Spermidine – a novel protector against neurodegeneration in *Drosophila melanogaster*

Insights into Parkinson's and Alzheimer's disease from the fruitfly model

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Content

List of abbreviations

Aβ	amyloid-beta protein
Aβ42	amyloid-beta protein – truncated: amino acids 1-42
AD	Alzheimer's disease
α-syn	alpha-synuclein
ATG	autophagy-related genes
CNS	central nervous system
Mn^{2+}	manganese hydrochloride
ODC1	Ornithine-Decarboxylase 1
PD	Parkinson's disease
spd	spermidine

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The sky is the limit...

Abstract

Parkinson's and Alzheimer's disease are amongst the neurodegenerative diseases that have the highest incidence of cases in people above the age of 60 As one mechanistic hallmark, recent data suggest that the cellular self-clearance process autophagy might be impaired in both diseases. Previous studies in laboratory models have shown that the natural substance spermidine is able to induce the cellular mechanism of autophagy. Same time, recent studies identified that this natural substance Spermidine is able to induce the cellular mechanism of autophagy in several organisms, including yeast, fruit flies (Drosophila) and mice. It could be proven that the uptake of spermidine leads to a prolongation of the lifespan of these animals and ameliorates the symptoms caused by Parkinson's and Alzheimer's disease. Moreover, Spermidine feeding recently was shown to protect Drosophila from age-induced memory impairment. Thus, we wanted to get an insight into an eventual therapeutic approach using well established stressors to monitor the resulting disruption of cellular pathways and the symptom progression in this model organism. In our fly experiments we could show analogous effects of symptom amelioration in the fruit fly through different approaches. Hence, our hypothesis consisted of the presumption that administration of spermidine would enhance autophagy and thus improve the performance of the flies, including delay of organismal death.

To test these assumptions, we first determined the survival rates of the animals that were expressing the main proteins of the illnesses Aβ-42 (for Alzheimer's) and α-synuclein (for Parkinson's) in a stress setup with and without administration of spermidine. Here, animals were stressed with manganese, which is known to induce neurodegeneration, and the animals that were fed with spermidine showed a better survival rate over the period of the experiment than the ones without spermidine. In a second step, we asked for protective effects concerning manganese -induced impairments of locomotor ability of the animals from the aforementioned stress setup, because one of the pathological hallmarks in patients suffering from Parkinson's disease is locomotion difficulties. Here, we could also find that spermidine acts as a substance that improves the existing locomotor dysfunction and reduces the strong decline in the climbing ability over time. Mechanistically, we observed reduced amounts of both Aβ-42 (for Alzheimer's) and α-synuclein in the brain of flies treated with Spermidine, in both immunofluorescence stainings followed by whole mount confocal brain scanning as as well as by western blot analysis of fly head extracts. Animals that were born on spermidine containing food had a similar reduction in protein levels than the ones that were given

spermidine acutely under manganese stress. Flies that did not get spermidine at any timepoint showed the highest staining levels (with both methods). We also tested the effect of spermidine on the lifespan of *D. melanogaster*, since Alzheimer's patients have a dramatically reduced life expectancy. Results, however, were controversial. On the one hand, spermidine seemed to help the flies in early stages but it also seemed to kill the animals the older they became.

These results might be a first step into a development of a natural treatment for these serious illnesses and need to be proceeded. We think that we have a great chance of helping patients to live a better life with these results, assuming that this substance will be approved as a food supplement in future times.

Deutsche Zusammenfassung

Die zwei neurodegenerativen Erkrankungen Parkinson und Alzheimer treten weltweit besonders hervor, da sie die höchsten Fallzahlen bei Menschen über 60 Jahren haben. Eine nicht mehr funktionstüchtige Autophagie-Maschinerie in der Zelle wird mit zu den Verursachern der Krankheiten gezählt. Vorhergegangene Studien haben gezeigt, dass ein natürliches Polyamin namens Spermidin in der Lage ist, die Autophagie in den Zellen zu induzieren. Vierschiede einfache eukaryotische Modelle, wie Hefe, Würmer und die Fruchtfliege wurden verwendet, um die grundlegenden Mechanismen von Neurodegeneration zu erforschen. Es konnte gezeigt werden, dass die Aufnahme von Spermidin zu einer Verlängerung der Lebensspanne und zu einer Linderung der Symptome von Alzheimer und Parkinson in diesen Tieren führt. Um einen möglichen therapeutischen Ansatz zu entwickeln, haben wir bereits etablierte Stressoren verwendet, um ihre Auswirkungen auf den Modellorganismus unter verschiedenen Aspekten zu untersuchen. In unseren Versuchen mit Fliegen, konnten wir analoge Effekte der Symptombesserung feststellen. Unsere Hypothese besteht deshalb aus der Annahme, dass eine Aufnahme von Spermidin zur Induktion von Autophagie und dadurch zu einer Verbesserung der Performance der behandelten Fliegen führt.

Um diese Vermutungen zu testen, haben wir die transgenen Fliegen, welche die Hauptproteine der Erkrankungen (α-synuclein (Parkinson) und Amyloid-β-42 (Alzheimer)) exprimierten, auf durch Spermidingabe verbessertes Überleben unter Mangan-Stress, was Neurodegeneration auslöst, untersucht. Hierbei wurde die Rate der überlebenden Fliegen über einen bestimmten Zeitraum gemessen und es konnte festgestellt werden, dass Mangan-gestresste Fliegen, die Spermidin über das Futter aufnahmen, eine signifikant verbesserte Überlebensrate aufwiesen als die Gruppe, die kein Spermidin erhielt. Weiters waren wir interessiert daran, die Auswirkungen von Spermidin auf die Bewegungsfähigkeit der Tiere zu untersuchen; da Parkinson durch einen Verlust der Neuronen im Bewegungszentrum der Gehirns gekennzeichnet ist und bei Alzheimer in späteren Stadien ebenso motorische Defizite bemerkbar sind. Hierbei konnten wir, durch Climbing Assays unter Mangan-Stress und Spermidingabe, eine signifikante Verbesserung dieser Defizite erkennen. Ebenso konnten wir auf zellulärer Ebene, eine Reduktion der Proteinmenge in den betreffenden Gehirnarealen der Fruchtfliegen feststellen. Hierzu wurden ebenso Fliegen unter Mangan-Stress mit und ohne Spermidin getestet und die Ergebnisse mittels konfokaler Mikroskopie und Western Blot Analyse bestätigt. Tiere die auf mit Spermidin versetztem Futter geboren wurden, wiesen eine

ähnlich starke Proteinreduktion auf, wie Tiere, die im Mangan-Stress mit Spermidin in Kontakt kamen. Fliegen, die keinen Zugang zu Spermidin hatten, wiesen die höchsten Level sowohl in der Mikroskopie, als auch im Western Blot auf. Wir waren auch an der Verlängerung der mittleren Lebensspanne der Tiere interessiert, da Patienten mit Alzheimer eine deutlich verkürzte Lebenserwartung haben. Hierbei konnten wir jedoch gegensätzliche Ergebnisse erhalten, welche in den frühen Stadien einen Vorteil in der mit Spermidin gefütterten Gruppe, im späteren Verlauf jedoch einen Überlebensnachteil zeigten. Spermidin scheint in jungen Fliegen eher positiv zu wirken, je älter die Tiere werden, desto toxischer scheint die Substanz zu sein.

Diese Erkenntnisse können in Zukunft wichtige Schritte hin zur Entwicklung neuer Therapien bzw. zur Testung von Spermidin in anderen Modellorganismen sein. Durch die bis heute erhaltenen Ergebnisse, hoffen wir, dass Spermidin in Zukunft als Nahrungsergänzungsmittel zugelassen wird und somit viele Patienten ein längeres und gesünderes Leben, trotz ihrer Erkrankung, genießen können.

1. Introduction

1.1 Neurodegenerative diseases

Neurodegenerative diseases (from greek νέυρο-, *néuro-*, „nerve(s)-“ and latin *dēgenerāre*, „decay”, “degenerate“) have become one of the most widespread causes of senile dementia in modern society. With the dramatic increase in life expectancy in the last decades more and more people reach the age where neurodegenerative diseases are common and so we face a dramatically increased need for therapies and novel treatments. Affected patients face different stages of the illness, every stage worsening the symptoms leading to a huge increase in home-nursery and elevated funding from the health-care systems worldwide.

The mentioned progressive disorders are characterized by age-dependent impairment of several cognitive and locomotional functions in humans, including memory impairment accompanied by morphological changes in distinct regions of the human brain. The affected regions mostly show selective death of distinct populations of neurons depending on the pathological conditions. These brain lesions are characterized by extracellular proteins that form so-called senile plaques and neurofibrillary tangles (in the case of Alzheimer’s disease) or dense aggregates called Lewy bodies (in Parkinson’s disease).

As scientist got to know more and more about these illnesses, a need for classifications emerged. In 1998, Hardy et al. came up with the first arrangement of these diseases grouping them in two distinct clusters, namely the Polyglutamine Diseases, Tauopathies and Synucleinopathies based on their molecular origin. Polyglutamine diseases, like Huntington’s disease and Friedreich’s ataxia have a pathological DNA-stretch of triplet repeats that code for the amino acid glutamine which disturbs the physiologic function of the protein as a characteristic feature. Proteins with this stretch are more prone to aggregate and to form intranuclear inclusions which are connected to neuronal malfunction[1].

In this classification Alzheimer‘s and Parkinson’s disease (AD and PD) were integrated into the group of Tauopathies and Synucleinopathies, as both of them are characterized by extracellular tangles and inclusion bodies, consisting of either amyloid-ß and/or tau or α-synuclein[2].

Also Creutzfeld-Jacob's disease, Frontotemporal dementia, Amyotrophic lateral sclerosis, Pick's disease and many other illnesses are counted to the neurodegenerative diseases[3] but these will not be mentioned any further in this master thesis.

The factors that can cause a neurodegenerative disease to manifest are variegated and can often not be narrowed to one single condition. Reaching from simple overexpression of a protein or mutation(s) in a single protein and altered physiological properties to inhibition or induction of the cellular self-clearance machinery called autophagy and apoptosis[4], even chemicals can play an important role. Some of the cases even show a higher familial occurrence indicating genetic risk factors, but often scientist do not even know if the disease was inherited or reveals a rare sporadic condition[5]. Since the first description of these pathologic health conditions, researchers have developed several therapies to treat the mental and physiologic symptoms, as the illnesses per se are non-curable.

In the following parts of the introduction I want to present some more information about the topics that are treated in this thesis, especially about the diseases mentioned throughout the work and the underlying hypothesis that form the basis of all the experiments described.

1.1.1 Parkinson's disease

Parkinson's disease (PD), first described as "An essay on the shaking palsy" by James Parkinson in 1817[6], is estimated to affect more than 4 million people in Europe's and the world's most densely populated countries in 2005, with this number estimated to double by 2030[7], making it to the second place of the most common neurodegenerative diseases after Alzheimer's disease (AD). This fact leads to an increased need for patient's care and medications. Clinical symptoms include Bradykinesia (slowness of motion), resting tremor, rigidity and postural instability and deformity and a high percentage of patients also suffer from cognitive disturbances[8]. There are several forms of the disease that need differential diagnosis like idiopathic Parkinsonism, vascular Parkinsonism, Parkinson-plus syndromes and drug-induced Parkinsonism that all show the same class of symptoms. Also essential tremor and normal ageing are part of this differential diagnosis of PD[9].

