# Investigating the effects of Aβ42-oligomer interacting peptide (AIP) on Aβ42-induced toxicity in *Drosophila melanogaster*

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**ABSTRACT**

Alzheimer's disease (AD) is one of the most widespread neurodegenerative diseases in the world, and is currently estimated to affect over 35 million people globally. With the continued rise in life expectancy, the number of afflicted patients will likely double in number every 20 years. The amyloid-β (Aβ) peptides is likely the primary perpetrator in the pathogenesis of AD. Aβ peptides are produced from the amyloid precursor protein (APP) through β- and γ-secretase processing. One form of the Aβ peptide, which has 42 amino acid residues (Aβ42), is highly aggregative and can become neurotoxic. Soluble oligomers of the Aβ42 peptide composed of 42 amino acid residues (Aβ42), in particular, are currently believed to be the principal effectors of synaptic dysfunction and neuronal loss in AD.

As a result, potential therapeutics that specifically targets these toxic oligomers has been a focus of drug development recently. While antibody therapies have positioned Aβ oligomers as a viable therapeutic target for AD, the high manufacturing and administration costs associated with an antibody-based therapeutic raise concerns over the general accessibility of such a treatment for patients. Alternatively, we have focused on investigating the efficacy of a small, peptidic inhibitor of toxic Aβ42-oligomers known as the Aβ42-oligomer interacting peptide (AIP). We have previously successfully demonstrated that AIP was able to specifically target Aβ42 oligomers *in vitro*, as well as ameliorate the Aβ42-induced loss of synaptic spine density and long-term potentiation (LTP) in organotypic hippocampal slices (Barucker et al. 2015).

Now, we have developed and characterized transgenic *Drosophila* models that express human Aβ42 in different tissues such as the eye and neurons, essentially allowing us to generate animals that acquired significant and observable Aβ42-induced deficits either in morphology and function. We hypothesized that AIP can neutralize Aβ42-induced toxicity in these *in vivo* models. We subsequently evaluated the longitudinal effects of chronic AIP administration through the oral route on transgenic *Drosophila* models, and assessed the efficacy of AIP administration via both morphological and functional assays. Our studies show that the protease resistant D-AIP was overall non-toxic to the animals even when administered in longitudinal experiments. We were also able to successfully rescue the Aβ42-induced morphological damages in the eyes of the flies with D-AIP supplementation, albeit only in the female animals. This sex-specific discrepancy can likely be attributed to the non-specific binding of D-AIP to a *Drosophila* male-specific sex peptide (Acp70A), which potentially sequestered and reduced the

overall bioavailability of D-AIP in the male animals. In a separate transgenic model, where Aβ42 is expressed in a pan-neuronal manner and impairs functional behaviour, the long-term locomotor activities of the flies were significantly rescued by D-AIP. Interestingly, the female transgenic animals with pan-neuronal Aβ42 expression were not affected by the expression of neuronal-specific Aβ42. Further studies showed that the heads of female transgenic flies have significantly increased insoluble Aβ42 content, which may have contributed to an overall decrease in toxicity (i.e. toxicity is generally caused by the *soluble* oligomers) and thus rending them resistant to Aβ42-induced changes in locomotor behaviour.

Overall, D-AIP appears to be a non-toxic, stable, and promising modulator of toxic Aβ42 oligomers *in vivo*. Going forward, future studies in more advanced vertebrate systems such as transgenic mouse and rat AD models will help us to shed more light on the potential of D-AIP as a therapeutic agent. Ultimately, we strongly hope and anticipate that the highly beneficial Aβ42-oligomer neutralization effects of D-AIP will be a formidable strategy for early intervention in AD.

**ABRÉGÉ**

La maladie d'Alzheimer (MA) est l'une des maladies neurodégénératives les plus répandues au monde. On estime actuellement qu'elle affecte plus de 35 millions de personnes dans le monde. Avec croissance continue de l'espérance de vie, le nombre de patients atteints devrait doubler tous les 20 ans. Les peptides bêta amyloïdes (Aβ) sont probablement les principaux responsables de la pathogenèse de la maladie d'Alzheimer. Ces peptides Aβ sont produits à partir du clivage séquentiel de la Protéine Précurseur de l'Amyloïde (APP) par les β- et γ-sécrétases. Une forme du peptide Aβ, qui possède 42 résidus d'acides aminés (Aβ42), est fortement agrégative et peut devenir neurotoxique. Les oligomères solubles de cette forme du peptide Aβ (Aβ42) sont actuellement considérés comme les principaux effecteurs du dysfonctionnement synaptique et de la perte neuronale dans la MA.

En conséquence, le développement de médicaments a récemment porté sur des thérapies potentielles ciblant spécifiquement ces oligomères toxiques. Alors que les traitements par anticorps ont positionné les oligomères Aβ en tant que cible thérapeutique viable pour la MA, les coûts de fabrication et d'administration élevés associés à un traitement à base d'anticorps soulèvent des préoccupations quant à l'accessibilité générale d'un tel traitement pour les patients. Alternativement, nous nous sommes concentrés sur la recherche de l'efficacité d'un petit inhibiteur peptidique d'oligomères toxiques de Aβ42 connu sous le nom de peptide interagissant avec les oligomères Aβ42 (AIP). Nous avons déjà démontré avec succès que l'AIP était capable de cibler *in vitro* les oligomères de Aβ42, ainsi que d'améliorer la perte de densité des épines dendritiques et la potentialisation à long terme (PLT) induites par l'Aβ42 (Barucker et al. 2015).

À travers cette étude, nous avons développé et caractérisé des modèles transgéniques de drosophile qui expriment l'Aβ42 humain dans différents tissus tels que l'œil et les neurones, ce qui nous permet essentiellement de générer des animaux qui acquièrent des déficits significatifs et observables induits par l'Aβ42, que ce soit des déficits morphologiques ou bien fonctionnels. Nous avons émis l'hypothèse que l'AIP peut neutraliser la toxicité induite par l'Aβ42 dans ces modèles *in vivo*. Nous avons ensuite évalué les effets longitudinaux de l'administration chronique d'AIP par voie orale sur des modèles de drosophiles transgéniques, et évalué l'efficacité de l'administration d'AIP par des tests à la fois morphologiques et fonctionnels. Par ailleurs, nos études montrent que le D-AIP résistant à la protéase était globalement non toxique pour les animaux, même administré longitudinalement. Nous avons

également pu sauver avec succès les dommages morphologiques induits par l'Aβ42 avec une supplémentation en D-AIP dans les yeux des mouches, même si ce n'est que chez les femelles. Cette divergence spécifique au sexe peut probablement être attribuée à la liaison non spécifique de D-AIP à un peptide spécifique de Drosophila de sexe mâle (Acp70a), qui pourrait potentiellement séquestrer et réduire la biodisponibilité globale de D-AIP chez les animaux mâles. En outre, dans un modèle transgénique séparé où Aβ42 est exprimé de manière pan-neuronale et altère le comportement fonctionnel, les activités locomotrices à long terme des mouches ont été significativement sauvées par D-AIP. Curieusement, les animaux transgéniques femelles présentant une expression pan-neuronale de Aβ42 n'étaient pas affectés par l'expression de Aβ42 dans les neurones. Des études ultérieures ont montré que les têtes de mouches transgéniques femelles présentaient une augmentation significative de la teneur en Aβ42 insoluble, ce qui aurait pu contribuer à une diminution globale de la toxicité (la toxicité étant généralement causée par les oligomères solubles), ainsi rendant les drosophiles femelles résistantes aux modifications de la locomotive induites par l'Aβ42.

Globalement, la D-AIP semble être un modulateur non toxique, stable et prometteur des oligomères toxiques de la Aβ42 *in vivo*. Des futures études sur des systèmes plus avancés de vertébrés, tels que les modèles MA transgéniques de souris et de rats, nous aideront à mieux comprendre le potentiel de la D-AIP en tant qu'agent thérapeutique. En fin de compte, nous espérons vivement et anticipons que les effets extrêmement bénéfiques de la neutralisation des oligomères de l'Aβ42 par D-AIP constitueront une stratégie formidable pour une intervention précoce dans la MA.

## CONTRIBUTION OF AUTHORS

As dictated by the McGill Thesis Guidelines, this thesis is composed of 5 chapters in a manuscript-based format. The chapters are composed of a literature review, three original manuscripts, and a discussion. One manuscript has been submitted to be published, while the other two are in preparation for publication. I am the first author of all three manuscripts. A detailed list and description of the contribution of individual authors are listed below:

**Manuscript I: The differential effects of human Aβ42-induced toxicity in the eye and central nervous system of *Drosophila melanogaster***
Yifei Zhong and Gerhard Multhaup

I designed and performed all experiments, analyzed the data, prepared and compiled the figures, and wrote the manuscript. GM led the research, designed experiments, and wrote the manuscript. All authors approved the final version of this manuscript.

**Manuscript II: The D-enantiomeric peptide AIP neutralizes toxic Aβ oligomers *in vivo* and rescues rough eye defects in transgenic *Drosophila melanogaster***
*Submitted to Journal of Neurochemistry*
Yifei Zhong, Adeola Shobo, Mark Hancock, and Gerhard Multhaup

I designed and performed all *Drosophila* and biochemical experiments, analyzed the data, prepared and compiled the figures, and wrote the manuscript. AS performed the MALDI-MSI and mass spectrometry experiments, analyzed data, and helped to compile figures and write the manuscript. MH generated the SPR data, analyzed data, and helped to compile figures, write, and edit the manuscript. GM led the research, designed experiments, and wrote the manuscript. All authors approved the final version of this manuscript.

**Manuscript III: The Aβ42-oligomer Interacting Peptide (D-AIP) attenuates amyloid-β42 induced decreases in *Drosophila melanogaster* locomotion**
Yifei Zhong, Adeola Shobo, Mark Hancock, and Gerhard Multhaup

I designed and performed all *Drosophila* and biochemical experiments, analyzed the data, prepared and compiled the figures, and wrote the manuscript. AS performed the MALDI-MSI and mass spectrometry experiments, analyzed data, and helped to compile figures and write the manuscript. MH generated the SPR data, analyzed data, and helped to compile figures, write, and edit the manuscript. GM led the research, designed experiments, and wrote the manuscript. All authors approved the final version of this manuscript.

## CONTRIBUTION TO ORIGINAL KNOWLEDGE

**I.** I successfully established two temperature-controlled human Aβ42-expressing transgenic models of *Drosophila melanogaster* to analyse the effects of human Aβ42-induced toxicity *in vivo* (Manuscript I).

**II.** Using climbing assays, I showed that human Aβ42 has minimal effects on the longevity and locomotor activity of eye-specific human Aβ42-expressing transgenic animals (Manuscript I).

**III.** I demonstrated that the expression of eye-specific human Aβ42 induced the rough eye phenotype in adults, but only when the transgenic protein is expressed during larval stages of the animals (Manuscript I).

**IV.** With this data, I proposed that the mature structure of the adult *Drosophila* eye is resistant to human Aβ42-induced toxicity (Manuscript I).

**V.** I concluded that human Aβ42 must be expressed during developmental stages in order to induce the rough eye phenotype (Manuscript I).

**VI.** I showed that human Aβ42 was generally neurotoxic and affected the longevity and locomotor activities of the transgenic animals when expressed in a neuronal-specific manner, regardless of the stage of development of the animal (Manuscript I).

**VII.** I propose that *Drosophila* neurons are generally susceptible to human Aβ42-induced toxicity (Manuscript I).

**VIII.** My data suggests that overall, different cells appear to have different susceptibilities to human Aβ42-induced toxicity (Manuscript I).

**IX.** I demonstrated that both L- and D-AIP supplementation have minimal effects on the longevity and locomotor activity of transgenic, eye-specific human Aβ42-expressing animals (Manuscript II)

**X.** Using live confocal microscopy methods, I showed that only D-AIP was able to ameliorate the human Aβ42-induced rough eye phenotype in the transgenics at day 5 post eclosion, albeit only in the female animals in a sex-specific manner (Manuscript II).

**XI.** Using advanced MALDI-MSI, I showed that L-AIP was rapidly degraded and undetectable in the animals, while D-AIP was stable and present at all times (Manuscript II).

**XII.** I propose that the stability and increased *in vivo* bioavailability of D-AIP contributed to the rescue of the eye phenotype (Manuscript II).

**XIII.** Comparing the MALDI-MSI data, I demonstrated that D-AIP was localized to the gut of the male transgenic animals at day 5 post eclosion, compared to the females which had D-AIP localization mainly in the thorax and head regions (Manuscript II).

**XIV.** Confocal data from day 28 post eclosion showed that the rescue of the human Aβ42-induced rough eye phenotype in the female transgenics was ameliorated, indicating that the effects brought on by D-AIP supplementation were reversible (Manuscript II).

**XV.** I found that at day 28 post eclosion, the distribution of D-AIP in the male transgenic animals have localized to the heads of the animals (Manuscript II).

**XVI.** I found an unknown protein that co-localized with human Aβ42 in the bodies of the male transgenic animals, and proposed that it likely sequestered D-AIP in a non-specific manner and lowered its bioavailability in the male animals (Manuscript II).

**XVII.** I identified the unknown protein to be Acp70A, a male-specific *Drosophila* sex peptide (Manuscript II).

**XVIII.** I demonstrated that Acp70A and D-AIP co-localized in the bodies of the male animals at day 5 post eclosion, and in the heads at day 28 post eclosion using MALDI-MSI (Manuscript II).

**XIX.** From these results, I proposed that Acp70A non-specifically sequestered D-AIP in the male transgenic animals only, thus limited its bioavailability and hindered its ability to rescue human Aβ42-induced eye degeneration in the males (Manuscript II).

**XX.** Using the neuron-specific human Aβ42-expressing strain, I showed that D-AIP supplementation was able to rescue defects in the locomotor behaviour of the transgenics, but only in the male animals in a sex specific manner (Manuscript III).

**XXI.** Comparing the climbing abilities of the female transgenics raised on non-supplemented or D-AIP supplemented food, I determined that the female neuron-specific human Aβ42-expressing animals were not susceptible to human Aβ42-induced toxicity (Manuscript III).

**XXII.** Immunoprecipitation data from fly head and body lysates show significantly increased amounts of non-soluble human Aβ42 in only the heads of the transgenic females (Manuscript III).

**XXIII.** With this data, I proposed that the increased pool of aggregated and non-toxic human Aβ42 in the heads of the female transgenic likely protected the animals from human Aβ42-induced toxicity (Manuscript III).

**XXIV.** Overall, I showed that D-AIP supplementation was able to attenuate human Aβ42-induced defects in both eye morphology and behaviour, and that D-AIP is well tolerated and efficacious in our transgenic fly models.

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# I. INTRODUCTION

# 1. Alzheimer's disease

## 1.1 History of AD and current statistics

In the early 1900s, Alois Alzheimer first described a particular form of dementia. His observations were based on a patient named Auguste D, a 51-years-old woman who had a striking cluster of symptoms that included reduced comprehension and memory, aphasia, disorientation, and paranoia amongst others symptoms (Maurer et al., 1997). Almost 70 years would pass after Alzheimer's first characterization of the disease before it would be recognized as an incredibly common cause of dementia and a major cause of death in the aged population (Katzman, 1976). Today, AD is estimated to affect 5.7 million people in the United States alone, and this number is expected to more than double by 2050 (Hebert et al., 2013; Alzheimer's, 2018).

There are currently no curative treatments that exist for AD, and available therapeutics are mostly used symptomatically to manage the disorder. Additionally, people aged 65 and older tend to survive an average of 4 to 8 years after AD diagnoses (Ganguli et al., 2005; Tom et al., 2015) – with some living as long as 20 years with AD (Brookmeyer et al., 2002; Larson et al., 2004). These individuals will likely spend an average of 40% of this time in the most severe stage of dementia, and approximately 75% of them will be significantly incapacitated and require placement in nursing homes by age 80 (Arrighi et al., 2010). The lack of cure and the long duration of illness before death adds significant burden to both personal caretakers and national agencies, and as patient population is projected to increase in the coming years, the situation could potentially precipitate into a global public health crisis. As such, it is now of paramount importance to strongly focus on the development of therapeutics to reduce symptoms, prevalence, or slow the onset of dementia in AD.

## 1.2 Neuropathology of AD

It is usually upon the histological examinations of the brain specimens that a definitive diagnosis of AD can be made. Here, the major pathologies identified in the autopsied brains of AD patients are the presence of neurofibrillary tangles (NFTs) and senile or amyloid plaques (Ramirez-Bermudez, 2012).

### 1.2.1 Neurofibrillary tangles

The NFTs consist of abnormal intraneuronal accumulations of hyperphosphorylated tau protein (Mandelkow and Mandelkow, 1998; Iqbal and Grundke-Iqbal, 2002; Crews and Masliah, 2010), which is a microtubule-associated protein (MAP) that stabilize neuronal microtubules in axons during cell process development and transport (Drewes et al., 1998). Many protein kinases are involved in tau hyperphosphorylation (Wang et al., 2013). Two kinases in particular – glycogen synthase kinase-3β (GSK-3β) and cell division protein kinase 5 (cdk5) – have been shown to phosphorylate tau at most of the known sites associated with AD and are currently targets of interest in the developments of therapeutics (Wang et al., 1998; Godemann et al., 1999; Liu et al., 2002; Porzig et al., 2007). It is thought that the abnormal hyperphosphorylation of tau (Mandelkow et al., 1995; Trojanowski and Lee, 1995; Delacourte and Buee, 1997) causes its dissociation from the microtubule, which then in turn instigates the breakdown of intracellular traffic and axonal dystrophy (Mandelkow and Mandelkow, 1998). The free, hyperphosphorylated tau can then sequester normal tau and other MAP proteins, and aggregates first into paired helical filaments (PHFs) then bundle into NFTs (Kidd, 1963, 1964; Wisniewski et al., 1976; Crowther, 1991). NFTs themselves have a stereotypic progression pattern in the brain that correlates with the severity of the cognitive decline, and its topographic staging is widely used for the pathological diagnosis of AD (i.e. Braak staging) (Braak and Braak, 1991).

### 1.2.2 Amyloid plaques

Amyloid or senile plaques are defined as extracellular deposits of the amyloid beta (Aβ) protein in the brain parenchyma (Kumar et al., 2015). They consist of a central core of Aβ, a small 4 kDa peptide, that is surrounded by abnormally configured neuronal processes or neurites (Perl, 2010) (**Figure 1**). There are some studies that suggest Aβ plaques can also enhance the formation of NFTs (Gotz et al., 2004; Seino et al., 2010). For example, Delacourte et al. suggested that there might be a synergistic interaction between Aβ- and tau-related pathologies despite differences in their spatiotemporal distribution where the detection of Aβ aggregates is linked to the appearance of tau pathology, but advanced tau pathology did not lead to the manifestation of Aβ aggregates (Delacourte et al., 1999; Delacourte et al., 2002).

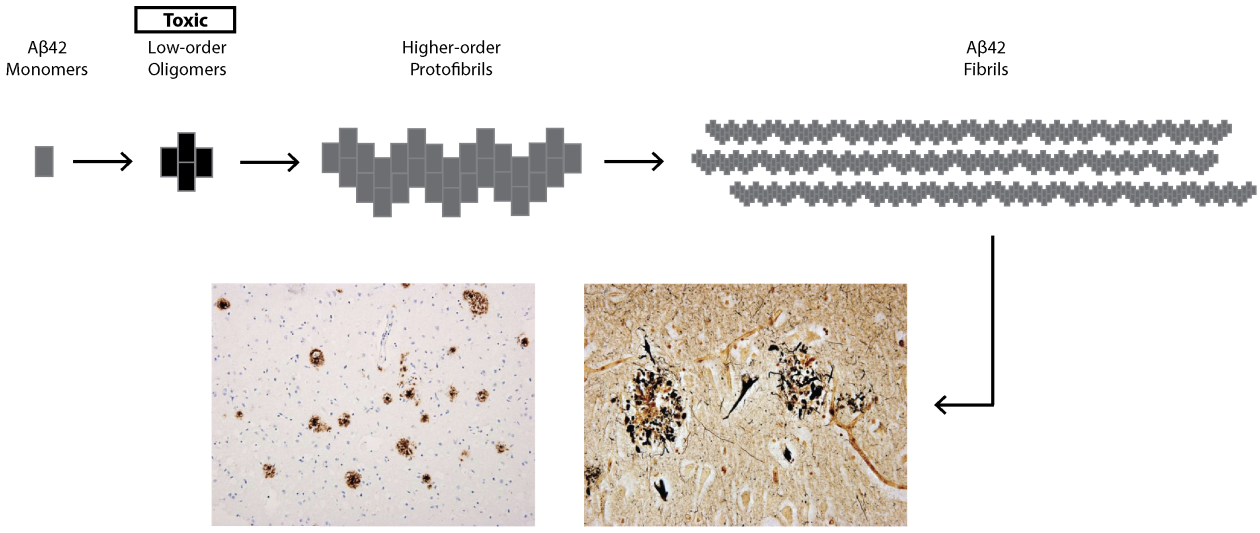


**Figure 1. Amyloid plaque formation from Aβ aggregation**
Simplified schematic of the Aβ aggregation pathway, Aβ monomers can aggregate into soluble, neurotoxic low-n oligomers. The oligomeric complex then undergoes rearrangement to assemble into protofibrils, which then go on to form mature fibrils and subsequently insoluble plaques. These plaques can be visualized in brain tissue of AD patients using stains or amyloid specific antibodies (dark brown stained patches). Micrographs taken from (Perl, 2010).

## 2. Aβ and the amyloid precursor protein (APP)

The process by which Aβ is generated has been well studied, and involves the sequential proteolytic cleavage of the amyloid precursor protein (APP). APP is a member of a family of conserved type I membrane proteins (Coulson et al., 2000). It has several isoforms derived from alternative splicing, where peptides with 695, 751, and 770 amino acids are the major forms. Of these, APP695 is the primary species produced by neurons, whereas APP751 and APP770 are produced by cells in the periphery such as platelets and leukocytes (Li et al., 1999).

### 2.1 Aβ production and APP processing

Full-length APP is first cleaved by β-secretase or β-site APP cleaving enzyme (BACE1), which generates a large, secreted derivative (sAPPβ) and a membrane bound fragment of 99 amino acids (β-CTF). β-CTF then undergoes a second cleavage, this time via γ-secretase (i.e. with active presenilin-1 or -2 (PSEN1/2)), to generate Aβ fragments and a cytosolic element, the APP intracellular domain (AICD). γ-secretase cleavage, however, is imprecise, which results in the production of Aβ species of various lengths. Of the Aβ species that are produced, the moiety with 40 amino acids (Aβ40) is the most abundant, while the form with 42 amino acids (Aβ42) is more hydrophobic, fibrillogenic, and is the primary species that are deposited in AD brains (**Figure 2**). Alternatively, APP can also be cleaved by α-secretase in a non-amyloidogenic fashion to produce α-CTF and sAPPα, which can then undergo γ-secretase cleavage to produce a small p3 peptide and AICD (reviewed in (Selkoe, 2001; Hardy and Selkoe, 2002; Zheng and Koo, 2006; Murphy and LeVine, 2010)).

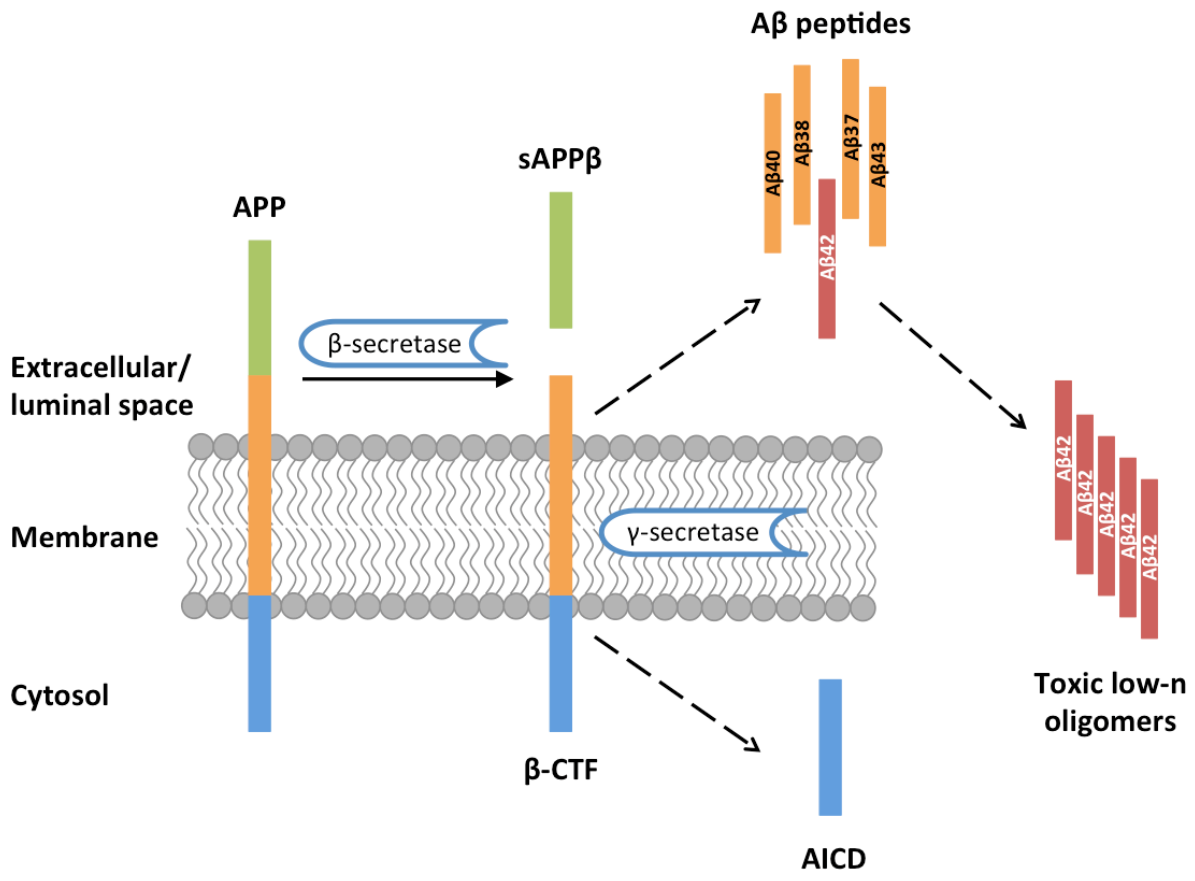


**Figure 2. The amyloidogenic processing of APP by secretases**
APP is sequentially cleaved first by β-secretase to produce soluble sAPPβ (green) and β-CTF (orange and blue), which is subsequently cleaved by γ-secretase to generate AICD (blue) and Aβ species of various lengths (orange). Aβ42 (red) in particular, is highly hydrophobic and prone to aggregation. Specifically, low order soluble oligomers of Aβ42 have been identified as the primary cause of synaptic dysfunction and cell death.

Aβ is mainly produced in the brain by astrocytes and neurons; however, non-neuronal tissues such as skin, skeletal muscle, and the intestinal epithelium have also been shown to secrete Aβ (Puig and Combs, 2013). It is normally soluble and secreted into the extracellular space of the brain and then cleared by the cerebrospinal fluid (CSF) and vascular system. The most abundant isoform of Aβ in the human brain is Aβ40 and the second most common being Aβ42 (Ida et al., 1996; Mo et al., 2015). The turnover of soluble Aβ is rapid in experiments with transgenic mouse models, and shows that it is generally cleared from the extracellular spaces and CSF with a half-

life of just 0.7-2.0 hours (Savage et al., 1998; Abramowski et al., 2008). The overall clearance of Aβ has been shown to be mediated in the periphery by the capillary beds of the kidneys, liver, gastrointestinal tract, and the skin (Xiang et al., 2015).

## 2.2 The amyloid hypothesis

The suspicion that Aβ was the causative component of AD was first initiated in the early 1980s (Glenner and Wong, 1984). Since then, the amyloid hypothesis has been the dominant model of AD pathogenesis (reviewed in (Hardy and Selkoe, 2002; Selkoe and Hardy, 2016)). Genetic mutations that cause familial AD (FAD) have also been discovered in three genes – *APP*, *PSEN1*, and *PSEN2* – which are all integrally involved in Aβ production (Bettens et al., 2013). Other genetic studies have also shown that duplication of the *APP* locus on chromosome 21, as well as extra copies of the chromosome itself in Down syndrome causes patients to develop Aβ plaque deposits and early-onset dementia (Prasher et al., 1998; Rovelet-Lecrux et al., 2006). Some FAD mutations are known to cause accelerated accumulation of amyloid plaques and early-onset dementia (Levy et al., 1990; Goate et al., 1991; Tsubuki et al., 2003; Tomiyama et al., 2008; Bettens et al., 2013), and have been shown to either increase the level of APP processing by secretases or increase the production of the amyloidogenic Aβ42 species (Citron et al., 1992; Eckman et al., 1997; Chavez-Gutierrez et al., 2012). Other APP mutations in the middle of the Aβ coding sequence can also increase the aggregation propensity or inhibit degradation of Aβ peptides (Tsubuki et al., 2003; Tomiyama et al., 2008). Furthermore, a recent mutation that was discovered in APP, which diminished amyloidogenic Aβ production, appears to be protective against the development of AD (Jonsson et al., 2012). Overall, this evidence seem to largely indicate that it is Aβ production and aggregation that drives FAD.

While the genetics of sporadic AD – the vast majority of disease cases – are more complex, the apolipoprotein *APOE* ε4 allele has been found to have the greatest increase in risk for AD (Corder et al., 1993; Chiang et al., 2010; Verghese et al., 2011). ApoE4 appears to exert a variety of effects in the brain, but it has been shown to be a strong modulator of A $\beta$ pathology. For example, knockin mice expressing human ApoE4 with APP and PSEN1 FAD mutations have greatly increased Aβ plaque pathology and reduced Aβ clearance (Castellano et al., 2011;

Verghese et al., 2011), while transgenic mice expressing both human APP with FAD mutations and the protective ApoE2 protein have decreased amyloid plaque pathology (Fagan et al., 2002). Additionally, the reductions of ApoE levels have also been shown to reduce A $\beta$ plaque burden in mice (Bales et al., 1997; Kim et al., 2011; Bien-Ly et al., 2012; Kim et al., 2012).

The evidences from studies on both sporadic and familiar AD suggest that Aβ production and aggregation have strong effects on disease pathogenesis. Aβ is likely the key initiator of AD pathogenesis, which forms the central tenet of the amyloid hypothesis.

### 2.3 The role of Aβ in AD pathogenesis

While there is an undeniable linkage between Aβ and AD pathogenesis, the correlation is poor, both temporally and anatomically, between the deposition of Aβ, neuronal death, and the appearance of clinical symptoms in AD. Aβ deposition seems to occur first and more severely in brain regions such as the precuneus and frontal lobes, while neuronal death has generally been reported to begin and occur most readily in the entorhinal cortex and hippocampus, where there are few Aβ plaques (Braak and Braak, 1991; Serrano-Pozo et al., 2011). Tau pathology actually correlates much more closely with the pattern of neuronal loss, in both spatial and temporal manners, compared to amyloid plaque deposits (Arriagada et al., 1992b; Arriagada et al., 1992a; Gomez-Isla et al., 1997; Serrano-Pozo et al., 2011). This has led to the criticism and suggestion that perhaps Aβ is not the true mediator of neurodegeneration in AD. FAD cases, however, have helped to shed some light on the situation. Aβ is known as the main driver of disease pathology in FAD, and interestingly, the pathology in FAD closely resembles what is observed in the sporadic disease, where Aβ plaque deposition is anatomically disconnected from areas of severe neuronal loss (Shepherd et al., 2009; Bateman et al., 2012). While the mechanism for this disconnection between Aβ deposition, tau pathology, and neuron death is not yet clear, genetic data shows that it is possible for Aβ to drive tau pathology and neuron loss without obvious colocalization between plaques and neurodegeneration (Musiek and Holtzman, 2015).

Interestingly, it has been shown previously that aggregated and phosphorylated tau pathology can be observed in both the brainstem and entorhinal cortex of asymptomatic people (Braak and Del

Tredici, 2011), and with age, hippocampal neurofibrillary tau pathology is almost ubiquitous in the region, but seems to be confined to limbic regions in cognitively normal and amyloid-free individuals (Price and Morris, 1999; Elobeid et al., 2012). However, tau seems to be able to spread into neocortical regions only in people with coexistent Aβ pathology, who will then generally go on to develop AD dementia (Price and Morris, 1999; Knopman et al., 2003). This and other studies examining the cognitively normal or AD patients suggests that Aβ is required for tau pathology and toxicity (West et al., 1994; Gomez-Isla et al., 1996; Petersen et al., 2006).

### 2.4 Soluble Aβ drives AD pathology

Soluble Aβ – more specifically, the neurotoxic Aβ42 species – can be purified from brain regions that are subjected to intense neuronal loss such as the hippocampus (McLean et al., 1999; Tomic et al., 2009; Esparza et al., 2013). Soluble oligomers of Aβ42 have been identified in AD brain lysates that can exert a wide variety of pathogenic effects both *in vitro* and *in vivo* (Walsh et al., 2002; Shankar et al., 2008; Mucke and Selkoe, 2012). Shankar et al. showed that amyloid plaque cores isolated from AD brains did not impair long-term potentiation (LTP) *in vitro*, but the Aβ42 oligomers that were released via solubilizing plaques with harsh denaturants could impair LTP (Shankar et al., 2008). As well, they found that oligomeric Aβ42 isolated directly from AD cortex could decrease synaptic number and function, and impair memory in healthy adult rats (Shankar et al., 2008). In studies with post-mortem human brain tissues, it was also found that non-demented brains that were plaque-rich had much lower oligomer-to-plaque ratios compared to the mildly demented plaque-rich patients (Esparza et al., 2013). These and other results demonstrating the toxicity of soluble Aβ42 oligomers led to the hypothesis that amyloid plaques are likely a protective response where toxic soluble oligomers are temporarily sequestered to prevent further toxicity (Hong et al., 2014).

There is also some evidence indicating that Aβ42 oligomers can drive tau pathology. For example, crossing human APP expressing transgenic mice with human Tau expressing transgenic mice significantly enhanced tau deposition but did not change overall Aβ deposition (Lewis et al., 2001). As well, treatment with soluble Aβ42 oligomers isolated from AD cortex induced neuritic dystrophy and tau hyperphosphorylation in primary rat hippocampal neurons (Jin et al., 2011).

Generally, the expression of tau seems to enable and amplify the neurotoxic downstream consequences of Aβ42 (Roberson et al., 2011; Maruyama et al., 2013).

Taken as a whole, Aβ peptides and specifically soluble Aβ42 oligomers seem to play a central role in AD pathogenesis by facilitating neuronal loss, synaptic dysfunctions, downstream tau dysfunctions, and other AD-related pathologies. As such, Aβ42 oligomers have become viable drug targets in the development of therapeutics for AD.

