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Role of Mipp1 in a *Drosophila melanogaster* model of Alzheimer's disease

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AFFIDAVIT

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly indicated all material which has been quoted either literally or by content from the sources used.

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Abstract

Due to a steadily increasing improvement of general health and medical treatment, a prolongation of the overall life span in western countries has been noticeable. Alzheimer's disease is the most common form of dementia in the western civilization. Aβ42, as the disease-relevant (peptide-)species is formed by an intramembrane proteolytic processing of its precursor, the β-amyloid precursor protein (APP) through the amyloidogenic pathway. Recently, spermidine as an autophagy inducer was found to have an impact on Aβ42-related plaque formation and the autophagic pathway. Therefore, it is seen as a potentially interesting therapeutic polyamine in fighting Alzheimer's disease. Due to the neuroprotective effect of spermidine in previous studies, this thesis examines the impact of dietary spermidine supplementation on aged, Aβ42-overexpressed *Drosophila melanogaster* model organism for Alzheimer's disease and the role of the autophagic pathway. An untargeted proteomics analysis on Aβ42 overexpressing flies with and without spermidine supplementation resulted in the identification of Mipp1 as a potential target/mediator of spermidine-associated neuroprotection.

The effect of spermidine and the role of possible modulators such as Mipp1 on an Alzheimer's disease model was investigated in this master thesis. An expression of Aβ42 peptide is sufficient to increase the flies' susceptibility to manganese induced metal stress. However, the protective effect of external spermidine supplementation on those flies and a possible chelating of manganese by spermidine could not be shown in this experiment. The analysis of possible polyamine effects on a cross of *elavX*-GAL4 x UAS-*Mipp1*;Aβ42 measuring locomotive performance, showed an enhanced toxicity of manganese in flies carrying the co-expression compared to solely Aβ42 expressing flies and a decreased locomotor activity. Also, a decrease of cellular Mipp1 levels could be observed after spermidine treatment alongside an increase in autophagic activity whereas the co-expression of Mipp1 in Aβ42 flies resulted in a deactivated autophagic machinery.

All in all the external spermidine supplementation has in fact a protective effect on AD flies in some cases through the decrease of Mipp1 as well as an induction of autophagy. However, the conduction of functional tests such as the survival assay as well as the climbing performance did not concur with the findings on a protein level.

Zusammenfassung

Aufgrund der stetig zunehmenden Verbesserung der allgemeinen Gesundheit und medizinischen Behandlung ist eine Verlängerung der Gesamtlebensdauer in westlichen Ländern zu beobachten. Die Alzheimer-Krankheit ist die häufigste Form der Demenz in der westlichen Zivilisation. Aβ42-, als krankheitsrelevante (Peptid-)Spezies wird durch proteolytische Prozessierung seines Vorläufers APP, gebildet. Kürzlich wurde festgestellt, dass Spermidin als Autophagie-Induktor einen Einfluss auf die Aβ42-assoziierte Plaqueformation und den autophagischen Signalweg hat. Daher wird es als potenziell interessantes therapeutisches Polyamin bei der Bekämpfung der Alzheimer-Krankheit angesehen. Aufgrund der neuroprotektiven Wirkung von Spermidin in früheren Studien untersucht die aktuelle Forschung die Auswirkungen der Supplementierung mit Spermidin auf den gealterten, Aβ42-überexprimierten Modellorganismus für die Alzheimer-Krankheit *Drosophila melanogaster* und die Rolle des autophagischen Signalweges. Eine ungerichtete Proteomic-Analyse von Aβ42-überexprimierten Fliegen mit und ohne Spermidin-Supplementierung führte zur Identifizierung von Mipp1 als möglichem Ziel / Vermittler von Spermidin-assoziierter Neuroprotektion. Die Wirkung von Spermidin und die Rolle möglicher Modulatoren wie Mipp1 auf ein Alzheimer-Modell wurde in dieser Masterarbeit untersucht. Eine Expression des Aβ42-Peptids ist ausreichend, um die Anfälligkeit der Fliegen gegenüber Mangan-induziertem Metallstress zu erhöhen. Die protektive Wirkung einer externen Spermidin-Supplementierung auf diese Fliegen und ein möglicher Chelat-bildender Effekt von Mangan durch Spermidin konnte in diesem Experiment jedoch nicht gezeigt werden. Die Analyse einer möglichen Wirkung des Polyamins an einer Kreuzung von elavX-GAL4 × UAS-Mipp1;Aβ42, die die Fitness der Fliegen misst, zeigte eine erhöhte Toxizität von Mangan und eine verringerte lokomotorische Aktivität bei Fliegen, welche die Co-Expression aufweisen. Auch eine Abnahme des zellulären Mipp1-Spiegels konnte nach Spermidinbehandlung beobachtet werden, einhergehend mit einer Zunahme der autophagischen Aktivität, während die Co-Expression von Mipp1 in Aβ42-Fliegen zu einer deaktivierten autophagischen Maschinerie führte.

Zusammenfassend hatte die externe Spermidin Gabe durch die Abnahme von Mipp1, sowie einer Induktion der Autophagie eine protektive Wirkung auf AD-Fliegen. Allerdings konnte dieses Ergebnis mit Hilfe der Funktionstest nicht bestätigt werden, was eine Anpassung des weiteren Versuchsaufbaus erforderlich macht.

Table of contents

1. Introduction

Due to a steadily increasing improvement of general health and medical treatment, a prolongation of the overall life span in western countries has been noticeable. According to the World Health Organization (WHO) the life expectancy in Austria rose from 78.1 years (both sexes) in 2000 up to 81.5 years in 2015 (World Health Organization, 2016). With this demography of aging the incidence and prevalence of age-associated, in specific, neurodegenerative diseases (e.g. Alzheimer's Disease) are continuing to escalate. Thus, it has become a growing societal burden for patients as well as caregivers with an urgent need of research in this field.

1.1. Alzheimer's disease (AD) and its relevance in modern society

Alzheimer's disease is the most common form of dementia in the western civilization. This brain illness affects around 47 million people worldwide with currently no effective cure or treatment of its progress (Cope, 2018). This form of dementia is clinically marked by a progressive loss of memory and cognitive functions, with pathological deteriorations including substantial atrophy of the hippocampus and frontal cerebral cortex (Hyman, 2015). There are two major causes for the outbreak of this illness: a build-up of two proteins, hyperphosphorylated tau and amyloid-β in brain tissue which cause a formation of fibrils (Bharadwaj, 2018). Accumulations/aggregations of amyloid-β (Aβ) are only associated with AD and are located in extracellular plaques. The predominant variants of Aβ peptide are 40 or 42 amino acids in length. Aβ42 as the disease-relevant species is formed by an intramembrane proteolytic processing of its precursor, the β-amyloid precursor protein (APP) through the amyloidogenic pathway (Haass C, 1992). APP occurs as a transmembrane protein with its N-terminus within the extracellular site and the corresponding C-terminus in the cytosol (Kang J, 1987). A major step in the production of Aβ is the cleavage by α-, β-, and γ-secretase embedded in the neuronal cell membrane (van Maanen, 2018). Cleaved Aβ may occur as a formation of extracellular deposits as well as an intracellular accumulation within neurons through re-absorption following the permeabilization of the cell membrane and receptor mediated uptake. An imbalance of Aβ42 production and clearance followed

by an extracellular accumulation has been assumed to be causative for Alzheimer's disease (Tarasoff-Conway, 2015).

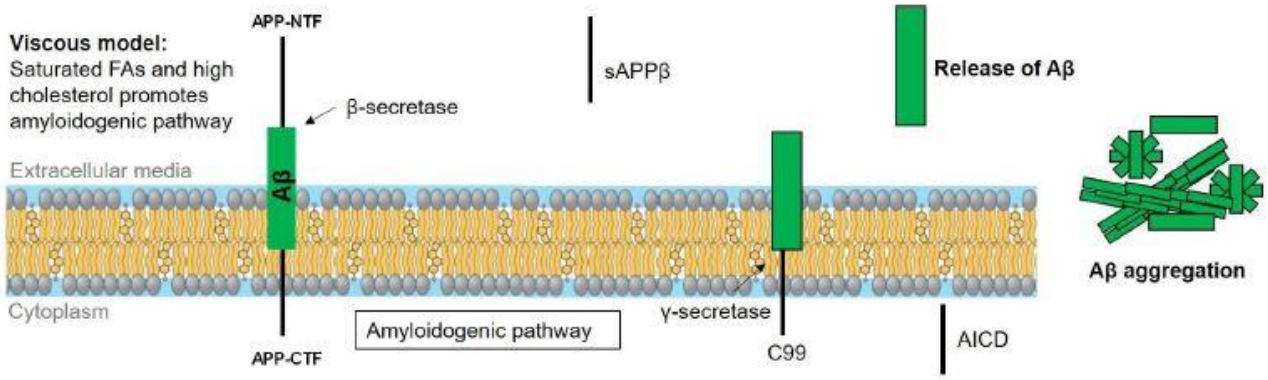

Figure 1: Membrane fluidity modulates APP processing and production of amyloid-beta. The amyloidogenic peptide Aβ is synthesized by proteolytic processing of the transmembrane protein APP. Cleavage of the β-secretase routes into the amyloidogenic pathway, which is accomplished by the γ-secretase complex and results in the generation of Aβ peptides *(Bharadwaj, 2018)*.

Furthermore, the presence of smaller intracellular Aβ42 oligomers was connected with disrupted neuronal calcium homeostasis, impairment of axonal transport and increased oxidative stress (Haass, 2007). Alzheimer's disease may manifest as a consequence of both the proteotoxicity of soluble small oligomers and the impairment of degradative pathways by excessive aggregate formation.

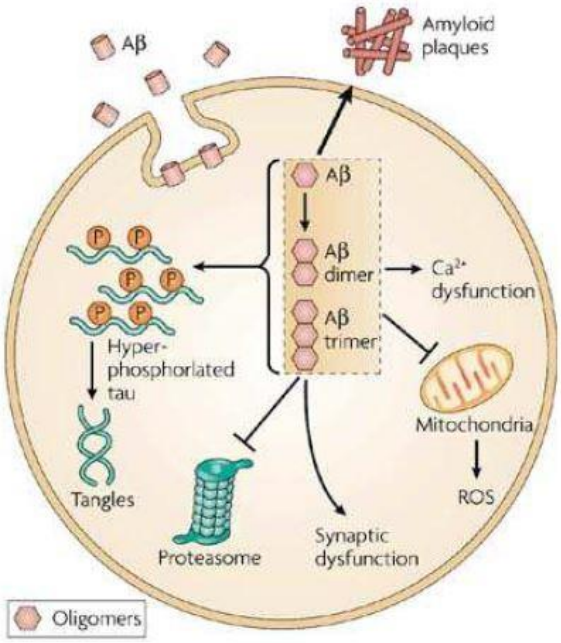

Figure 2: Aβ42 generation confers intracellular toxicity associated with AD. In addition to amyloid-β plaques and neurofibrillary tau tangles, intracellular enrichment of monomeric or oligomeric Aβ42 contributes to AD pathology. Soluble A42 peptides are proposed to disrupt cellular calcium homeostasis and impair mitochondrial or proteasomal function and thus, leading to increased oxidative stress, synaptic dysfunction and neuronal cell death *(LaFerla, 2007)*.

Seeing AD as an increasing health burden in our society it is crucial for researchers to devote more effort to improving the understanding of this disease as well as finding effective methods against Aβ42 plaque formation and thus, provide medical treatment. Recently, spermidine as an autophagy inducer was found to have an impact on plaque formation and the amyloidogenic pathway, therefore, it is seen as a potentially interesting therapeutic polyamine in fighting Alzheimer's disease.