Now, after decades of nescience about the substantial causes of PD, it is stated that it is predominantly caused by formation of filamentous intraneuronal inclusions consisting of protein aggregates in the so-called Lewy-bodies and the dystrophic neurites (Lewy neurites) followed by the consecutive loss of dopaminergic neurons by induction of apoptosis of the

affected neuronal cells in the substantia nigra of the human brain[10]. In this process, more than 70 % of dopaminergic neurons can be lost before first symptoms of PD can be clinically estimated[11].

These inclusions are immunopositive for the protein α-synuclein (α-syn) as one of the major components in the aggregated protein clusters. The protein is encoded by the SNCA-locus on the long arm of chromosome 4, 4q21-q22 region of the human genome (NCBI Entrez gene 6622) leading to a translated protein of 140 amino acids (monomer) in size which occurs naturally unfolded within the cell. Also the oligomer-form containing alpha-helices occurs naturally and prior to neurologic symptoms and in affected, as well as unaffected regions of the brain[12]. α-syn is normally localized at the presynapse[13] around synaptic vesicles in the presynaptic terminal of dopaminergic neurons where it is thought to be involved in the refilling and trafficking of synaptic vesicles from the reserve pool to the readily releasable pool at the site of vesicle release[14,15]. Upon neuronal activity or environmental stress conditions, the above mentioned monomers aberrantly aggregate into β-sheet like structures and further on, to insoluble fibrils which form the Lewy-bodies[16].

In 1997, Polymeropoulos and colleagues identified a point mutation in the SNCA-locus, leading to an exchange of the amino acid alanine to threonine at position 53. This single mutation is responsible for a dominant form of PD causing a stronger aggregation phenotype and can additionally be dominantly inherited within a family. In their studies, the group could reveal a penetrance of the gene (the proportion of people with the genotype who actually show signs of the disease) of 85 %, suggesting that this single defect is sufficient for the PD-phenotype to occur[17].

Also other factors can lead to an abnormal production of α-syn in the cell, namely there is the possibility of a duplication[18] or triplication[19] of the SNCA-locus itself. These multiplications of the locus are causal for the development of PD.

Normally the expression of α-syn is very tightly regulated in the physiologic conditions in the cell. But if these control mechanisms fail, because of epigenetic and transcriptional modifications[20], the protein is thought to be more likely to be expressed in a higher amount if there are additional promoter modifications.

In PD many factors act together that can cause the illness to become manifest, including ageing, genetic predisposition and environmental influences. These elements can rarely be avoided and are therefore crucial to the recent development of increased incidence in the aging population of man countries.

1.1.2 Alzheimer's disease

As the german neuropathologist Alois Alzheimer met the first patient suffering from the "Disease of Forgetfulness" in 1911, he witnessed "strange behavioral symptoms" including unconsciousness of time and place and a loss of short-term memory. Alzheimer also described the physiological changes in the brain of these patients using staining techniques to identify the core component of observed pathological changes in the brain structure of these patients[21]. These observations were scientifically proved in 1985, where the morphological hallmarks of the illness, neurofibrillar tangles and senile plaques, were authenticated and could be shown to consist of an amyloid beta protein (Aβ) that forms these protein aggregates[22].

The gene coding for this protein - APP (NCBI Entrez gene 351) - is located on the human chromosome 21 (location:21q21.3) and is the precursor of the human Aβ, which encodes a

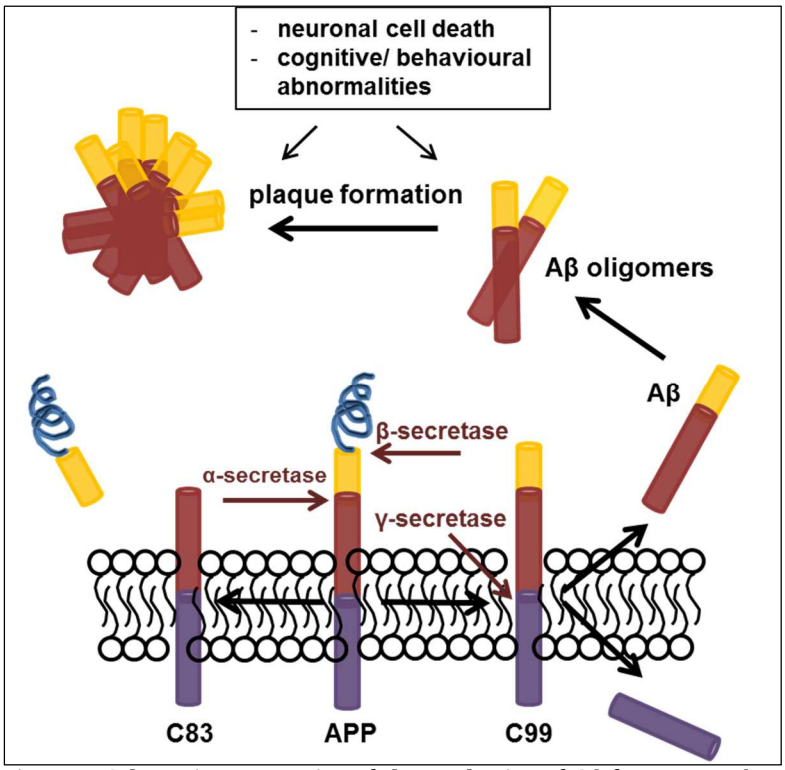

Figure 1: Schematic presentation of the production of Aβ fragments. The precursor protein APP is translated and then incorporated in the cell membrane. Then, α- and β-secretases cleave the first fragment whose function will not be explained further. The γ-secretase is the one that generates the Aβ-fragment that is physiologically relevant to this work. If this γ-secretase is mutated, it forms fragments of Aβ that are more prone to aggregate and form harmful oligomers which can then accumulate to plaques. Picture courtesy of MSc. Cornelia Sommer, Institute of Molecular Biosciences, University of Graz Humboldtstraße 50, 8010 Graz, Austria.

cell surface receptor and gets cleaved by secretases to form a high number of different peptides (see Figure 1). If the Aβ protein gets cleaved by mutated γ-secretase (regulators PSEN1 and PSEN2), it results in a higher amount of the $A\beta_{42}$ fragment which then aggregates and forms the typical plaques in the affected patient's brain[23].

APP has several homologue genes in organism including *D. melangoster, C. elegans* and all mammals[24]. Alzheimer's disease (AD) is grouped into the pathologies of Tauopathies, as the observed neurofibrillary tangles mainly contain, additionally to the Aβ-protein, the small protein tau, that is normally responsible for axon formation and stabilization[25], but was found to form helical and/or straight bundles of abnormally hyperphosphorylated tau protein in the case of AD[26].

Later, it was discovered that there are several forms of AD that are caused by different genetic factors. In 1997, Dennis Selkoe summarized the most important genetic loci that cause AD in different patient age groups[27]. He stated that the early onset form of the disease is caused by a gene called Presenilin 1 (PSEN1) in chromosome 14 which causes a higher production or deposition of the Aβ-protein already in the late fourth and fifth decade of the patient's life. Later onset forms of AD include mutations in the Presenilin 2 (PSEN2) locus on chromosome 1 and in the βAPP locus on chromosome 21 that lead to the same increased production of Aβ.

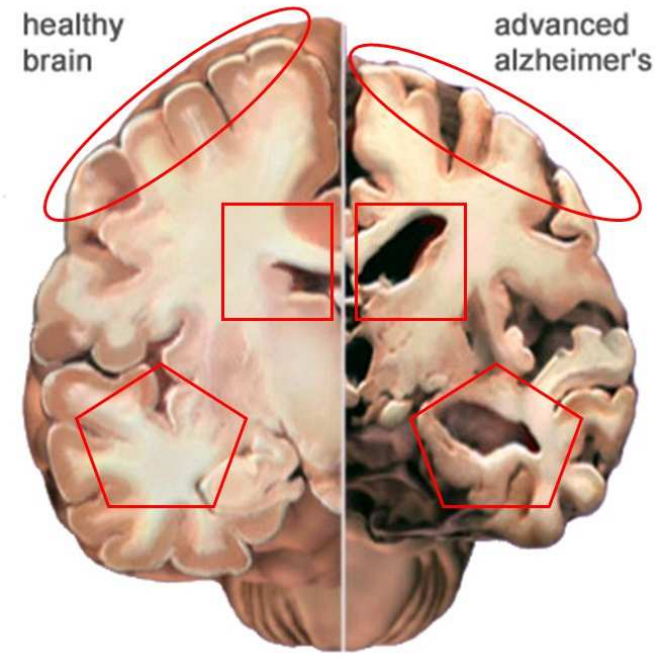

Figure 2: Differences between normal brain and brain in an advanced stadium of Alzheimer's disease. Several regions are remarkably reduced in an AD brain (right). The cortex (oval marking) and the hippocampus (rectangular marking) show a high level of neuronal loss including an overall reduced weight of the whole brain. The fluid-filled compartments (ventricles – pentagonal marking) get bigger due to loss of compact and healthy brain substance. Picture modified from: http://www.alz.org/braintour/healthy_vs_alzheimers.asp 04.06.2014

The late forms of AD are caused by a polymorphism in the locus for the allele ε4 of the Apolipoprotein E (apoE) but also by other environmental factors which are not mentioned here[28].

As mentioned before, AD is caused by abnormal amount of fragments the protein Aβ, which causes plaques in the patient's brain. The accumulated proteins then lead to damage and death of nerve cells in the areas responsible for memory formation and orientation (hippocampus)[29,30] and cognitive function (cortex)[31] (see Figure 2) via unknown mechanisms.

Up to date there are only symptomatic therapies available for AD as the progress of the illness cannot yet be reversed or stopped. The therapies deal with the difficulties of cognitive impairment in the patients including pharmacologic and psychotherapeutic treatments[32].

In recent years, inhibitors of the cholinesterase became the standard therapeutic approach, as they ameliorate the behavioral symptoms through inhibition/delay of the breakdown of the neurotransmitter acetylcholine in the human body[33,34].

The incidence rates of AD are very high in the ageing population with over 5 million American citizens being affected in 2013[35] and this number will dramatically increase, reaching a total estimated number of more than 13 million patients in 2050 with a high percentage of these being over 85 years of age[36].

1.1.3 Modelling neurodegeneration in *Drosophila melanogaster*

Since I could not test human beings in my master thesis, I used a well-established model organism to show the detrimental effects of α-synuclein (α-syn)[37] and amyloid-β-protein (Aβ)[38,39] on neurons using the fruit fly *Drosophila melanogaster*[40,41]. Even though being a small invertebrate animal, the fruit fly still has a well-developed central nervous system (CNS), including an accumulation of neuronal tissue in the head, what we would call a brain. This organism has several advantages in modeling neurodegenerative diseases like AD and PD – it has a short life span, a great number of progeny, a well-known anatomy and can easily be manipulated via simple molecular methods and genetic techniques.

In this Master thesis I used flies expressing the wild-type form of α-syn in their CNS - modeling PD - and a truncated construct of the human Aβ-gene (amino acids 1-42 – Aβ42) - for AD - under the control of the GAL4-UAS system. This system has several conveniences that help in the design of *Drosophila* studies. The proteins being under the control of this

system can be expressed in a time-dependent manner, meaning the tight control of the eventual detrimental effects of the translated transgenic construct[42].

