutations in metabolic pathways have been studied (Green, 2010).

Lately, *Drosophila melanogaster* has become an increasingly important model in understanding the mechanisms by which physiology adapts in response to the environment (Cooper et al., 2010). The membranes are mainly composed of glycerophospholipids, sphingolipids, and sterols (Dominguez et al., 2011b; Overgaard et al., 2008). Sphingolipids are found in all eukaryotic cells and are implicated in an assortment of cellular processes. The structural diversity of sphingolipids stems from over 300 known distinct head groups, as well as modifications of the hydrophobic ceramide moiety (Alderson et al., 2004; Taylor et al., 2010). One of the common modifications of the ceramide moiety is 2-hydroxylation of the N-acyl chain. Sphingolipids with 2-hydroxy fatty acid are found in most organisms including plants, yeast, worms, vertebrate animals, and some bacterial species (Alderson et al., 2004). There are different studies that discriminate the lipid composition of *Drosophila* membranes, (Dominguez et al., 2011a), which are reported to consist of approximately 50% phosphatidylethanolamine (PE), 25% phosphatidylcholine (PC), with the remainder consisting of a number of other lipid classes (Fast, 1966). The fatty acid content in polar and non-polar lipid TLC fractions was found to be dominated by oleic (18:1), palmitoleic (16:1), palmitic (16:0), linoleic (18:3), and stearic (18:0) acids. Contrary to mammals, myristic acid (14:0) was found in low abundance (~1% of total phospholipid fatty acids), (Jones et al., 1992). In mammals, omega-3 and omega-6 PUFAs are synthesized through elongation and desaturation of the essential α-LNA and LA fatty acids, respectively (Figure 22), (Ibarguren et al., 2014). *Drosophila* melanogaster does not possess the ability to synthesize ARA, EPA and DHA; however, they have the catabolic machinery for the shortening of DHA into EPA. The mass spectrometry supplies a powerful approach for profiling lipid composition (Han et al., 2004; Serrano-Pozo et al., 2011). Even though human metabolomics is necessarily observational, studies of simpler organisms offer the prospect of linking levels of gene expression with their impact on tissue metabolomes (Chintapalli et al., 2013), whereas a metazoan, *Drosophila* has multiple, functionally distinct tissues; and as the FlyAtlas.org resource has shown, that gene expression can vary markedly between tissues (Chintapalli et al., 2007). The biologists are interested more and more in how this membrane architecture is modified in the model organism *Drosophila melanogaster* during genetic, physiological or environmental perturbations (Overgaard et al., 2008)

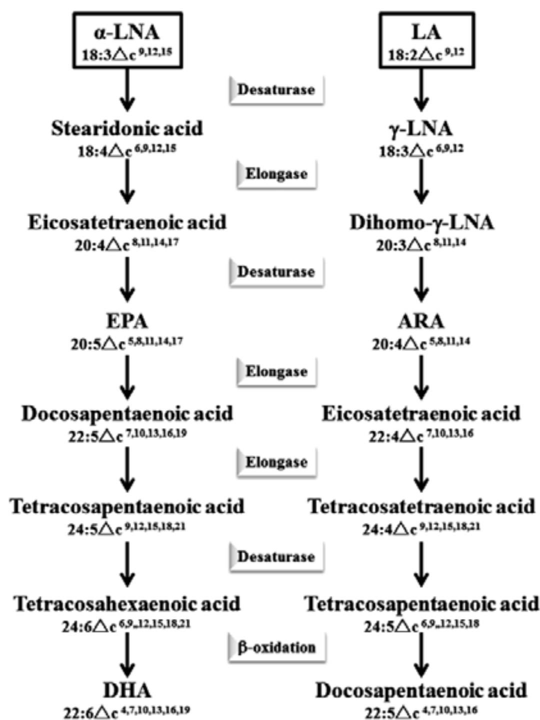


Figure 22. Metabolism of omega-3 and omega-6 fatty acids in mammals. Different omega-3 and omega-6 fatty acids are produced through elongation and desaturation of the essential α-LNA and LA fatty acids, respectively. The addition of double bonds and the elongation of the acyl chains occur in the endoplasmic reticulum, while the final step in the synthesis of the omega-3 DHA and the omega-6 docosopentaenoic acid consists of a single reaction from β-oxidation in the peroxisome. Adapted from (Ibarguren et al., 2014).

Different treatments for Alzheimer disease

Currently there is no cure for Alzheimer's disease (AD), however, there are multiple drugs that have proven to slow disease progression and treat symptoms. The current treatments for (AD) are used to reduce the cognitive decline, and the central role of these drugs is to stabilize and thereafter minimize disruption of two key neurotransmitters, acetylcholine (ACh) (the cholinergic hypothesis of (AD), and glutamate. One of the earliest pathological events in (AD) is a loss of cholinergic basal forebrain neurons and a reduction in cortical acetylcholine (ACh) (Perez et al., 2007). AChE inhibition is used to protect the cholinergic neurons and glutamate (Klafki et al., 2006). The compounds which are work on the basis of AChE inhibition are the cholinergic drugs, donepzil, rivastigmine and galantamine. All three compounds are efficacious in reversing and improving memory and global cognition, in mild to moderately demented patients (Birks, 2006). These drugs enhance the remaining cognitive function, but they do not delay the disease progression by preventing senile plaque or neurofibrillary tangle formation. The drugs aimed to treat Alzheimer's disease modulate the fibrillation pathway of Aβ, by targeting molecular sites in order to prevent Aβ production,

prevent the formation of toxic forms of Aβ, or prevent toxic effects of Aβ (Findeis, 2007). These drugs have been shown to have modest clinical benefits in AD by temporarily slowing down the rate of cognitive decline by 6-12 months (van Marum, 2008). Although measurable, the beneficial effects of these drugs are not large.

Moreover, treatment of the symptoms of AD consists of a wide range of unspecific pharmacological (Alves et al., 2012), and non-pharmacological interference (Ballard et al., 2011), but can only palliate the encumbrance of symptoms for patients and caregivers, encounter behavioral and psychological symptoms of the disease, and help to afford activities of daily living as long as possible (Hort et al., 2010). However, the work continues into the development of other Aβ peptide vaccines along with novel strategies such as γ-secretase inhibition (Basi et al., 2010). Anyhow, it is clear that a better understanding of the initiation and progression of AD is needed, to allow future treatments to be developed against novel drug targets.

Lipids and Alzheimer's disease

In addition to the pathological features of Alzheimer's disease (AD) senile plaques, neurofibrillary tangles, and lipid granule accumulation, many studies reported a link between lipids and AD (Tajima et al., 2013).

Membrane lipids broadly supply a milieu for transmembrane proteins and can modulate their function. However, γ-secretase activity is affected by the lipid composition of the membrane with sphingolipids and cholesterol increasing and phosphatidylinositol decreasing its activity (Holmes et al., 2012; Osenkowski et al., 2008). The activity of γ-secretase is affected by lipid carbon chain length and double bond position (Tajima et al., 2013).

γ-activity can be increased by increasing fatty acyl (FA) carbon chain length ($14<16<18<20$), and reduced longer Aβ species and reduced Aβ42/40 (Holmes et al., 2012). The most numerous omega-3 PUFA in the brain is DHA (22:6 n-3) and it is tightly implicated in the performance of the central nervous system (Torres et al., 2014), especially in neurogenesis, synaptogenesis and synaptic transmission (Salem et al., 2001).

In the AD patients the docosahexaenoic acid (DHA) content of phospholipids (PLs) is lower in brain tissue and plasma compared to those without cognitive impairment (Cunnane et al., 2012; Green et al., 2007). The neuroprotectin D1 that is derived from DHA was related to suppression of Aβ42-induced neurotoxicity (Lukiw et al., 2005). The primary component of membrane phospholipids in the brain is the n-3 polyunsaturated fatty acid docosahexaenoic acid (DHA; 22:6n-3), (Morris et al., 2003).

Omega 3 fatty acids and Alzheimer’s disease.

Alzheimer’s disease (AD) is mostly correlating with lower omega-3 fatty acid absorption from fish but, in spite of varied studies, it is still indistinct if there are differences in omega-3 fatty acids in plasma or brain. There is different studies showing that the fish consumption decreases the risk of dementing illnesses such as AD (Cunnane et al., 2012). Fatty acids function as energy substrates and integral membrane components together essential for proper neuronal and brain function (Cole et al., 2009).

The essential role of brain omega-3 polyunsaturated fatty acids (n-3 PUFA) in the maintenance of learning ability and memory is exceedingly recognized (Cao et al., 2009).

Omega-3 PUFA may act to alter amyloidogenic processing in several distinct and possibly interrelated ways including:

1. simplify the interaction of α-secretase with APP to produce nontoxic fragments and prevent the formation of Aβ;
2. armor the major recognition sequence and intramembrane cleavage site for γ-secretase;
3. deed as a local sink for free radicals that reduce the enzymatic augmentation of γ-secretase activity, that can be induced by free radical damage to the protein complex, which is important for the regulation of normal γ-secretase function;
4. directly inhibit fibrillation and formation of toxic oligomeric species of Aβ (Figure 23) (Jicha and Markesbery, 2010).

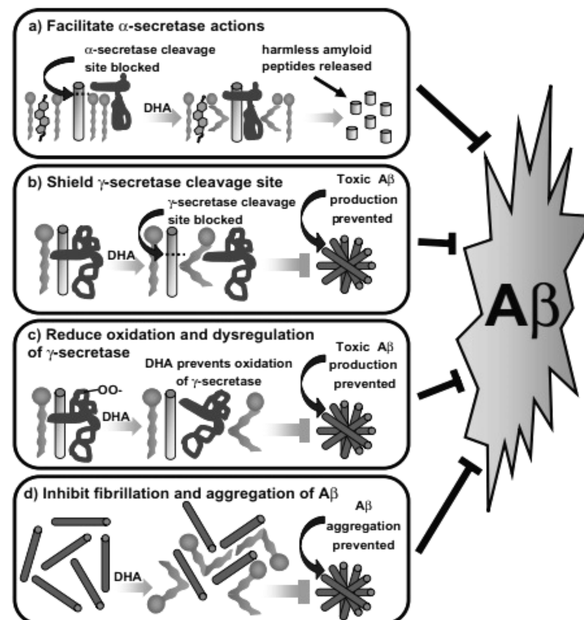


Figure 23. The effect of omega-3 PUFAs in amyloidogenic processing. During several featured and interconnected mechanisms. Adapted from (Jicha and Markesbery, 2010).

The roles throughout Omega-3 fatty acids are essential for brain growth and development. They play an important role in the life, as critical modulators of neuronal function and regulation of oxidative stress mechanisms, in brain health and disease.

Humans lack the ability to synthesize omega-3 from omega-6 fatty acids, and vice versa, and they lack Δ12- and Δ15-desaturase activities, which are responsible for the formation of a double bond in the carbon 12 and 15 of an acyl chain, respectively. Therefore, the omega-3 fatty acid α-LNA (18:3Δc9, 12, 15) and the omega-6 fatty acid LA (18:2Δc9, 12) are the two essential fatty acids in humans (i.e., they must be incorporated into the metabolism through the diet), (Ibarguren et al., 2014).

Docosahexanoic acid (DHA), the major omega-3 fatty acid found in neurons, has been taken on a central role as a target for therapeutic intervention in AD (Jicha and Markesbery, 2010). DHA is a polyunsaturated fatty acid (PUFA) which can influence bodily functions through different ways, including the regulation of neuroinflammation, neuro transmission, gene expression, fluidity of neuron membranes and ATP-generating machinery (de Urquiza et al., 2000). Moreover, the deficiency of DHA also contributed to the development of normal ageing (Florent-Bechard et al., 2007; Plourde et al., 2007). About one third of the essential constituents during normal ageing, necessary for membrane formation, especially phospholipids rich in DHA, is missing in frontal cortex and hippocampus (Plourde et al., 2007). Decreased level of DHA may imply to an increased risk of age dependent cognitive decline (Cole et al., 2009). Thus, the dietary supplementation of DHA could be of great importance for the prevention of ageing and ageing-related diseases. It has been notified that early dietary supply of DHA improves later cognitive development in human infants (Birch et al., 2000).

In fish, the nutrients most obviously related with protecting brain function in the elderly are the omega-3 fatty acids, particularly docosahexaenoic acid (DHA). Levels of DHA are found highly in more metabolically active area of the brain, including the cerebral cortex, mitochondria, synaptosomes, and synaptic vesicles (Morris et al., 2003).

Drosophila melanogaster is an ideal model for nutrigenomics, especially for FA metabolism (Shen et al., 2010).

Naturally Occurring 2-Hydroxylated Fatty Acids

Fatty acids are part of the fat contained in food products and they contribute to their flavor and consistency, and lead to the feeling of fullness when eating. Furthermore, fat, and fatty acids in particular, are a major source of energy and they aid the absorption of lipophilic substances like vitamins A, D, E and K (Ibarguren et al., 2014). Naturally occurring 2-

hydroxylated fatty acids exist. For instance, C22 to C26 saturated and monounsaturated 2-hydroxy fatty acids have been found as major lipid components of the cell wall in three marine chlorophytes (Gelin et al., 1997), and detritus from the sea-grass *Zostera muelleri* is a source of 2-hydroxy acids (0.6 mg/g) that range from C_{18} to C_{28}, including different mono- and polyunsaturated derivatives (Volkman et al., 1980). Seed oils from *Thymus vulgaris* are enriched in 2-hydroxylinolenic acid (13%) (Smith and Wolff, 1969), while the seed oil of *Salvia nilotica* contains 0.6% 2-hydroxyoleic, 4.2% 2-hydroxylinoleic and 5.4% 2-hydroxylinolenic acids (Bohannon and Kleiman, 1975). Moreover, hydroxylated DHA derivatives may also be found among resolvins (Weylandt et al., 2012).

During the last few years, a number of 2-hydroxylated fatty acid derivatives other than 2OHOA have been rationally designed for the treatment of cancer, inflammation, AD, obesity, diabetes, spinal cord injury, etc. Indeed, the data available indicate that the mechanism of action of these compounds is related to their capacity to modulate the lipid structure of the membrane (Barceló et al., 2004; Escribá, 2006b; Yang et al., 2005). In this context, 2OHOA has proved effective in reducing blood pressure in hypertensive rats through a mechanism that involves the modulation of membrane lipid composition, and of the membranes biophysical properties (Prades et al., 2008). Also, 2OHOA is currently being studied in phase I/IIa clinical trials for the treatment of solid tumors (code NCT01792310) (NCT01792310, 2013) and it is under preclinical development for the treatment of spinal cord injury. 2OHARA has been described as a new non-steroidal anti-inflammatory drug that can inhibit COX1 and COX2 activity, thereby reducing the synthesis of pro-inflammatory mediators. Molecular dynamics have predicted binding competition between 2OHARA and the proinflammatory fatty acid ARA to COX1 and COX2. Moreover, in addition to the *in vitro* inhibition of COX1 and COX2 activity, 2OHARA decreased plasma TNFα levels *in vivo* (López et al., 2013). Finally, 2OHDHA has arisen as an interesting candidate to revert the cognitive deficiencies associated with neurodegeneration, such as in AD. This fatty acid derivative decreases Aβ accumulation in parallel with a recovery of cognitive scores in animal models. These results are consistent with the reduced binding of oligomeric and fibrillar Aβ lipid raft-like vesicles in the presence of 2OHDHA (Torres et al., 2013). All these 2-hydroxylated compounds are thought to act by regulating signal transduction through membrane lipid therapy, an approach that aims to regulate membrane lipid organization through structure-function principles (Escribá, 2006a). Changes in the membrane's physico-chemical properties, such as the lateral pressure, membrane fluidity or phase behavior may regulate the localization and activity of relevant signaling proteins, resulting in the regulation of gene expression and a reversion of pathological states within cells.

AIM OF THE STUDY

This thesis is the result of several years of work as a graduate student within the research group of Prof. Pablo Escribá and Prof. Xavier Busquets in collaboration with Prof. José Aurelio Castro. The work has focused on the validation of a model of Alzheimer's disease in *Drosophila melanogaster* and to study the effect, in this system, of food supplementation with the non-hydroxylated and 2-hydroxylated forms of DHA, ARA and EPA.

The objectives of this study were:

In *Drosophila melanogaster*

1. Determine the potential toxicity of the compounds used in the present study.
2. Validate a *Drosophila melanogaster* model of human Alzheimer's disease and a method to evaluate the cognitive decline.
3. Study the effect of 2-hydroxylated omega-6 and omega-3 fatty acids (OHDHA, OHEPA and OHARA, TGMs, LP103A1, LP183A2) on the behavior of flies bearing human genes with mutations causing Alzheimer's disease.
4. Analyze the lipid profile in *Drosophila melanogaster* head membranes after treatment with DHA and OHDHA, by using gas chromatography.

MATERIALS AND METHODS

Drosophila Stocks

All fly stocks were maintained at 25°C and crosses were carried out and maintained at 25°C in a 12:12 light:dark cycle at 60% humidity unless otherwise indicated. *Drosophila* flies strains were maintained by serial transfers in 150 ml bottles containing 30 ml of standard food and active yeast powder on the surface. Normal food consisted of a standard corn meal, yeast, and molasses recipe (Chakraborty et al., 2011). Oregon-R strain was used as wild-type control. BL# refers to Bloomington Stock Center stock number (http://flystocks.bio.indiana.edu/bloomhome.htm). The flies stocks are:

1. (33801). Expresses the C99 fragment of APP with the human APP signal peptide and a C-terminal myc tag and human MAPT (Microtubule-associated protein tau) under UAS control. (Insertion chromosome (s) 2).
2. (33803). Expresses the C99 fragment of APP with the human APP signal peptide and a C-terminal myc tag and human MAPT (tau) under UAS control. (Insertion chromosome (s) 3).
3. (33799). Expresses human MAPT (tau), human BACE1, and the 695 amino acid isoform of human APP under the control of UAS. (Insertion chromosome (s) 3).
4. (33771). Expresses the human Aβ 42 fragment of APP and human MAPT (tau) under the control of UAS. Expresses GFP (green fluorescent protein), under actin control only in males and hid under heat shock control only in females. (Insertion chromosome (s) 1;2).
5. (8760). Express GAL4 in the nervous system. (Insertion chromosome (s) 3).

Pharmacological treatments in F1 generation flies

The following compounds have been used for pharmacological treatments:

Omega-3 fatty acids:

1. DHA (docosahexaenoic acid-Na salt).
2. OHDHA (2-hydroxy docosahexaenoic acid-Na salt).
3. EPA (eicosapentaenoic acid-Na salt).
4. OHEPA (2-hydroxy eicosapentaenoic acid-Na salt).
5. DHA lifort (mixture of 33% fish oil, 66% 226FFA (OHDHAFFA), and 1% α-tocophenol as antioxidant).
6. 226FFA (free fatty acid form of OHDHAA1).
7. LP183A1 (2-hydroxy-α-linolenic acid-Na salt).

Omega-6 fatty acids:

1. ARA (arachidonic acid-Na salt)
2. OHARA (2-hydroxy arachidonic acid-Na salt).
3. LP183A2 (2-hydroxy-γ-linolenic acid-Na salt).

Triacylglycerol mimetics (TGM). The basic structure of these compounds is that of a triglycerol, where 2-hydroxy fatty acids are esterified with glycerol. TGMs can be differentiated according to the 2-hydroxy fatty acids that were esterified with glycerol:

1. TGM0 (triacylglycerol containing 2-hydroxyheptanoic acid).
2. TGM1 (triacylglycerol containing 2-hydroxyoleic acid).
3. TGM2 (triacylglycerol containing 2-hydroxylinoleic acid).
4. TGM3 (triacylglycerol containing 2-hydroxylinoleic acid).
5. TGM4 (triacylglycerol containing 2-hydroxyarachidonic acid).
6. TGM5 (triacylglycerol containing 2-hydroxyeicosapentaenoic acid).
7. TGM6 (triacylglycerol containing 2-hydroxydocosahexaenoic acid).
8. TGM12 (triacylglycerol containing 1xLP181A1 and 2xLP182A1 or 2xLP181A1 and 1xLP182A1).
9. TGM14 (triacylglycerol containing 1xLP181A1 and 2xLPARAA1 or 2xLP181A1 and 1xLPARAA1).
10. TGM16 (triacylglycerol containing 1xLP181A1 and 2xLPHDHAA1 or 2xLP181A1and 1xLPHDHAA1).
11. TGM46 (triacylglycerol containing 2xLPHARAA1 and 1xHDHAA1 or 1xLPHARAA1 and 2xHDHAA1).
12. TGM146 (triacylglycerol containing 1xLP181A1 and 1xLPHARAA1 and 1xHDHAA1).

All the compounds used during this study were kindly provided by Lipopharma Therapeutics (Palma de Mallorca, Spain).

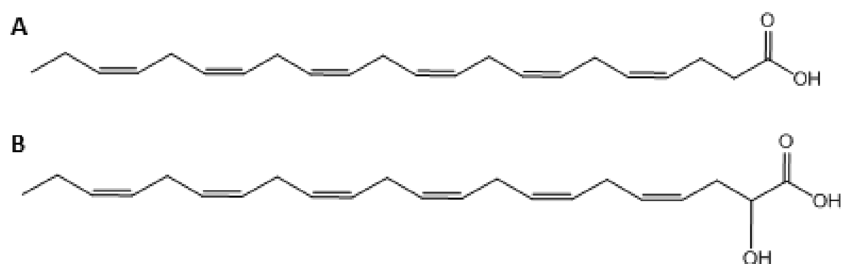


Figure 24. Chemical structure of compounds used. (A) docosahexaenoic acid or DHA (B) 2-hydroxy-acid docosahexaenoic OHDHA (LP226).

Drosophila **food supplements and fly culture maintenance**

The standard food was prepared as described (Chakraborty et al., 2011), (25 g yeast, 1000 ml H_2O, 60 g corn powder, 60 g sugar, agar 10 g, methyl 4-hydroxybenzoate 3 g, absolute ethanol 69% 10ml, propionic acid 5ml). F1 fly treatments were carried out at different concentrations, and the flies food was supplemented with 1, 3, 10, 30, 100 and 250 µg/ml of DHA, OHDHA, EPA, OHEPA, ARA, OHARA and the mixture (10 µg/ml OHDAH+10 µg/ml OHARA), (10 µg/ml OHDHA+30 µg/ml OHARA), (30 µg/ml OHDAH+10 µg/ml OHARA), (30 µg/ml OHDHA+30 µg/ml OHARA), and mixture of (10 µg/ml OHEPA+10 µg/ml OHARA), (30 µg/ml OHEPA+10 µg/ml OHARA), (10 µg/ml OHEPA+30 µg/ml OHARA), (30 µg/ml OHEPA+30 µg/ml OHARA), and 30 µg/ml of TGM0, TGM1, TGM2, TGM3, TGM4, TGM5, TGM6, TGM12, TGM14, TGM16, TGM46, TGM146, and 10, 20, 30 µg/ml of LP183A1 and LP183A2, during the larva and adult stage, The compounds were dissolved in absolute ethanol and it was used to prepare food vials for AD model flies at initial concentration of 25 mg/ml. No food supplement was used for control flies.

Flies were culture on media prepared by mixing an equal weight of the required concentration of the compounds with instant fly food power (Phillips Scientific).Twenty days after adult formation, fly heads were collected and stored at -80 ºC until use.

Transgenic model of Alzheimer's Disease in *Drosophila melanogaster*

This study was conducted in a transgenic *Drosophila melanogaster* model. This model is based on the expression system UAS/Gal4, which has been widely used for the study of many human diseases in *Drosophila* (Crowther et al., 2005). It is based on the use of two strains: one that expresses the Gal4 transcriptional activator under a tissue-specific promoter; and the other containing the current UAS upstream of the gene to be studied. By crossing these two strains, the gene of interest is expressed only in those cells expressing Gal4 (Figure 25).

In our case, the parental strain carrier (code 33771) contained the human inserts corresponding to the nucleotide sequence of human amyloid peptide of 42 amino acids (AB42) and the sequence encoding 2N4R isoform of human Tau protein, both under control UAS promoter. Furthermore, the activating parental strain (code 8760) expresses the factor of Gal4 transcription exclusively on neuronal cells because it is under the control of ELAV promoter, a feature of the nervous system protein. Accordingly, the cross of both strains will result in an F1 generation of flies expressing the peptide AB42 and Tau protein exclusively in

neural cells, which in turn would lead to a phenotype like pathology including own cognitive deficits of the disease.

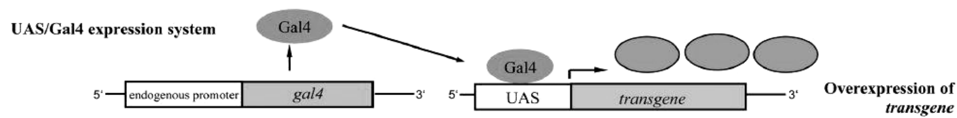


Figure 25. Diagram of the operation of the GAL4/UAS system in F1 generation. Adapted from (Prussing et al., 2013)

The model used was obtained from the Bloomington *Drosophila* Stock Center at Indiana University.

Genotyping of the parental strains

Before carrying out the parental strains crossing each other to result in generation F1, it is necessary to genotype in order to ensure that human inserts AB42 and Tau as well as the Gal4 activator gene are present in the corresponding strains. Checking the correct genotype is a measure that allows us to ensure that, after parental crossing, the next generations will have the desired genotype and phenotype. Flies were used females and males of each parental strain, the carrier (33771) and the activator (8760).
The genotyping of the parents was made by PCR. To test each inserts present in these strains different pairs of primers were used leading to a PCR products of different sizes (Table 2). As an endogenous control in the reaction it was also amplified the Actin5C gene constitutively present in the *Drosophila* genome (Table 2).

Briefly, whole flies were homogenized in a lysis buffer (10 mM Tris-HCl pH 8.2, 1 mM EDTA, 25 mM NaCl, proteinase K 200 µg/ml). Five flies were mechanically disrupted in 250 µl of lysis buffer and incubated at 37°C half an hour to allow tissue digestion by proteinase K. Then samples were heated to 95°C for 10 minutes to inactivate proteinase K and diluted 1/50 before performing PCR reactions. One µl of each sample was added to 24 µl of the a PCR Mix containing 1 mM Mg2 +, 2.5 mM dNTPs Mix (each one dATP, dGTP, dCTP, dTTP), primers UP and LO 0.4 mM (each) and Taq polymerase (Biotools) 5 U. Once prepared the reaction, different protocols were used for DNA amplification. The protocol 1 was used for amplification of the products corresponding to Aβ42, Tau and Actin5C (Table 3), and the protocol 2 to Gal4 (Table 4).

Once amplification was completed, the reaction volume (25 µl) was mixed with a loading buffer 6x (0.25% bromophenol blue, 30% glycerol in H_2O), for electrophoresis. For each

reaction, 20 µl of sample were loaded in a 2% agarose gel (containing 0.005% ethidium bromide in TBE (45 mM Tris-borate, 1 mM EDTA)), for performing the horizontal electrophoresis. Electrophoresis conditions were 120 V (voltage constant) and 20 minutes. Subsequently the gels were irradiated with ultraviolet light in a transilluminator to observe the banding pattern obtained.

Table 2. Information on the primers used for genotyping of the parental strains.

Primer	sequence	The length of amplified sequence
Actin 5c UP Actin 5c LO	GCACCACACCTTCTACAATGAGC TACAGCGAGAGCACAGCCTGGATG	171 pb
Gal 4 UP Gal 4 LO	TGGAACAAAGACGCCGAATT TATGGTGGGACCTGTTGTGGT	152 pb
Aβ42 UP Aβ42 LO	CAGAATTCCGACATGACTCAGG CATGAGTCCAATGATTGCACCTT	101 pb
Tau UP Tau LO	ATCTCCCCTGCAGACCCCCA TGCCTGCTTCTTCAGCTGTGGT	188 pb

Table 3. Program 1 for amplification of the products corresponding to AB42, Tau and Actin5C.

1	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7
temperature	94ºC	94ºC	60ºC	72ºC	Repeat 34 times steps 2, 3 and 4	72ºC	4ºC
time	3 min	30 sec	1 min	1 min		2 min	∞

Table 4: Program 2 for the amplification of the product corresponding to Gal4.