1.2. Spermidine as a potential therapeutic against AD

Spermidine belongs to a ubiquitous family of polycations called polyamines. The spermidine synthase catalyzes the reaction of putrescine to spermine with spermidine as a precursor. Polyamines play a vital role in cell proliferation and differentiation as well as survival and cell death/apoptosis (Heby, 1981). The intracellular levels of polyamines are regulated by biosynthesis, degradation and transportation with

remarkably high levels of spermidine during cell growth. Recent studies have shown that a naturally occurring decrease in polyamine levels is linked to the aging process and the pathophysiology of neurodegenerative diseases such as Alzheimer's disease. Spermidine activates the autophagic machinery and therefore, counteracts age associated cell death and exhibits beneficial effects in fighting oxidative stress, apoptosis and inflammatory cell stress (Jamwal, 2016). These findings were manifested in several model organisms such as *S. cerevisiae*, *C. elegans* and *D. melanogaster*. Beside neuroprotection, further research on this protein uncovered a protective effect on survival of the fruit fly upon challenging stress scenarios (Minois, 2012) as well as a reduction of age-related oxidative protein damage in mice (Zhang M. T., 1997). Furthermore, a dietary administration of spermidine could improve cognitive functions in aged *Drosophila melanogaster* and effectuate a life extension which underlines the neuroprotective effect of polyamines (Gupta, 2013). Current investigations on this specific polyamine demonstrated that it does not only influence lifespan, spermidine supplementation also seems to reverse and inhibit age-related myocardial morphologies in rats (Zhang H. J., 2017), enhance cardiac autophagy (Tobias Eisenberg, 2016) and has a blood-pressure lowering effect in mice which most likely results from a higher arginine bioavailability and protection from renal damage (Eisenberg T. A., 2017). These effects are partially mediated by regulating the autophagic flux.

Due to the neuroprotective effect of spermidine in previous studies, current research examines the impact of dietary spermidine supplementation on aged, Aβ42-overexpressed fruit flies and the role of the autophagic pathway in flies with Alzheimer's disease.

1.3. Autophagy

Autophagy is known to be a major route of depletion and removal of misfolded, damaged or simply unwanted proteins and other cellular components like peroxisomes, mitochondria and the endoplasmic reticulum. Overdue proteins with altered metabolic needs due to nutrient stress are forming vast, aggregated particles and therefore hinder proteasomal degradation. Hence, these proteins are recycled by

autophagy (Glick, 2010). Besides its cellular clearance process with supporting a cell's survival, autophagy also promotes cellular senescence and cell surface antigen presentation. Therefore, autophagy obtains a key role in preventing certain diseases, including late-onset neurodegenerative diseases, called proteinopathies, such as Alzheimer's disease.

Autophagy of aforementioned aggregate-prone proteins and cellular components is conducted by engulfing respectives by double-layered membranes, called autophagosome and consequently transported to the lysosome where both components fuse into the final form – the autolysosome. Upon fusion, the intralysosomal pH lowers. Hence, functional proteases get activated which essentially degrade the entailed cargo to its basic molecules such as nucleotides and amino acids. These can be further recycled into the anabolic pathway (Menzies, 2015). Depending on the cargo transporting pathways to the lysosome, three major forms of autophagy are currently distinguishable: macroautophagy is the formation of double-membraned autophagosomes around the cytoplasmic cargo and then transferring it to the lysosome; microautophagy is the direct engulfment of cellular particles by the lysosome and lastly, chaperone-mediated autophagy (CMA) which requires the support of Hsp70 chaperones recognizing and transferring proteins to the lysosomal membrane where they are unfolded and translocated into the degradative environment of the lysosome (Menzies, 2015). The complex underlying machinery of macroautophagy – herein referred to as autophagy – is illustrated in Figure 3, and can be dissected into distinct steps, including induction of autophagy, cargo selection, autophagosome nucleation, expansion and the final cargo packaging, further transport to the lysosome, docking and fusion, and the ultimate vesicle break down and cargo recycling within the autolysosome. The presence of autophagic ubiquitin-dependent receptors and additional adaptor proteins raise the assumption of a linkage between the pathways of autophagy and proteasomal degradation. These receptors include p62 (among other proteins) which recognize ubiquitinated proteins and tether them to the core autophagic machinery in which the microtubule-associated protein light chain 3 (LC3) acts as an acceptor (Shaid, 2013).

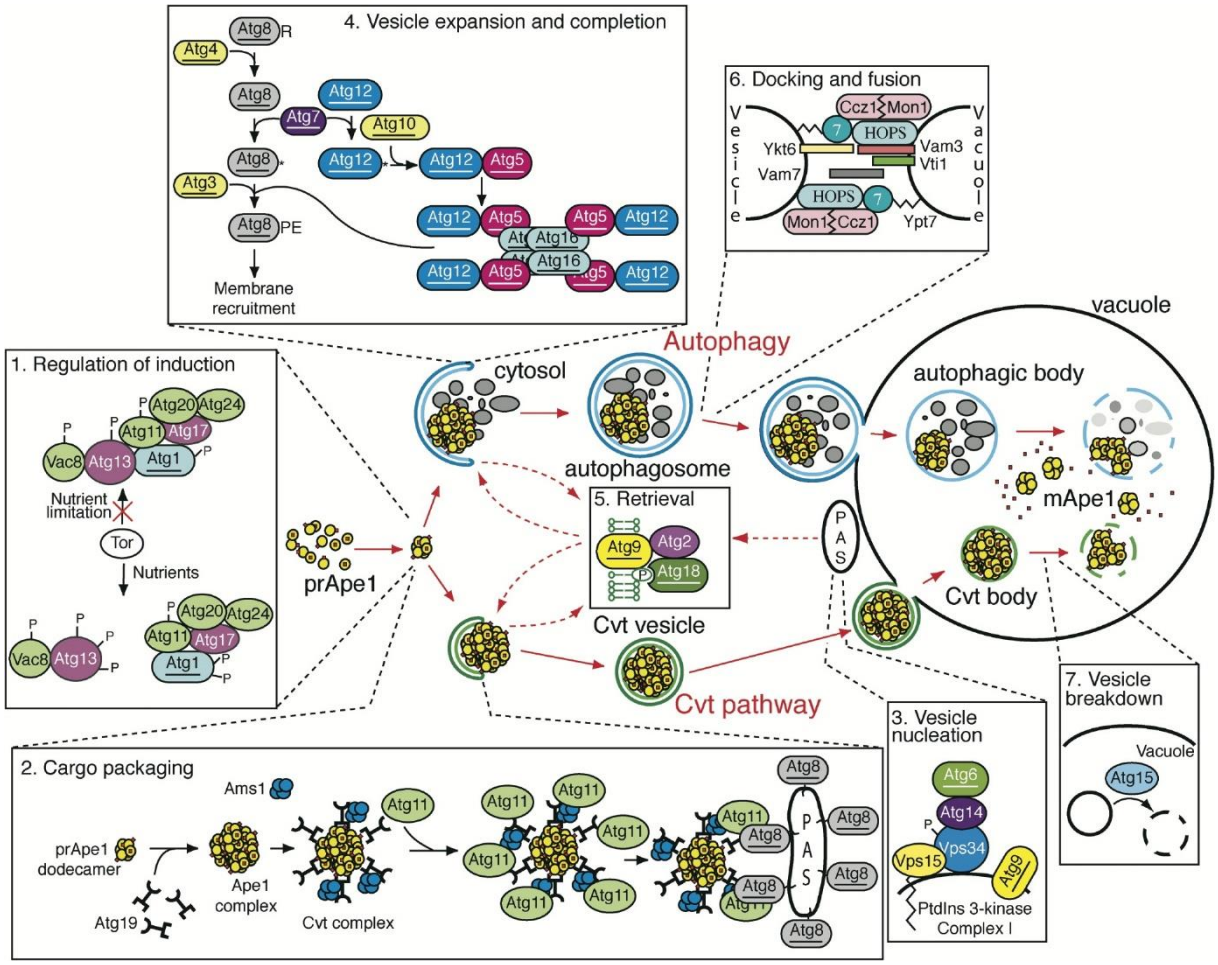

Figure 3: Schematic representation of the autophagy and cytoplasm to vacuole targeting pathways in yeast. The process can be divided into distinct steps as indicated. Induction is mediated by the key regulator Tor by phosphorylation status of Atg13, which influences the affinity to Atg1. Modulated Atg1 kinase activity results in the formation of precursor aminopeptidase 1 (prApe1) complexes by yet undefined routes. PrApe1, Atg11 and Atg19 are required for cargo packaging and the formation of the preautophagosomal structure (PAS). After vesicle nucleation it has to be expanded, which is mediated by an orchestrated interaction of E1 ubiquitin activating enzymes (Atg7), ubiquitin-like proteins (Atg8, Atg12) and Atg5, forming a transient coat complex. Retrieval of the vesicle is mediated by Atg9, Atg2 and Atg18. The completed autophagosome or Cytoplasm to Vacuole Targeting (Cvt) vesicle finally dock to the vacuole with the help of SNARE proteins and release their cargo into the acidic lumen of the lysosome *(Levine B, 2014)*.

Autophagic deficiencies are assumed to be implicated in neurodegenerative diseases such as Alzheimer's disease. The CNS - and neurons in particular - rely on a precisely functioning repair machinery due to an absent mitotic cell division. A basal autophagic level is crucial to maintain neuronal proteostasis by removing age-related neurotic fibrils or aggregated proteins.

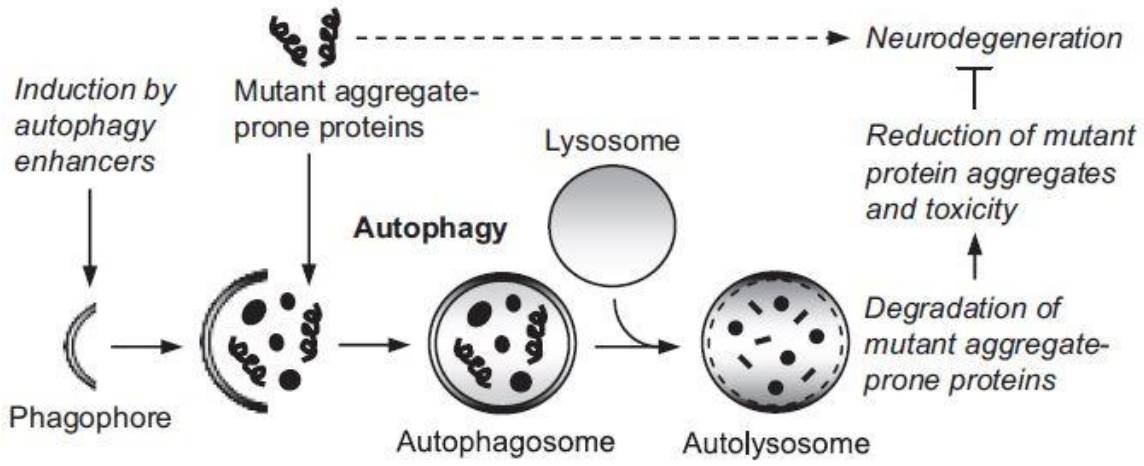

Figure 4: Autophagy as a protective pathway for neurodegenerative diseases. Toxic aggregate-prone proteins which are associated with neurodegenerative diseases such as Alzheimer's disease are degraded and cleared by the autophagic pathway. The process is induced by autophagy enhancers and therefore triggers the formation of a double-membrane called phagophore. An engulfment of mutant aggregate-prone proteins leads to the formation of the autophagosome which later forms the autolysosome after fusion with the cellular lysosome. This formation ultimately degrades the content of the autolysosome and enhances autophagic clearance of the containing target proteins. Hence, the degradation results in reduction of toxicity and therefore shows a protective effect in neurodegenerative diseases *(Renna, 2010)*.

A pathological acceleration of protein-aggregation is therefore linked to neurodegenerative diseases (Marino, 2011). In order to comprehend the complexity of the linkage between the autophagic pathways and Alzheimer's disease and further investigate the role of pathological markers, a model organism with a thoroughly evolved CNS is required. *Drosophila melanogaster* has therefore become a widely used and well established model organism in the research on neurogenerative diseases.

