# CAN ALTERNATIVE OXIDASE (AOX) COMPLEMENT THE DELETERIUS PHENOTYPE OF *DROSOPHILA MELANOGASTER* MODELS OF ALZHEIMER´S DISEASE? PREPARATORY GENETIC CROSSES

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LASSILA AINO KATARIINA: AOX-oksidaasin rooli Alzheimerin taudissa: transgeenisten Drosophila melanogaster -linjojen muokkaus

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On todettu, että Alzheimerin tautiin liittyy Aß-geenien lisääntynyt ilmentyminen ja sitä kautta amyloidiplakkien muodostuminen. Tämä saattaa kuitenkin olla ainoastaan seuraus jollekin muulle mekanismille, joka aiheuttaa neuronien solukuoleman. Koska mitokondrioiden elektroninsiirtoketjun toiminnan on huomattu muuttuvan Alzheimerin tautia sairastavien soluissa, ajateltiin tutkia, voisiko AOX-oksidaasigeenin sijoittaminen tautia mallintavaan banaanikärpäsmalliin vaikuttaa taudin ilmenemiseen, sillä AOX-entsyymi kykenee toimimaan ketjun osana ja siten nostaa ketjun laskenutta toimintatasoa.

Tämän työn tavoitteena oli muokata Aß-siirtogeenisiä tautimalleja siten, että niiden kanssa toimiminen olisi mahdollisimman helppoa ja käyttökelpoista. Malleja muokattiin risteyttämällä mallien banaanikärpäsiä sellaisten siirtogeenisten kärpästen kanssa, joiden fenotyyppien kanssa toimiminen olisi huomattavasti helpompaa ja tulevien tutkimusten tekeminen siten sujuvampaa. Muokkauksen kohteina olivat sekä siirtogeenisiä Alzheimerin tautia mallintavia linjoja että siirtogeeninen, AOX-oksidaasigeenin sisältävä linja.

Tutkimuksessa huomattiin, että mallien muokkaaminen oli mahdollista ja valitut geeniyhdistelmät eivät olleet tappavia. Jotta mallien toiminnasta sekä kärpäslinjojen genotyyppien oikeellisuudesta voitaisiin varmistua, genotyypit tulisi selvittää muilla menetelmillä.

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# 1 INTRODUCTION

## 1.1 Alzheimer’s disease

Alzheimer’s disease is the most common form of dementia in Finland (Scheinin ym. 2011). Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive deterioration of cognitive skills together with the reduction of the ability to perform daily activities and neuropsychiatric symptoms or behavioral changes. (Readnower 2011, Saykin ym. 2010, Seeman 2011, Sperling ym. 2010, Zhang 2011). Disease begins mildly often by the loss of short-term memory and mild forgetfulness and gradually proceeds to intellectual impairment, which extends to the domains of language, skilled movements, recognition and to those functions related to the frontal and temporal lobes of the brain (e.g. planning and making decisions). This disease is described with pathological cortex changes and appears mainly as a neuronal loss or atrophy in the temporoparietal cortex and in the frontal cortex. (Saykin ym. 2010, Wang 2010). This disease is roughly divided into three stages: at the early stage of the disease, patients are usually less energetic or spontaneous, although changes are often unnoticed as they might be very mild. As the disease progresses to the middle stage, the patient might still be able overcome daily activities independently, but may need assistance with more complicated activities. (Seeman 2011, Sperling ym. 2010). As the disease progresses from the middle to late stage, the patient cannot perform a simple task independently and will need constant supervision. They may even lose the ability to walk or eat without assistance. (Sperling ym. 2010, Wang 2010).

Alzheimer's disease has been claimed to be a protein misfolding disease but also other theories or hypotheses exist. The oldest theory is known as the "cholinergic hypothesis", which suggests that AD is caused by reduced biosynthesis of the neurotransmitter acetylcholine. (Filosto 2011, Readnower 2011, Seeman 2011). This theory has not maintained its support as new investigations are increasingly supporting those theories which are associated with the formation of Aβ peptides and tau protein. Later on these hypotheses has been proposed to be related to the aggregation and formation of amyloid

plaques. However, most currently available medications for AD are designed to treat the acetylcholine deficiency and there is evidence that they only relieve the symptoms, not have halted or reversed disease progression. At the moment, there is no curative treatment to Alzheimer's disease. (Seeman 2011).

## 1.2 Role of amyloid-protein

The role of amyloid precursor protein, APP, coded by the gene APP in chromosome 21 in humans, in the functions of the human body has not been fully elicidated. This integral membrane protein of 365–770 amino acids, is expressed in several tissues but is mainly concentrated in the synapses of neural cells. (Readnower 2011, Seeman 2011, Zhang 2011). Its function has thought to be related to functions in the brain and nervous system (e.g. synapse formation) or iron export (Eckert 2011, Readnower 2011, Seeman 2011). Its individual domains have been successfully crystallized and their structures determined: APP seems to contain at least a growth factor-like domain, a copper-binding domain and complete E1 and E2 domains (Readnower 2011). The complete structure of the protein has not yet been solved (Readnower 2011, Zhang 2011).

The reason why this protein is intensively investigated is that its endoproteolysis produces an abnormal amount of small peptides called beta amyloid (Aß) peptides. The enhanced production of these peptides seems to cause their accumulation and formation of plaques in the brain of AD patients. (Naslund 2000, Wang 2010, Zhang 2011). Alois Alzheimer himself found extracellular deposits of a substance in specific brain regions in postmortem examinations, and at the end of the 1980s it was discovered that this substance, nowadays described as amyloid plaques, consisted of Aß aggregates. Alzheimer also found another pathognomic lesion in the brain, described as neurofibrillary tangles (NFT), which accumulate intraneuronally. (Santos ym. 2010). At the beginning of the 1990s NFTs were discovered to be also composed of peptide aggregates caused by an abnormal hyperphosphorylation of tau protein (an abundant protein in the brain and nervous system functioning as a stabilizer of microtubules). (Eckert 2011, Filosto 2011).