## 3. Animal models of AD

The progress of AD research over the past two decades have uncovered much information regarding the susceptibility and causative genes associated with AD, as well as proteins involved in the pathogenic process such as the secretases and APP (reviewed in (Van Cauwenberghe et al., 2016)). This has profoundly facilitated the development and availability of genetically altered and/or transgenic animal models. These animal models of AD have played major roles in defining disease-related mechanisms as well as in the evaluation of novel therapeutic approaches. As there are a large variety of AD animal models – both in invertebrates and vertebrates – currently used in research, selection of the model that is most appropriate and useful typically depends on the scientific questions to be answered.

### 3.1 Mammalian models of AD

Neurodegeneration related to specific human diseases such as AD is not a global event and usually initiates in specific brain compartments. This makes the complex brains structures of higher order mammalian vertebrates (i.e. with greater homology to human physiological processes and anatomical structures) particularly excellent *in vivo* models of human neurodegenerative diseases.

### 3.2 Transgenic rodent models

Mammalian rodent models are commonly used in AD studies, as they are easier and more cost effective to maintain compared to non-human primates (NHPs) and other large mammalian models. They also have relatively short life spans to facilitate long-term studies, and are amenable to transgenic techniques. Although rodents are genetically more distant to humans compared to the NHPs, their gene expression profiles have been shown to be similar to humans (Strand et al., 2007; Chan et al., 2009; Zheng-Bradley et al., 2010).

### 3.2.1 Mouse

Murine models are currently the most popular animal choice for modeling AD *in vivo*, as transgenic techniques are well established in the mouse and it is relatively easy to generate transgenic lines (Doyle et al., 2012). The cognitive abilities of mouse models are well characterized and have been shown to accurately emulate neurodegeneration in humans along with the associated cognitive and behavioural changes (Webster et al., 2014).

A wide variety of transgenic mouse models have been developed for AD. FAD-associated mutations (such as in *APP*, *PSEN*, and/or *MAPT* (tau)) can be introduced to the existing murine genetic background, and driven by strong neural-specific promoter such as the platelet derived growth factor-beta (PDGP) promoter (Hsia et al., 1999; Mucke et al., 2000), hamster prion promoter (PrP) (Borchelt et al., 1996; Hsiao et al., 1996; Chishti et al., 2001), or thymocyte antigen promoter (Thy1.2) (Lewis et al., 2000; Richards et al., 2003) which allows up to 30-fold overexpression of the AD associated transgene (depending on the promoter and genetic background of the strain) (Jankowsky et al., 2005). Artificial chromosome-based technologies can also allow for the induction of transgenes – which have large genomic fragments such as the *cis*-acting elements that are required to regulate gene expression – under the control of native promoter to more accurately mimic the normal expression patterns of the endogenous gene (Newman, 2017).

### 3.2.2 Rat

While mouse models of AD are more commonly used, much effort has also been made to establish various transgenic rat models of AD. They offer an advantage over mouse models in that they are much closer to humans in terms of genetics, physiology, and neurohistology (Jacob and Kwitek, 2002; Gibbs et al., 2004; Tesson et al., 2005). Rats are also better performers in behavioural tests compared to mice, and their larger bodies are more amenable to procedures such as surgery and neuroimaging (Tesson et al., 2005). Transgenic rats expressing human APP (with or without FAD mutations) have been developed to model Aβ deposition (Folkesson et al.,

2007; Flood et al., 2009; Kloskowska et al., 2010; Leon et al., 2010). However, a transgenic rat model that exhibits both amyloid plaques and NFT deposition has yet to be developed.

### 3.2.3 Pitfalls of rodent models

While rodent models are easier and more cost effective to maintain compared to NHPs and other complex mammalian models, it can still take anywhere from months up to years for pathology to manifest depending on the model used. As such, it is generally not considered to be an appropriate model system for high-throughput screening studies of potential therapeutic compounds. It must also be noted, however, that rodents themselves are poor natural models of AD, and do not typically exhibit pathological hallmarks of the disease, due to perhaps the differences between mouse and human Aβ and tau species, as well as the aggregation propensity and states of these proteins (reviewed in (Newman, 2017)). Transgenic animals expressing multiple human FAD-associated genes are commonly used instead to study FAD related mutations in genes involved in Aβ metabolism (i.e. *APP*, *PSEN*, *MAPT* (tau)), but as there are currently no known human AD patients with multiple pathogenic FAD mutations in AD-associated genes, none of the transgenic mouse strains can truly claim to mimic the genetics of FAD to the full extent.

The relevance of these models to the much more widespread, sporadic, and late-onset AD is also unclear. Although genetic predisposition is indeed associated with sporadic AD, environmental, metabolic, as well as lifestyle risk factors are also thought to play important roles in disease pathogenesis (Stozicka et al., 2007; Piaceri et al., 2013; Chakrabarti et al., 2015). Recently, a publication by Hargis and Blalock suggested that these AD transgenic mouse models are really models of amyloid deposition only (Hargis and Blalock, 2017). They examined the concordance between brain gene expression changes in transcriptome data from humans and rodents during normal aging, as well as in human AD patients and in five distinct transgenic mouse models of AD. Their results indicate that there was very little concordance between human AD and mouse models, or even between the various mouse models themselves (Hargis and Blalock, 2017). As a result, current efforts are underway to investigate and develop better mammalian models of AD that takes into consideration the complex etiology and progression of the sporadic disease.

### 3.3 Invertebrate models of AD

Invertebrates have become increasing popular models for human degenerative disorders in the last few decades (Alexander et al., 2014; Fernandez-Funez et al., 2015). They tend to be much more inexpensive and easy to maintain compared to the rodent models, reproduce quickly, and have a relatively short life span, which allows for the study of neurodegeneration in a timeline of weeks instead of months to years compared to even the rodent models. Additionally, a plethora of molecular and genetic techniques are available in invertebrate systems, and their development and anatomy have been well studied and described. As such, invertebrate models are often used in unbiased, large-scale genetic screens, which makes them potent tools for investigating the genetic mechanisms and pathways underlying neurodegenerative diseases such as AD (reviewed in (Newman, 2017)).

#### 3.3.1 *Drosophila melanogaster*

The fruit fly *D. melanogaster* is a widely used invertebrate model for the study of neurodegenerative diseases. There is a high degree of gene conservation between humans and flies (Wangler et al., 2015), and it has a much more complex nervous system – compared to other commonly used invertebrate models such as *Caenorhabditis elegans* – that is composed of approximately 200,000 neurons (Zars et al., 2000), and organized into distinct brain regions with specific functions similar to the vertebrate brain nuclei. The fly brain also contains several types of morphologically distinguishable glial cells, including some that form an invertebrate blood-brain barrier (BBB) (Parker and Auld, 2006; Hartenstein, 2011), which greatly facilitates the testing of drug permeability in the brain. Additionally, a wide array of learning and memory assays are available to assess neurodegeneration in both short and long term studies in the fly.

A variety of genetic tools have been developed for *Drosophila*, such as the use of P-elements, which are transposable elements that allow stable integration of foreign genes into the fly genome (Rubin and Spradling, 1982). The most widely used system for transgenic expression of proteins, however, is the binary expression system developed by Brand and Perrimon in the early 1990s, which employs the use of the yeast transcription factor Gal4 in a manner similar to the Cre/lox

system commonly used in transgenic mice (Brand and Perrimon, 1993). This method allows for the ectopic expression of human proteins in the fly in a cell- or tissue-specific manner by performing a simple genetic cross (**Figure 3**). Individual fly promoters (i.e. with expression specific to a cell type or tissue of interest) were fused to Gal4 to create the promoter or driver strains. Transgenic genes of interest such as human A $\beta$ s and other AD-related proteins were cloned downstream of the upstream-activating sequence (UAS) (i.e. the yeast Gal4 binding region) to generate UAS-human transgene containing strains. The subsequent crossing of these strains can then generate transgenic F1 flies with the desired transgene expression in the tissue of interest.

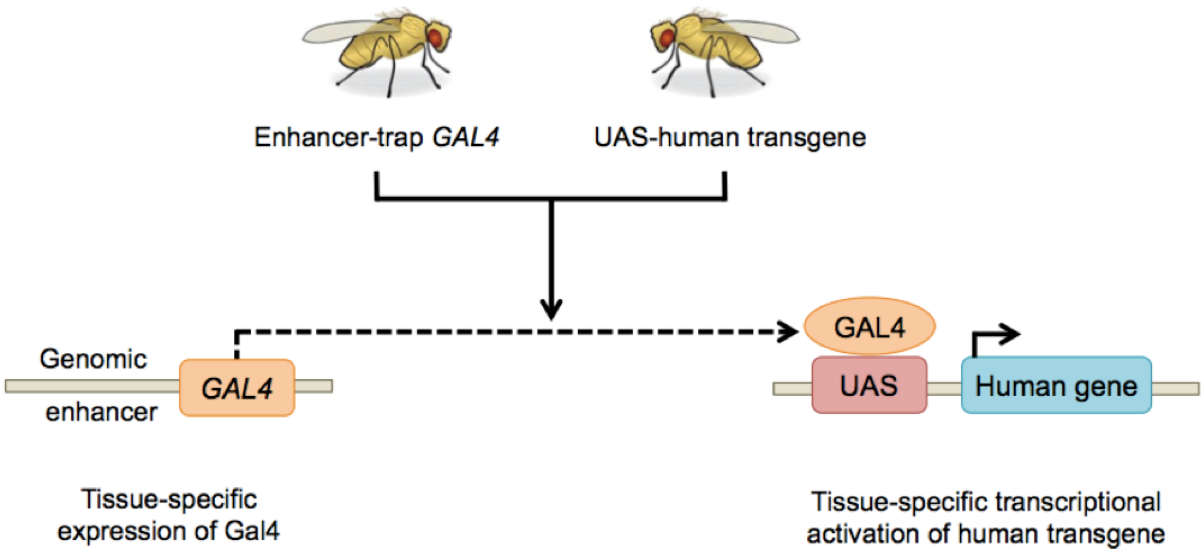


**Figure 3. The *Drosophila* UAS/Gal4 expression system**
The binary *Drosophila* UAS/Gal4 system requires the generation of two strains: the cell/tissue-specific driver or enhancer-trap Gal4 strain, and the upstream-activation sequence (UAS) containing strain with transgene inserted downstream. Genetic crossing of these two strains results in the expression of the transgene in the cell or tissue of choice of the F1 offsprings, driven by the promoter linked with Gal4.

Transgenic *Drosophila* have been created using the UAS/Gal4 system that express wildtype human Aβ40, Aβ42, and Aβ associated with familial forms of AD (Fossgreen et al., 1998; Gunawardena and Goldstein, 2001; Iijima et al., 2004; Crowther et al., 2005). These studies have shown that only the induction of Aβ42 caused progressive degeneration and plaque formation in these animals, where Aβ40 induction did not (Finelli et al., 2004; Iijima et al., 2004; Crowther et al., 2005). Other studies also showed that the *in vivo* toxicity observed in these animals correlated with the levels of soluble Aβ42 oligomers (Speretta et al., 2012). Additionally, Aβ42 expression in transgenic flies induced other AD-related phenotypes such as synaptic deficits (Jang and Chung, 2016; Zhang et al., 2016) and neuronal hyperactivity (Ping et al., 2015).

However, even though the fly brain is much more complex than brains of other lower order invertebrates, it still lacks the complexity found in mammalian brains. As a result, their behavioural repertoire is comparatively small and cannot mimic the complex behaviour patterns found in mammals. Flies also have open vascular systems that lack vessels, preventing the study of vascular effects of AD. However, while invertebrate systems such as *C. elegans* and *Drosophila* cannot model the full spectrum of AD pathology seen in humans, they still make excellent models of specific disease processes (i.e. Aβ42-induced toxicity). Combined with their ease of use and cost effectiveness, invertebrate systems are often used as a high- to medium-throughput and economical system for drug screening (Gunawardena and Goldstein, 2001; Greeve et al., 2004), often prior to more complex and costly studies in mammalian models.

Divergent results on gene activation, cellular processes, and neurophysiological events obtained from current AD models – particularly the transgenic rodents – have demonstrated that it may be wise to use multiple models for testing of potential therapeutics, rather than relying on a single species. For example, it may be beneficial to first screen for compound efficacy in fast but relatively reductive invertebrate models such as *Drosophila*, then subsequently further validate the resulting hits in more complex mammalian models such as rodents. The use of multiple model systems would allow for the cross validation of potential therapeutics, and hence increase the predictive therapeutic value of the compound.

## 4. Therapeutics in development for AD

Over the past decade, the focus of drug discovery and development effects for AD has been mostly on disease modifying therapies – therapies that aim to affect the underlying process by impacting one or more of the many brain changes characteristic of AD.

### 4.1 Secretase inhibitors and modulators

Amyloidogenic, APP processing secretases such as β- and γ-secretase are considered to be particular interesting AD drug targets, as they are central to the generation and modulation of Aβ and can be directly targeted by small compounds both *in vitro* and *in vivo* (De Strooper et al., 2010).

*β-secretase*

BACE1 has been postulated to be an ideal therapeutic target for AD since its inhibition should decrease all forms of Aβ. Indeed, Ohno et al. showed in 2007 that BACE1-deficient 5XFAD mice (with APP Swedish, London, Florida, and PS1 with two FAD mutations) do not produce Aβ or develop amyloid plaques and memory deficits compared to 5XFAD mice carrying wildtype BACE1 genes (Ohno et al., 2007). There are, however, some potential issues with BACE1 targeting and inhibition. Since the active site of BACE1 is quite large, compounds that target BACE1 are also quite large and as a result, have low membrane permeability and difficulties crossing the BBB (Ghosh et al., 2012). As well, BACE1 is also involved in the processing of many other substrates other than APP that may give rise to more adverse side effects if the enzyme is inhibited. While BACE1 knockout mice have abrogated Aβ expression, they unfortunately also display a variety of defects such as cognitive and memory problems, as well as higher mortality rates early on in life (Laird et al., 2005).

A multitude of BACE inhibitors are in development with varying results. Verubecestat, developed by Merck, was perhaps the most high profile BACE inhibitor in recent development. It

had great safety profiles in two Phase 1/2 trials, and proceeded to Phase 2/3 trial in late 2012. However, by 2017, the trial was prematurely ended after an interim analysis due to lack of efficacy (Egan et al., 2018). Interestingly, treatment with Verubecestat was found to significantly decrease the concentrations of Aβ40, Aβ42, and sAPPβ in the CSF of patients in the treatment group compared to the placebo group, which confirms that the drug had the intended action of reducing Aβ production (Egan et al., 2018). This suggests that once dementia is present, AD progression may become independent of Aβ. Since Aβ deposition begins years before any clinical symptoms are present, these failed trials have fuelled the debate as to how early these BACE inhibitors would need to be given to be effective. Still, there is hope that advances in technological diagnostic tools will allow for the better identification of patients at risk of developing AD, and will enable clinical trials at preclinical stages when BACE1 inhibitors are expected to have the largest impact on disease progression (Voytyuk et al., 2018).

*γ-secretase*

The γ-secretase has been characterized as a high molecular weight complex that consists of four subunits: presenilin (PS1 or PS2), nicastrin, anterior pharynx defective-1 (APH-1), and presenilin enhancer-2 (PEN-2) (De Strooper, 2003). The presenilins seems to be the active core of the protease. Similar to BACE1, the γ-secretase is an extremely attractive therapeutic target for AD due to its essential role in Aβ generation. However, since γ-secretase is also known to have multiple substrates other than APP – the most important one being Notch – severe consequences are sometimes observed when it is inhibited completely. Semagacestat, a γ-secretase inhibitor developed by Eli Lilly, entered Phase III trials but was terminated before completion as the drug not only failed to improve the cognitive status of patients, but was associated with a host of adverse side effects such as skin cancers and infections which were attributed to concomitant Notch inhibition (Doody et al., 2013). As well, the γ-secretase inhibitor avagacestat that was developed by Bristol-Myers-Squibb was abandoned after Phase II trials for similar reasons (Coric et al., 2012). From these negative results, it was clear that perhaps the total inhibition of γ-secretase activity was not a feasible therapeutic target.

As a result, currently there are two strategies to hopefully circumvent the issue: one is to develop APP selective secretase inhibitors that are able to spare Notch signaling, and the second is to develop γ-secretase modulators, which shifts the production of Aβ to shorter and less toxic forms as well as spare Notch (Zhang et al., 2014). The results so far are not very promising: ELND006 from the Elan Corporation was reported to be more selective against inhibiting APP processing than semagacestat, but it was halted in the clinical trial stage due to liver toxicity (Hopkins, 2011). A different Notch-sparing γ-secretase inhibitor called Begacestat developed by Wyeth was discontinued after Phase I trials (ClinicalTrials.gov Identifier: NCT00959881). As for the γ-secretase modulators, one drug that had much promise was tarenflurbil that was developed by Myrexis. It showed positive results in Phase II trials where it slowed the rate of decline in patients with mild AD, but was abandoned after Phase III trials after due to lack of efficacy (Green et al., 2009). It is interesting to note that as of early 2018, there are currently no γ-secretase modulators and/or modulators that are in any phase of clinical trials (Cummings et al., 2018a).

### 4.2 Anti-Aβ therapies

The most popular anti-Aβ treatments that are currently in development are immunotherapies or the passive administration of exogenous antibodies (a selected list is summarized in **Table 1**). These anti-Aβ monoclonal antibodies (mAbs) have generally been engineered to bind and clear Aβ, and many have proceeded through to clinical trials. There are some major drawback of mAb treatments, namely the need for repeated administrations and the associated cost of their production, which could affect their potential accessibility to the general global public (Lemere, 2013).

Bapineuzumab (epitope at Aβ 1-5) was one of the first mAb to enter human testing that recognized Aβ monomers, oligomers, and fibrils; it was found to be generally safe and well tolerated in Phase 1 trials (Black et al., 2010). Interestingly, in the study, 3 out of 10 participants in the highest dose group developed magnetic resonance imaging (MRI) abnormalities that were consistent with vasogenic edema, which later resolved. These events prompted the formation of a Workgroup in July 2010 by the Alzheimer's Association Research Roundtable, which coined the

term amyloid-related imaging abnormalities (ARIA) to refer to the MRI signal alterations that are associated with Aβ-modifying therapies (Sperling et al., 2011). Bapineuzumab went on to Phase 2 and 3 trials, but was discontinued in August 2012 due to high rates of symptomatic ARIA occurrences (Salloway et al., 2009; Rinne et al., 2010; Salloway et al., 2014).

Next, solanezumab and gantenerumab were developed to target the central amino acids of Aβ (epitope at Aβ 16- 26, and Aβ 3-12/18-27 respectively) (van Dyck, 2018). Both mAbs proceeded through to Phase 2 and 3 trials, but ultimately no significant slowing of decline as well as reduction of Aβ and tau PET biomarkers were found, and the trials were subsequently terminated (Ostrowitzki et al., 2017; The Lancet, 2017). However, as both mAbs had excellent safety profiles and showed encouraging trends in mild AD cases, they are currently being evaluated in prevention trials by the Dominantly Inherited Alzheimer Network (DIAN) for its phase 2/3 trial in individuals at risk for and with early-stage autosomal-dominant AD (Bateman et al., 2017). Crenezumab, another antibody that targets the mid-domain of the Aβ peptide (epitope at Aβ 13-24) and had high affinity for oligomers (Adolfsson et al., 2012; Ultsch et al., 2016), is also currently under evaluation after no significant treatment benefits were observed in Phase 2.

**Table 1.** A selection of Aβ-targeting monoclonal antibodies and their trial status

| Antibody | Epitope (Aβ) | Recognized Aβ conformation | Status | References |
|---|---|---|---|---|
| **Bapineuzumab** | 1-5 | Monomers, oligomers, and fibrils | Not in development | (Salloway et al., 2009; Rinne et al., 2010; Salloway et al., 2014) |
| **Solanezumab** | 16-26 | Monomers | Phase 2/3 | (Doody et al., 2014; Bateman et al., 2017) |
| **Gantenerumab** | 3-12, 18-27 | Monomers, oligomers, and fibrils (high affinity for oligomers) | Phase 2/3 | (Ostrowitzki et al., 2012; Bateman et al., 2017) |
| **Crenezumab** | 30-40 | Monomers, oligomers, and fibrils | Phase 3 | (Reiman et al., 2011; Cummings et al., 2018b) |
| **BAN2401** | Protofibrils | Protofibrils | Phase 2b | (Lannfelt et al., 2014) |
| **Aducanumab** | 3-6 | Oligomers and fibrils | Phase 3 | (Sevigny et al., 2016) |

Lastly, BAN2401 and aducanumab are currently considered to be the most promising mAb treatments in development. BAN2401 is a humanized IgG1 mAb that selectively binds and clears soluble Aβ protofibrils (Tucker et al., 2015). It was shown to be well tolerated in Phase 1/2a study, and is currently in Phase II trials (Lannfelt et al., 2014). Aducanumab is known to selectively react with Aβ aggregates, including both soluble oligomers and insoluble fibrils (Sevigny et al., 2016). It was developed by screening libraries of memory B cells from healthy elderly individuals for reactivity against aggregated Aβ. A Phase 1b trial of aducanumab has been completed with prodromal or mild AD patients, and exploratory analysis of clinical assessments demonstrated encouraging, dose-dependent slowing of disease progression at 1 year of treatment (Sevigny et al., 2016). Based on this promising Phase 1b study, aducanumab is now currently in Phase 3 trials as of August 2015 (ClinicalTrials.gov Identifier: NCT02477800 and NCT02484547).

As with secretase modulation, there are concerns that immunotherapy trials may be started too late in disease when too much Aβ has accumulated. Again, early intervention – before the Aβ cascade is irrevocably initiated – is likely key to prevent the onset of neurodegeneration.

### 4.3 Other targets of treatment

Most therapies that are currently in development for AD are amyloid-targeting agents, but there is an increase in non-amyloid mechanisms of action for drugs in earlier phases of development (Cummings et al., 2018a). For example, there are also increased numbers of agents that are directed at tau-related targets. Previous anti-tau therapies were largely disappointing, but there are some interests now to revisit these agents (reviewed in (Bakota and Brandt, 2016)). More recently, immunotherapy strategies have been a more popular approach, with seven tau immunotherapies entering Phase 1 or 2 testing as of 2018 (Cummings et al., 2018a). The development of tau radioligands that are detectable by PET is also expected to provide insights to how well these compounds are able to target the protein (Maass et al., 2017).
A variety of compounds are also under development for symptomatic therapies. They are of particular interest as there are few agents in development that will target moderate to advance stages of AD, where symptomatic treatment may have the best chance at improve the quality of life for patients at these advance levels of disease.

### 4.4 Peptidic inhibitors

A class of therapeutics that has been given renewed consideration in the last ten years are therapeutic peptides, which are defined as proteins of 50 amino acids or less. They are often designed to inhibit protein-protein interactions, as proteins typically interact via large surfaces, which is a challenge for small molecules to address and often require the use of high molecular sized inhibitors (i.e. MW 1-2 kDa) (Nevola and Giralt, 2015; Tsomaia, 2015). Also, most interaction surfaces of proteins are relatively featureless and lack pre-formed and well-defined hydrophobic cavities which can fully accommodate a small molecule ligand (Tsomaia, 2015). Peptides can bind to large protein targets with high potency and great selectivity, which can translate into fewer off-target side effects and potential for toxicity compared to small molecule

drugs (Craik et al., 2013). This is because unlike small molecules that often trigger side effects by producing toxic metabolites that accumulate (Ahrens et al., 2012), peptides generally degrade into amino acids, which can minimize the risk of toxicity. It is also much cheaper to manufacture peptides compared to recombinantly produced antibodies; they are more stable at room temperature, and have a better ability to penetrate tissues due to their smaller size (Tomlinson, 2004; Vlieghe et al., 2010; Miller et al., 2013). Moreover, peptidic sequences can often be altered to incorporate non-natural building blocks and various chemical scaffolds and modifications to easily alter their functionality and diversity.

### 4.4.1 Development of peptidic inhibitors for AD

Since the protein aggregation of Aβ is a primary feature of AD, the inhibition of both oligomer and fibril formation has remained a goal of treatment (**Figure 4**). Peptide-based inhibitors of amyloid growth have been the subject of much attention in the last two decades (reviewed in (Sciarretta et al., 2006). In the late 90s, Tjernberg et al. showed that a short Aβ fragment (KLVFF; Aβ 16-20) was able to bind to full-length Aβ and prevent its assembly into amyloid fibrils (Tjernberg et al., 1996). Continuing on this work, Pallitto et al. developed a more effective hybrid molecule using the KLVFF sequence by adding a lysine hexamers-disrupting element, and showed that it was able to fully protect PC-12 cells from Aβ-induced toxicity (Pallitto et al., 1999). They then went on to develop a series of KLVFF peptide derivatives with a range of protective effects against Aβ treatment (Lowe et al., 2001; Gibson and Murphy, 2005).

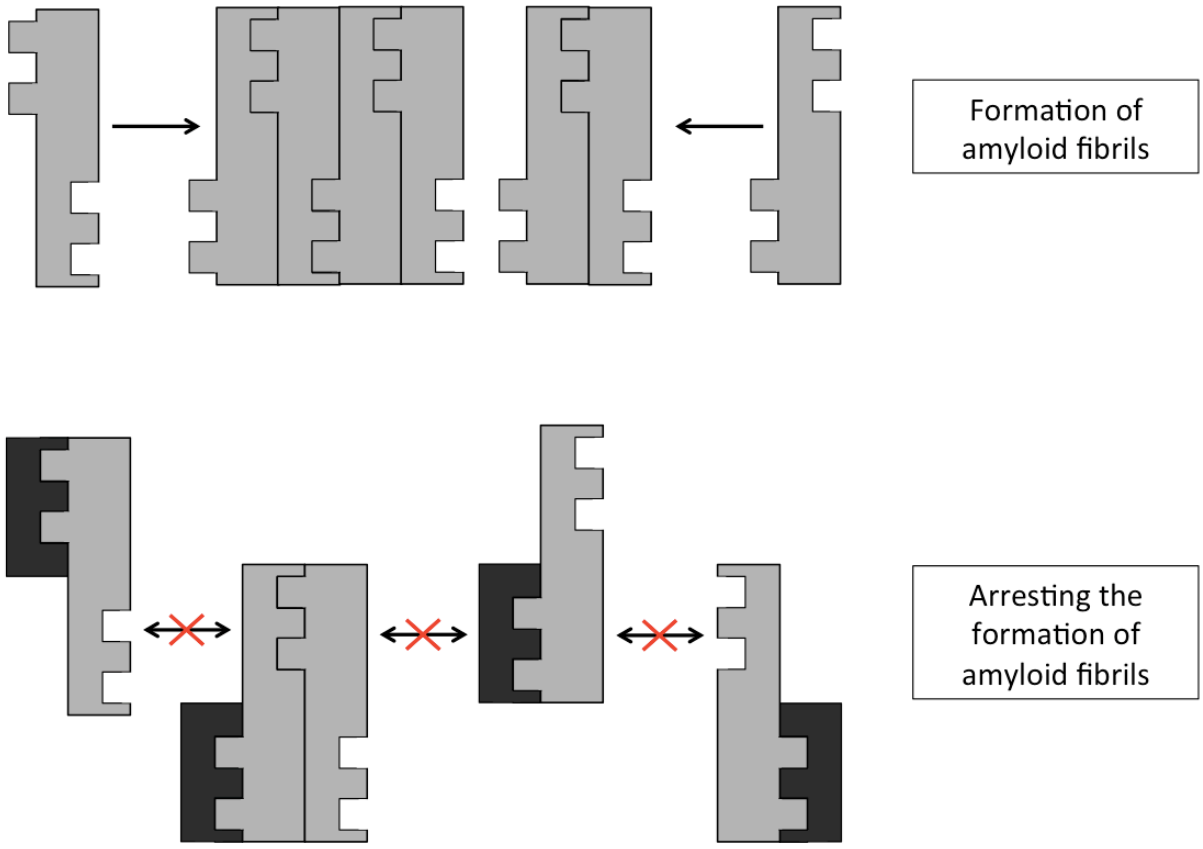


**Figure 4. The mechanism of action of peptidic inhibitors on amyloid aggregation**
Aβ molecules (shown in gray) can interact with each other to form oligomers and larger aggregates, which may lead to the eventual formations of large amyloid fibrils and plaques. Small peptidic inhibitors (shown in black) may be able to bind to key sequences or motifs in the Aβ molecule that are important for polymerization and arrest their aggregation to higher order oligomers, fibrils, and plaques. (Adapted from (Tjernberg et al., 1996))

Later, Gronwall et al. discovered a variety of homologous Aβ-binding affibody molecules (small antibody mimetics of about 6 kDa) from a phage display library (Gronwall et al., 2007). It was later shown that Aβ binding by one of such affibody molecule (name ZAβ3) was able to inhibit amyloid fibril formation as well as further aggregation when it was added to solutions in which amyloid was already being formed (Hoyer et al., 2008; Luheshi et al., 2010). Coexpression of ZAβ3 in the brains of *Drosophila melanogaster* with Aβ42 or the FAD associated mutation Aβ42 E22G abolished the detrimental effects of the Aβ42 peptides on the longevity of the animals, and seemed to be able to both capture monomeric Aβ42 as well as dissolve oligomers (Luheshi et al.,

2010). Our lab has been interested in the three consecutive repeats of the GxxxG motif that encompasses Aβ residues Gly33 to Gly37, and has showed previously that this motif is particularly important in the oligomerization and toxicity of Aβ42 (Munter et al., 2007; Harmeier et al., 2009). The GxxxG motif forms molecular ridges and grooves on the amyloid surface, which are proposed to facilitate the sheet-to-sheet stacking of aggregated Aβ. Using this motif, Liu et al. were able to design short, inhibitory peptides that acted to break the compatibility between the amyloid fibril surfaces by targeting their glycine grooves (Liu et al., 2005; Sato et al., 2006). Our lab has previously successfully tested the capacity of one such inhibitor peptide (with a sequence of RGTFEGKF) to attenuate Aβ42 toxicity and aggregation (Barucker et al., 2015), and the further characterization of this inhibitor will be the main focus of this thesis.

### 4.4.2 Challenges associated with peptidic inhibitors

Therapeutic peptides have the capacity to combine beneficial properties of both biologics (i.e. antibodies) and small molecules by having high specificity and affinity of proteins, coupled with low production costs, toxicity, and increased potential to permeate the cell. There are, however, some challenges in making peptidic inhibitors into successful therapeutics. Peptides that are made of natural amino acids are generally considered to be poor drug candidates due to their pharmacokinetic profiles. Since many proteolytic enzymes recognize common structural features of peptides, unmodified peptides generally have poor *in vivo* stability against proteases. Additionally, they are rapidly cleared from the body by the liver and kidneys – half-lives for some peptides can be in the range of only minutes (reviewed in (Tsomaia, 2015; Wojcik and Berlicki, 2016)). Regardless, even though further research using advanced drug discovery tools and new platform technologies are still needed to engineer better peptidic inhibitors with more desirable properties, the development of peptide therapeutics will likely allow us to reach what are currently considered “undruggable” targets.

### 4.4.3 D-amino acids peptide therapeutics

Dextrorotatory (D)-amino acids are enantiomers of their levorotatory (L) counterparts – they share identical chemical and physical properties, except for the ability to rotate plane-polarized

light in opposite directions. Synthetic proteins composed of D-amino acid are generally more costly to synthesize compared to those composed of L-amino acids, but their increased resistance to proteolytic degradation makes them very attractive potential therapeutics (reviewed in (Feng and Xu, 2016)). D-amino acids rarely act as the substrates of endogenous proteases – which can serve to increase the stability and circulation half-time of D-peptides *in vitro* and *in vivo* – making the D-peptide based drug delivery system more attractive and efficient than their L-peptide counterparts (D'Amours et al., 1999; Liang et al., 2009; Li et al., 2012b).

Due to their improved stability and bioavailability, D-peptidic inhibitors of Aβ aggregation have been of great interest in the field (reviewed in (Kumar and Sim, 2014)). In a study by Soto et al. in the late 1990s, they demonstrated that their D-peptide candidate has a similar inhibitory effect as the L-peptide version on fibril formation of full-length Aβ42, but the D-peptide was more stable against proteolysis *in vitro* (Soto et al., 1996). Following up on studies regarding the inhibitory peptide KLVFF (originally described by (Tjernberg et al., 1996)), Findeis et al. showed in 1999 that a similar D-pentapeptide modified from KLVFF can also successfully inhibit fibril formation and cytotoxicity while demonstrating improved stability in monkey CSF (Findeis et al., 1999). Our lab has also demonstrated in 2015 that our small D-peptide inhibitor was able to attenuate the rough-eye phenotype in a transgenic Aβ42 *Drosophila* model and significantly prevent the functional degeneration of photoreceptors from human Aβ42-induced toxicity, while the L-peptide did not exhibit any rescue effects *in vivo* (Barucker et al., 2015).

## 5. Aim and rationale of this thesis

The identification of soluble, low-n Aβ42 oligomers as the potential culprit of cell death and synaptic dysfunction in AD has made them promising and viable targets for the development of therapeutics. We have previously studied a small synthetic Aβ42-oligomer interacting peptide (AIP), and have demonstrated that AIP could specifically target low-n Aβ42 oligomers to neutralize their toxicity. Specifically, D-amino acid AIP (D-AIP) appeared to be more effective *in vivo* compared to the L-amino acid counterpart.

We now intent to further study the effects of this therapeutic peptide in longitudinal experiments using *in vivo* transgenic *Drosophila* models as a screening system, due to their low cost, rapid generation time, and ease of use. Here, we first aimed to develop two transgenic *Drosophila* models, which expressed human Aβ42 under eye- and neuron-specific promoters (**manuscript 1**). By fully characterizing the toxic effects of Aβ42 on both of these systems in terms of morphological and functional changes in embryonic and adult stages, we established clear phenotype baselines and usage guidelines upon which future rescue studies can measure and abide by.

Next, we aimed to assess in detail the neutralization effects of AIP on Aβ42-induced toxicity on both gross morphology (i.e. eye structure) as well as more complex aspects of behaviour (i.e. locomotor activity) of *Drosophila*. We first used the eye-specific expression model in food supplementation studies with AIP (both L and D) to evaluate the longitudinal effects of AIP administration on both Aβ42-induced changes in eye morphology and general physiology of the flies (**manuscript 2**). Importantly, using matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI), we analyzed and confirmed the uptake, distribution, as well as stability of label-free L- and D-AIP *in vivo*. Finally, we used the neuron-specific expression model, which expresses Aβ42 in a pan-neuronal manner, to assess AIP rescue in more complex functional studies such as climbing or locomotor activity assays (**manuscript 3**). MALDI-MSI was also employed here to characterize the uptake and distribution of AIP in these animals.

Overall, the results from our transgenic models allowed us to gain more insight into the Aβ42-neutralizing effects of AIP in different physiological systems (i.e. eye morphology and locomotor behaviour). We believe our studies in *Drosophila* provided strong *in vivo* evidence of the efficacy of AIP treatment, and will allow us to move forward to the testing of AIP in more complex and advanced rodent models of AD in the future.