On the long way to new therapeutic agents against the aforementioned neurodegenerative illnesses, this model system is a very useful approach towards the development of new substances to ameliorate the most severe symptoms of the diseases. Recently, a new polyamine compound named Spermidine (spd) was tested under conditions of elevated cellular stress, leading to induction of autophagy and increase of the lifespan of several model organisms including yeast (*Saccharomyces cerevisiae*), nematodes (*Caenorhabditis elegans*) and fruit flies[43].

In the experimental work this thesis is based on, we wanted to show the beneficial effects the polyamine spd has on the affected/damaged brain cells in the model to regain a status of functionality or at least alleviate the symptoms.

1.2 Spermidine – a natural polyamine

Spermidine (spd) is a natural biogenic polyamine (CAS number: 124-20-9), precursor of Spermine, that was discovered more than 300 years ago in samples of human semen[44] (molecular structure – see Figure 3). It can be found in various food sources like leguminous plants (beans, peas, lentils), especially in soy beans and derived products[45]. In the human body it is produced from a conversion of the amino acid L-Ornithine (or L-Arginine, or S-Adenosyl-L-Methionine) via the enzyme Ornithine-Decarboxylase1 (ODC1, or Arginine-Decarboxylase (ADC), or S-Adenosylmethionine Decarboxylase (AdoMetDC)) to Putrescine and via the Spermidine-Synthase to Spermidine[46].

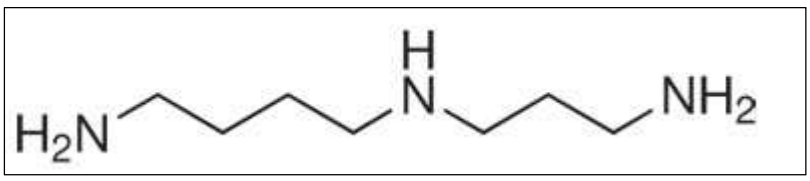

Figure 3: Molecular structure of the natural polyamine Spermidine. Picture modified from: http://www.nature.com/nature/journal/v464/n7288/box/nature08981_BX2.html, 7 June 2014

Spd has an important role in several cellular processes such as cell growth, proliferation and survival. Alterations in the cellular polyamine level have been shown to be connected to organismal aging and several diseases[47].

This substance also reduces age-related oxidative protein damage in the fruit fly *Drosophila* indicating that spd might be a general factor against decline of cellular functions[48]. Spd also mediates longevity through induction of autophagy in yeast (*S. cerevisiae*), worms (*C. elegans*) and flies[49].

Based on these findings, expectations are also directed towards assistance of the substance to alleviate the most severe symptoms of neurodegenerative diseases via the mentioned pathways.

1.3 Autophagy – a two sided sword in neurodegeneration

As mentioned earlier, neurodegenerative diseases often start with an abnormal amount of certain proteins that accumulate and form aggregates which are then toxic to the cell. In many scenarios, self-digestion of particular parts of the affected cell itself can help to avoide a rapid progress of the illness. This self-digestion is called autophagy - from the Greek auto-, "self" and phagein, "to eat" – and is a basic cellular mechanism to get rid of unnecessary or damaged cell compartments or proteins.

There are some distinct forms of autophagy dependent on their substrate. Macro-autophagy, Micro-autophagy and chaperone-mediated autophagy. These three forms share the mechanism of the proteolytic degradation of cellular organelles and proteins in the autolysosome, which is a special form of the cellular lysosome. In Macroautophagy, the cell engulfs cytoplasmic components via this autophagosome-pathway, in Microautophagy the cellular cargo is directly taken up by the lysosome and in chaperone-mediated autophagy proteins and other cargo are first targeted for breakdown by chaperone proteins (e.g. Hsc-70)[50].

Up to date there are more than 30 autophagy-related genes (ATG) identified in yeast and many of these genes have conserved domains or are completely conserved in molds, worms, plants, flies and mammals[50].

If the regulation of autophagy does not work properly, this might lead to several serious health problems, including cancer and neurodegenerative diseases. In neurodegenerative illnesses like AD, PD, Huntington’s disease and Amyotrophic lateral sclerosis at least one decisive step in the autophagic pathway seems blocked. In AD and PD autophagosome-like structures accu-

mulate in the cell which indicates impaired downstream processing of these vesicles. Induction of autophagy in the affected cells reduces toxicity of Aβ and α-syn and can lead towards neuroprotection[51].

There are several substances known to inhibit or induce this cellular "clean-up" mechanism including natural and synthetic substances. On the one hand, there are drugs that are used to inhibit high levels of autophagy (e.g. in cancer cells) like Chloroquine diphosphate[52] (CAS No: 50-63-5), 3-Methyladenine[53] (CAS No: 5142-23-4), Bafilomycin A1[54] (CAS No: 88899-55-2), on the other hand, impaired or malfunctioning autophagy can be induced by substances like Rapamycin[55] (CAS No: 53123-88-9), Metformin[56] (CAS No: 1115-70-4), Resveratrol[57] (CAS No: 501-36-0) and Spermidine (CAS number: 124-20-9).

Given these connections we came to the following hypothesis that led us to the experiments that are described in this master thesis.

1.4 Induction of Autophagy via Spermidine ameliorates symptoms of Neurodegeneration in flies?

With the knowledge of the aforementioned facts, we came up with the following hypothesis:

"Can the induction of the autophagic machinery via administration of spermidine ameliorate the symptoms of neurodegeneration in the model organism of the fruit fly?"

To test this hypothesis, we combined the connections pointed out earlier in the introduction. If neurodegeneration was at least in parts caused by impaired autophagy, we assumed that boosting autophagy might protect from toxic effects of protein aggregates. Therefore we came up with the substance that already showed similar effects in yeast cells – namely spermidine (spd). Spermidine is able to induce autophagy via transcriptional activation of several ATG-genes that encode core-autophagic proteins in yeast and other organisms.

In our experiments we wanted to determine the effect spd has on the whole organism of the transgenic fruitfly. Thus, we designed experiments that gave us the opportunity to test several scenarios that could elucidate the impact of spd administration on the organismal level (in contrast to the cellular level that could be investigated in yeast before).

We assume that with the supply of spd to transgenic flies expressing amyloid-β protein or α-synuclein, the loss of important neuronal cells can be stopped or at least be decreased. If the autophagy can be induced, we hope to find a reduced amount of staining in the central nervous system (CNS) – especially the brain – of the treated flies.

Also the hallmark symptoms of the individual illnesses – decreased lifespan, impaired locomotion and memory impairment – should be diminished upon addition of spd to the fly food.

2. Materials and Methods

2.1 Materials

Chemicals, ingredients, fly food constituents and lab equipment were ordered from the following companies: Biorad, Covance, Dianova, GE Healthcare, Invitrogen/Life Technologies, Merck-Millipore, Roche, Roth, Sigma Aldrich.
For all the experiments we used standard laboratory equipment, alongside with fly incubators (25 °C, 12h:12h light:dark cycle) and a confocal laser scanning microscope (SP8 from Leica).

2.1.1 Antibodies

The Antibodies used for the staining of the dissected flybrains are listed in the Table 1below.
The stained brains were scanned with a confocal laser scanning microscope (SP8 from Leica).

Table 1: Used Antibody for staining of dissected flybrains. To show protein expression in the adult flybrain, we dissected brains and stained them with a commonly used fast protocol. To compare intensities in the staining we always used a co-staining with an ubiquitinous synaptic protein called Bruchpilot.

Primary antibodies	company
Rabbit-α-synuclein	Sigma-Aldrich
Mouse-α-Aβ-42 6E10	Covance
Mouse-α-Nc-82	CD Creative Diagnostics, Wagh et al. 2006
Rabbit-α-BRP N-term	Fouquet et al 2009
Secondary antibodies	company
Goat-α-rabbit Cy3	Dianova
Goat-α-mouse Cy3	Dianova
Goat-α-rabbit Alexa 488	Invitrogen/Life Technologies
Goat-α-mouse Alexa 488	Invitrogen/Life Technologies

2.1.2 Fly food

The normal fly food was prepared according to Bloomington media recipe (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/media-recipes.htm) with minor

modification. We used the standard corn meal medium supplemented with dry yeast. Spermidine (spd) was prepared as a 2 M stock solution in sterile distilled water, aliquoted in 4 mL portions and stored at −20 °C until they were thawn for use. After food has cooled down to 40 °C, spd was added to normal food to a concentration of 5 mM.

Spd-manganese-solution (spd- Mn^{2+}-solution) was prepared as follows:

10 % w/v sucrose was dissolved in distilled water (Millipore), spd (from 2 M stock solution) to a concentration of 5 mM was added and the pH was measured. The alkaline pH of 10,8 was adjusted using 1 M HCl until it reached the pH of 5,6 (exact pH of the spd- Mn^{2+}-solution). Afterwards, Mn^{2+} (from 2 M stock solution) to the concentration of 20 mM was added to the solution. The solution must be clear to be used, there should not be any yellow/brown precipitate, which shows that Mn^{2+} and spd formed a complex which titrates away both of the substances. For the sucrose-Mn^{2+} solution, 10 % w/v sucrose was dissolved and Mn^{2+} to the concentration of 20 mM was added.

2.2.3 Fly stocks

Flies used in the experiments were all with w1118 background used normally in the Lab of Prof. Dr. S.J. Sigrist. Used UAS-lines and GAL4 lines are listed in Table 2 below. All flies used in the experiments are from the F1 generation.

Table 2: List of used UAS- and GAL4 lines in the experiments.For all the crosses, parental flies of these specific lines were mated and the F1 progeny was used.

UAS-line	reference	Sigrist-Lab Stock number
UAS - $\frac{A\beta-42}{TM3\ Ser}$	Iijima et al. 2008	2361
UAS - $\frac{A\beta-42}{Sbtb}$	Iijima et al. 2008	2362
UAS – α-synuclein	Bonini et al. 2005	1904
GAL4-lines		
$Elav^{C155}$-GAL4	Lin and Goodman, 1994	10
Appl-GAL4 (10G outcrossed)	Torroja et al. 1999	2252
nSyb-GAL4*	Bushey et al. 2009, Yu et al. 2010	1008

w1118 – Hazelrigg et al, 1984

*… This stock was used with light modifications: the TM3 Sb – balancer that was on the 3rd chromosome before was selected out so that the nSyB-GAL4 got homozygous.

2.2 Methods

2.2.1 Survival in Carousels

For the survival experiments in carousels, two schemes were used: in schema YOUNG, one-to-three day old flies were collected from big bottles, males and females separated (40 flies per vial) under CO_2 anesthesia on the same day, let regenerate for 24hrs at 25 °C, then put at 29 °C for 24hrs and filled into vials of carousels by tapping. Then dead flies were counted every 24hrs at the beginning (in the first 2 days), then every 12 hrs (for the following days). In schema OLD, one-to-three day old flies were collected from big bottles, males and females separated (40 flies per vial) under CO_2 anesthesia on the same day, then aged at 25 °C for 13 days, subsequently put at 29 °C for 24hrs and then tapped into carousels – dead flies were counted every 12hrs from the beginning. Each dead fly was then subtracted from the total amount of flies in the vial to determine the survival rate over a period of 6 to 8 days.