1.4. *Drosophila melanogaster* as a model organism

The study of complex human neurodegenerative diseases exceeds the possibilities of *S. cerevisiae* in many ways, thus making the use of a more complex organism necessary. *Drosophila melanogaster* – the fruit fly - provides a fully annotated genome, a sophisticated yet well studied brain and nervous system and an established collection of experimental tests to analyze complex behavior and cognitive

phenotypes. Besides its apparent advantages, including a short generation time and a cheap and easy handling, the fly's little redundant genome prompted the development of dedicated tools for genetic engineering, thus enabling large-scale and high throughput experiments for genetic and pharmaceutical screenings. Its genome consists of eight chromosomes: 2 sex chromosomes and two sets of autosomes in both sexes (second, third and fourth chromosome) (Gallant, 2008). The fusion of a yeast upstream activating sequence (UAS) to a transgene and the integration of the corresponding Gal4p transcription factor under the control of an endogenous promoter allows the temporal and spatial control of transgene expression in the progenitors emerging from a cross between the UAS and the GAL4 fly line. This genetic tool has widely been used to create flies that mimic complex human diseases by transgenic expression of affected proteins (Elliott, 2008).

Different invertebrate models to study Alzheimer's disease have evolved within the last years, recapitulating most but not all of the criteria for this disease. *Drosophila* as a model for AD is limited in terms of loss of function studies, as in fact many proteins involved in disease pathogenesis, including the fly amyloid precursor protein like (APPL) and presenilins, are evolutionary conserved from flies to humans, yet functional homologs of the β-secretase as well as the amyloidogenic Aβ42 sequence at the C-terminus of APP are missing. More recently fly models have been designed as transgenic animals directly expressing the human Aβ42 peptide specifically in the eye resulting in a roughened eye surface, which serves as a model to reconstitute cell degeneration (Cao, 2008) (Finelli, 2004) and elegantly fulfills the needs of a high-throughput screening model to test genetic or pharmacologic modifiers of Alzheimer's disease. Furthermore, neuronal expression of human Aβ42 as well as a more toxic disease-relevant mutant (Aβ42 E22G, also Aβ42-Arc) and the extracellular plaque formation in the brain was facilitated by fusing the amyloid nucleotide sequence to a secretion sequence (Iijima K. L., 2004) or an N-terminal signal sequence targeting the peptide for the secretory pathway (Iijima K. C., 2008). These strategies resulted in the generation of extracellular amyloid plaques and allowed a substantial study concerning the neurotoxicity in AD. Besides biochemical and histological approaches following AD pathogenesis *Drosophila* depicts a smooth and convenient organism to study behavioral consequences of Aβ42 toxicity. The fruit fly bears a complex central nervous system (CNS) including a functional homolog of the human hippocampus

(Heisenberg, 2003). Taking all the advantages of *Drosophila melanogaster* as a model organism into consideration, it offers a variety of techniques to study specific genomic modifications and learn about yet unknown functions of certain proteins.

For example, an untargeted proteomics analysis on Aβ42 overexpressing flies with and without spermidine supplementation resulted in the identification of Mipp1 as a potential target/mediator of spermidine-associated neuroprotection. The concentration of this protein showed a significant decrease in *Drosophila* brains after spermidine supplementation in contrast to untreated Aβ42 overexpressed flies. Therefore, Mipp1 was further investigated.

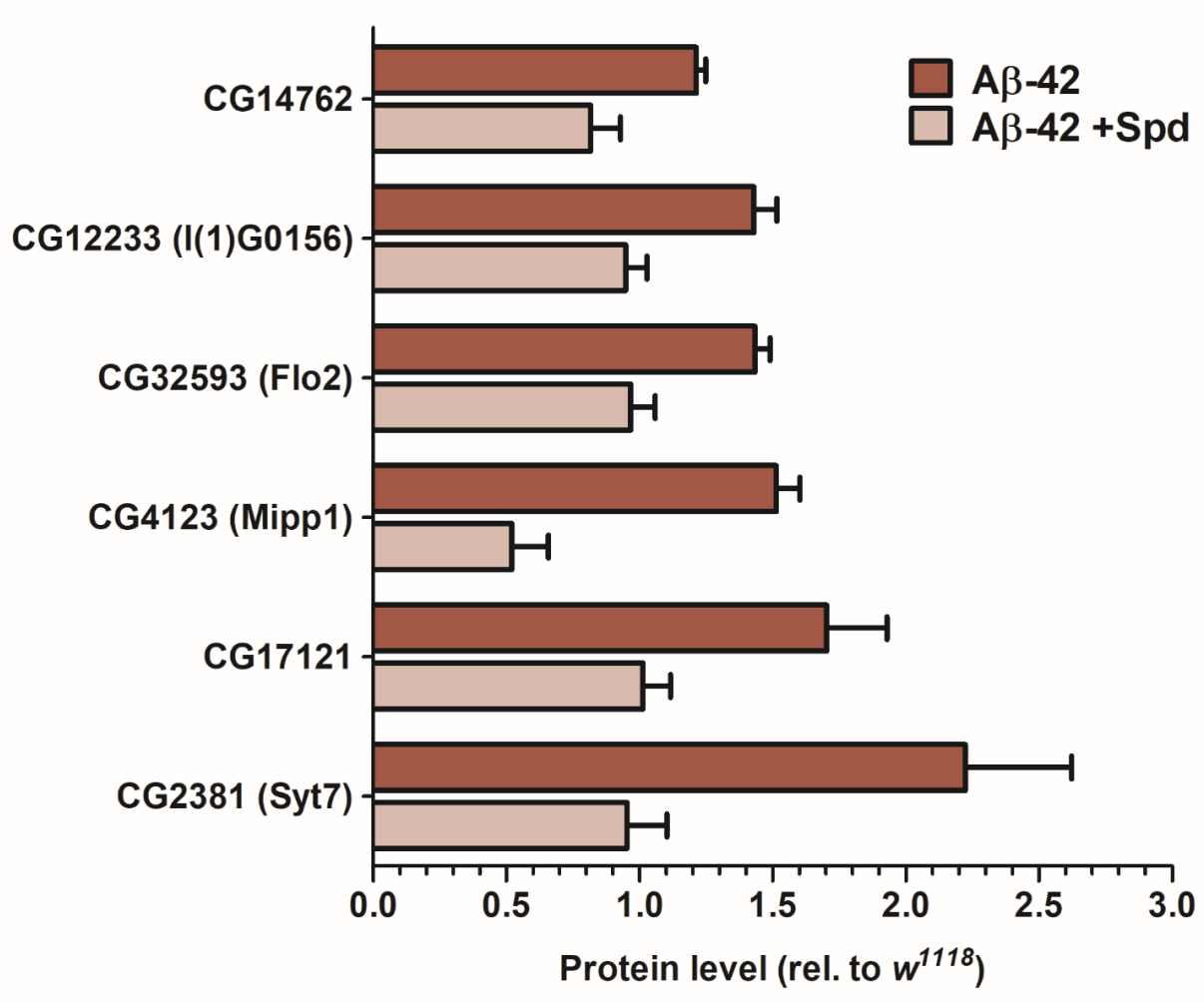

Figure 5: Decrease of Mipp1 levels in Aβ42 overexpressed flies after spermidine supplementation. Aβ42 was overexpressed in *Drosophila melanogaster* strains and aged for 14 days. After 48h of spermidine treatment (versus no spermidine treatment) fly heads were extracted and an untargeted proteomics analysis was performed. Mipp1 levels were increased in Aβ42 flies compared to the wild type strain, whereas a significant decrease of Mipp1 levels after spermidine treatment was measured (Preliminary data: Cornelia Sommer-Ruck).

1.5. Mipp1 and neuroprotection

Mipp1 (multiple inositol polyphosphate phosphatase 1) and Mipp proteins in general are highly conserved and are known to convert inositol phosphates such as IP6 to IP3. The characterization of *Mipp1* showed a high level of expression mainly located in the developing embryonic trachea of *Drosophila melanogaster*, especially in cells of its migrating branches to allow an advantage in cell migration (Cheng, 2015). During tube elongation, Mipp1 relocates to the plasma membrane of the cell and then dephosphorylates IP6 to IP3 where it further promotes filopodia formation and aids the rearrangement of cells. Earlier studies found that *Mipp1* is ubiquitously expressed in mammalian tissues which indicates the potentially important role of this protein. Also, its involvement in the inositol pathway suggests an interference in the autophagic pathways and therefore, a potential target of spermidine supplementation. According to Renna *et al*. intracellular IP3 levels negatively regulate autophagy and autophagy can be induced by lowering intracellular inositol or IP3 levels in specific, independently of mTOR (Renna, 2010).

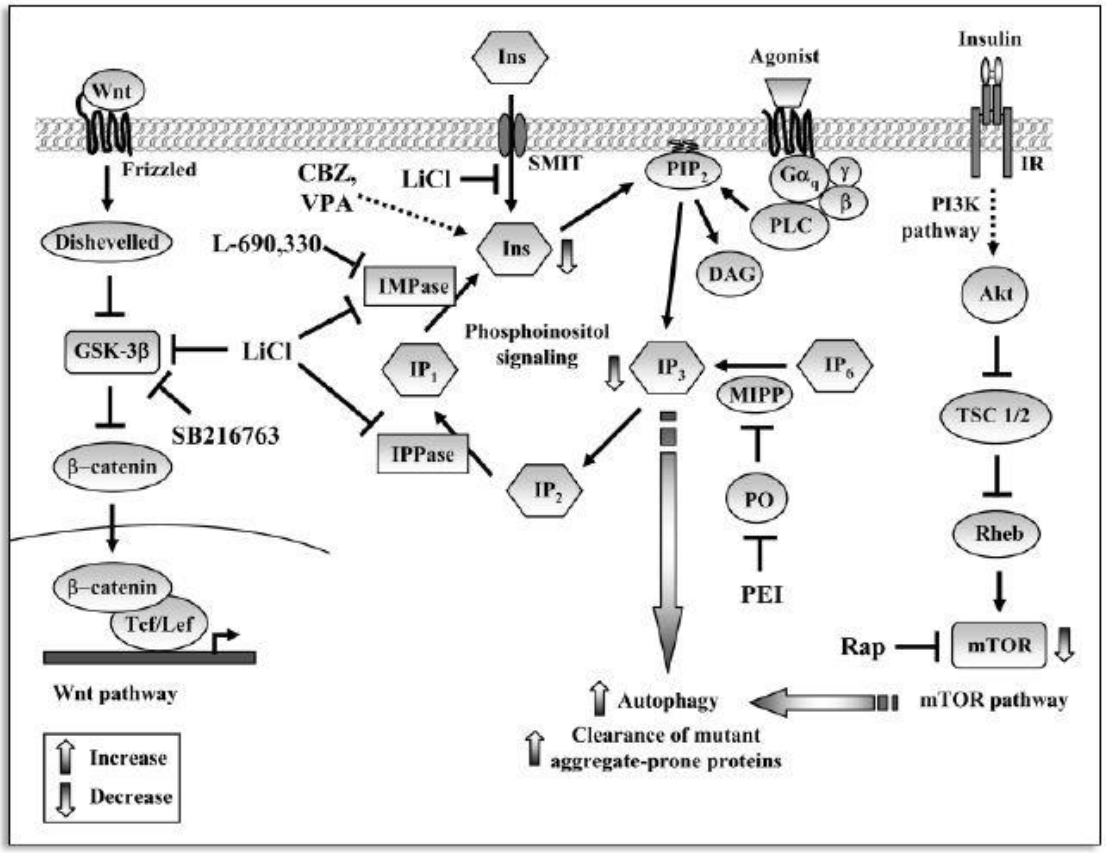

Figure 6: Mipp1 elevates intracellular IP3 levels and therefore negatively regulates autophagy, independently of mTOR. Multiple inositol polyphosphate phosphatase (Mipp) converts inositol-6-phosphate to inositol-3-phosphate which is an inhibitor of the autophagic pathway and therefore leads to a reduced clearance of mutant aggregate-prone proteins *(Sarkar S. D., 2006)*.