# II. EXPERIMENTAL SECTION

**MANUSCRIPT 1**

## The differential effects of human Aβ42-induced toxicity in the eye and central nervous system of *Drosophila melanogaster*

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## 1.1 Foreword

In this manuscript, we described the generation and characterization of both the eye- and neuronal-specific human A $\beta$ 42-expressing transgenic *Drosophila* models. We confirmed and described the longitudinal effects of eye- and neuronal-A $\beta$ 42 expression on both eye morphology and functional locomotor behaviour in the flies by performing confocal microscopy of fly retinae, rapid iterative negative geotaxis (RING) assays to assess climbing behaviour, and Western blotting to confirm the expression of A $\beta$ 42. Overall, this manuscript aims to define the effects of A $\beta$ 42 expression on both different tissues of interest (i.e. eye and neurons) as well as periods of development (i.e. larval and adult stages) in *Drosophila*, which will allow us to accurately employ and assess these transgenic *Drosophila* models in future rescue experiments with AIP supplementation.

## 1.2 Abstract

Amyloid-beta (Aβ) peptides have long been considered as one of the primary culprits in the pathogenesis of Alzheimer's disease (AD). The Aβ peptide with 42 amino acid residues is known to be the key Aβ species that is neurotoxic and prone to aggregation. *Drosophila melanogaster* is one of the most commonly used invertebrate model systems for the studies of neurodegenerative diseases such as AD, where the binary UAS-Gal4 system of conditional gene expression is used to transgenically express human Aβ42 in *Drosophila* tissues of interest. Two of the most commonly used drivers for this system are *elav* (pan-neuronal) and *GMR* (eye-specific). Both drivers, however, are strongly expressed starting at embryonic and larval stages of the animals, and to date no systematic analysis has been conducted to examine whether there are in fact downstream differences between Aβ42-induced toxic effects during developmental and adult stages of *Drosophila*. To this end, we studied the effects of *GMR*- and *elav*-driven Aβ42 expression induced in either embryonic or adult animals, and observed that eye-specific expression of Aβ42 preferentially disrupts eye structure only when it is expressed during embryonic stages, while neurons in both developmental and adult stages of the animals were equally affected by Aβ42 expression. These findings show that (i) while neurons are susceptible at all stages of development, the mature eye structure is immune to Aβ42-induced toxicity, and (ii) these observed differences in downstream effects of Aβ42 illustrate the importance of thorough characterization of transgenes in animal models prior to using them in research studies.

## 1.3 Introduction

*Drosophila melanogaster* or the fruit fly is a commonly used invertebrate animal model for neurodegenerative diseases such as Alzheimer's disease (AD) (Jeibmann and Paulus, 2009; Pandey and Nichols, 2011; Prussing et al., 2013). The most widely used technique to drive gene expression in *Drosophila* is the binary UAS/Gal4 system (Brand and Perrimon, 1993). Using the UAS-Gal4 system, amyloid beta-42 (Aβ42) – the primary culprit in dysregulating neural synapses as well as facilitating neuronal death in AD (Masters et al., 1985; Miller et al., 1993; Walsh et al., 2002) – can be expressed in a tissue-specific manner in the fly via the use of specific promoters to isolate and characterize its aggregation dynamics and toxicity. Two of the most common *Drosophila* promoters that are used in the UAS-Gal4 system are *elav* (embryonic lethal, abnormal vision; pan-neuronal expression) and *GMR* (Glass Multimer Reporter; eye-specific expression) (Fischer et al., 1988). Using these promoters, neuron- and eye-specific expression of Aβ42 have both been previously characterized and shown to cause neuronal dysfunction/neurodegeneration and eye degeneration, respectively, in a multitude of studies (Fortini and Bonini, 2000; Finelli et al., 2004).

It is worth noting, however, that both the *elav* and *GMR* promoters have been shown to be expressed starting at the embryonic level of development in the animals. The expression of the elav protein, for example, has been shown to coincide with the birth of the first neurons at around stage 9 of development (i.e. 4-5 hours after fertilization of the egg). Also, the *elav* locus appears to have the highest transcription level early in the life of a neuron and continues at a lower level as the neuron ages (Robinow and White, 1988, 1991). The *GMR* driver is also believed to be expressed from the mid-third-instar larval stage through to pupal development exclusively in all cells that are posterior to the morphogenetic furrow in the differentiating larval eye discs, as well as in the photoreceptor cells of adult animals (Freeman, 1996; Hiesinger et al., 2001; Chin et al., 2014).

The heterogeneity in the temporal expression of the two driver proteins during different aspects of *Drosophila* development represents a significant confound: since the vast majority of studies

that use *elav* and/or *GMR* promoters to drive the expression of human Aβ42 normally elect to express Aβ42 from the embryonic stages of the insects, it is generally impossible to determine whether the detrimental phenotype observed from Aβ42 expression is due to process disruptions in the developmental or adult stages of the animals. Furthermore, since Aβ42 has so far been shown to only accumulate in mid to later stages of life in humans (with the exception of individuals with Down syndrome as well as early-onset AD caused by familial mutations) (Gyure et al., 2001; Netzer et al., 2010), it is important to elucidate and separate the effects of Aβ42 on development and adult stages in flies to better recapitulate the dynamics of Aβ42 in the human disease.

To date, despite the large number of studies using human Aβ42-expressing transgenic flies, there has not been a systematic analysis of whether human Aβ42 expression, using either the *elav* or *GMR* driver, preferentially affects *Drosophila* during development or adult stages. Therefore, we examined the effects of *elav-Gal4* or *GMR-Gal4* driven expression of the UAS-Aβ42 construct on locomotor activity, eye morphology, and survival in this study. Expressions of the drivers were controlled via simple temperature alterations to allow the induction of Aβ42 expression in different developmental stages of the animals. Our experiments indicate that the locomotor activity and survival of the animals were significantly and negatively affected only when Aβ42 was expressed in a neuron specific manner using *elav*. This effect was present regardless of whether A $\beta$ 42 was expressed during development or adulthood, and so seems to indicate a general susceptibility of neurons to A $\beta$ 42-induced toxicity. Eye-specific A $\beta$ 42 expression with *GMR*, however, only caused deformations in eye morphology when it is expressed during development, which implies that the established adult eye structures in *Drosophila* – composed of cells such as the various pigment cells that make up each ommatidia (Kumar, 2012) – are potentially resistant to A $\beta$ 42 toxicity. We conclude that the degree of toxic effects induced by transgenic A $\beta$ 42-expression is likely intrinsically linked to the stage of development (i.e. larval vs. adult) of the animal, while specific tissue (i.e. neurons vs. eyes) may also differ in their susceptibility to Aβ42-induced toxicity. These diverse responses should be evaluated and considered when over-expression analysis is performed using *GMR* or *elav* as drivers.

## 1.4 Materials and Methods

### 1.4.1 Transgenic *Drosophila*

UAS-Aβ42 flies were generated as described previously (Harmeier et al., 2009). *GMR-Gal4*/*Bc, Gla*; *Gal80ts*/*Tb, Hu* and *elav-Gal4; Gal80ts*/*Sb* strains were a gift from Dr. Yong Rao. They were crossed with UAS-Aβ42 flies to induce temperature-sensitive Aβ42 expression in either an eye- or neuron-specific manner respectively. Canton S flies were crossed with *GMR-Gal4* flies to obtain Gal4-only controls. Flies are reared in an incubator (Tritech) with 12hr light/dark cycle, 50% constant humidity, and at 22°C.

### 1.4.2 Temperature-induced A $\beta$ 42 expression

Gal80ts, a negative regulator of Gal4, is highly activated at 18°C and inactivated at 29°C (Zeidler et al., 2004). This essentially allows us to induce the expression of Aβ42 in F1 animals at any point during development simply by elevating the ambient temperature. Transgenic Gal80ts flies were crossed on Jazz-Mix Drosophila food (Fisher) and separated into three groups. Two groups were place in an 18°C incubator (Tritech) while one group was placed in a 29°C incubator, both with 12hr light/dark cycle and 50% humidity. Parental generation flies were removed after the appearance of F1 3$^{rd}$ instar larvae, which were left to incubate in the respective incubators. F1 transgenic flies were separated according to sex and collected into fresh tubes as they eclosed. Adult transgenic flies collected from the 29°C incubator were kept under the same condition through the experiments. Out of the two groups that were placed in the 18°C incubator, eclosed adult transgenic flies collected from one group were removed and placed in the 29°C incubator, while the transgenic flies collected from the other group remained at 18°C.

### 1.4.3 Confocal imaging

Confocal imaging of fly retinae were performed at the Imaging & Molecular Biology Platform Department of Pharmacology & Therapeutics, McGill Life Sciences Complex). Flies were anesthetised with $CO_2$ and the heads of the animals were removed with fine dissection tweezers (Dumont #5). The heads were then briefly dipped in 70% ethanol to remove the cuticle wax, and placed on an agarose-lined petri dish. A small amount of PBS was then dripped on top of the

heads, and the retinae were dissected in the liquid. The dissected retinae were subsequently kept in a 3cm petri dish filled with PBS, and once all dissections were complete, dried and mounted on glass slides (Globe Scientific) using superglue. The retinae were then imaged using a TCS SP8 confocal microscope (Leica; oil-immersion objective, 40X 1.3NA HC PL APO; Cat. #506358; 552 laser at 12% power). Z-stacks were subsequently made of the whole eye of the animal to fully visualize the compound eye structure. The autofluorescence which illuminates the compound eye under confocal microscopy is thought to derive from the conversion of rhodopsin to its photoproduct metarhodopsin upon light stimulation in the rhabdomeres or photoreceptors of each ommatidia (Franceschini et al., 1981).

### 1.4.4 Locomotor assay

Rapid iterative negative geotaxis assays (RING assay) were performed as described (Barone and Bohmann, 2013). Briefly, groups of 15-18 transgenic animals were aged 21 days post eclosion, as Aβ42-induced locomotor dysfunction has been previously shown to be discernible after this period (Iijima et al., 2004). Measurements were then taken every 3 days, at the same time of the day for each group of 10-15 flies separated according to sex, genotype, and treatment. Each group of flies were placed in empty clear plastic tubes and allowed to recover from $CO_2$ anaesthesia at room temperature for 45mins. Flies were then forced to the bottom of the tube by firmly tapping against the bench for 10 seconds. After being allowed to climb up the sides of the vials for a further 10 seconds, the number of flies that walked above the 2 cm mark was recorded. There was a one minute rest period between assays. Assays were repeated 15 times to obtain the average climbing activity for the day, and the percentage climbing activity (number of flies over 2cm line/number of total flies x 100) was plotted as a function of age using GraphPad Prism.

### 1.4.5 Tissue homogenates

Sixteen flies (8 male and 8 female) of each genotype were snap-frozen in liquid nitrogen. The heads were collected and homogenized in 100µl of PBS-PI (PBS buffer with Complete EDTA-free Protease Inhibitor Cocktail Tablets (Roche)) (Helmfors et al., 2015). The resulting mixture was centrifuged at 12,000g for 10mins at 4°C, and the supernatant was extracted as the soluble fraction. The pellet was resuspended in 50µl of harsh extraction buffer containing guanidinium HCL (5M GnHCl, 50mM Hepes pH 7.3, 5mM EDTA, Protease inhibitor (Roche)) (Caesar et al.,

2012) and centrifuged again to obtain the supernatant as insoluble fraction. Alternatively, isolated fly heads were homogenized in whole cell extract (WCE) buffer (Sayre et al., 1992) and then centrifuged at 10,000g for 10min at 4°C.

### 1.4.6 Immunoprecipitation and Western blot

Aβ42 was immunoprecipitated from the insoluble and soluble fractions of fly head homogenates using an in-house antibody W0-2 (anti-Aβ, epitope residues 5-8) and protein G Sepharose beads (GE Healthcare #17-0618-01). Samples were then separated by SDS-PAGE on 4-20% tris-tricine gels (BioRad) and transferred to 0.45μm nitrocellulose membranes and probed with W0-2 and anti-mouse secondary antibody (Promega) for Aβ42 detection. Without immunoprecipitation, fly head homogenates were separated directed by SDS-PAGE on 10-20% tris-tricine gels (BioRad), transferred to 0.45μm nitrocellulose membranes, and probed with W0-2 and an anti-actin antibody (Millipore #MAB1501).

### 1.4.7 Statistical analyses

Statistical analyses were performed using GraphPad Prism. Kaplan-Meier survival curve analysis was used to compare survival curves. All results are expressed as mean ± SEM (Standard Error of Mean). P values $< 0.05$ were considered to be significant. Sample sizes are reported in figure legends, and no statistical method was used to predetermine sample size.

## 1.5 Results

### 1.5.1 Adult *D. melanogaster* eye structure is resistant to structural defects induced by human Aβ42 toxicity

The expression of human Aβ42 has been shown previously to cause the disorganization and degeneration of the *Drosophila* compound eye subunits or ommatidia, resulting in a "rough eye" phenotype (Finelli et al., 2004; Tan et al., 2008; Barucker et al., 2015). We generated flies with temperature-controlled 'switches' for eye-specific Aβ42 using the UAS/Gal4 system along with Gal80ts, then monitored the long-term effects of 'turning on' Aβ42 expression on eye morphology in either developmental or adult stages of the flies. Retinae from temperature-activated eye-specific Aβ42-expressing flies were imaged via confocal microscopy using autofluorescence (Franceschini et al., 1981) **(Figure 5)**. As expected, transgenic flies raised at 18°C (i.e. with no Aβ42 expression) showed no changes in eye morphology over time **(Figure 5, left column)**, while transgenic animals raised at 29°C (i.e. with constitutively activated eye-specific Aβ42 expression) showed a gradual and severe loss of the compound eye structure, resulting in a total loss of all eye morphology at day 28 post eclosion **(Figure 5, right column)**. Interestingly, we observed that flies which pupated at 18°C but are then transferred to 29°C post eclosion (i.e. as adult animals) to activate Aβ42 expression in the adult stage showed no eye degeneration throughout the time points **(Figure 5, middle column)** despite having ongoing, eye-specific Aβ42-expression as detected by Western blot **(Figure 6)**.

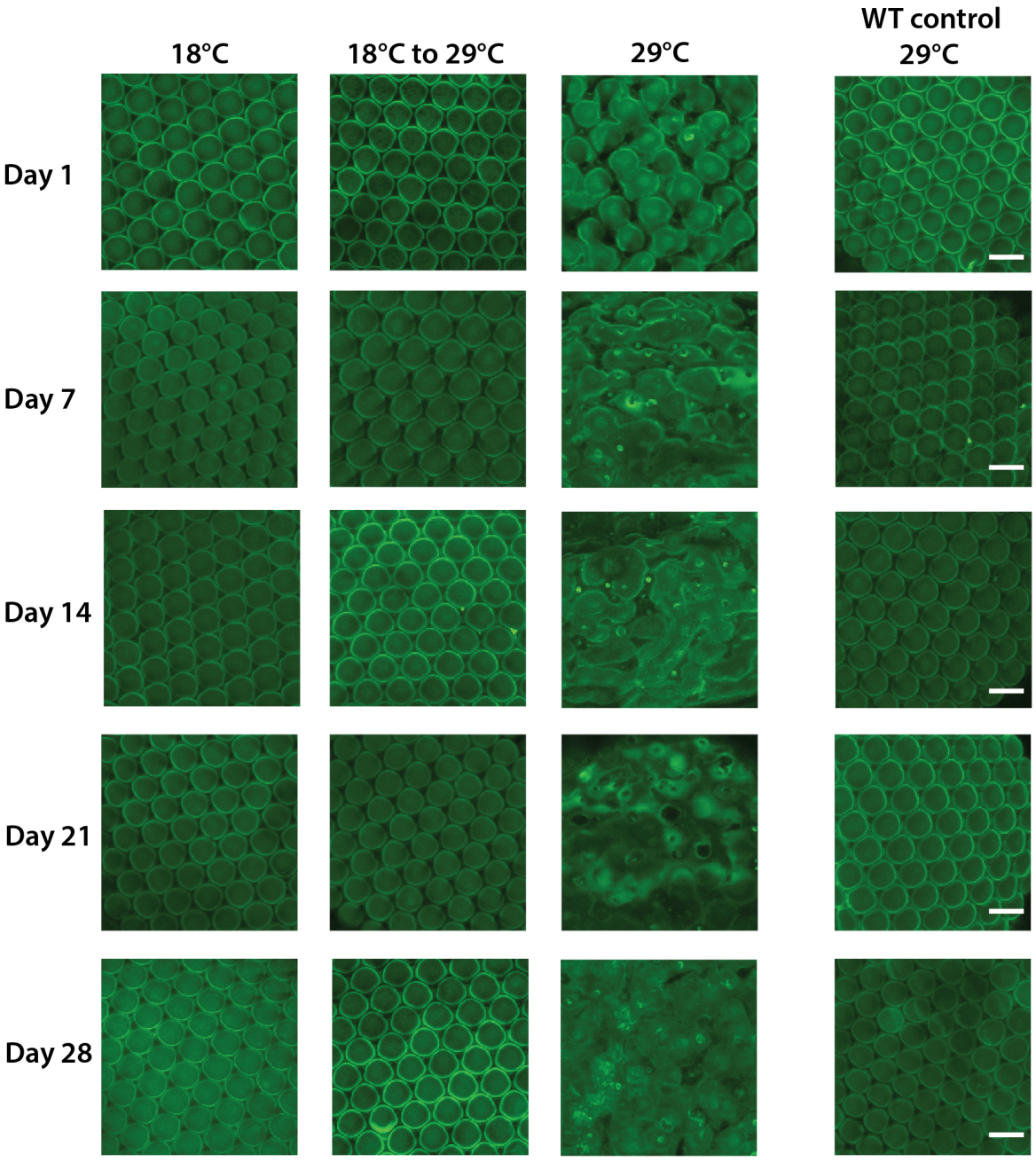


**Figure 5. The adult *Drosophila* compound eye structure is resistant to Aβ42-induced toxicity**

Representative images of compound eyes of temperature-sensitive Aβ42-expressing strains. Flies were dissected and retinae were mounted post eclosion at the dates indicated. Eye morphology was assessed with confocal microscopy using autofluorescence. Transgenic animals raised at 18°C exhibited no morphological defects in the eye, regardless of whether they were moved to 29°C during adult stages. However, flies raised at 29°C showed extensive damage in eye structure, with gradual losses of ommatidia definition, which culminated in a total loss of eye structure on day 28. $n \geq 4$ for each group. Scale bar = 100μm.

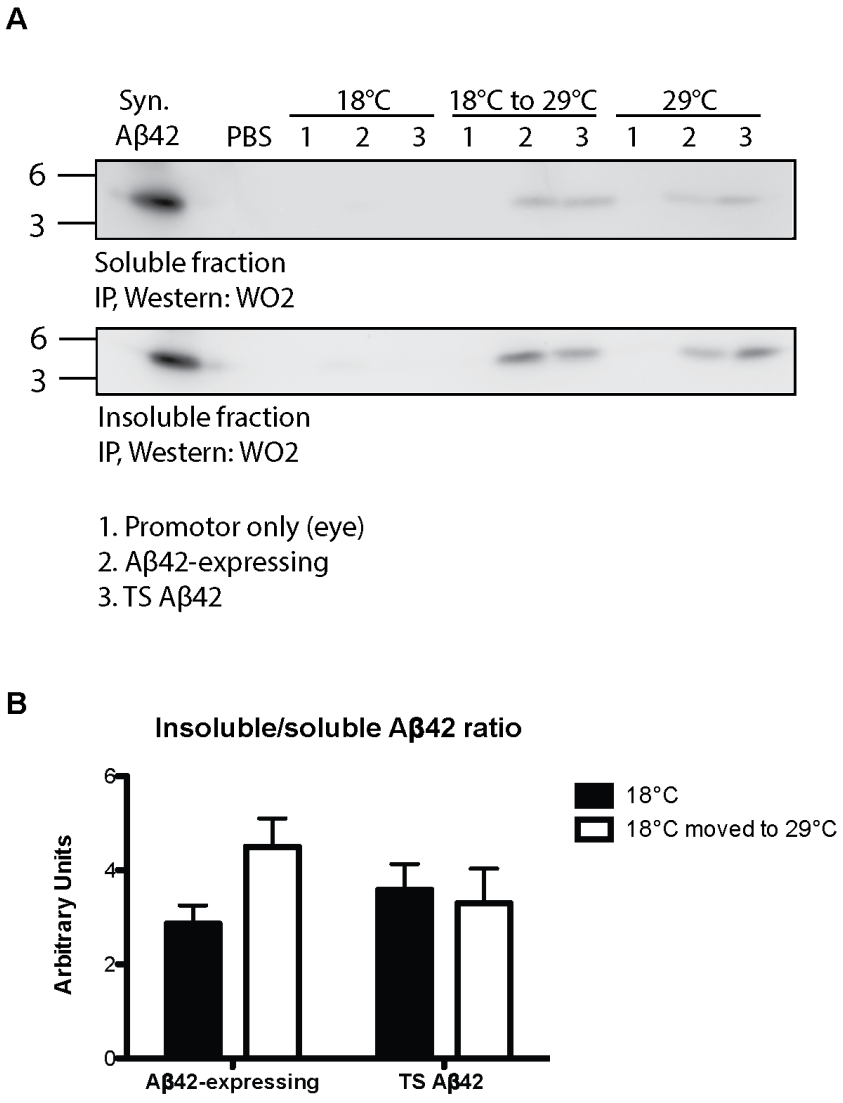


**Figure 6. Expression of human Aβ42 in transgenic flies is activated at 29°C**
**A.** Immunoprecipitation and Western blot of human Aβ42 expression in the eyes of transgenic *D. melanogaster*. Fly head homogenate were obtained from promoter control, Aβ42-expressing, and temperature-sensitive Aβ42-expressing strains (TS Aβ42) bred at different temperatures. Aβ42 was detected in both TS Aβ42 and Aβ42-expressing strains regardless whether they were raised at high temperatures or moved to high temperatures as adults. No Aβ42 expressing was detected at 18°C in any strain. 16 heads were used per genotype. **B.** Western blot quantification of the ratio between expression of Aβ42 in the insoluble and soluble fraction. Two way ANOVA was performed and no significant difference was detected between the groups. n = 3.

Classical immunoprecipitation and Western blotting was used to verify the temperature-induced Aβ42 expression in the transgenic flies **(Figure 6)**. No Aβ42 expression was detected in flies bred and raised at 18°C, while both the constitutive and temperature-sensitive strains showed strong Aβ42 when they were bred and raised at 29°C. Both Aβ42-expressing strains that were born and pupated at 18°C and subsequently moved to 29°C after eclosion showed temperature-activated expression of Aβ42. The overall ratio between the insoluble and soluble fractions of Aβ42 is not significantly different between the groups **(Figure 6B)**.

### 1.5.2 Locomotor activity and survival of transgenic *D. melanogaster* are minimally affected by the eye-specific expression of human Aβ42

Aside from the eye, recent research has shown that *GMR* is also active in a variety of other tissue types such as the wing, brain, trachea, and leg imaginal discs (Li et al., 2012a; Ray and Lakhotia, 2015). Since this 'leaky' expression of *GMR* (and consequently Aβ42) have the potential to affect the physiology of the flies aside from morphological changes in the eye, we next decided to investigate the long-term locomotor activity and survival of the flies. Promoter control and temperature-sensitive transgenic flies raised at 18°C **(Figure 7A, blue lines)** showed minimum declines in climbing activity over 47 days, with a slightly steeper decline observed in the transgenic flies compared to the promoter controls. Flies raised at 29°C, however, showed a more drastic decline over time **(Figure 7A, red lines)**, where both strains dropped similarly to almost 0% climbing activity by day 40. Animals raised at 18°C then moved to 29°C during adulthood **(Figure 7A, black lines)** showed an intermediate phenotype and exhibited a slower decline in their locomotor activity compared to flies raised at 29°C. We observed similar trends for the survival of the animals **(Figure 8A)**. Animals of either strain raised at 18°C had no mortalities, while flies raised at 29°C showed significantly decreased levels of survival. Animals raised at 18°C then moved to 29°C during adulthood again exhibited an intermediate phenotype with small declines in survival rate over the course of 47 days.

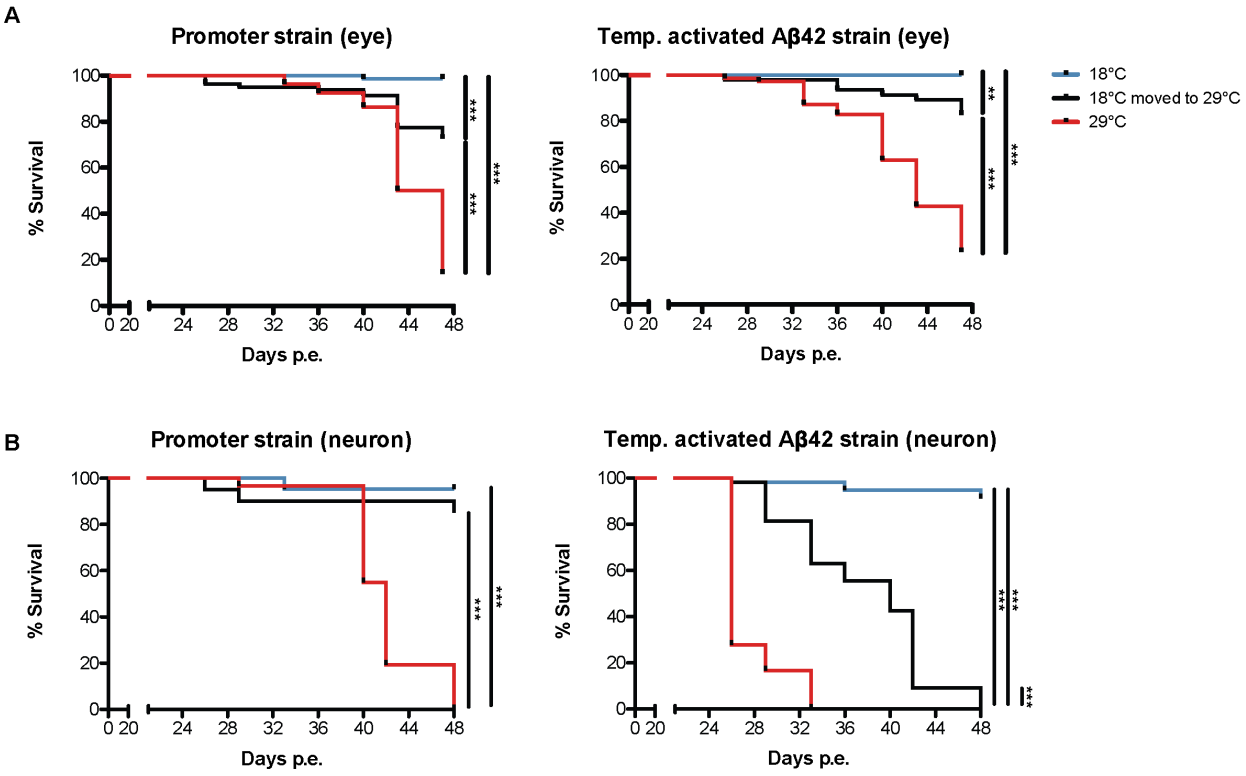


**Figure 7. Neuron-specific expression of human Aβ42 negatively affects the locomotor behaviour of transgenic animals**

**A**. RING assays showed a gradual decline in climbing activity of transgenic flies raised at 29°C regardless of genotype (red lines). Promoter control flies raised at 18°C or moved from 18°C to 29°C exhibited minimum changes in locomotion, while temperature activated, eye-specific human Aβ42 expressing flies showed larger, temperature dependent declines in locomotion (black lines). n ≥ 30 for each strain/group. **B**. RING assays show that higher temperatures mildly reduced the locomotor activity of the promoter control animals (left panel, red line). In contrast, temperature activated, neuron-specific human Aβ42 expressing flies exhibited complete deterioration of locomotor activities when bred at 29°C (right panel, red line), and also showed rapid and steep decline in climbing activity when the animals were moved to 29°C (right panel, black line). n ≥ 18 for each strain/group.

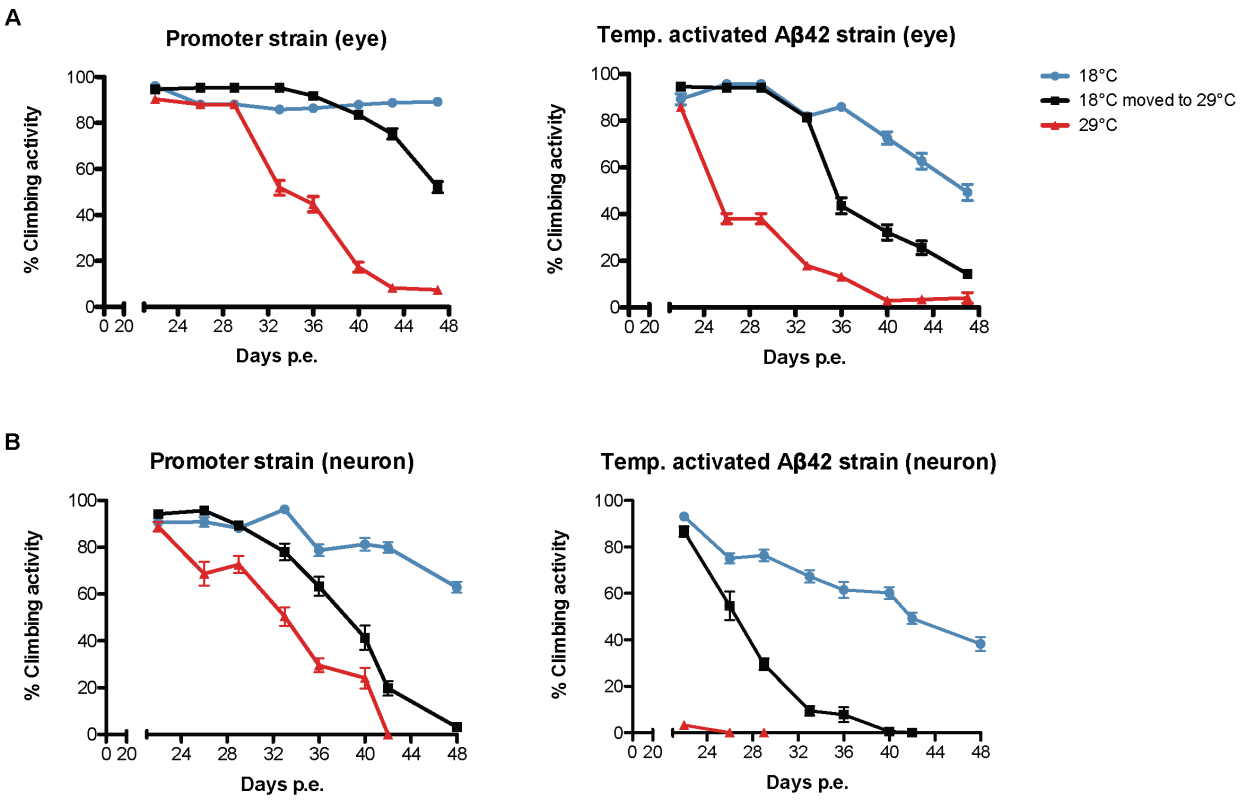


**Figure 8. Neuron-specific expression of human Aβ42 has major impacts on the longevity of transgenic *D. melanogaster***

**A**. Kaplan-Meier survival curves indicate that the lifespan of transgenic animals raised at 29°C of either genotype (i.e. either with eye-specific promoter or Aβ42 expression) was significantly decreased (red lines). n ≥ 30 for each strain/group. Kaplan-Meier survival curves. **p<0.005, ***p<0.0001. **B**. Kaplan-Meier survival curves show that animals raised at 29°C of either genotype showed increased mortality (red lines). A rapid decline in survival was observed in temperature activated, neuron-specific human Aβ42 expressing flies that were moved to 29°C (bottom right panel, black line). n ≥ 18 for each strain/group. Kaplan-Meier survival curves. ***p<0.0001.

### 1.5.3 *D. melanogaster* neurons are generally sensitive to human Aβ42-induced toxicity

We next analyzed the locomotor activity and survival of the temperature-activated, neuron-specific Aβ42-expressing transgenic flies. Animals raised at 18°C showed little decline in their locomotor activity regardless of genotype **(Figure 7B, blue lines)**. Promoter control flies raised at 18°C then transferred to 29°C showed a mild decline in climbing ability that was similar to flies strictly raised at 29°C **(Figure 7B, left panel, comparing black line to red line)**. The temperature activated neuron-specific Aβ42-expressing flies raised at 29°C, however, had completely abolished climbing activity from day 21 **(Figure 7B, right panel, red line)**. Additionally, when these transgenic flies were raised at 18°C then transferred to 29°C, their climbing activity declined drastically starting on day 26, and was completely absent by day 42 **(Figure 7B, right panel, black line)**. We observed similar patterns in the survival of these animals: flies of both strains survive well at 18°C but did poorly at 29°C, with the complete loss of the neuron-specific Aβ42 strain by day 33 compared to the loss of the promoter control flies by day 48 **(Figure 8B, red lines)**. It is interesting to note that when the flies are moved from 18°C to 29°C, the survival of the promoter control flies were completely unaffected, while the temperature-activated neuron-specific Aβ42 strain exhibited an intermediate survival phenotype where the survival of the animals significantly decreased from day 30 to 48 **(Figure 8B, right panel, black line)**, which potentially indicates a susceptibility of the adult neurons to Aβ42-induced toxicity.

### 1.5.4 Neuron-specific expression of human Aβ42 in *D. melanogaster* has little effect on gross eye structure

The pan-neuronal driver *elav* has been shown to also be essential in the development of the eye and the optic lobe (Campos et al., 1985; Campos et al., 1987). As such, we examined the effects of temperature-activated *elav* driven Aβ42 expression on the eye structure of flies. The retinae from the transgenic animals were imaged via confocal microscopy using autofluorescence **(Figure 9)**. While mild deformations in ommatidia shape can be observed on day 28 in transgenic flies raised at 29°C, no morphological deformities were present in the eyes of the animals at any other time point or incubation temperature. Relative expression levels of Aβ42 were verified in transgenic flies via classical Western blotting **(Figure 10)**.