## 1.3 Aß peptide

Aß peptides are formed through the cleavage of APP by enzyme complexes termed α-, β- and γ-secretases. APP can be processed in two ways: either through amyloidogenic or non-amyloidogenic pathway. The amyloidogenic pathway starts with proteolysis by ß-secretase and secretion of one part of APP (sAPPß) to the extracellular space. The other intracellular fragment (termed C99) is cleaved by γ-secretase forming Aß peptides with various lengths. The non-amyloidogenic pathway is quite similar to the amyloidogenic pathway except that the first cleavage, forming extracellular (sAPPß) and intracellular (C83) fragments, is performed by α-secretase. (Readnower 2011, Seeman 2011, Zhang 2011). As mentioned, the length of Aß peptide varies from 38 to 43 amino acids, but most of the residues are 40 amino acids long and approximately 10% are 42 amino acids long. It is still not known whether the formation of Aß peptides or the formation of extracellular plaques cause the neurogenerative characteristics of AD whether the disease triggered by some completely different mechanisms.(Maruszak 2011).

It has been shown that intraneuronal formation of these peptides precedes the formation of extracellular plaques during the development of neuropathology in the brain. When 3xTg-AD mice, (one type of AD animal model which overexpresses the $APP_{Swe}$ variant and taup301L in addition to a knock-in mutation of PS1M146V) were treated by immunotherapy, it was found that the levels of extracellular Aß peptides decreased before the levels of intracellular Aß peptides. Without immunotherapy the intraneuronal immunoreactivity of Aß peptides appeared before the formation of extracellular plaques suggesting that these peptide pools may depend on each other. (Winton ym. 2011). It might be possible that secreted Aß could move back and forth between intra- and extracellular spaces as putative Aß reseptors have been discovered. (Snyder ym. 2005). On the other hand, in an investigation of 36 AD patients and 32 control subjects, intraneuronal Aß immunoreactivity was found to be constantly high also in completely healthy brains. It seemed that the amount of Aß inside neuronal cells appeared already at an early age, increased during childhood and remained relatively high in adult brains

implying that the amount of intraneuronal Aß is not a sufficient indicator of plausible amyloidosis and hence, of AD. (Wegiel ym. 2007).

It has been found out that the relationship of the amount of these two isoforms affects the severity of AD. A Larger relative amount of $Aß_{42}$ to $Aß_{40}$ seemed be more harmful and also the absolute amount of $Aß_{42}$ seems to cause more pronounced symptoms in the patients. $Aß_{40}$ was predominant in milder cases. (Mayeux ym. 2003). On the other hand, the overall amount of amyloid plaques in the brain does not seem to correlate with the severity of the symptoms among AD patients. (McLean ym. 1999, Naslund ym. 2000). It might be that the neurogenerative effect would not be due to extracellular plaques but rather due to the intraneuronal Aß. (Kienlen-Campard ym. 2002). Nevertheless, the main mechanisms of secretion and uptake of Aß remain unclear.

Other theories suggest that Aß peptides could cause cellular dysfunction due to their cytotoxicity. It's been found that Aß peptides have a role in the induction of apoptosis by disrupting the cell's calcium ion homeostasis. Aß seems to be involved in the increase of calcium release from the ER, leading to increased intracellular calcium levels. This leads to an increase of calcium uptake by mitochondria and causes mitochondrial dysfunction and possibly the induction of apoptotic signaling. (Csordas ym. 1999, Maruszak 2011) It has also been shown that Aß interacts with several lipid bilayers. (Davis ja Berkowitz 2009). It also has been shown to localize in many cell organelles suggesting their participation in the modification of Aß peptides. Preventing the transport of the APP-protein sAPPß from ER to Golgi Apparatus and initiating the retrograde transport from Golgi apparatus to ER by brefeldin A (a lactone antibiotic) stopped the production of $Aß_{40}$ but increased the production of $Aß_{42}$ suggesting that the production of $Aß_{42}$ could occur in the ER. (Wild-Bode ym. 1997). It has also been shown that $Aß_{40}$ can be produced in the Golgi apparatus but it only took place in neurons. (Hartmann ym. 1997). Further investigation is required in order to elucidate the mechanisms through which Aß peptides act on neurons.

Recent studies have shown that different mutations cause or increase the risk of AD. It was shown that apolipoprotein E (APOE) receptors, which belong to the group of low-density lipoprotein receptors (LDLR), take part in the production of Aß and in the

modulation of Aß cellular uptake. (Maruszak 2011, Readnower 2011, Seeman 2011, Zhang 2011) It was discovered that the accumulation of Aß was due to the interaction of Aß, APOE and some other components, revealing that having a pathogenically active APOE-gene is a genetic risk factor for AD. (Maruszak 2011, Seeman 2011, Ye ym. 2005). On the other hand, AD has also been observed in patients lacking this allele suggesting that it is not the only factor causing the disease. (Filosto 2011, Maruszak 2011, Santos ym. 2010). It has also been shown that other mutations in other genes related to APP processing and cleavage (e.g. gene APP, genes PS1 and PS2, which code for sub-units of the enzyme γ-secretase), affecting the metabolism and stability of Aß peptides may cause AD and these mutations have been used to model AD in animal models. (Cowan 2010, Eckert 2011, Iijima-Ando 2010, Maruszak 2011, Seeman 2011, Zhang 2011).

## 1.4 *Drosophila* model: Aß expression

*Drosophila melanogaster* (species: Diptera, family: Drosophilae), commonly known as the fruit fly, is an excellent target for genetic investigation. It contains four pairs of chromosomes; a x/y-pair and three chromosome pairs (2,3 and 4), which can be effectively used for the insertion of genes in order to create transgenic models (chromosome pair 4 is very small and cannot be used in modification, so is often neglected). Drosophila is also a good model since its maintenance is easy and manageable, having an average developmental period of approximately 7 to 20 days depending on the line and the growth temperature. Normally flies are cultured in 25°C but in order to vary the speed of the development, they can be kept from 18°C to 29°C. (Ashburner ym. 2005).