As demonstrated in Figure 5, Aβ42 expressing flies showed elevated levels of cellular Mipp1 compared to wild type fruit flies whereas an external spermidine supplementation on those flies led to a significant decrease in cellular Mipp1 levels below wild type baseline. The human homolog of *Drosophila melanogaster*'s Mipp1 is the mammalian 3-phytase also called MINPP1 which was identified to be essentially involved in both the extracellular as well as the lysosomal IP6 dephosphorylation process in the lumen of the endoplasmic reticulum (ER) (Windhorst, 2013). Also, the expression of *MINPP1* is known to be modulated by the transcription factor (TF) EP300 (Harmonizome, 2008). EP300 is a histone acetyltransferase which regulates transcription via chromatin remodeling and is important in the processes of cell proliferation and differentiation. The activation of this transcription factor can be inhibited by spermidine and therefore leads to an induction of autophagy which supports the idea of EP300 being an endogenous repressor of autophagy (Pietrocola,

2015). Growing evidence shows that the autophagic induction can be triggered by nutritional or pharmacological interventions and therefore reduce age-associated diseases such as AD and extend longevity (Eisenberg T. K.-G., 2009).

Taking all recent studies on Mi(n)pp1 into consideration, the aim of this thesis was to investigate whether this protein might be causative for spermidine-mediated neuroprotection in Aβ42 overexpressing fruit flies.

2. Materials

2.1. *Drosophila melanogaster* strains

The GAL4 driver line *elav*^{C155}-GAL4 (Bloomington stock number 105921) (Lin, 1994) was obtained from the Bloomington Stock Center (Indiana, USA). UAS-Aβ42 (Aβ42) flies were gifted from Dr. Koichi Iijima (Thomas Jefferson University, USA) (Iijima K. C., 2008). UAS-*Mipp1* (II) (further referred to as *Mipp1*) was kindly provided by Deborah Andrew (Andrew, 2015) and UAS-*Mipp1*;UAS-Aβ42/TM3, Sbtb stocks (further referred to as Mipp1;Aβ42) were established in the lab.

2.2. Buffer and Solutions

Deionized water was used for the preparation of all buffers and solutions. Given pH-values of all solutions are referred to room temperature conditions.

Laemmli buffer (1x)

50 mM Tris/ HCl pH 6.8, 2 % SDS, 10 % (v/v) glycerol, 0.1 % bromphenol blue, 2 % β-mercaptoethanol

10% Tris-Tricine gel

Stacking gel

3 M Tris/ HCl/ 0.3% SDS, pH 8.45, 30 % acrylamide, 10 % ammonium peroxodisulfate (APS), 0.1% N.N.N‘.N‘-tetramethylethylenediamine, 0.1% bromphenol blue

Separating gel

3 M Tris/ HCl/ 0.3% SDS, pH 8.45, 30 % acrylamide, 10 % ammonium peroxodisulfate (APS), 0.1% N.N.N‘.N‘-tetramethylethylenediamine

10x cathode buffer

1M Tris, 1M Tricine, pH 7.95, 1% SDS, stored at 4°C

10x anode buffer

1M Tris, pH 8.9, stored at room temperature

CAPS buffer

10mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS), 1N sodium hydroxide, pH 11.0, 10% methanol

25x Complete (Sigma-Aldrich)

Phenylmethylsulfonyl fluoride (PMSF) (200mM in EtOH)

RIPA buffer

50mM Tris/HCl, pH 8.0, 150mM NaCl, 0.5% Na-Deoxycholate (DOC), 1% Triton X-100, 1% SDS, 1x Complete, 2mM PMSF

1x TBS (Tris-buffered saline)

20 mM Tris, 150 mM NaCl, pH 7.6

1x TST (Tris-HCl, saline, 0.05% Tween 20)

1x TBST (Tris-buffered saline, 0.1% Tween 20)

Stripping buffer

2% SDS, 62.5mM Tris/HCl, β-Mercaptoethanol (Sigma-Aldrich)

2.3. Antibodies

Antibodies were prepared in 1x TBS, 5 % non-fat dry milk or 1x TST 5% bovine serum albumin (BSA) and used as followed: polyclonal serum α-Mipp1 antiserum prepared and gifted by Deborah Andrew (Andrew, 2015) 1:2500 in 5% milk powder (MP), Mouse α-FLAG 1:10,000 (Sigma-Aldrich), mouse α-Aβ42 1-16 6E10 1:750 (Biolegend, 803001), α-GABARAP/LC3 1:500 (MBL International, PM037) in 5% BSA, α-ref(2)P 1:250 (Cell Signaling Technology) in 5% MP and α-tubulin 1:5000 (Sigma Aldrich, T9026) in 5% MP. Secondary antibodies were goat α-guinea pig 1:5000 (Abcam, ab6908) in 5% MP for Mipp1, α-rabbit-POD 1:10000 (Sigma-Aldrich, S3062) in 5% BSA for GABARAP/LC3, α-rabbit-POD 1:10000 in 5% MP for Ref(2)p and α-mouse-POD 1:10000 (Sigma-Aldrich, T9026) in 5% MP for tubulin.

3. Methods

3.1. *Drosophila melanogaster* maintenance and treatment

Unless otherwise specified standard laboratory breeding was carried out at 25°C, 65-70% humidity and a 12:12 hours light:dark cycle (Sigrist, 2003). Standard fly food was prepared according to the Bloomington recipe as a semi-defined cornmeal-molasses medium with slight modifications (0.92 l H_2O, 4.2 g agar-agar, 85.3g molasses, 7.5g dry yeast, 8.3 g soy, 66.7 g cornmeal, 1.33 g p-hydroxy-benzoic acid methyl ester dissolved in 4.2ml 100% ethanol, 5.25 ml propionic acid).

3.1.1. Food preparation

The standard fly food for all experiments was cornmeal-molasses-food, modified from Bloomington. All ingredients as well as the recipe are listed below:

Ingredients
Baker's yeast (Lesaffre, France)
Agar (Difco)
Propionic acid (Sigma-Aldrich, ~99% pure, suitable for insect cell, P5561)
Molasses (Demeter)
Cornmeal
Soy flour
Nipagin/methyl 4-hydroxybenzoate (Sigma-Aldrich, ≥99% pure, 111988)
ddH2O
Ethanol, abs. (Sigma-Aldrich)

Double distilled H_2O was mixed with agar. After the water-agar solution started boiling, molasses was added. When it reached 100°C, dry yeast was added and boiled for at least 10 minutes. Then, corn and soy meal were added and boiled for another 10 minutes. When the food reached a temperature of approximately 55°C nipagin and propionic acid were subjected.

Food was poured into vials and air dried over night at room temperature and later stored at 4°C for a maximum of 2 weeks for usage.

3.2. Mating scheme

3.2.1. Harvesting male and female virgin flies

For spatially and temporally controlled expression of Aβ42, male flies harboring the UAS-Aβ42 construct were crossed with GAL4 driver line virgins in a ratio of 1:5 – 1:4. The same procedure applies for the wild type w^{1118} as well as the expression of Mipp1 and Mipp1,Aβ42 co-expressing flies. ElavX-Gal4 virgin female flies were collected over a maximum period of 10 days prior to mating with males of desired genotype. For each crossing, an estimate of 2000 female virgin flies was harvested. There flies were incubated at 18°C with a 12:12 light:dark cycle until setting up the crossings. Male flies of each genotype were collected on the day of the crossing.

3.2.2. Mating pattern

Approximately 25 males were crossed with 100-125 female virgin flies per vial on standard fly food and incubated at 25°C and 70% humidity. Parental flies were transferred to new vials every third day for a maximum of four times, and only 1 to 3 day old progenitor flies from the F1 generation were used for experiments. In order to determine specific genotypes for the experiments, females from the F1 generation had to be sorted accordingly. The flies were anesthetized on a porous pad by CO_2 application for a maximum time of 5 minutes. Phenotypical markers have been used

for the selection: Aβ42 - as well as Mipp1;Aβ42 co-expressing flies were divided into wild type hair on their heads and backs or stubble hair. The desired F1 generation females had normal hair. All Mipp1 overexpressing flies and w^{1118} wild type flies had normal hair, therefore, only female flies were collected for the following experiments.

3.3. Survival Assay

To assess resistance against bivalent metal ion stress, approximately 30 female F1 progenitor flies from an *elavX*-GAL4 x w^{1118} , *elavX*-GAL4 x UAS- Aβ42, *elavX*-GAL4 x UAS-*Mipp1* and *elavX*-GAL4 x UAS-*Mipp1*;Aβ42 cross were collected one to three days after eclosion and transferred to fresh vials containing standard fly food. Flies were shifted to fresh food every three days for a period of 14 days to let them pre-age prior to the transfer onto manganese and spermidine media. At least 120 flies per were tested for a single experiment. To expose the animals to manganese they were subsequently shifted to empty vials that were fixed on the cover of a petri dish containing filter papers that reach from inside the vial through the spongy plug to the inner part of the petri dish. To induce manganese stress 30ml of a 10% (w/v) sucrose/ 10 mM $MnCl_2$ solution was provided in each petri dish so the soaking filter paper allowed the flies to consume sucrose and Mn^{2+}. Each petri dish contained a maximum of six vials. Furthermore, half of the fly-containing vials were provided with a 10% (w/v) sucrose/ 10 mM $MnCl_2$/ 3.75 mM spermidine solution (adjusted to pH 6.1 - 6.3 prior to $MnCl_2$ addition). The flies were incubated at 25°C and 70% humidity. Dead individuals were counted at a 12 hour rhythm until all flies were dead. The experiment has been performed blinded.

All data was analyzed and visualized using GraphPad Prism 5 software and Microsoft® Excel (2016). For a statistical analysis a two-way ANOVA followed by a post hoc (Bonferroni) test was performed.

3.4. Climbing Assay

Locomotor activity was measured by assessing the climbing performance. Therefore, approximately 20 female flies which were pre-aged for 14 days, subsequently stressed with manganese for 48h or 72h – either supplemented with spermidine or not -, and were transferred into each slot of a climbing apparatus which was built by a colleague.

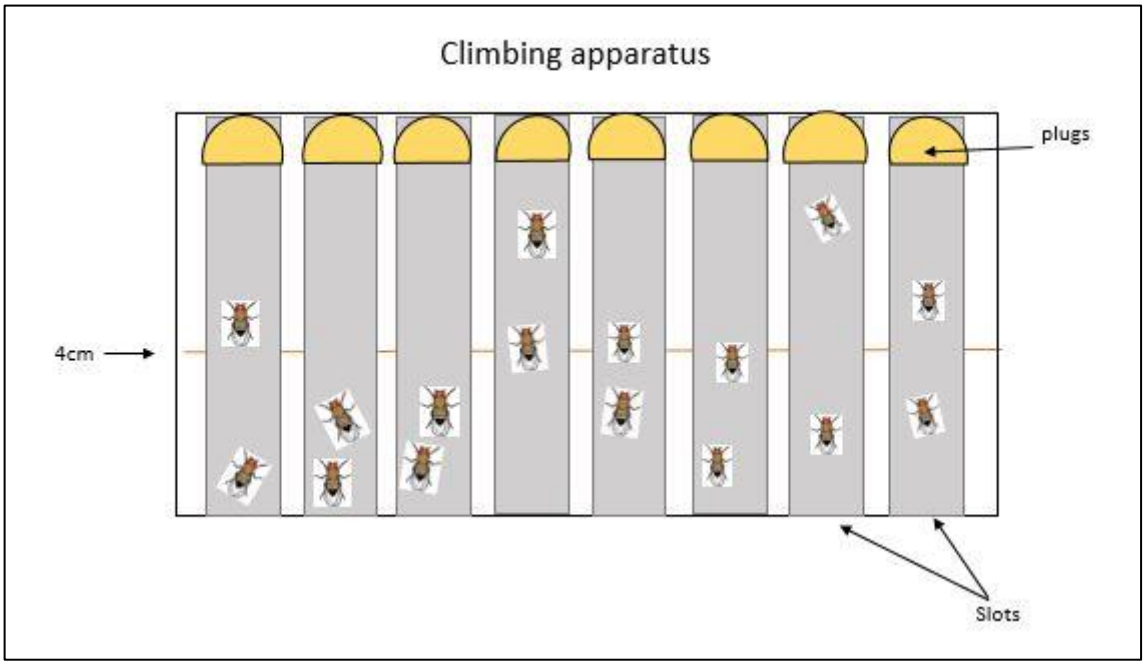

Figure 7: Schematic representation of the Climbing apparatus to manifest locomotor activity of *Drosophila melanogaster*. The Climbing apparatus consists of 6 to 9 slots with a height of 10 cm and depth of 1 cm. Each slot has the capacity of containing approximately 20 flies, closed with a foam plug to enable oxygen intake. Performance index threshold was set to 4 cm and marked by a line. Locomotion was measured for 15 seconds, repeated for three times.