2	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7
temperature	94ºC	94ºC	60ºC	72ºC	Repeat 34 times steps 2, 3 and 4	72ºC	4ºC
time	3 min	30 sec	1 min	1 min		2 min	∞

Negative geotaxis (climbing) assay

Negative geotaxis is a commonly assessed behavior in *Drosophila* that is used as a proxy measure of neuronal dysfunction. It is an innate escape response mechanism elicited by banging flies to the bottom of a container; the flies respond to the mechanical stimulation by climbing up the container wall. This feature has been widely used to assess the cognitive decline that occurs in these models work (Chakraborty et al., 2011; Gargano et al., 2005). In our case, this test was used to test the skills cognitive of the various treatment groups: flies untreated F1 generation (group control), treated with different compounds.

To perform the assay, flies were transferred to plastic vials containing 5 ml of food (+/-drug) medium at a density of 20 flies per vial. Two vials of 20 flies were typically set up per

genotype/condition. Climbing ability was then assayed every 2-3 days. All assays were performed at the same time of day. Flies were moved to the test area at least an hour prior to the beginning of each assay, to allow the flies to adjust to changes in the environment. For the assay, 20 adult flies were placed at the bottom of a vertical column (70 ml) (Figure 26) and genteelly taped to the bottom allowed to climb for 20 s. The number of flies at the top and at the bottom was determined as described previously (Moloney et al., 2010).

The number of flies reaching the 7 cm (labelled on the column) was recorded after 20 second period, were counted separately, and three trials were performed at 1 min intervals for each experiment. Scores recorded were the mean number of flies at the top (ntop), the mean number of flies at the bottom (nbottom) and the total number of flies assessed (ntot). A performance index (PI) defined as ½ (ntot + ntop – nbottom)/ ntot) was calculated (Rogers et al., 2012).

In the present work, data are presented as the PI mean ± SEM obtained in three independent experiments for each group, and analysis of variances (ANOVA).

Figure 26. Picture of the device used for climbing assays. In the 70 ml cylinder assay the number of flies reaching the top, middle (area between top and bottom) and remaining at the bottom were scored after 20 second.

Survival Assays

Survival assays were performed as described by (Crowther et al., 2005). Briefly, 40 flies of each genotype were collected, divided into tubes of 20 flies each tube, kept at 25 °C, and transferred to fresh food containing 0, 3, 10, 30, 100 and 250 µg/ml of DHA, OHDHA, ARA, OHARA, EPA, OHEPA, and 10 and 30 µg/ml of the mixture of OHDHA+OHARA, and the mixture of OHEPA+OHARA and 30 µg/ml of TGMs and 10, 20, 30 µg/ml of LP183A1 and LP183A2 respectively every 2–3 days. Regarding the toxicity, it is evaluated taking into account the concentration of drug and deaths of flies. Every 2-3 days, the total number of live

flies were quantified in each condition until all died. Survival curves were analyzed using Kaplan-Meier plots and log-rank statistical analysis.

Isolation of RNA and proteins from fly heads

RNA and protein extraction protocol is based in Tripure isolation Reagent® protocol. The starting sample consisted of 150-200 heads of each condition including control and treated F1 generation and both parental strains. Samples were homogenized in 500 µl of Tripure isolation Reagent® (Roche) and mechanically homogenized with a teflon pestle. After 10 minute incubation at room temperature (RT), 100 µl of chloroform was added and shacked vigorously. Next the samples were incubated at RT, for 10 minutes and centrifuged (10,000 g, 15 minutes, at 4°C) and the upper aqueous phase was transferred to an RNase free tube. This phase was used for the purification of RNA while the interface and lower phase organic were used for protein isolation.

Isolation and quantification of RNA

Two hundred fifty µl of isopropanol were added to the upper aqueous phase and incubated for 20-30 minutes at RT. Later, the upper phase was transferred to a silica column (RNAeasy Mini Spin Column Omega Bio-Tech) and centrifuged (10000 g, 1 min, RT). Then, the columns were washed with RNA Wash Buffer (Omega Bio-tech), and 70% ethanol to remove organic products used in the extraction phase of the sample. Finally, the RNA adhered column was eluted with 60 µl of RNase-free water. (RNase-free water was produced by incubating ultrapure water (Milli-Q; Millipore) DEPC (Sigma) (0.1% v/v) for 24 hours.). This RNA was subjected to further digestions with DNase (Omega Bio-tek) to prevent contamination by genomic DNA at the time of performing the real time PCR. This digestion was performed with RNase-free DNase kit Set Qiagen®.

DNA buffer was prepared mixing 73.5 µl of DNase Digestion Buffer with 1.5 µl of RNase Free DNase in a final volume of 75 µl.

A Nano drop spectrophotometer (Thermo Fisher) was used to determine the concentration of RNA and to determine the ratios 260/280 and 260/230 nm, which were considered correct when their value was 1.8-2. The first ratio gives an idea of protein contamination (absorbance at 280 nm) and the second ratio detects the presence of the aromatics compounds (absorbance at 230 nm) used in the extraction phase. The samples used in this work **always** showed 260/280 and 260/230 ratios higher than 1.8.

Isolation and quantification of proteins

Protein isolation was performed following the instructions of the supplier (Tripure Isolation Reagent® from Roche®). Starting from the aforementioned two phases (intermediate and organic), 150 µl of absolute ethanol and 750 µl isopropanol were added, mixed and incubated for half an hour at RT, allowing the precipitation of the proteins. Then centrifuged (12000 x g, 10 min, and 4°C) and the supernatant were discarded. After two washes the pellet was resuspended in 1 ml guanidine hydrochloride.

Guanidine hydrochloride is a chaotropic agent that maintains the denaturation proteins and allows washing the precipitate of organic debris. Each wash was carried out for 20 minutes with stirring, then centrifuged (7500 x g, 5 min, and 4°C) and the supernatant was discarded. One ml of absolute ethanol precipitated protein was added vortexed and then incubated for 20 minutes at RT, centrifuged (7500 x g, 5 minutes, and 4°C) and the supernatant discarded. Subsequently the excess ethanol was removed heating the samples at 60°C for 20 minutes then, the protein precipitate was dissolved adding 200 µl of 8M urea, 4% SDS, 50 mM Tris-HCl pH 7.5 and incubated overnight. Finally the sample was sonicated (3 pulses 10 s, 100 W) and centrifuged (12000 x g, 10 min, RT) to precipitate insoluble debris from the exoskeleton of the flies.

Protein concentration was determined by the Lowry method (BC Bio-Rad Protein Assay Kit). Ten µl of each sample was used together with a standard curve with a known amount of BSA.

Tau and Aβ determination by Western Blot

Protein samples (120 µg per lane) were resolved on 12% polyacrylamide gels (for Tau), (table 5), or 16% polyacrylamide gels (for Aβ), (table 6), using Tris-tricine or Tris-glycine electrophoresis buffer, respectively. The proteins were then transferred to methanol-activated PVDF (only for Aβ; Bio-rad) or nitrocellulose membranes (for Tau), (GE Healthcare) that were subsequently blocked with 5% (w:v) non-fat dry milk in 0.1% (v:v) Tween-20 TBS. These membranes were then probed overnight at 4ºC with the corresponding primary antibody: mouse monoclonal anti-human-β-amyloid 1-16 (clone 6E10; 1:2000; Signet Labs), rabbit polyclonal anti-human-Tau (Tau46; 1:1000; Thermo Scientific), anti-phosphorylated Tau (anti-phospho-Ser202-Tau; CP13 clone, 1: 1000 and anti-phospho-Ser202-Thr205-PHF; clone AT8; 1:1000) and mouse monoclonal anti-α-tubulin (1:5000; Sigma). Antibody binding was detected with horseradish peroxidase conjugated anti-mouse/rabbit IgG (1:2000; GE

Healthcare). Membrane chemiluminescence was detected by ECL (GE Healthcare). The intensity of the bands was quantified by densitometry using Quantity One software (Bio-rad) and normalized to α-tubulin.

Table 5. Composition of the gels and buffers used for Western blotting for protein Tau

A Running gel Reactive	Quantity for 2 gels	B Stacking gel Reactive	Quantity for 2 gels
Acrylamide 20% Bisacrylamde 0.8%	6 ml	Acrylamide 20% Bisacrylamide 0.8%	600 µl
Tris HCl 2.5 M pH 8.8	4.5 ml	Tris HCl 1 M pH 6.8	1 ml
Water	5.25 ml	Water	4.2 ml
SDS 10%	150 µl	SDS 10%	60 µl
APS 50%	100 µl	APS 50%	140 µl
TEMED	20 µl	TEMED	10 µ1

Table 6. Composition of the gels and buffers used for Western blotting for β-amyloid Peptide

Running gel Reactive	Quantity for 2 gels	Stacking gel Reactive	Quantity for 2 gels
Acrylamide 48% Bisacrylamde 1.5%	3,23 ml	Acrylamide 48% Bisacrylamide 1.5%	404 µl
Tris HCl 2.5 M pH 8.45	3.6 ml	Tris HCl 2.5 M pH 8.45	1.24 ml
Water	3.04 ml	Water	3.28 ml
SDS 10%	94 µl	SDS 10%	60 µl
APS 50%	20 µl	APS 50%	15 µl
TEMED	20 µl	TEMED	10 µ1

Real time quantitative polymerase chain reaction (RT-qPCR)

Reverse Transcription

This phase was carried without the High Capacity cDNA Reverse Transcription kit (Aplied Biosystems). Nucleotides, primers and transcriptase solution was prepared with the Master Mix 2X provided by the manufacturer (RT Buffer 1X, dNTP Mix 4 mM (each), random primers 1X, transcriptase 2.5 U /µl) in a final volume of 10 µl of RNase-free water. Samples (0.8 micrograms of RNA) were prepared in a final volume of 10 µl of RNase-free water.

Table 7. Program reverse transcription of the RNA samples obtained from fly head.

Reverse Transcriptase	Step 1	Step 2	Step 3	Step 4
Temperature	**25ºC**	**37ºC**	**85ºC**	**4ºC**
Time	**10 minutes**	**120 minutes**	**5 minutes**	**∞**

Before performing reverse transcription, samples were pre-treated with a heat shock of 10 minutes at 65°C in order to prevent the formation of structures in secondary RNA that can

interfere in the process of retro-transcription. Once finished this heat shock, 10 µl of 2X Master Mix were added to each sample and they were subjected to the following temperature program to occur reverse transcription RNA to cDNA (complementary DNA) (Table 7).

Real-time PCR

This technique was used in order to quantify transcriptional expression of human inserts Tau and Aβ42 contained in the fly transcriptome. The quantification of the expression of human inserts of Aβ42 and Tau can be interfered with genome cDNA. This is because it was included a DNase digestion to remove traces of DNA which can be co-purified with the RNA sample. It has been found that this digestion was not entirely efficient and still there were traces of gDNA in the RNA sample. It was Inserted human transgenes were constructed as mini genes (not containing introns), meaning that there is no process of maturation of the RNA, and therefore, it is not possible to distinguish the PCR product from mRNA (or cDNA), from the remains of genomic DNA contaminant through the design of primers that link specific mRNA in different exons. Consequently, the final signal intensity obtained from Aβ42 or Tau primers does not derive exclusively from mRNA (or cDNA) but also from gDNA amplification.

Unfortunately, the level gDNA contamination from one sample to another may vary, especially if the samples were obtained in separate purification processes, so it is necessary to estimate the degree of contamination of gDNA in each sample to make a correction on the obtained results. In this work, has been used as endogenous reference (housekeeper) the expression of the gene Actin 5C of the fly itself. Expression of Actin 5C was quantified with two different pairs of primers in two different reactions. The first two primers hybridize within the same exon of the actin 5C gene, which implies that the detected signal derived from the mRNA amplification (or cDNA) 5C actin, but in addition, it can also be amplified from gDNA remaining in the sample. For that reason the PCR product obtained with these primers was called Total Actin 5C.

Furthermore, the product was amplified using another Actin 5C primer couple, which hybridizes each on a separate exon. This implies that the product detected can only come from mRNA (or cDNA) from the transcription of the actin 5C gene, but no from traces of genomic DNA contaminating the RNA sample. For this reason, this PCR product is called Actin 5C ExEx (exon-exon) (Figure 27).

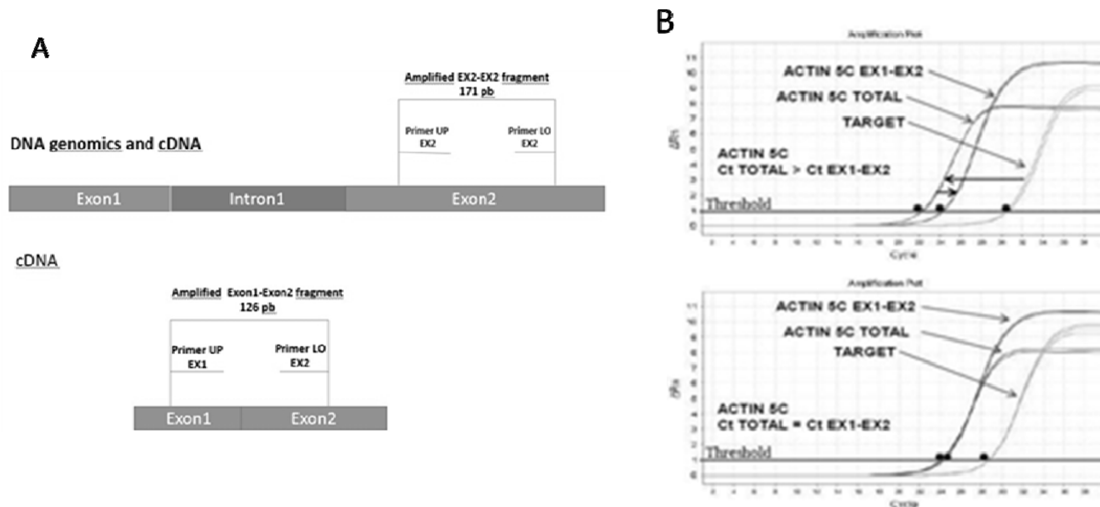


Figure 27. Determination of amplification of the different actin product. (A) Outline of binding primers: for determining the total actin PCR product (genomic DNA + cDNA) and for determining only the Actin 5C Actin 5C cDNA (Actin 5C ExEx). (B) Schematic explanation for determining the degree of contamination. In top panel an example in which no contamination is observed by genomic DNA as the amplification of the total Actin begins cycles before amplification of Actin cDNA. In the panel less the case in which no DNA contamination is observed genomic since total actin and actin cDNA start amplified in the same cycle.

By comparing the results obtained with both sets of primers, can be estimated the degree of contamination by genomic DNA that originally exists in the RNA sample object of study. To perform the qPCR it was used the reagent SYBR® Premix Ex.Taq ™ (TAKARA) at 2X. This mix contains Tli RNaseH (RNase that minimizes the inhibition of the PCR due to residual traces of RNA), dNTPs, Mg2 + SYBR Green I, which is a fluorescent intercalating agent that allows monitoring the reaction and the "hot-start" Taq polymerase. Primers were further added at a final concentration of 150 nM (Table 8) designed to determine the genes and Rox Dye, a component, which corrects the fluorescence between wells. The samples were diluted 1/100 and 5 µl of these samples were mixed with 5 µl of water containing primers and the Rox Dye and 10 µl of the SYBR-Premix 2X (Table 9).

Table 8. Components of the mix reaction used to perform real time PCR.

Components of the Mix (15µl) each reaction	
Reagents	**Amount per reaction**
SYBR® Premix Ex Taq™ $_{(2x)}$	10 µl
Primer UP 10 µM	0.3 µl
Primer LO 10 µM	0.3 µl
Rox Dye	0.4 µl
H_2O for PCR	4 µl

To carry out the calculation of the expression levels of we used the method of Livak, which sets the units expression relating through the formula $2^{-\Delta\Delta Ct}$, where Ct is the cycle threshold, that explain in which cycle starts the exponential phase of amplification. We analyzed the Ct gene of the problem, which we will rest the Ct Total actin by setting the ΔCt1. On the other

hand the Ct in total actin we rest the CT of the actin Exex establishing the ΔCt2. The result of the subtraction ΔCt1-ΔCt2 established the ΔΔCt that made it possible to implement the method formula of Livak commented at the beginning of this explanation. All of the qPCR assay in this study were performed according to MIQE Guidelines (Taylor et al., 2010).

Table 9. Primers used to perform qPCR.

Primers used for qPCR			
Primer	**Sequence**	**Amplicon size**	**Melting temperature**
Actin 5c Ex2-Ex2 UP	GCACCACACCTTCTACAATGAGC	171 pb	85.7ºC
Actin 5c Ex2-Ex2 LO	TACAGCGAGAGCACAGCCTGGATG		
Actin 5c Ex1-Ex2 UP	GCCAGCAGTCGTCTAATCCAG	126 pb	74ºC
Actin 5c Ex1-Ex2 LO	CGACAACCAGAGCAGCAACTT		
Tau UP	ATCTCCCCTGCAGACCCCCA	188 pb	87.6ºC
Tau LO	TGCCTGCTTCTTCAGCTGTGGT		
Gal 4 UP	TGGAACAAAGACGCCGAATT	152 pb	79ºC
Gal 4 LO	TATGGTGGGACCTGTTGTGGT		
Aβ42 UP	CAGAATTCCGACATGACTCAGG	101 pb	76ºC
Aβ42 LO	CATGAGTCCAATGATTGCACCTT		

Fatty acid analysis of lipids from heads of *Drosophila melanogaster* after treatment with DHA and OHDHA

Materials

HPLC-grade chloroform, hexane and methanol are generous gift from Scharlab (Barcelona, Spain). Margaric acid, acetyl chloride and N, O-bis (trimethylsilyl) acetamide was purchased to Sigma-Aldrich (St. Louis, MO). DHA were provided by BASF Pharma (Callanish, UK).

Lipid extraction and gas chromatography analysis

Approximately 20 mg of fly heads (200 flies heads), were maintained in 3 ml chloroform: methanol (2:1; v:v) for 48 hours under an inert atmosphere at room temperature (Yoshioka et al., 1985). After 48 hours, all the heads had sunk to the bottom of the vial and they were homogenized at 4ºC using a Kinematica Polytron PT3100 homogenizer (Luzern, Switzerland). Samples were centrifuged at1000 x g for 10 minutes at 4ºC and the pellet was washed with 3 ml chloroform: methanol (2:1; v: v) for 1 hour.

The lipid-containing supernatants were combined with 0.2 volumes of 0.9% NaCl, the mixture was vortexed and centrifuged at 1000 x g for 10 minutes at 4 ºC. The lower, organic phase was evaporated in a pre-weighed tube and it was placed under vacuum for 12 hours to eliminate traces of solvents. The mass of extracted lipid was calculated by difference in

weights and, afterwards, the lipid mixture was supplemented with 1 µmol margaric acid as internal standard for GC analysis. The lipid film was resuspended in 166 µl hexane to dissolve the least polar lipids, such as triglycerides. The transmethylation of fatty acids was performed by incubating the lipid mixture in 3 ml methanol:acetylchloride (10:1, v:v) at 100ºC, for 90 minutes, in an inert atmosphere in pyrex screwed-capped tubes (Christie, 1993).

Fatty acids subjected to methylation or to methylation/trimethylsilylation were analyzed by an Agilent 7890A GC system equipped with a FID and a 7693 auto-injector (Santa Clara, CA).

An Agilent J&W HP-88 capillary column (30 m x 0.25 mm x 0.20 µm) was used with 1.3 ml/min of helium as a carrier and the split ratio was 5:1.

The column was equilibrated at 130ºC for 5 minutes, the temperature was increased up to 160ºC at 2.5 ºC/min and then, up to 220ºC at 2ºC/min. Finally the column was left at 220ºC for 5 minutes. The injector and flame ionization detector (FID) temperatures were kept at 250ºC. Areas of peaks were quantified using margaric acid as internal standard and corrected using the calculated weight of extracted lipid. The identification of peaks was performed using standards of the different hydroxylated and non-hydroxylated fatty acids.

Statistics.

Statistical analyses were performed using GraphPad Prism for PC. Results are reported as mean±SEM.

For statistical analysis of the results of Western blotting and real time PCR it was carried out multiple sample comparison by ANOVA followed by Tukey post-hoc test. Differences were considered statistically significant in confidence interval of 95% ($p < 0.05$).

RESULTS

Genotype characterization

In order to obtain F1 flies that overexpress both APP/Abeta and Tau proteins, I performed a series of crossing of males flies stocks carriers; with a female stock (8760) that expresses GAL4 in the nervous system. The following carriers were characterized in the present work prior to selection for additional experiments: (33801, 33802, 33799, 33771, Material and Methods, Drosophila stocks).

All these transgenic strains were designed to mimic AD pathology by over-expression of human Aβ peptide and Tau protein. To check if F1 flies from all these strains express Aβ and Tau, Western blot analysis was addressed.

Only the crossing of 33771 with 8760 lead to F1 flies expressing Aβ42 and Tau (Figure 28). The over-expression of these human proteins was reflected in the climbing and survival test where it was found a significant different between F1 and the parental strain as shown in (Figure 39), and (Table 13), for climbing test and (Figure 40) and (Table 14), for survival test. As a consequence it was decided to use this genotype for the present work.

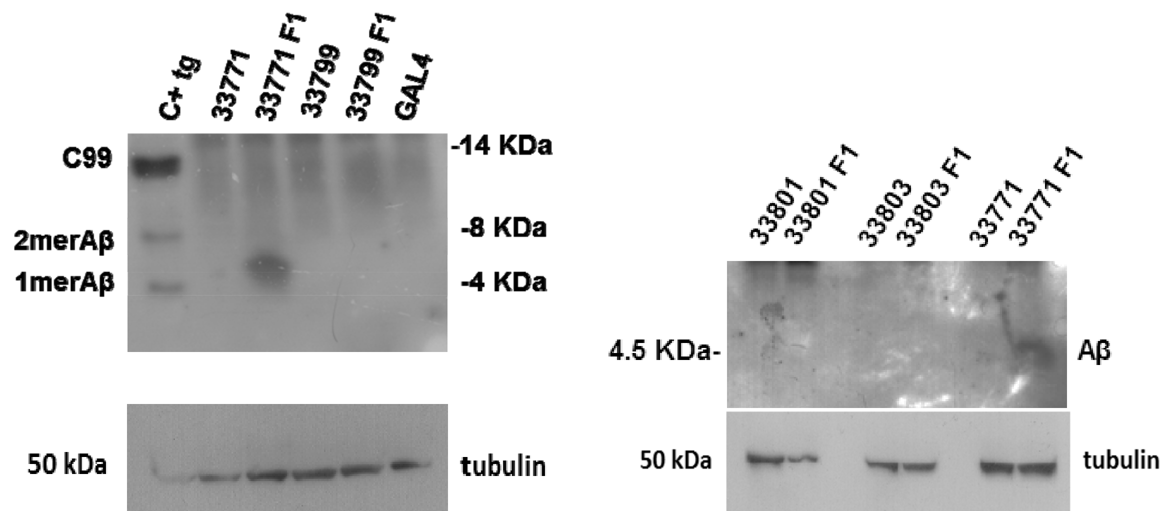


Figure 28. Western blot, showing the expression of Aβ of all flies.

Genotyping of 33771 and 8760 parental strains

The result of the progenitor flies genotyping it shown in Figure 29, lane 1 and 2 display band from parental carrier strain samples (33771) while lane 3 and 4 display band from parental activator strain samples (8760). First PCR product analyzed was that of the transgene corresponding to human Aβ42 (100 bp) (Figure 29A). This PCR product was only observed in lanes 1 and 2. Similarly, the PCR product from human Tau transgene is also showed only

in lanes 1 and 2 (188pb band) (Figure 29C). Therefore, these two results confirmed the correct genotype of the parental carrier strain. Finally, (Figure 29B) shows results for Gal4 genotyping in the same samples aforementioned. In this case, only lanes 3 and 4 showed the band corresponding to Gal4 (152pb) demonstrating the correct genotype of this strain.

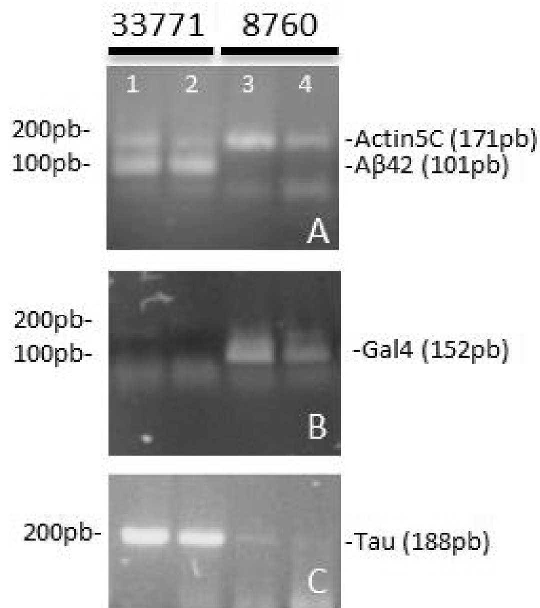


Figure 29. Genotyping of 33771 (carrier) and 8760 (activator) progenitor strains by PCR. (A) Human Aβ42 transgene was denoted by one band at 101bp that appeared only in 33771 (lanes 1 and 2) and not in 8760 (lanes 3 and 4) flies whereas Actin C5 as endogenous control was equally amplified in both progenitor samples (171 bp). (B) Gal4 transcription factor transgene was detected as a band of 150 bp only in 8760 but not in 33771 flies. (C) Human tau transgene was denoted by one band at 188 bp that appeared only in 33771 and not in 8760 samples.

Expression of human Aβ and Tau in non-treated transgenic *Drosophila melanogaster*

Flies expressing Aβ42 and Arctic Aβ42 in neurons have been shown to accumulate toxic non amyloid aggregates, firstly intracellular and then extracellular, causing neuronal dysfunction and then neurodegeneration. Crowther and Colleagues described the Aβ expression in the brain of various flies that were analyzed day 20 after hatching distributed throughout the brain of files (Crowther et al., 2006). This result was confirmed by Western blot using 6E10 antibody that detects the existence of soluble and aggregated forms of the peptide (Figure 30). Both in flies grown in the absence and in the presence of the drugs, showing that the drugs do not interfere with Aβ expression. Gene expression was controlled by an anti-human 6E10 antibody, and by SDS- PAGE followed by Western blotting (Figure 30) of brain sections corresponding to Aβ and Tau expressing flies.

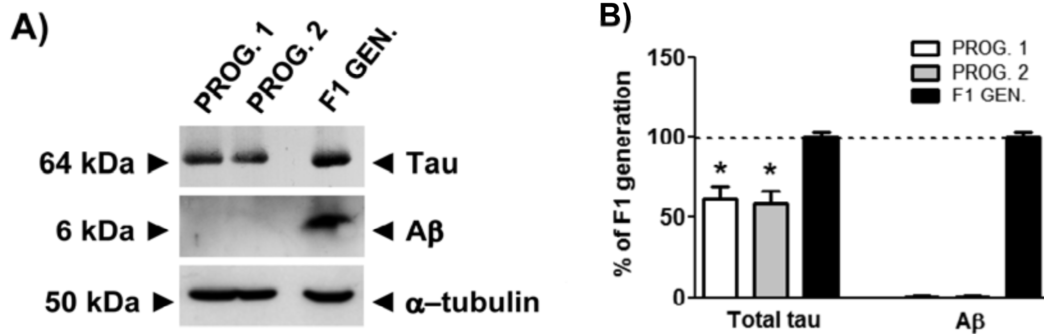


Figure 30. Molecular characterization of the transgenic fly model of AD. A) *Drosophila melanogaster* overexpressing human β-amyloid and Tau protein were sacrificed and decapitated. Protein samples were isolated from fly heads and used to test Ab and Tau expression by Western blot. B) Quantitative analysis showed presence of Tau protein in both progenitors (PROG. 1: Gal4-expressing flies; PROG. 2: flies expressing Ab and Tau under UAS promoter control) and flies from the F1 generation (F1 GEN.) thus indicating presence of a human homolog Tau protein in *D. melanogaster*. As expected, Tau and Ab expression was significantly elevated in F1 flies as compared with their progenitors. The bars represent the mean ± SEM. Statistical analysis was performed by ANOVA followed by Tukey post-hoc test. The asterisks indicate a significant difference as compared with F1 GEN. flies: * p<0.05

Detection of the peptide Aβ42 and Tau protein (total and phosphorylated) in treated transgenic *Drosophila melanogaster*

Various Western blot assays for determining the Aβ42 peptide and protein Tau (total and phosphorylated) were performed.