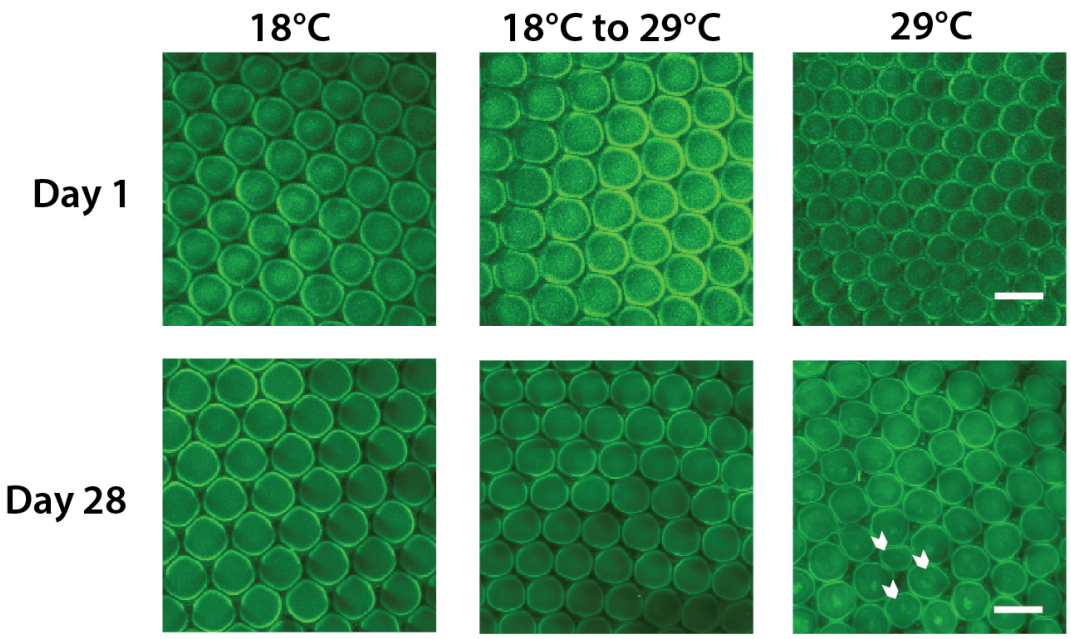


**Figure 9. Compound eye morphology is not affected by neuron-specific expression of human Aβ42**
Representative images of compound eyes of transgenic flies expressing human Aβ42 driven by neuron-specific driver. Eye morphology was assessed with confocal microscopy using autofluorescence. Mild disturbances in eye structures were detected on day 28 only in transgenic flies raised at 29°C (white arrows indicate locations of misshapen and deformed ommatidia). No other changes are observed. $n \geq 4$ for each group. Scale bar = 100μm.

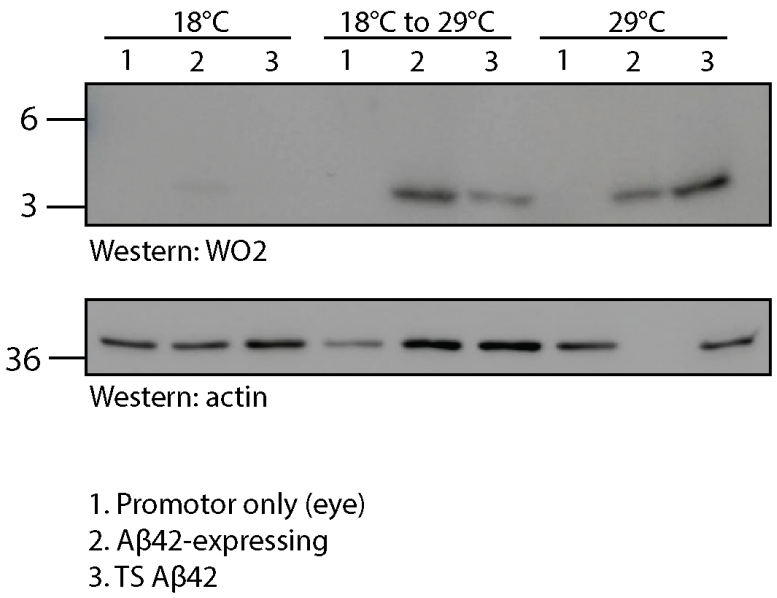


**Figure 10. Neuron-specific expression levels of Aβ42 in transgenic flies**
Western blot quantification of human Aβ42 with neuron-specific expression in transgenic *D. melanogaster*. Fly head homogenate from transgenic animals raised at 29°C were blotted using Aβ specific antibody (W0-2).

## 1.6 Discussion

*D. melanogaster* is one of the most common invertebrate animal models of AD. It is more often used in research compared to other popular invertebrates such as *Canorhabditis elegans*, as *Drosophila* are more compatible with assays that measures behavioural and memory outcomes compared to the nematode models (Mhatre et al., 2013). The *GMR* and *elav* drivers, in particular, have been extensively used by researchers to observe the effects of amyloid expression in *Drosophila* eyes (Harmeier et al., 2009) and neurons (Chakraborty et al., 2011; Speretta et al., 2012). Our observations show that the downstream effects of human Aβ42 expression driven by *GMR* and *elav* very much depend on the temporal expression of A $\beta$ 42 (i.e. during developmental or adult stages of the animals). Our study further proposes that the selection of drivers for expression may also play a major role in influencing downstream effects of A $\beta$ 42 expression, as tissues (i.e. eye and neuron) seem to differ in their overall susceptibility to A $\beta$ 42-induced toxicity.

*GMR*, as previously reported, drives the expression of target genes in all cells posterior to the morphogenetic furrow in the developing eye (Freeman, 1996). Interestingly, the toxic effects of *GMR* driven expression of A $\beta$ 42 in our studies seems to be entirely limited to embryonic stages of *Drosophila*, as eye-specific A $\beta$ 42 expression in adult animals does not seem to affect overall eye morphology **(Figure 5)**. We suspect that this diverging effect may be potentially due to the differential sensitivity of embryonic and adult cells to A $\beta$ 42-induced toxicity, since the total ratio of A $\beta$ 42 between soluble and insoluble fractions are consistent between genotypes that express A $\beta$ 42 in embryonic as well as adult stages **(Figure 6)**. We showed in a previous study that eye-specific A $\beta$ 42-expression can also negatively influence the electroretinography (ERG) readings taken from transgenic animals (Barucker et al., 2015), indicating that *GMR*-driven A $\beta$ 42-induced toxicity can affect both the structure and the function of the eye – possibly by negatively affecting the photoreceptors, as *GMR* has been shown to be expressed in the rhabdomeres (Coelho et al., 2013). Our current experiments showed that the structure of the adult eye was resistant to A $\beta$ 42-induced morphological changes, but the function of these A $\beta$ 42-

expressing but morphological normal organs are unknown. We speculate that if the function of the eye were also unaffected by A $\beta$ 42-induced toxicity in the adult animals, it would likely indicate a general resistance of the adult organ to A $\beta$ 42-induced toxicity. However, if eye function is impaired while the structure is intact, it may indicate that the functional competences of the cells in the compound eye are separate from their structural capabilities (i.e. overall morphology can be preserved even when function is impaired).

Additionally, while *GMR* has been shown to also be expressed in tissues other than the eye such as the larval brain, trachea, and leg discs (Li et al., 2012a), *GMR*-driven A $\beta$ 42 expression does not seem to affect neuron function in terms of locomotor behaviour in adult flies. This is likely due to the differential expression of *GMR* in adulthood where it is mainly found in the photoreceptors (Hiesinger et al., 2001; Chin et al., 2014), where it would have minimal effect on neuronal activity that are responsible for locomotor behaviour. Previous works using *eyeless* strains and mutants with optic lobe defects have also shown that vision defects does not affect general locomotor activity, but can instead influence the overall pattern of behaviour that is usually dictated by light/dark cycles (Helfrich, 1986) which our studies did not measure.

*elav* has been previously described to be expressed exclusively in postmitotic neurons (Robinow and White, 1988, 1991). It is also a widely used driver line for the neuron-specific expression of Aβ species such as Aβ40 and Aβ42, where only Aβ42 expression induced decreases in the climbing behaviour and longevity of the transgenic animals (Iijima et al., 2004; Chakraborty et al., 2011; Speretta et al., 2012). Although *elav* function has been previously shown to be important in both the development of the eye and optic lobe (Campos et al., 1985; Campos et al., 1987), we did not observe any morphological changes in the eyes of *elav*-driven A $\beta$ 42 expressing flies. This indicates that the presence and function of *elav* does not likely affect the development of the structural elements of the eye. Overall, our data indicates that A $\beta$ 42 expression in a neuron-specific manner seems to affect both the flies' locomotor activities and survival regardless whether the expression takes place during development or adulthood. This may indicate a general susceptibility of neurons to A $\beta$ 42-induced toxicity, which has been

shown previously in both *in vitro* and *in vivo* studies (Shiwany et al., 2009; Krantic et al., 2012; Popugaeva et al., 2015; Ungureanu et al., 2016).

## 1.7 Conclusion

In summary, our experiments provided a systematic analysis of the effects of eye- and neuron-specific A $\beta$ 42 expression in either adult or embryonic *Drosophila*, and demonstrated that the temporal expression of A $\beta$ 42 in either developmental or adult stages of the animals have different influences on its downstream effects. The tissue-specific drivers are also important factors in determining A $\beta$ 42-induced toxicity, as the drivers' differential expression patterns in embryonic and adult stages seems to greatly influence the susceptibility of the specific tissue to A $\beta$ 42 expression. These results emphasizes the need for careful confirmation of the drivers used in *Drosophila* in terms of their expression profiles in embryonic and adult stages, as well as more detailed experimental planning of the temporal expressions of transgenes of interest.

## 1.8 Acknowledgements

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**MANUSCRIPT 2**

**The D-enantiomeric peptide AIP neutralizes toxic A$\beta$ oligomers *in vivo* and rescues rough eye defects in transgenic *Drosophila melanogaster***
*Submitted to Journal of Neurochemistry*

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## 2.1 Foreword

Our previous characterization of the transgenic flies showed that both eye- and neuronal-specific expression of Aβ42 had significant and measurable negative impacts on the eye morphology and behaviour of the animals, respectively. The eye-specific Aβ42-expressing model, in particular, had an overt and easily detectable change in morphology that appeared to be highly conducive to rescue experiments. In the following manuscript, we used the eye-specific A $\beta$ 42-expressing transgenic model in longitudinal food supplementation experiments with AIP. We first investigated the toxicity and stability of AIP – composed of either L-amino acids or D-amino acids – during longitudinal experiments on the general physiology of the flies (i.e. survival and locomotor activity). Next, to further analyze the rescue of A $\beta$ 42-induced changes in morphology by D-AIP, we utilized live imaging techniques to visualize the eyes of the same cohort of flies over a month long period. In addition, we have also validated the expression of A $\beta$ 42 in the transgenic animals via immunoprecipitation and Western blotting.

We have also performed MALDI-MSI to detect the presence and localization of AIP within the animals. This method enabled us to verify the uptake and distribution of AIP *in vivo* without the need to alter the peptide, and to our knowledge, is the first time such experiments have been performed using *Drosophila* specimens. Overall, this second manuscript provides evidence that D-AIP is highly stable in the animals throughout longitudinal experiments, and can effectively attenuate Aβ42-induced morphological deficits *in vivo* (i.e. rough-eye phenotype).

## 2.2 Abstract

Soluble oligomers of the 42-amino acid amyloid-beta (Aβ42) peptide are highly toxic and suspected as the causative agent of synaptic dysfunction and neuronal loss in Alzheimer's disease (AD). We have previously shown that a small, eight amino acid Aβ42-oligomer interacting peptide (AIP) was effective at neutralizing human Aβ42-mediated toxicity using both *in vitro* and cell-based studies. In the present work, we have utilized advanced techniques including live confocal imaging and mass spectrometry imaging (MALDI-MSI) to show that AIP – the D-enantiomeric form in particular (D-AIP) – can effectively attenuate Aβ42 toxicity *in vivo*, using transgenic *Drosophila melanogaster* models. We showed that the Aβ42-induced "rough eye" phenotype was significantly rescued in the female transgenics upon AIP treatment, and D-AIP was indeed found co-localized with the transgenic human Aβ42 in the female fly heads. Interestingly, a male-specific sex peptide (Acp70A) was discovered via MALDI-MSI to have co-localized with D-AIP in the gut of the male transgenics, and potentially impaired rescue of the eye-specific phenotype. Our longitudinal study demonstrates two important outcomes: (i) observed sex differences between the flies illustrate the need to include both males and female specimens in cell, animal, and pre-clinical research, and (ii) D-AIP is a safe, stable, and highly effective neutralizer of toxic Aβ42 peptides *in vivo*.

## 2.3 Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease in the world and currently accounts for 50-70% of all neurodegenerative dementia cases (Winblad *et al.*, 2016). To date, no effective preventative or treatment strategies exist due to the complexity of its pathogenesis (Alzheimer's, 2018). One of the key hallmarks of the disease is the abnormal processing and deposition of amyloid beta (Aβ) peptides (Wang *et al.*, 2017) which are produced from the proteolytic processing of the amyloid precursor protein (APP) by sequential β- and γ-secretase cleavages (Selkoe and Hardy, 2016).

Soluble Aβ oligomers containing 42-amino acid long peptides (Aβ42) are highly toxic as evidenced by their ability to impair synaptic structure and function both *in vitro* and *in vivo* (Walsh et al., 2002; Oddo et al., 2003; Glabe and Kayed, 2006; Shankar et al., 2008; Koffie et al., 2009; Zhao et al., 2012; Esparza et al., 2013). Consequently, neutralization and elimination of these neurotoxic Aβ oligomers has become a promising avenue for therapeutic intervention. Antibody-based candidates that target Aβ oligomers have generated encouraging preliminary results (Salahuddin *et al.*, 2016; Sevigny *et al.*, 2016), but the high manufacturing and distribution costs of such biologics have raised concerns over their general accessibility. As such, protease-resistant D-amino acid peptides represent a more cost-effective alternative to neutralize toxic Aβ oligomers (Kumar and Sim, 2014; Fosgerau and Hoffmann, 2015).

*Drosophila melanogaster*, or the "common fruit fly", is the traditional model of choice for genetic research (Jeibmann and Paulus, 2009; Pandey and Nichols, 2011). More recently, the *Drosophila* "rough eye" model has become a well-established system to study toxicity as related to cancer and human neurodegeneration (Green et al., 2012; Stochmanski et al., 2012; Ashton-Beaucage et al., 2014; Yoshida et al., 2018). The compound fly eye is comprised of well-organized ommatidia (hexagonal structures) each containing seven rhabdomeres (circular structures) (Ready *et al.*, 1976). When toxicity disrupts the natural gross morphology, the "rough eye" phenotype (i.e. distorted ommatidia and rhabdomeres) can be visualized (Harmeier *et al.*, 2009).

We reported recently that a small synthetic Aβ42-oligomer interacting peptide (AIP), initially designed to disrupt sheet-to-sheet packing of Aβ40 and Aβ42 (Sato *et al.*, 2006), can specifically target and neutralize the toxicity of low-order Aβ42 oligomers (Barucker *et al.*, 2015). We previously showed that the short AIP peptide (RGTFEGKF) was able to rescue Aβ42-induced neurotoxicity in SH-SY5Y cells, as well as ameliorate the Aβ42-induced loss of synaptic spine density and long-term potentiation (LTP) in organotypic hippocampal slice cultures (Barucker *et al.*, 2015). We have now examined the *in vivo* efficacy of AIP in a full longitudinal study using transgenic *D. melanogaster* models. Traditionally, the visualization of the "rough eye" phenotype is commonly done via electron microscopy, which required flies to be sacrificed at each imaging time point (Prussing et al., 2013; Chouhan et al., 2016). Instead, we utilized a live confocal microscopy method in our current study, which permitted the live imaging of the flies (i.e. no time-dependent sacrifices). In parallel, we also developed a novel mass spectrometry imaging (MALDI-MSI) method to detect the uptake of label-free AIP into the flies and test for its co-localization with Aβ42 in the fly heads in separated groups of male and female flies.

Overall, our present study demonstrates that orally administered AIP in the D-enantiomeric form (D-AIP) is highly stable and has the potential to cross the invertebrate blood brain barrier (BBB) to rescue Aβ42-induced toxicity *in vivo* without detrimental side effects.

## 2.4 Materials and Methods

### 2.4.1 Transgenic *Drosophila*

UAS-Aβ42 flies were generated as described previously (Harmeier *et al.*, 2009). Transgenic flies containing the UAS-Aβ42 construct were crossed with *GMR-Gal4*-containing flies to induce the expression human Aβ42 in an eye-specific manner. Transgenic UAS-BACE1 flies were generated using the pUAST-BACE1 construct and crossed with *GMR-Gal4*-containing flies to induce the expression of BACE1 in an eye-specific manner. Canton S flies were used as wildtype controls and were also crossed with *GMR-Gal4* flies to obtain Gal4-only controls. Flies are reared in a 25°C incubator (Tritech) with 12hr light/dark cycle and 50% humidity.

### 2.4.2 Peptides

Label-free AIP (L- or D-amino acids, RGTFEGKF, 940.5 Da) and scrambled AIP (L- or D-amino acids, EFRKFTGG, 940.5 Da) were purchased from BioBasic (Markham, ON, Canada) and verified by mass spectrometry at the McGill SPR-MS Facility (Department of Pharmacology & Therapeutics, McGill Life Sciences Complex). AIP peptides were prepared fresh for each experiment, and were solubilized at 50mg/ml in deionized water containing 0.1% ammonia, vortexed, and then sonicated at 37hz and 100% power for 10min at 4°C. Resuspended AIP peptides were diluted to 1mg/mL in TA50 (acetonitrile : 0.1% (v/v) TFA = 50:50) for testing by MALDI-MS (verify intact mass via Bruker UltrafleXtreme system) or ESI-MS (verify sequence via Bruker Impact II system) according to the manufacturer's instructions.

### 2.4.3 Fly food and AIP supplementation

Flies were bred on Jazz-Mix *Drosophila* fly food according to the manufacturer's instructions (Fisher Scientific), freshly prepared in 15 ml centrifuge tubes (Sigma-Aldrich) to conserve volume and prevent drying. For AIP supplementation, freshly resuspended peptides were added to the bottom of the tubes (5mM AIP final concentration), then overlaid with 1ml of freshly prepared fly food cooled to 45°C. The mixture was thoroughly blended and left to solidify for over 12hrs at room temperature. Parental generation flies were crossed on regular or AIP-

supplemented food, then removed with the appearance of F1 3rd instar larvae. F1 transgenic flies were separated according to sex and collected into fresh tubes as they eclosed.

### 2.4.4 Stability of AIP in the feed and flies analysed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry

Prior to the confocal imaging and MALDI-MSI experiments, feed slurries from the 15mL conicals containing male and female flies were assessed for the long-term stability of both L-AIP and D-AIP. After 28 days at room temperature, 200µl of each slurry was precipitated with 300µl methanol, vortexed for 30 sec, and centrifuged for 10 min at 18,000g. Similarly, homogenates for the wildtype male and female flies fed with both forms of AIP for 28 days were prepared. Each sample (50µl) was precipitated with methanol, water and chloroform (3:1:1), vortexed for 30 sec and centrifuged at 18,000g. At the McGill SPR-MS Facility, supernatants were mixed 1:1 with CCA matrix (20mg/mL α-cyano-4-hydroxycinnamic acid (Sigma #70990) in TA50) and spotted on ground steel targets using the dried droplet method. MALDI spectra were acquired using a Bruker UltrafleXtreme MALDI-TOF/TOF system in reflectron positive ionization mode (calibrated mass range of 500 – 5,000 *m/z*; FlexControl v3.4 software). Ion intensities were evaluated by averaging three measurements of 4,000 shots each (i.e. 12,000 shots total per sample).

### 2.4.5 Tissue homogenates

Three flies of each sex (male/female), genotype (wildtype/transgenic), and treatment (vehicle/AIP) were snap-frozen in liquid nitrogen. The heads were collected and homogenized in 100µl of PBS-PI (PBS buffer containing Complete EDTA-free Protease Inhibitor Cocktail Tablets (Roche)) (Helmfors *et al.*, 2015). The resulting mixture was centrifuged at 12,000g for 10min at 4°C, and the supernatant was extracted as the "soluble fraction". The pellet was further resuspended in 50µl of harsh extraction buffer (5M guanidine HCl, 50mM HEPES pH 7.3, 5mM EDTA, Protease inhibitor (Roche)) (Caesar *et al.*, 2012), centrifuged, and the supernatant extracted as the "insoluble fraction". Alternatively, isolated heads, isolated bodies, or whole flies were homogenized in whole cell extract (WCE) buffer (Sayre *et al.*, 1992) and then centrifuging at 10,000g for 10min at 4°C. For each of the three different tissue homogenates, the supernatants

were isolated and centrifuged twice more to remove additional impurities and insoluble contaminants.

### 2.4.6 Immunoprecipitation and Western blot

Aβ42 was immunoprecipitated from the soluble and insoluble fractions of fly head homogenates using antibody W0-2 (anti-Aβ, epitope residues 5-8; Millipore #MABN10) coupled to protein G Sepharose beads (GE Healthcare #17-0618-01). Samples were then separated by SDS-PAGE on 10-20% tris-tricine gels (BioRad) and transferred to 0.45μm nitrocellulose membranes and probed with W0-2 and anti-mouse secondary antibody (Promega) for Aβ42 detection. Without immunoprecipitation, 15μl aliquots of soluble and insoluble fractions were separated directed by SDS-PAGE on 10-20% tris-tricine gels (BioRad), transferred to 0.45μm nitrocellulose membranes, and probed with an anti-actin antibody (Millipore #MAB1501). In the similar manner, fly head homogenates were probed with anti-actin (Millipore #MAB1501) and anti-BACE1 (Cell Signaling #D10E5) antibodies. Colloidal Coomassie staining of gels was performed as described (Neuhoff *et al.*, 1988; Neuhoff *et al.*, 1990). Briefly, acrylamide gels were fixed in a solution of 10% (v/v) acetic acid and 40% (v/v) methanol for one hour, and then stained overnight with Coomassie Brilliant Blue G.

### 2.4.7 Live confocal imaging

At the Imaging & Molecular Biology Platform (Department of Pharmacology & Therapeutics, McGill Life Sciences Complex), longitudinal confocal imaging of live flies was performed as described previously (Dourlen *et al.*, 2013). Briefly, our non-transgenic (wildtype) and transgenic flies (eye-specific Aβ42 expression) were immobilized sagittally using 35mm plates half-filled with 2% low melting point agarose (Fisher Scientific) at 45°C, where half of the body and head of each fly was embedded into the agarose. After the plates were placed on ice to solidify the agarose, the flies were covered with ice-cold water for anaesthesia and cornea neutralization. The eyes were then imaged using a TCS SP8 confocal microscope (Leica; water-immersion objective, #506323, 25X 0.95NA Water HCX IRAPO L; 552 green laser at 18% power) and z-stacks were made for the whole eye of each fly. Post-imaging, the flies were gently retrieved from the agar using forceps and dried on Kimwipes (Kimberly-Clark). They were then placed back into their original tubes and allowed to recover overnight with the tubes placed on their side to prevent the

flies from drowning/sticking to the food. The autofluorescence which illuminates the compound eye under confocal microscopy is thought to derive from the conversion of rhodopsin to its photoproduct metarhodopsin upon light stimulation in the rhabdomeres or photoreceptors of each ommatidia (Franceschini *et al.*, 1981). The percent of defective ommatidia is quantified by dividing the number of defective ommatidia, defined as either merged or misshapen subunits, by the total number of observable ommatidia. The count was performed manually using the Cell Counter plugin in ImageJ.

### 2.4.8 Locomotor assay

Rapid iterative negative geotaxis assays (RING assays) were performed as described (Barone and Bohmann, 2013). Flies were aged to three weeks (21 days) at the beginning of the experiments, as Aβ42-induced locomotor dysfunction has been previously shown to be discernible after this period (Iijima *et al.*, 2004). Measurements were then taken every 3 days, at the same time of the day for each group of 10-15 flies separated according to sex, genotype, and treatment. Each group of flies was placed in empty clear plastic tubes and allowed to recover from $CO_2$ anaesthesia at room temperature for 45 min. Flies were then forced to the bottom of the tubes by firmly tapping them against the lab bench for 10 sec at the start of each assay. After being allowed to climb up the sides of the vials for a further 10 sec, the number of flies that walked above the 2cm mark was recorded. There was a 1 min rest period between assays which were repeated 15 times to obtain the average climbing activity for the day, and the percentage climbing activity (number of flies over 2cm line/number of total flies x 100) was plotted as a function of age using Excel.

### 2.4.9 Mass spectrometry imaging (MALDI-MSI)

At the Histology Facility (Goodman Cancer Research Centre, McGill Life Sciences Complex), 10μm serial sections were prepared from flash-frozen flies using a cryostat (-14°C) and thaw-mounted on conductive, indium titanium oxide (ITO)-coated glass slides (Bruker Daltonics #8237001). Stored at -80°C before analysis, the mounted slides were transferred to vacuum desiccator and dried overnight before matrix application. At the McGill SPR-MS Facility, the dried sections were co-crystallized with super-DHB matrix (Sigma #50862; 9:1 (w/w) mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) using an ImagePrep sprayer

(Bruker Daltonics; 10mg/mL super-DHB in TA50). MSI spectra were acquired using a Bruker UltrafleXtreme MALDI-TOF/TOF system in reflectron positive ionization mode (calibrated mass range of 500 – 5,000 *m/z* and 20μm laser diameter; FlexControl v3.4 software). Spectra were processed using FlexImaging v4.1 software and normalized to total ion current (TIC).

### 2.4.10 Surface plasmon resonance (SPR)

At the McGill SPR-MS Facility, binding between D-AIP (941 Da) and Aβ42 (4512 Da) was examined using a BIACORE T200 system (GE Healthcare Bio-Sciences AB, Upsala, Sweden; Control software v2.0 and Evaluation software v1.0). Experiments were performed on S-series CM5 sensors (Biacore) at 25°C using filtered (0.2μm) and degassed PBS-T buffer (10mM phosphate, pH 7.4; 150 mM NaCl; 0.05% (v/v) Tween-20). Immobilized Aβ42 surfaces (50 μg/mL in 10 mM sodium acetate pH 4.0) were prepared using the Biacore Amine Coupling Kit as recommended by the manufacturer (1000 – 3000 RU final density); corresponding reference surfaces were prepared in the absence of Aβ42. D-AIP (positive: RGTFEGKF) or an inverse peptide (negative: FKGEFTGR) were titrated in tandem over reference and active Aβ42 surfaces in single-cycle mode (25μl/min × 60sec association + 60–600 sec dissociation). Between titration series, the surfaces were regenerated at 50μl/min using two 30 sec pulses of PBS-T containing 1M NaCl and 0.1% (v/v) Empigen detergent (Anatrace). Since the single-cycle data deviated from the simple "1:1 titration" binding model (i.e. likely due to mixed Aβ42 oligomeric states coupled to sensor), the "separate ka/kd" tool (BIAevaluation software) was used to predict the dissociation rate constant ($k_d$) at the beginning of the dissociation phase (i.e. between 625-645 sec to avoid rebinding effects).

### 2.4.11 Statistical analysis

Statistical analyses were performed with one way ANOVA followed by Tukey's multiple-comparison tests as appropriate, and individual p values are reported in figure legends. Kaplan-Meier survival curve analysis was used to compare survival curves. All results are expressed as mean ± SEM (Standard Error of Mean). All statistical tests are performed using GraphPad Prism. P values $< 0.05$ were considered to be significant. Sample sizes are reported in figure legends, and no statistical method was used to predetermine sample size.

## 2.5 Results

### 2.5.1 A $\beta$ 42-induced toxicity is rescued by D-AIP only in female transgenic flies at 5 days post-eclosion

Wildtype and Aβ42 transgenic flies were crossed and reared on food supplemented with AIP, synthesized with protease-sensitive L-amino acids (L-AIP) or protease-resistant D-amino acids (D-AIP). Human Aβ42 expression was verified in the transgenic flies via Western blot, and L- or D-AIP supplementation was found to have little effect on the overall longevity, locomotor behaviour, and overall Aβ42 levels of the animals (**Supplemental Figure 1 and 2**). At 5 days post-eclosion, the flies were individually imaged via live confocal microscopy to examine the morphology of the adult compound eye. No morphological changes in the shape of each ommatidium or the overall structure of the eye itself was observed in wildtype flies, irrespective of sex or AIP supplementation (**Figure 11A**). Eye-specific Aβ42 transgenic flies, however, exhibited severe deformations in ommatidia structure – especially around the outer boundary of the eye, where many fused and misshapen ommatidia could be observed. This detrimental effect on ommatidia structure appears to be Aβ42-specific, since eye-specific BACE1-expressing flies do not exhibit similar morphological deficits **(Supplemental Figure 3)**. In the absence of AIP, quantification of the present micrographs indicates that 30% of the total ommatidia were misshapen or deformed in Aβ42 transgenics at 5 days post-eclosion, regardless of sex (**Figure 11B**). Interestingly, female Aβ42 transgenic flies supplemented with D-AIP showed a significant decrease in ommatidia deformation at day 5, with an average of 16% rescue compared to all other sexes and/or treatments. This rescue was not observed in male transgenic flies treated with D-AIP or any other flies of either sex treated with L-AIP.

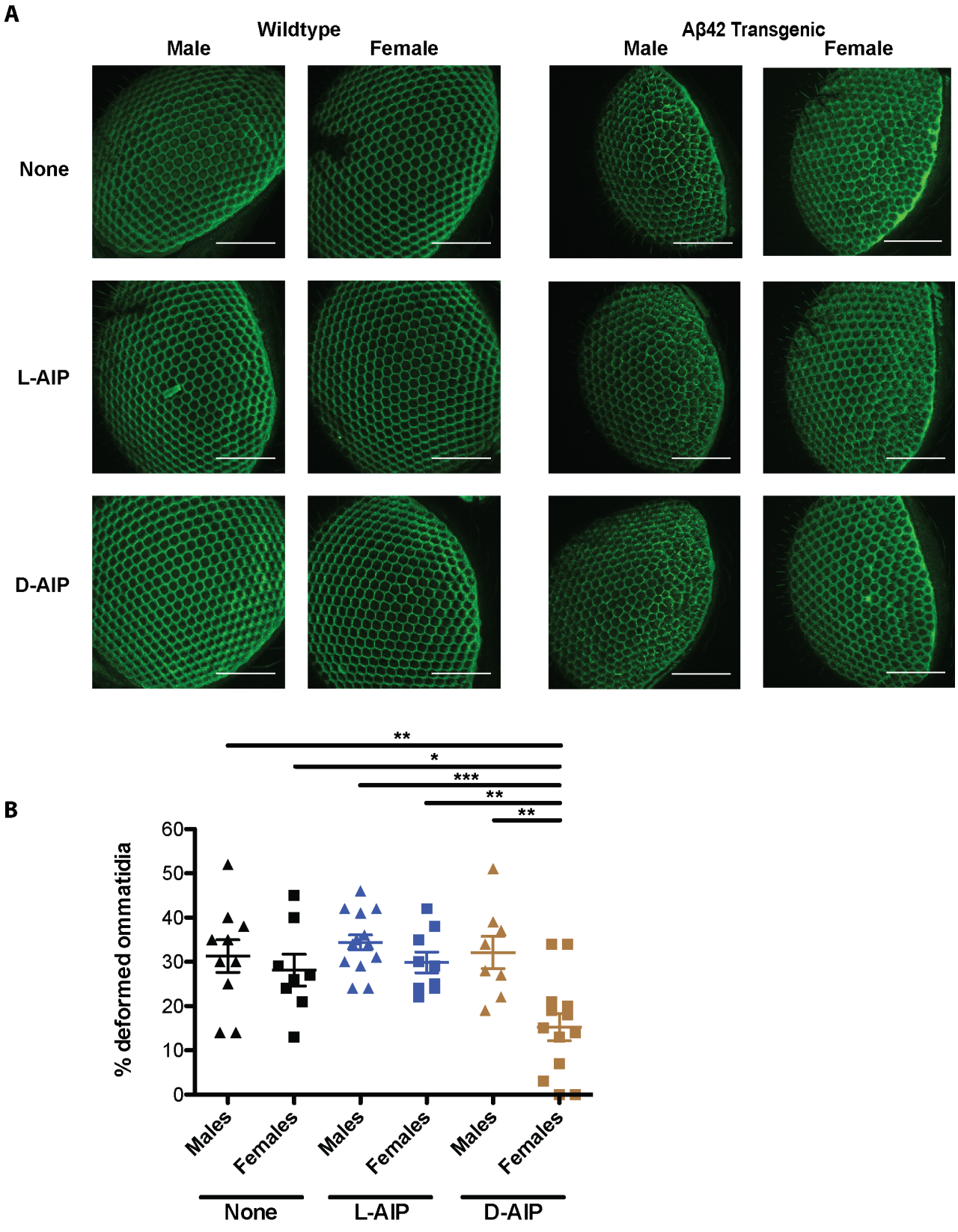

A
Wildtype
Male
Female
Aβ42 Transgenic
Male
Female
None
L-AIP
D-AIP
B
**
*
***
**
**
% deformed ommatidia
60
50
40
30
20
10
0
Males
Females
Males
Females
Males
Females
None
L-AIP
D-AIP

**Figure 11. Aβ42-induced "rough eye" phenotype is rescued in female transgenic flies at day 5 post eclosion by D-AIP supplementation**
**A**. Representative live confocal images of compound eye at day 5 post eclosion for wildtype flies (normal morphology) and Aβ42 transgenics (disrupted morphology) in the absence and presence of L- or D-AIP; scale bar: 200μm. **B**. Quantification of the percent of deformed ommatidia in Aβ42 expressing flies raised on none, L-AIP, and D-AIP supplemented food; data is represented as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA ($F(5, 57) = 6.679$, $p < 0.0001$, ANOVA) followed by Tukey's multiple comparison test; p values starting from the leftmost comparison are as follows: **p = 0.0029, *p = 0.0152, ***p < 0.0001, **p = 0.0023, and **p = 0.0025.