Wild-type fruit-flies (no genetic mutation applied) have round red eyes and a yellow-brown body with transverse black rings across their abdomen. Female flies are approximately 2.5 mm long and males are slightly smaller, having a darker body and more distinctive black rings. Also males can be distinguished from females by the hairs (claspers) around the reproductive organs and by the row of dark, thick hairs (sex

combs) on the front legs. Virgin females (females not yet fertilized by males) are much lighter in color (pale-gray) and often have a black spot in the ventral abdomen. (Ashburner ym. 2005)

As the *Drosophila* genome contains recognizable analogs for approximately 80 % of human genes known to cause various diseases, *Drosophila* models are widely used to model numerous pathologies including neurogenerative diseases such as Huntington's and Alzheimer's disease. (Possidente 2010, Reiter ym. 2001, Sang ja Jackson 2005) *Drosophila* models are produced by insertion of genes typical of a certain disease into the genome. Alzheimer's disease models typically contain a combination of genes for Aß expression ($Aß_{40}$ and $Aß_{42}$). Also Aß-genes which contain the so-called arctic mutations have been used. (Crowther ym. 2005, Ling 2011) Other genes have also been used in AD models e.g. contain genes coding for tau protein. These genes have been used either alone in the genome or they have been combined with Aß-genes. (Talmat-Amar ym. 2011)

*Drosophila* models are often created so that genes inserted into the genome of the fly are combined with phenotypic characteristics, balancer chromosomes, which act as markers of the chromosomes in which the inserted gene is present. Balancers help to distinguish if the fly is heterozygous or homozygous for the inserted gene. Balancers are also excellent tools for the control of the inheritance of transgenes i.e. thus prevent genetic recombination at meiosis) and many balancers are homozygous lethal. Common balancers contain markers such as curly wings (CyO), shorter hair on the dorsal side of the thorax (Sb) and possessing slightly more hair on the superolateral part of the humeral joint (Hu). Crossing is used for the production of flies with a desired genotype. (Ashburner ym. 2005, Cowan 2010).

The expression of inserted genes in *Drosophila* models is often controlled. This makes it possible to maintain the model so that inserted genes are not continuously expressed and do not affect the properties of the animal (e.g. lifespan, reproductive ability). One type of control mechanism is the GAL4/UAS-system. (Crowther ym. 2005, Sang ja Jackson 2005) In this system, a UAS-regulator has been inserted next to the transgene in the fly genome. Without any transcriptional activator, the inserted gene is not expressed, but in

the presence of expressed GAL4, a transcriptional activator of the UAS element, gene is expressed. (Alberts ym. 2002) In order to activate the expression of the inserted gene, flies possessing the GAL4 are crossed with flies possessing the inserted genes with UAS-regions. Flies of the F1 generation, which have both GAL4 and the inserted transgene in their genome, will express the inserted gene as desired, since GAL4 is automatically expressed and it acts as a transcription activator for the inserted gene. There are many different GAL4-lines ("driver lines"). Without the presence of UAS-associated transgenes, the expression and hence, presence of GAL4 in such flies has little or no effect on their properties. (Sang ja Jackson 2005)

## 1.5 Mitochondrial dysfunction in Alzheimer's disease

In addition to Aß interacting with lipid bilayers and accumulation in neurons, it has been shown to localize and accumulate inside mitochondria. Mitochondria are important eukaryotic cell organelles responsible for major metabolic processes, citric acid cycle and fatty acid ß-oxidation. In addition to the processes taking place in the mitochondrial matrix, the respiratory chain is situated in the mitochondrial inner membrane. The chain consists of five enzymatic complexes (I, II, III, IV, V) through which electron flow causes production of ATP via oxidative phosphorylation via the formation of an electrical gradient across the inner membranes.(Eckert 2011, Filosto 2011, Maruszak 2011) Mitochondria contain a multiple copies of mitochondrial DNA, and replication is catalyzed by DNA polymerase gamma. It has been proposed that ROS (reactive oxygen species), which are produced as toxic by-products of oxidative phosphorylation, can cause mutations to the mitochondrial DNA since they are produced in close proximity to the DNA itself. (Filosto 2011, Maruszak 2011, Sastre ym. 2000)

It has been found that Aß peptides can cause mitochondrial dysfunction due to their ability to bind to several molecules important for mitochondrial function. Aß accumulation has been shown to be associated with decreased activity of many enzymes involved in the respiratory chain (e.g. complexes III and IV) and apoptosis by the initiation of a caspase-independent pathway involving DNA fragmentation and

chromatin condensation.(Filosto 2011, Maruszak 2011) It is debated whether Aß are directly interacts with these components or whether decreased activity may be caused by mutations in the mitochondrial DNA. Several other mechanisms are have been suggested. (Filosto 2011, Maruszak 2011, Readnower 2011)

## 1.6 Accumulation of Aß peptide: Cause or effect of mitochondrial dysfunction?

Other studies have suggested that the production of Aß may not be a direct cause of mitochondrial dysfunction but rather a side-effect caused by the dysfunction itself. This theory is based on the formation of ROS, which seems to precede the formation of Aß. It has been suggested that oxidative stress caused by the increased production of ROS would be responsible of the neuronal impairment and hence, the symptoms of AD. (Goldsbury ym. 2008a, Sastre ym. 2000) It has been found that at low physiological Aß-peptide concentrations, the decreased function of the respiratory chain was associated with increased activity of complex III. This suggests that the increased formation and the accumulation of Aß peptides could be a response to the stress of mitochondrial dysfunction. (Tillement ym. 2011) It was also found that Aß peptides seem to be oxidized in the extracellular amyloidal plaques, which would support this theory. (Maruszak 2011, Tillement ym. 2011) However, further investigation is needed in order to understand the mechanisms involved.

## 1.7 Cytochrome c oxidase (COX) dysfunction in mitochondrial dysfunction

Cytochrome c oxidase (COX) is an enzyme in the respiratory chain within mitochondrial membranes. This enzyme, complex IV, takes part in the electron transport in the OXPHOS-system and as electrons pass through each complex and finally reach complex IV, they are passed to oxygen in order to form water. During the transfer of electrons via these complexes, protons are simultaneously translocated to the mitochondrial

intermembrane space by complexes I, III and IV. The formation of an electrochemical proton gradient is utilized by complex V (ATPase synthase) in order to produce ATP via proton movement from the intermembrane space back to the matrix. (Coskun ym. 2010, Cottrell ym. 2001, Fukui ym. 2007) It has been shown that numerous mitochondria are needed in neural cells in order to produce sufficient amount of energy for synaptic transmission and they play an important role in neurotransmitter release, regulation of calcium levels, vesicle cycling in presynaptic terminals, repolarization after depolarizing stimulation, and in growth cone formation. (Diaz 2010, Maruszak 2011) Hence the dysfunction of a single part of the OXPHOS system might have a severe effect on the function of neurons and thus, on cortical processes in the brain. (Cottrell ym. 2001)