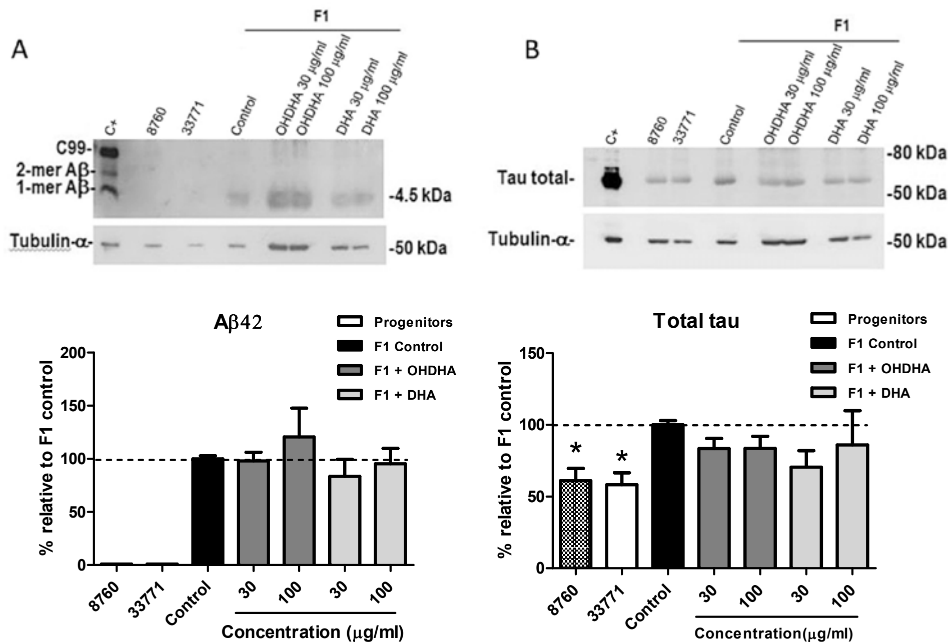


Figure 31. Results of Western blotting for the total Tau and AB42 peptide. (A) Western blot showing representative Aβ42 peptide with α-tubulin as loading control (upper panel). Graph of the quantification of western blotting performed on F1 percentages relative to controls (bottom panel). Observe that there are no significant differences between all generation flies F1. (B) Representative Western blot showing total Tau protein α-tubulin as a loading control (upper panel). Graph of quantification of the Western blot conducted in percentages relative to the control F1. Significant differences were observed between parental and all the F1 generation flies, but not when comparing F1 samples among them. (lower panel). Statistical analysis ANOVA, * p <0.05.

In (Figure 31A) and (Figure 31B), it is showed the most representative results observed from western blot that was conducted for peptide Aβ42 and total Tau protein. In the chart associated with (Figure 29A) Aβ42 peptide expression relative to control are shown F1, which was established as 100%. No significant differences between the F1 control and treated samples OHDHA or DHA. As expected for the carrier (8760) and the activator (33771) progenitors, was not detected the peptide in either cases, because the expected result should not express: in first case because it contains and the second because the transgene cannot express without the Gal4 transcription factor. Moreover, the graph in (Figure 29C) shows the expression of Tau protein respect to Control F1, which was considered 100%. The Tau protein expressed by human transgene corresponds to a protein of about 45 kDa, however, a band was observed around 60 kDa corresponding to the Tau protein posttranslational (generally, phosphorylation in several residues). It is noted that in the treated groups no significant differences compared to control F1.

Determination of the transcriptional expression of human Aβ42 and Tau.

Expression levels of the inserted human transgenes were assessed by real time PCR. It was measured Aβ42 and tau gene expression from cDNA samples obtained by reverse transcription from total RNA samples (Materials and Methods; RT-qPCR). In (Figure 32) it can be observed the representation of these results. In both cases, a high variability in expression levels was observed. Our statistical analysis revealed that there are no statistically significant differences between the F1 control and the rest of the F1 generation groups treated with OHDHA or DHA. As expected, both human transgenes, Tau and Aβ42, exhibited similar expression profiles as a result of both are expressed together under the control of the same UAS promoter. In addition, since our model was designed to over-express the human inserts depending on the previous expression of the Gal4 transcription factor, no treatment associated expression differences would be expected.

On the other hand, it may be observed remarkable expression differences in both Aβ42 and tau between progenitors and F1 flies. Parental activators (8760) showed no specific signal (they do not contain the human inserts in their genome) but, unexpectedly, parental carriers (33771) did show detectable levels of human Aβ42 and tau genes (see figure 32). Since human Aβ42 peptide was not detected in this progenitor by western-blot (see figure 31), this signal has been attributed to partial genomic DNA contamination of cDNA samples (see Materials and Methods; RT-qPCR). Accordingly, the signal increase observed between 33771 and F1 control samples was statistically significant since F1 generation should contain

expression levels as consequence of Gal4-induced transcription plus this partial gDNA contamination. Furthermore, it can be seen that the difference in expression between 33771 parental line and F1 generation and it is not as great as might be expected. If the signal detected in 33771 belongs just to residual contamination by gDNA that would be present in cDNA samples (no Gal4 transcriptional expression mediated), we suggest that the difference in intensity between this parental sample and F1 flies is relatively low. Because the human inserts Aβ42 and tau have inducible expression, presumably they should show an expression evel such that it should not be confused with the background noise (due to amplification of the insert from gDNA).

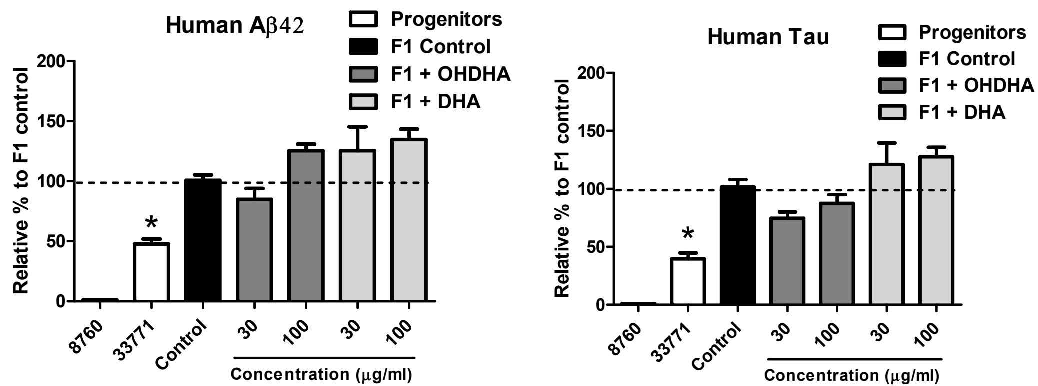


Figure 32. Expression levels of the inserted human transgenes Aβ42 and tau. (A) Expression level of human Aβ42. No significant differences were observed between F1 control and treated samples. (B) Expression of the human tau. No significant differences were observed between F1 control and treated samples. Unexpectedly, human Aβ42 and tau gene expression was detected in 33771 progenitor but significant differences between this and F1 control were observed. Statistical analysis ANOVA, * $p < 0.05$.

Evaluation of the toxicity of compounds by median survival analysis

The evaluation of the toxicity of the various treatments was carried out by median survival (MS) establishment of each group. The MS is the day in which each group presented a 50% of its population alive. The comparison between the group of F1and the parental (33771) carrier is shown in Figure 39 and Table 14. The group F1 that express the transgene, the model being studied and the parental group carrier (33771), which does not express the transgene, serves as healthy control. The F1 group has a lower survival rate compared to parental. The MS for F1 was established on the day 14.8 while for parental carrier established on the day 19.4. Once performed comparison between our F1 model and the parental group, we proceeded to compare the F1 groups treated with different compounds.

Figures 42, 43 and Table 16 shows the survival rates after treatments were applied on the F1 flies, (DHA and OHDHA) respectively. In Figure 43E it is showed that the longevity of OHDHA-treated flies 100 μg/ml is about 10 days higher compared to the control group, the

value MS calculated for the group treated with 30 µg/ml OHDHA Figure 43 D, was 16.6 days compared to 16.8 days in the treated group with 100 µg/ml and 14.4 days for group F1. These results demonstrate that OHDHA treatment with 100 µg/ml increases the longevity of the flies. For the group treated with 30 or 100 µg/ml DHA Figure 42 D, E the MS value calculated was 15 and 15.8 days respectively so there is no significant different between F1 and DHA group. For flies treated with 30 or 100 µg/ml of ARA or OHARA the MS was 16.2, 16.1, 17.6, 16.5 days respectively Figure 46 D, E, 47 D, E and Table 18. This result demonstrates that the flies treated with 30 µg/ml OHARA increase the longevity. Flies treated with 30 or 100 µg/ml EPA or OHEPA the MS was 16.4, 16.6, 16.9, 17,7 days respectively Figure 50D, E, 51 D, E, Table 20. The result demonstrated that the F1 treated with 100 µg/mg OHEPA was the best compound to increase the longevity. The result for 250 µg/ml of the compounds above shows toxicity in the flies, decreasing its longevity Figure 42F, 43F, 44F, 45F, 50F, 51F. For flies treated with the mixture of OHDHA and OHARA (30 µg/ml+30 µg/ml), the MS was 21.5 days Figure 54D and Table 22. When the mixture was OHARA and OHEPA (30 µg/ml+30 µg/ml), the MS was 21.7 Figure 56D and Table 24. Theses result demonstrated that the mixture of OHDHA and OHARA and the mixture of OHARA and OHEPA increased the longevity.

On the other hand, the MS for F1 treated with 30 µg/ml of TGM4 was 24.1 days Figure 58C and TGM3 23.5 days Figure 58A, TGM6 23.5 days Figure 60D, TGM14 19.8 days Figure 62A, and TGM16 19.5 days Figure 62B, TGM46 20.3 days Figure 62C, TGM146 20.5 days Figure 62D, DHAlifort 21 days Figure 62F and Tables 26, 28, 30. Theses result demonstrated that the TGMs increased the longevity.

Finally, 30µg/ml of LP183A1 showed MS value of 19.6 days Figure 64C and Table 32. The mixture of LP183A1 and OHARA (10 µg/ml+ 30 µg/ml) showed a MS of 20 days Figure 64E and when changing proportions of LP183A1 and OHARA (30 µg/ml+ 10µg/ml), the obtained MS value was 20.6 days Figure 65F and Table 32. These results demonstrate that when OHARA is combined with LP183A1, there was no improvement in F1 flies longevity. When 30 µg/ml of LP183A2 were used the MS value was 19.3 days Figure 66C, and the mixture of LP183A2 10µg/ml and OHDHA30 µg/ml MS was 21 days Figure 66F, and the mixed of LP183A2 30µg/ml and OHDHA30 µg/ml MS 21.8 days Figure 66G and Table 34. These results demonstrate that when OHDHA is combined with LP183A2, there was no improvement in F1 longevity.

Climbing assay

The effect of expressing human amyloidogenic proteins in the central nervous system on the climbing behavior of transgenic flies, in comparison with a control group, was used as a biomarker for potential neurological impairment. In order to study the cognitive status of transgenic *Drosophila* as a model for Alzheimer's disease. It was carried out the so-called "climbing test". When the flies tapped to the bottom of a vial, flies will normally orient themselves rapidly begin to climb vertically up the vial. This behavior has been used to assess CNS function in fly models of AD (Chakraborty et al., 2011; Long et al., 2009; Llorens et al., 2007).

It is observed that F1 flies, that express human Aβ42 and human Tau in the nervous system showed a consistent and significant decrease in climbing ability as compared the control with the carrier 33771, figure 37A and the activator 8760, Figure 38B, Table 13. All groups of flies, where tested every two days. Results obtained with DHA and OHDHA is shown in Figure 40, 41 and Table 15. Results from F1 treated with ARA and OHARA are shown in Figure 44, 45 and Table 17, EPA and OHEPA in Figure 48, 49 and Table 19, the mixed drugs of OHDHA and OHARA in Figure 53 and Table 21, the mixed drugs of OHEPA and OHARA in Figure 55 and Table 23, and all tested TGMs in Figure 57, 59, 61 and Table 25, 27, 29. Finally, Results from LP183A1 and LP183A2 treatments are shown in Figure 63 and Table 31, and in Figure 65 and Table 33, respectively.

Moreover, it can be seen that the untreated group F1 show a cognitive decline slightly earlier than those that were treated with DHA or OHDHA. In treated over time flies that manage to overcome the test in higher proportion when they have been treated with 30 or 100 µg/ml OHDHA Figure 41. Furthermore, DHA treatments are represented in Figure 40; it shows that the group treated with 30 or 100 µg/ml DHA flies expressed the test for a longer period of time. These results demonstrate that OHDHA promotes longevity and cognitive improve in F1 flies, while the same dose of DHA does not improve cognitive state of F1 flies. Comparison of the F1 strain with the parental strain 33771, as a control, we observe that cognition declines more rapidly in the F1 group, as expected due to the expression of human transgenes. Comparison of OHDHA and F1, the best results is F1 treated with OHDHA 30 or 100 µg/ml. Also, both treated groups have higher test improvement as compared with control. In this context, F1 treated with 30 or 100 µg/ml of OHARA or OHEPA showed more improvement in climbing test as compared to native form of ARA or EPA at same doses.

To see the clearer effect for those compounds in the curve, it was used, median effective dose ED50 a dose that produces the desired effect in 50 per cent of a population, as shown in

Figure 52. In the case of TGMs, the best result takes place with TGM0, TGM1, TGM2, TGM3, TGM4, TGM5, TGM6, TGM12, TGM14, TGM16, TGM46, TGM146, as compared with control F1 flies. Finally, in the case of treatments with LP183A1 and LP183A2, it was found that the treatment improved cognitive performance in F1 treated flies as compared with F1 controls.

Life span assay

The effect of expressing human amyloidogenic proteins in the central nervous system on the lifespans of transgenic flies, in comparison with a control group, was used as a marker of terminal disease. Expression of Aβ in the F1 flies central nervous system resulted in reduced lifespan when compared to parental control flies Figure 39.

The life span for the F1 flies as a control was 40 days while the life span for or-R (wild type), was 47 days. In this sense, lifespan of flies treated with 30 or 100 μg/ml of OHDHA, displayed a rescue effect on genotypes displaying the strongest neurodegenerative phenotype. The increased lifespan following treatments was striking for flies expressing Aβ42 and Tau. The survival of flies treated with OHDHA Figure 43, D, E, OHARA (Figure 47, D, E), OHEPA Figure 51, D, E and LP183A1 Figure 64, C, E, F and LP183A2 Figure 66, E, F, G and TGMs 30 μg/ml Figure 58, 60, 62, was increased as compared with F1 control. The median survival among flies treated with 30 or 10μg/ml concentration increased to 25%. However, the F1 flies suffered a toxic effect when treatment dose was increased up to 250 μg/ml Figures 42 F, 43 F, 46 F, 47 F, 50F, 51F.

Fatty acid analysis of lipids from heads of *Drosophila melanogaster* after treatment with DHA and OHDHA

Food supplementation with either hydroxylated or non-hydroxylated compounds induced changes in the head's fatty acid profile of *Drosophila melanogaster* Table 10, Figure 33. Transgenic control flies F1, were generated by crossing female flies (carrying GAL4 on their X chromosome), with male carrying upstream activation sequence promotor (UAS). Heads from untreated F1 flies contained approximately 56 mol% of short chain fatty acids
(12-16 carbon atoms), while this amount decreased to 51, 36 and 49 mol % after ARA, EPA and DHA treatments, respectively. Regarding the hydroxylated fatty acids, the reduction in the levels of short chain fatty acid (SCFA) was similar to that of the non-hydroxylated fatty acids (42, 39 and 51 mol% for OHARA, OHEPA and OHDHA, respectively). Moreover, all food supplement tested induced an increase of long chain fatty acids (≥ 18C). Interestingly,

the latter increase did not only account for the presence of the fatty acid supplemented but also for other long chain fatty acids. This implies that these compounds regulate lipid metabolic pathways that are involved in either catabolism of the incorporated food supplements and/or the *de novo* synthesis of fatty acids.

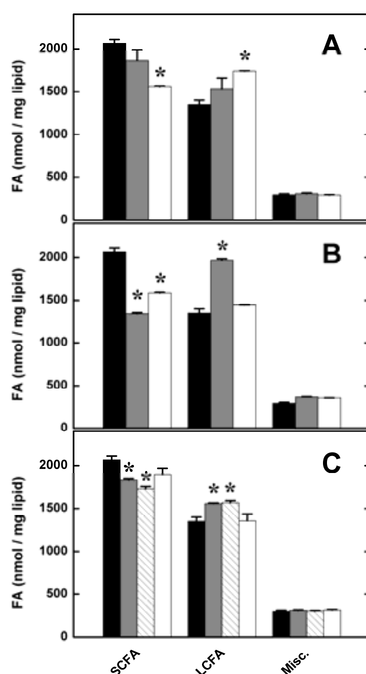


Figure 33. Fatty acid profile of lipid extracts from heads of *Drosophila melanogaster*. *Drosophila* being fed with (**A**) ARA and OHARA; (**B**) EPA and OHEPA; (**C**) DHA, DHAlifort and OHDHA. In all cases, control are depicted as filled bars; non-hydroxylated FA food supplementation as grey bars; OHFA food supplementation as empty bars and treatment with DHAlifort, as dashed bars. A group of 20 minor chromatographic peaks present in all lipid extract are gathered in a "miscellaneous" (Misc.) bar for simplicity. Data are mean values ± SEM of 2-4 independent experiments. * indicates statistical differences (*p* value at least ≤ 0.05) compared to control samples.

Control (untreated F1 flies), samples lacked C20 and C22 polyunsaturated fatty acids (PUFAs), which correlates with previous works (Shen et al., 2010); however, ARA, EPA and DHA were present in the fatty acid profile of flies treated with non-hydroxylated fatty acids. This fact proves the absorption and incorporation of dietary PUFAs into the body tissues. Besides, DHA-treated flies showed not only the presence of the omega-3 DHA (22:6n-3), but also the omega-3 EPA (20:5n-3). Interestingly, the amount of DHA was much lower compared to that of EPA (1.6 ± 0.3 and 42.6 ± 1.5 nmoles/mg lipid, respectively), which indicates a fast conversion of 22:6n-3 into the 20:5n-3 form. In mammals, omega-3 and omega-6 PUFAs are synthesized through elongation and desaturation of the essential α-LNA and LA fatty acids, respectively (Ibarguren et al., 2014). *Drosophila melanogaster* does not possess the ability to synthesize ARA, EPA and DHA; however, they have the catabolic machinery for the shortening of DHA into EPA. The presence of other shorter fatty acids suggest that *Drosophila* is also able to catabolize ARA and EPA, however, whether flies possess a degradation system for C20 PUFAs requires further investigation.

Table 10. Fatty acid composition of heads of *Drosophila melanogaster*. The flies fed with 100 µg/ml of different hydroxylated and non-hydroxylated fatty acids**.** Values are mean ± SEM of 2-4 separate experiments and are expressed as nmol/mg lipid. OHARA, 2-hydroxyarachidonic acid; OHDHA, 2-hydroxydocosahexaenoic acid; OHEPA, 2-hydroxyeicosapentaenoic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; DHAlifort, nutraceutic formula enriched in omega-3 fatty acids and OHDHA; EPA, eicosapentaenoic acid; ND, not detected. * statistically significant decrease compared to control samples (p at least ≤ 0.05). # statistically significant increase compared to control samples (p at least ≤ 0.05).

FA	Control	ARA	OHARA	EPA	OHEPA	DHA	DHALifort	OHDHA
12:0	157.9 ± 35.7	110.5 ± 30.6*	69.6 ± 3.3*	80.4 ± 13.5*	83.5 ± 3.1*	98.1 ± 7.6*	82.2 ± 16.2*	120.5 ± 27.2*
14:0	668.3 ± 25.4	515.1 ± 75.7	326.9 ± 1.5*	274.5 ± 2.9*	383.9 ± 2.3*	484.6 ± 4.3*	436.7 ± 15.1*	592.1 ± 64.9
14:1n-5	34.5 ± 3.0	27.6 ± 3.9	15.8 ± 0.1*	17.5 ± 0.8*	20.1 ± 0.2*	24.7 ± 0.6*	20.1 ± 0.7*	30.7 ± 3.7
16:0	704.3 ± 16.2	706.9 ± 21.7	715.1 ± 1.6	555.9 ± 1.4*	660.7 ± 5.8	709.9 ± 1.9	755.9 ± 10.0*	649.5 ± 19.6
16:1n-7	502.2 ± 4.2	507.4 ± 8.2	437.4 ± 0.5*	413.6 ± 2.2*	438.5 ± 4.2*	516.1 ± 0.9	435.7 ± 7.5*	502.4 ± 3.0
18:0	106.8 ± 1.8	138.9 ± 9.6#	142.6 ± 0.1#	226.3 ± 4.0#	126.7 ± 1.6#	175.8 ± 0.6#	167.6 ± 16.5#	156.1 ± 4.6#
18:1n-9	571.5 ± 22.4	653.3 ± 47.1	698.1 ± 0.6#	758.8 ± 6.3#	679.1 ± 5.4#	687.3 ± 3.1#	695.7 ± 14.6#	627.8 ± 41.6#
18:2n-6	542.2 ± 19.8	519.6 ± 47.9	749.7 ± 2.2#	667.4 ± 9.6#	508.8 ± 5.8	512.3 ± 3.3	541.1 ± 17.1	428.4 ± 38.2*
18:3n-3	115.9 ± 9.2	120.3 ± 9.8	134.5 ± 2.1	173.7 ± 0.1#	88.5 ± 0.1	120.3 ± 2.8	105.2 ± 2.1	123.5 ± 3.1
20:0	12.2 ± 0.4	17.1 ± 0.9#	16.6 ± 0.6#	26.28 ± 0.3#	16.9 ± 8.3#	16.8 ± 0.2#	16.9 ± 0.7#	18.0 ± 1.7#
20:4n-6	ND	71.7 ± 8.5#	ND	ND	ND	ND	ND	ND
20:5n-3	ND	ND	ND	115.2 ± 5.0#	ND	42.6 ± 1.5#	42.7 ± 2.5#	ND
22:6n-3	ND	ND	ND	ND	ND	1.6 ± 0.3#	ND	ND

On the other hand, food supplementation with 100 µg/ml of OHARA, OHEPA and OHDHA did not induce their own presence in the head of flies, which suggests that *Drosophila melanogaster* possesses the enzyme machinery necessary for rapid metabolization of these compounds. This degradation was not related with the production of their non-hydroxylated derivatives either (20:4n-6, 20:5n-3 and 22:6n-3, respectively). The analysis of the fatty acid profile of these samples showed a group of minor chromatographic peaks which were not present in control or in any treatment with non-hydroxylated lipids (Figure 34) (Figure 35). These peaks, of heterogeneous retention times, were not hydroxylated, which indicated that OHFAs were metabolized into non-hydroxylated metabolites, varying in the number of carbon atoms and degree of unsaturation. It is worth mentioning the use of DHALifort, a mixture of OHDHA and fish oil enriched in omega-3 PUFAs (1:1; v/v), as food supplement. In this case, chromatographs did not show any peak with the same retention time of OHDHA. However, the metabolic derivative peaks detected in the treatments with OHFAs peaks of both omega-3 DHA and EPA were observed. Moreover, the omega-3 DHA and EPA were present in the lipid extract, which correlates with previously shown results.

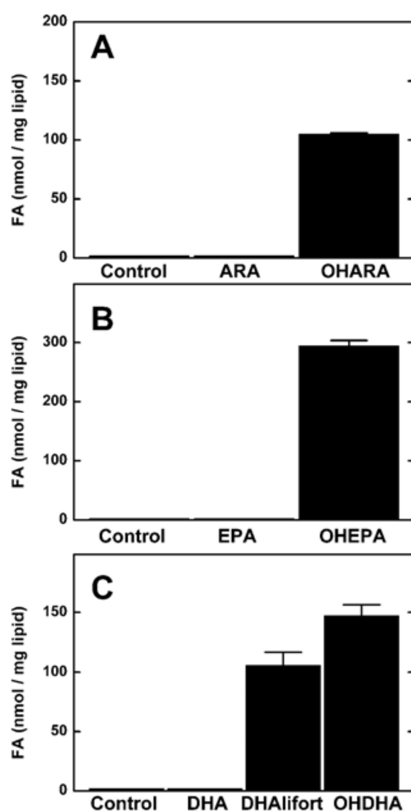


Figure 34. Levels of unusual FA peaks found after treatment with OHFAs. The sum of chromatographic peaks present in lipid extracts of flies fed with 100 µg/ml OHARA, OHEPA, DHAlifort and OHDHA were quantified and compared to lipid extracts of control or their non-hydroxylated FA treatments. Data show mean values ± SEM of 2-4 independent experiments. On the wright side shows the chromatograms.

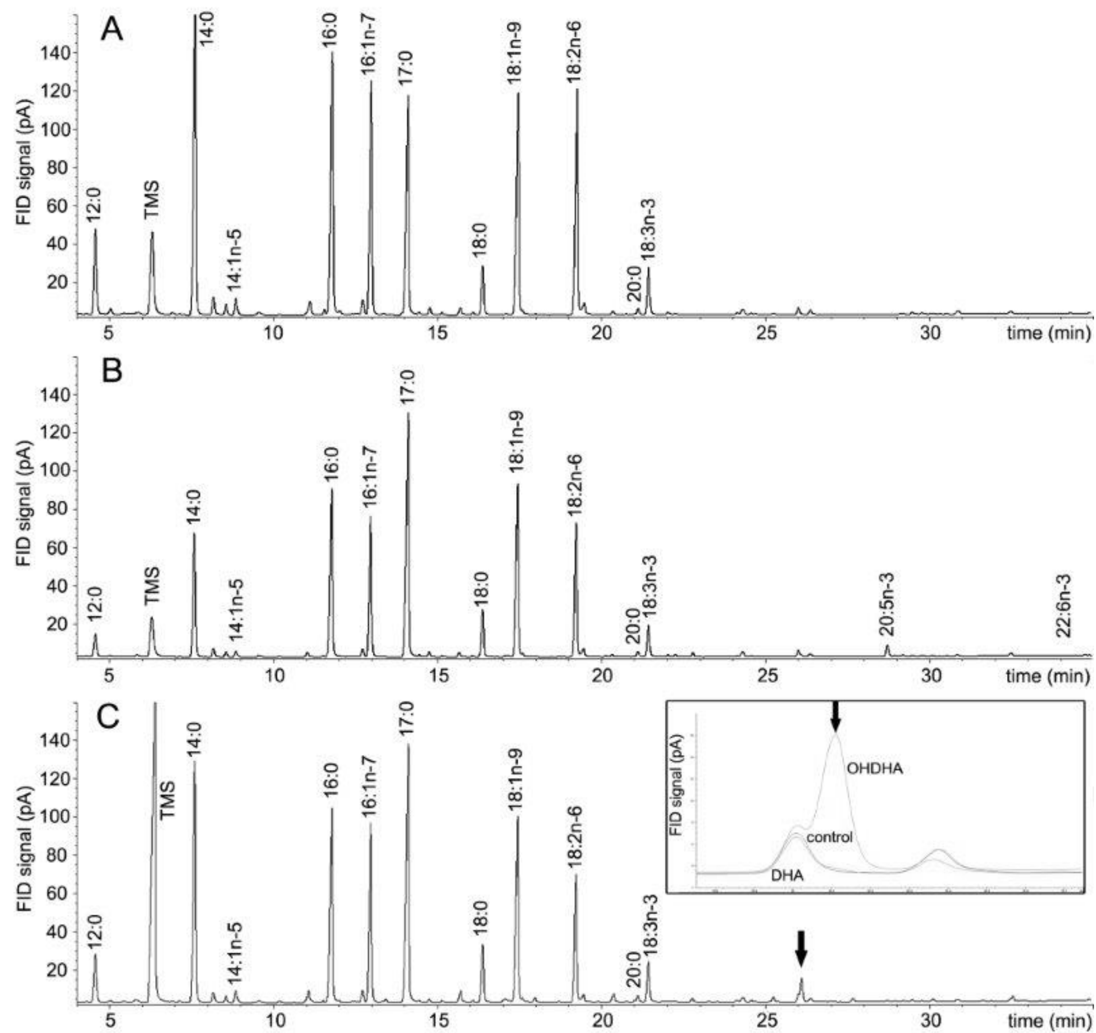


Figure 35. Representative chromatograms of the FA composition in *Drosophila melanogaster*. *Drosophila* fed with (A) base diet, (B) base diet supplemented with 100 µg/ml DHA and (C) base diet supplemented with 100 µg/ml OHDHA. Total lipids from heads of flies were extracted and transmethylated with acetyl chloride in methanol. Further derivatization with *N,O* –bis (trimethylyl) acetamide was performed in half of the samples to distinguish hydroxylated from non-hydroxylated fatty acids. Fatty acids were analyzed by an Agilent 7890A GC system with an HP88 capillary column. Peaks were identified by comparison with FA standards. The arrow in panel C indicates the presence of one of the non-hydroxylated peaks present in the OHDHA, but not in control or DHA fed flies. Inset in panel C shows the three chromatograms overlapped in the amplified region were the indicated peak is located.