The climbing apparatus consists of 6 to 9 slots in which an average of 20 flies were placed. The animals were tapped to the bottom of the climbing chamber and the number of individuals that could climb upwards within 15 seconds was determined. This was repeated for three times. The threshold for the performance index was set to 4cm and was calculated as the percentage of living animals that could reach the defined height within 5 seconds. All climbing experiments were performed in the dark under red dim light with the flies being adjusted to the absence of light for at least 30 minutes prior to testing. Between 60 and 80 flies per genotype and condition (with or without spermidine supplementation) were tested in an individual experiment. Again, the process of Mn-stress as well as the locomotion experiment was performed blinded

and flies were randomized to avoid investigator's bias.

All data was analyzed and visualized using Fiji/Image J software, GraphPad Prism 5 software and Microsoft® Excel (2016). For a statistical analysis a two-way ANOVA followed by a post hoc (Bonferroni) test was performed.

3.5. Western Blot Analysis

An approximate of 60-90 female flies per genotype and condition were pre-aged for 14 days and then stressed with Mn^{2+} or Mn^{2+} supplemented with spermidine for 48h and frozen in liquid nitrogen prior to Western Blot analysis.

3.5.1. Protein extraction

For fly head protein extraction 20-30 fly heads per genotype and condition were collected by vortexing deep-frozen flies (-80°C). The heads were collected and incubated with 2µl RIPA buffer per fly head, 200mM PMSF, 1x Complete and immediately squished (while on ice) using a pistill. Head extracts were incubated at room temperature for 15 minutes before they were centrifuged for 10 minutes at 8°C (pre-cooled) and 14.000rpm so fly head debris was removed. The supernatant was then transferred into a fresh vial and incubated with 15µl of 5x Laemmli buffer (250 mM Tris/HCl pH 6.8, 20% SDS, 60 % glycerol and 10% β-mercaptoethanol). Samples were then heated at 95°C for 5 minutes. Samples were either frozen at -20°C to perform an SDS-PAGE the following day or directly used. 15µl (equivalent for 6 heads) were subjected to a denaturing SDS-PAGE using a 10% Tris-Tricine gel.

3.5.2. SDS-PAGE

Proteins were fractionated using a standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to an established system (Bio-Rad). 6 fly heads were subjected to a 10% Tris-Tricine gel and blotted onto PVDF membranes in cold CAPS buffer at 220mA for 90 minutes. The membranes were cut at a height of

70kDa and around 30kDa. Each membrane piece was blocked in either 5% MP or 5% BSA for 30 minutes prior to antibody incubation. Primary antibodies were applied over night at 4°C. Secondary antibodies were applied after 3 washing steps in PBS/1%Tween-20 for 1 hour at room temperature. For detection, the enhanced chemiluminescence (ECL) substrate (GE Healthcare/Amerham) is applied onto the membranes which causes light emission upon reaction with the peroxidase coupled to the secondary antibody. The emitted signal was captured using ChemiDoc Imaging Systems (Bio-Rad).

Western Blot images were analyzed and quantified using Image Lab software (Bio-Rad) and Microsoft® Excel (2016).

4. Results

4.1. Spermidine shows no protective effect in Aβ42-expressing flies

Given the known link between industry-prompted manganese exposure and neurodegeneration in general as well as a recent link to Alzheimer's Disease in specific (Schneider, 2009), we investigated the effect of Mn^{2+} on a *Drosophila melanogaster* model of AD. As dysfunctional autophagy is a hallmark of pathological AD conditions we were furthermore interested in a possibility of a protective impact of polyamines – such as spermidine - on affected neurons. For this purpose, w^{1118} wild type flies or transgenic flies carrying an inducible human Aβ42 nucleotide sequence were crossed with $elav^{C1115}$-Gal4 driver flies in order to encompass temporally and spatially controllable expression of the Aβ42 peptide in all neurons of larvae as well as in the adult fly. To assess resistance against bivalent metal ion stress, approximately 30 female F1 progenitor flies were collected one to three days after eclosion and transferred to fresh vials containing standard fly food. Flies were then shifted to fresh food every three days for a period of 14 days to let them pre-age prior to the transfer onto 30ml of a 10% (w/v) sucrose/ 10 mM manganese medium in each vial as a control or with an additional 5mM spermidine supplementation, aiming at a protective effect. To determine the specific survival Aβ42-expressing flies in comparison to wild type flies and to provide evidence for a neuroprotective effect of spermidine, dead flies were counted every 12 hours until all flies were deceased. As shown in figure 8, we could demonstrate that an expression of Aβ42 peptide is sufficient to increase the flies' susceptibility to manganese induced metal stress compared to wild type flies. However, the protective effect of a 5mM spermidine supplementation on those flies could not be shown in this experiment on either fly population (Fig. 8, 15). These results do not match previously performed survival assays performed by Cornelia Sommer-Ruck (Fig. 16) in which concomitant supplementation with 5 mM spermidine could revert the deleterious effects significantly.

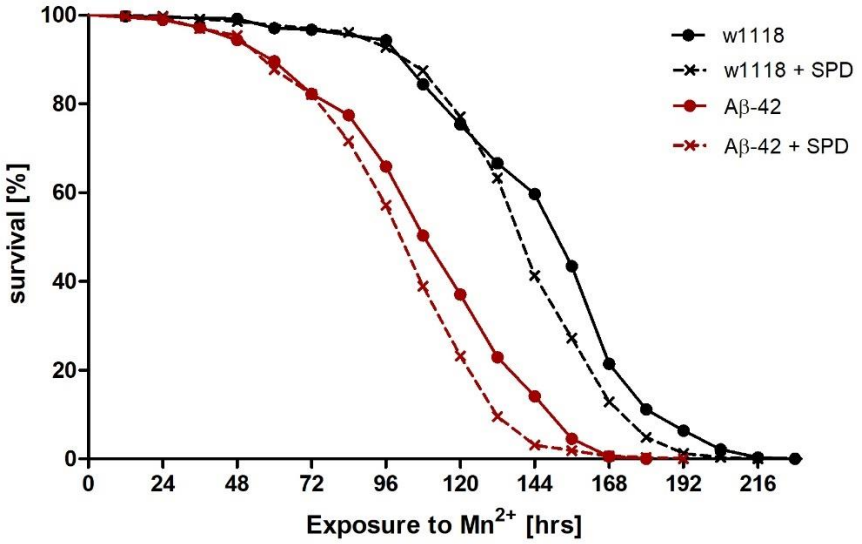

Figure 8: Spermidine administration shows no protective effect in manganese stressed flies representing an Alzheimer's disease model. 14 days-old female flies expressing Aβ42 and w^{1118} control flies are fed with a sucrose solution containing 10 mM $MnCl_2$ to induce metal stress and a comparable of flies is supplemented with additional 5 mM spermidine. Dead flies are counted in a 12 hours cycle and the survival is calculated relatively to the start of manganese exposure. The mean of 4 experiments, including approximately 500 flies per condition, is illustrated.

This data suggests that the toxicity of Aβ42 peptide, though merely present in neuronal cells, can be exacerbated by the presence of manganese ions and reduces the resistance during metal stress conditions. In order to further examine the toxicity of manganese in diverse AD *Drosophila melanogaster* models, a co-expression of Aβ42 and *Mipp1* was generated and spermidine supplementation was also investigated.

4.2. Co-expression of *Mipp1* in Aβ42 flies enhances toxicity

Since aging is a main risk factor for the development of Alzheimer's disease the prevalence of this neurodegenerative disorder lies in the elderly population at the age of 60 and above (Bekris, 2010). An age-dependent fall in autophagic processes as well as in the efficacy of the ubiquitin-proteasome system are major implications in normal aging as well as pathogenic conditions of neurodegenerative disorders. Cells are usually able to cope with moderate levels of misfolded or aggregate-prone proteins by removing them through different yet overlapping lysosomal and proteasomal

degradation pathways. These age-related risk factors can induce pathological conditions and cause an excessive, degradative process. The outcome is an aggregation of toxic Aβ42 peptides and hyper-phosphorylated neurofibrillary tau tangles accumulate in the brain which are caused by a failure in sequestering mechanisms that store intracellular soluble Aβ42 mono- and oligomers into their less deleterious bulky aggregates (Haass, 2007).

In order to examine not only the effects of spermidine on Aβ42 flies but also on a co-overexpression of the ubiquitously occurring *Mipp1*, which is assumed to play a role in the inositol thus, autophagic pathway, a survival assay has been performed.

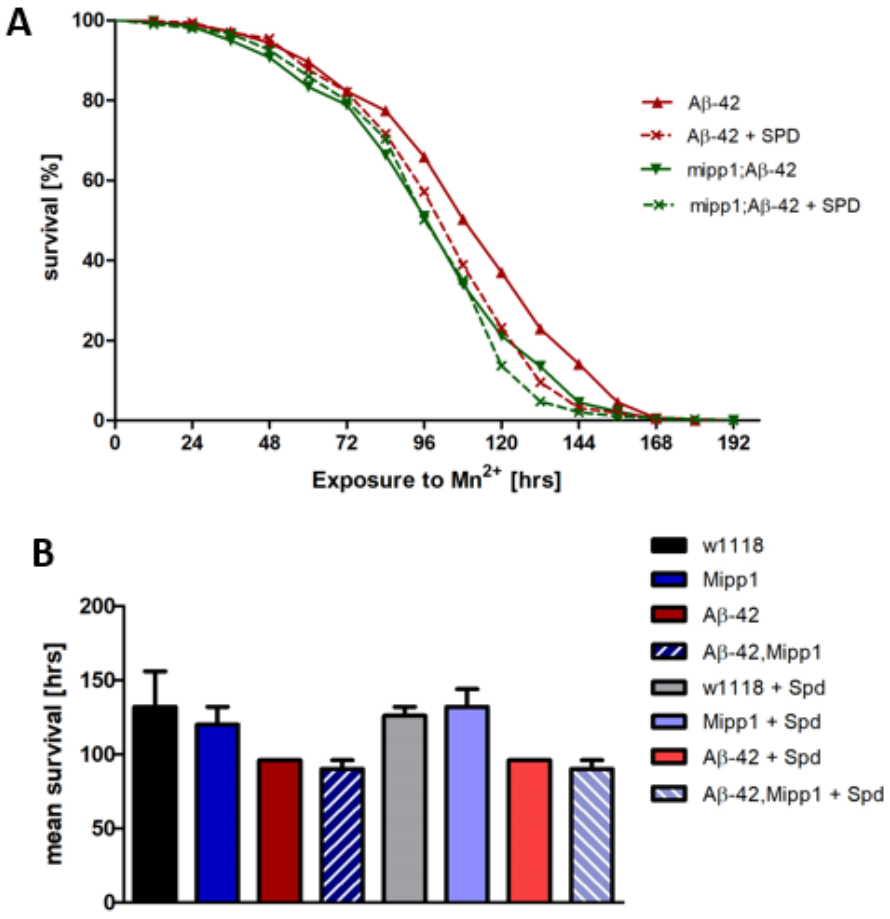

Figure 9: Co-expression of *Mipp1* in Aβ42 flies enhances toxicity in manganese stressed pre-aged flies representing an Alzheimer's disease model. 14 days-old female flies neuronally expressing Aβ42 or co-expressing Mipp1, Aβ42 are fed with a sucrose solution containing 10mM $MnCl_2$ as an induction of metal stress. A comparable set of flies is treated with a 5mM spermidine supplementation. Deceased flies are counted every 12 hours and the survival is calculated relatively to the start of manganese exposure (A). The same experimental set-up is performed with an additional fly population of *elavX-GAL4 x UAS-Mipp1*. The mean of 4 experiments including approximately 500 flies per group is illustrated with error bars representing SEM. 2-way ANOVA with Bonferroni correction is used (B).