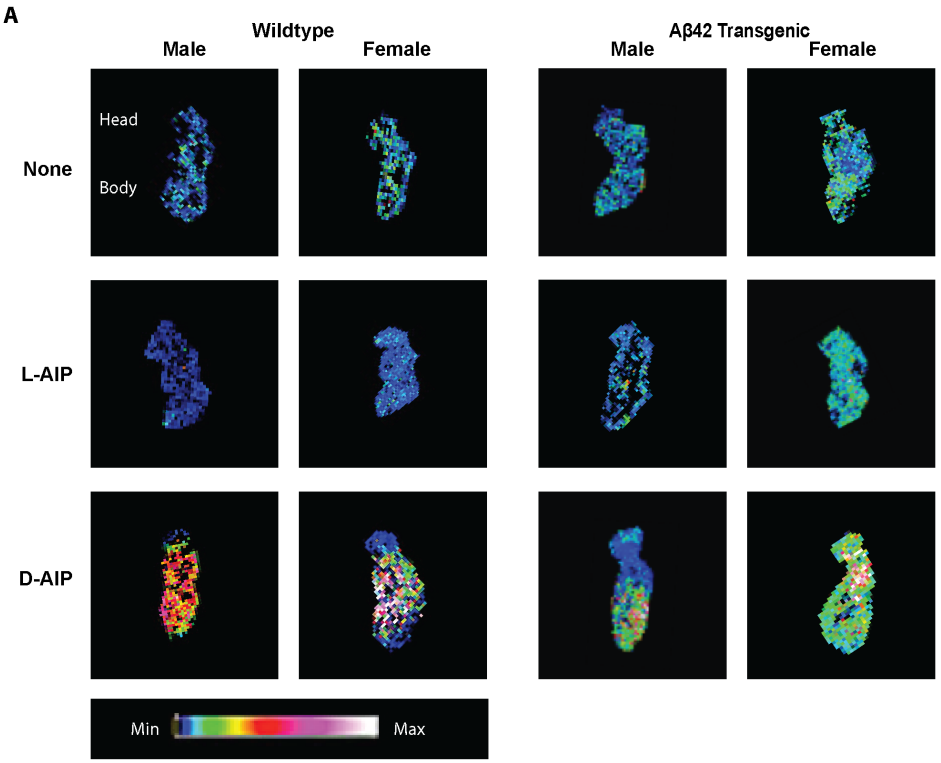
### 2.5.2 Differential localization of label-free D-AIP in male and female A$\beta$42 transgenic flies at 5 days post-eclosion

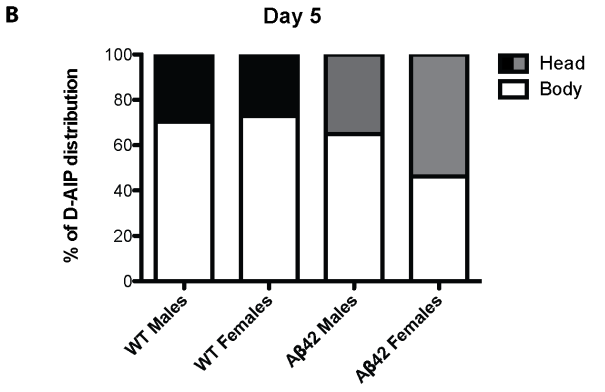
To determine if AIP was taken up from the supplemented fly food and crossed the invertebrate BBB, we used innovative MALDI-MSI to examine the distribution of label-free AIP in the flies at day 5 post-eclosion. As evidenced by the blue-coloured heat maps (i.e. low signal intensity), little or no L-AIP was detectable in both wildtype and Aβ42 transgenic flies bred and raised on L-AIP supplemented food (**Figure 12A**). For flies raised on D-AIP supplemented food, a generalized distribution of D-AIP was detectable in the wildtype male and female flies (mid-range colours and signal intensities). As evidenced by the white-coloured heat maps (i.e. high signal intensity), D-AIP was detected predominantly localized to the gut (body) of male transgenic flies in contrast to the head region of females (**Figure 12B**). Based upon the stable, direct binding interaction that we have detected between D-AIP and Aβ42 *in vitro* (**Figure 12C**), proximity of the eye-specific Aβ42 expression (i.e. heads not bodies; **Supplemental figure 1**) and head-specific D-AIP distribution was our first thought as to why neutralization of the "rough eye" phenotype (i.e. D-AIP-bound Aβ42 oligomers are trapped and rendered non-toxic) was evident in the female transgenics but not males at day 5 post-eclosion.

### 2.5.3 Altered rescue efficacy and localization of D-AIP in A$\beta$42 transgenic flies at 28 days post-eclosion

In our current study, the flies were bred and raised on a single, continuous dose of L- or D-AIP in their food (i.e. no new administrations of AIP were added to their food). To assess the long-term efficacy of the AIP rescue, both wildtype and transgenic flies were imaged again at 28 days post-eclosion. While there were no changes in the native morphology of the ommatidia in wildtype flies, the "rough eye" phenotype appeared to be modestly worse in the Aβ42 transgenics by day 28 (**Figure 13A**). Quantification of the ommatidia showed that in male transgenics, regardless of food supplementation, the percentage of deformed ommatidia had increased slightly at day 28 compared to day 5 post-eclosion (**compare Figure 11B vs. Figure 13B**). Compared to day 5, rescue of the "rough eye" morphology by D-AIP in female transgenics was no longer evident at day 28. At this point, the D-AIP-treated female transgenics exhibited similar magnitude of ommatidia defects as male transgenics in the absence/presence of L-AIP or D-AIP. As an added control, wildtype and Aβ42 transgenic flies were raised in the absence and presence of a scrambled form of the D-AIP peptide. While the long-term presence of scrambled D-AIP did not alter the native morphology of the compound eye in male or female wildtype flies by day 5 or 28, there also was no rescue of the "rough eye" phenotype in the male/female Aβ42 transgenic flies by scrambled D-AIP (**Figure 14**). Therefore, this control helped confirm that the observed rescue of the female transgenics was specifically dependent upon D-AIP supplementation in the food.

Next, MALDI-MSI was used to examine the distribution of label-free AIP and label-free Aβ42 in the flies at day 28 post-eclosion. Similar to the day 5 results (**Figure 12**), D-AIP was localized within the body of the wildtype flies, whereas in the female Aβ42 transgenics, it was localized towards the heads of the animals (**Figure 15A**). Notably, there was an increase in signal intensity for the amount of D-AIP detected in the heads of male Aβ42 transgenics at day 28 when compared to day 5 (**compare Figure 12B vs. Figure 15B**). As an added control, we examined the food slurries after days 5 and 28 to examine the stability of AIP during the time course of the fly feedings (**Figure 15C**). Having detected the degradation of intact L-AIP (941 *m/z*) to a smaller metabolite (750 *m/z*) in fly head homogenates at day 28 (**Figure 15D**), this likely explains why there is little or no intact L-AIP in the coloured heat maps of day 28 sagittal fly sections (**Figure 15A**). In contrast, peaks of the intact protease-resistant D-AIP (941 Da) were still detected at day 28 in food and sagittal fly sections.


A
Wildtype
Male
Female
Aβ42 Transgenic
Male
Female
None
L-AIP
D-AIP
Head
Body
Min
Max


B
Day 5
% of D-AIP distribution
100
80
60
40
20
0
Head
Body
WT Males
WT Females
Aβ42 Males
Aβ42 Females

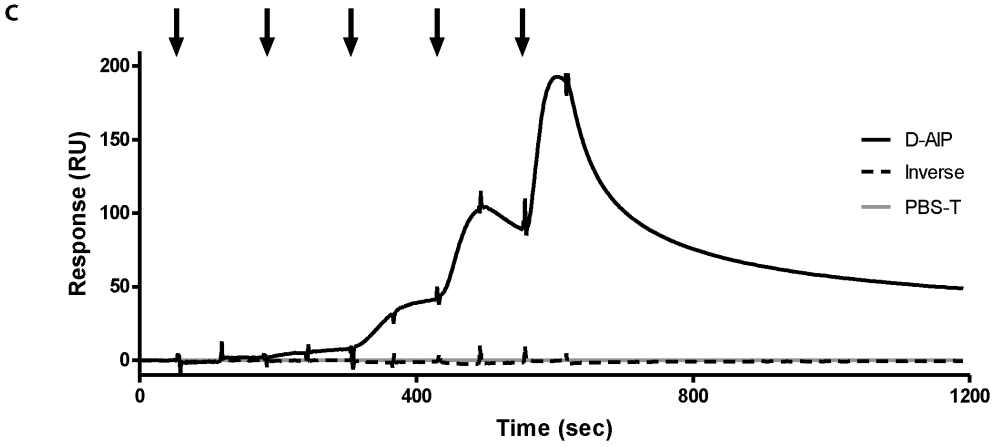
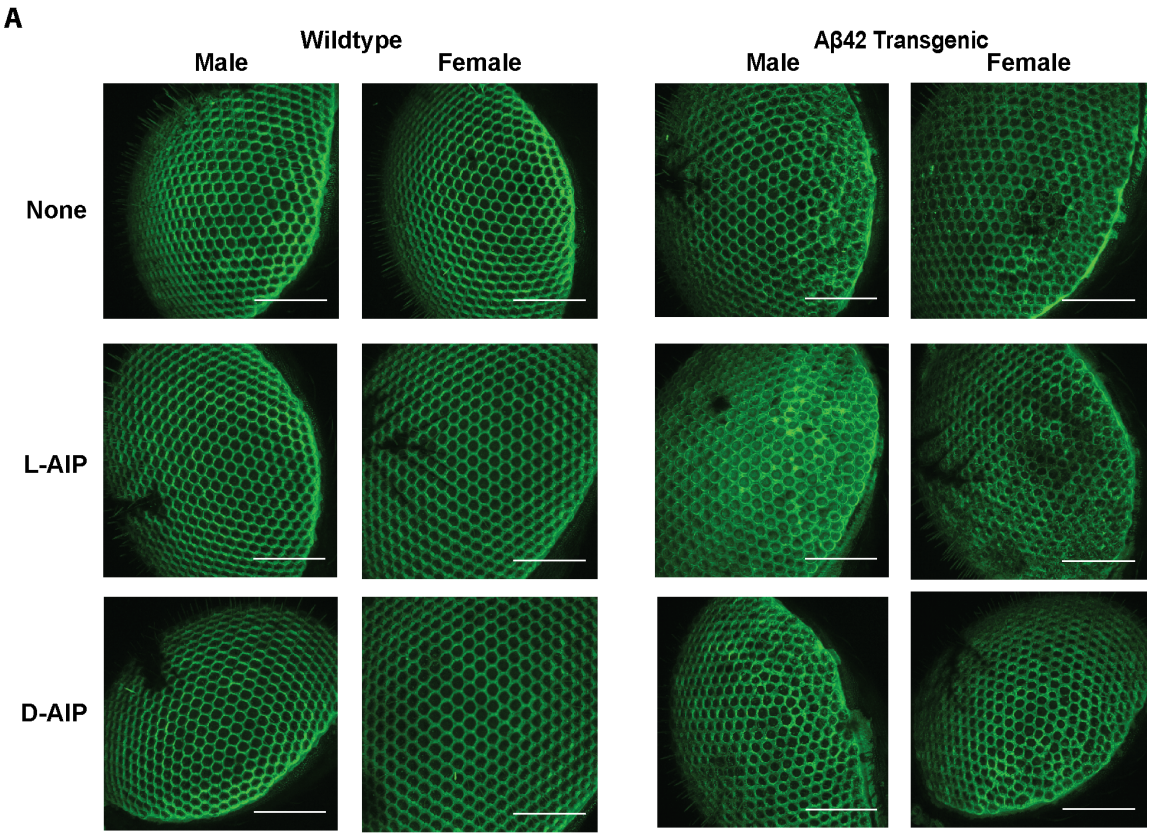

C
Response (RU)
200
150
100
50
0
0
400
800
1200
Time (sec)
D-AIP
Inverse
PBS-T

**Figure 12. Distribution of label-free AIP in wildtype and transgenic A$\beta$42 flies at day 5 post eclosion, as detected by MALDI-MSI at 20 $\mu$m resolution**

**A.** Wildtype and transgenic Aβ42 flies were raised on fly food in the absence and presence of L- or D-AIP. Sagittal sections of the flies (at day 5 post eclosion) were analyzed by label-free MALDI-MSI. Using the appropriate mass filter (AIP = 941 *m/z*), the resultant "heat maps" indicate the relative intensity (low (*blue*) to high (*white*)) and position of L- or D-AIP within the fly heads and/or bodies. **B**. Relative quantification of D-AIP signal intensity in head vs. body for wildtype and Aβ42 transgenic flies. **C**. Representative SPR data in which 156, 313, 625, 1250, and 2500μM D-AIP (low to high injections depicted by black arrows from left to right, respectively) specifically binds in a dose-dependent manner to 2220 RU amine-coupled Aβ42 at 25mL/min; under identical conditions, no binding with inverse peptide (negative control) was observed. While sample heterogeneity (i.e. mixed Aβ42 oligomeric states coupled to sensor) prevented calculation of the overall equilibrium dissociation constant ($K_D$), the observed dissociation rate constant (representative $k_d$ = 0.012 $s^{-1}$) demonstrates stable complex formation between D-AIP and Aβ42.


A
Wildtype
Male
Female
Aβ42 Transgenic
Male
Female
None
L-AIP
D-AIP

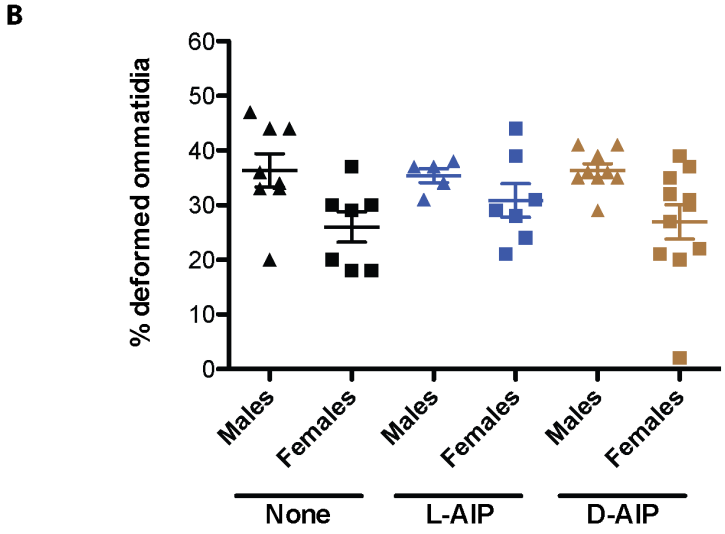

B
% deformed ommatidia
60
50
40
30
20
10
0
Males
Females
Males
Females
Males
Females
None
L-AIP
D-AIP

**Figure 13. D-AIP supplementation cannot rescue Aβ42-induced "rough eye" phenotype in female transgenic flies at day 28 post eclosion**

**A**. Representative live confocal images of compound eye at day 28 post eclosion for wildtype flies (normal morphology) and Aβ42 transgenics (disrupted morphology) in the absence and presence of L-AIP or D-AIP; scale bar: 200μm. **B**. Quantification of the percent of deformed ommatidia in Aβ42 expressing flies raised on none, L-AIP, and D-AIP supplemented food; data is represented as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA ($F(5, 41) = 3.043$, $p = 0.0199$, ANOVA) followed by Tukey's multiple comparison test; no significant difference was found between the samples.

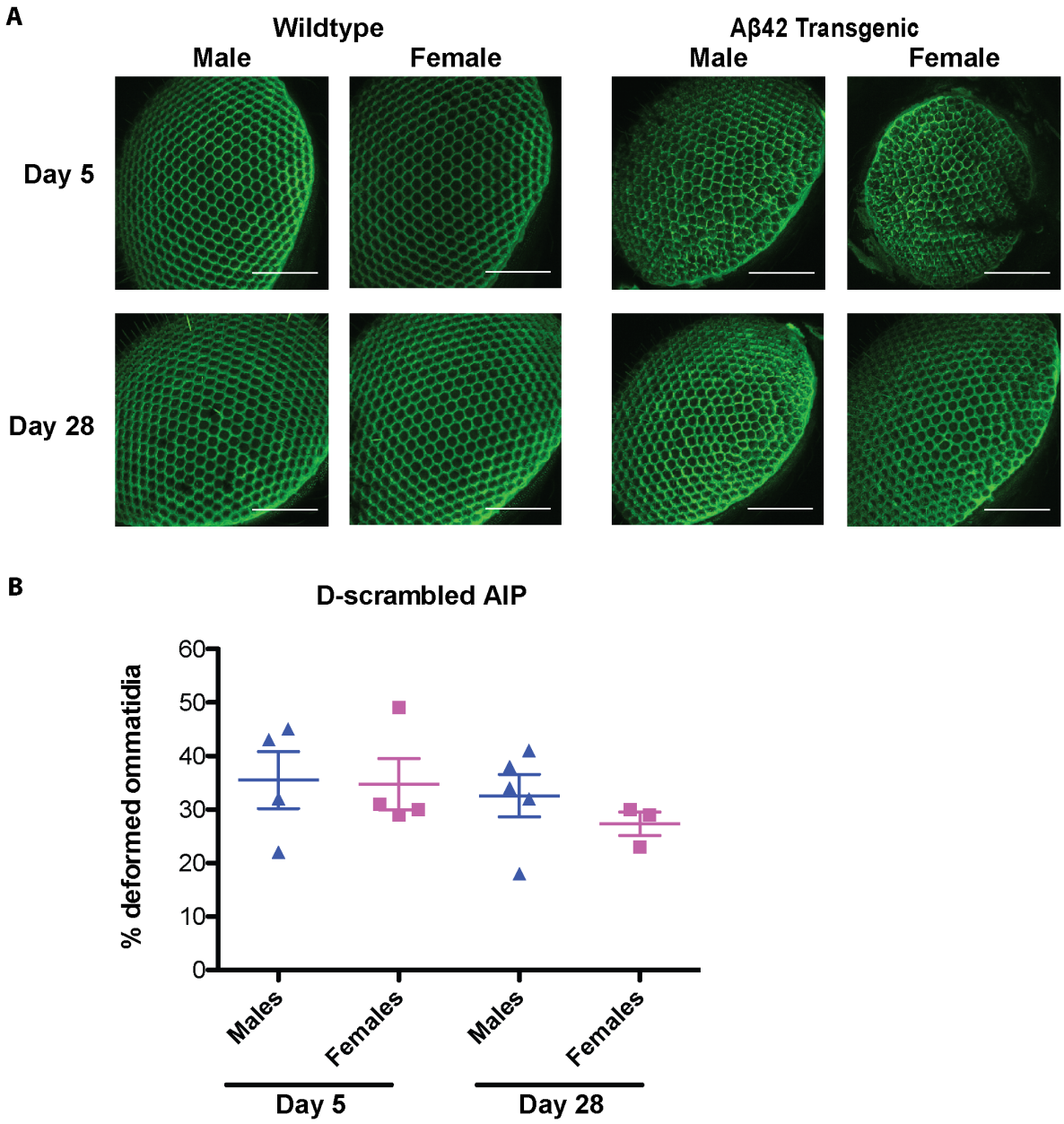
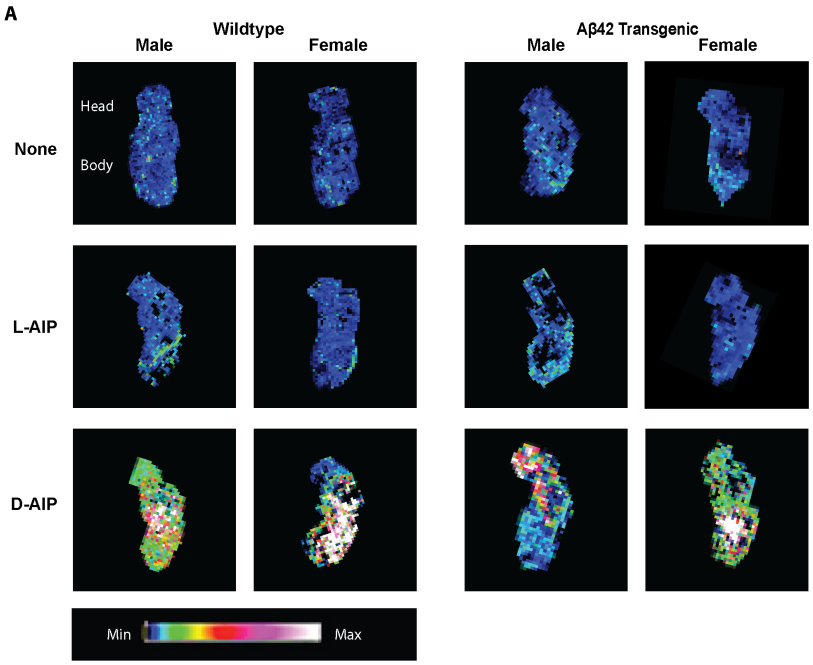
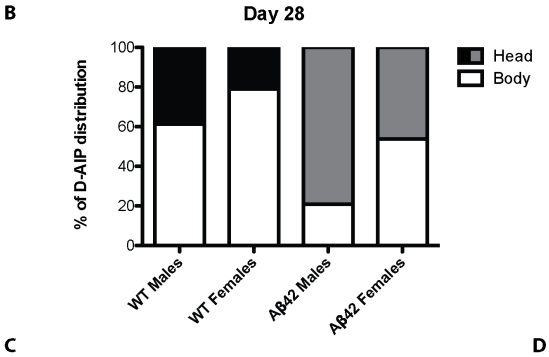



**Figure 14. Supplementation with scrambled D-AIP peptide has no effect on eye morphology in wildtype and Aβ42-expressing flies**

**A**. Representative live confocal images of compound eye at days 5 and 28 post eclosion for wildtype flies (normal morphology) and Aβ42 transgenics (disrupted morphology) in the presence of scrambled D-AIP (EFRKFTGG); scale bar: 200μm. **B**. Quantification of the percent of deformed ommatidia in Aβ42 expressing flies raised on food supplemented with scrambled D-AIP; data is represented as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA ($F(3, 12) = 0.5603$, $p = 0.6513$, ANOVA) followed by Tukey's multiple comparison test, no significant difference was found between the samples.


A
Wildtype
Male
Female
Aβ42 Transgenic
Male
Female
Head
Body
None
L-AIP
D-AIP
Min
Max


B
Day 28
% of D-AIP distribution
100
80
60
40
20
0
Head
Body
WT Males
WT Females
Aβ42 Males
Aβ42 Females

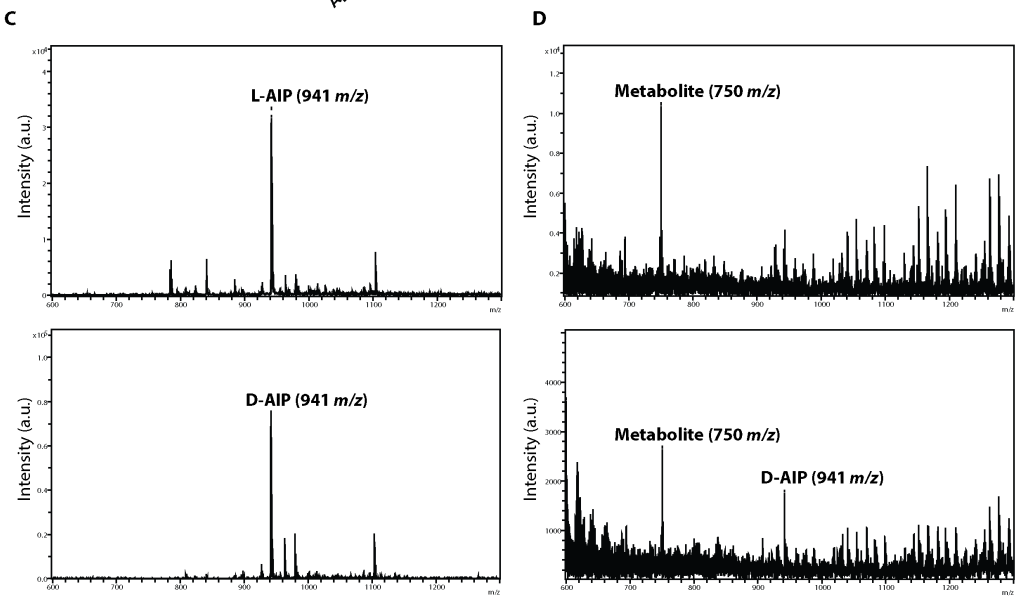
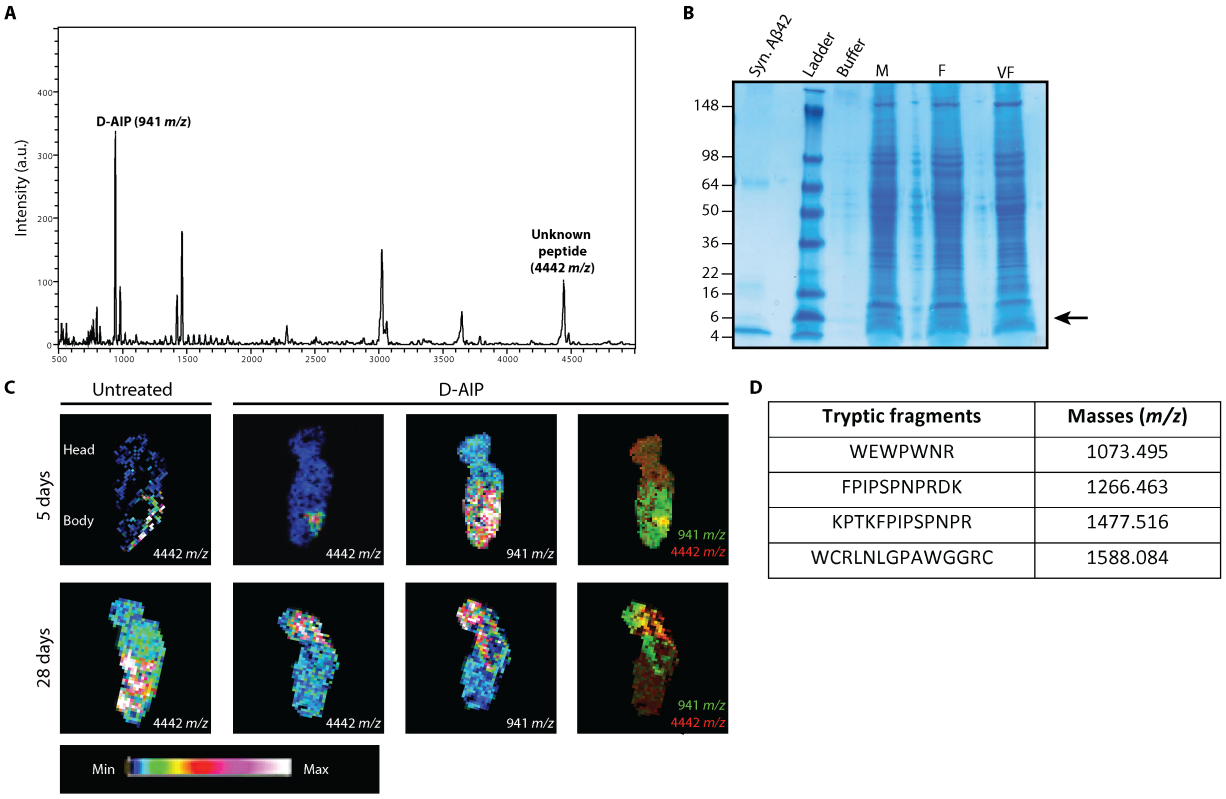

C
L-AIP (941 m/z)
Intensity (a.u.)
D-AIP (941 m/z)
Intensity (a.u.)
D
Metabolite (750 m/z)
Intensity (a.u.)
Metabolite (750 m/z)
D-AIP (941 m/z)
Intensity (a.u.)

**Figure 15. D-AIP is localized to the heads of male transgenic flies by day 28 post eclosion, as detected by MALDI-MSI at 20 $\mu$ m resolution**

**A.** Wildtype and transgenic Aβ42 flies were raised on fly food in the absence and presence of L- or D-AIP. Sagittal sections of the flies (at day 28 post eclosion) were analyzed by label-free MALDI-MSI. Using the appropriate mass filter (AIP = 941 *m/z*), the resultant "heat maps" indicate the relative intensity (low (*blue*) to high (*white*)) and position of L- or D-AIP within the fly heads and/or bodies. **B**. Relative quantification of D-AIP signal intensity in head vs. body for wildtype and Aβ42 transgenic flies. **C.** MALDI mass spectrometry of food slurry samples from 28-day old supplemented food. Both L- and D-AIP are readily detected (intact peptides at 941 *m/z* in both spectra). **D.** Wildtype flies were raised on L- or D-AIP supplemented food for 28 days; by MALDI mass spectrometry, the corresponding fly head homogenates reveal that L-AIP is protease-sensitive (metabolite detected at 750 *m/z*) whereas D-AIP is protease-resistant (intact peptide at 941 *m/z*).




| Tryptic fragments | Masses (*m/z*) |
|---|---|
| WEWPWNR | 1073.495 |
| FPIPSPNPRDK | 1266.463 |
| KPTKFPIPSPNPR | 1477.516 |
| WCRLNLGPAWGGRC | 1588.084 |

**Figure 16. Endogenous, male-specific *Drosophila* peptide co-localizes with D-AIP**
**A**. Representative single point detection of unique peptide (4442 *m/z*) that co-localizes with D-AIP (941 *m/z*) in the body of male wildtype/transgenic flies raised on D-AIP supplemented food. **B**. Whole fly body lysates of wildtype males, females, and virgin females (VF) separated by SDS-PAGE and subjected to MS-friendly colloidal Coomassie staining; unique peptide observed in males and females (as indicated, 4-5 kDa range) is absent in virgin females. **C.** Representative MALDI-MSI heat maps show co-localization of 4442 Da peptide (male-specific) and 941 Da peptide (D-AIP) in sagittal sections of male transgenic flies at days 5 and 28 post eclosion. **D.** Representative MALDI-TOF data obtained from in-gel digestion of candidate 4442 Da peptide (male wildtype/transgenic flies only) by trypsin; UniProt BLAST search for each tryptic fragment yielded consistent identification as mature Acp70A (UniProt #P05623).

### 2.5.4 A male-specific sex peptide alters D-AIP localization and thereby impairs rescue of "rough eye" phenotype

Since the rescue of the "rough eye" phenotype by D-AIP was specific to the female Aβ42 transgenic flies (i.e. live confocal imaging) and the distribution of D-AIP differed between the sexes, we then investigated the male Aβ42 transgenics in greater detail. Re-analyzing our

MALDI-MSI spectra, a peptide (4442 *m/z*), which was only found in the male wildtype/transgenic flies, also co-localized with D-AIP in the fly bodies (**Figure 16A**). To isolate and identify this unknown protein in the flies, male, female, and virgin female body lysates were separated by SDS-PAGE and subjected to MS-friendly colloidal Coomassie staining (**Figure 16B**). The bands of interest (i.e. 4-6 kDa range) were excised for in-gel digestion with trypsin. The resulting peptide fragments were analyzed by MALDI mass spectrometry and searched against the UniProt database to identify the unknown *Drosophila* protein as accessory gland protein-70A (Acp70A; **Figure 16D**). Acp70A is a male-derived sex peptide that co-localized with D-AIP in the gut of the male wildtype/transgenic flies by day 5 post-eclosion, and later co-localized to the heads by day 28 post-eclosion (**Figure 16C**). To account for the sex-specific rescue in the female transgenic flies (**Figure 11**), we thought that the differential distribution of D-AIP (i.e. male gut, female heads) impacted its efficacy to neutralize the eye-specific Aβ42 toxicity at day 5 post-eclosion. Acp70A may have sequestered D-AIP in the gut of the male flies, thereby impairing the rescue of the "rough eye" phenotype in the male Aβ42 transgenic flies at day 5 post-eclosion.

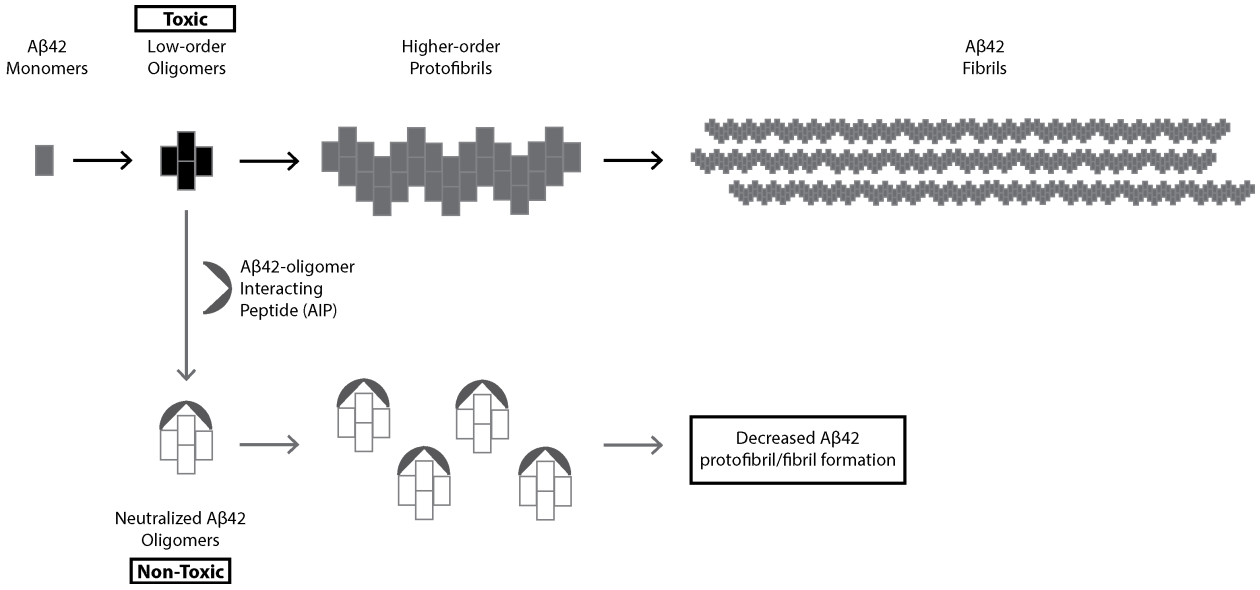



**Figure 17. Development of AIP-Trap as a novel intervention to prevent toxic amyloid formation during early stages of AD**

Proposed mechanism of action in which AIP-Trap (synthesized using protease-resistant D-amino acids) specifically binds to toxic low-order Aβ42 oligomers rendering them non-toxic (without creating larger aggregates in the process). By example, our current study demonstrates that *Drosophila* transgenics expressing human Aβ42 in the compound eye exhibit a "rough eye" phenotype (i.e. toxic) whereas D-AIP administration can render Aβ42 non-toxic (i.e. rescued phenotype) without obvious side effects (i.e. high dosage D-AIP does not alter overall longevity, structure, or function of wildtype flies).

## 2.6 Discussion

We have previously shown that a short, 8-residue Aβ42-oligomer interacting peptide (AIP) can suppress the aggregation of low-order Aβ42 oligomers into fibrils *in vitro*, as well as neutralize Aβ42-induced neurotoxicity in cell cultures and rat hippocampal slices (Barucker *et al.*, 2015). We have now used the *Drosophila* "rough eye" model to investigate the long-term efficacy of AIP in a full longitudinal study comparing male to female flies.

For our current study, individual flies had to be monitored for changes in their compound eye morphology over time. Therefore, we elected to use confocal microscopy since live imaging techniques are not compatible with electron microscopy (EM), the traditional approach used to image compound fly eyes (Pignoni *et al.*, 1997; Schreiber *et al.*, 2002; Iyer *et al.*, 2016). Faster and cheaper than TEM or SEM, live confocal imaging allowed us to monitor multiple cohorts of flies over 28 days (post-eclosion) for changes in eye morphology in the absence or presence of Aβ42 toxicity, with or without AIP supplementation in the fly food. It is well established that the expression of human Aβ42 in the *Drosophila* compound eye induces dramatic morphological changes (Crowther *et al.*, 2006; Jeon *et al.*, 2017; Sarkar *et al.*, 2018), resulting in deformed or merged ommatidia instead of the highly organized lattice array in wildtype flies (Ready *et al.*, 1976).