The group of metabolic derivatives that appear upon treatment with any OHFA accounted for *ca.*4 mol% of all fatty acids and they might be involved in the differential effects of hydroxylated FAs compared to other FAs used in the present study. The OHFAs increased the lifespan of flies and improved the results of the climbing test compared to non-hydroxylated FA treatment. This suggests that either OHFAs or their metabolic derivatives are involved in the restoration of cognitive abilities and other parameters related to health. Thus, the analysis of these lipid derivatives could be useful in the treatment of neurodegenerative disorders such as Alzheimer's or Parkinson's disease.

DISCUSSION

During the first years of the XXth century, Dr. Alois Alzheimer discovered and described a neuropathological process that was named after him, Alzheimer's disease (AD). Despite the current relevant knowledge about the molecular bases underlying AD, there are no plausible therapies to treat this condition. NMDA channel blockers and acetylcholinesterase inhibitors constitute the current therapies for patients with AD but these drugs only modestly delay the neurodegenerative process for a limited period of time. Similarly, the omega-3 fatty acid, DHA, also delays AD progression for some time.

Alzheimer's disease (AD) is the most common form of dementia, and one of the principal causes leading to death around the world (Dominguez et al., 2011b). There is robust evidence that genetic factors trigger development of early-onset AD (EOAD), but the cause of late-onset AD (LOAD) remains unclear. Yet, LOAD accounts for over 90% of AD cases (Bertram and Tanzi, 2012).

Many neurodegenerative diseases, including Alzheimer's disease, share in common the accumulation of toxic proteins and a late age of onset (Mattson and Magnus, 2006). However, the mechanisms linking protein aggregation and the onset of disease symptoms are not fully understood. Aging may either be necessary for the accumulation of damaged proteins to cause neuropathology or increase the vulnerability of neurons to protein toxicity. In fact, the presence of senile plaques has been detected in brains of humans who have not developed dementia or neurodegeneration, arguing against the involvement of amyloid peptides in the etiology of AD (Serrano-Pozo et al., 2011).

This fact and the late onset of AD suggest that other pathophysiological processes might be involved in the development of this condition. A decrease in the levels of DHA has been associated with neurodegeneration, suggesting that neuron lipids are crucial for the development of age-related cognitive decline (Astarita et al., 2010; Fraser et al., 2010).

Transgenic model systems can help understand the cellular and molecular bases of complex disorders. Numerous model organisms such as bacteria, nematodes (*C. elegans*), arthropods (*Drosophila*), fish (*Dario renio*), rodents (*Mus musculus*) as well as non-human primates (*Rhesus monkeys*) are used to study neurodegenerative diseases (Gama Sosa et al., 2012).

An ideal model would allow to understand the biology of the disease in a relatively short span of time. A major advantage of model organisms is that they can be used for answering fundamentally unbiased and unconstrained questions. Most of the AD transgenic models utilize the disease driven approach and are based on the amyloid hypothesis, which argues that amyloid deposits are the initiating factor for pathogenesis of AD. Although transgenic mice models engineered to overexpress mutant forms of genes associated with AD are very

useful, they are expensive and time consuming (Kawarabayashi et al., 2001; Oakley et al., 2006; Oddo et al., 2003b).

Before the inception of these models, it was difficult to establish a robust correlation between amyloid plaque load, neurodegeneration, and cognitive decline in AD patients, but with recent advances in technology and *ante mortem* Aβ detection tools (Cohen et al., 2012). It is known that Aβ deposition frequently precedes neuronal degeneration and cognitive decline (Jack et al., 2013; Jack et al., 2012). Additionally, it is also been seen that amyloid deposition occurs at a slower rate while neurodegeneration accelerates (Jack et al., 2009).

Drosophila melanogaster has emerged as an excellent invertebrate model system for studying human neurodegenerative diseases like AD (Chakraborty et al., 2011; Gama Sosa et al., 2012; Jackson et al., 1998; Mhatre et al., 2013). Orthologues of AD-related genes are endogenously expressed in *Drosophila* (Allinson et al., 2003; Carmine-Simmen et al., 2009; Francis et al., 2002; Rosen et al., 1989), and are well studied. Moreover, it is shown that the human form of APP can be proteolytically processed by endogenous fly secretases.

These evolutionarily conserved functions make *Drosophila* an attractive model to study AD. Most of the fly AD models rely either on expression of the toxic Aβ42 peptide in the developing eye (Finelli et al., 2004; Greeve et al., 2004; Guo et al., 2003) or in the nervous system (Iijima et al., 2004; Sofola et al., 2010) or in wings (Fossgreen et al., 1998). Other fly models express human APP and BACE ubiquitously (Greeve et al., 2004).

In order to study AD, it is of great importance to develop models that recapitulate AD like phenotypes with age being the primary factor. A previously published adult-onset AD fly model used a GeneSwitch induction system (Sofola et al., 2010). It has been successfully characterized an adult fly model for AD (Chakraborty et al., 2011). To modulate the expression of transgenes it was used the temperature-dependent GAL4/UAS system (Duffy, 2002). *Drosophila* model system was used to study the motor behavior, lifespan and senile plaque formation in flies expressing Aβ42 and Tau. It was showed that expression of human Aβ42 and human Tau in *Drosophila* gives rise to motor deficits and reduced lifespan. Therefore, this animal model of AD proved to reproduce similar symptoms to those of human AD.

In addition, the short life span of these flies allows assessment of cognitive features and overall survival of flies treated with vehicle or several compounds at different doses for a life time. The use of other models (e.g., transgenic mice) to carry out the research here shown, would have taken longer time of research. As described elsewhere for *Drosophila* models of AD, this study shows interesting cellular similarities between the alterations observed in the fly and the pathologies described in mice models of AD and in the brains of patients with this condition.

Furthermore, it was confirmed the expression of the amyloid peptide in *Drosophila* along with progressive neuronal loss and reduced longevity. This neurodegeneration process is characteristic of patients of AD, but it is rarely seen in mice models. In addition, this work observed in *Drosophila* what it had already been previously demonstrated both in patients and transgenic mice (Coleman et al., 2004; Heinonen et al., 1995; Masliah and Terry, 1993; Oddo et al., 2003a; Selkoe, 2002): synaptic degeneration in early stages of the disease following the presence of amyloid peptide.

This neuronal alteration is only observable in adult stages and its severity increases with age. This model of AD was used to investigate a therapeutic strategy based on the use of synthetic lipid analogues that might reduce Aβ toxicity and other cellular or behavioral alterations associated with AD. In our model, it was observed that the expression of the human AD Aβ42 peptide and human Tau protein specifically in the fly's central nervous system. Indeed we were able to see a decline of motor behavioral score and a reduction in the fly's lifespan in association with gradual accrual of Aβ42 and Tau in the central nervous system (CNS). Thus, age appeared to be a key factor in the behavioral and neuroanatomical phenotypes that were observe in this animal model of AD. In the present study, it was showed that the AD phenotypes could be pharmacologically reversed with novel synthetic lipids and it was also demonstrated the utility of this fly model for potential AD drug testing. This pioneering work in *Drosophila* must be validated in mammalian model systems and could hopefully contribute to the development of effective treatments against neurodegeneration. The lifespan and climbing behavior in AD *Drosophila* flies was markedly and significantly increased after treatments with some of the compounds added to food at a final concentration 30 or 100 μg/ml (Ford et al., 2007).

It was found that the carrier 33771 and activator 8760 fly strains, which do not express the Aβ42 and human proteins Tau, constituted a good negative control for F1 flies. Moreover, treatments with 30 or 100 μg/ml of OHDHA, OHARA and OHEPA, and the mixture of OHARA+OHDHA and the mixture of OHARA+OHEPA, as well as treatments with 30 μg/ml of all TGMs, LP183A1, LP183A2 and DhaLifort did not show apparent toxic effects. Note that 30 or 100 μg/ml were the best concentrations to improve the life span in control flies (F1). In addition, the dose of 100 μg/ml increase expectancy of the F1 treated with OHDHA in 10 days.

The same molecules at the same doses that caused longer life span without apparent toxicity also improved the motor behavior function in *Drosophila* with AD. Although once again it has to be emphasized that the effects of the hydroxylated forms were significantly higher than those of the natural lipids in terms of life expectancy and cognitive score

improvements. This improvement could be due to an enhancement of neurogenesis or to neuroprotective effects or both, as described in other studies for OHDHA using mouse models (Fiol-deRoque et al., 2013).

One might think that this cognitive improvement would be accompanied by a decrease in levels Aβ42 peptide detected. This was not the case, possibly because this transgenic model is designed to overexpress the 42 amino acid peptide directly (without the processing of the precursor). As discussed in the Introduction section, in humans the Aβ peptide is not expressed, but it is produced from the processing amyloid precursor protein (APP) which is cleaved by secretase to yield the Aβ42 peptide.

In principle, it would be possible that the administered drug would affect the amount of the peptide produced in *Drosophila* (Prussing et al., 2013). However, since this fly model is based on the insertion of a gene constantly overexpressed by the activation of an exogenous transcription factor (Gal4), it would be unlikely that the treatments would reduce the overall levels of Aβ42 peptide.

The treatment could modulate clearance of accumulated peptide in the CNS. However, the results do not support this hypothesis because it was not found significant differences between different treatments and F1 controls. In fact, it was no changes detected in expression of Aβ (in the protein or mRNA). Although this fact argues against the relevance of amyloid peptides in AD etiology, there are other reduce the intracellular amyloid load, as described for OHDHA in mice with AD (Torres et al., 2014). In any case, all the potential explanations suggest that Aβ42 peptide production is a downstream event that follows earlier events that would trigger the neurodegenerative process.

Membrane lipid alteration could be the upstream event that may cause later alterations of APP, Tau, inflammation, and eventually neuronal death and cognitive decline. Therefore, therapeutic approaches based on the use of lipids could be an efficacious alternative to treat this devastating condition. Treatments with polyunsaturated lipids and especially with their hydroxylated forms alternative explanations that would justify these findings. As previously seen, OHDHA (and possibly other synthetic lipids) reduces the binding of Aβ42 peptide aggregates (fibrils) to membranes, so that it would reduce the effects ensuing formation of senile plaques (e.g., tau protein phosphorylation (Torres et al., 2014).

It could also be possible that the food supplements/drugs used in the present study could (mainly at the dose of 30 or 100 μg/ml) showed to be therapeutically effective, improving cognitive status and longevity in F1 flies. Regarding the levels of total and phosphorylated Tau, we could only detect total Tau levels. It would be expected that a model like this, which overexpresses Aβ peptide and Tau, would also show increased levels of Tau protein

phosphorylation (Zheng et al., 2002). Nevertheless, we could not detect Ser202 or Thr205 phosphorylation, using specific antibodies for the phosphorylated epitopes (CP13 and AT8 clones, Material and Methods section, Western blotting for Tau).

In principle, the undetectable levels of phospho-Tau could be originated by one of the following reasons. It could be because the expression levels of human Aβ42 are not high enough to induce Tau hyperphosphorylation. Furthermore, it could be possible that the phosphorylated residues (and/or the sequence in which different residues are phosphorylated) were different in human and in *Drosophila*.

Total Tau levels detected in F1 are higher compared to the parental strains, because the transgene is expressed at lower extent under the UAS promoter control. Also the Aβ42 expression levels are very different between parental strain and F1 flies because the parental strain do not produce endogenous Aβ42 (Prussing et al., 2013), and therefore it is not expected that the endogenous levels of protein interfere with the detection, as it was observed for Tau (Heidary and Fortini, 2001). Nevertheless, it is possible that same non-detectable Aβ42 expression occurred in parental strain flies because this peptide and Tau expression were under control of the same promoter. This could explain the same differences between the present study and other studies using models less demanding than this model (Marcora et al., 2014).

The F1 flies have shown important differences in longevity compared to the parental healthy carrier 33771, suggesting that the modest differences in Tau expression between the parental strains, 33771, 8760, and F1 justify the pathological status. In addition, the present data indicate that *Drosophila* has a protein homologous to human Tau (Heidary and Fortini, 2001). The homology between the endogenous fly tau and the human counterpart occurs in the Tau46 antigen domain (anti total tau; Material and Methods section Western blotting for Tau) (Wittmann et al., 2001).

The studied carried out indicates Tau protein detected in F1 flies by WB is combination of endogenous and human Tau protein. This cross reactivity could be prevented in the future using other antibodies with different immunoreactivity against the human and the *Drosophila* Tau protein homologs (possibly Alz50, MC1 or 12E8 clones (Wittmann et al., 2001). On the other hand, other authors have used the AT8 antibody to detect phosphorylated Tau (as in this work), but in a model that also transfers a mini-gene for human GSK3 kinase into the *Drosophila* genome, perhaps suggesting that phosphorylation carried out by endogenous *Drosophila* kinases do not follow the same pattern as human kinases, which prevented the detection of the phosphorylated Tau epitope in the present

study. However, there are studies using similar models where phosphorylated Tau was detected using the AT8 antibody (Iijima et al., 2010).

The latter findings support the hypothesis that expression levels of the human genes inserted in the *Drosophila* genome is not sufficient to induce Tau hyperphosphorylation. The possibility that expression levels of human inserts in our model are insufficient to induce Tau hyperphosphorylation is supported by the relatively modest overexpression levels observed in F1 flies. In these flies, Tau expression levels detected were about twice those observed in the parental healthy carrier 33771 (Figure 32).

Lipid analysis from heads of *Drosophila melanogaster* revealed that *Drosophila* does not possess the ability to synthesize ARA, EPA and DHA. However, they have the catabolic machinery for shortening of DHA into EPA, because treatments with DHA caused increased in EPA levels in the fly head. By contrast, food supplementation with 100 µg/ml of OHARA, OHEPA and OHDHA did not induce their presence in the head of flies, which suggest that *Drosophila* possesses the enzyme machinery necessary for a rapid metabolization of these hydroxylated compounds.

Furthermore it was found that the amount of short chain fatty acids (SCFA), from the heads of F1 treated with ARA, EPA and HAD was less than that from untreated F1 flies. Concerning the hydroxylated fatty acids, the reduction in the levels of short chain fatty acid (SCFA) was similar to that of the non-hydroxylated fatty acids.

The fact that OHFAs increased the lifespan of flies and improved the results of the climbing test obtained upon non-hydroxylated FA treatment, suggests that either OHFAs or their metabolic derivatives are involved in the restoration of cognitive abilities. Thus, the future analysis of these lipid species could be useful to design and produce molecules with potential interest in the treatment of neurodegenerative disorders, such as Alzheimer's or Parkinson's disease.

APPENDIX

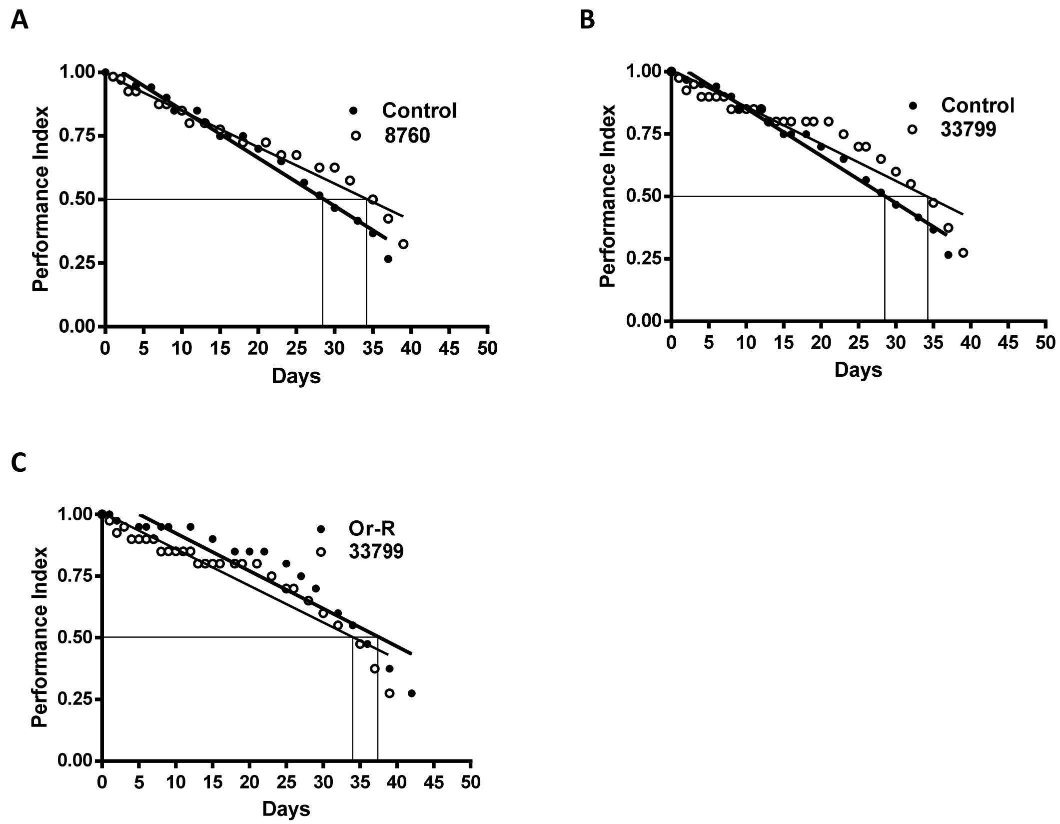


Figure 36. Climbing assay of wild type (Or-R), carrier 33799 and activator 8760.

Table11. Climbing assay of wild type (Or-R), carrier 33799 and activator 8760.

	Median Climbing (days)
Control	28.5
Or-R	37.6
33799	34.4
8760	34.3

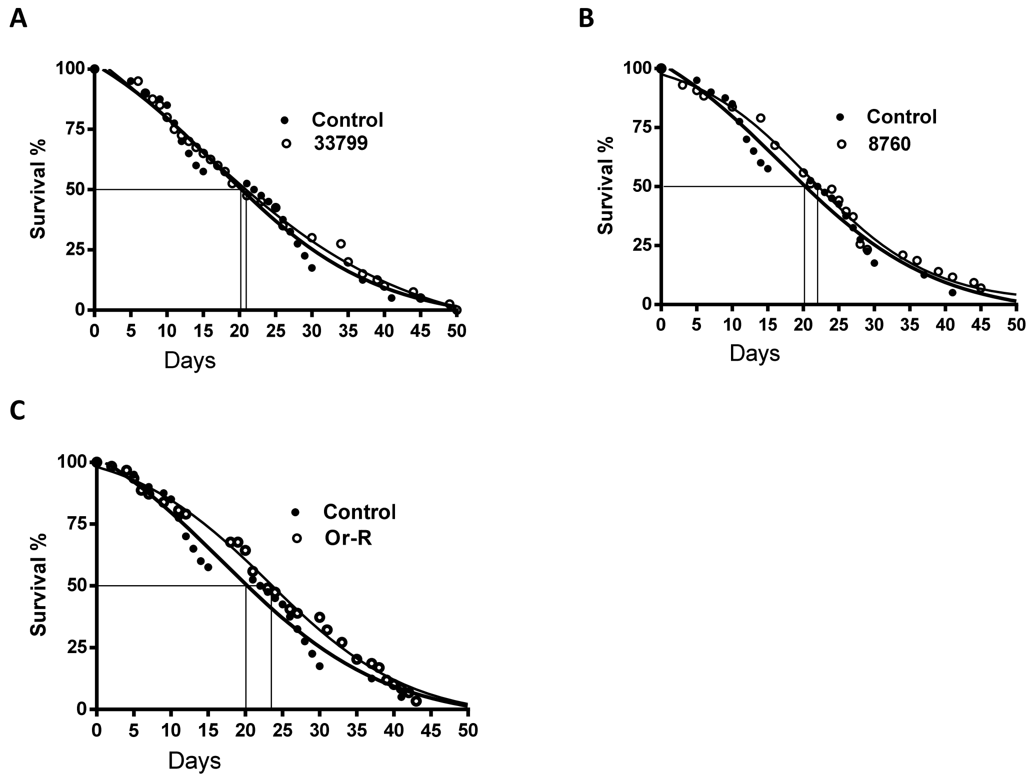
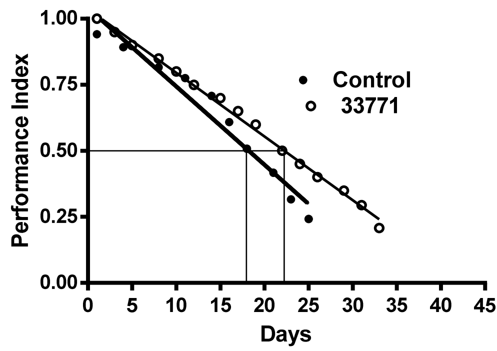


Figure 37. Survival assay. Panel A to C, wild type (Or-R), carrier 33799 and activator 8760.

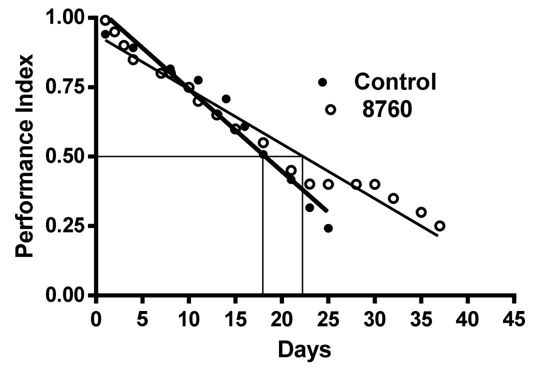
Table 12. Survival assay. Panel A to C, wild type (Or-R), carrier 33799 and activator 8760.

	Median Survival (days)
Control	20.3
Or-R	23.6
33799	21
8760	22

A



B



C

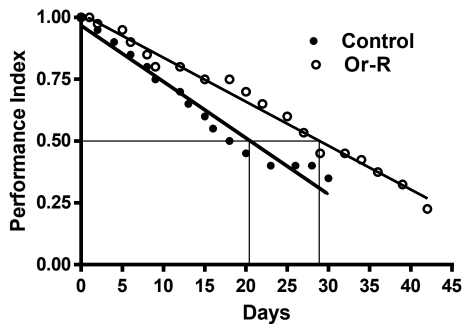


Figure 38. Climbing assay of wild type (Or-R), carrier 33771 and activator 8760.

Table 13. Climbing assay of wild type (Or-R), carrier 33771 and activator 8760.

	Median Climbing (days)
Control	17.9
Or-R	29
33771	22
8760	22.4

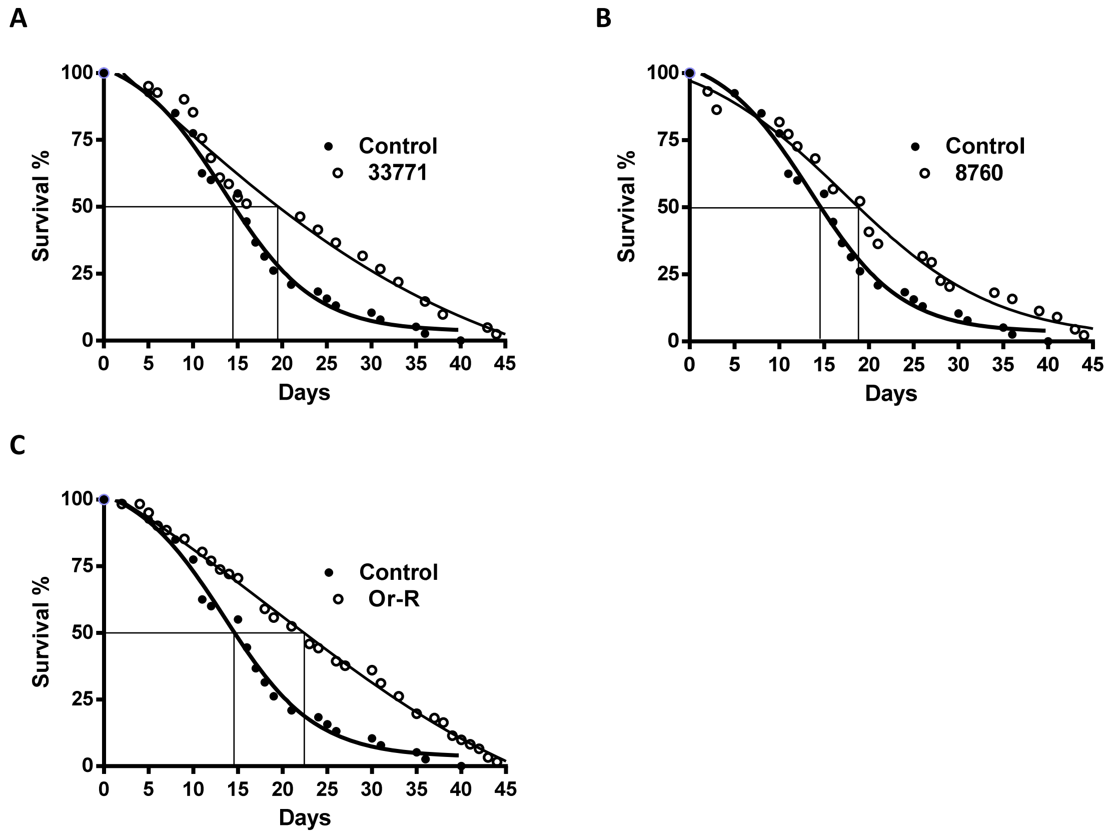


Figure 39. Survival assay. Panel A to C, wild type (Or-R), carrier 33771 and the activator 8760.

Table 14. survival assay of wild type (Or-R), carrier 33771 and the activator 8760.