The analysis of possible polyamine effects on an additional cross of *elavX*-GAL4 x UAS-*Mipp1*;Aβ42 using the same survival set-up showed an enhanced toxicity of manganese in flies carrying the co-expression compared to solely Aβ42 expressing flies (Fig. 9, A). Comparing the overall experimental set-up, a co-expression of *Mipp1*;Aβ42 showed a similar mean survival towards manganese stress induction as well as spermidine treatment. The same effect of mean survival between wild type flies and *Mipp1* overexpressing flies was observed (Fig. 9, B).

4.3. Decreased locomotive performance of co-expressing as well as Aβ42 flies compared to wild type *D. melanogaster*

Even though spermidine had only little or no protective effects on the general survival rate, it was examined for its effect on the drastic decline in locomotive performance of Aβ42 as well as Aβ42;*Mipp1* co-expressing flies. As Aβ42 is under the control of the *elav*^{C1115} promoter it is over-expressed in the brain region and thus, has an impact on motor neurons as well. Since neurons as non-regenerative cells strongly rely on various mechanisms sustaining homeo- and proteostasis, an age-dependent decline in these pathways is of vast noxiousness. As illustrated in figure 10, pre-aged Aβ42 expressing flies showed a strong reduction in climbing performance after 48 hours and 72 hours of manganese stress, similarly. A further decrease in locomotive performance was observed in flies co-expressing Aβ42 and *Mipp1*. However, external spermidine supplementation could not prevent a decrease of locomotor activity in this study population and thus, had no significant neuroprotective effect on Aβ42 expressing flies. Interestingly, an overexpression of *Mipp1* alone showed no decline in locomotive performance compared to wild type flies at either timepoint of the experiment.

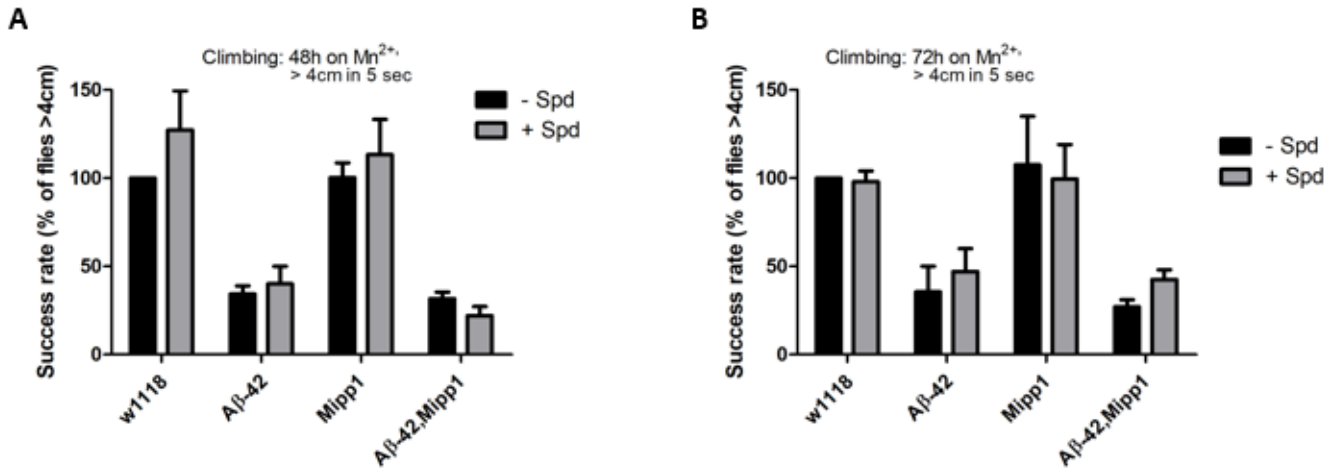

Figure 10: Decreased locomotive performance of Aβ42;*Mipp1* co-expressing as well as Aβ42 pre-aged flies representing an Alzheimer's disease model after manganese stress treatment. 14 days-old female flies neuronally expressing either *Aβ42* or *Mipp1* or co-expressing Aβ42*;Mipp1* are fed with a sucrose solution containing 10mM manganese for 48 hours (A) or 72 hours (B) in order to induce metal stress. A comparable set of flies is supplemented with 5mM spermidine. The climbing activity as the percentage of flies that can climb a height of 4 cm within 5 seconds. The mean of 3 experiments including approximately 240 flies per group (A) and the mean of 2 experiments including approximately 160 flies per group (B) is illustrated with error bars representing SEM. 2-way ANOVA with Bonferroni correction is used.

4.4. Spermidine treatment lowers cellular Mipp1 levels

In order to understand the role of a basal expression of *Mipp1* in AD flies, a western blot analysis of Aβ42 flies was performed, measuring endogenous Mipp1 protein levels compared to a possible change after spermidine treatment.

Earlier studies found that *Mipp1* is ubiquitously expressed in mammalian tissues which indicates the potentially important role of this protein. Also, its involvement in the inositol pathway suggests an interference in the autophagic pathways and therefore, a potential target for a polyamine such as spermidine. After performing a western blot analysis on head extracts collected from Aβ42 flies using a *Mipp1* antibody, a decrease of cellular Mipp1 levels could be observed after spermidine treatment (Fig. 11A). A repetition of this experiment did not show concur with the previous results however, it shows a slight tendency (Fig 11C), which makes further repetitions necessary.

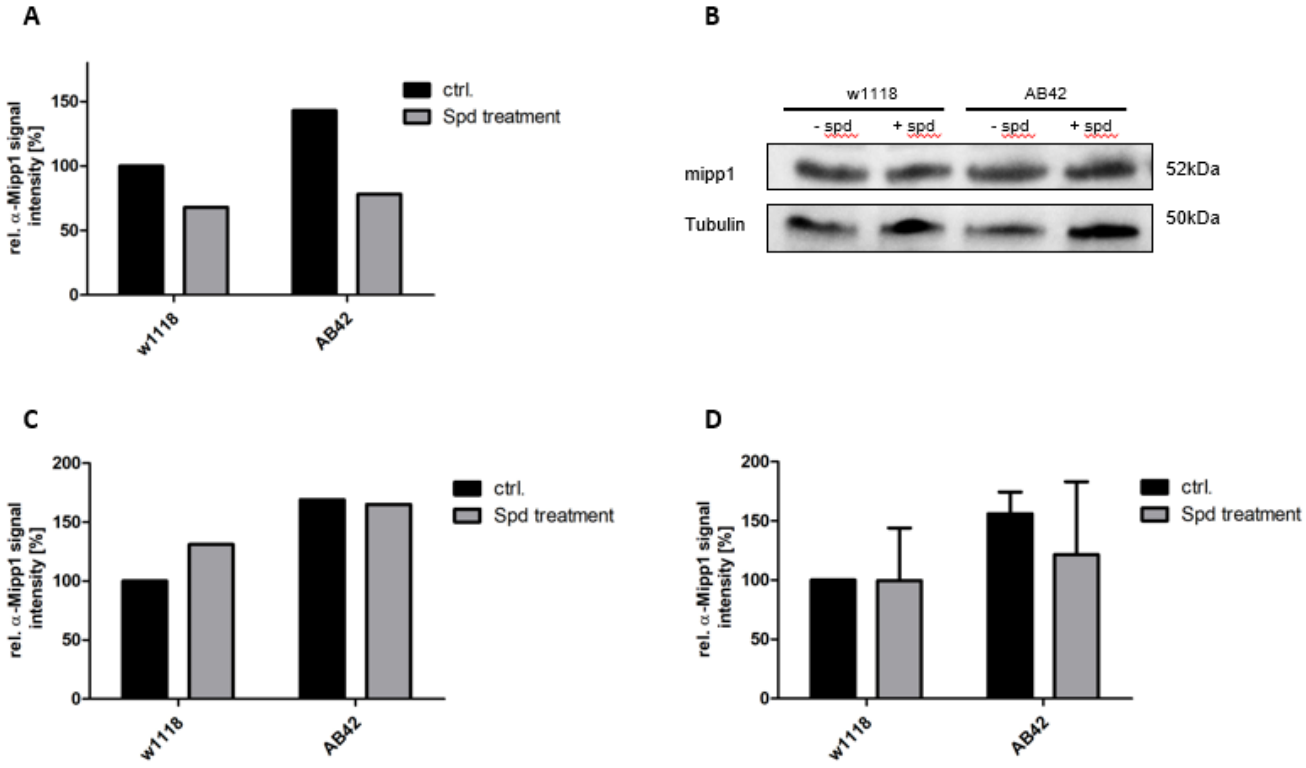

Figure 11: Spermidine treatment lowers cellular Mipp1 levels in Aβ42 expressing fruit flies. Head extracts from control (*elavX* x *w*^{1118}) as well as Aβ42 expressing flies were taken from pre-aged and Mn^{2+} stressed animals at 48 hours (A, C, D) of manganese exposure. Extracts were subjected to SDS PAGE and incubated with a secondary antibody goat α-guinea pig 1:5000 in 5% MP for *Mipp1.* A representative western blot for quantification using α-mouse-POD 1: in 5% MP for tubulin is shown in B.

After observing a tendency of reducing cellular Mipp1 levels after spermidine treatment and therefore, being aware of the possible link between Mipp1 and autophagy, we subsequently targeted the ability of spermidine to induce autophagy in Aβ42 flies.

4.5. Spermidine induces autophagy in Aβ42 overexpressing *Drosophila melanogaster* as a model for Alzheimer's disease

It is commonly accepted that spermidine increases the life span of different model organisms and it does so by inducing the autophagic pathway (Eisenberg T. K.-G., 2009). Assuming Mipp1 is involved in the inositol and therefore, possibly the autophagic pathway, we aimed at determining autophagic activity in spermidine-fed Aβ42 flies. Autophagy comprises the engulfment of cellular waste by a double-membraned vacuole, the subsequent transport to the lysosome and the final

degradation of its cargo by the emerging autophagolysosome (Klionsky, 2013). A huge amount of proteins are involved in the manifold steps, with Ref(2)p being one amongst those that regulate the initial steps towards autophagy. Ref(2)p is the *Drosophila melanogaster* homolog of human p62 and can be considered as an autophagic receptor targeting ubiquitinated cargo to the newly forming phagophore (Liu, 2016). After the induction of autophagy commenced, cellular levels of free p62 decrease due to the successful engulfment and further degradation by the autophagosome. Therefore, we tested the relative protein amounts of Ref(2)p from 14 days-old flies exposed to either 10mM manganese or manganese plus 5mM of spermidine. Figure 12 illustrates a reduced level of Ref(2)p/p62 upon supplementation with spermidine in the *elav*^{C155} X Aβ42 cross (Aβ42) compared to the untreated Aβ42 control which shows a 100% increase of p62 build-up, consequently hinting at an impaired autophagic machinery. Spermidine treatment on wild type flies however, showed no change in Ref(2)p/p62 levels, whereas the relative decrease of p62 is prominent (100%) in Aβ42 flies.