## 1.8 COX and Alzheimer's disease

It's been proposed that the formation of amyloid plaques and the activity of the OXPHOS system could be related and the decreased activity of COX may cause neuronal death, possibly through the formation of ROS and the induction of apoptotic mechanisms. COX activity has been observed to be decreased in several cell types (e.g. neurons, fibroblasts) of AD patients and its function has been detected to be abnormal compared to the cells of control patients. (Maruszak 2011, Valla ym. 2006) In an investigation of mtDNA cybrid cell lines from AD patients, increased oxidative stress, reduced COX activity and increased expression of $Aß_{40}$ and $Aß_{42}$ was seen (Khan ym. 2000, Maruszak 2011). It has been suggested that COX deficiency causes the increased formation of Aß peptides and could be related to the onset of AD, since it was shown that the inhibition of COX drives the APP processing towards the amyloidogenic pathway (Maruszak 2011, Webster ym. 1998) On the other hand it has been argued that since the formation of ROS precedes the formation of amyloid, the production of Aß proteins could be a compensatory mechanism by neural cells in response to the stress caused by ROS. (Goldsbury ym. 2008b) If the mechanism of Alzheimer's disease lies in the function of COX protein in the OXPHOS system, its decreased activity would be the major underlying cause of the pathology. It remains unclear whether neural degeneration

is caused by the decreased activity of COX or by the formation of amyloid plaques, and how these events are related to each other.

Contrary to previous findings, it has been argued that decreased COX activity may be related to Alzheimer's disease and in fact, the expression of COX may significantly increase in the course of the disease. (Maruszak 2011, Rhein ym. 2009) It has also been suggested that COX deficiency could be a consequence of the formation of Aß peptides, because the knockout of COX in neural cells did not have an effect on the course of amyloid plaque formation. (Fukui ym. 2007, Maruszak 2011) It has also been shown that $Aß_{42}$ peptides localize and interact with COX suggesting that they may actually cause decreased activity of COX and the development of AD simultaneously. (Tillement ym. 2006, Tillement ym. 2011) Thus, it is vital to investigate how the function of COX is related to the development of Alzheimer's disease and whether the mechanism of the disease be related to this specific protein.

## 1.9 Alternative oxidase (AOX) and COX: remedy for Alzheimer's disease?

The alternative oxidase (AOX) is an enzyme, found mainly in plants, protozoa, fungi and some bacteria. This integral membrane protein can function in the ubiquinone pool of the OXPHOS system of mitochondria by offering an alternative pathway to the electron flow. It is situated after the complex II in the electron transport chain and accepts electrons in order to reduce oxygen to water. This decreases electron flow to the complexes III and IV, which leads to a decreased electrochemical proton gradient formation between the cellular spaces in mitochondria and a decreased amount of ATP produced by the complex IV. This causes the extra energy to be released as heat. Its expression is usually triggered by some kind of stress, such as cold or the increase of the levels of ROS. (Fernandez-Ayala ym. 2009)

It has been shown that the gene coding for this enzyme expressed by *Ciona intestinalis* could be inserted in fly genome and expressed in flies without lethal consequences. It

was also shown that AOX expression was able to complement electron transport chain defects (AOX expression complemented transport chain activity in a knockdown of complex IV assembly factors). AOX expression also rescued locomotor defect and excess ROS mitochondrial formation of a *Drosophila* model of Parkinson's disease containing mutated Parkinson's disease gene DJ1 (Fernandez-Ayala ym. 2009). Thus, AOX seems to offer remedy in OXPHOS disorders. It might be possible that if the mechanism of Alzheimer's disease would include the decreased function of COX, and as it has been shown that AOX can complement defects in mitochondrial OXPHOS system, the expression of AOX could prevent the progress of the disease through the prevention of neuronal death and could possibly offer a remedy to the disease.

The purpose of this investigation is to create balanced, transgenic *Drosophila* models for the investigation of Alzheimer's disease, which can be used to find out whether AOX is able to change the course of AD in the fly model by compensate for COX deficiency.

# 2 MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Transgenic Alzheimer *Drosophila* lines

Transgenic lines with the phenotypic characteristics shown in Table 2.1. were used in the experiments. The lines mainlycontained genes coding for Aß peptides ($Aß_{40}$ and $Aß_{42}$). The flies of one line (line 1) were artic mutants (AlzArc2E) incorporate the Glu22Gly mutation in the $Aß_{42}$ peptide sequence.

Table 2.1: Genotypes and phenotypes of transgenic Alzheimer model *Drosophila* lines

| Genotype | Phenotype |
|---|---|
| $1: w^{1118};+;AlzArc2$ | Wild-type, bright-red eyes |
| $2: w^{1118};\frac{Alz^{40.3}}{CyO};+$ | Wild-type, orange eyes, curly wings |
| $3: w^{1118};\frac{Alz^{42.2}}{CyO};+$ | Wild-type, orange eyes, curly wings |
| $4: w^{1118};\frac{If}{CyO};\frac{Alz^{42.3}}{Tm6,Hu}$ | Wild-type, rough-surfaced light-brown eyes, curly wings, humeral hair |
| $5: w^{1118};\frac{Alz^{42.2}}{Alz^{42.2}};\frac{Alz^{42.3}}{Tm6,Hu}$ | Wild-type, dark-red eyes, humeral hair |

### 2.1.2 Transgenic AOX-UAS$^{F6}$ and elav-GAL4 *Drosophila* lines

Lines containing the gene coding for the enzyme AOX and the elav-GAL4-lines are also stabilized. Line 6 contains the elav-GAL4 insertion at the chromosome X. Line 7 has this insertion in chromosome 3.

Table 2.2: Genotypes and phenotypes of transgenic elav-Gal4-lines and AOX-line

| Genotype | Phenotype |
|---|---|
| $6 : elav-Gal4;+;+ \otimes \frac{elav-Gal4}{y};+;+$ | Wild-type, bright-red eyes |
| $7 : w^{1118};+;elav-Gal4$ | Wild-type, bright-red eyes |
| $8 : w^{1118};UAS-AOX^{F6};+$ | Wild-type, bright-red eyes |

### 2.1.3 Control and transgenic balancer chromosome *Drosophila* lines

Table 2.3 shows the lines used in crosses with Alzheimer, elav-GAL4 and AOX -lines in order to create lines with easier characteristics to distinguish (e.g. marker Tm6, Hu (slightly more hair in the superolateral part of the humeral joint) is quite difficult and especially time-consuming to distinguish). Line 9 was used as a control line.