	Median Survival (days)
Control	14.8
Or-R	22.4
33771	19.4
8760	18.8

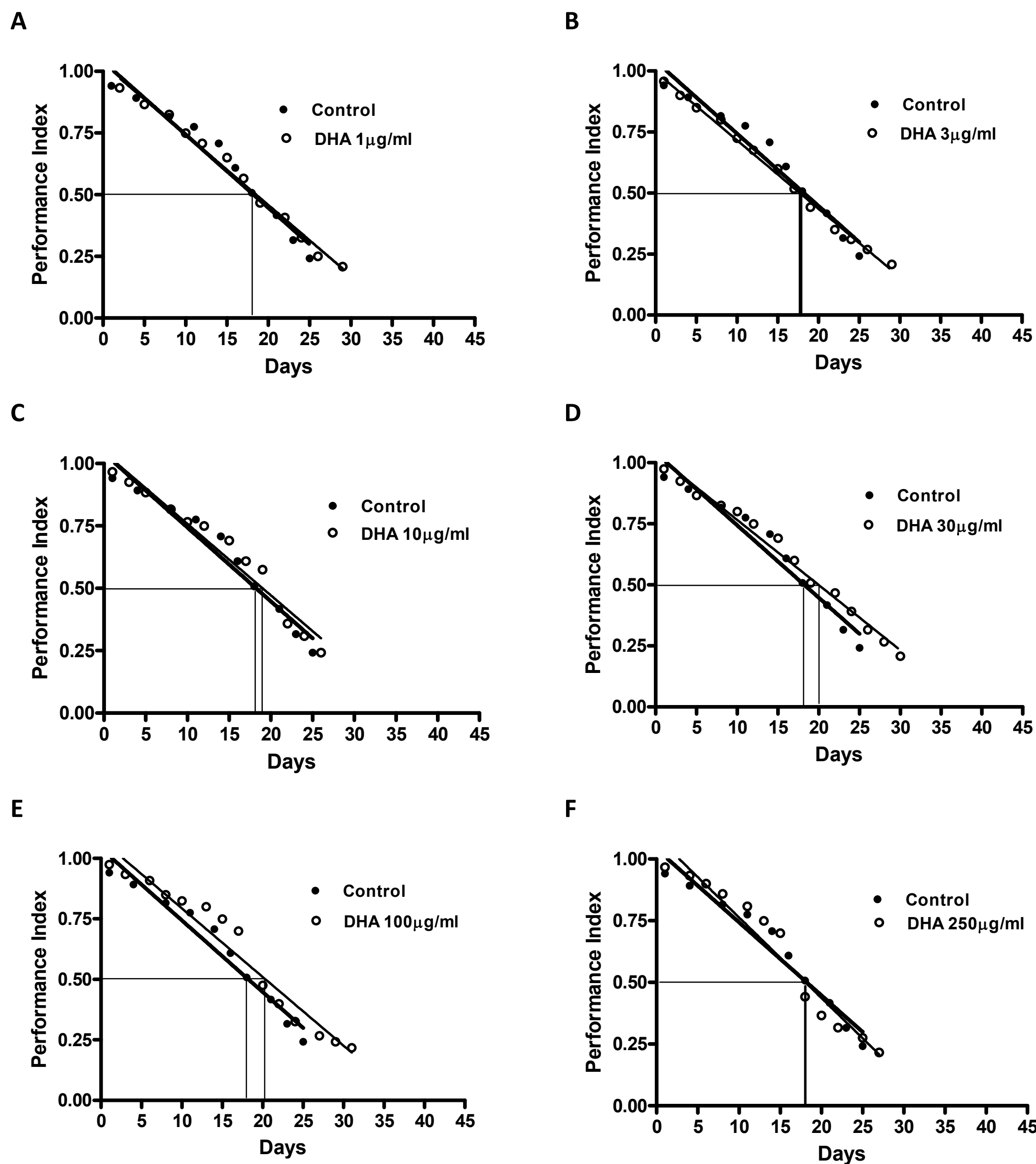


Figure 40. Effect of DHA in climbing assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 μg/ml DHA (Panels A to F, respectively) was added to the food.

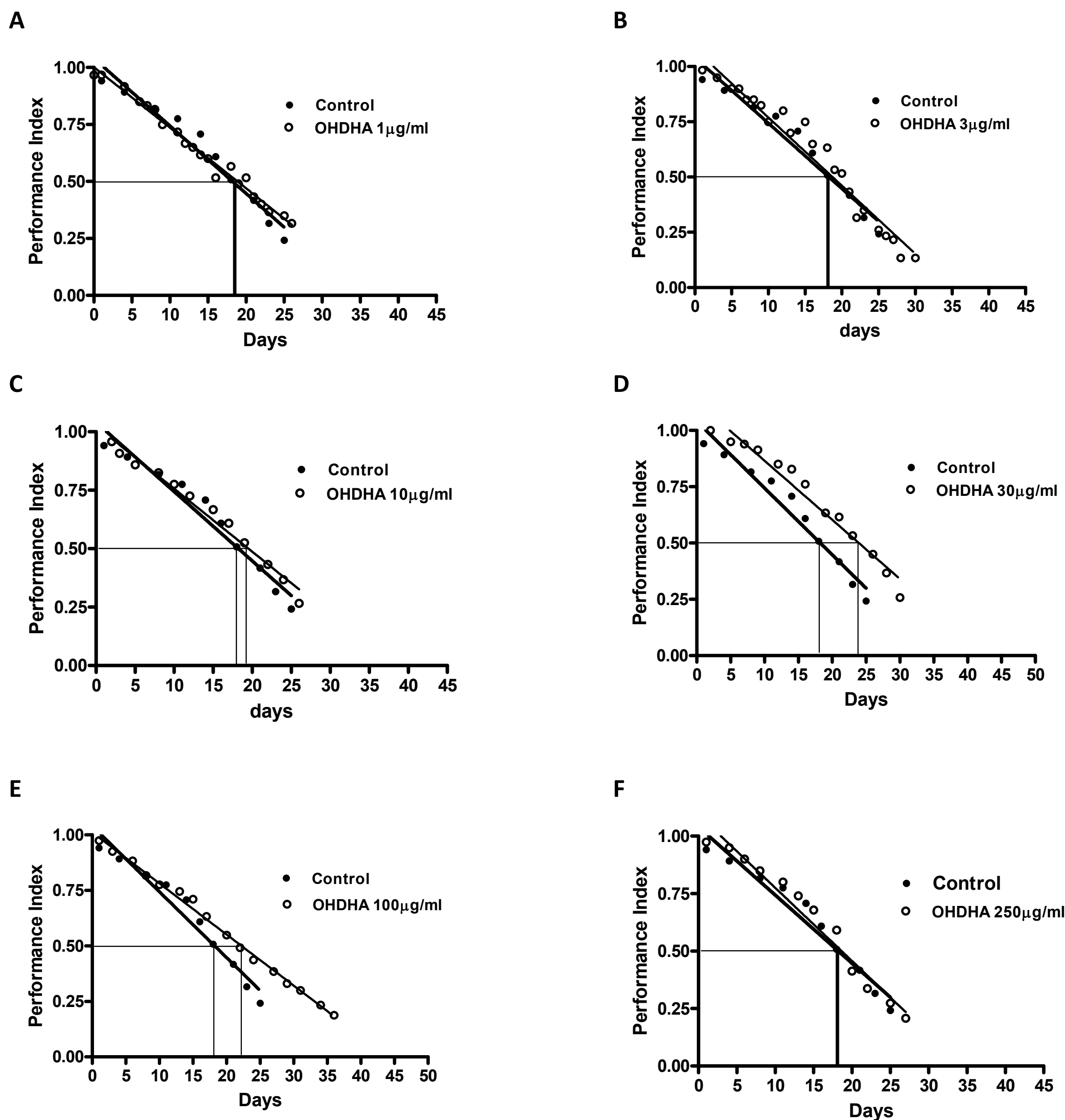


Figure 41. Effect of OHDHA in climbing assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 μg/ml OHDHA (Panels A to F, respectively) was added to the food.

Table 15. Effect of DHA and OHDHA in climbing assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 μg/ml DHA, OHDHA respectively was added to the food.

	Median Climbing (days)	
μg/ml	**DHA**	**OHDHA**
0 (control)	17.5	17.5
1	17.6	17.5
3	17.8	17.6
10	18	18.5
30	19	22.5
100	18.8	21.8
250	17.7	17.4

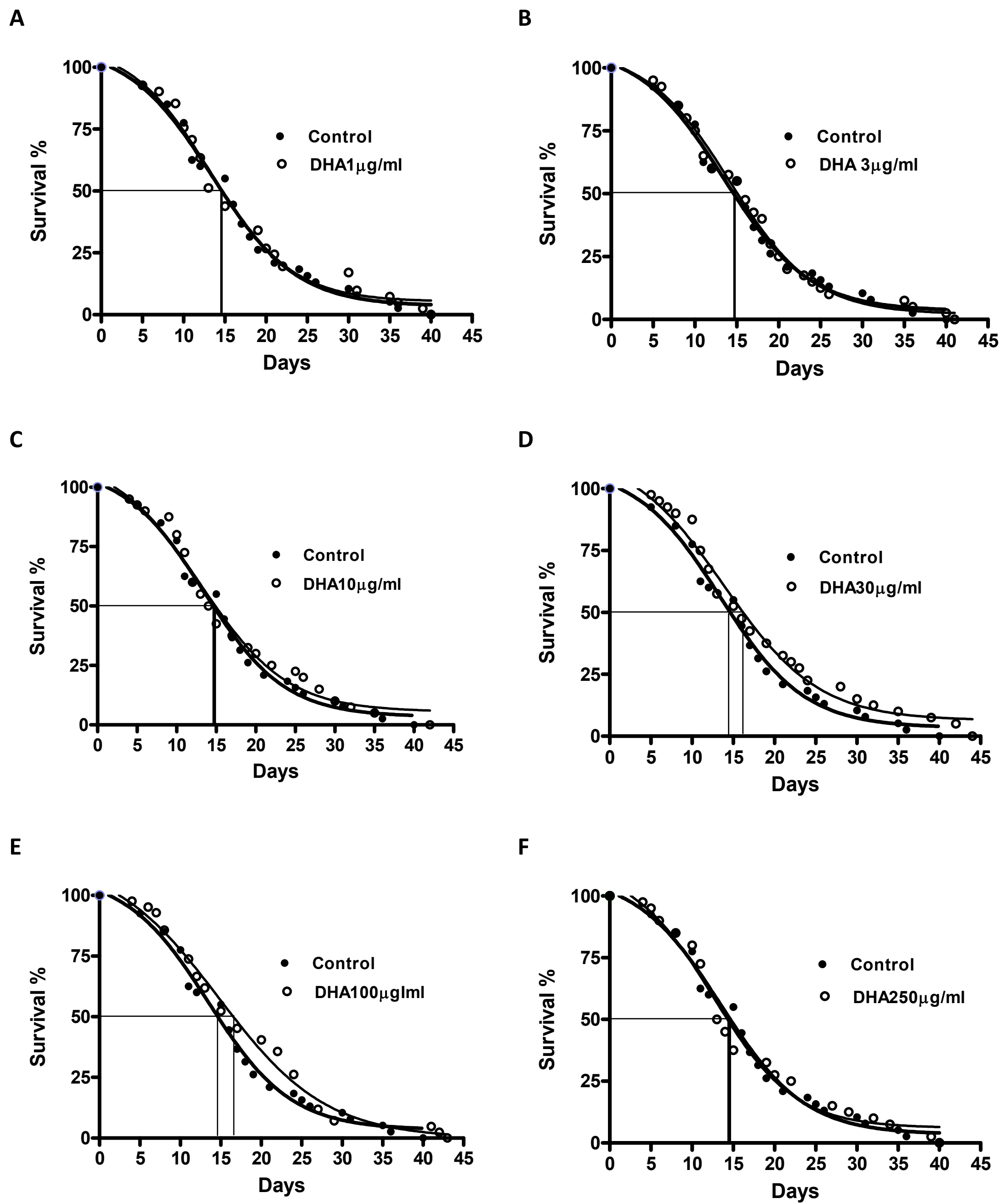


Figure 42. Effect of DHA in survival assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 µg/ml DHA (Panels A to F, respectively) was added to the food.

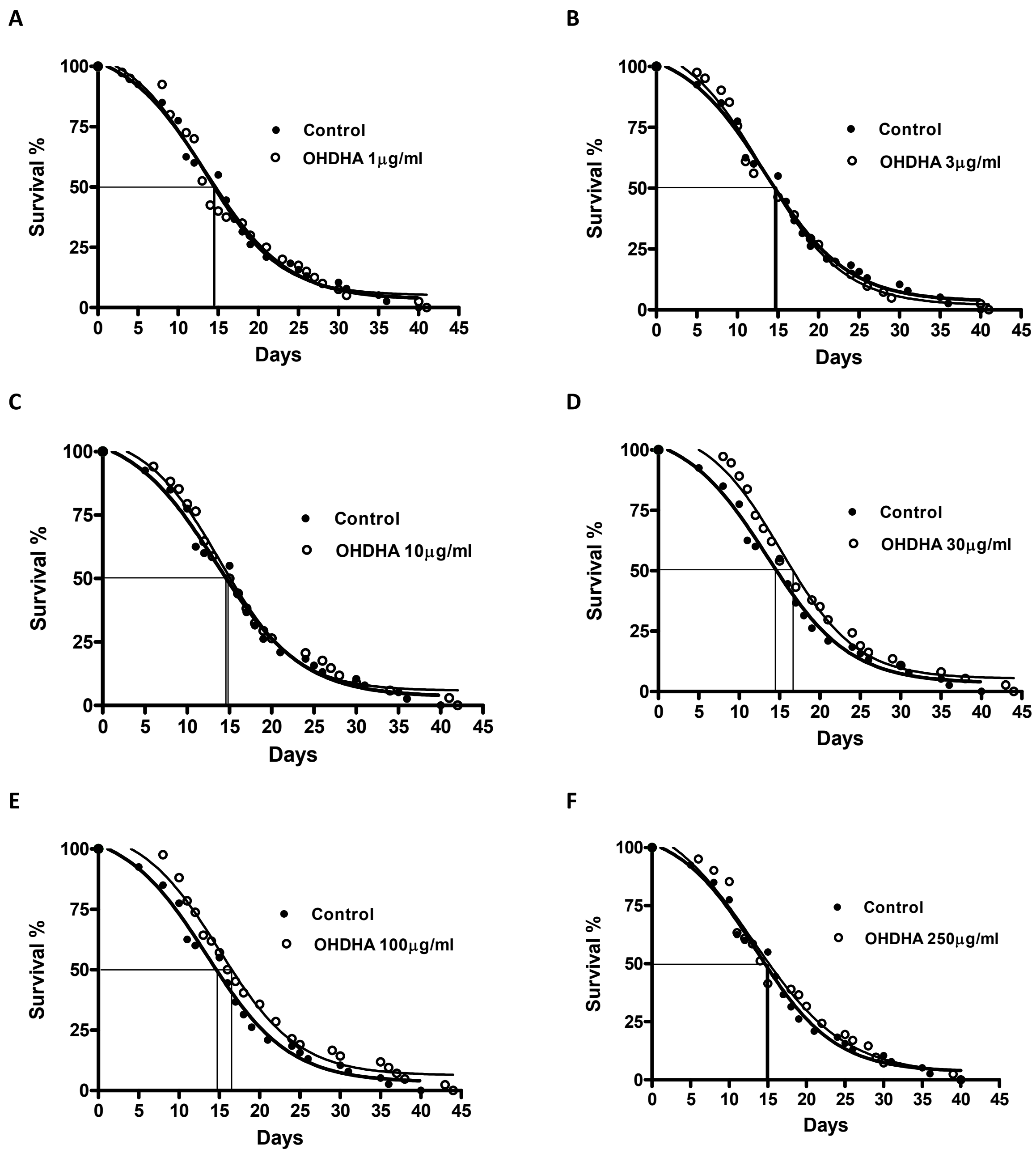


Figure 43. Effect of OHDHA in survival assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 µg/ml OHDHA (Panels A to F, respectively) was added to the food.

Table 16. Effect of DHA and OHDHA in survival assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 µg/ml DH A, OHDHA respectively was added to the food.

	Median Survival (days)	
µg/ml	DHA	OHDHA
0 (control)	14.4	14.4
1	14.4	14.5
3	14.5	14.6
10	14.5	14.8
30	15	16.6
100	15.8	16.8
250	14.5	14.6

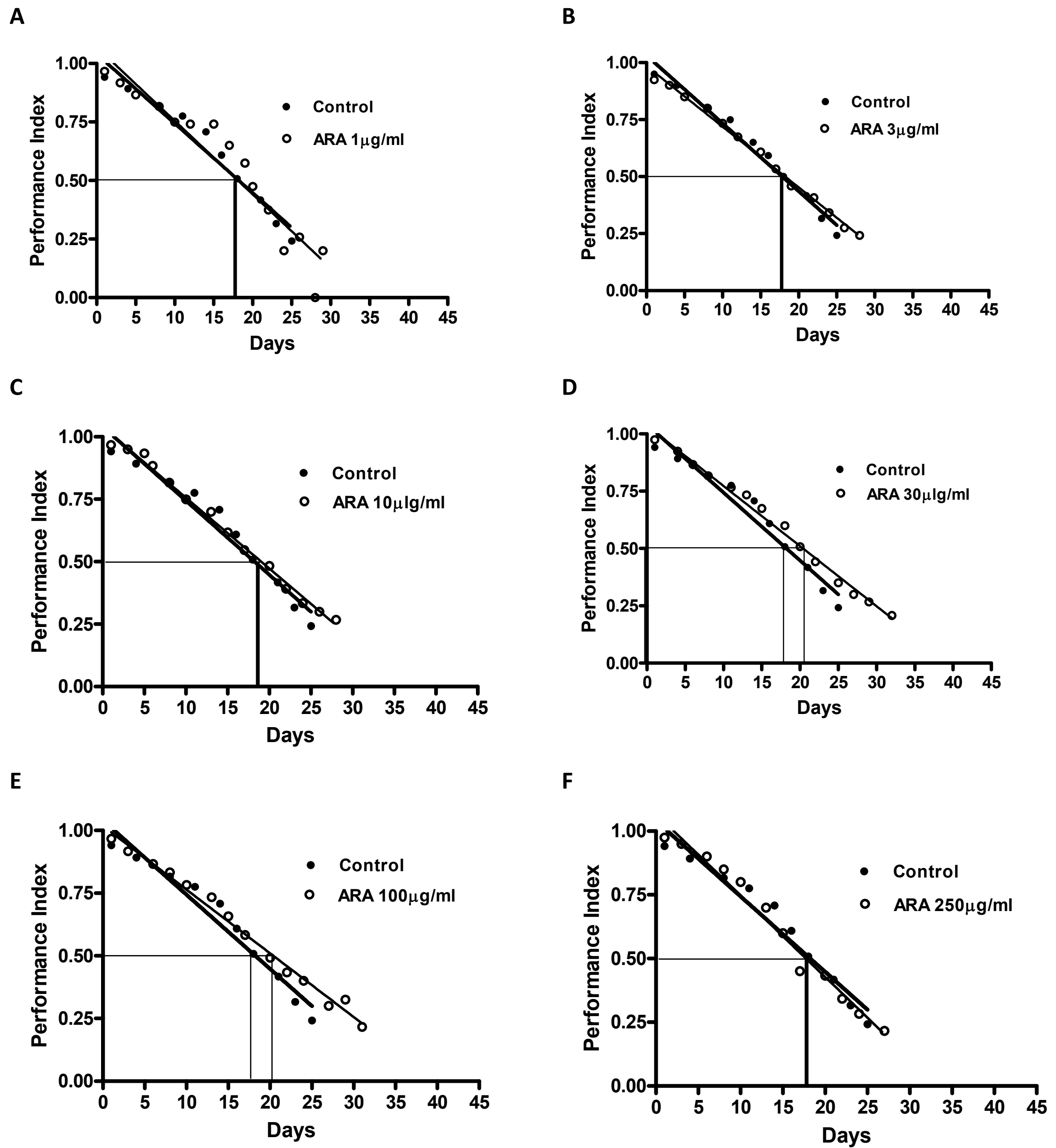


Figure 44. Effect of ARA in climbing assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 µg/ml ARA (Panels A to F, respectively) was added to the food.

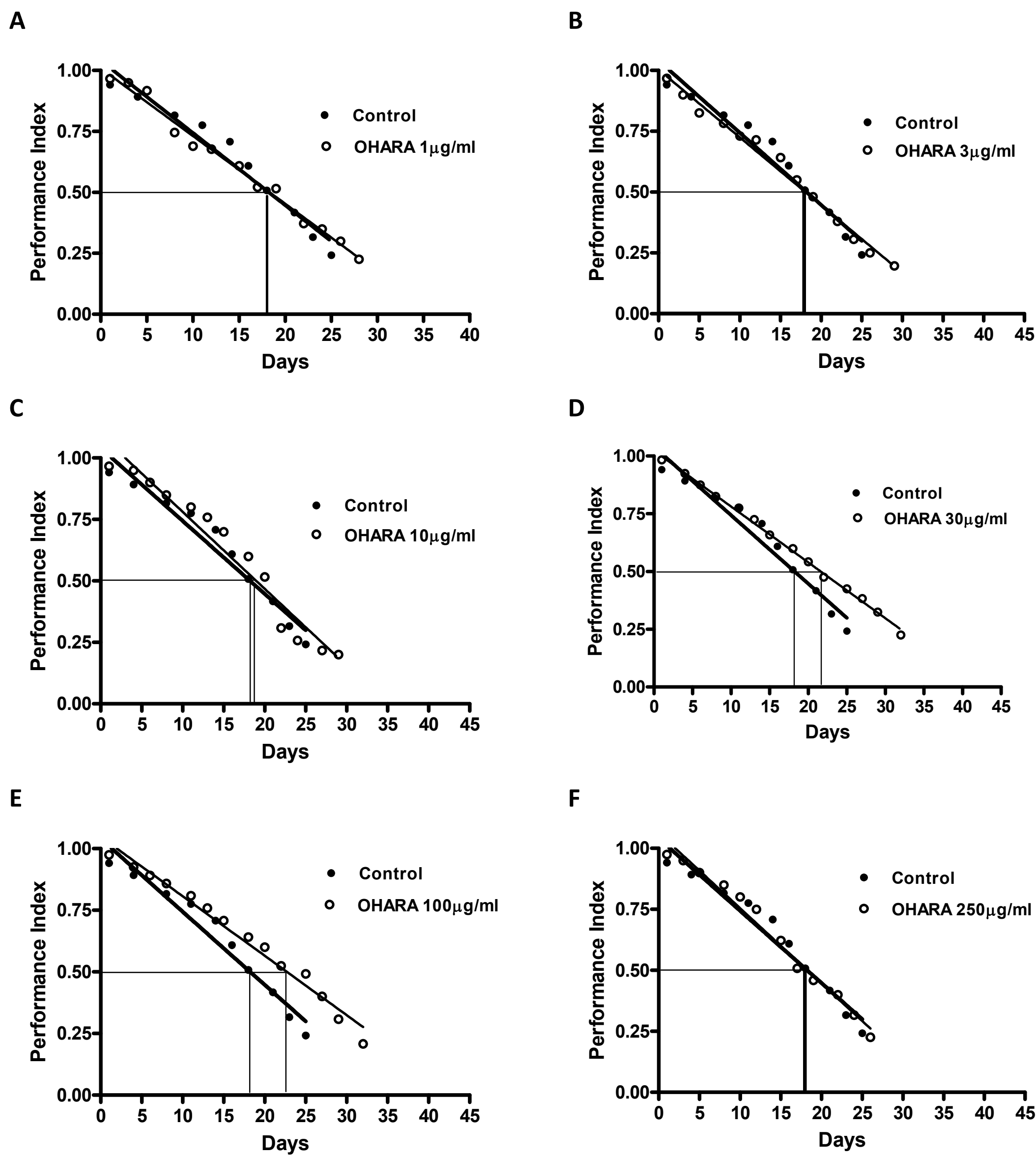


Figure 45. Effect of OHARA in climbing assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250µg/ml OHARA (Panels A to F, respectively) was added to the food.

Table 17.Effect of ARA and OHARA in climbing assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 µg/ml ARA, OHARA respectively was added to the food.

	Median Climbing (days)	
µg/ml	**ARA**	**OHARA**
0 (control)	17.7	17.7
1	17.7	17.8
3	17.8	17.9
10	14.5	14.8
30	18	18.3
100	20.1	22.1
250	17.8	17.9

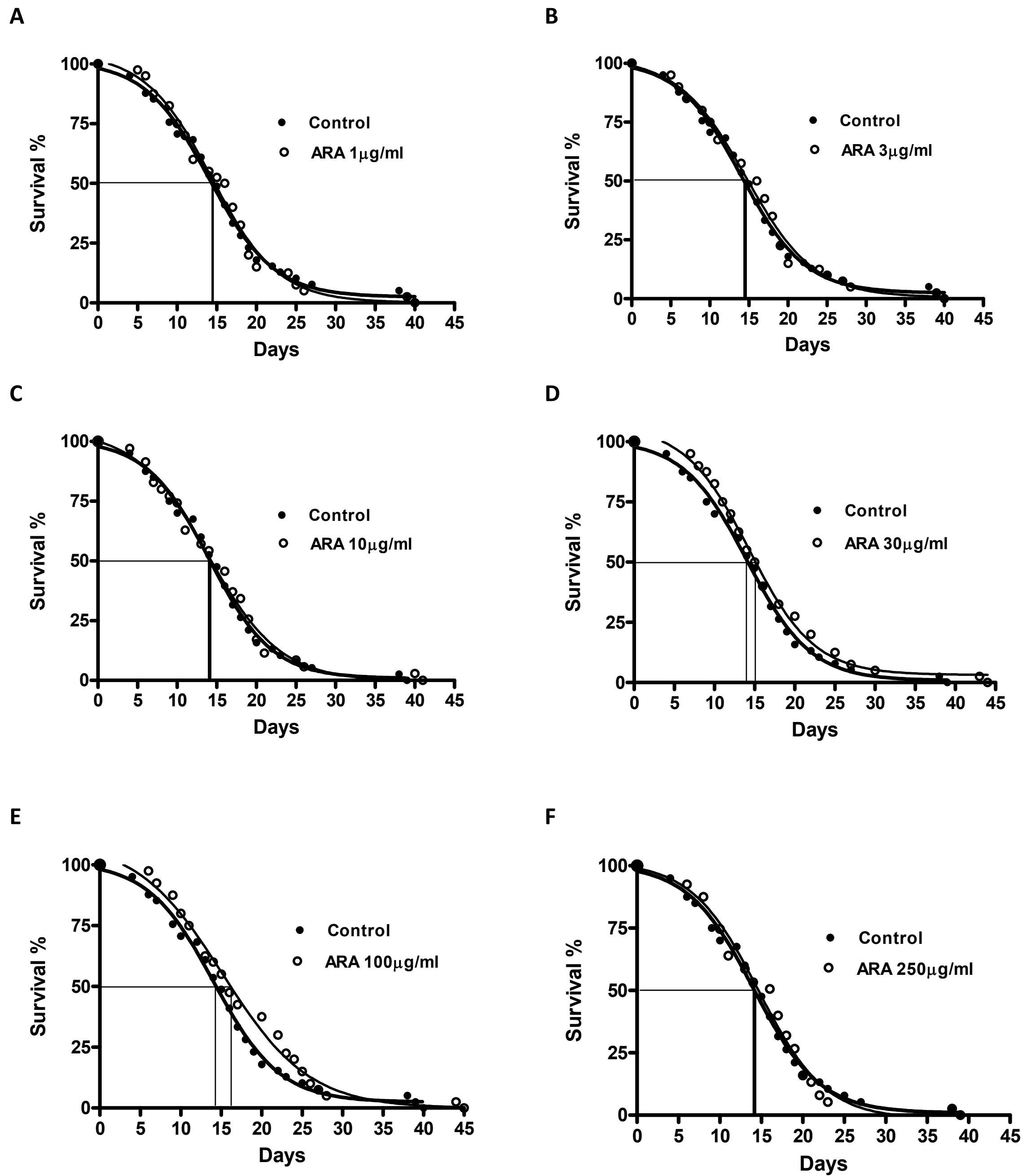


Figure 46. Effect of ARA in survival assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 µg/ml ARA (Panels A to F, respectively) was added to the food.