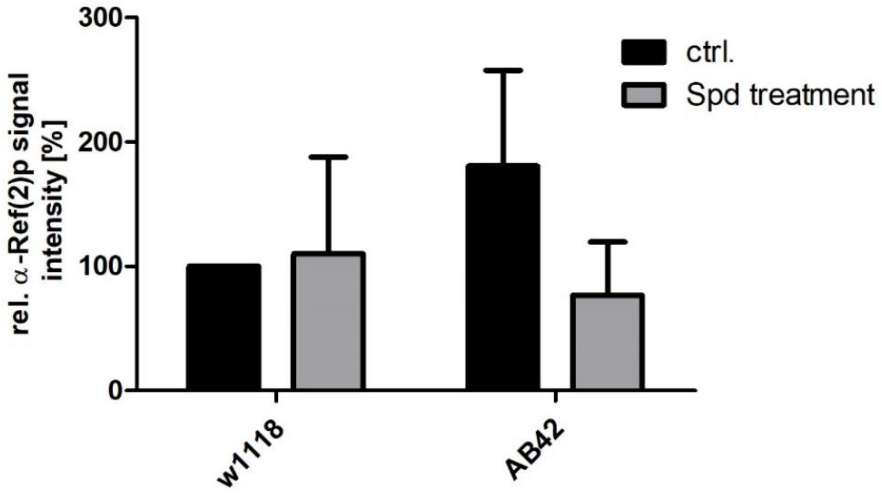

Figure 12: Spermidine induces autophagy in the brain of Aβ42 overexpressing flies. Head extracts from control (*elav*^{C1115} X *w*^{1118}) as well as Aβ42 expressing flies were taken from pre-aged and manganese stressed animals at 48 hours of Mn^{2+} exposure. Extracts were subjected to SDS PAGE and incubated with α-Ref(2)P. As demonstrated by quantification, spermidine increases autophagy and thus reduces Ref(2)p (p62).

Following the analysis of autophagy induction on Aβ42 flies after spermidine supplementation, we decided to investigate the effects of spermidine supplementation on *Mipp1* and Aβ42;*Mipp1* co-expressing fruit flies in order to find a possible connection between *Mipp1* and autophagy.

4.6. Autophagy is not induced after spermidine supplementation in *Mipp1* and Aβ42;*Mipp1* co-expressing fruit flies

As mentioned before, the presence of autophagic ubiquitin-dependent receptors and additional adaptor proteins raise the assumption of a linkage between the pathways of autophagy and proteasomal degradation. These receptors include the previously described p62 as an autophagic marker (among other proteins) which recognizes ubiquitinated proteins and tether them to the core autophagic machinery in which the microtubule-associated protein light chain 3 (LC3) acts as an acceptor. In order to measure the autophagic outcome, we decided to perform a western blot analysis of *Mipp1* and Aβ42;*Mipp1* co-expressing fruit fly head extracts using both markers, namely Ref(2)p and additionally, LC3.

LC3 is used to measure the overall autophagic turnover. Given the low signal levels of LC3 in *Mipp1* and Aβ42;*Mipp1* co-expressing flies (as seen in figure 13) compared to wild type *Drosophila melanogaster*, both over-expressing fly populations show a similarly decreased level of basal autophagy. Adding a 5mM spermidine supplementation for a comparable set of flies did not induce autophagy in this experimental set-up.

Measuring Ref(2)p signal levels in those fly populations showed a similar result (Fig. 14A). While autophagy is induced in Aβ42 after a 5mM spermidine treatment, the induction of autophagy within *Mipp1* as well as Aβ42;*Mipp1* flies is abolished. Since spermidine is able to induce autophagy in Aβ42 flies but fails to induce this pathway once *Mipp1* is co-expressed or expressed alone, it seems that an over-expression of *Mipp1* other than its basal level, inhibits autophagy and therefore minimizes the protective effects of spermidine in fruit flies.

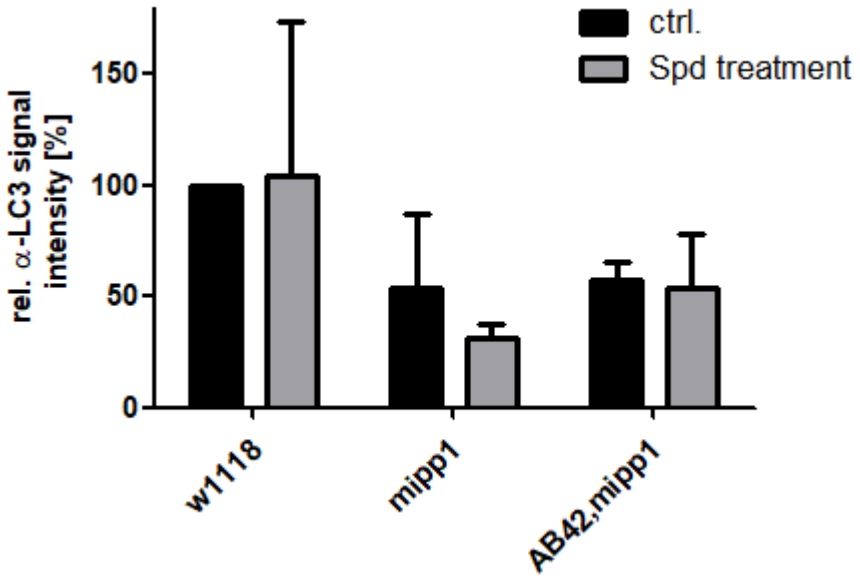

Figure 13: Spermidine does not induce autophagy in Mipp1 and Aβ42;Mipp1 co-expressing fruit flies. Head extracts from control (*elav*^{C1115} X *w*^{1118}), *Mipp1* overexpressing flies (UAS-*Mipp1*) and Aβ42,*Mipp1* co-expressing fruit flies were taken from 14 days pre-aged and manganese stressed animals at 48 hours of Mn^{2+} exposure. Extracts were subjected to SDS PAGE and incubated with α-GABARAP/LC3 1:500. As demonstrated by quantification, spermidine decreases LC3 levels in Mipp1 overexpressed as well as Aβ42;Mipp1 co-expressed fruit flies and therefore, decreases autophagy in both populations compared to wild type flies. The mean of 2 experiments including approximately 180 flies per group is illustrated with error bars representing SEM. 2-way ANOVA with Bonferroni correction is used.

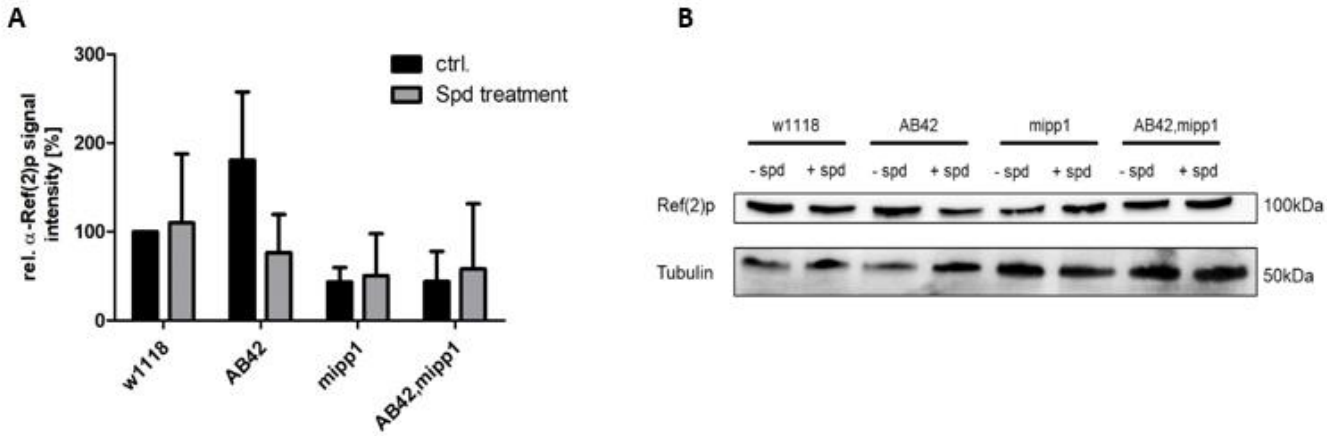

Figure 14: Spermidine does not induce autophagy in *Mipp1* and Aβ42;*Mipp1* co-expressing fruit flies. Head extracts from control (*elav*^{C1115} X *w*^{1118}), Aβ42 expressing flies (UAS-Aβ42), *Mipp1* overexpressing flies (UAS-*Mipp1*) and Aβ42;Mipp1 co-expressing fruit flies were taken from pre-aged and manganese stressed animals at 48 hours of Mn^{2+} exposure. Extracts were subjected to SDS PAGE and incubated with α-Ref(2)P. As demonstrated by quantification, spermidine does not decrease Ref(2)p (p62) and thus, does not increase autophagy in *Mipp1* and Aβ42,*Mipp1* (A). The mean of 2 experiments including approximately 180 flies per group is illustrated with error bars representing SEM. 2-way ANOVA with Bonferroni correction is used. A representative western blot for quantification using α-mouse-POD 1: in 5% MP for tubulin is shown in (B).

5. Discussion

Aging is an unstoppable process one cannot escape. However, over the last decade immense research has been taking place to minimize age-related demise and attenuate late-onset disorders and ease the burden of aging. Very common diseases that come with age are neurodegenerative diseases like Alzheimer's disease. It gradually impairs one's locomotive activity, memory and other neuronal functions. Research mainly focused on two possible targets which were thought to be causative for AD: intracellular tau tangles and extracellular amyloid plaques (Arbor, 2016). However, pharmacological novelties made attempts on degrading the Aβ42 plaques enzymatically to inhibit a fast progress of the disease with a lot of research maintaining to be done. Another approach was to tackle neuronal symptoms, which failed to improve patient's cognitive abilities at late stages of the disease and moreover, had various side effects (Allgaier, 2014). Promising studies focused on alterations of the brain's metabolism such as higher spermidine levels in the temporal cortex and low levels in the frontal cortex of Alzheimer's disease patients (Morrison, 1995). These early finding showed an involvement of polyamines such as spermidine in neurodegenerative disease like AD and opened doors for further research on spermidine and its effects on various diseases. Various groups were able to show that external spermidine supplementation through culture media has positive effects concerning longevity as well on the overall health span (Madeo F, 2018) (Madeo & Eisenberg, 2018). Low chances of recovery after age-related memory loss were observed in old flies which were fed with spermidine while being Atg7-deficient which indicates the importance of autophagy mediated by spermidine (Gupta, 2013) (Eisenberg T. K.-G., 2009). . The ability of spermidine to cross the mammalian blood-brain barrier remains to be investigated despite its neuroprotective effects on various model organisms which makes further research crucial. Polyamines in general, have shown a beneficial effect in AD pathology. Initially, spermidine was demonstrated to drive Aβ peptide fibrillation and aggregation, which led to the assumption of a neurotoxic induction (Luo J, 2013). However, briefly after those findings were published, the same research group reported that spermidine mediated fibrillation led to a decreased hydrophobic surface of the peptide and therefore, making it less toxic (Luo J., 2014). This concurs with earlier findings that support the hypothesis of small

hydrophobic oligomers being the more toxic kind of Aβ, whereas a protective disposal structure was formed by bigger aggregates (Haass, 2007).

The effect of spermidine and the role of possible modulators such as Mipp1 on an Alzheimer's disease model was investigated in this master thesis. Expression of Aβ42 peptide, which recapitulates certain aspects of AD pathology, is sufficient to increase the flies' susceptibility to manganese induced metal stress – a bivalent cation that is implicated in neurodegenerative diseases. However, the protective effect of external spermidine supplementation on those flies and a possible mitigation of manganese toxicity by spermidine could not be shown in this experiment on either fly population. Since these results do not match previously performed experiments with spermidine providing a significant protective effect on Aβ42 flies the experimental set-up has to be adjusted in further experiments.