For the survival setup we used self-made carousel constructions made of empty fly food vials and petridishes with a hole in the bottom part of the vial and in the top part of the petridish. A stripe of filter paper was led through both holes and reached the lower part of the petridish which was filled with approximately 30 mL of the sucrose-Mn^{2+}solution. The filter paper-stripes sucked the solution from this lower reservoir to provide it to the flies in the top vial. In this apparatus, 7 vials, each containing 40 flies, could be tested in one run.

2.2.2 Longevity LO

For the longevity experiments, the following two setups were used: for the schema I, one-to-three day old flies were collected from big bottles of normal fly food and food with 5 mM spd. Through the following steps, all flies collected from spd-containing food were always kept at that food and flies from normal food bottles were kept at normal food. Males and females were kept together in big vials for 24 hrs to mate, then separated under CO2 anesthesia and put in small vials (5-10 vials, 20flies each), kept at 25 °C in a humidity controlled incubator (70% humidity) with 12hrs:12hrs light:dark cycle and let age. Flies were flipped on fresh food every 2-3 days and dead flies were recorded for up to 80 days total. Flies that got stuck to the food but were alive were transferred into the fresh vial by using a brush, but also flies that flew away were recorded and considered as censored objects in the computational analysis.

For the schema II, one-to-three day old flies were again collected from big bottles of normal fly food, males and females were kept together in big vials for 24 hrs to mate, then separated under CO2 anesthesia and put into small vials (5-10 vials, 20 flies each). Again, the flies were flipped every 2-3 days and dead flies were recorded for up to 80 days total. Flies that got stuck to the food but were alive were treated like mentioned above and also flies that flew away were recorded and considered as censored objects. For this schema, one half of the total number of vials was flipped on 5 mM spd-containing food on day 29/30. The other half of the vials was continuously kept on normal food. Flies were kept in an incubator as mentioned before.

2.2.3 Climbing (Negative Geotaxis)

In the climbing experiments, one-to-three day old flies were collected from big bottles of normal fly food, separated on the same day (40 flies per vial) and used in the same schema than mentioned in "survival in carousels". According to the survival schemes, flies were tested at different timepoints after stress appliance. For schema YOUNG, climbing abilities were tested after 24, 72 and 96 hrs and for schema OLD, flies were tested after 24, 48 and 72 hrs. For this experiment, the 40 flies were anesthetized with CO_2 in the vial of the carousel, separated on a pad in portions of 10-15 flies, put into climbing vials (see fig. X) and let regenerate for 30 minutes in a dark room with red light turned on.

After regeneration, flies were tested for locomotion and negative geotaxis in the red light by tapping them on the bottom of the vial and let them climb the vertical vial for 15 sec and 30 sec. The flies that could cross the height of 7 cm (schema YOUNG) or 4 cm (schema OLD) were recorded in the mentioned time.

These experiments were also performed with flies from the longevity setup, were we tested the flies on d2, d5 and then every 5 days. Again, flies that could cross the height of 4 cm were recorded. Each trial was at least triplicated. At least 100 flies in for each genotype were tested.

2.2.4 Recombination of Aβ-42/α-synuclein with ODC1 in flies

In order to generate flies which contain the two desired genes ODC1 combined with Aβ-42 or α-synuclein, we needed to perform a recombination, given the fact that both genes are on the same chromosome (III) but in different flies. The schema for the crosses is shown in Figure 4.

The flies were collected right after hatching to have the chance to get the ones with the darkest eye color, which have a high possibility to have the two desired genes recombined on their third chromosome.

The following crosses (Figure 4) were set to obtain the final recombined flies:

$\frac{\boldsymbol{ODC1,\ A\beta-42}}{\boldsymbol{TM3\ Sbtb}}$ and $\frac{\boldsymbol{ODC1,\ \alpha-Syn}}{\boldsymbol{TM3\ Sbtb}}$

As an example for both genotypes, crosses for these flies include:

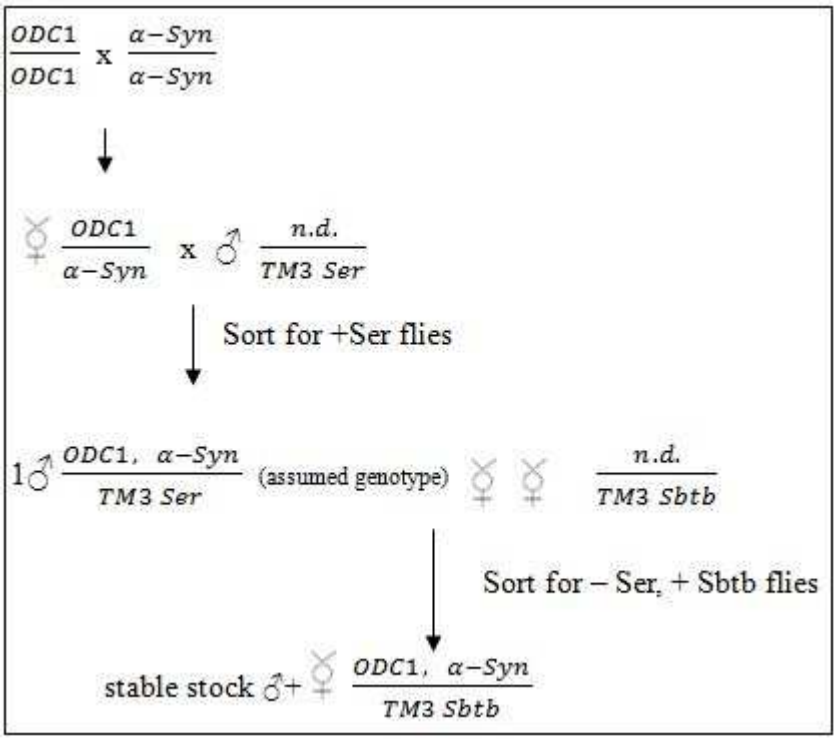

Figure 4: Crosses for the generation of recombinant flies. To obtain recombinant flies containing two desired genes, we needed to cross the single genotypes and, for getting a stable stock, stabilizing them with a balancer chromosome for the desired locus.

To verify the correct insertion of the desired genes after the recombination, we performed a PCR-analysis. The primers generated and used are listed in Table 3 below. The primer for the ODC1 gene were used from the Sigrist-Laboratory primer list with the numbers: ODC1 fwd = #61, ODC1 rev = #62.

Table 3: Primer list for the verification of the correct generation of the recombined flies.

Name/number	sequence	Tm	Fragment lenght
Syn fwd	atctGATGTATTCATGAAAGGACTTT	54,7	422 nt
Syn rev	atctTTCAGGTTCGTAGTCTTGATA	55,5	
Graz AB42 fwd	ATCTgatgcagaattccgacatga	56,4	132 nt
Graz AB42 rev	ATCTctatgacaacaccgccca	56,1	

The PCR programs that were used to confirm the correct recombination are listed in Table 4 below.

Table 4: PCR-programs used for the genetic verification of the recombined flies

Genotype	Fragment length (nt)	Time (min)	Temperature (°C)	cycle
ODC1	1182	5	95	
		0,5	94	30 x
		1	53	
		1	72	
		10	72	
α-synuclein	420			
Aβ-42	126	5	95	
		0,5	94	30 x
		1	53	
		0,5	72	
		10	72	

2.2.5 Brain dissections, staining and wholemount brain scanning

To determine, whether the level of protein amount stained by the specific antibodies (mentioned in 2.1.1 Antibodies, Table 1) in the fly brain changes with time and stress, we dissected the brains of adult flies (4- to 5-day-old adult males and 13- to 15-day-old adult females) according to a fast protocol. Dissected brains were collected in cold HL3, then fixed

in PBS with 0,1 % Triton X-100 and 4% Formaldehyde for 20 min at room temperature (RT), washed in PBS with 1% Triton-X100 for 30 min at RT. Wash brains with PBS with 0,3% Triton-X100 (PBST) (4x15 min) at RT, pre-incubate them in PBST with 10% NGS (1h at RT on shaker) and incubate with desired primary antibody in PBST with 5% NGS and Sodiumazid (1:100) for 48 hrs at RT. Wash brains again in PBST (3x20 min), incubate with desired secondary antibody in PBST with 5% NGS and Sodiumazid (1:100) for 24 hrs at RT, then wash in PBST (4x20 min) at RT and incubate in Vectashield (Vector Laboratories LTD., Peterborough, United Kingdom) for 24 hrs (to dehydrate the tissue) before mounting them on glass slides sealed with nail polish. Keep brains at 4 °C for short term or at -20 °C for long term storage. Three brains of each genotype were scanned by Confocal Microscopy (Leica SP8), then pictures were analyzed by comparing the intensities of stained areas in the central brain for Aβ-42 or counting the dopaminergic neurons for α-synuclein.

2.2.6 Western blot analysis

To determine the expression of our proteins with the utilized driver lines, we performed Western blot analysis with extracts from adult fly heads.

We followed the head extraction protocol from the Sigrist-Laboratory from Dr. H. Depner. We collected the flies, immediately froze them for several minutes in liquid nitrogen and then decapitated them by vortexing for 1 minute. On a pre-chilled metal plate (ice/liquid nitrogen), the fly heads were collected with pre-chilled forceps and transferred into pre-chilled 1,5 mL Eppendorf cups. Then 1 µL of extraction buffer (2 % SDS with 10 % Triton X-100 in water) per fly head was added and the heads were squished with a micropistil in the 1,5 mL cup until the head is completely decomposed. Then 1 µl of Laemmli-sample buffer per head was added and the mix was heated for 5 minutes at 95 °C. After this, incubate mix for 5 minutes at room temperature, then centrifuge it for 2 minutes at full speed (approx.. 17.000rpm) to pellet head debris. Now the extract is ready for SDS-page.

We used W1118-extract as a negative, and sequence-verified α-syn/Aß42-flies as positive controls. Because of the different size of the protein, we had to use different extraction buffer for Aβ-42 and α-syn. We got the recipe for the modified lysis/extraction buffer from Christine Quentin, member of the Sigrist Lab group. We had to use a buffer with a very high concentration of detergent - 500 mM TrisHCl (pH 9,0), then we added 1 % Sodium-Deoxycholate and 0,1 % Triton X-100.

We made 20 % Acrylamide-gels (with 1 % SDS) and as a running buffer, we used a solution of 25 mM TrisHCl (pH 8,3) with 192 mM Glycine and 0,2 % SDS. The transfer buffer was the same but without the 0,2 % SDS added.

We used the Lämmli-buffer of the Lab in Graz which contains 250 mM TrisHCl (pH 6,8), 20 % SDS, 60 % glycerol, a bit of bromphenolblue and before use 1/10 volume of β-mercapto-ethanol has to be added. For detection, we used the Amersham ECL Prime Western Blotting Detection Reagent from GE Healthcare.

3. Results

3.1 α-synuclein

3.1.1 Spermidine rescues manganese-induced death of α-synuclein-expressing flies

Both environmental as well as genetic risk factors have been shown to contribute to the pathology of Parkinson's disease (PD). In this case, flies expressing human α-synuclein (α-syn) under the control of the UAS/Gal4-system have been shown to die within 5-7 days upon additional exposure to manganese (Mn^{2+}), a known risk factor for PD[58]. We applied this setup to analyze whether spermidine (spd), which has shown to promote longevity in several model organisms, is able to ameliorate α-syn-induced demise.