Table 2.3: Genotypes and phenotypes of other lines used in the experiments

| Genotype | Phenotype |
|---|---|
| $9: w^{1118};+;+$ | Wild-type, white eyes |
| $10: w^{1118};\frac{CyO}{+};\frac{Sb}{+}$ | Wild-type, white eyes, curly wings, stubble hair |
| $11: FM7;+;+ \otimes \frac{FM7}{y};+;+$ | Wild-type, yellow bar-shaped eyes, yellow body |
| $12: \frac{FM7}{w^-};\frac{CyO}{+};+ \otimes \frac{FM7}{y};\frac{CyO}{+};+$ | Females: Wild-type, red heart-shaped eye, curly wings. Males: Wild-type, yellow bar-shaped eye, curly wings, yellow body |

## 2.2 Methods

### 2.2.1 Crosses

Flies were produced in plastic bottles which contained approximately 35 ml of fly food (1 % tayo agar, 1,5 % sucrose, 3 % glucose, 3,5 % active dried yeast, 1,5 % corn flour, 1 % wheat germ, 1 % soya flour, 3 % treacle, 0,5 $\%_{vol}$ propionic acid, 0,1 $\%_{vol}$ Nipagin M (in EtOH) and 83,9 % $H_20$), which contains all the nutrients essential to flies in order to grow and develop. *Drosophilae* were produced by crosses in which approximately 20 female virgin flies and 10 male flies are brought together in a container. After copulation females start to lay eggs on the bottom of the container. Crossings were expanded by putting the flies into new bottles three times (1$^{st}$ time: two days from the initial day, 2$^{nd}$ time: one day from last change, 3$^{rd}$ time: one further day from previous change).

### 2.2.2 Collection

As the flies started to hatch, they were observed under 3D-microscope and those with desired combinations of markers were collected three times per day into vials containing 3.5 ml of fly food for approximately five days. Female flies were collected as virgins and male flies as young as possible. Flies were collected by sedation with carbon dioxide. Via sedation, flies can be easily handled and flies with desired phenotype distinguished. When a sustainable amount of certain flies was collected in order to carry out crosses, new crosses were made. Crosses were carried out as many times as required for the final, desired genotype. Once the genotype was achieved, the transgenic line was maintained as a stock, which can be then used for future experiments.

# 3 RESULTS

## 3.1 Crossings A & B: elav-GAL4 (in chromosome 3) and UAS-AOX$^{F6}$

### 3.1.1 Cross A: Modification of elav-Gal4-line 7

Modification of line 7 was carried out by crossing female flies from line 7 with males from line 10 (Figure 3.1, Cross 1A). As line 10 contains markers CyO (curly wings) and Sb (stubble hair), flies possessing these markers were collected and crossed (Figure 3.1, Cross 2A). As the marker "Sb" situates in Chromosome 3, flies possessing just marker CyO were collected from Cross 2A in order to get flies which are homozygous to elav-GAL4 -gene.

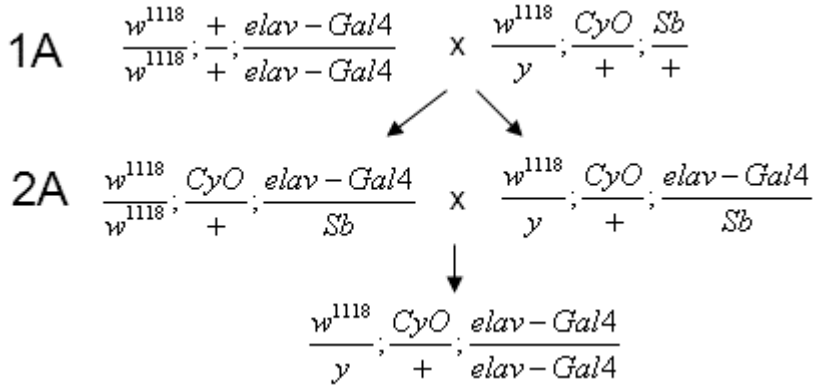


Figure 1: Modification of elav-GAL4-line 7 (original lines: 7 (females) and 10 (males))

### 3.1.2 Cross B: Combination of genes elav-GAL4 and UAS-AOX$^{F6}$

In order to create a fly line with a combination of elav-GAL4 and UAS-AOX$^{F6}$, many combinations of different markers were used in crosses. First females from line 8 were crossed with males from line 10 (Figure 2, Cross 1B). Cross 2B was carried out by crossing flies with markers "CyO" and "Sb" in order to achieve a fly line homozygous to UAS-AOX$^{F6}$-gene (Figure 2: Cross "B). These flies have only marker "Sb" and these

females were used in cross 3B (Figure 2, cross 3B). Females homozygous to UAS-AOX$^{F6}$-gene were crossed with males from Cross A as they are homozygous to elav-GAL4 -gene (Figure 1). This cross was carried out by crossing females with Sb-marker from cross 2B with males with CyO-marker from cross A. The next generation with both markers CyO and Sb are flies having both genes, elav-GAL4 and UAS-AOX$^{F6}$.