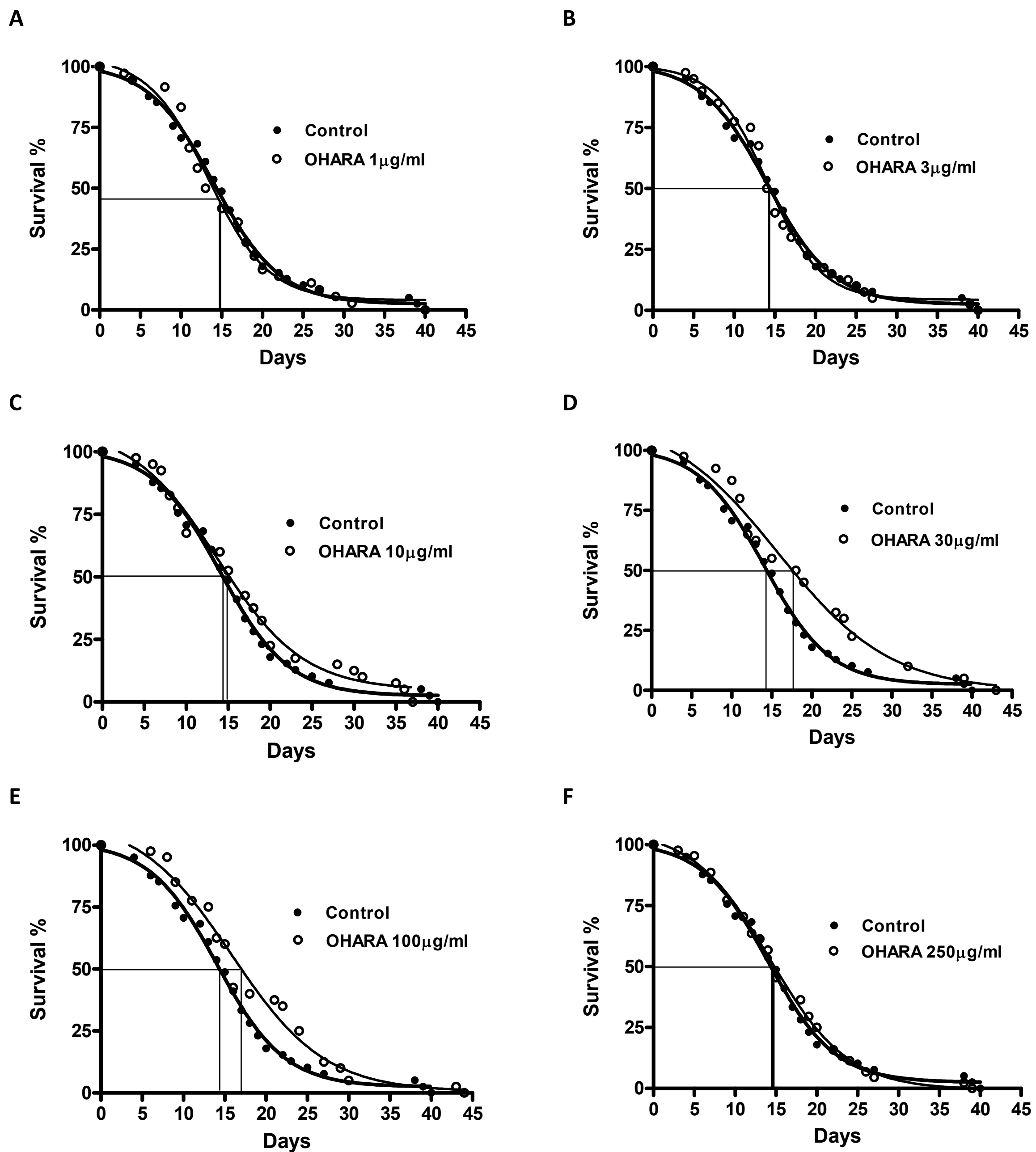


Figure 47. Effect of OHARA in survival assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 μg/ml OHARA (Panels A to F, respectively) was added to the food.

Table 18. Effect of ARA and OHARA in survival assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 μg/ml ARA, OHARA respectively was added to the food.

μg/ml	Median Survival (days) ARA	OHARA
0 (control)	14.5	14.5
1	14.6	14.6
3	14.7	14.8
10	15	15.3
30	16.2	17.6
100	16.1	16.5
250	14.8	15

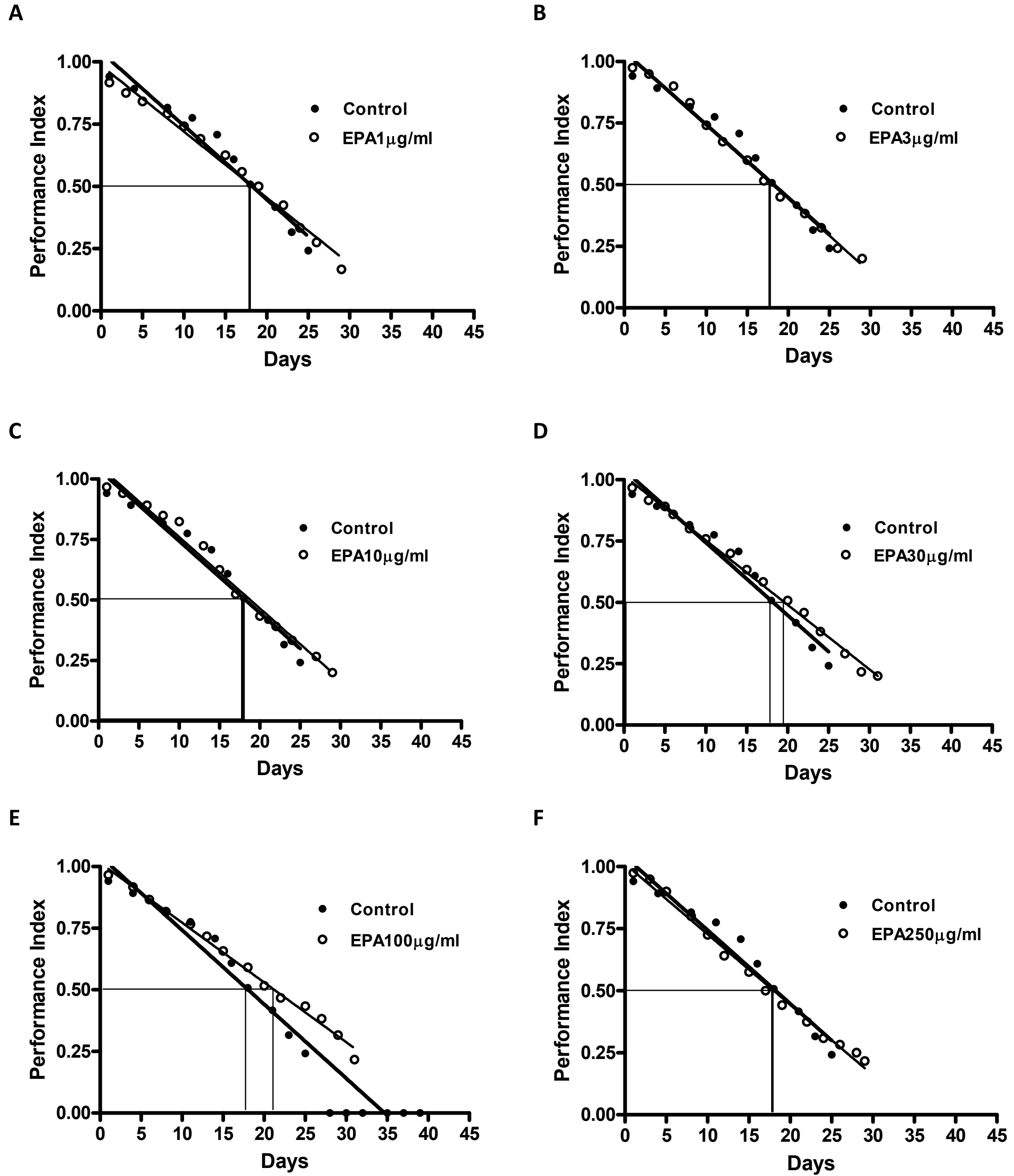


Figure 48. Effect of EPA in climbing assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 µg/ml EPA (Panels A to F, respectively) was added to the food.

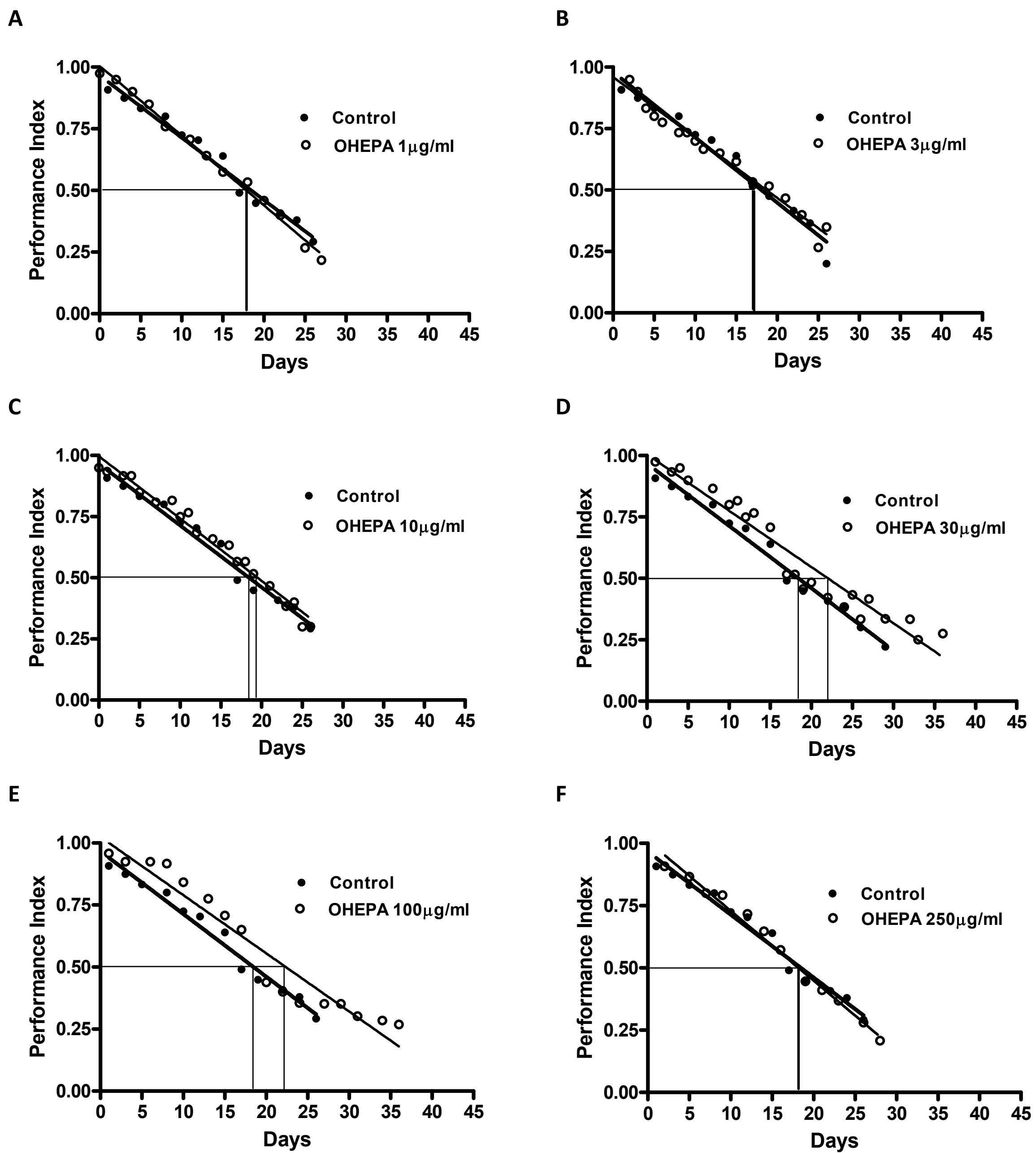


Figure 49. Effect of OHEPA in climbing assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 μg/ml OHEPA (Panels A to F, respectively) was added to the food.

Table 19. Effect of EPA and OHEPA in climbing assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 μg/ml EPA, OHEPA respectively was added to the food.

,	Median Climbing (days)	
μg/ml	**EPA**	**OHEPA**
0 (control)	17.5	17.5
1	17.6	17.7
3	17.7	17.8
10	15	15.3
30	19.4	21.4
100	20.5	22.1
250	17.6	17.8

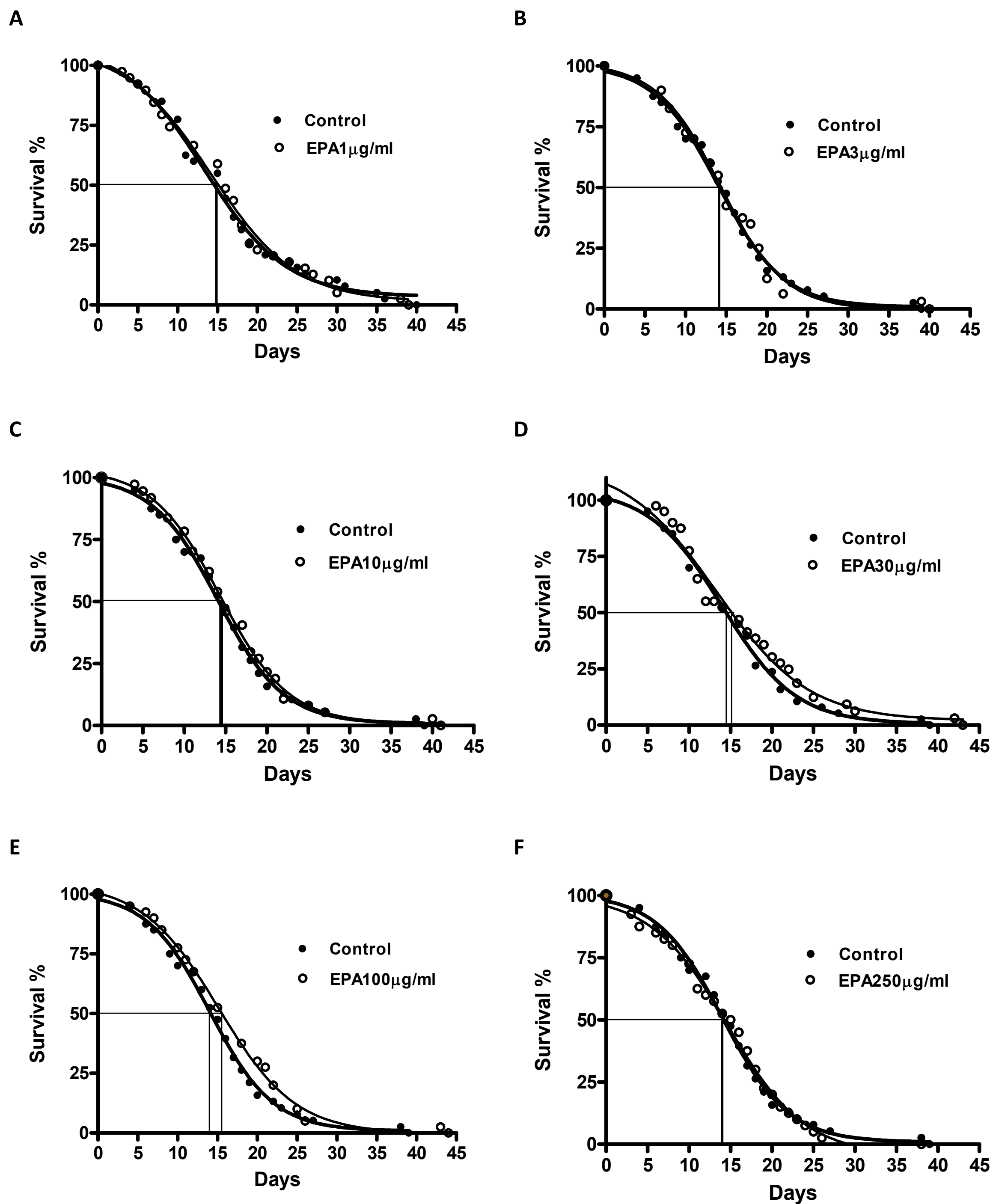


Figure 50. Effect of EPA in survival assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 μg/ml EPA (Panels A to F, respectively) was added to the food.

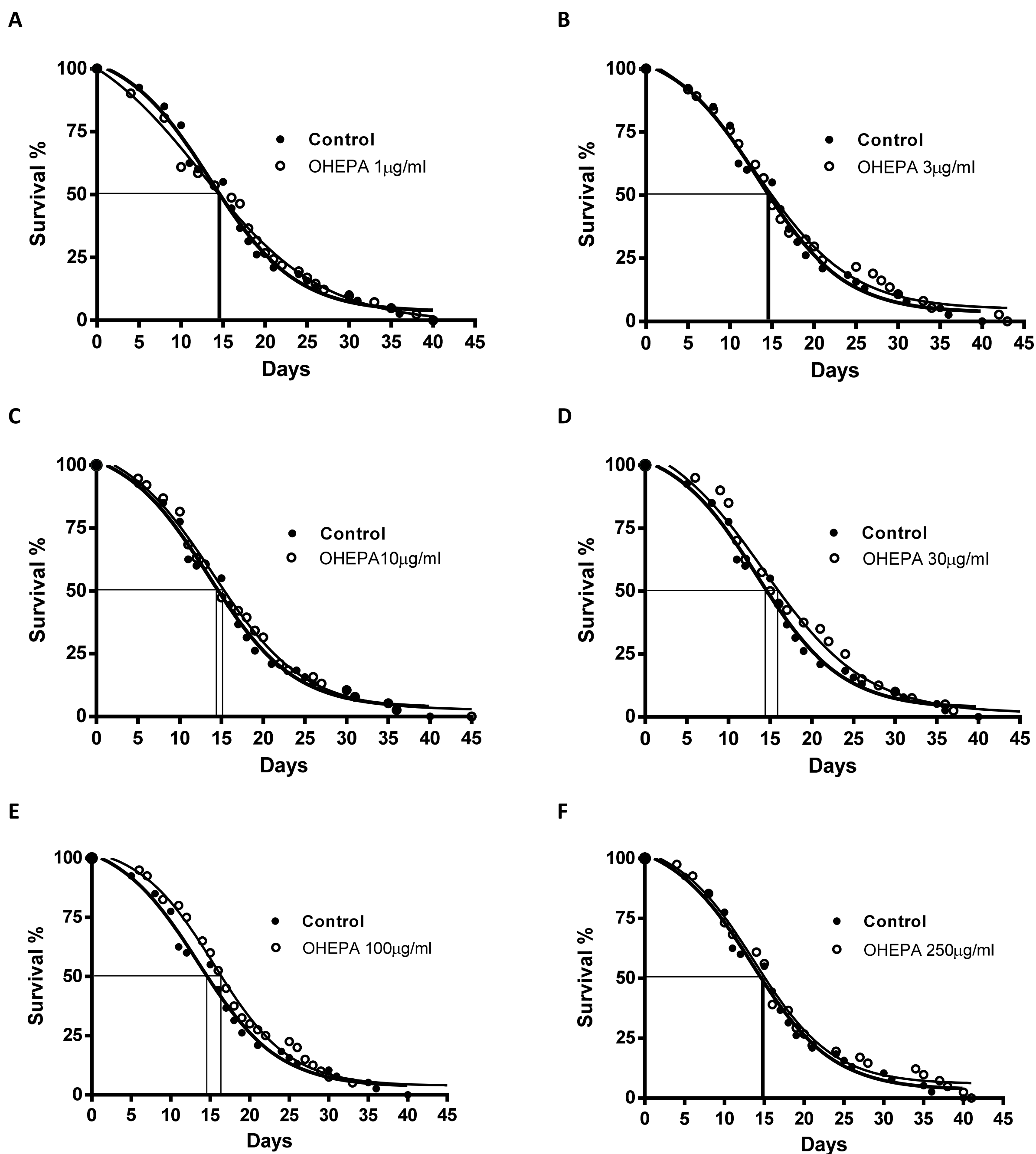


Figure 51. Effect of OHEPA in survival assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 µg/ml OHEPA (Panels A to F, respectively) was added to the food.

Table 20. Effect of EPA and OHEPA in survival assay of F1 transgenic *Drosophila*. 1, 3, 10, 30, 100, 250 µg/ml EPA, HEPA respectively was added to the food.

	Median Survival (days)	
µg/ml	**EPA**	**OHEPA**
0 (control)	14.5	14.5
1	14.6	14.7
3	14.7	14.8
10	14.9	15.3
30	16.4	16.9
100	16.6	17.7
250	14.7	14.9

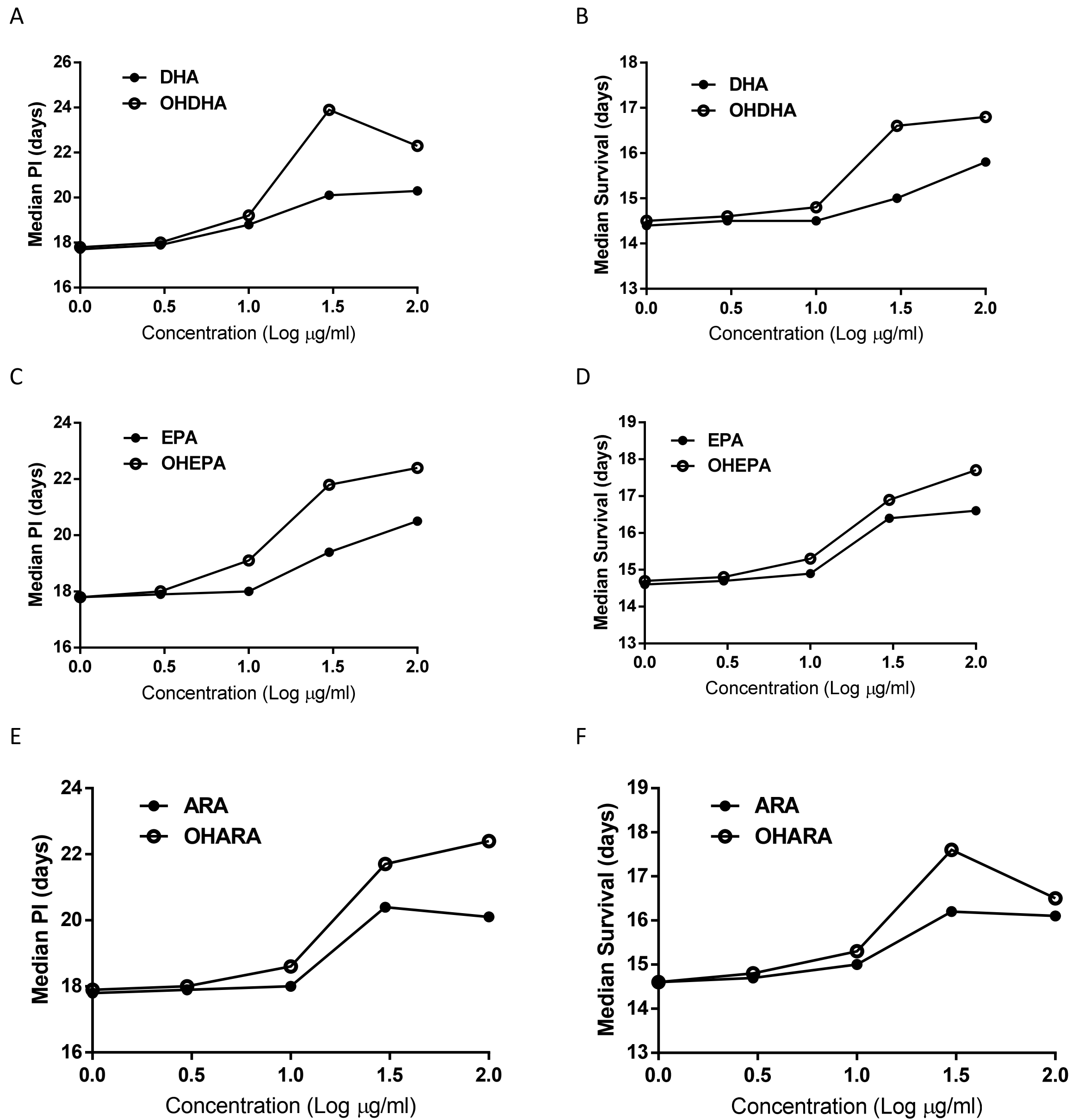


Figure 52. ED50 for polyunsaturated fatty acid of F1 transgenic Drosophila. Median locomotor performance index plots (panels A, C, and E) and median survival plots (panels B, D, and F) of F1 flies fed with DHA, OHDHA, EPA, OHEPA, ARA, or OHARA, as indicated in the graphs. In general, food supplementation with hydroxylated acids displayed better effectivity compared to their non-hydroxylated fatty acid forms in terms of increasing the locomotor performance, as well as the life-span.

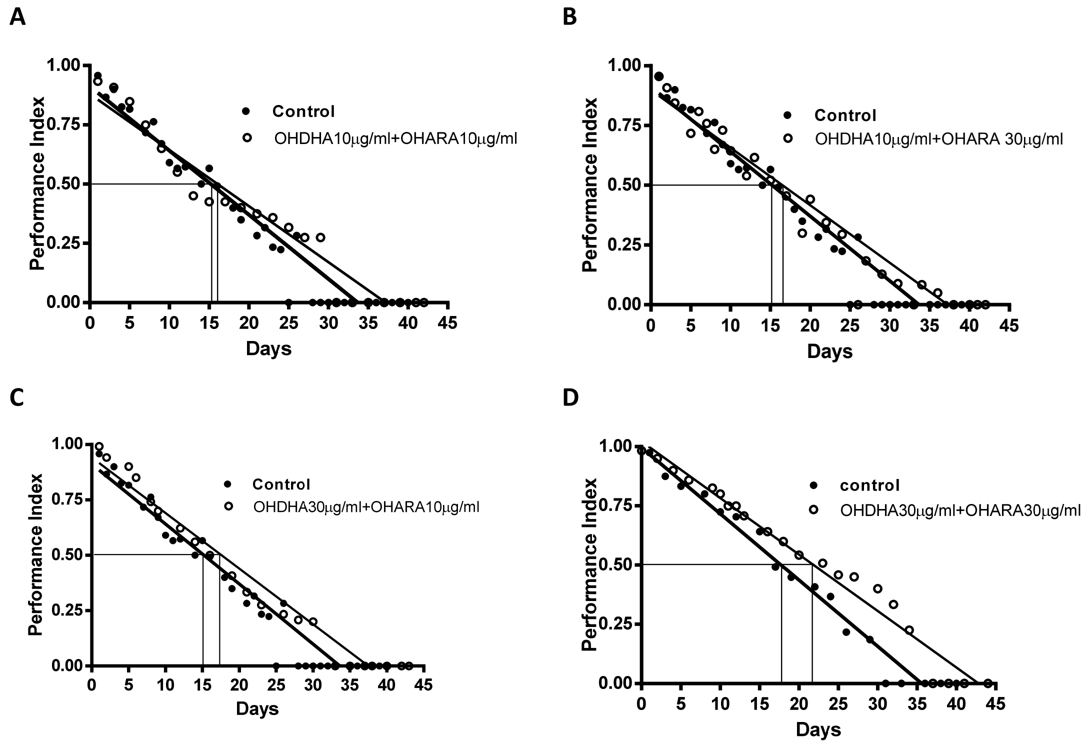


Figure 53. Effect of OHDHA+OHARA in climbing assay of F1 transgenic *Drosophila*. 10 and 30 µg/ml OHDHA+OHARA was added to the food.

Table 21. Effect of OHDHA+OHARA in climbing assay of F1 transgenic *Drosophila*. 10 and 30 µg/ml OHDHA+OHARA was added to the food.