Using different pan-neuronal driver lines, we found that the supplementation of food with 5 mM spd could delay organismal death induced by α-syn. However, the extent of the protection is clearly depended on the respective driver used, which will be given attention to later in the discussion section (see 4.1.1).

We performed the experiments with male and female flies separated between 1-3 days after eclosion. Exposure of young flies (2-5 days) to Mn^{2+} enhanced death upon elav(x)-Gal4-driven expression of α-syn as compared to similar treated wild type flies both in males and in females (Figure 5). Simultaneous application of spd could largely restore the survival of α-syn-expressing flies. The periods of the highest rescue rate were mostly found in the middle part of the experimental time-course. Between 72 hrs and 144 hrs the difference between the spd-treated animals and the ones without spd (and control flies) was most prominent and found with a P-value of less than 0, 01.

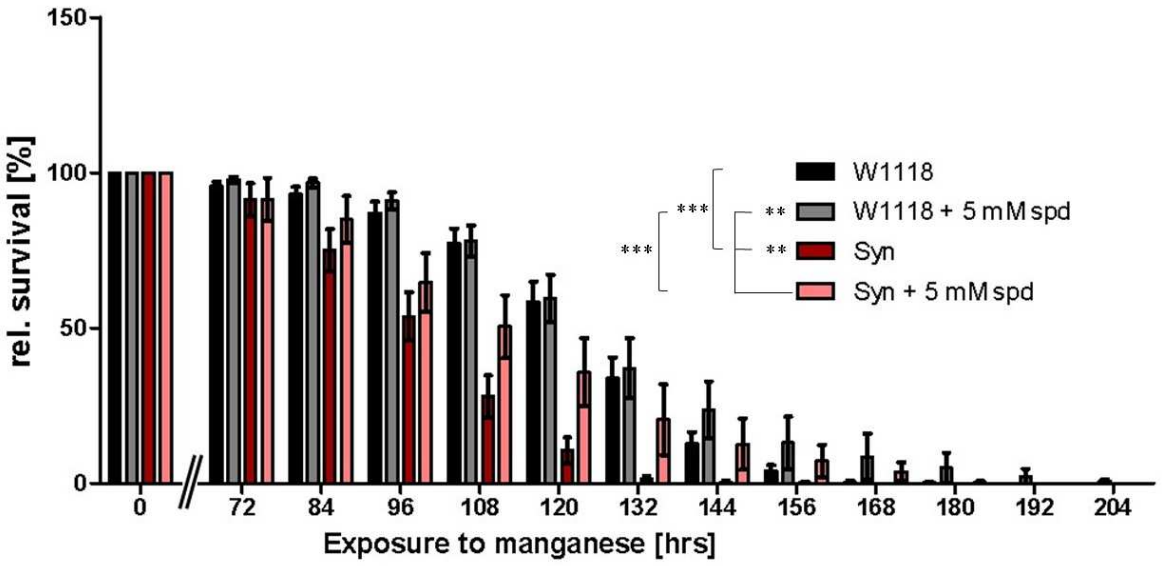

Figure 5: Spermidine (spd) treatment rescues α-synuclein expressing flies upon manganese (Mn^{2+}) stress. Young wild type and α-syn-expressing flies (2-5 days) were kept on 10% sucrose supplemented with 20 mM Mn^{2+} with and without addition of 5 mM spd and survival was determined at indicated time points. Expression of α-syn was achieved using the UAS/Gal4 system and the pan-neuronal driver line elav(x)-Gal4. P-values of less than 0,01 (Syn vs. Syn + spd at 108 hrs and 120 hrs and Syn + spd vs. w1118 + spd at 120 hrs) and less than 0,001 (Syn vs. w1118 at 96 - 132 hrs and Syn + spd vs. w1118+ spd at 96 hrs and 108 hrs) could be calculated with the Two-way ANOVA with Bonferroni correction with an n=4 experiments (approximately 400-480 flies per genotype). The comparison of w1118 vs. w1118 + spd was not significant at any timepoint. Here, we show data of young male flies.

Concerning 14 days-aged flies, we could obtain a high evidence for a protective role of spermidine for the female flies. We also tested the young animals (as mentioned before) that could be rescued by spd-treatment, even though they did not show a specific rescue for only the α-syn-expressing animals in comparison to the control flies (see Figure 6). Nevertheless, we were able to calculate a P-value of less than 0,05 for the aged flies. Expression of α-syn was achieved by using the nSyB-Gal4 driver line which is more consistent over a longer period of the lifetime of flies than the elav(x)-Gal4 driver line.

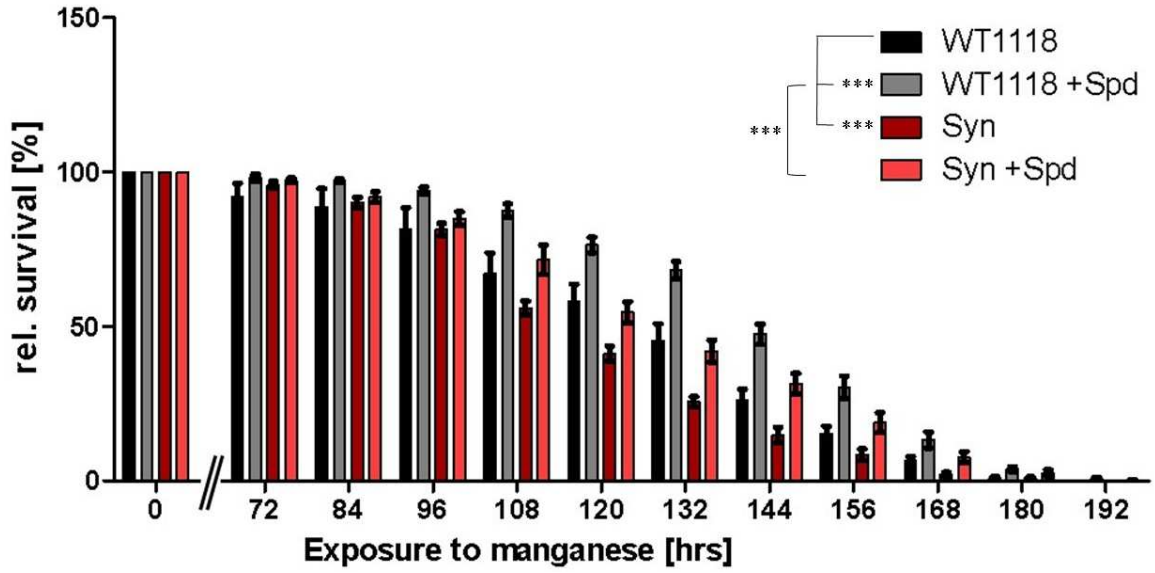

Figure 6: 14 days-aged α-synuclein expressing flies are able to survive manganese (Mn^{2+}) stress better when treated with spermidine (spd). Young α-syn expressing flies were separated 1-3 days after eclosure, subsequently kept at 25 °C for 13 days and then fed with a solution containing 10 % sucrose with 20 mM Mn^{2+} with and without 5 mM spd. The survival rates were determined at several timepoints indicated above. Expression of α-syn was performed using the UAS/Gal4 system and the pan-neuronal driver line nSyB-Gal4. P-values of less than 0,01 (Syn vs. Syn + spd at 120 hrs) and less than 0,001 (Syn + spd vs. w1118 + spd at 108 - 144 hrs, Syn vs. w1118 at 120 hrs and 132 hrs and w1118 vs. w1118 + spd at 108 – 144 hrs) could be calculated with the Two-way ANOVA with Bonferroni correction from an n=3 experiments (approximately 480-560 flies per genotype). Here, data of aged female flies are shown.

3.1.2 Establishing α-synuclein expression in transgenic flies

To analyze whether the improved survival of α-syn expressing flies upon supplementation of food with spd is accompanied by a change in α-syn expression levels, we performed western blot analyses of whole brain protein extracts.

In order to additionally prove the functionality of the used driver line we obtained a head extract (following the head extraction protocol in 2.2.6) of the F1 generation flies, which could then be tested in a Western blot using a specific anti-α-syn antibody for detection. As can be seen in Figure 7, we were able to detect the α-syn protein specifically in the extracts of the used flies with the applied antibody. The protein runs at the right height (around 14 kDa) in the sample of the transgenic flies and the band is not visible in the w1118-control flies' samples.

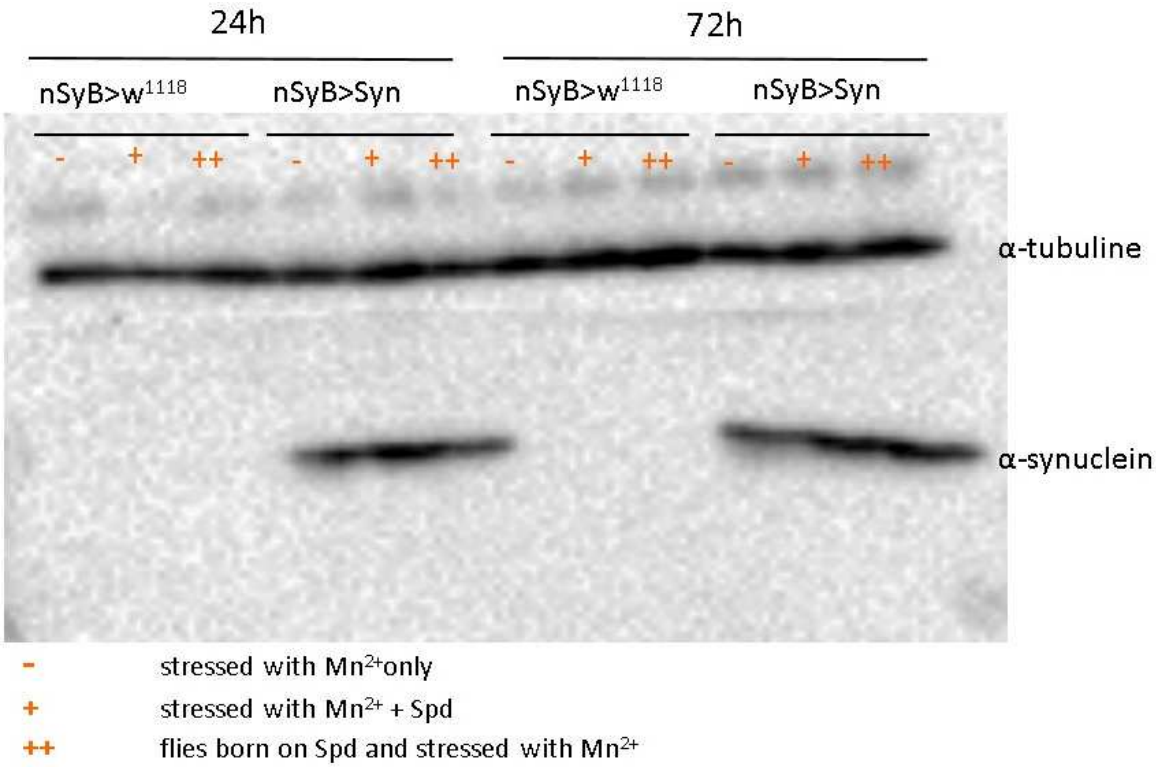

Figure 7: Change in expression-levels of α-synuclein upon spermidine (spd) treatment. Head extract samples from distinct timepoints from flies that were treated in the manganese (Mn^{2+}) stress setup were obtained and the protein amount was determined via Western Blot analysis. We used anti-α-tubulin as a reference protein and a specific α-syn antibody to detect the band in the samples. We also included samples from flies born on spd containing fly food and subsequently stressed with manganese in this analysis.