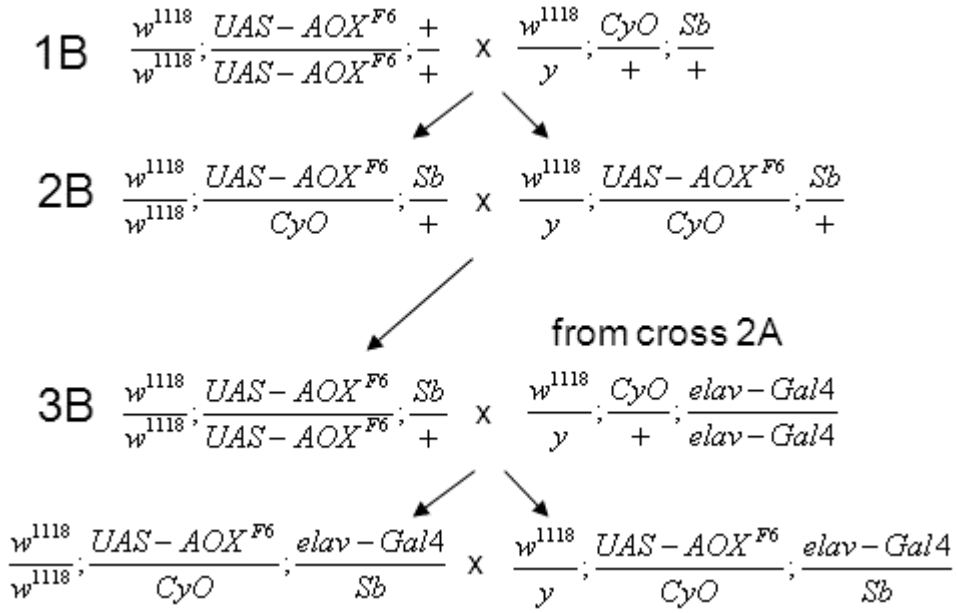


Figure 2: Combination of transgenes elav-GAL4 and UAS-AOX$^{F6}$ with original fly lines 7, 8 and 10.

## 3.2 Crossings C & D: Modification of balancers in transgenic Alzheimer lines

### 3.2.1 Cross C: Modification of line 4

Line 4 with transgene Alz$^{42.3}$ was decided to modificate to a phenotype easier and faster to distinguish. The original line 4 contains marker "Tm6, Hu", which causes the flies to have slightly more denser area of hair in the superolateral part of the humeral joint. Searching these type of flies under microscope is slow and difficult to inexperienced as the marker is not easy to observe.

First females from line 4 were crossed with males from line 10 (Figure 3, Cross 1C). Next cross was carried out by crossing flies with markers CyO and Sb (Figure 3, Cross 2C). The final cross 3C was done with flies with just marker Sb. This fly linecan also be used if flies homozygous to the transgene $Alz^{42.3}$ are needed. These flies are one outcome from cross 3C and they don't have the marker Sb, as in this case this marker is situated in chromosome 3.

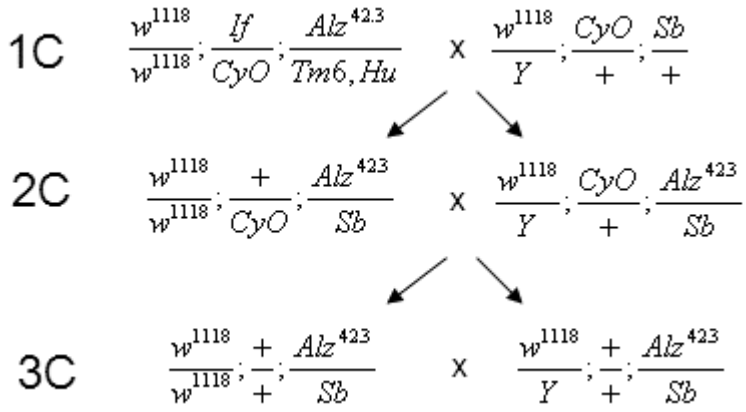


Figure 3: Modification of fly line 4: Change of balancer from “Tm6, Hu” to “Sb” with original lines 4 and 10.

### 3.2.2 Cross D: Modification of line 5

Cross D (Figure 4) was carried out in a similar way as the cross C, for same reasons and with same markers, although the original fly line 5 contained two transgenes, $Alz^{42.2}$ and $Alz^{42.3}$. This line was originally homozygous to $Alz^{42.2}$ in chromosome 2 and heterozygous to $Alz^{42.3}$ in chromosome 3. And in a similar way as in cross C, if flies homozygous to both transgenes are desired, cross 3D will be carried out and flies without marker Sb are chosen.

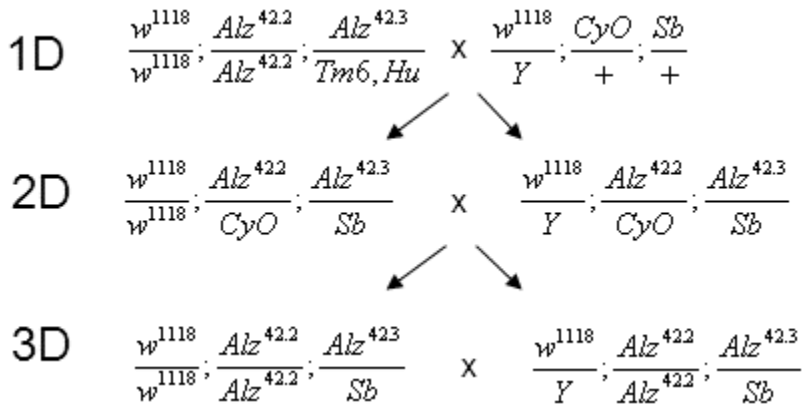


Figure 4: Modification of fly line 5: Change of balancer from “Tm6, Hu” to “Sb” with original lines 5 and 10.

## 3.3 Crossings A’ and B’: Combination of genes elav-GAL4 (in X chromosome) and UAS-AOX$^{F6}$

### 3.3.1 Cross A’: Modification of elav-GAL4-line 6

Line 6, in which the transgene elav-GAL4 lies in the sex chromosome, was modified to include markers so that its usage would be more convenient. This cross was done by crossing females from line 6 with males from line 12, which have markers FM7 and CyO (Figure 5: Cross 1A'). FM7, which is a balancer chromosome found in this case in the sex chromosome causes female flies to have red, heart-shaped eyes. In males however, this balancer causes them to have yellow, bar-shaped eyes and also a yellow-colored body.

From the first generation, females with heart-shaped eyes and curly wings and males with just curly wings were selected to be kept as a stock line. It is also possible to receive female flies homozygous to elav-Gal4 and these would look like just wild type flies as the balancer FM7 is situated in the sex chromosome and homozygosity to CyO-marker is lethal.

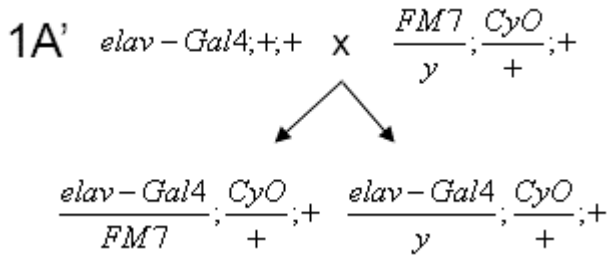


Figure 5: Modification of elav-GAL4-line 6 with original lines 6 and 12.