Supplement	Median Climbing (days)
Control 0	16
OHDHA 10 µg/ml+OHARA 10 µg/ml	16.5
OHDHA 10 µg/ml+OHARA 30 µg/ml	16.8
OHDHA 30 µg/ml+OHARA 10 µg/ml	17.2
OHDHA 30 µg/ml+OHARA 30 µg/ml	17.5

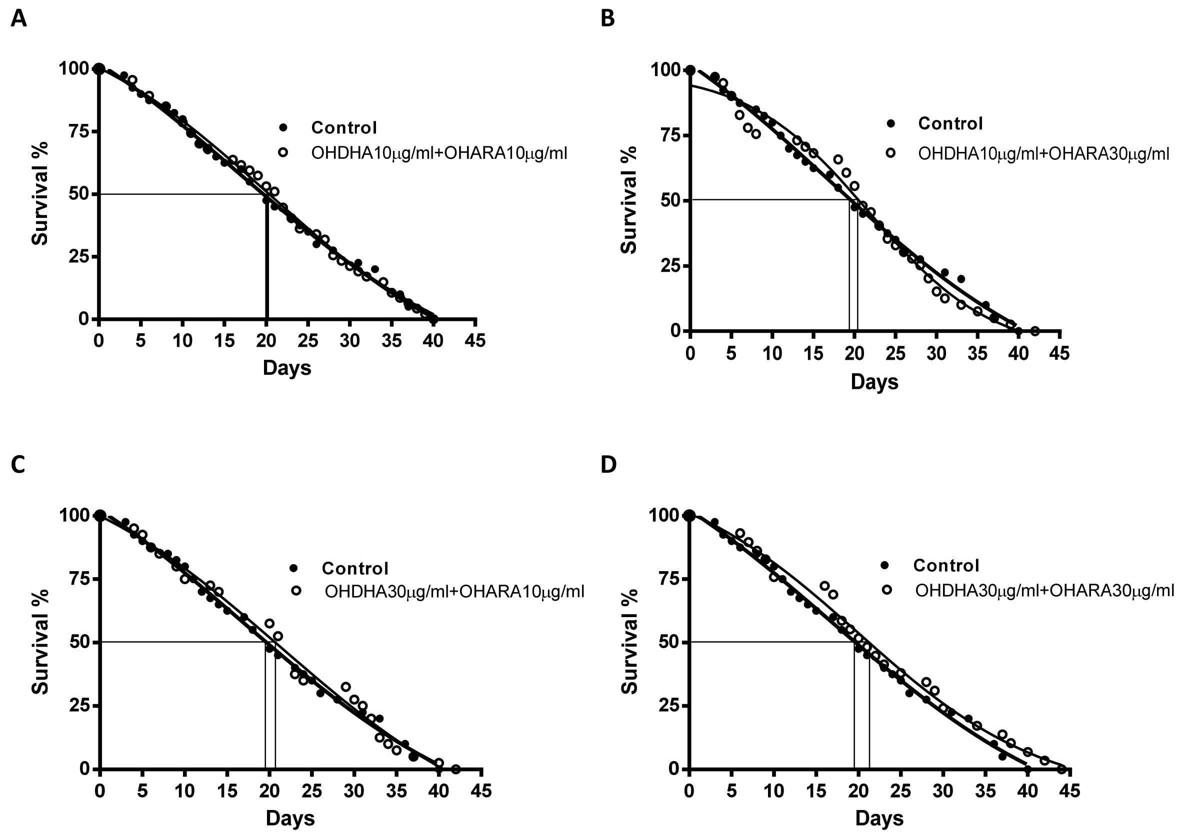


Figure 54. Effect of OHDHA+OHARA in survival assay of F1 transgenic *Drosophila*. 10 and 30 µg/ml OHDHA+OHARA was added to the food.

Table 22. Effect of OHDHA+OHARA in survival assay of F1 transgenic Drosophila. 10 and 30 µg/ml OHDHA+OHARA was added to the food.

Supplement	Median Survival (days)
Control 0	19.3
OHDHA 10 µg/ml+OHARA 10 µg/ml	20.2
OHDHA 10 µg/ml+OHARA 30 µg/ml	20.6
OHDHA 30 µg/ml+OHARA 10 µg/ml	21
OHDHA 30 µg/ml+OHARA 30 µg/ml	21.5

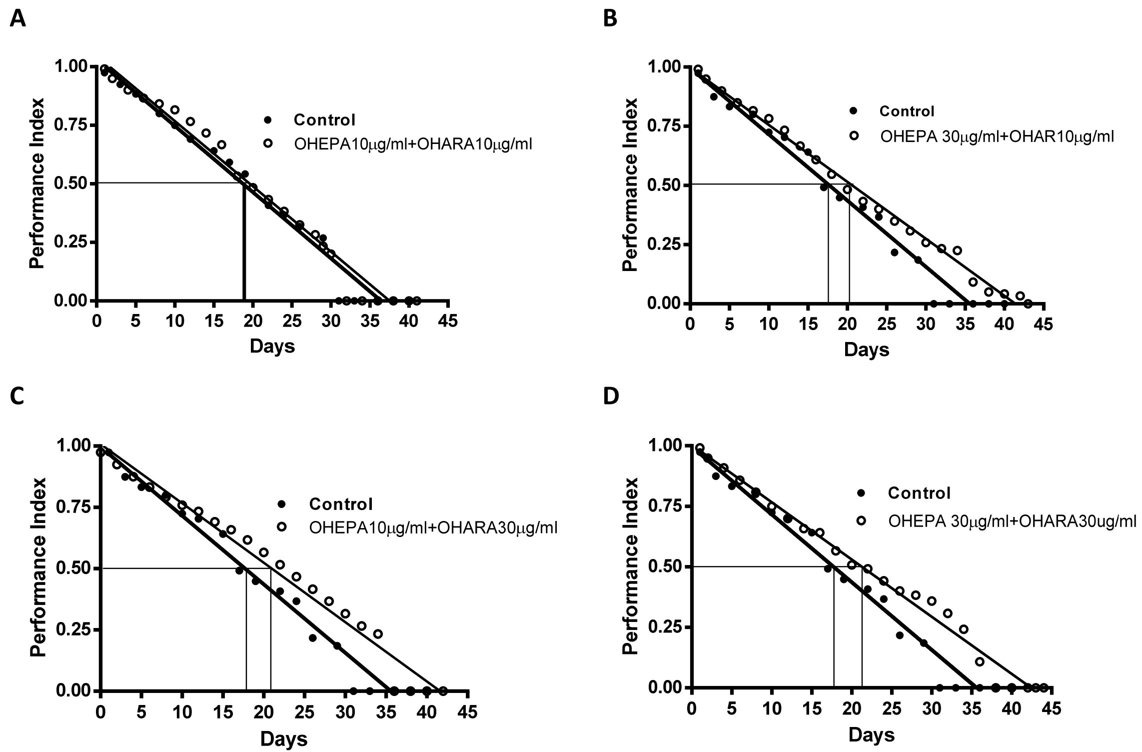


Figure 55. Effect of OHEPA+OHARA in climbing assay of F1 transgenic *Drosophila*. 10 and 30 µg/ml OHEPA+OHARA was added to the food.

Table 23. Effect of OHEPA+OHARA in climbing assay of F1 transgenic *Drosophila*. 10 and 30 µg/ml OHEPA+OHARA was added to the food.

Supplement	Median Climbing (days)
Control 0	18
OHEPA 10 µg/ml+OHARA 10 µg/ml	19
OHEPA 30 µg/ml+OHARA 10 µg/ml	20.4
OHEPA 10 µg/ml+OHARA 30 µg/ml	20.9
OHEPA 30 µg/ml+OHARA 30 µg/ml	21.3

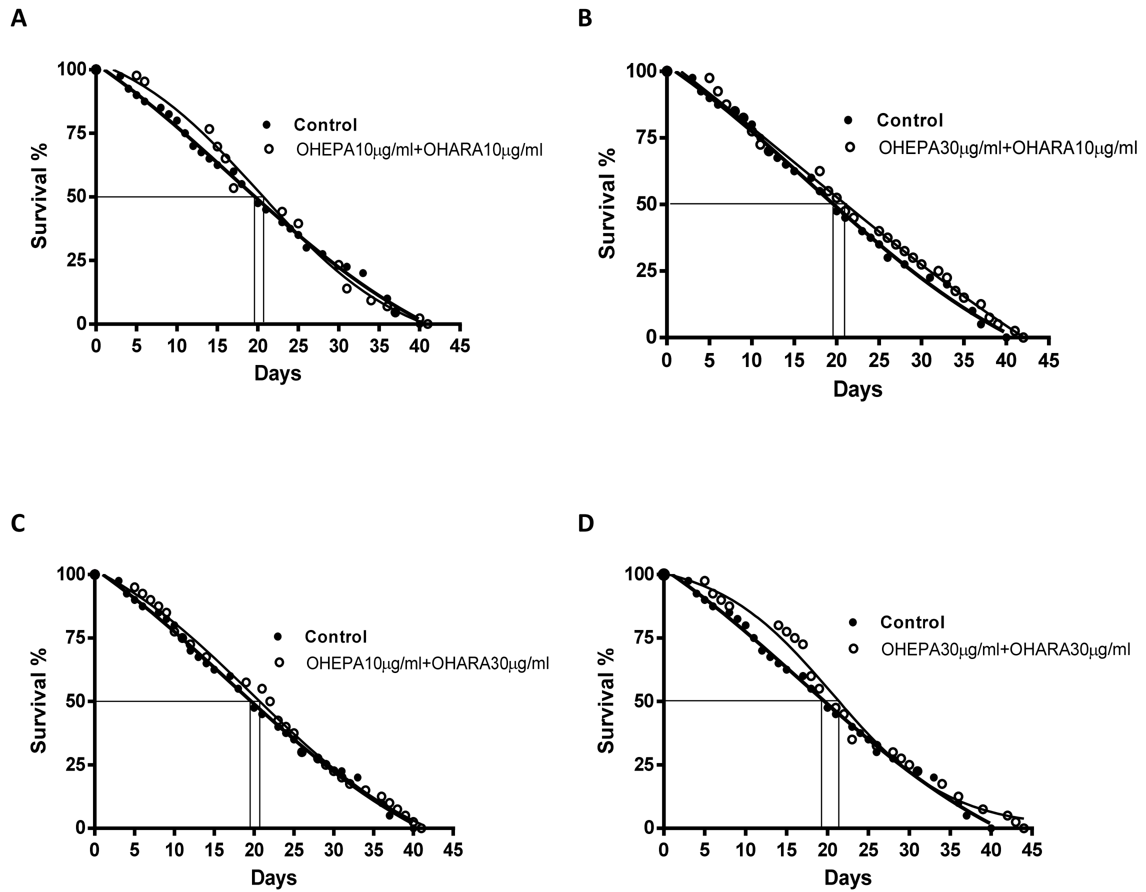


Figure 56. Effect of OHEPA+OHARA in survival assay of F1 transgenic *Drosophila*. 10 and 30 µg/ml OHEPA+OHARA was added to the food.

Table 24. Effect of OHEPA+OHARA in survival assay of F1 transgenic *Drosophila*. 10 and 30 µg/ml OHEPA+OHARA was added to the food.

Supplement	Median Survival (days)
Control 0	19
OHEPA 10 µg/ml+OHARA 10 µg/ml	20.1
OHEPA 30 µg/ml+OHARA 10 µg/ml	21.2
OHEPA 10 µg/ml+OHARA 30 µg/ml	20.8
OHEPA 30 µg/ml+OHARA 30 µg/ml	21.7

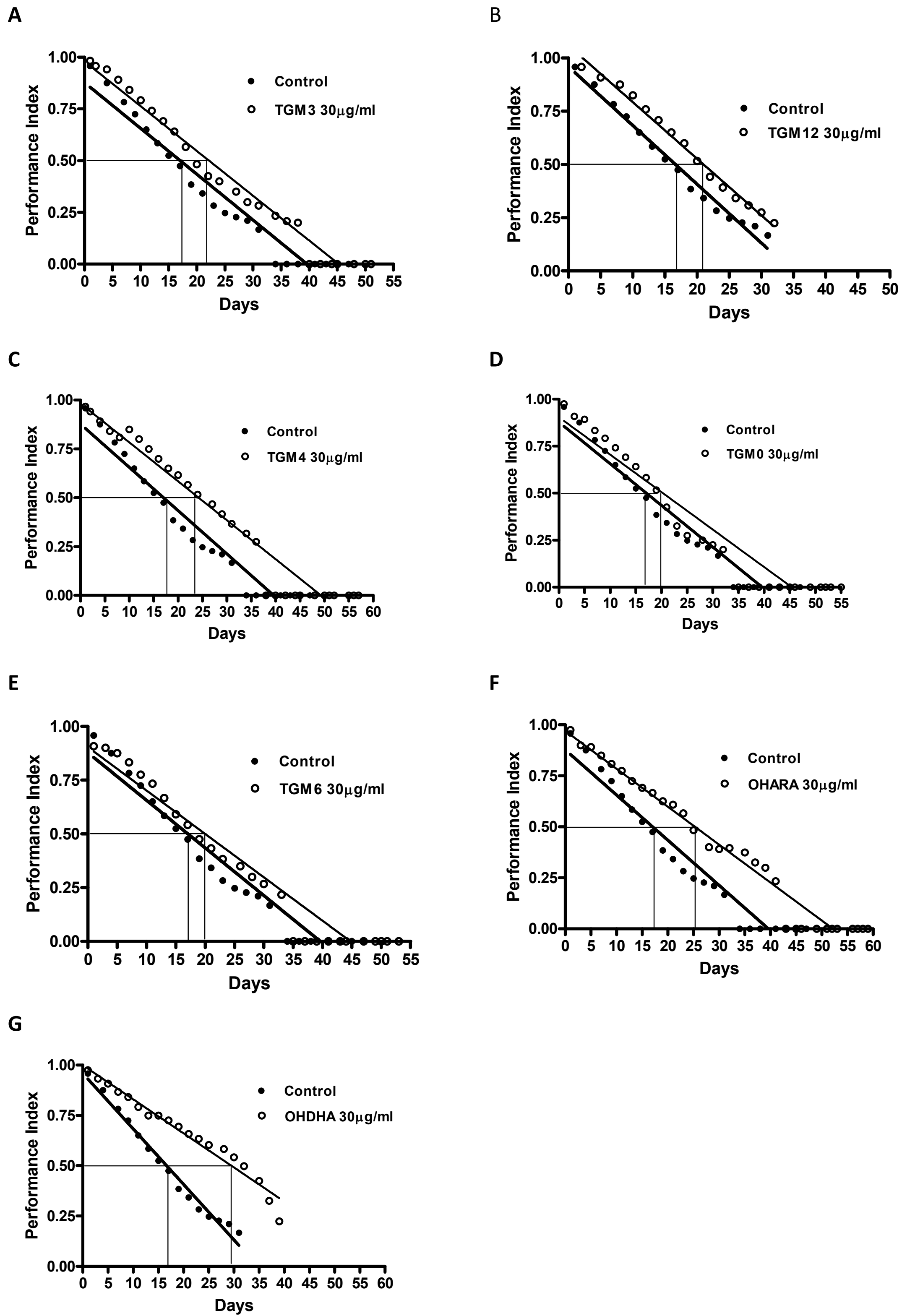


Figure 57. Effect TGMs in climbing assay of F1 transgenic *Drosophila*. 30 μg/ml TGMs (Panels A to G) was added to the food.

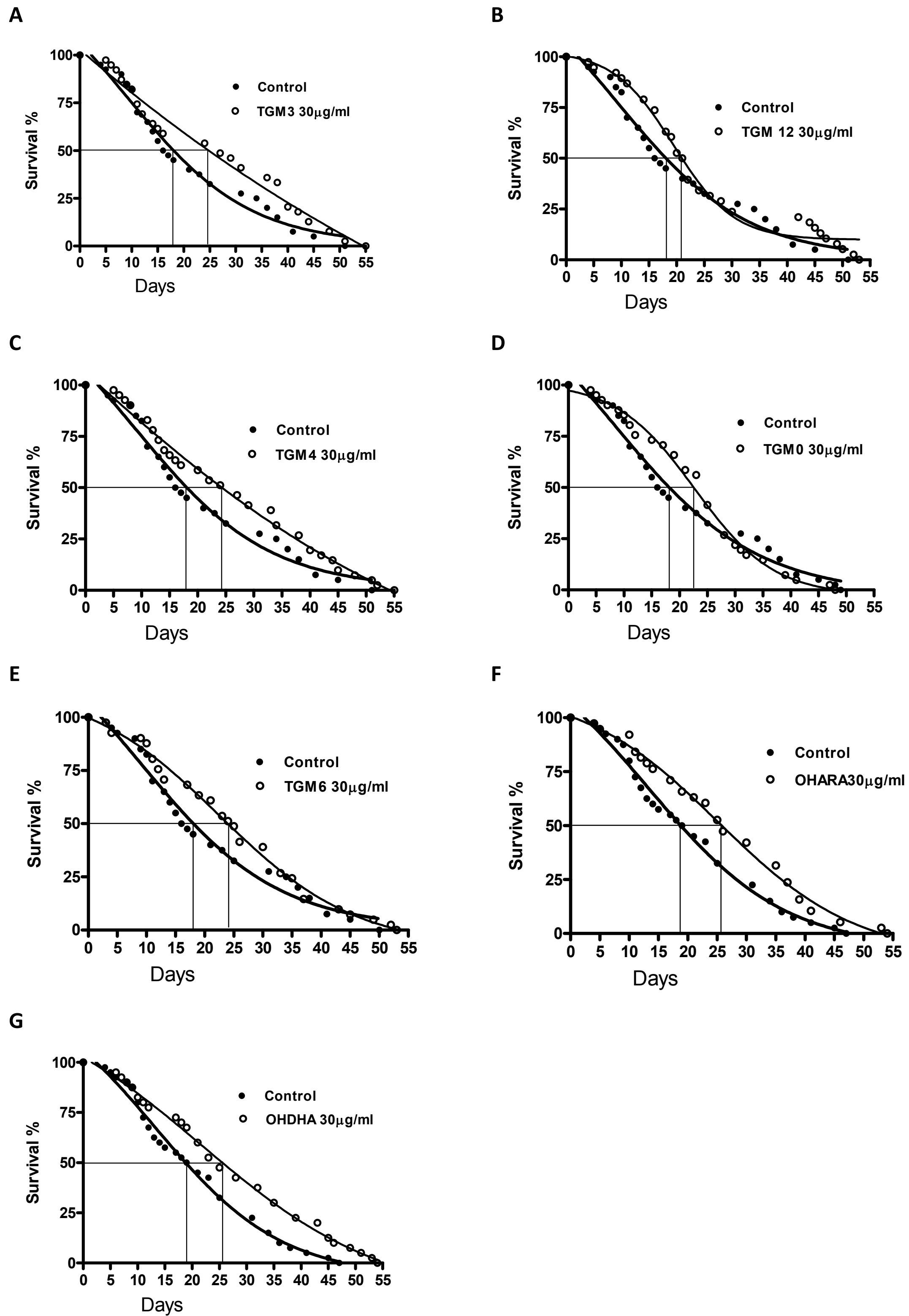


Figure 58. Effect TGMs in survival assay of F1 transgenic *Drosophila*. 30 µg/ml TGMs (Panels A to G) was added to the food.

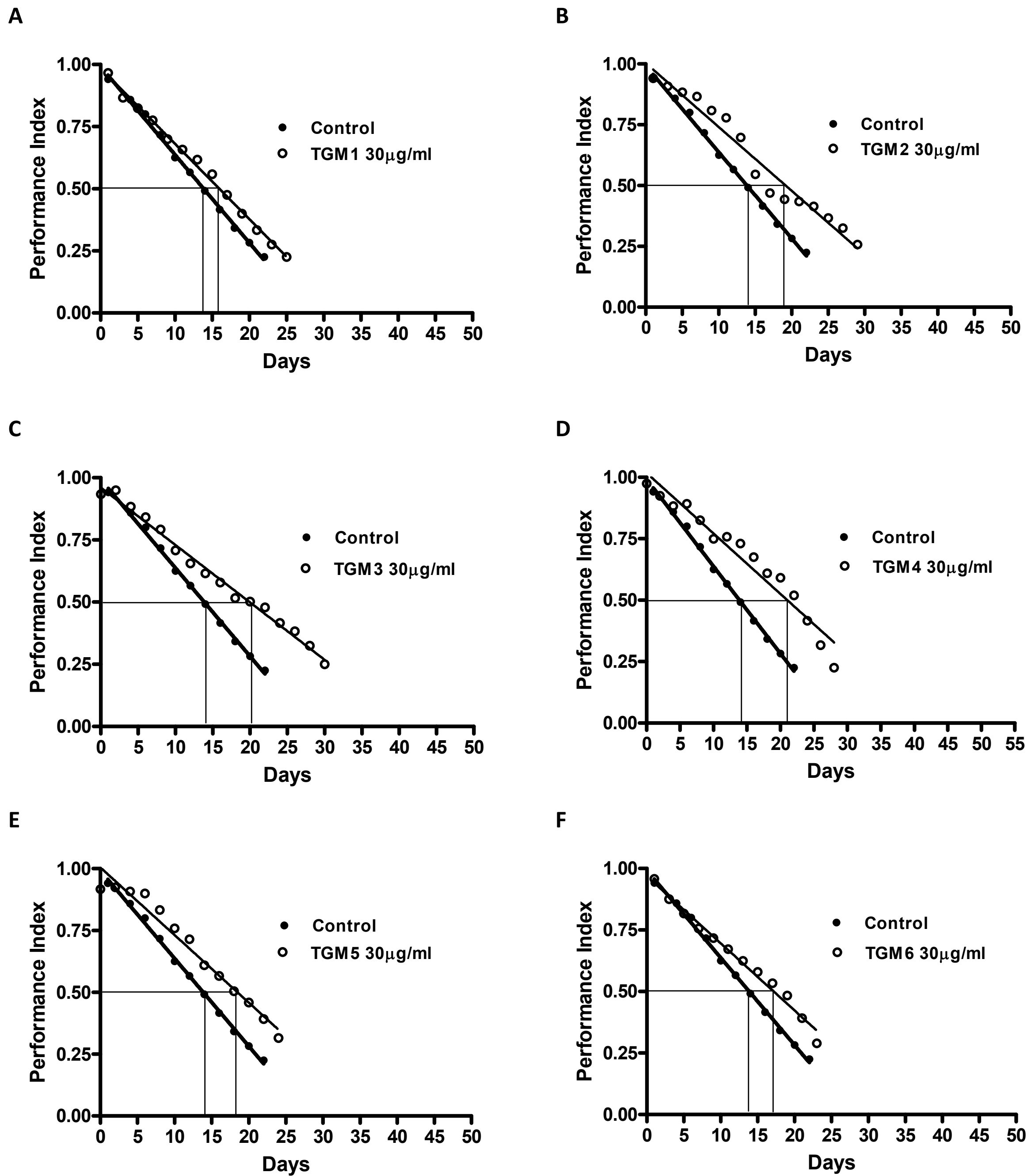


Figure 59. Effect TGMs in climbing assay of F1 transgenic *Drosophila*. 30 μg/ml TGMs (Panels A to F) was added to the food.

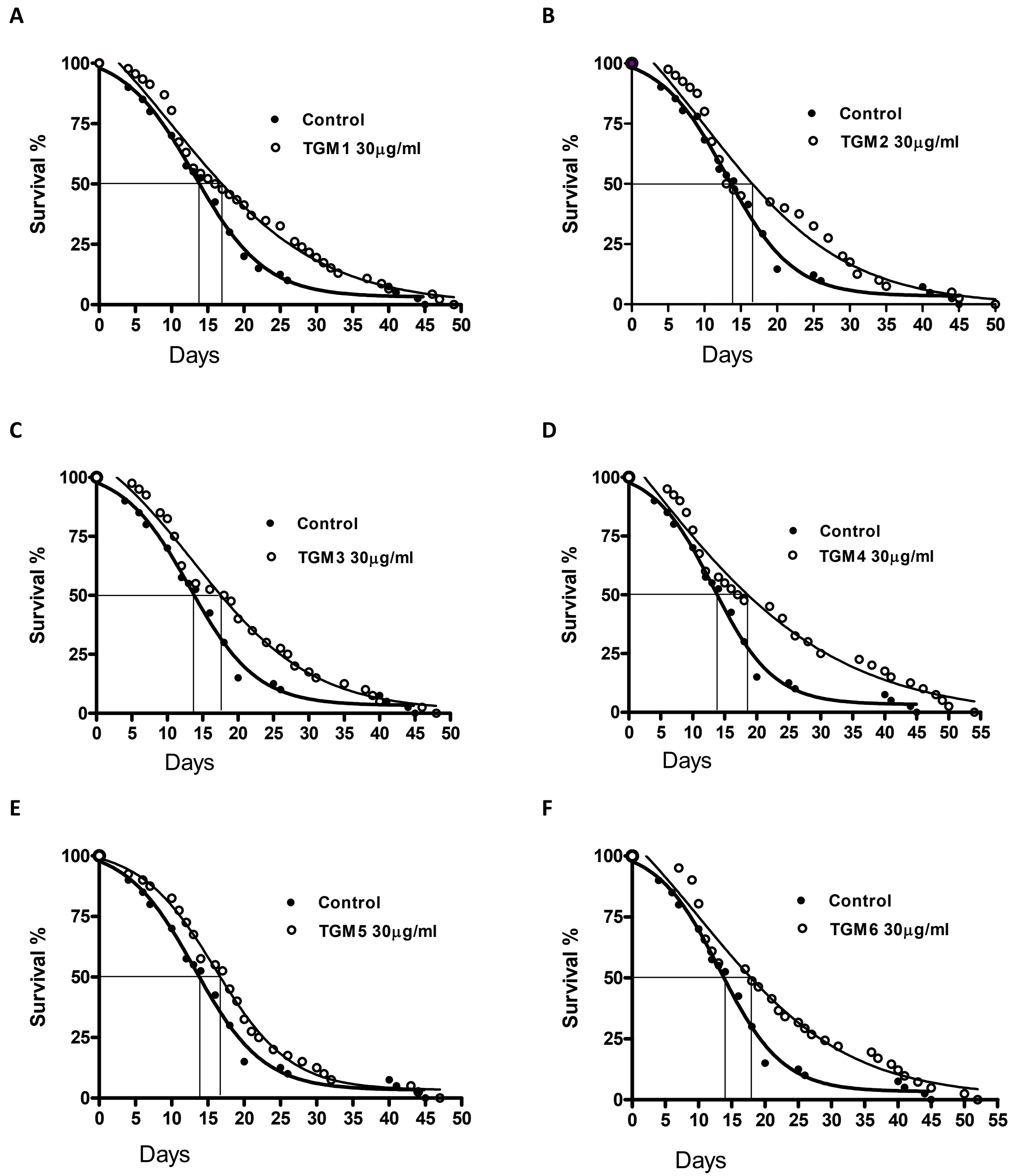


Figure 60. Effect TGMs in survival assay of F1 transgenic *Drosophila*. 30 µg/ml TGMs (Panels A to F) was added to the food.

A

B

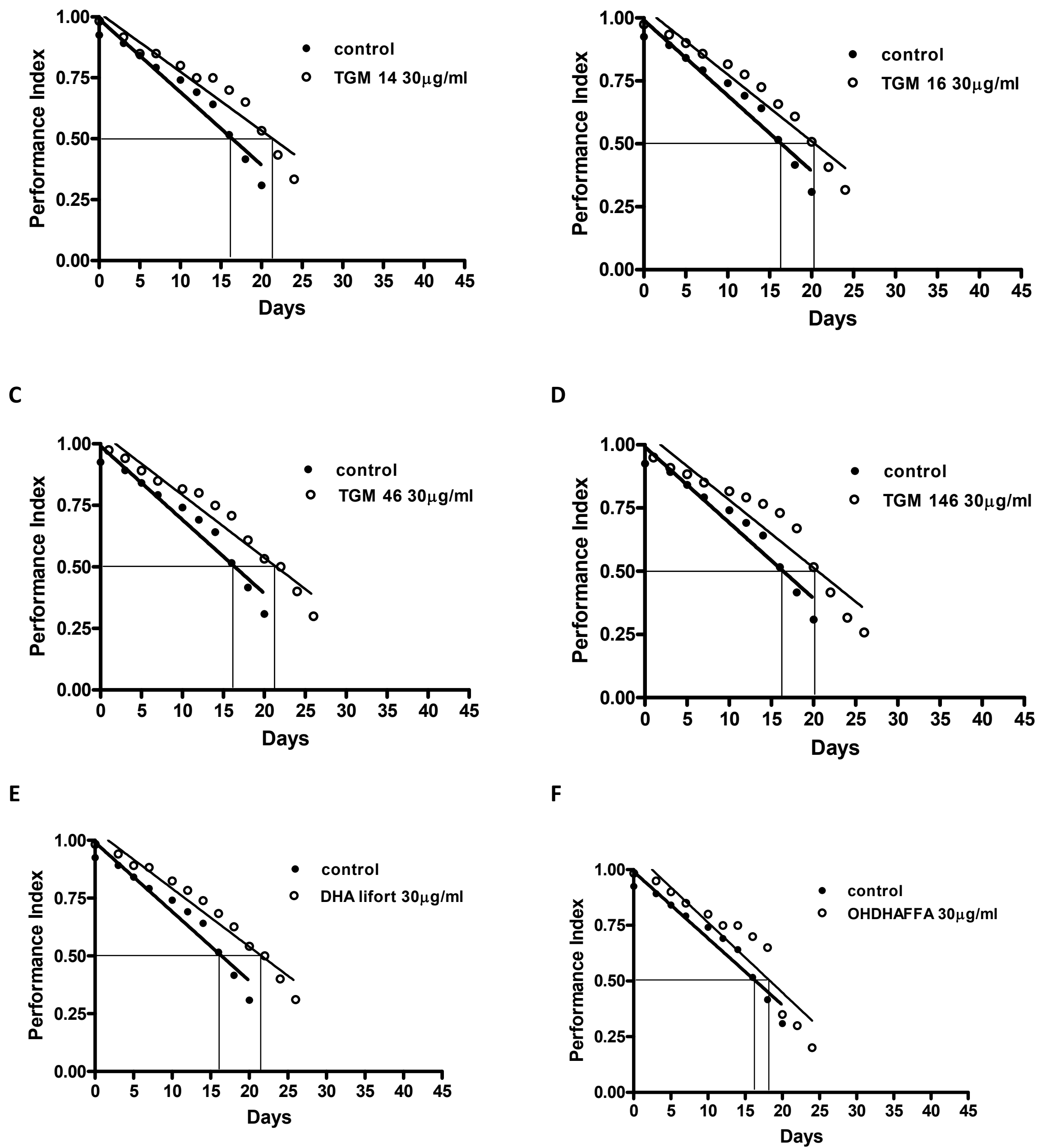


Figure 61. Effect TGM14, 16, 46, 146, OHDHAFAA, DHAlifort in climbing assay of F1 transgenic *Drosophila*. At 30 µg/ml (Panels A to F) was added to the food.