3.1.3 Spermidine does not prolong the lifespan of *D. melanogaster*

Due to the fact that PD is a late-onset disease and leads to a rapid worsening of life-quality, we wanted to establish a probable scenario in which spd can help to considerably improve the lifespan and or the detrimental impact in a transgenic fly model expressing human α-syn under the control of the UAS/Gal4-system and the pan-neuronal driver line APPL-Gal4.

We performed the experiments with mated male and female flies separated two days after eclosion and kept in vials containing normal fly food or food with 5 mM spd. In this longevity setup, dead flies were recorded every second day, these data were computed and graphs were calculated to show the possible effects spd has on aging and aged flies.

In the graph in Figure 8 below the survival curves of the different genotypes over a period of approximately 80 days are shown. Here, we show some curves as examples for the rescue of the α-syn expressing flies upon spd treatment, more results will be dealt with in the discussion section.

Some experiments did not show a consistent rescue but rather showed a possible detrimental effect of spd when it was applied from the beginning of the aging. As can be seen in Figure 8 A, B below we could on one hand show a possible rescuing effect of spd on the α-syn expressing flies but on the other hand we had a detrimental effect on the control flies which were killed earlier in time by spd. We could show this effect in both sexes as you can see in A for the females and in B for the male flies. The graphs are the result of one single experiment with 180 flies per sex and genotype, which was repeated several times (data not shown).

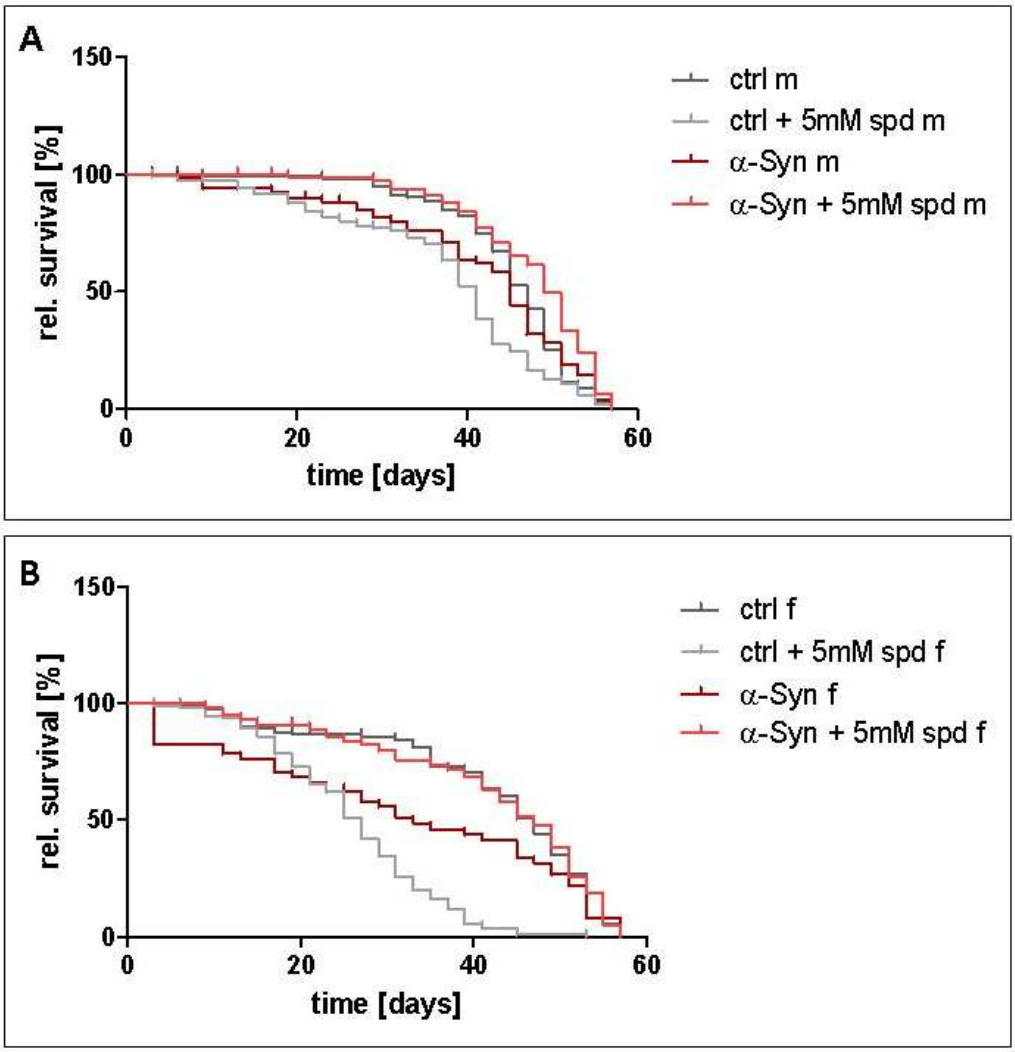

Figure 8: No increase of the lifespan of α-synuclein expressing flies upon administration of 5 mM spermidine (spd) via fly food. In the experiments we collected male and female flies one day after eclosion, let them mate for 24 h and separated them. Flies were kept in vials containing fly food with or without 5 mM spd and survival was determined when flies were flipped on fresh food. Every second day, dead flies were counted until the last which took approximately 80 days. α-syn expression was obtained by using the pan-neuronal APPL-Gal4 driver line. These curves resulted from one single experiment with 180 flies per sex and genotype, which was repeated several times.

3.1.4 Ability to overcome a certain height in negative geotaxis is enhanced upon spermidine-treatment

As several environmental parameters play a critical role in the development of PD, we tried to find an elaborate model for one of the most characteristic symptoms of the disease, namely the locomotion difficulties e.g. tremor and coordination problems. Given the fact that there are already existing tests for locomotion in the fruitfly model we decided to establish the assay for negative geotaxis in α-syn expressing *D. melanogaster*. Here we examined the flies for their ability to overcome a certain vertical distance in a transparent plastic vial (we needed to conduct the experiments under red light, as normal light would influence the results).

We used young (3-5 d old) and aged (12-14 days old) animals based on the data obtained from the Mn^{2+}-survival assays before. We used flies expressing human α-syn under the control of the UAS/Gal4-system using the elav(x)-Gal4 driver line for experiments with young animals and the nSyB-Gal4 driver line for the experiments with aged animals. The optimal driver line for each setup was subject to prior tests analogous to the pre-experiments for the Mn^{2+} stress setup.

We tested different conditions to find the optimal height for the flies to start with a percentage of at least 75% of flies that could overcome that level in the first run of the experiment. For the young flies we set a height of 7 cm, but for the aged flies we had to lower that height to 4 cm to obtain the desired starting values. While processing the acquired raw data, we found that the locomotor activity was enhanced in flies which were treated with 5 mM spd in addition to being stressed with Mn^{2+} compared to the ones stressed with Mn^{2+} only.

In Figure 9 below the obtained graphs are shown; in the top graph, the absolute climbing ability is shown, which declines over time, and in the graph below there is the normalized relative climbing ability. We wanted to show the relative rescue of the locomotion of the α-syn flies upon spd treatment which should be above the level of the control group to have a significant restoration in the α-syn disease model flies.

For the tested aged flies, the restoration upon spd treatment is very prominent, even though the young tested animals showed a less notable effect. We could still see a difference in the spd-treated group (see Figure 9C and D at 72 h timepoint), which this was nearly significant. In the aged flies, the restoration is especially seen at the 48 h-timepoint for male flies and at the 72 h-timepoint for female flies (see Figure 10C and D). The graphs for the young animals (Figure 9) are the result from 2 independent experiments with approximately 320 flies per sex and per genotype. For the experiments with aged animals we used approximately 360 flies per sex and per genotype.

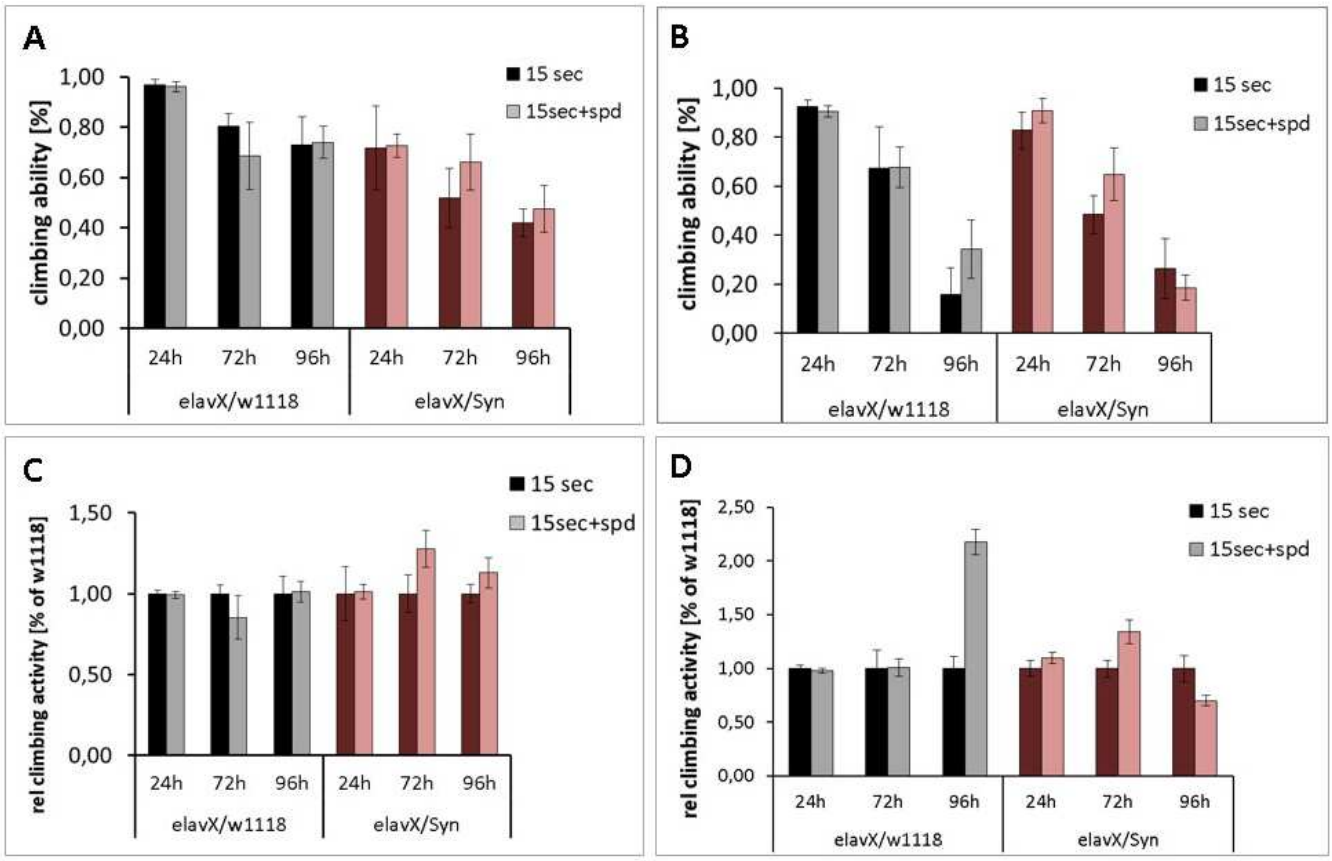

Figure 9: No significant restoration of the climbing ability in young α-synuclein flies upon acute manganese (Mn^{2+}) stress and spermidine (spd) treatment. Young flies were separated 1-3 days after eclosion and tested on their ability to overcome a height of 7 cm in a vertical vial after being stressed with 20 mM Mn^{2+} in a 10 % sucrose solution with and without addition of 5 mM spd. The ability to overcome the height-level was tested in a red-lit chamber at the indicated timepoints. Expression of α-syn was achieved using the UAS/Gal4 system and the pan-neuronal driver line elav(x)-Gal4.. In (A) and (B) the absolute climbing ability to surpass the 7 cm line is shown, whereas the normalized locomotion ability is shown in (C) and (D). These graphs are the result of 2 independent experiments with approximately 320 flies per sex and per genotype.