### 3.3.2 Cross B: Combination of genes elav-GAL4 and UAS-AOX$^{F6}$

In order to create a fly model, in which the UAS-AOX$^{F6}$-system can be activated, gene for the pruduction of the activation protein must be included into the genome and this was done according to figures 6.1 and 6.2. First marker FM7 was included into the fly genome which contains transgene UAS-AOX$^{F6}$. This was done by crossing females from line 8 with males from line 12. With crosses 1B' and 2B', flies homozygous to UAS-AOX$^{F6}$-transgene with marker FM7 were received. Flies received after cross 2B' have a phenotype otherwise like wild-type except the females have red, heart-shaped eyes and males have bar-shaped yellow eyes and yellow body.

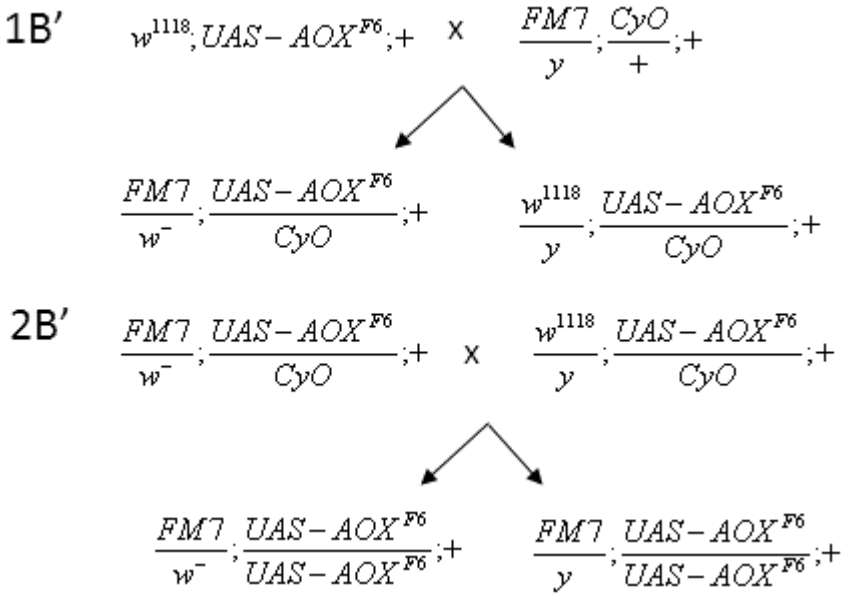


Figure 6.1: Modification of fly line 8 by the addition of marker FM7 to the genome.

Crosses were continued with cross 3B' seen from the figure 6.2.In order to include the transgene elav-Gal4 to the genome of a transgenic fly line containing transgene UAS-AOX$^{F6}$, proper markers are used in order to be able to distinguish and cross flies with correct genotypes. In cross 3B', females received from cross 2B' were crossed with males received earlier from cross 1A'.

To cross 4B' (figure 6.2), females heterozygous to both transgenes Elav-Gal4 and UAS-AOX$^{F6}$ were used and the distinguishing was possible, because these flies contain both markers FM7 and CyO (heart-shaped red eyes, curly wings and otherwise wild-type form). Males with same markers were chosen as these males have only the transgene UAS-AOX$^{F6}$, because the transgene Elav-Gal4 is situated in the x -chromosome, but in this case there is a marker FM7 in the x -chromosome of the chosen males to cross 4B'.

From cross 4B', flies homozygous to UAS-AOX$^{F6}$ -transgene were chosen as these flies do not have the marker CyO, so these flies do not have curly wings. Flies with this genotype were kept as a stock line for future experiments (line 5B', figure 6.2).

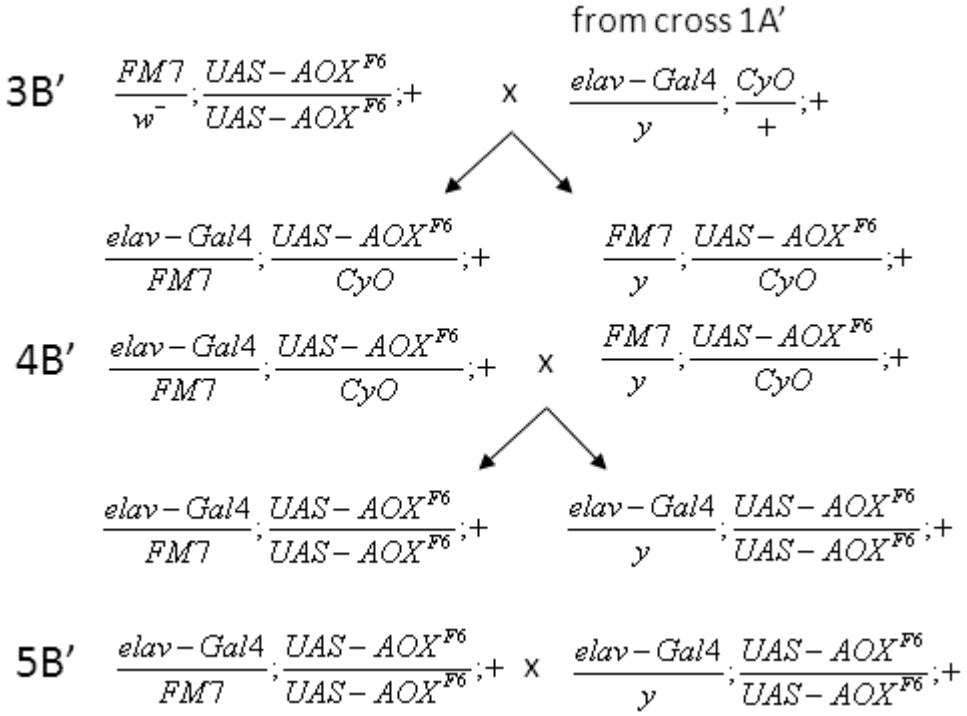


Figure 6.2: Combination of transgenes UAS-AOX$^{F6}$ and elav-Gal4 with flies received from cross 2B' (figure 6.1) and cross 1A' (figure 5). Original fly lines used: 6, 8 and 12.