The analysis of possible polyamine effects on an additional cross of *elavX*-GAL4 x UAS-*Mipp1*;Aβ42 using the same survival set-up as well as measuring locomotive performance, showed an enhanced toxicity of manganese in flies carrying the co-expression compared to solely Aβ42 expressing flies and a decreased locomotor activity. Assumedly, the co-expression of Mipp1 in already impaired Aβ42 flies potentiates cellular stress conditions which diminishes the protective effect of spermidine supplementation due to ER stress induction (Kilaparty, 2016). Increased levels of Mipp1 catalyze the conversion of IP6 to IP3 which subsequently leads to an accumulation of IP3 and thus, possibly inhibits autophagy. (Sarkar & Floto, 2005). Yet, the elevated levels of IP3 have to be confirmed via mass spectrometry. After performing a western blot analysis on head extracts collected from Aβ42 flies, a decrease of cellular Mipp1 levels could be observed after spermidine treatment which could be a possible target to counteract the accumulation of IP3 in AD flies.

An induction of autophagy was highlighted by immunoblot assays, that showed a reduction of the autophagy receptor Ref(2)p in AD representing flies after spermidine treatment. Ref(2)p represents the fly homolog of human p62, an important recognition factor of ubiquitinated proteins which are labelled for autophagic degradation (Lippai, 2014). Furthermore, protein levels are decreased in the AD disease model upon spermidine feeding which indicates an increase of autophagic activity. This hints at a spermidine-mediated induction of autophagy in AD flies. The co-expression of Mipp1 in Aβ42 flies resulted in a low signal of LC3 in a western blot assay as well as elevated levels of Ref(2)p,

representing a deactivated autophagic machinery.

Taken all findings together we showed that external spermidine supplementation has in fact a protective effect on AD flies in some cases through the decrease of Mipp1 as well as an induction of autophagy. However, the conduction of functional tests such as the survival assay as well as the climbing performance did not concur with the findings on a protein level. In order to get more accurate results, a set-up adaptation of functional tests concerning the application of spermidine (concentration gradient) or the choice of different timepoints for the observation of flies is necessary. Also, different fly strains and fly gender could be used to repeat the experiments.

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7. Abbreviations

Aβ	amyloid-β
AD	Alzheimer's Disease
APP	β-amyloid precursor protein
APPL	amyloid precursor protein like
BSA	bovine serum albumin
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
C. elegans	*Caenorhabditis elegans*
CMA	chaperone-mediated autophagy
CNS	central nervous system
Cvt	Cytoplasm to Vacuole Targeting
D. melanogaster	*Drosophila melanogaster*
ECL	enhanced chemiluminescence
ER	endoplasmic reticulum
Hsp70	heat shock protein 70
IP6	Inositol-6-phosphate
IP3	Inositol-3-phosphate
kDa	kilo Dalton
LC3	light chain 3
MINPP1	mammalian 3-phytase
Mipp1	multiple inositol polyphosphate phosphatase 1

MP	milk powder
mTOR	mammalian target of rapamycin
NMDA	N-methyl-D-aspartate
PAS	pre-autophagosomal structure
PMSF	Phenylmethylsulphonyl fluoride
S. cerevisiae	*Saccharomyces cerevisiae*
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Spd	spermidine
TBS	Tris-buffered saline
TST	Tris saline Tween20
TF	transcription factor
UAS	upstream activating sequence
WHO	World Health Organization

8. Supplementals

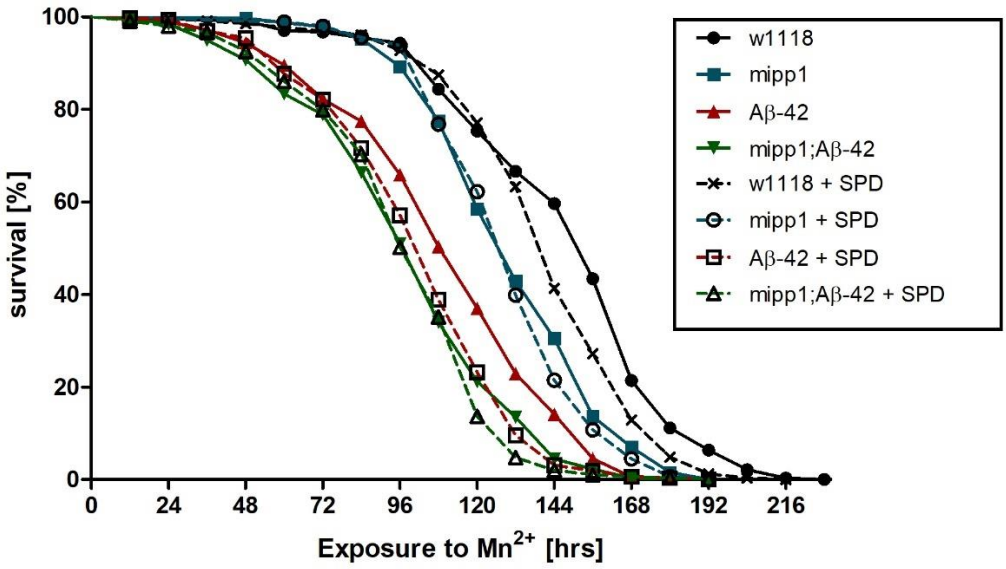

Figure 15: Spermidine administration shows no protective effect in manganese stressed flies representing an Alzheimer's disease model. 14 days-old female flies expressing Aβ42 and w^{1118} control flies as well as Mipp1 expressing and Mipp1,Aβ42 co-expressing flies are fed with a sucrose solution containing 10 mM $MnCl_2$ to induce metal stress and a comparable of flies is supplemented with additional 5 mM spermidine. Dead flies are counted in a 12 hours cycle and the survival is calculated relatively to the start of manganese exposure. The mean of 4 experiments, including approximately 500 flies per condition, is illustrated.

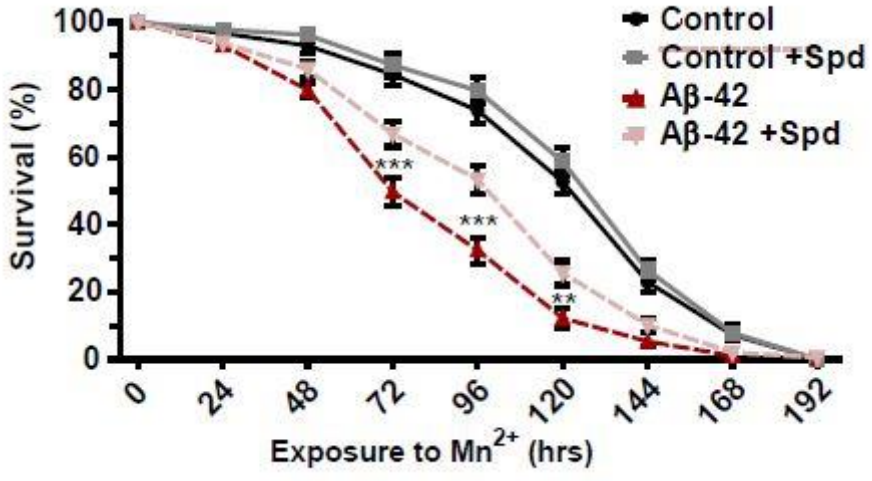

Figure 16: Spermidine administration exerts protective effects in manganese stressed pre-aged flies representing an Alzheimer's disease model. 14-16- days-old female flies pan-neuronally expressing Aβ42 are fed with a sucrose solution containing 20 mM MnCl2 to induce metal stress. A comparable set of flies is supplemented with additional 5 mM spermidine. Dead flies are counted every 12 hours and the survival is calculated relatively to the start of manganese exposure

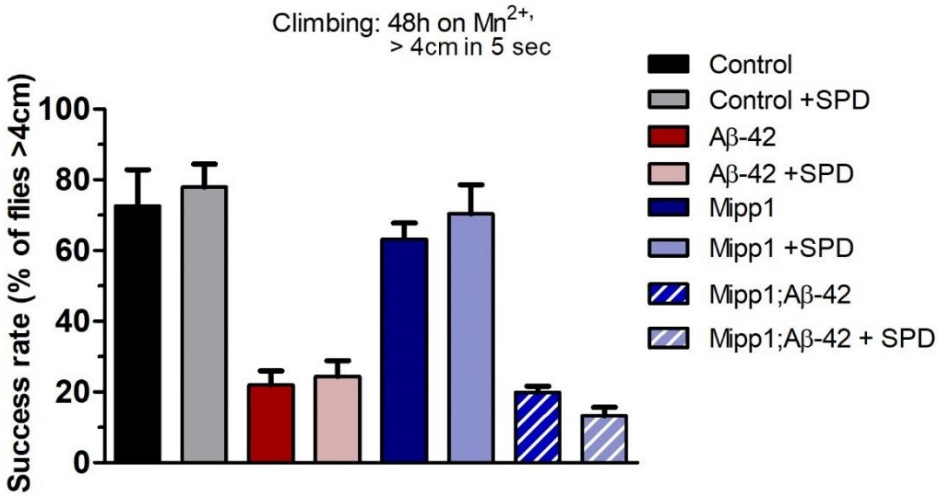

Figure 17: Further decreased locomotive performance of Aβ42, *Mipp1* co-expressing pre-aged flies representing an Alzheimer's disease model after manganese stress treatment. Raw data of 14 days-old female flies neuronally expressing either *Aβ42* or *Mipp1* or co-expressing Aβ42*, Mipp1* are fed with a sucrose solution containing 10mM manganese solution for 48 hours in order to induce metal stress. A comparable set of flies is supplemented with 5mM spermidine. The climbing activity as the percentage of flies that can climb a height of 4 cm within 5 seconds. The mean of 3 experiments including approximately 240 flies per group is illustrated with error bars representing SEM. 2-way ANOVA with Bonferroni correction is used.

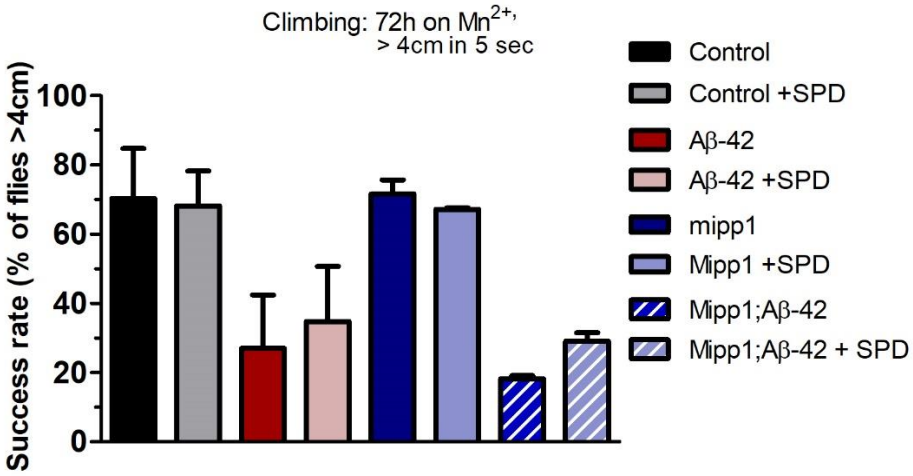

Figure 18: Further decreased locomotive performance of Aβ42, *Mipp1* co-expressing pre-aged flies representing an Alzheimer's disease model after manganese stress treatment. Raw data of 14 days-old female flies neuronally expressing either *Aβ42* or *Mipp1* or co-expressing Aβ42*, Mipp1* are fed with a sucrose solution containing 10mM manganese solution for 72 hours in order to induce metal stress. A comparable set of flies is supplemented with 5mM spermidine. The climbing activity as the percentage of flies that can climb a height of 4 cm within 5 seconds. The mean of 2 experiments including approximately 160 flies per group is illustrated with error bars representing SEM. 2-way ANOVA with Bonferroni correction is used.

9. Table of figures