Wildtype and Aβ42 transgenic flies were raised on food in the absence or presence of synthetic peptide supplementation (L-AIP, D-AIP, or scrambled D-AIP control). While intact masses for L- and D-AIP were readily detectable in the starting fly food by MALDI-MS (**Figure 15C**), protease-sensitive L-AIP was less stable compared to protease-resistant D-AIP especially by day 28 in the fly head homogenates (**Figure 15D**). While the intact mass for D-AIP (941 *m/z*) was still evident at this point, the conversion of intact L-AIP (941 *m/z*) to a smaller metabolite (750 *m/z*) appeared to correlate with the loss of the functionally important N-terminal arginine (Barucker *et al.*, 2015). Knowing that L-amino acid peptides typically exhibit decreased biostability compared to D-amino acid peptides (Tugyi et al., 2005; Li et al., 2012c; Fosgerau and Hoffmann, 2015), the *in vivo* degradation of L-AIP and loss of its N-terminal arginine residue

likely explains why L-AIP was unable to rescue the "rough eye" phenotype in our longitudinal study. Notably, other anti-amyloid peptide candidates have exhibited superior *in vivo* stability when synthesized using D-amino acids (e.g. "Aβ1-$6_{A2V}$-TAT" candidate (Diomede et al., 2016)). In addition to enhanced stability, we showed that our lead D-AIP candidate can directly interact with Aβ42 in a specific, dose-dependent manner as assessed by label-free, real-time SPR. The stable complex formation observed between our short, 8 amino acid D-AIP peptide and Aβ42 (i.e. dissociation rate constant, $k_d = 10^{-2}$ $s^{-1}$; **Figure 12C**) is consistent with other small inhibitors analyzed by SPR: e.g. $k_d = 0.022$ $s^{-1}$ for 19 amino acid cyclic peptide ("MGSADGA-u2" serine protease inhibitor candidate) binding to immobilized uPA (Jiang et al., 2011); $k_d = 0.008$ $s^{-1}$ for single-chain antibody fragment ("I2" anti-amyloid candidate) binding to immobilized Aβ42 fibrils (Munke et al., 2017); $k_d = 0.026$ $s^{-1}$ for 7 amino acid cyclic peptide ("CP2" anti-infective candidate) binding to immobilized hSPSB2 (Sadek et al., 2018). Overall, our present longitudinal study demonstrates that a single, continuous dose of D-AIP in the fly food is (i) well tolerated *in vivo* over a significant portion of the *Drosophila* lifespan, (ii) not likely to generate potentially toxic secondary metabolites like L-AIP, and (iii) likely rescues the "rough eye" phenotype through its direct, stable binding interaction with low order Aβ42 oligomers.

At day 5 post-eclosion, it was evident that the "rough eye" phenotype was only ameliorated in the female Aβ42 transgenics fed with D-AIP supplemented food (**Figure 11**). To further investigate this sex-specific outcome, we first examined the distribution of D-AIP in male and female flies. In the absence of D-AIP-specific antibodies and not wanting to adversely affect the therapeutic potential of the peptide with non-native tags and/or modifications, we elected to use innovative, label-free MALDI tissue imaging to determine the distribution of L- and D-AIP in the sagittal fly sections – to our knowledge, this is the first time that MALDI-MSI has been used to track a label-free therapeutic candidate in *Drosophila* sections. D-AIP localized more to the heads of female transgenics compared to the gut in male transgenics at day 5 post-eclosion (**Figure 12**). The differential D-AIP distribution between males and females is likely what contributed to the sex-specific rescue of the "rough eye" phenotype in female Aβ42 transgenics only (**Figure 11**). Sequestration of the peptide in the gut of male flies may have impeded D-AIP from binding to eye-specific Aβ42 in the heads of the transgenics flies and, for this reason, the Aβ42-induced "rough eye" phenotype persisted in the male transgenics only.

We also investigated the long-term effect of single, continuous dose AIP administrations on the wildtype and Aβ42 transgenic flies. By day 28 post-eclosion, the ability of D-AIP to rescue the Aβ42-induced toxicity in female transgenic flies was no longer observed. Since the AIP-supplemented food was not refreshed throughout the duration this longitudinal study and the AIP distributions by MALDI-MSI are not quantitative, we expect that the bioavailability of D-AIP would be significantly decreased by day 28 (e.g. increased proteolysis or degradation of D-AIP in the flies and/or decreased uptake of D-AIP from food) when compared to day 5 post-eclosion. Although *Drosophila* possesses an endogenous γ-secretase complex (Hong and Koo, 1997; Periz and Fortini, 2004), as well as β- and α-secretase-like enzymes (Rooke *et al.*, 1996; Carmine-Simmen *et al.*, 2009), these proteins only share about 50% homology with their human counterparts. The *Drosophila* APP ortholog, named dAPPl, also lacks significant homology in the region corresponding to the Aβ peptide sequence compared to vertebrate APP family members (Luo *et al.*, 1992). Therefore, the non-native, human Aβ42 that we introduced in our "rough eye" transgenics was likely not readily degraded by the flies and, with increasing age, accumulated to the point that decreasing D-AIP levels could no longer rescue the more advanced phenotype (i.e. potentially more higher-order Aβ42 oligomers by day 28, compared to low-order Aβ42 oligomers at day 5 post-eclosion).

In the male Aβ42 transgenic flies, it was observed that the distribution of D-AIP was different at day 28 compared to day 5 post-eclosion (**compare Figure 15 vs. 12, respectively**). Despite the overlap of D-AIP with eye-specific Aβ42 in the heads of the male transgenics by day 28, there still was no rescue of the more advanced "rough eye" phenotype (i.e. potentially more unaffected higher-order Aβ42 oligomers) at this point. Upon further investigation, we discovered a male-specific 4.4 kDa peptide, a mass consistent with the male-specific *Drosophila* sex peptide accessory gland protein-70A (Acp70A) (**Figure 16**), that seemed to colocalize with D-AIP on both days 5 and 28 post-eclosion (**Figure 16C**). Acp70A is produced exclusively in the male *Drosophila* and passed onto females in the seminal fluid during mating to enhance reproductive success (Liu and Kubli, 2003; Lawniczak and Begun, 2004; McGraw *et al.*, 2004; Mack *et al.*, 2006; Pilpel *et al.*, 2008). It is composed of 36 amino acids and is only produced by an accessory gland found in the gut of male flies (Yapici *et al.*, 2008). Since the low concentration of Acp70A in female flies is rapidly degraded (Pilpel *et al.*, 2008), we suspect that the high concentration of

Acp70A in male flies somehow sequestered D-AIP in the gut of the male transgenics by day 5 post-eclosion, thus impeding D-AIP from reaching the eye-specific Aβ42 in the heads and rescuing the "rough eye" phenotype. Since Acp70A is a gut-specific peptide (i.e. day 5 detection in body by MALDI-MSI; **Figure 16**), its differential localization in the male Aβ42 transgenics by day 28 post-eclosion suggests that it somehow co-migrated with D-AIP to the heads of the adult, day 28 flies. While this is intriguing, the unexpected outcome with Acp70A illustrates the importance of including both male and female *Drosophila* in the design of *in vivo* fly models. Nevertheless, this confounding factor does not impact our future directions since we now need to establish the efficacy of D-AIP in more advanced transgenic mouse/rat models of AD where fly physiology (i.e. Acp70A) will not be a factor.

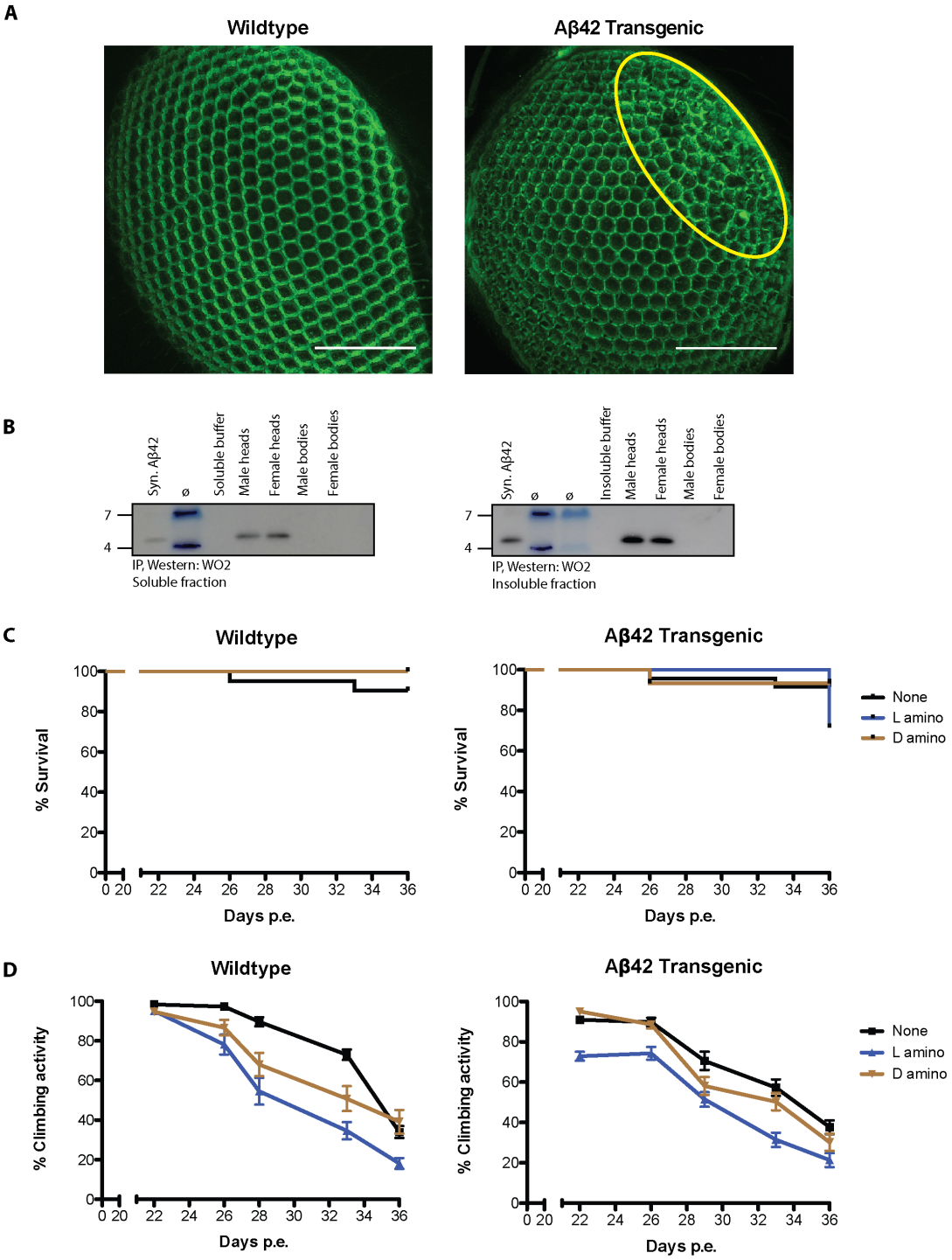
## 2.7 Conclusion

In closing, this longitudinal study demonstrates that orally administered D-AIP has the potential to cross the invertebrate BBB and neutralize the Aβ42-induced toxicity without obvious side effects (i.e. rescue of "rough eye" phenotype without impacting survival or locomotor behaviour in transgenic *Drosophila* model). In the absence of the confounding Acp70A sex-peptide issue (male flies only), we expect that rescue of the male transgenics would also have been possible at day 5 post-eclosion like the female transgenics. Now that we need to establish the efficacy of D-AIP in more complex rodent models of AD such as the 3xTg model (Oddo et al., 2003), we are encouraged that many other groups have reported that their small D-amino acid peptides are stable (i.e. protease-resistant), non-toxic, and can readily penetrate the blood brain barrier in wildtype mice via different routes of administration (Jiang *et al.*, 2016; Leithold *et al.*, 2016). Overall, our D-AIP peptide provides a novel mechanism of action to reduce amyloid toxicity (**Figure 17**), thus providing a promising new prophylactic strategy to delay and/or prevent the onset of AD.

## 2.7 Acknowledgements

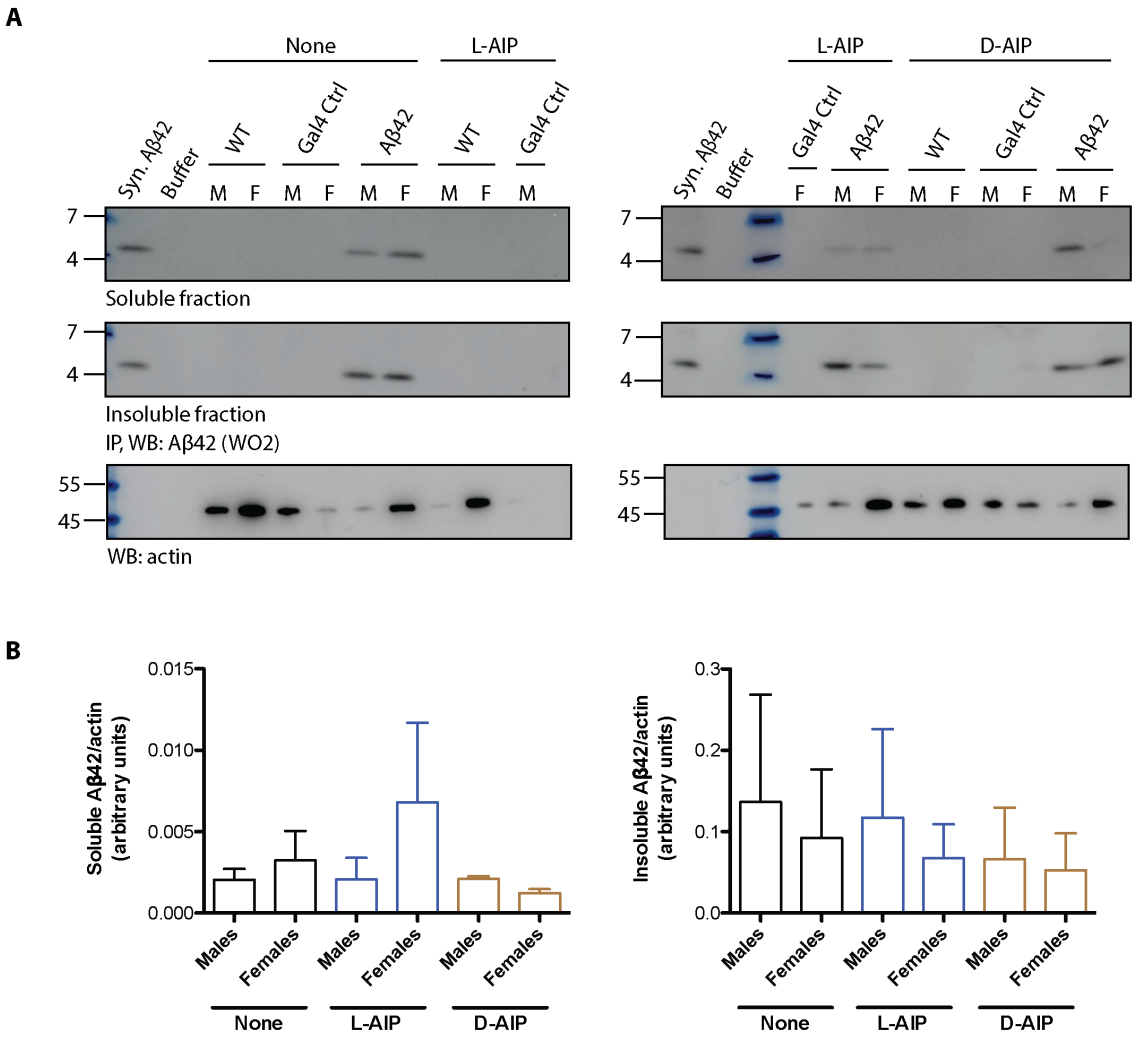
This study was supported by a grant from the Canadian Institutes of Health Research (MOP-133411). GM holds both a Canada Research Chair in Molecular Pharmacology and a Canada Foundation for Innovation (CFI) grant. The McGill Life Sciences Complex thanks the CFI for infrastructure support. Thanks also to Dr. Filip Liebsch for establishing the transgenic *Drosophila* strains and scientific guidance, and to the McGill Life Sciences Histology facility for their technical support.

## 2.8 Supplemental Figures



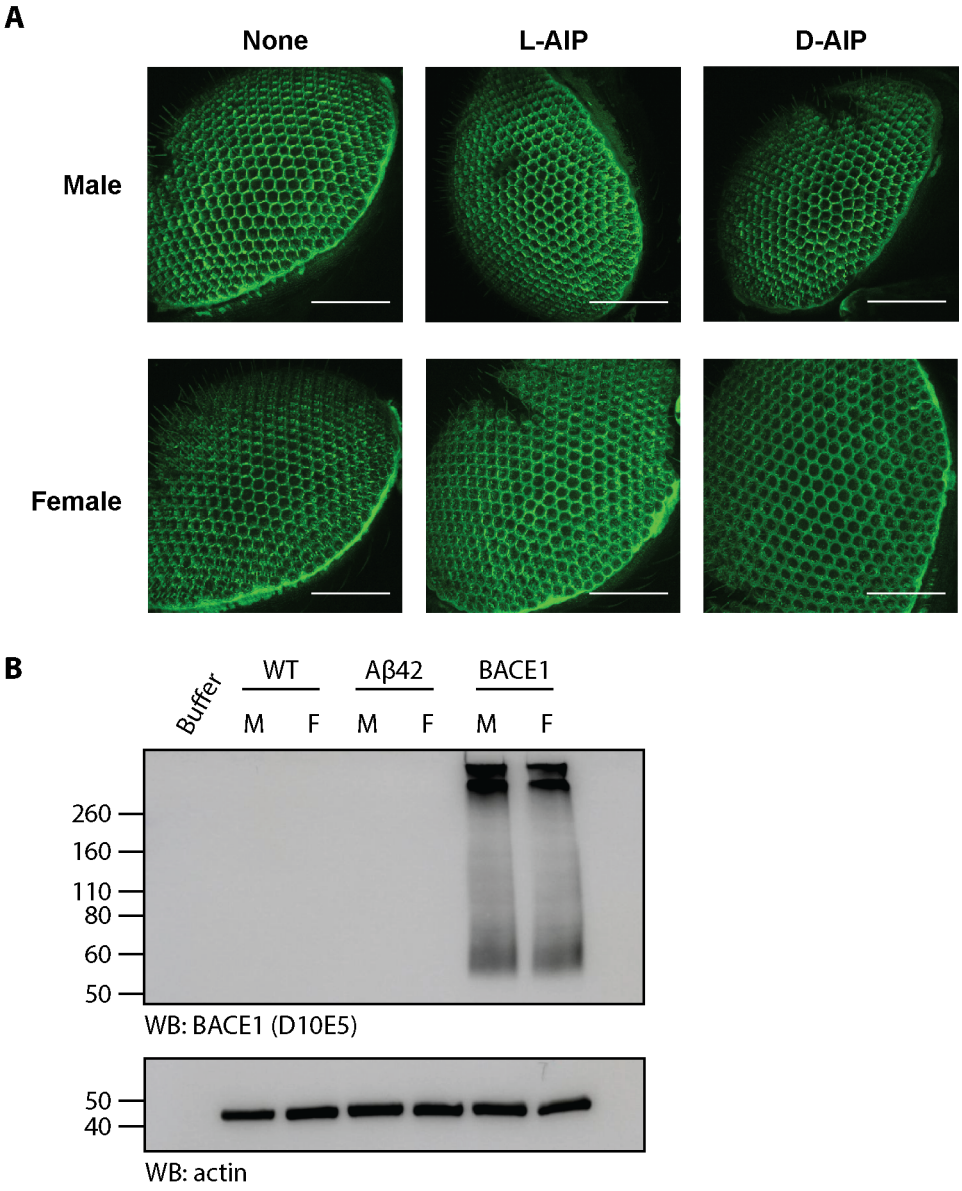
**Supplemental Figure 1. AIP-supplemented food does not affect survival or locomotor behaviour of transgenic Aβ42 flies**

**A**. Representative live confocal images of compound eye at day 5 post-eclosion for wildtype flies (normal morphology: hexagonal ommatidia) and Aβ42 transgenics (disrupted "rough eye" morphology: oval to highlight fused and/or misshapen ommatidia). **B**. Soluble and insoluble fractions from transgenic *Drosophila* heads and bodies were immunoprecipitated with the Aβ-specific antibody W0-2. Compared to molecular weight markers and a known synthetic Aβ42 standard (far left lane, 4.5 kDa, 5 ng), representative Western blot analysis shows that eye-specific expression of human Aβ42 was only detectable in the heads of male and female flies as anticipated. Ø represents lanes loaded with protein ladder, 3 heads or bodies of each sex was used per sample. **C**. Representative Kaplan-Meier survival curves show that longevity of wildtype ($df = 2$, $p = 0.3589$) and Aβ42 ($df = 2$, $p = 0.2107$) transgenic flies was unaltered in the presence of added AIP. (13 animals per group, $n = 3$) **D**. Representative RING assays show that locomotor activity of wildtype and Aβ42 transgenics flies was unaltered in the presence of added AIP; 13 animals per group, $n = 3$; data is represented as mean ± SEM. Linear regression was used to establish and compare slopes for wildtype ($F = 4.04113$, $p = 0.01823$, statistically significant difference between slopes) and Aβ42 transgenics ($F = 1.81889$, $p = 0.1634$, no significant difference between slopes).




**Supplemental Figure 2. Expression of human A $\beta$ 42 is similar in both male and female transgenic flies regardless of food supplementation**

**A.** Western blot of human Aβ42 expression in transgenic *D. melanogaster* with eye-specific driver. Animals were treated with unsupplemented, L-AIP supplemented, and D-AIP supplemented food. **B.** Quantification of Western blot data. Aβ42 expression levels appears to be similar in both the soluble and insoluble fraction of both sexes regardless of food supplementation. The addition of L- and D-AIP does not seem to affect the overall levels of Aβ42 accumulation. Data is represented as mean ± SEM. Two way ANOVA was performed and no significant difference was detected between the groups. n = 3.




**Supplemental Figure 3. Eye-specific BACE1 expression has no effect on gross morphology of ommatidia in male (M) or female (F) transgenic flies**

**A**. Representative confocal images of eye-specific human BACE1-expressing flies supplemented with food containing L-, D-, and D-scrambled AIP at day 5 post-eclosion; scale bar: 200μm. **B**. Western blot analysis of BACE1 expression in eye-specific BACE1 expressing flies. Lysates from isolated fly heads of wildtype, Aβ42-expressing, and BACE1-expressing transgenics were probed for human BACE1.

**MANUSCRIPT 3**

## The D-enantiomeric Aβ42-oligomer Interacting Peptide (D-AIP) attenuates amyloid-β42 induced defects in *Drosophila melanogaster* locomotion

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## 3.1 Foreword

Our results from the previous manuscript (**manuscript 2**) showed D-AIP was highly effective at rescuing A $\beta$ 42-induced defects in the gross morphology of the *Drosophila* compound eye. We are next interested as to whether D-AIP can also rescue A $\beta$ 42-induced functional deficits in more complex physiological systems such as the *Drosophila* central nervous system (CNS). To this end, we have decided to utilize the neuron-specific A $\beta$ 42-expressing model that we have also previously characterized in our first manuscript (**manuscript 1**). Using these transgenic animals, we performed food supplementation experiments with AIP and measured the locomotor activities of the flies in longitudinal experiments to assess functional changes and rescue. We also employed label-free MALDI-IMS techniques to determine and analyze the presence and localization of D-AIP in sagittal fly slices. Overall, this third manuscript suggests that A $\beta$ 42-induced functional deficits in *Drosophila* could also be ameliorated with long-term administration of D-AIP, and in conjunction with the second manuscript, shows that D-AIP is a stable, non-toxic, and effective *in vivo* neutralizer of toxic A $\beta$ 42.

## 3.2 Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is characterized by the loss of neurons and the formation of amyloid plaques. These AD plaques are a result from the aggregation of amyloid beta (A $\beta$ ) peptides, and small soluble oligomers of the A $\beta$ moiety with 42 amino acids (A $\beta$ 42) are believed to be the main effector of synaptic dysfunction and neuronal loss. We have demonstrated previously that a small A $\beta$ 42-oligomer interacting peptide synthesized from D-amino acids (D-AIP) can ameliorate A $\beta$ 42-induced eye deformations *in vivo* in a transgenic *Drosophila* model. Now, to further investigate the neutralizing effects of D-AIP on A $\beta$ 42-induced toxicity in functional studies, we have conducted longitudinal food supplementation in transgenic *Drosophila* models where human A $\beta$ 42 is expressed in a pan-neuronal manner and induces defects in locomotor activity of the transgenic animals. Treatment with D-AIP was able to significantly rescue the decline of climbing activity driven by A $\beta$ 42 expression, and mass spectrometry imaging (MALDI-MSI) data confirmed the robust distribution and uptake of D-AIP *in vivo*. Surprisingly, this rescue was only evident in male transgenic animals, as the climbing activities of the females were not generally affected by the pan-neuronal expression of human A $\beta$ 42. Our longitudinal study demonstrates that D-AIP can successfully attenuate A $\beta$ 42-induced defects in functional behaviour (i.e. climbing), and the observed sex difference in rescue further illustrates the need to include sex as a variable in both *in vitro* and *in vivo* experiments.

## 3.3 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, and is currently the most common cause of dementia in the world (Alzheimer's, 2010). A common pathological feature of the disease is the presence of amyloid plaques, which are extracellular deposits of a small protein named amyloid beta (Aβ) (LaFerla and Oddo, 2005; Nelson et al., 2009). Aβ peptides are generated via the sequential proteolysis of the amyloid precursor protein (APP) by β- and γ-secretase, where Aβ of a variety of lengths can be produced (De Strooper and Annaert, 2000). Of which, the Aβ peptide with 42 amino acid residues (Aβ42) has been shown to be the most prone to oligomerization and neurotoxicity (Tanzi and Bertram, 2005). Soluble Aβ42 oligomers have been suggested to be the main toxic agent that is culpable for the development and progression of AD (Ferreira et al., 2015; Selkoe and Hardy, 2016). As such, they have become interesting therapeutic targets for the development of AD interventions (Salahuddin et al., 2016).

Previously, we have reported that the small synthetic Aβ42-oligomer interacting peptide (AIP) is able to ameliorate the aggregation and toxic behaviours of Aβ42 oligomers in *in vitro* experiments, as well as rescue the Aβ42-induced loss of synaptic spine density and LTP in organotypic hippocampal slice cultures (Barucker et al., 2015). Additionally, using transgenic *Drosophila melanogaster* – which have been demonstrated as an important and influential *in vivo* model system in the study of neurodegenerative diseases such as AD (Iijima et al., 2004; Jeibmann and Paulus, 2009) – we showed that AIP supplementation was able to prevent the formation of the "rough-eye" phenotype caused by Aβ42 expression (Barucker et al., 2015). Specifically, we showed that AIP composed solely of D-amino acids (D-AIP) was able to rescue the "rough eye" phenotype in induced by Aβ42 expression in a sex-dependent manner, where eye malformations caused by Aβ42 expression was ameliorated by D-AIP only in female animals (**manuscript 2**).

We now aim to further characterize the effects of D-AIP in more complex *Drosophila* physiological systems. Notably, *Drosophila* models that express the toxic human Aβ42 peptide in the brain and/or CNS have been extensively used to reliably characterize its toxic downstream

effects at both molecular and physiological levels (Finelli et al., 2004; Crowther et al., 2005; Luheshi et al., 2007; Iijima et al., 2008; Iijima-Ando et al., 2008). For our study, we have elected to use a *Drosophila* model with pan-neuronal Aβ42 expression in order to assess the effects of D-AIP on Aβ42-induced functional defects. To this end, we demonstrated that D-AIP was able to rescue the locomotor deficits in the transgenic animals. However, this effect was limited to the male animals, as the female animals appeared to be resistant to Aβ42-induced toxicity. We also used novel, label-free matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) technique to confirm the uptake and distribution of D-AIP in the transgenic animals. Our study shows that orally administered D-AIP is readily bioavailable and can reliably rescue Aβ42-induced functional defects *in vivo*.

## 3.4 Materials and Methods

### 3.4.1 Transgenic *Drosophila*

UAS-Aβ42 flies were generated as described previously (Harmeier et al., 2009). Transgenic flies that contain the UAS-Aβ42 construct were crossed with flies containing *elav-Gal4* to induce Aβ42 expression in a neuron-specific manner. Transgenic UAS-BACE1 flies were generated using the pUAST-BACE1 construct. They were crossed with *elav-Gal4* containing flies to obtain control transgenic flies that expressed BACE1 in a neuron-specific manner. Canton S flies were used as wildtype controls and were also crossed with *elav-Gal4* flies to obtain Gal4-only controls. Flies are reared in an incubator (Tritech) with 12hr light/dark cycle, 50% humidity, and at 25°C.

### 3.4.2 Peptides

Label-free AIP (L- or D-amino acids, RGTFEGKF, 940.5 Da) and scrambled AIP (L- or D-amino acids, EFRKFTGG, 940.5 Da) were purchased from BioBasic (Markham, ON, Canada) and verified by mass spectrometry at the McGill SPR-MS Facility (Department of Pharmacology & Therapeutics, McGill Life Sciences Complex). Prepared fresh for each experiment, AIP peptides were solubilized at 50 mg/ml in deionized water containing 0.1% ammonia, vortexed, and then sonicated at 37hz and 100% power for 10min at 4°C. Resuspended AIP peptides were diluted to 1 mg/mL in TA50 (acetonitrile : 0.1% (v/v) TFA = 50:50) for testing by MALDI-MS (verify intact mass via Bruker UltrafleXtreme system) or ESI-MS (verify sequence via Bruker Impact II system) according to the manufacturer's instructions.

### 3.4.3 AIP food supplementation

Flies were bred on Jazz-Mix *Drosophila* fly food (Fisher Scientific). Food supplemented with AIP peptides was prepared in 15ml centrifuge tubes (Sigma-Aldrich) to conserve volume and prevent drying. Dissolved peptides were added to a final concentration of 50mM to the bottom of the centrifuge tubes, then 1ml of freshly prepared fly food cooled to 45°C was added on top. The mixture was thoroughly blended using a 1ml pipette with cut tip and left to solidify for over 12hrs at room temperature. Parental generation flies were crossed on supplemented food, and

were removed with the appearance of F1 3rd instar larvae. F1 transgenic flies were separated by sex and collected into fresh tubes as they eclose.

### 3.4.4 Live confocal imaging

Live confocal imaging studies have been described previously (Dourlen et al., 2013). Briefly, each animal is immobilized sagittally on a 35mm plate half-filled with 2% low melting point agarose (Fisher Scientific) at 45°C, where half of the body and head of the fly is embedded into the agarose. The plate is placed on ice for the agarose to solidify. The fly is then covered with ice-cold water for anaesthesia and cornea neutralization, and imaged using a TCS SP8 confocal microscope (Leica; water-immersion objective, #506323, 25X 0.95NA Water HCX IRAPO L; 552 green laser at 18% power) at the Imaging & Molecular Biology Platform (Department of Pharmacology & Therapeutics, McGill Life Sciences Complex. A z-stack is subsequently made of the whole eye of the animal. The flies are gently retrieved from the agar using forceps post imaging and dried on Kimwipes (Kimberly-Clark). They are then placed back into their original tubes and allowed to recover overnight with the tube place on its side to prevent the animals from drowning/sticking to the food. The autofluorescence which illuminates the compound eye under confocal microscopy is thought to derive from the conversion of rhodopsin to its photoproduct metarhodopsin upon light stimulation in the rhabdomeres or photoreceptors of each ommatidia (Franceschini et al., 1981).

### 3.4.5 Locomotor assay

Rapid iterative negative geotaxis assays (RING assay) was performed as described (Barone and Bohmann, 2013). Flies were aged to three weeks (21 days) at the beginning of the experiments, as Aβ42-induced locomotor dysfunction has been previously shown to be discernible after this period (Iijima et al., 2004). Measurements were taken every 3 days, at the same time of the day for each sex/genotype. Groups of 10-15 animals, separated by sex, genotype, and treatment, are placed in empty clear plastic tubes and allowed to recover from $CO_2$ anaesthesia at room temperature for 45mins. Flies are then forced to the bottom of the tube by firmly tapping against the bench for 10 sec. They are then allowed to climb up the sides of the vials for a further 10 sec, and the number of flies that walked above the 2cm mark was recorded. They were allowed one minute to rest between assays. This was repeated 15 times to obtain the average climbing activity

for the day, and the percentage climbing activity (number of flies over 2cm line/number of total flies x 100) was plotted as a function of age using Excel.

### 3.4.6 Tissue homogenates

Five flies of each sex, genotype, and treatment were snap-frozen in liquid nitrogen. The heads were collected and homogenized in 100µl of PBS-PI (PBS buffer with Complete EDTA-free Protease Inhibitor Cocktail Tablets (Roche)) (Helmfors et al., 2015). The resulting mixture was centrifuged at 12,000g for 10mins at 4°C, and the supernatant was extracted as the soluble fraction. The pellet was further resuspended in 50µl of extraction buffer containing guanidinium HCl (5M GnHCl, 50mM Hepes pH 7.3, 5mM EDTA, Protease inhibitor (Roche)) (Caesar et al., 2012) and centrifuged again to obtain the supernatant which constitutes the insoluble fraction. Alternatively, whole head, body, or fly homogenates were prepared by crushing 15 fly heads into whole cell extract (WCE) buffer (Sayre et al., 1992) and then centrifuging at 10,000g for 10mins at 4°C. The supernatant was isolated and spun down twice more to further separate out impurities and insoluble contaminants.

### 3.4.7 Immunoprecipitation and Western blot

Aβ42 was immunoprecipitated from insoluble and soluble fractions of fly head homogenates via incubation with antibody W0-2 (Aβ residues 5-8) and protein G Sepharose beads (GE Healthcare #17-0618-01). Samples were then separated by SDS-PAGE on 10-20% tris-tricine gels (BioRad) and transferred to 0.45µm nitrocellulose membranes and probed with W0-2 and anti-mouse secondary antibody (Promega) for Aβ42 detection. 15µl aliquots of insoluble and soluble fractions were conserved and directly separated by SDS-PAGE on 10-20% tris-tricine gels (BioRad) and transferred to 0.45µm nitrocellulose membranes, and subsequently probed with anti-actin (MAB1501, Millipore). Whole head homogenates were directly separated by SDS-PAGE on 12.5% polyacrylamide gels and transferred to 0.45µm nitrocellulose membranes, and subsequently probed with W0-2, anti-actin (MAB1501, Millipore) and anti-BACE1 (D10E5, Cell Signaling).

### 3.4.8 Mass spectrometry imaging (MALDI-MSI)

At the Histology Facility (Goodman Cancer Research Centre, McGill Life Sciences Complex), 10μm serial sections were prepared from flash-frozen flies using a cryostat (-14$^{o}$C) and thaw-mounted on conductive, indium titanium oxide (ITO)-coated glass slides (Bruker Daltonics #8237001). Stored at -80$^{o}$C before analysis, the mounted slides were transferred to vacuum desiccator and dried overnight before matrix application. At the McGill SPR-MS Facility, the dried sections were co-crystallized with super-DHB matrix (Sigma #50862; 9:1 (w/w) mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) using an ImagePrep sprayer (Bruker Daltonics; 10 mg/mL super-DHB in TA50). MSI spectra were acquired using a Bruker UltrafleXtreme MALDI-TOF/TOF system in reflectron positive ionization mode (calibrated mass range of 500 – 5,000 *m/z* and 20μm laser diameter; FlexControl v3.4 software). Spectra were processed using FlexImaging v4.1 software and normalized to total ion current (TIC).