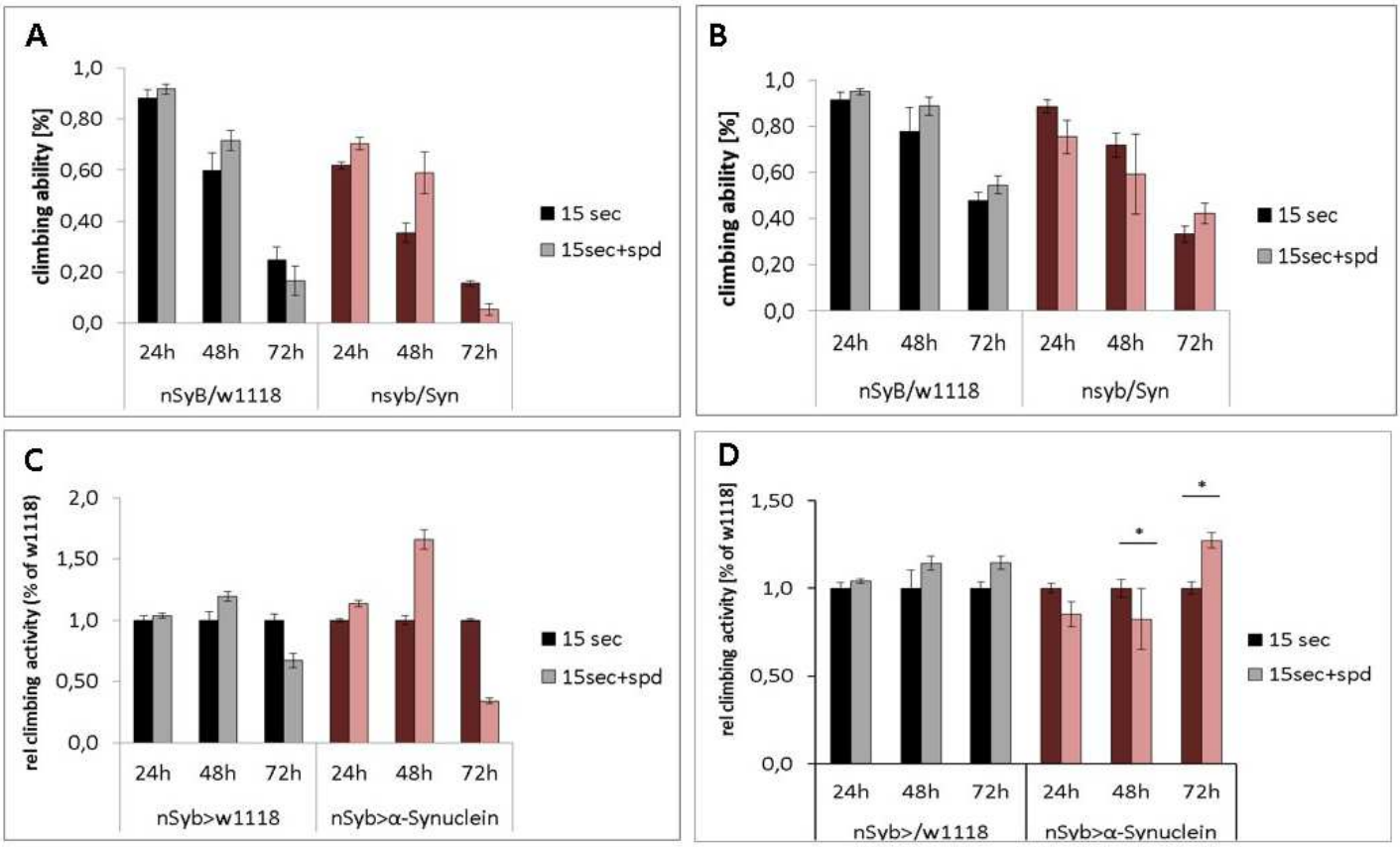

Figure 10: Spermidine (spd) treatment significantly improves locomotion ability in aged α-synuclein expressing flies. Young flies were separated 1-3 days after eclosion, let age for 13 days and then kept in the stress carousels containing 20 mM Mn^{2+} in a 10 % sucrose solution with and without addition of 5 mM spd. Aged flies were tested in their ability to overcome a height of 4 cm in a vertical vial in a red-lit chamber at the indicated timepoints. Expression of α-syn was achieved using the UAS/Gal4 system and the pan-neuronal driver line nSyB-Gal4. In (A) and (B) the absolute climbing ability to surpass the 4 cm line is shown, whereas the normalized locomotion ability is shown in (C) and (D). These graphs are the result of 2 independent experiments with approximately 360 flies per sex and per genotype.

3.1.5 Staining intensity of wholemount brains of α-synuclein-flies is not significantly changed upon spermidine administration

In PD, patients have to face progressive deficiencies in several fields of their daily life. During pathogenesis of the illness, accumulating aggregates of α-syn in the central nervous system lead to damage of dopaminergic neurons and substantial loss of this neuronal population in the substantia nigra of the brain. To investigate this process in more detail we used the fruitfly *D. melanogaster* as model organism which has a very simple but elaborated central nervous system (CNS) including a specialized brain structure. We hoped to find areas in the brain which are connected to the various behavioral patterns of the flies and are

severely affected by α-syn expression showing signs of eventual damage of population of neuronal cells, especially dopaminergic neurons.

After dissecting whole fly brains (see materials and methods - 2.2.5) we stained them with appropriate antibodies (here: anti-α-syn) to scan them using a SP8 Leica confocal microscope. Given the non-constant staining signal of the BRP in the fly brains, we decided to analyze the signal of the anti-α-syn antibody alone to see whether the absolute intensities change upon spd administration. In the picture (Figure 11) below we could see that the intensity of staining of α-syn was reduced in the brain samples that were obtained from spd-fed flies (we show both channels here). The level of staining was quite high in the α-syn expressing flies that were treated with Mn^{2+} only and was reduced in flies fed with spd (either acutely together with Mn^{2+} or constantly via the fly food). Here, we show data from samples obtained from aged female flies that were treated in the stress setup. Certainly, we performed the same analyses also with flies from the other conditions mentioned before, but the acquired data sets are not yet fully validated, therefore we will not give further information here.

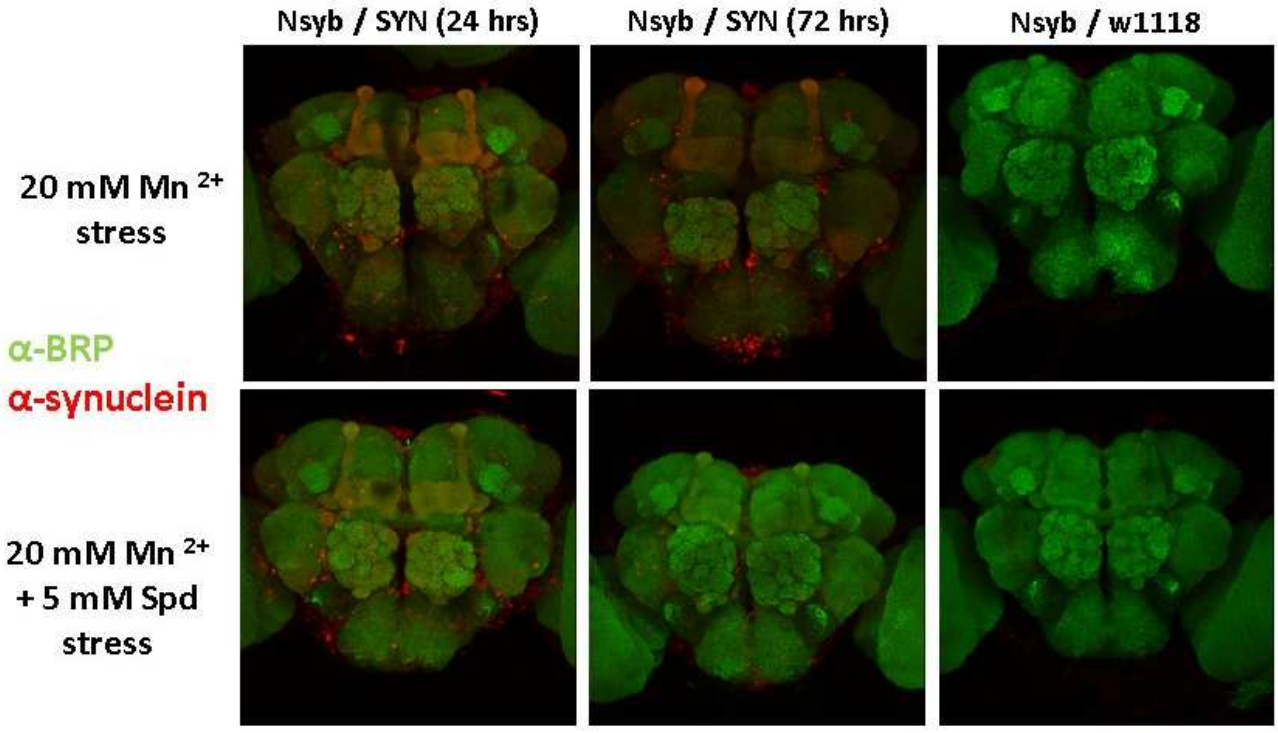

Figure 11: Signal intensity of α-Synuclein decreases upon spermidine (spd)-administration in samples of manganese (Mn^{2+}) stressed fruit flies. Flies were treated according to the Mn^{2+} stress setup protocol followed by brain dissections at 24 hrs and 72 hrs after treatment. Fly brains were stained with anti-α-syn antibody and anti-BRP antibody and were scanned in a Leica SP8 confocal microscope.

3.2 Amyloid-β-42 (Aβ-42)

3.2.1 Spermidine rescues Aβ-42 expressing flies in a manganese-dependent survival setup

In an illness such as Alzheimer's disease (AD), environmental and genetic risk factors have been shown to contribute to the development of pathological symptoms. Here, we used fruit flies expressing the human Amyloid-β-42 protein under the control of the UAS/Gal4-system in an animal research approach. The flies have been shown to die within 5-7 days after being additionally exposed to Mn^{2+}, a known factor that increases physical stress in the flies' metabolic system[59].