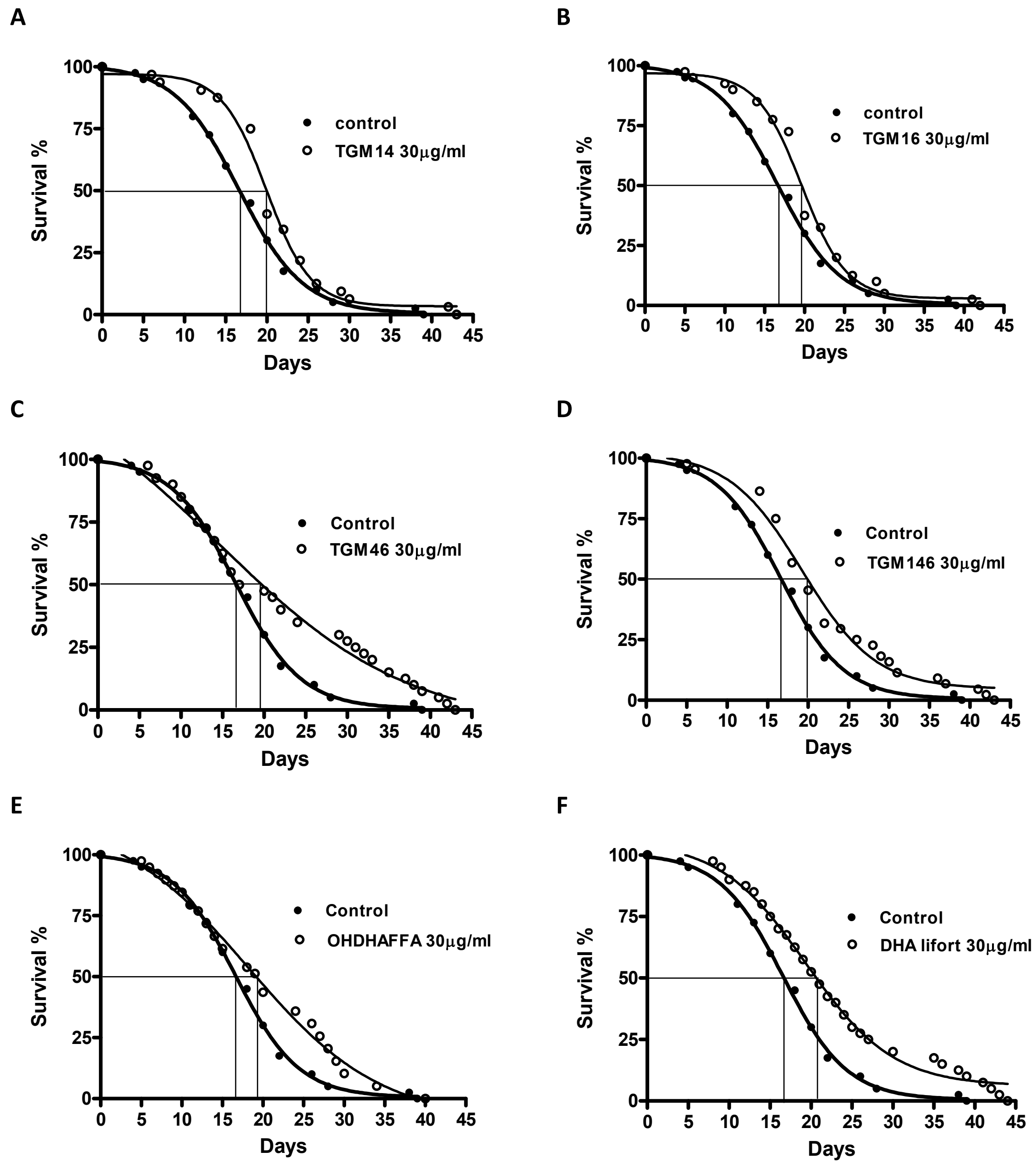


Figure 62. Effect TGM14, 16, 46, 146, OHDHAFAA, DHALifort in survival assay of F1 transgenic *Drosophila.* At 30 μg/ml (Panels A to F) was added to the food.

Table 25. Effect TGMs in climbing assay of F1 transgenic *Drosophila*. 30 μg/ml TGMs was added to the food.

Supplement	Median Climbing (days)
Control 0	17.4
TGM3 30μg/ml	21.5
TGM12 30μg/ml	20.6
TGM4 30μg/ml	23.4
TGM0 30μg/ml	19.9
TGM6 30μg/ml	20
OHARA 30μg/ml	25
OHDHA 30μg/ml	26.5

Table 26. Effect TGMs in survival assay of F1 transgenic *Drosophila*. 30 μg/ml TGMs was added to the food.

Supplement	Median Survival (days)
Control 0	18.4
TGM3 30μg/ml	23.5
TGM12 30μg/ml	21.3
TGM4 30μg/ml	24.1
TGM0 30μg/ml	22.5
TGM6 30μg/ml	23.5
OHARA 30μg/ml	25.5
OHDHA 30μg/ml	25.6

Table 27. Effect TGMs in climbing assay of F1 transgenic *Drosophila*. 30μg/ml TGMs was added to the food.

Supplement	Median Climbing (days)
Control 0	13.6
TGM1 30μg/ml	15.9
TGM2 30μg/ml	18.4
TGM3 30μg/ml	18.8
TGM4 30μg/ml	21
TGM5 30μg/ml	17.8
TGM6 30μg/ml	16.1

Table 28. Effect TGMs in survival assay of F1 transgenic *Drosophila*. 30μg/ml TGMs was added to the food.

Supplement	Median Survival (days)
Control 0	13.8
TGM1 30μg/ml	16.5
TGM2 30μg/ml	16.4
TGM3 30μg/ml	17.5
TGM4 30μg/ml	18.5
TGM5 30μg/ml	16.9
TGM6 30μg/ml	17.8

Table 29. Effect TGM14, 16, 46, 146, OHDHAFAA, DHAlifort in climbing assay of F1 transgenic *Drosophila*. 30µg/ml TGMs was added to the food.

Supplement	Median climbing (days)
Control 0	16
TGM14 30µg/ml	21
TGM16 30µg/ml	20
TGM46 30µg/ml	21.3
TGM146 30µg/ml	20
DHA lifort 30µg/ml	21.5
OHDHAFFA 30µg/ml	18.2

Table 30. Effect TGM14, 16, 46, 146, OHDHAFAA, DHAlifort in survival assay of F1 transgenic *Drosophila*. 30µg/ml TGMs was added to the food.

Supplement	Median Survival (days)
Control 0	16.7
TGM14 30µg/ml	19.8
TGM16 30µg/ml	19.5
TGM46 30µg/ml	20.3
TGM146 30µg/ml	20.5
DHA lifort 30µg/ml	21
OHDHAFFA 30µg/ml	19

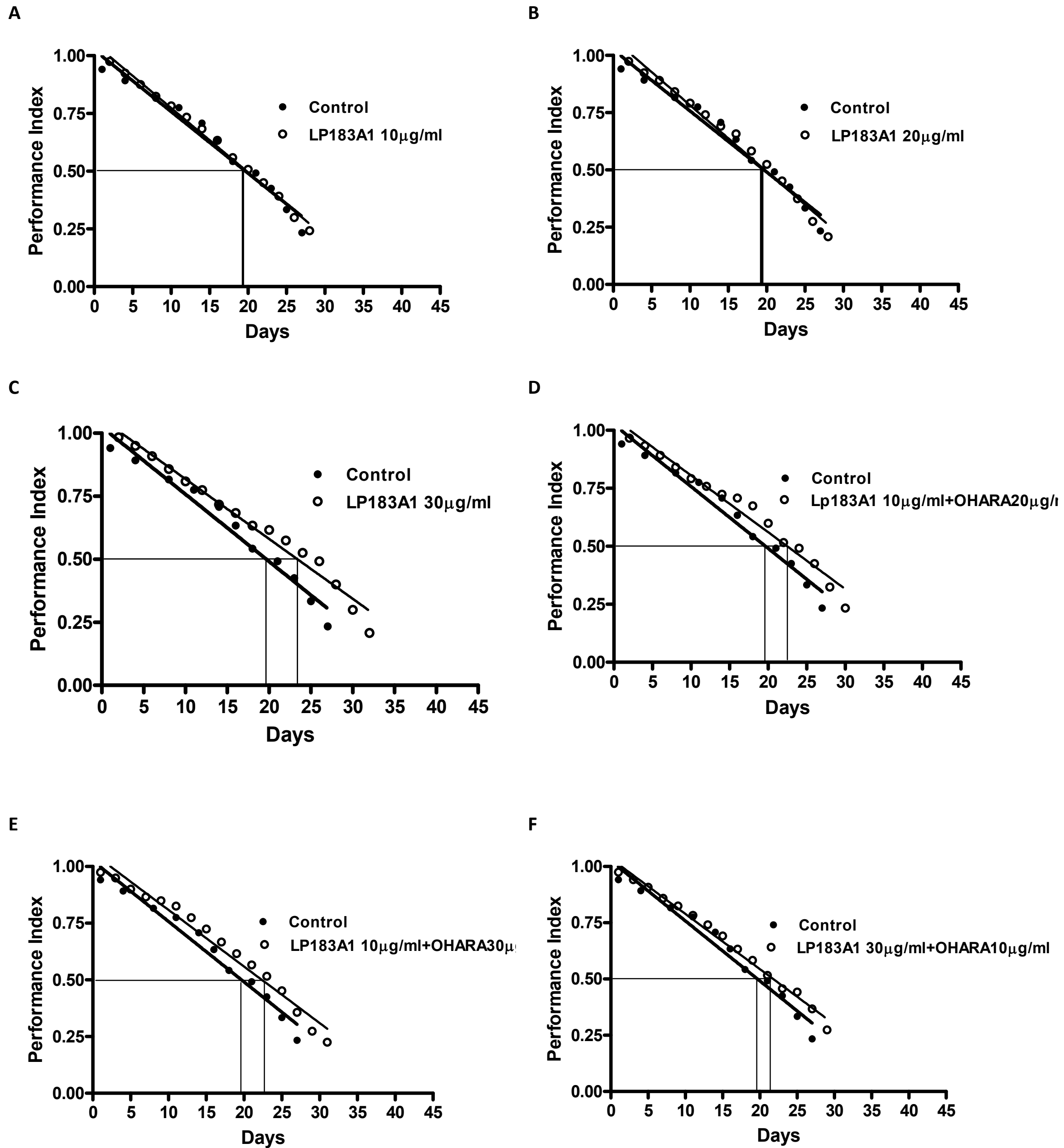


Figure 63. Effect LP183A1 in climbing assay of F1 transgenic *Drosophila*. 10, 20 30µg/ml LP183A1 (Panels A to F), respectively was added to the food.

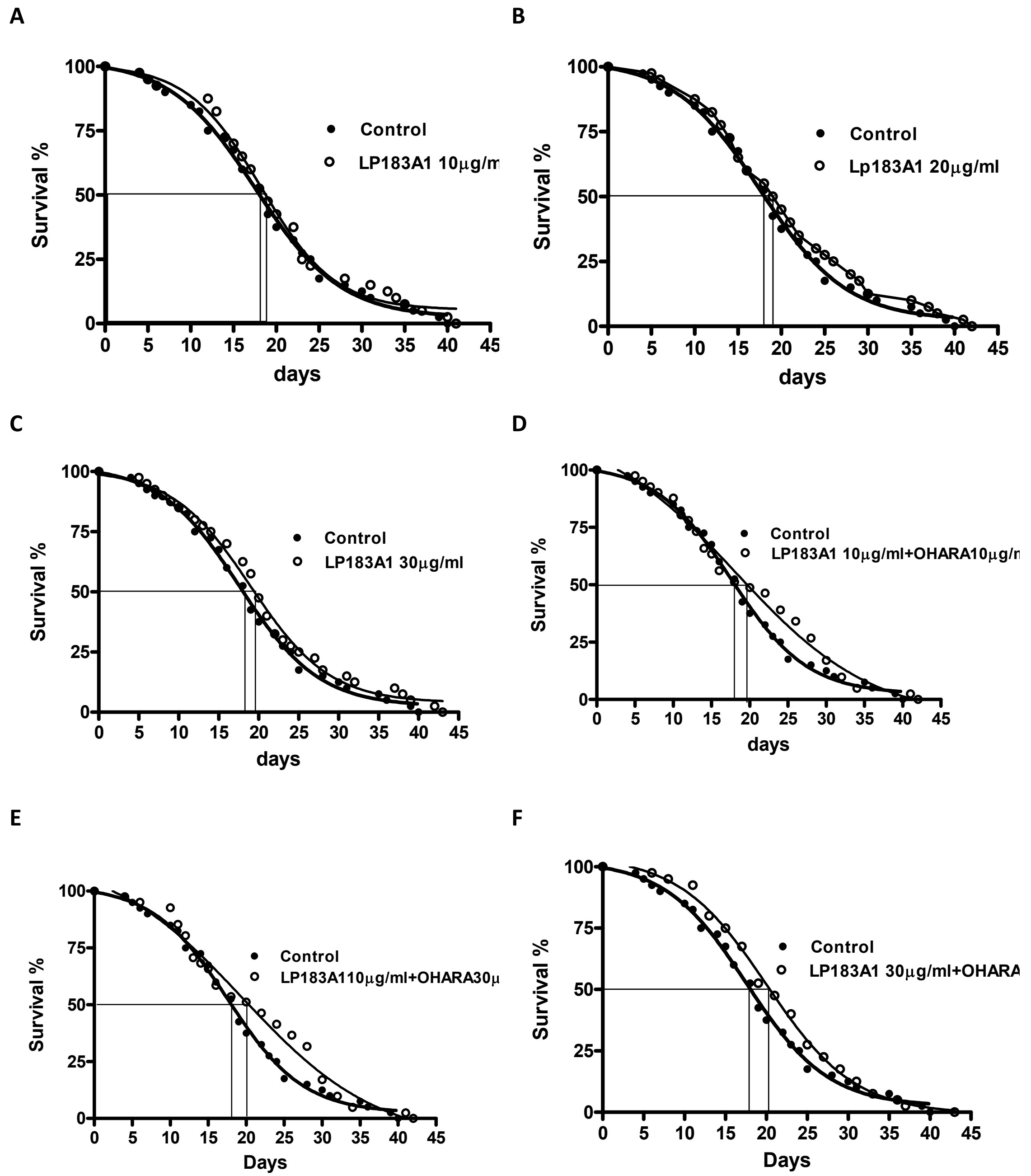


Figure 64. Effect LP183A1 in survival assay of F1 transgenic *Drosophila*. 10, 20, 30 µg/ml LP183A1 (Panels A to F), respectively was added to the food.

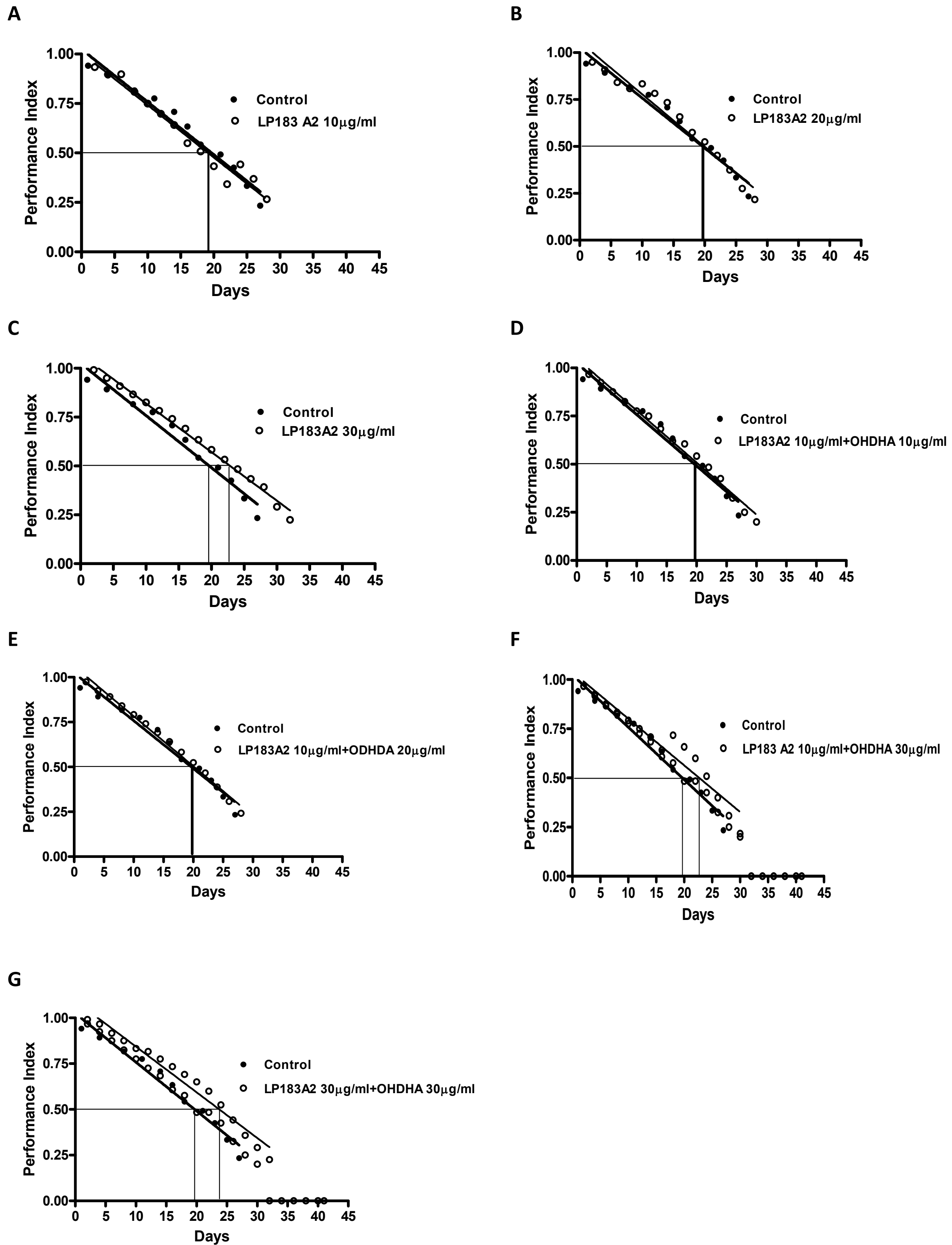


Figure 65. Effect LP183A2 in climbing assay of F1 transgenic *Drosophila*. 10, 20, 30µg/ml LP183A2 (Panels A to G), respectively was added to the food.

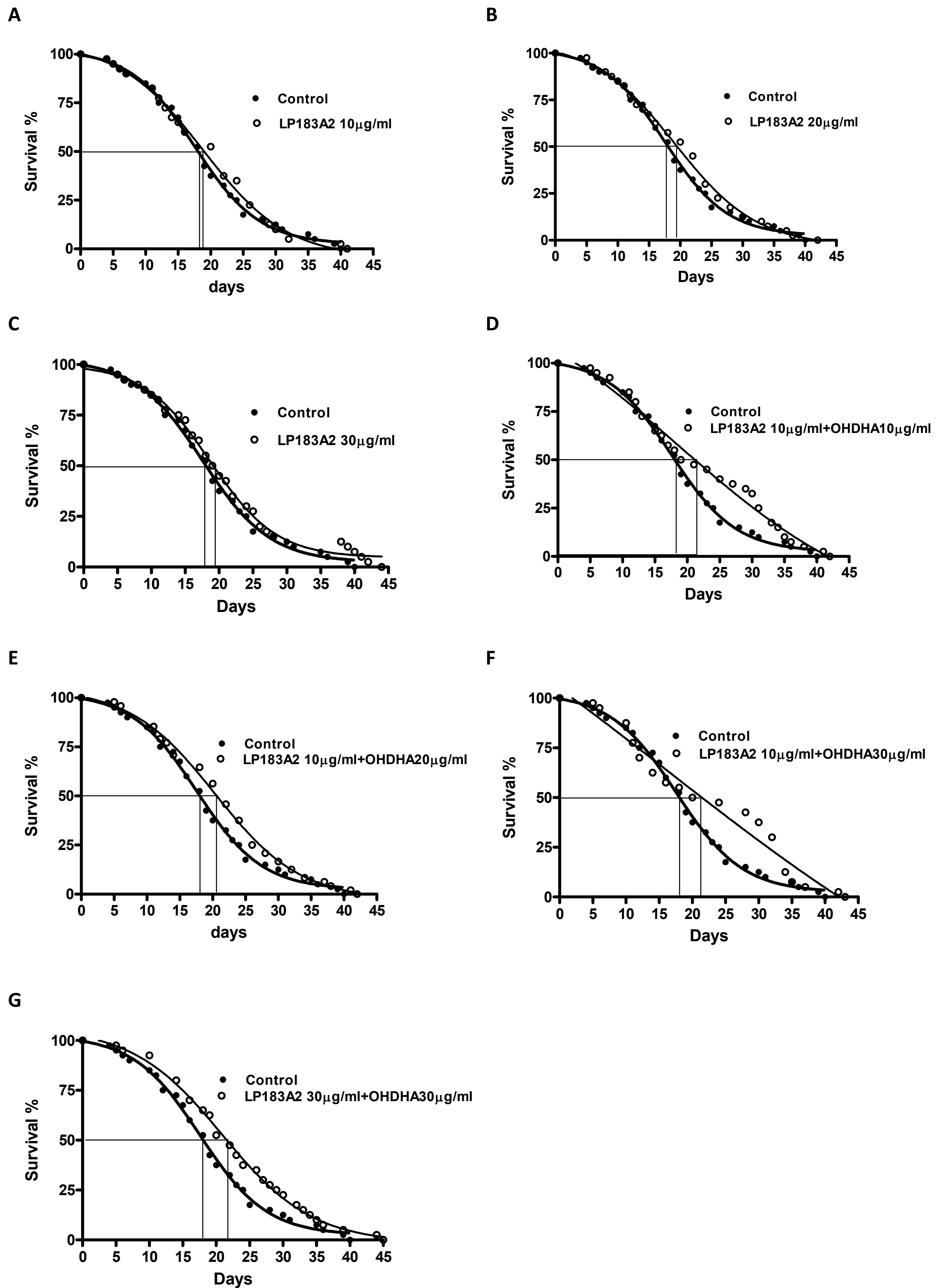


Figure 66. Effect LP183A2 in survival assay of F1 transgenic *Drosophila*. 10, 20, 30µg/ml LP183A2 (Panels A to G), respectively was added to the food.

Table 31. Effect LP183A1 in climbing assay of F1 transgenic *Drosophila*. 10, 20, 30 µg/ml LP183A1 respectively was added to the food.

Treatment	Median Climbing (days)
Control 0	18
LP183A1 10 µg/ml	18.3
LP183A1 20 µg/ml	18.6
LP183A1 30 µg/ml	22.4
LP183A1 10 µg/ml+OHARA 20 µg/ml	22.5
LP183A1 10 µg/ml+OHARA 30 µg/ml	22.8
LP183A1 30 µg/ml+OHARA 10 µg/ml	21.5

Table32. Effect LP183A1 in survival assay of F1 transgenic *Drosophila*.10, 20, 30 µg/ml LP183A1 respectively was added to the food.

Treatment	Median Survival (days)
Control 0	18.1
LP183A1 10 µg/ml	18.6
LP183A1 20 µg/ml	19
LP183A1 30 µg/ml	19.6
LP183A1 10 µg/ml+OHARA 20 µg/ml	19.7
LP183A1 10 µg/ml+OHARA 30 µg/ml	20
LP183A1 30 µg/ml+OHARA 10 µg/ml	20.6

Table 33. Effect LP183A2 in climbing assay of F1 transgenic *Drosophila*. 10, 20, 30 µg/ml LP183A2 respectively was added to the food.

Treatment	Median Climbing (days)
Control 0	18
LP183A2 10 µg/ml	18.4
LP183A2 20 µg/ml	18.8
LP183A2 30 µg/ml	21
LP183A2 10 µg/ml+OHDHA 10 µg/ml	18.9
LP183A2 10 µg/ml+OHDHA 20 µg/ml	19.3
LP183A2 10 µg/ml+OHDHA 30 µg/ml	20.8

Table 34. Effect LP183A2 in survival assay of F1 transgenic *Drosophila*. 10, 20, 30 µg/ml LP183A2 respectively was added to the food.

Treatment	Median Survival (days)
Control 0	18.1
LP183A2 10 µg/ml	18.4
LP183A2 20 µg/ml	18.9
LP183A2 30 µg/ml	19.3
LP183A2 10 µg/ml+OHDHA 10 µg/ml	19.8
LP183A2 10 µg/ml+OHDHA 20 µg/ml	20.4
LP183A2 10 µg/ml+OHDHA 30 µg/ml	21
LP183A2 30 µg/ml+OHDHA 30 µg/ml	21.8

CONCLUSIONS

In this thesis I concluded that,

1. The *Drosophila melanogaster* model shown in this thesis represents a good post-cell and pre-rodent system to test the efficacy and safety of potential therapeutic agents for AD.
2. The F1 generation from crossing the carrier (33771) and the activator (8760) fly strains expressed both Aβ and Tau and mimicked an AD-like phenotype, with significant reductions in lifespan and motor behavior function.
3. The lifespan and motor behavior function in AD *Drosophila* flies treated with hydroxylated fatty acids and other synthetic lipids showed marked and significant improvements with respect to untreated flies of flies treated with non-hydroxylated lipids.
4. Among all the concentrations tested, 30 and 100 µg/ml induced the highest cognitive improvement in AD flies for treatment with DHA, EPA, ARA, and their hydroxylated derivatives.
5. No toxicity was observed at therapeutic doses (30-100 µg/ml). The first toxic effects were seen at 250µg/ml after supplementation for all tested compounds.
6. The F1 generation of Alzheimer´s Disease *Drosophila melanogaster* model used in the present study was able to absorb hydroxylated (e.g., OHARA, OHEPA, OHDHA) and non-hydroxylated (e.g., ARA, EPA, DHA) fatty acids, inducing changes in the brain lipid profile of the flies. While non-hydroxilated fatty acids were detected in the organism after treatment, hydroxylated fatty acids were not. Instead, a series of non-hydroxylated metabolites with unusual gas chromatography retention times were found following treatment with hydroxylated fatty acid derivatives. These non-hydroxylated metabolic derivatives might be related with improvement of the cognitive abilities and increased survival of flies.
7. This work shows the potential of 2-hydroxylated fatty acids and triacylglycerol mimetics for the treatment of AD.

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