## 3.4 Genotypes and phenotypes of final transgenic *Drosophila* lines

After modifications, the fly lines were renumbered. Genotypes and phenotypes of new, modified fly lines can be seen from table 4.

Table 4: Genotypes and phenotypes of the final modified transgenic fly lines.

| Genotype | Phenotype |
|---|---|
| $1: w^{1118};+;AlzArc2$ | Wild-type, bright-red eyes |
| $2: w^{1118};\frac{Alz^{40.3}}{CyO};+$ | Wild-type, orange eyes, curly wings |
| $3: w^{1118};\frac{Alz^{42.2}}{CyO};+$ | Wild-type, orange eyes, curly wings |
| $4: w^{1118};\frac{+}{+};\frac{Alz^{42.3}}{Sb}$ | Wild-type, orange eyes, stubble hair |
| $5: w^{1118};\frac{Alz^{42.2}}{Alz^{42.2}};\frac{Alz^{42.3}}{Sb}$ | Wild-type, dark-red eyes, stubble hair |
| $6: elav-Gal4;+;+ \otimes \frac{elav-Gal4}{y};+;+$ | Wild-type, bright-red eyes |
| $7: w^{1118};+;elav-Gal4$ | Wild-type, bright-red eyes |
| $9: w^{1118};+;+$ | Wild-type, white eyes |
| $10: w^{1118};\frac{UAS-AOX^{F6}}{CyO};\frac{elav-Gal4}{Sb}$ | Wild-type, red eyes, curly wings, stubble hair |
| $11: \frac{elav-Gal4}{FM7};\frac{UAS-AOX^{F6}}{UAS-AOX^{F6}};+ \otimes \frac{elav-Gal4}{y};\frac{UAS-AOX^{F6}}{UAS-AOX^{F6}};+$ | Females: Wild-type, dark-red heart-shaped eyes<br>Males: Wild type, dark red eyes |

# 4 DISCUSSION

The aim of this investigation was to produce balanced, transgenic lines to model Alzheimer's disease with clearly distinguishable phenotypic characteristics from already existing transgenic lines in order to make the handling and controlling the lines easier. The lines produced were successfully produced and no clear obstacles concerning the properties of the lines were met (e.g. none of the genotypes, which were planned to be used in the crossings, were lethal, which then would have required to make new crossing plans in order to circulate the lethal combinations of genes). Every succeeding generation contained flies with required phenotypes enough to carry out crossings and final lines had expected phenotypes.

The results showed that it is possible to combine the gene elav-GAL4 with the alternative oxidase gene (UAS-AOX$^{F6}$) with two different ways: having the elav-GAL4 in the chromosome X or in the autosomal chromosome 3. The original AOX line, line 8, was modified so that the final lines contained the elav-GAL4-gene. Final line 10 is a modification from the line 8, so that it is heterozygous to UAS-AOX$^{F6}$ (chromosome 2) and to elav-GAL4 (chromosome 3) and it contains two balancer chromosomes: "CyO" on chromosome 2 (causing curly wings) and "Sb" on chromosome 3 (causing stubble hair). Final line 11 is also a modification from original line 8 so that female flies are heterozygous to elav-GAL4 in X-chromosome and homozygous to UAS-AOX$^{F6}$ in chromosome 3. The genome of female flies also contains one balancer chromosome "FM7" in X-chromosome (as heterozygous in female flies, it causes heart-shaped eyes). Male flies have elav-GAL4 in their X-chromosome and are homozygous to UAS-AOX$^{F6}$ on chromosome 2.

It was also shown that it is possible to change the phenotypes of two Alz-lines, original lines 4 and 5, to easier to distinguish. Both lines contained originally balancer chromosome "Tm6, Hu" in chromosome 3, which causes the flies having slightly more hair on the superolateral part of the humeral joint on the thorax and the observation of this characteristics is very time-consuming and even difficult for an observer unaccustomed to work with *drosophila*. Line 4 was modified so that the balancer

chromosome “CyO” was removed from the genome and the balancer “Tm6, Hu” on chromosome 3 was replaced with balancer “Sb”. Chromosome “If” (causing rough-surfaced eyes) originally found in chromosome 2 was also replaced with a wild-type chromosome (+). This chromosome is not a balancer chromosome, just a marker, so it was relevant to remove it from the genome. Line 5 was modified so that the balancer chromosome “Tm6, Hu” in the chromosome 3 was replaced with a balancer easier to distinguish (“Sb”).

Results reflect the method of crossing and the usage of drosophila in genetics to be a good way to investigate the effect and expression of genes to various aspects. As the heritage of transgenes (genes for Aß expression) are controlled with the help of balancer chromosome which provide certain specific phenotypic characteristics to drosophila having a certain genotype, the probability to choose and collect flies with wrong genotype is minimized. On the other hand, flies with wrong genotypes could still be involved in the crossings and to produce a line with a wrong genotype although the phenotypes would be as expected as the balancers might still be inherited in a similar way as expected. Contamination of a line in a laboratory conditions is also extremely possible as different drosophila lines are continuously handled in same environment and flies continuously escape from their containers during laboratory work. Already an involvement of a single fly with wrong genotype to a crossing can contaminate the line and cause severe mistakes in the heritage of genes in the next steps of crossing. In addition to more careful laboratory working, the accuracy of this investigation could be substantially improved by verifying the genotypes of the final lines with other methods. The conduction of PCR would directly reveal whether some drosophila line has the expected genotype as if the expression of transgenes is induced, the expression levels of the products of these genes can be detected.

This study created very applicable tools in the field on Alzheimer’s disease investigation and tools to be used in the experiments with the effect of AOX to the expression of Aß peptides in transgenic *drosophila* models. As these models contain easily distinguishable characteristics, the control and working with these lines is clear and convenient. With clear phenotypes, it is also possible to make first conclusions based on the phenotypes of the flies in the actual experiments with AOX and Aß as the combination of the induced

expression of both AOX-gene and Aß-gene might have an effect on the phenotype of the flies if there is an effect on the molecular level. However the actual genotypes of the drosophila lines should be verified with molecular methods and also the inducibility of the expression of transgenes (both AOX and Aß-genes) should be carefully tested before clear conclusions can be drawn how AOX affects to the drosophila models of Alzheimer's disease.

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