### 3.4.9 Statistical analyses

All results are expressed as mean ± SEM (Standard Error of Mean). Statistical comparisons are made by unpaired Student's t-test with Bonferroni correction, and Kaplan-Meier survival curve analysis was used to compare survival curves. All statistical tests are performed using GraphPad Prism. P values $< 0.05$ were considered to be significant. Sample sizes are reported in figure legends, and no statistical method was used to predetermine sample size.

## 3.5 Results

### 3.5.1 D-AIP administration ameliorates Aβ42-induced locomotor deficits only in male animals

To assess the effects of AIP rescue on more complex aspects of *Drosophila* physiology, we have decided to use a pan-neuronal driver, *elav*. Neuron-specific expression of A $\beta$ 42 has been shown previously to cause locomotor dysfunction in *Drosophila*, whereas the expression of other amyloid species such as A $\beta$ 40 did not have an effect (Iijima et al., 2004). Here, the climbing behaviour of wildtype flies appeared to decline with age, which has been reported previously in the *Drosophila* literature (i.e. locomotor activity is negatively correlate with aging)(Arking and Wells, 1990; Gargano et al., 2005; Jones and Grotewiel, 2011). The male A $\beta$ 42 transgenic animals, however, showed a much steeper decline over time in comparison (**Figure 18, top and bottom left panels**). This defect in locomotor behaviour was significantly improved with the administration of D-AIP, showing a very pronounced rescue of Aβ42-induced behaviour deficits. Interestingly, the behaviour of the female transgenic flies does not seem to be affected by pan-neuronal Aβ42-expression or D-AIP administration compared to their wildtype counterparts (**Figure 18, top and bottom right panels**).

To fully characterize the effects of A $\beta$ 42 expression on our transgenic flies, we also performed live confocal imaging to visualize the eye morphology of the animals (**Figure 19A**). We did not observe any eye deformations at 28 days post eclosion in transgenic animals of either sex. Additionally, we also monitored the survival of all genotypes and sexes across treatments, and detected no major differences between them (**Figure 19B**). Next, to confirm that the detrimental effects are specific to the presence of Aβ42, we also generated human BACE1 neuron-specific expressing animals. We assessed their locomotor behaviour and found that neuron-specific transgenic BACE1 expression had no effect on either sex (**Figure 20A**). Surprisingly, female transgenic animals supplemented with D-AIP exhibited significantly reduced longevity (**Figure 20B**). We confirmed the expression of BACE1 in these animals via Western blot analysis (**Figure 20C**).

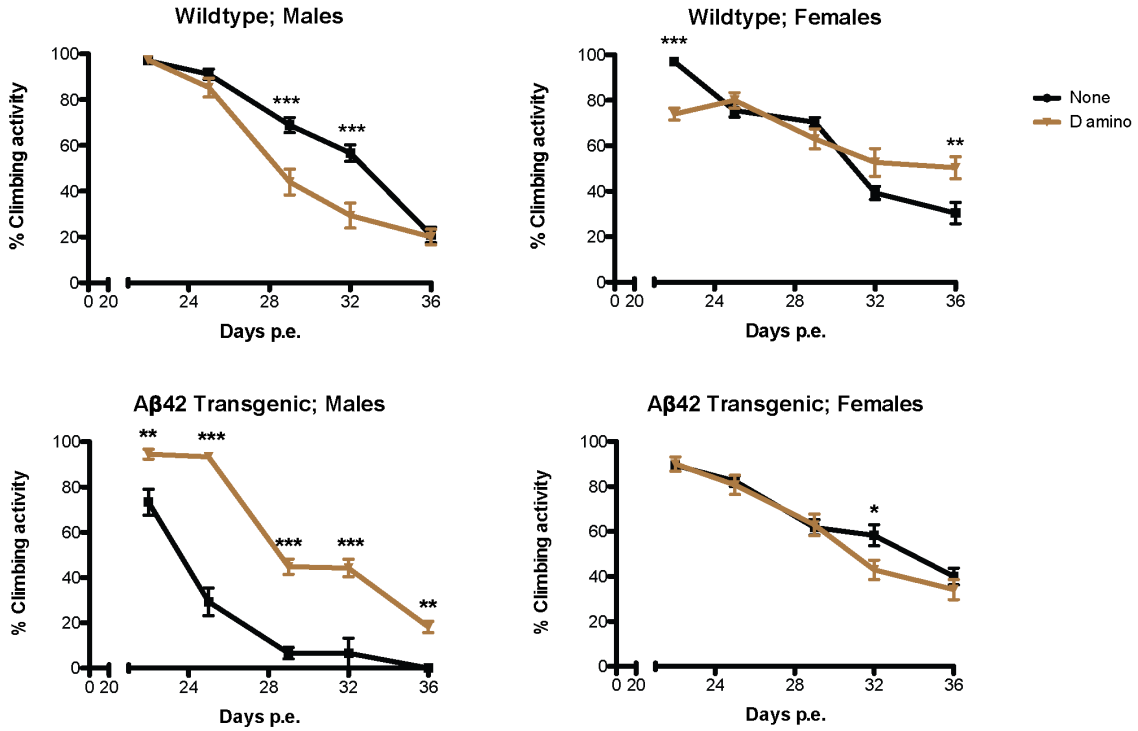



**Figure 18. D-AIP supplementation rescues Aβ42-induced locomotor deficits in male transgenic flies**

Representative RING assays show that locomotor activity of male A $\beta$ 42 transgenic flies are decreased compared to wildtypes. D-AIP supplementation significantly rescued this defect. 15 animals per group, n = 3; data is represented as mean ± SEM. Statistical analysis was performed using two way ANOVA with Bonferroni posttests; *p <0.05, **p <0.001, ***p < 0.0001.

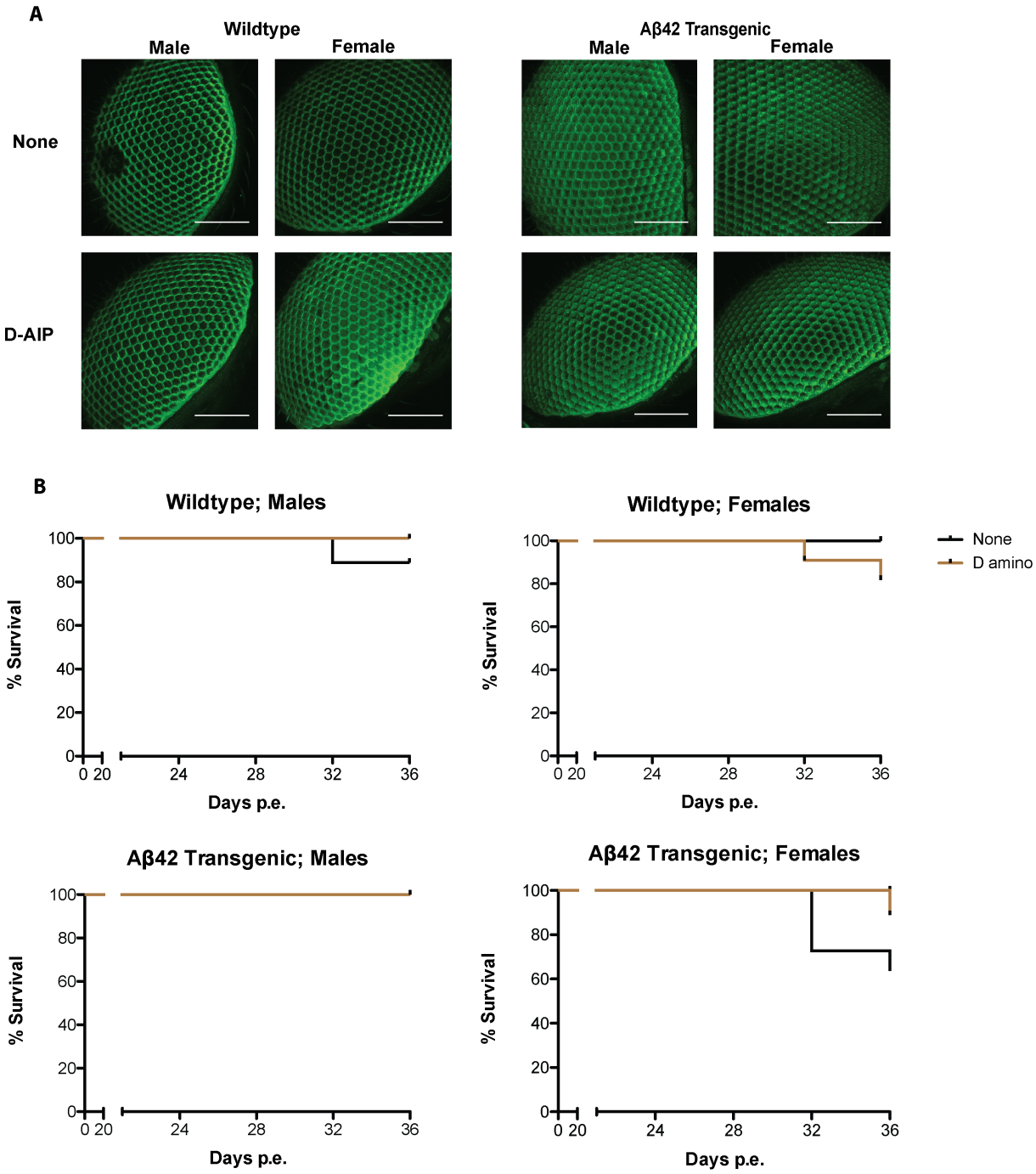

A
Wildtype
Male
Female
Aβ42 Transgenic
Male
Female
None
D-AIP
B
Wildtype; Males
Wildtype; Females
Aβ42 Transgenic; Males
Aβ42 Transgenic; Females
% Survival
Days p.e.
None
D amino
0
20
40
60
80
100
0 20
24
28
32
36

**Figure 19. AIP supplementation has no effect on the gross eye morphology and survival of transgenic Aβ42 flies**

**A**. Representative live confocal images of compound eye at day 28 post eclosion for wildtype flies and Aβ42 transgenics. Both exhibit normal morphology of hexagonal ommatidia subunits. scale bar: 200μm. **B**. Representative Kaplan-Meier survival curves show that longevity of wildtype and Aβ42 transgenic flies were unaltered in the presence of added D-AIP. No significance was detected between the groups. 15 animals per group, n = 3.

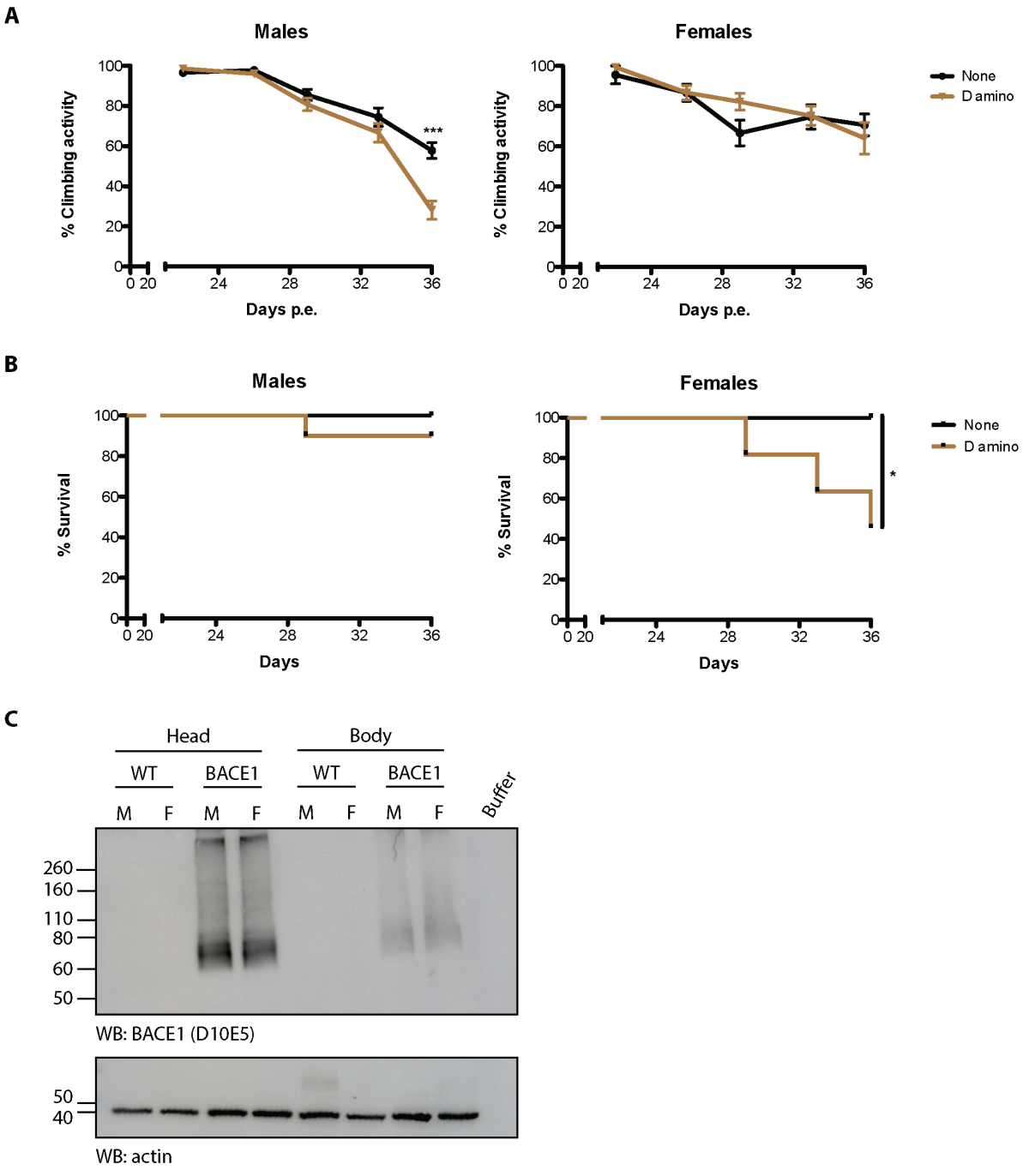

A
Males
Females
% Climbing activity
Days p.e.
None
D amino
***
B
Males
Females
% Survival
Days
None
D amino
*
C
Head
Body
WT
BACE1
M
F
Buffer
260
160
110
80
60
50
WB: BACE1 (D10E5)
50
40
WB: actin

**Figure 20. Neuron-specific BACE1 expression has no effect on the climbing behaviour of transgenic flies**

**A**. Representative RING assays show that locomotor activity of wildtype and BACE1 transgenic flies are unchanged by D-AIP supplementation. 15 animals per group, n = 3; data is represented as mean ± SEM. Statistical analysis was performed using two way ANOVA with Bonferroni posttests; ***$p < 0.0001$. **B**. Representative Kaplan-Meier survival curves show that longevity of wildtype flies and male BACE1-expression flies were unaltered in the presence of added D-AIP. Female BACE1 transgenic flies exhibited a decrease in survival upon D-AIP treatment. 13 animals per group, n = 3; *$p = 0.0142$. **C**. Western blot analysis of BACE1 expression in neuron-specific BACE1 expressing flies. Lysates from isolated fly heads of wildtype and BACE1-expressing transgenics were probed for human BACE1.

### 3.5.2 Distribution of label-free D-AIP is similar between male and female A $\beta$ 42 transgenic flies at 5 days post eclosion

We used MALDI-IMS as a label-free method to determine the uptake and distribution of AIP in whole animals slices between the sexes. At day 5 post eclosion, L-AIP was virtually undetectable in both wildtype and transgenic flies bred and raised on L-AIP supplemented food (**Figure 21A, second row**). In contrast, D-AIP was detectable throughout the body and the head of both wildtype as well as A $\beta$ 42-expressing transgenic animals bred and raised on D-AIP supplemented food (**Figure 21A, third row**). Overall, quantification of the signal localization of D-AIP shows a relatively constant distribution between genotypes and sexes, with the majority of D-AIP being found in the body of the animals (**Figure 21B**).

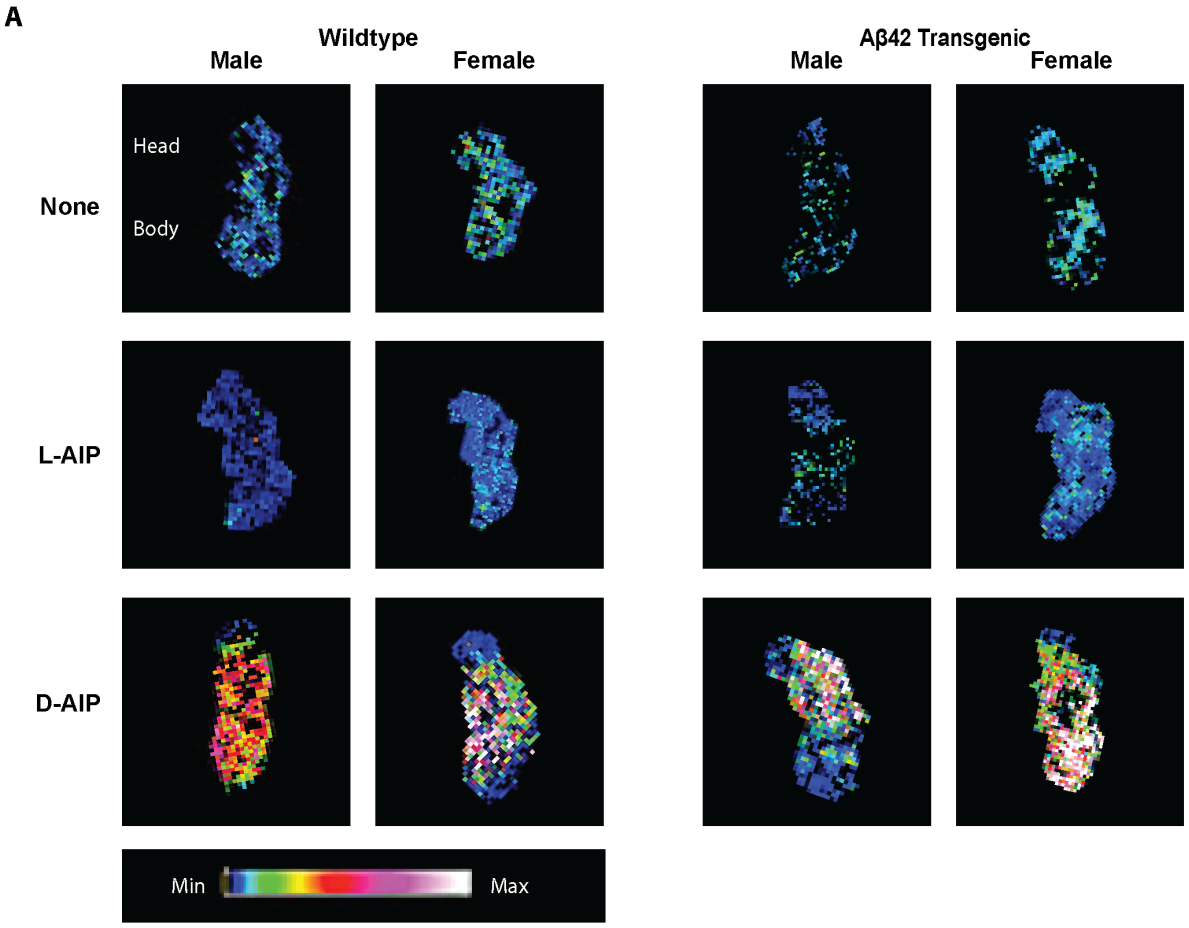
### 3.5.3 Altered localization of D-AIP in female A $\beta$ 42 transgenic animals at 28 days post eclosion

In our current study, the flies were bred and raised on a single, high dose of L- or D-AIP in their food (i.e. no new administrations of AIP were added to their food). To assess the long-term efficacy of the AIP rescue, both wildtype and transgenic flies were imaged again via MALDI-MSI at 28 days post-eclosion. The distribution of D-AIP in wildtype animals and male Aβ42 transgenic animals remained relatively consistent with day 5 results, where the majority of the peptide is found in the body of the insect (compare **Figure 21A** to **Figure 22A**). The levels of D-AIP in the heads of transgenic females, however, appears to have decreased dramatically from 31% on day 5 to 0% of the total on day 28 post eclosion (**Figure 22B**).

### 3.5.4 Insoluble deposits of A $\beta$ 42 is significantly increased in the heads of transgenic female animals

We next performed immunoprecipitation and Western blot experiments to ascertain the relative levels of A $\beta$ 42 expression in the animals. Analysis of day 28 post eclosion fly homogenates indicated a similar level of A $\beta$ 42 expression regardless of sex and AIP supplementation (**Figure 23**). Further analysis of head and body homogenates (i.e. with an increased number of animals) revealed a significant increase in the insoluble fraction of A $\beta$ 42 in only the heads of the female A $\beta$ 42 transgenic flies (**Figure 24**). This effect seems to be specific to A $\beta$ 42, as BACE1

expressing transgenic flies had similar levels of protein expression between the sexes (**Figure 20B**). We propose that the altered localization of D-AIP to the body with the increased insoluble A $\beta$ 42 levels in the heads of female transgenic flies may be likely to explain why female transgenic flies are resistant to A $\beta$ 42 neurotoxicity, since insoluble A $\beta$ 42 aggregates have been previously shown to be less toxic compared to oligomers (reviewed in (Selkoe and Hardy, 2016)).


A
Wildtype
Male
Female
Aβ42 Transgenic
Male
Female
None
L-AIP
D-AIP
Head
Body
Min
Max

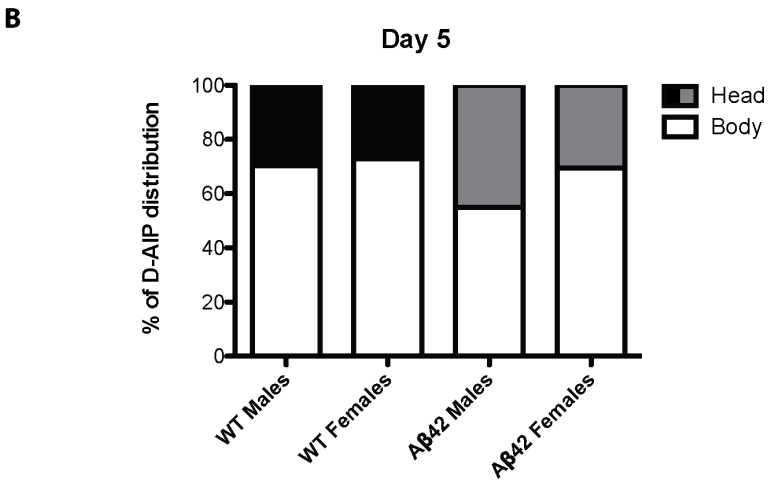
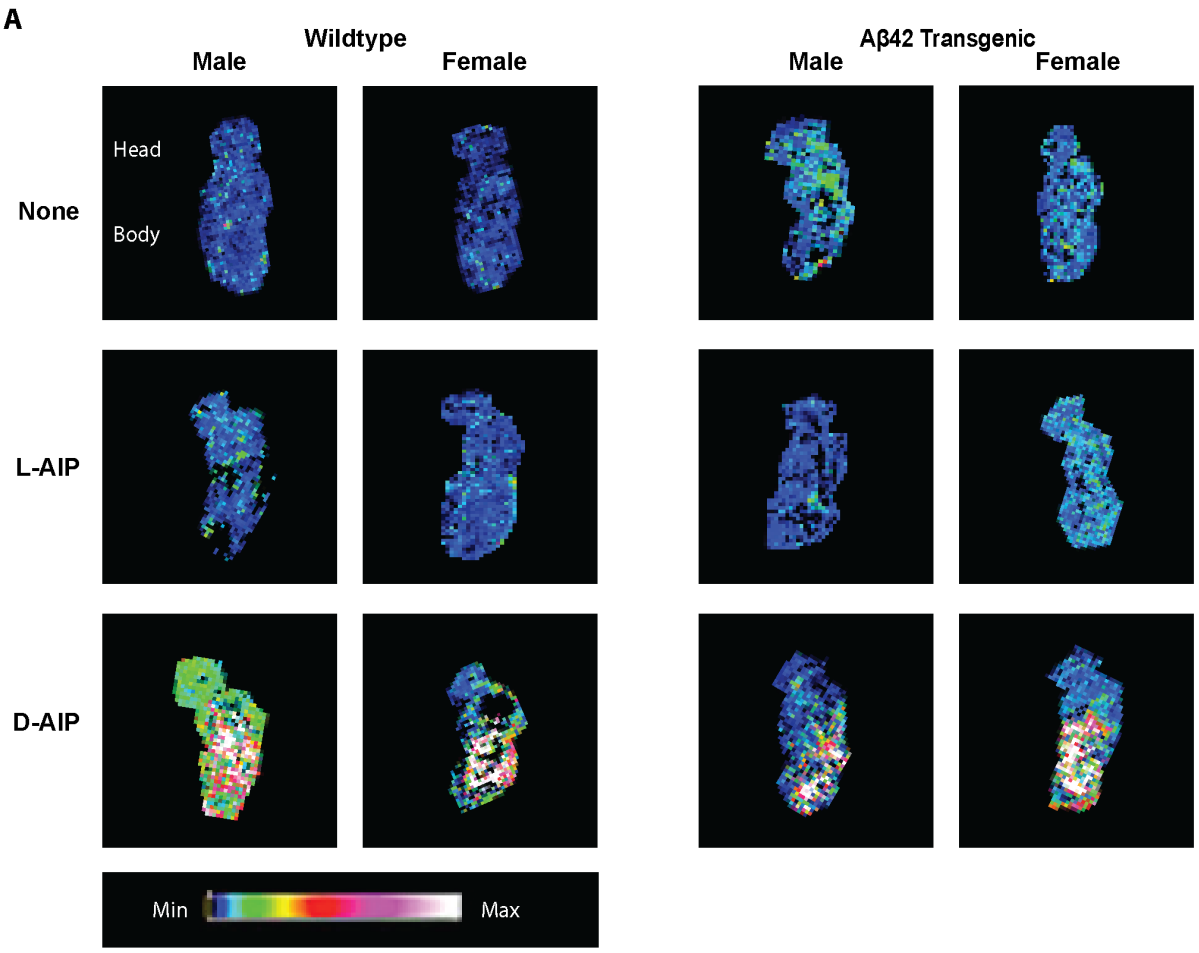

B
Day 5
% of D-AIP distribution
100
80
60
40
20
0
WT Males
WT Females
Aβ42 Males
Aβ42 Females
Head
Body

**Figure 21. Distribution of label-free AIP in wildtype and transgenic Aβ42 flies are similar at day 5 post eclosion**

**A.** Wildtype and transgenic Aβ42 flies were raised on fly food in the absence and presence of L- or D-AIP. Sagittal sections of the flies (at day 5 post eclosion) were analyzed by label-free MALDI-MSI. Using the appropriate mass filter (AIP = 941 *m/z*), the resultant "heat maps" indicate the relative intensity (low (*blue*) to high (*white*)) and position of L- or D-AIP within the fly heads and/or bodies. **B**. Relative quantification of D-AIP signal intensity in head vs. body for wildtype and Aβ42 transgenic flies.


A
Wildtype
Male
Female
Aβ42 Transgenic
Male
Female
Head
Body
None
L-AIP
D-AIP
Min
Max

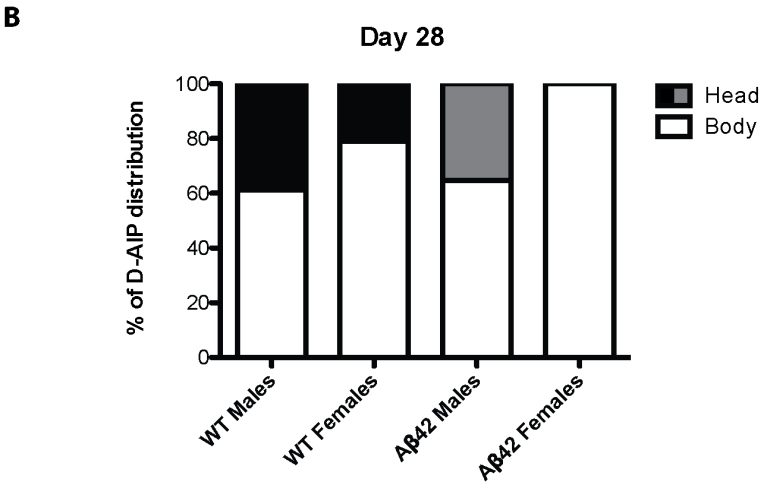

B
Day 28
% of D-AIP distribution
100
80
60
40
20
0
Head
Body
WT Males
WT Females
Aβ42 Males
Aβ42 Females

**Figure 22. D-AIP localizes to the body of female transgenic flies at day 28 post eclosion**
**A.** Wildtype and transgenic Aβ42 flies were raised on fly food in the absence and presence of L- or D-AIP. Sagittal sections of the flies (at day 28 post eclosion) were analyzed by label-free MALDI-MSI. Using the appropriate mass filter (AIP = 941 *m/z*), the resultant "heat maps" indicate the relative intensity (low (*blue*) to high (*white*)) and position of L- or D-AIP within the fly heads and/or bodies. **B**. Relative quantification of D-AIP signal intensity in head vs. body for wildtype and Aβ42 transgenic flies.

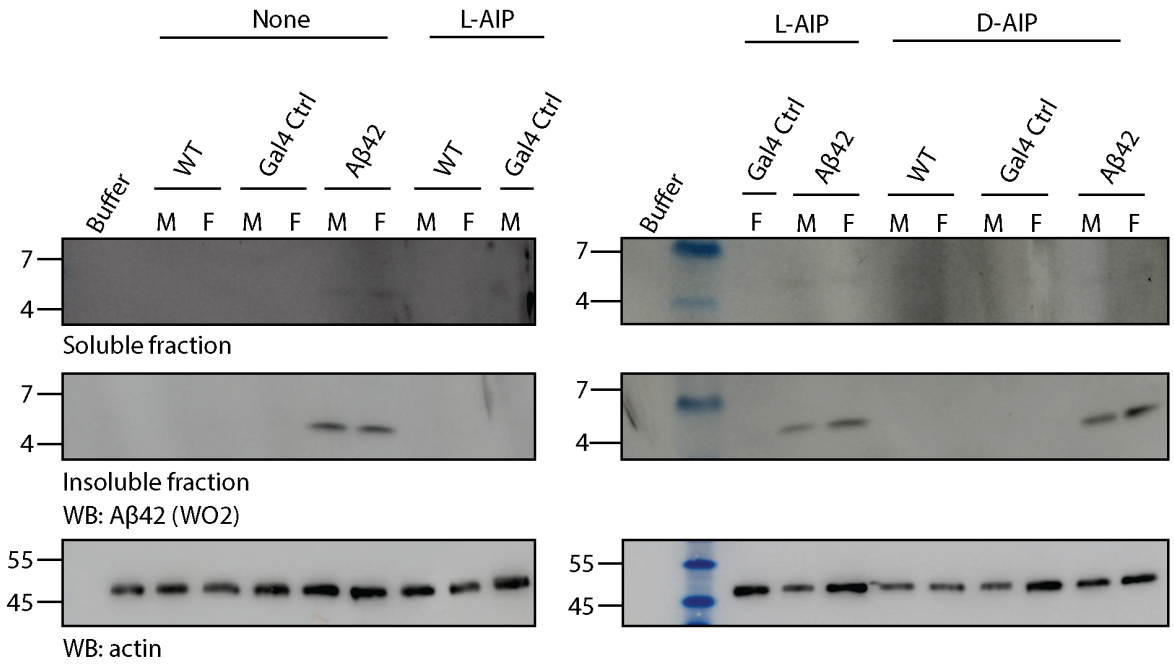
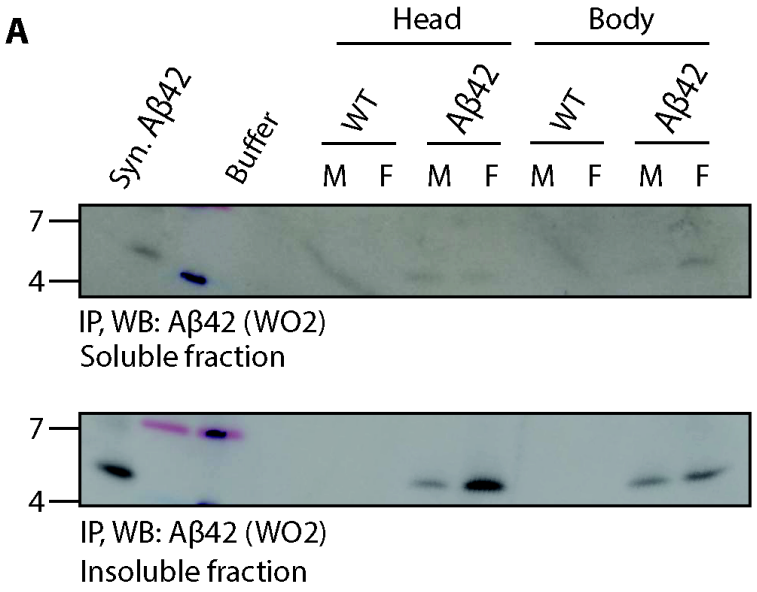



**Figure 23. The expression of Aβ42 is similar in transgenic animals regardless of sex or AIP treatment**

Soluble and insoluble lysates from whole transgenic *Drosophila* (aged 28 days post eclosion) were immunoprecipitated with the Aβ-specific antibody W0-2. Representative Western blot analysis shows that neuron-specific expression of human Aβ42 was detectable in the transgenic animals. 5 heads or bodies of each sex was used per sample.


A
Head
Body
Syn. Aβ42
Buffer
WT
Aβ42
WT
Aβ42
M F M F M F M F
7
4
IP, WB: Aβ42 (WO2)
Soluble fraction
7
4
IP, WB: Aβ42 (WO2)
Insoluble fraction

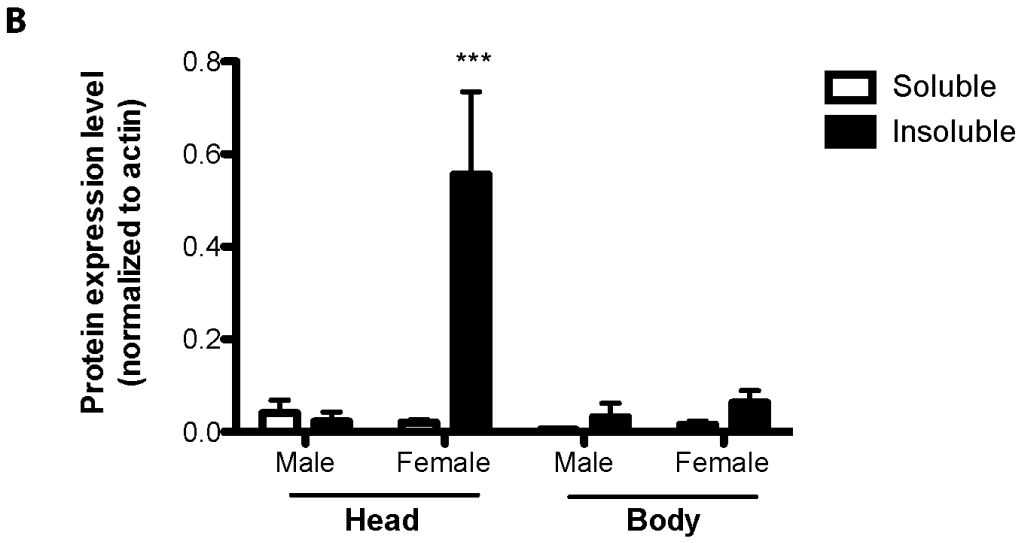

B
Protein expression level
(normalized to actin)
0.8
0.6
0.4
0.2
0.0
***
Male
Female
Male
Female
Head
Body
Soluble
Insoluble

**Figure 24. Insoluble Aβ42 levels are increased in the heads of transgenic female flies**
**A**. Soluble and insoluble lysates from transgenic *Drosophila* heads and bodies (aged 28 days post eclosion) were immunoprecipitated with the Aβ-specific antibody W0-2. Compared to molecular weight markers and a known synthetic Aβ42 standard (far left lane, 4.5 kDa, 5 ng), representative Western blot analysis shows that neuron-specific expression of human Aβ42 was detectable in both the heads and bodies of male and female flies. 10 heads or bodies of each sex were used per sample. **B.** Quantification of Western blot data, normalized to actin control. n = 4. Statistical analysis was performed using two way ANOVA with Bonferroni posttests; ***$p < 0.0001$.

## 3.6 Discussion

Decreases in both synapse function and number is one of the strongest quantitative pathological correlation to dementia in AD (Selkoe and Hardy, 2016), and Aβ42 oligomers, in particular, have been strongly implicated in the impairment of synaptic function and structures. We have previously shown that an inhibitor of Aβ42 oligomers – the small, 8-residue Aβ-oligomer interacting peptide (AIP) – was able to ameliorate the loss of synaptic spin density and LTP in hippocampal slice cultures, as well as neutralize Aβ42-induced morphological deficits in transgenic *Drosophila* (Barucker et al., 2015) (**manuscript 2**). In our current study, we further investigated the efficacy of AIP to neutralize Aβ42-induced toxicity in more complex physiological systems such as the brain and CNS of transgenic *Drosophila*.

We used *elav* as a driver to drive the expression of A $\beta$ 42 in a pan-neuronal manner, as it has been shown to be expressed pan-neuronally in all stages of development, and is present exclusively in all immature and mature neurons (Robinow and White, 1991; Koushika et al., 1996). While *elav* is not known to be highly expressed in the eyes of adult flies, its function has been shown to be essential in the development of the eye and the optic lobe (Campos et al., 1985; Campos et al., 1987), and especially in the proper development of photoreceptors (Koushika et al., 1996). However, we did not observe any gross eye deformations in the transgenic animals in our study, which indicates that *elav*-driven Aβ42 expression does not affect structural aspects or components of the eye (**Figure 19**). The detrimental effects of pan-neuronally expressed Aβ42 on both the longevity and locomotor behaviour of *Drosophila* have been broadly characterized by both our group and others (**manuscript 1**) (Finelli et al., 2004; Crowther et al., 2005; Luheshi et al., 2007; Iijima et al., 2008; Iijima-Ando et al., 2008). In our hands, the climbing activity of the wildtype flies declined overtime, which has been previously reported as a normal consequence of aging (Arking and Wells, 1990; Gargano et al., 2005; Jones and Grotewiel, 2011). The transgenic flies, however, showed a much more dramatic decrease over time in locomotor behaviour (i.e. RING assays) in excess of the normal decline observed with age. Interestingly, we were able to rescue this adverse behavioural phenotype in a sex specific manner via food supplementation with D-AIP. Specifically, male transgenic animals fed on the D-AIP supplemented food were

able to maintain a robust locomotor response that is comparable to those observed in wildtype flies in longitudinal studies (**Figure 18**). This result correlates with our previous observations where we used D-AIP to successfully rescue Aβ42-induced 'rough-eye' phenotypes, and shows that D-AIP can indeed rescue deficits in both morphological (i.e. eye structure) and functional (i.e. CNS) systems (**manuscript 2**).

Additionally, it is interesting to note that in our studies, female Aβ42 transgenic animals did not appear to be susceptible to Aβ42-induced defects in locomotor activity. This was quite an interesting finding for us, and so far there do not seem to be any detailed reports in the literature on this sex-specific discrepancy in response to pan-neuronal Aβ42 expression. Upon further investigation, we discovered that the insoluble fraction of Aβ42 was significantly increased in the female animals (**Figure 24**). Since it has been shown previously by other groups that insoluble amyloid plaque cores are not toxic and may in fact sequester the toxic soluble oligomers to a certain degree (Koffie et al., 2009; Hong et al., 2014), we suspect that this increase in insoluble Aβ42 may be the contributing factor which allows the female Aβ42 transgenic flies to be resistant to Aβ42-induced toxicity. We also detected a discrepancy in the D-AIP distribution (analyzed via MALDI-IMS) at 28 day post eclosion, where D-AIP became entirely located in the body of the female Aβ42 transgenic specimens (compare **Figure 21** to **Figure 22**). In our previous work, we have shown that AIP preferentially binds to soluble, low-order Aβ42 oligomers (i.e. mainly tetramers and hexamers) (Barucker et al., 2015) and not larger insoluble aggregates. As such, the increased insoluble Aβ42 fraction in the heads of transgenic females suggests that this is likely the reason D-AIP was not detected in the heads of the animals as it would not likely bind to the insoluble Aβ42 aggregates, which would explain the observed shift in distribution of AIP to mainly in the body of the female animals as we have detected via MALDI-MSI.

## 3.7 Conclusion

Overall, our study showed that orally administered D-AIP has the potential to rescue functional deficits induced by Aβ42 toxicity *in vivo* without obvious side effects (i.e. rescue of locomotor impairments in transgenic *Drosophila* without impacting survival). Taken together with our previous *in vitro* and *in vivo* results (Barucker et al., 2015) (**manuscript 2)**, we strongly believe that our D-AIP peptide is a promising potential therapeutic for AD and is an excellent candidate for further studies in more advance rodent models of AD.

## 3.7 Acknowledgements

This study was supported by a grant from the Canadian Institutes of Health Research (MOP-133411). GM holds both a Canada Research Chair in Molecular Pharmacology and a Canada Foundation for Innovation (CFI) grant. The McGill Life Sciences Complex thanks the CFI for infrastructure support. Thanks also to Dr. Filip Liebsch for establishing the transgenic *Drosophila* strains and scientific guidance, and to the McGill Life Sciences Histology facility for their technical support.

# III. GENERAL DISCUSSION

In this thesis, we have focused our efforts on elucidating the efficacy of AIP in ameliorating Aβ42-induced toxicity *in vivo* in transgenic models of *Drosophila melanogaster*. To this end, I have presented three individual manuscripts in this manuscript-based thesis. The first of which established and characterized novel human Aβ42-expressing *Drosophila* models to better study and mimic the temporal expression and downstream effects of human Aβ42 (**manuscript 1**). We then demonstrated that longitudinal oral treatment with D-AIP was able to effectively ameliorate the human Aβ42-induced toxicity in the morphology (**manuscript 2**) and behaviour (**manuscript 3**) of the animals with minimal negative side effects, albeit in interesting, sex-specific manners.

Overall, our findings show that i) the sex of the animals is an important variable in *in vivo* studies, and sex-specific effects (such as the presence of male- or female-specific proteins) can potentially offer key explanations on the differing efficacy of therapeutics in male and female animals, and ii) AIP is indeed a viable therapeutic candidate against Aβ42-induced toxicity *in vivo*, and our *Drosophila* studies of AIP efficacy are able to serves as proof-of-principle for future studies in more complex rodent models of AD.

## 1. Modeling Aβ42-induced toxicity *in vivo*

Our first goal of this study was to establish and characterize in detail the effects of human Aβ42 expression on the morphology (i.e. compound eye structure) and behaviour (i.e. locomotor activity) of transgenic *Drosophila*, in order to establish a pathological baseline that can be used as a comparison in future rescue studies **(manuscript 1)**.

In our hands, the constitutive transgenic expression of human Aβ42 in *Drosophila* neurons had significant negative impact on both the locomotor ability of the animals as well as their longevity (**Figure 7 and 8**), indicating that neurons are susceptible to Aβ42 expression in these transgenic flies. Others have also observed this previously where the induction of human Aβ42 in the neurons of *Drosophila* caused progressive degeneration and plaque formation in the animals (Finelli et al., 2004; Iijima et al., 2004; Crowther et al., 2005). This is by no means a specific susceptibility unique to *Drosophila*, but is indicative of the general detrimental effects of human Aβ42 on neurons – toxicity induced by the expression of human Aβ42 has been shown in a variety of animal models such as mice, rats, and other invertebrate models such as nematodes. For example, transgenic mice expressing Aβ42 develop compact amyloid plaques and cerebral amyloid angiopathy (CAA), while mice expressing Aβ40 do not show such pathology (McGowan et al., 2005). The expression of APP with Swedish and Indiana mutations in transgenic rats – which both increases the production of Aβ42 and the Aβ42/Aβ40 ratio (Citron et al., 1994; Johnston et al., 1994; Tamaoka et al., 1994; Scheuner et al., 1996) – showed extensive cognitive impairments in the animals as early as 3 months of age (Leon et al., 2010). Additionally, Aβ42 expression in the neurons of *C. elegans* resulted in deficits in odour preference learning and neuronal degeneration (Dosanjh et al., 2010; Treusch et al., 2011).

Non-neuronal cells and tissues outside of the brain and CNS in invertebrate models also appear to be somewhat susceptible to Aβ42-induced toxicity. Our studies in the *Drosophila* model have shown that eye-specific Aβ42 produced a 'rough eye' phenotype where the morphology of the compound eye is disrupted due to Aβ42-induced degeneration (**Figure 5**). Others have also consistently observed this phenotype in the literature as well (Finelli et al., 2004; Iijima et al., 2004). Alternatively, using a different invertebrate model and non-neuronal tissue, Link et al.

were able to also show that both constitutive and induced transgenic Aβ42 expression in the muscles of *C. elegans* caused a progressive paralysis and the accumulation of intracellular deposits (Link, 1995; Link et al., 2003).

Most of these previously described AD animal models – both vertebrate and invertebrates – express high levels of transgenic Aβ42 or AD-related proteins such as APP from embryonic stages, well before the protein's natural physiological accumulation in either early-onset human FAD and late-onset sporadic AD. This makes it very difficult to discern the true effects of Aβ42-induced toxicity (i.e. are the changes in morphology and/or behaviour stemming from disruptions in the developmental or adult cell pathways?). To this end, we next developed novel, temperature-induced transgenic Aβ42-expressing *Drosophila* models to better study the effects of Aβ42-induced toxicity during both development and adult stages.

### 1.1 The mature structure of the compound eye is resistant to Aβ42-induced toxicity

In our studies, we used Gal80 in our UAS/Gal4 system to manipulate the expression of Aβ42 expression in a manner that relied on simple temperature manipulations, and required no administration of extraneous compounds to activate transcription. Gal80ts, a negative regulator of Gal4, is highly activated at 18°C and inactivated at 29°C (Zeidler et al., 2004). The introduction of Gal80ts essentially allows us to induce the expression of Aβ42 in F1 animals at any point during development simply by elevating the ambient temperature. We were able to control the expression of Aβ42 in a temporal manner using this method, and used this system to drive the expression of human Aβ42 in the flies in either a constitutive manner, or only in the adult animal. Unexpectedly, the eye-specific expression of Aβ42 appeared to only affect eye structure and morphology when it is constitutively turned on (**Figure 5**). No degeneration was observed if Aβ42 was expressed in the eyes of the animals only when they reached adulthood. This was quite surprising as it suggests that the structure of the adult eye may be resistant to Aβ42-induced toxicity.

While there are currently no reports of tissues with similar immunities against Aβ42 toxicity, we speculate that the unique structure of the *Drosophila* compound eye likely contributed to its

ability to resist Aβ42-induced toxicity. The 'rough eye' phenotype we have used here is widely used in *in vivo* studies because of its high sensitivity to perturbations in the structure of each subunit or ommatidia – any defect that affects the hexagonal geometry of a single ommatidium will disrupt the positioning of all surrounding units, and disruptions in genes that govern the development of even a single cell within an ommatidium will come to affect the entire structure (Kumar, 2012). The reason for this sensitivity lies in the fact that the adult compound eye is derived from a monolayer epithelium called the eye-antennal disc, and during embryogenesis, the nascent eye-antennal discs only consists of 8-9 cells (Ouweneel, 1970; Haynie and Bryant, 1986; Kumar, 2012). It is easy to imagine then, that if Aβ42 were expressed constitutively in the eye, its negative effects on these progenitor cells would likely have wide-reaching consequences on the entire final structure. In the adult animals, however, the eye morphology is already well established and intact. It may be that at this point, perturbations of single cells in the established ommatidia may have minimal effects on the overall structure. It would be interesting to further ascertain the function of the eye in these adult-onset Aβ42 expression models via physiological measurements such as electroretinography (ERG) to investigate the relationship between structure maintenance and function.

### 1.2 Neurons have a general susceptibility to Aβ42-induced toxicity

In contrast to the eye-specific Aβ42 expressing model, we observed that the neuron-specific expression of Aβ42 – regardless of whether it was induced constitutively or only in the adult animals – caused a significant decrease in locomotor activity and longevity of the animals (**Figure 7 and 8**). This lead us to speculate that, unlike the mature eye structure, neurons likely have a general susceptibility (i.e. both developing and mature neuronal cells) to Aβ42-induced toxicity compared to non-neuronal tissues such as the eye.

This is not particularly surprising, as the overall susceptibility of specific neurons to Aβ42 toxicity has been noted previously by others (Braak et al., 2006). Described mainly in vertebrate models, these susceptible neurons tend to be projection neurons (Braak, 1980) and in particular, cells with disproportionately long and thin axons in relation to the size of the cell body or soma show an increased tendency to develop pathology (Morrison et al., 1998). Interestingly, short-

axoned local circuit neurons, on the other hand, usually remain untouched by the pathological processes involved in AD (Hof et al., 1991; Hof et al., 1993). Additionally, these vulnerable nerve cell types share another feature, where their long axons are also comparatively unmyelinated or only have a thin myelin sheath – such as the Meynert pyramidal cells in the striate area (Braak et al., 2006). In contrast, cortical projection neurons with a heavily myelinated axon are resistant to the formation of AD pathologies (Hof et al., 1991; Hof et al., 1993). This lack of mature myelin sheath likely contributed to the increased metabolic demands placed on the parent nerve cell for the transmission of impulses (Hildebrand et al., 1993). This way, these rapid-firing projection neurons with unmyelinated or incompletely myelinated axons may be subjected to higher energy turnovers and exposed to grater oxidative stress, which are probably contributory factors to their susceptibility to Aβ42 in AD (Beal, 1995; Sohal, 2002).

While it is unclear which type of neurons in the invertebrate system are the most vulnerable to human Aβ42 expression, it has been shown previously that peripheral nerves in the flies closely resemble that of unmyelinated peripheral axons (where nonmyelinating Schwann cells ensheath small-diameter axons) in mammals (Taveggia et al., 2005). This likely contributes to the susceptibility of these neurons to Aβ42 expression both in embryonic and adult stages. There is also some evidence that these *Drosophila* peripheral neurons may play important roles in governing locomotor activity (Song et al., 2007; Charng et al., 2014), which may explain the significant decrease in the climbing activity of the animals observed in our studies.

In future studies, perhaps Aβ42 could be selectively expressed in different brain regions – such as the mushroom body or central complex – to fully characterize the sensitivity of neurons in each region to Aβ42-induced toxicity. As well, since the locomotor functions of the animals were greatly impaired, it would have been interesting to further study the direct susceptibility of motor neurons to Aβ42 by staining or isolate them and analyse for markers of apoptosis or cell death.

### 1.3 Development of novel *in vivo* models with adult-onset Aβ42 production

In our studies, we have chosen to use the temperature-sensitive Gal80ts system to modulate the temporal expression of Aβ42 in *Drosophila*. This system has allowed us to successfully generate

transgenic animals with inducible human Aβ42 expression that can be 'turned on' via simple changes in temperature. While we chose the Gal80ts model specifically due to the fact that it is only reliant on temperature changes (i.e. no additional exogenous compounds are needed to trigger the transgene expression), there are, however, still some drawbacks to this model. The activation temperature for Aβ42 expression is 29°C in our experiments, which is much higher than *D. melanogaster*'s preferred temperature ($T_p$) of 25°C (Fogleman, 1979; Dillon et al., 2009). While we are able to achieve maximal A $\beta$ 42 expression at this temperature (Zeidler et al., 2004), there is some evidence that the overall fitness of the animal may be affected (Dillon et al., 2009), which may introduce variability into our studies. Indeed, we observed a much more profound eye degeneration in the A $\beta$ 42 transgenic animals (**Figure 5 compared to Figure 11**) as well as decreases in survival of all strains (**Figure 7 compared to Figure 19**) raised at 29°C compared to at 25°C in later studies. However, it should be noted that while control animals did show an increase in mortality at 29°C, they still performed better than the Aβ42 transgenic animals raised at the same temperature (i.e. the toxic effects of A $\beta$ 42 expression has not been subsumed by the temperature effects), indicating that the Gal80ts system was successful in both inducing A $\beta$ 42 expression and not introducing so much variability that differences between strains cannot be ascertained.

There are, of course, many alternative systems that may be used to achieve control over transgene expression in *Drosophila*. The GeneSwitch-Gal4 system is one system that is commonly used to gain spatial control of gene expression in *Drosophila*. It is derived from the UAS-Gal4 system, and uses a modified Gal4 protein fused to a progesterone steroid receptor, which allows the regulation of its Gal4 activity via the presence or absence of the synthetic progesterone analogue mifepristone (RU-486) (Osterwalder et al., 2001). However, we did not consider this system suitable as there are studies showing that RU-486 can interact with endogenous *Drosophila* proteins such as the Sex Peptide (SP) to induce significant downstream effects on survival (Landis et al., 2015; Tower et al., 2017), and have been shown to reduce the food consumption and longevity of the animals (Yamada et al., 2017). Since our goal is for these models to be used in the testing of potentially therapeutic compounds against Aβ42 – such as AIP, which is a small peptide and thus may non-specifically bind to RU-486 – we did not want to introduce RU-486 as an additional variable in our future studies.

## 1.4 Conclusion

Adding to the existing literature regarding the sensitivity of neurons to Aβ42-induced toxicity, our analysis showed that *Drosophila* neurons at all stages of development have a general susceptible to A $\beta$ 42 expression – especially compared to periphery tissues such as the mature compound eye. Additionally, the Gal80ts system proved to be quite useful in inducing the temporal expression of A $\beta$ 42 in our hands. While this system was complementary to our studies, there is likely not a single ‘perfect’ model of inducible gene expression in *Drosophila*, as the selection of models often depends on the question that needs to be answered. We believe that these inducible transgenic animals can offer us greater flexibility in terms of the design and interpretation of experiments, and allows us to separate the effects of a transgene on either development or adult processes.

## 2. Evaluating the efficacy of AIP on neutralizing Aβ42-induced toxicity *in vivo*

We used both the eye-specific **(manuscript 2)** and neuron-specific **(manuscript 3)** A $\beta$ 42 expression models in long-term experiments to analyze the effects of AIP supplementation on both morphology (i.e. eye structure) and complex behaviour (i.e. locomotor activity) respectively. We found that AIP composed of D-amino acids (D-AIP) was biostable when administered orally, and was able to rescue both A $\beta$ 42-induced eye defects and declines in locomotor activity. Interestingly, the rescue was also sex-specific in that only the eye anatomy of the female animals and the climbing activity of the male animals were protected from A $\beta$ 42-induced toxicity **(Figure 11 and 18)**. This phenomenon has not been described in recent literature, and we believe it demonstrates the importance of including sex as a variable in *in vivo* experiments.

### 2.1 The biostability of L- and D-AIP

In previous *in vitro* studies, we discovered that L-AIP performed similarly to other anti-Aβ42 short peptidic inhibitors reported by others (Tjernberg et al., 1996; Pallitto et al., 1999; Lowe et al., 2001) in terms of preventing fibril formation and rescuing A $\beta$ 42-induced toxicity in SY5Y cells *in vitro* (Barucker et al., 2015). However, in *in vivo* experiments, we found that supplementation with L-AIP had no effect on rescuing A $\beta$ 42-induced rough eye morphology, whereas the D-AIP peptide had significant effects **(Figure 11)**. In order to further determine whether the L or D peptide is physically present in the animals, we used MALDI-MSI as a label-free method of detection in sagittal fly slices. We showed that L-AIP could not be detected in the supplemented animals at any time point **(Figure 12 and 15)**. Additionally, while intact L-AIP can still be found in the supplemented feed of the flies at day 28 post eclosion, it could not be detected in the fly homogenates via MALDI-MS **(Figure 15)**. Compared to the D-AIP peptide – which is present in its intact form in both the 28-day food slurry, fly head homogenate, as well as the fly section themselves – it is clear that the L-AIP is likely degraded rapidly in the flies, and thus is extremely not biostable and likely has a poor pharmacokinetic profile *in vivo*. We surmise that the efficacy of L-AIP in previous *in vitro* experiments is likely due to the fact that cell cultures tend not to be an accurate representation of the complexity of *in vivo* environments, and cannot be used to accurately predict the stability of compounds in animal studies. It is clear from

both our experiments, and the experience of others with similar D-amino acid peptidic inhibitors (Soto et al., 1996; Findeis et al., 1999), that D-AIP is the far better therapeutic candidate *in vivo*. This is unsurprising, as one of the main weaknesses of conventional peptidic inhibitors is their poor pharmacokinetic profile. Unmodified L-amino peptides are generally not considered to be good drug candidates, since they may have poor *in vivo* stability against proteases and may be rapidly cleared from the body by the liver and kidneys (Tsomaia, 2015; Wojcik and Berlicki, 2016).

### 2.2 Evidence that D-AIP may have the ability to cross the invertebrate BBB

Similar to primitive vertebrates, *Drosophila* possesses a modified, invertebrate BBB. Instead of being coordinated by endothelial cells such as in vertebrates, the open circulatory system in *Drosophila* requires the usage of different types of glial cells to participate in BBB function (Limmer et al., 2014; Daneman and Prat, 2015). The entire nervous system of *Drosophila* is covered by an outer layer of perineurial glial cells and an inner layer of subperineurial glial cells, which form elaborate septate junctions and prevent paracellular diffusion (Bainton et al., 2005; Stork et al., 2008; Mayer et al., 2009).

Our transgenic animals produce Aβ42 in only either an eye- or neuron-specific manner, which means that the majority of transgenic Aβ42 expression is confined to the CNS of the animals. This has been verified by Western blotting using the head and body homogenates of both eye- or neuron-specific Aβ42-expressing animals **(Supplemental Figure 1 and Figure 24)**. Consequently, this means that in order for D-AIP to exert its neutralization effects of Aβ42, it would likely need to cross the *Drosophila* BBB to reach the eyes and neurons. Indeed, we were able to observe specific D-AIP mediated rescue of both eye phenotype and locomotor behaviour in the animals (while L- and D-scrambled AIP peptides had no effect) **(Figure 11 and 18)**, demonstrating that D-AIP is likely able to cross the BBB in flies to exert its therapeutic effects. The ability of small D-peptides to cross the BBB has likewise been demonstrated previously in the literature, with the most recent by Jiang et al. in 2016, showing that their small D-peptide candidate D3 was able to penetrate the BBB after oral administration in both wildtype and AD mouse models (Jiang et al., 2016).

Additionally, MALDI-MSI heat-maps also show the localization of D-AIP in the heads of eye-specific Aβ42-expressing animals, which again supports our conclusion that D-AIP is crossing the BBB to reach the eyes in these animals. Interestingly, no such localization was seen in the neuron-specific Aβ42-expressing male animals despite the rescue of locomotor activity by D-AIP. We suspect it may be due to the fact that a number of thoracic ganglions (i.e. clusters of neurons in the body of the insect) are also part of the *Drosophila* CNS (Matheson, 2002), and in our transgenic animals, these thoracic ganglions would also produce some Aβ42 in the body of the animals. We have confirmed this via Western blotting using fly body homogenates **(Figure 24**). These extra-CNS Aβ42 may be then binding to D-AIP in the body of the animals as seen in the MALDI-MSI results. Additionally, since *Drosophila* peripheral neurons have been shown to play important roles in governing locomotor activity (Song et al., 2007; Charng et al., 2014), the neutralization of Aβ42 in these ganglions may be contributing directly to the rescue in climbing activity seen in the animals.

### 2.3 D-AIP neutralizes the toxic effects of Aβ42

Our results showed that oral supplementation with D-AIP successfully neutralized Aβ42-induced defects in both eye morphology and climbing behaviour, with minimal side effects. This confirmed and corroborated with both our previous, proof-of-concept *in vivo* data as well as our *in vitro* rescue data in SH-SY5Y neuroblastoma cells. The rescue effects observed from our studies seems to be specific to the composition and sequence of the D-AIP peptide, as treatment with L- and D-scrambled peptides did not have any effects in either the eye- or neuron-expression models. Our results are in line with what many others have observed with other D-peptidic Aβ inhibitors in *in vivo* experiments using transgenic mouse models of AD, where these D-peptides were able to improve both the pathology and behaviours in animals (Aileen Funke et al., 2010; Parthsarathy et al., 2013). Overall, D-peptidic inhibitors of Aβ42 appear to be excellent drug candidates for the prevention of Aβ42-induced toxicity *in vivo*.

## 2.4 Rescue of Aβ42-induced toxicity by D-AIP is sex-specific

We observed striking, sex-specific rescue of both eye morphology and locomotor behaviour using D-AIP treatment as a potential therapeutic for Aβ42-induced toxicity. D-AIP supplementation rescued the 'rough eye' phenotype in female, eye-specific Aβ42-expressing animals but had no effect on the male flies **(Figure 11)**. Inversely, in the neuron-specific Aβ42-expressing animals, only the locomotor behaviours of the male flies were rescued by D-AIP supplementation **(Figure 18)**. This suggests that, perhaps, specific tissues in each sex may respond to treatment differently, which has been reflected in our results: we determined that a male-specific protein (Acp70A) was likely responsible for sequestering D-AIP in the bodies of the male, eye-specific Aβ42-expressing animals, thus preventing its distribution into the heads of the flies to exert its neutralization effects on Aβ42. Conversely, in the female, neuron-specific Aβ42-expressing animals, the increase of insoluble Aβ42 deposits via unknown, possibly sex-based mechanisms may have indirectly sequestered the toxic, soluble Aβ42 peptides in the heads of the female animals, leading to their resistance against Aβ42-induced declines in locomotor behaviour.

The results from our experiments further demonstrated the importance of the inclusion of sex as a variable in *in vivo* studies as many others have noted previously (Beery and Zucker, 2011; Tannenbaum et al., 2017). We believe this factor is especially crucial in diseases with sex-specific risks such as AD, where sex-based differences in response to drug treatment may be particularly vital to the analysis of drug dosage and efficacy.

It is generally known that men and women may differ in their responses to corresponding drug treatment due to a wide range of factors, such as differences in body weight, height, body surface area, total water, and the amount of extracellular and intracellular water (Soldin et al., 2011). Pharmacokinetics and pharmacodynamics have also been attributable to the differences seen between males and females (Soldin and Mattison, 2009). For example, Bebia et al. studied the *in vivo* activity of a variety of cytochrome P450 (CYP) enzymes in the liver of human subjects – which are important for determining the rate of elimination of lipid-soluble drugs – and concluded that many of these enzymes, including CYP3A4, had similar activities between men

and women (Bebia et al., 2004). Other groups, however, demonstrated instead a ~2-fold greater hepatic CYP3A4 in women compared to men (Wolbold et al., 2003), which was supported by a subsequent study indicating that clearance of 15 different CYP3A4 substrates were greater in women than in men (Greenblatt and von Moltke, 2008). These conflicting data suggest that much remains unknown regarding the differences between sexes in terms of drug pharmacokinetics.

### 2.5 Future validation of D-AIP in complex rodent AD models

The *Drosophila* model has proven to be an excellent system for the preliminary efficacy screening of potential therapeutics such as D-AIP. In our hands, we were able to rapidly assess the efficacy and overall safety of D-AIP using multiple expression models (i.e. eye and neuron), allowing us to validate the rescue of Aβ42-induced toxicity by D-AIP in both morphology and functional studies. Our positive results in our invertebrate model show that D-AIP has the potential to also be effective against Aβ42 toxicity in a mammalian system such as the mouse.

Compared to *Drosophila*, the mouse brain is far more complex and possesses a much larger behavioural repertoire. As such, it would be an excellent system to evaluate the ability of D-AIP to rescue Aβ42-induced defects in both structure and behaviours. We would also be able to investigate the effects of D-AIP on tau pathology in a mammalian model, as the *Drosophila* homologue of human tau (dTau) does not seem to potentiate human Aβ42-induced toxicity (i.e. no NFT formation and exacerbation of Aβ42 toxicity in transgenic Aβ42 flies) (Heidary and Fortini, 2001; Burnouf et al., 2016). As well, since flies do not have a closed circulatory system (i.e. no vascularization) and lack conventional adaptive immune responses (Hoffmann, 2003; Newman, 2017), the murine system will provide us with much more information in regards to the rescue effects of D-AIP on mammalian vascular systems as well as immune responses.

Transgenic mouse models of AD such as TgCRND8 and the 3xTg models will likely be ideal models for the testing of D-AIP. The TgCRND8 strain only has the overexpression of the human APP gene (containing Swedish and Indiana mutations), and has significant Aβ42 levels at just 3 months of age (Chishti et al., 2001). This model would also allow us to perform more long-term experiments, as within 6 months, amyloid deposition in the cortex and hippocampus of these

mice also leads to cognitive impairment, inflammation (reactive gliosis), decreased spine density, dystrophic neuropathy, and vascular impairments (Allemang-Grand et al., 2015). Essentially, the TgCRND8 strain would allow us to look at the effects of D-AIP on a relatively simple system – with only one transgene, APP – and in a comparatively short timespan.

Alternatively, the 3xTg strain is another promising model that we can potentially utilize. This strain expresses three transgenes – APP (Swedish), tau (MAPT P301L), and presenilin-1 (PSEN1 M146V) (Oddo et al., 2003). Since plaque formation (extracellular Aβ deposits by 6 months) precedes tau pathology (by 12 months) in this model, the increased complexity of the 3xTg mice will provide a more comprehensive assessment of D-AIP. For example, the presence of Aβ42 oligomers have been postulated to increase the levels of tau phosphorylation (Selkoe and Hardy, 2016). Using the 3xTg model, we would then be able to assess the downstream effects on tau from the neutralization of Aβ42 by D-AIP.

## 2.6 Conclusion

While L-AIP has been shown in our previous publications to be an effective treatment against Aβ42 toxicity and aggregation *in vitro*, we have fully demonstrated that it is highly unstable in *in vivo* environments and thus would be a poor Aβ42 inhibitor candidate. Alternatively, our analysis has shown D-AIP to be a biostable, safe, and highly effective neutralizer of human Aβ42 toxicity in our *in vivo Drosophila* models. Intact D-AIP was readily detectable in the animals in both short-term and long-term experiments. As well, the oral administration of D-AIP was able to successfully rescue the toxic effects of Aβ42 expression on both eye structure and morphology, as well as in more complex systems such as the locomotor behaviour of the insects. Prolonged treatment with D-AIP was also non-toxic and had minimal side effects on the animals. Going forward, further studies on the pharmacokinetics of D-AIP will need to be completed in mammalian models (such as the mouse) in order to fully analyze its characteristics *in vivo.* Overall, D-AIP appears to be a promising anti-Aβ42 therapeutic, and we are hopeful that it will represent a new prophylactic strategy to delay and/or prevent the onset of AD.

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**ABBREVIATIONS**

| | |
|---|---|
| α-CTF | α C-terminal fragment |
| AD | Alzheimer's disease |
| Acp70A | accessory gland peptide 70A |
| AICD | APP intracellular domain |
| AIP | Aβ42-oligomer interacting peptide |
| ANOVA | analysis of variance |
| APH-1 | anterior pharynx defective-1 |
| ApoE4 | apolipoprotein E4 |
| APP | amyloid precursor protein |
| ARIA | amyloid-related imaging abnormalities |
| Aβ | amyloid-β |
| Aβ40 | Aβ peptide with 40 amino acid residues |
| Aβ42 | Aβ peptide with 42 amino acid residues |
| BACE1 | β-site APP cleaving enzyme |
| BBB | blood-brain barrier |
| β-CTF | β C-terminal fragment |
| CAA | cerebral amyloid angiopathy |
| cdk5 | cell division protein kinase 5 |
| CNS | central nervous system |
| CSF | cerebrospinal fluid |
| CYP | cytochrome P450 |
| D-AIP | AIP composed of D-amino acids |
| D-amino acids | dextrorotatory amino acids |
| dAPPl | *Drosophila* APP-like |
| DHB | 2,5-dihydroxybenzoic acid |
| DIAN | Dominantly Inherited Alzheimer Network |
| dTau | *Drosophila* tau |
| EDTA | Ethylenediaminetetraacetic acid |
| *elav* | embryonic lethal, abnormal vision |
| ERG | electroretinography |
| FAD | familial AD |
| *GMR* | Glass Multimer Reporter |
| GnHCl | guanidinium hydrochloride |
| GSK-3β | glycogen synthase kinase-3β |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| ITO | indium titanium oxide |
| $K_D$ | overall equilibrium dissociation constant |
| $k_d$ | dissociation rate constant |
| L-AIP | AIP composed of L-amino acids |

| | |
|---|---|
| L-amino acids | levorotatory amino acids |
| LTP | long-term potentiation |
| mAbs | monoclonal antibodies |
| MALDI-MSI | matrix-assisted laser desorption/ionization mass spectrometry imaging |
| MALDI-TOF | matrix-assisted laser desorption/ionization time-of-flight mass spectrometry |
| MAP | microtubule-associated protein |
| MRI | magnetic resonance imaging |
| NFT | neurofibrillary tangle |
| NHP | non-human primates |
| PBS | phosphate buffered saline |
| PEN-2 | presenilin enhancer-2 |
| PHF | paired helical filaments |
| PSEN1/2 | presenilin-1 or -2 |
| RING | rapid iterative negative geotaxis assay |
| RU-486 | mifepristone |
| sAPPβ | soluble APPβ |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEM | Standard Error of Mean |
| SP | sex peptide |
| SPR | surface plasmon resonance |
| $T_p$ | preferred temperature |
| UAS | upstream-activating sequence |
| WCE | whole cell extract buffer |