We adopted the setup from previously conducted studies to analyze if spd, a known antioxidant which has already shown beneficial effects in several model organisms[60], is able to ameliorate the detrimental effects of Aβ-42-dependent decline in survival.

Using a pan-neuronal driver line, we could detect that supplementation of 5 mM spd, administered via food, is able to continuously improve the flies' ability to survive upon high metabolic stress rates, thus leading to a prolonged lifespan.

There is a prominent phenotype of antecent death of the Aβ-42 expressing flies and this effect is significant in flies aged for 12-14 days and is also seen in young (1-3d old – see Figure 12 males in A and females in B) animals. We could also show a clear difference between male and female flies, especially in young animals, where the males showed a more distinct rescue by spd than the females (see Figure 12). In the aged flies, females were the ones to show better survival upon spd administration (see Figure 13).

The presented graphs for young flies are the outcome of at least 8 independent experiments with a number of approximately 1.000 flies per sex and per genotype. For the aged flies, we had an n=4 experiments with approximately 480 flies per sex and per genotype. Here we were able to determine a P-value of less than 0,001 in Figure 13.

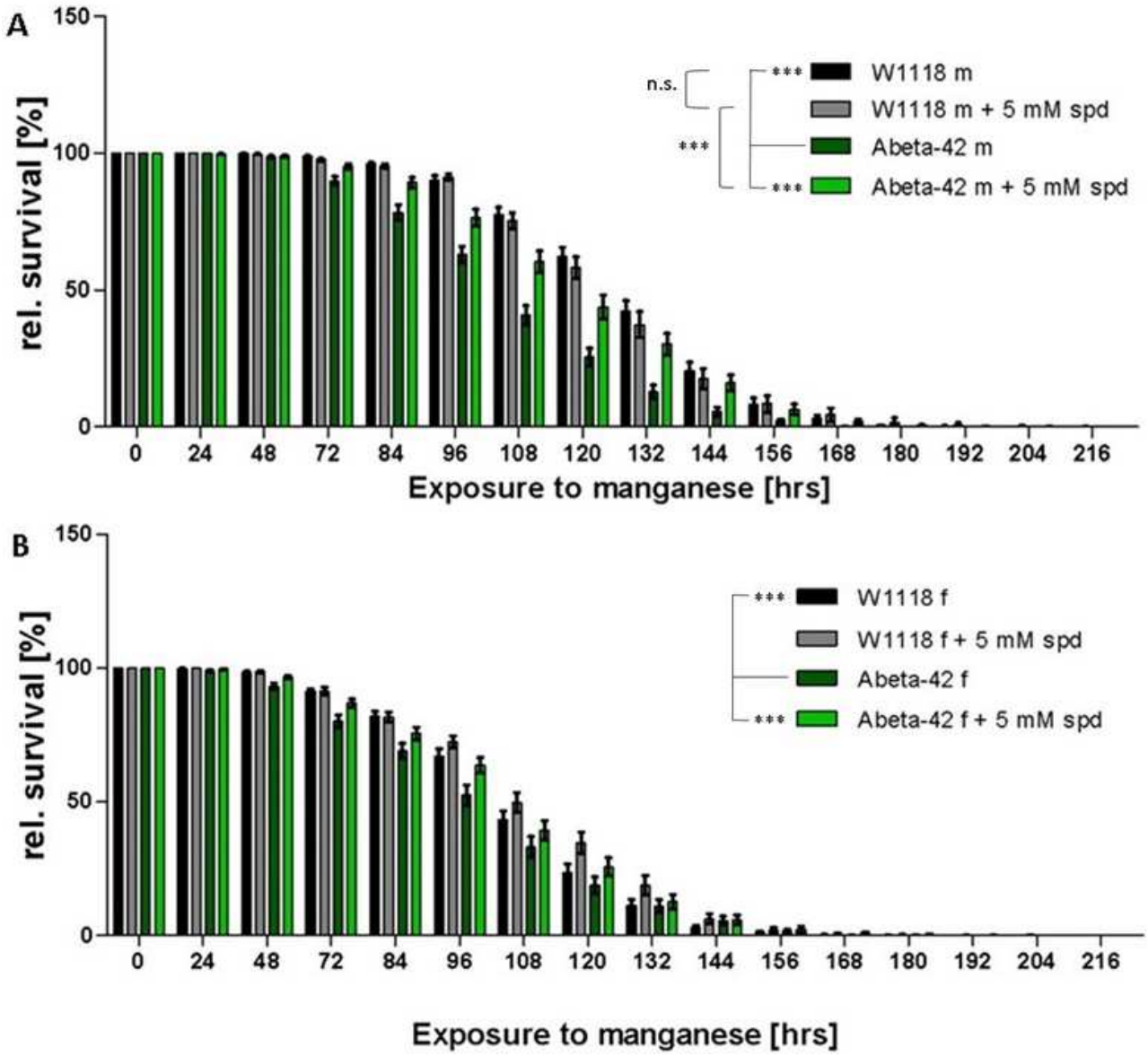

Figure 12: Administration of 5 mM spermidine (spd) rescues antecent death of young Aβ-42 expressing *D. melanogaster* upon acute manganese (Mn^{2+}) stress. Young wild type and Aβ-42-expressing flies (2-5 days) were kept on 10% sucrose supplemented with 20 mM Mn^{2+} with and without addition of 5 mM spd and survival was determined at indicated time points over a period of 5-7 days. Expression of Aβ-42 was achieved by using the UAS/Gal4 system and the pan-neuronal driver line elav(x)-Gal4. Male flies are shown in **(A)**, females are shown in **(B)**. The graphs were generated based on data of 8 independent experiments with approximately 1.000 flies per sex and per genotype. Statistical values: For **(A)**, we could calculate p-values of less than 0,05 (Aβ-42 vs. w1118 at 72 hrs), less than 0,01 (for Aβ-42 vs. Aβ-42 + spd at 84 hrs and 144 hrs) and less than 0,001 (for Aβ-42 vs. Aβ-42 + spd at 96 - 132 hrs, for Aβ-42 vs. w1118 at 84 – 144 hrs and for Aβ-42 + spd vs. w1118 + spd at 120 hrs) – the comparison between w1118 and w1118 + spd was not significant at any timepoint. For **(B)**, we could calculate the following p-values: less than 0,05 for Aβ-42 + spd vs. w1118 + spd at 96 hrs and 120 hrs, less than 0,01 for Aβ-42 vs. w1118 at 108 hrs, for Aβ-42 + spd vs. w1118 + spd at 108 hrs and a p-value less than 0,001 for Aβ-42 vs. Aβ-42 + spd at 96 hrs, for Aβ-42 vs. w1118 at 72, 84, 96 hrs and for w1118 vs. w1118 + spd at 120 hrs. These p-values were calculated using GraphPad with a Two-way ANOVA analysis with Bonferroni correction**.**

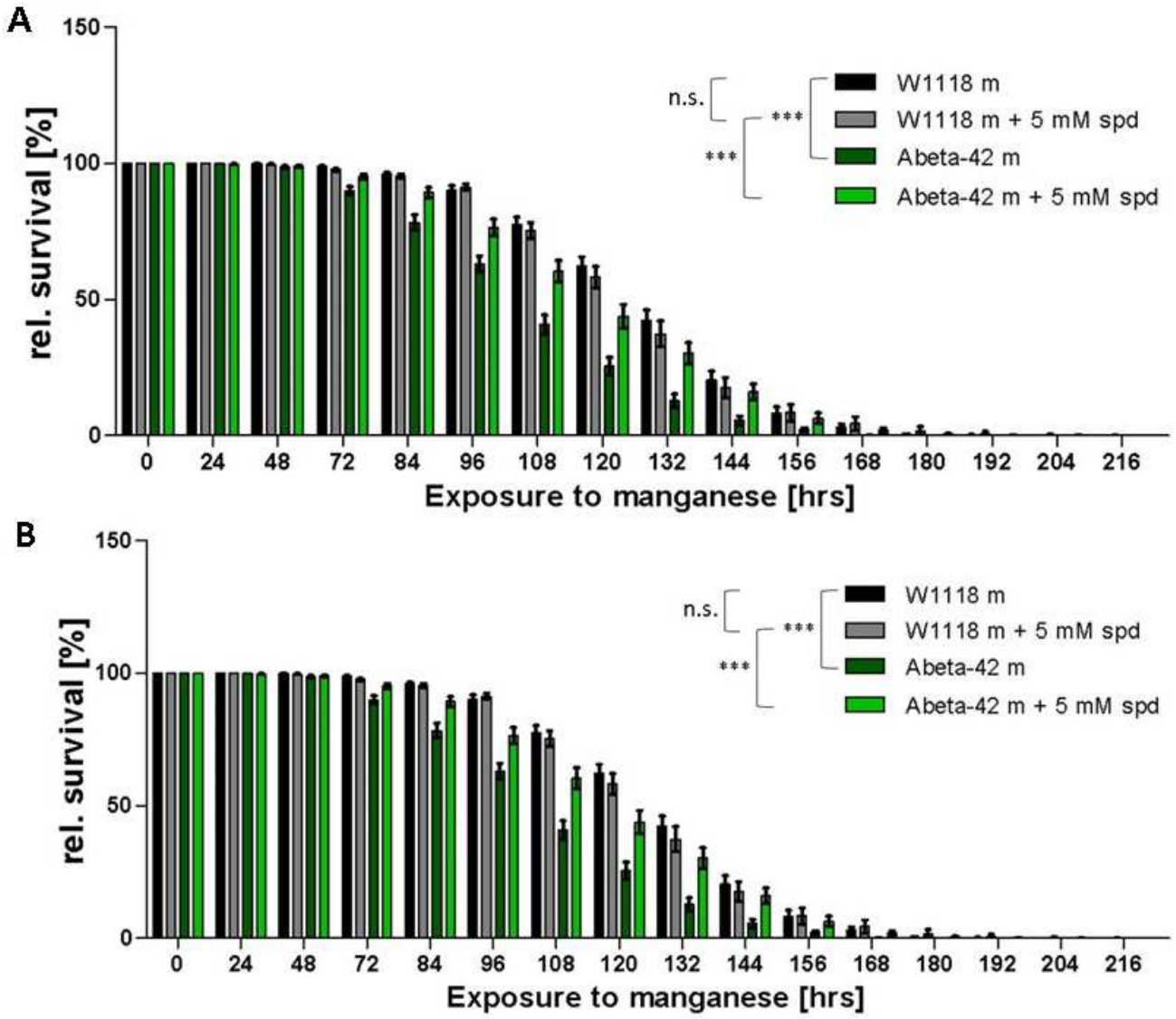

Figure 13: spermidine (spd) treatment significantly rescues 14 days-aged Aβ-42-expressing *D. melanogaster* in a manganese (Mn^{2+}) stress setup. Young Aβ-42-expressing flies were separated 1-3 days after eclosure, kept at 25 °C for 13 days and then fed with a solution containing 10 % sucrose with 20 mM Mn^{2+} with and without addition of 5 mM spd. The survival rates were determined at indicated timepoints over a period of 5-7 days as shown above. Expression of Aβ-42 was performed using the UAS/Gal4 system and the pan-neuronal driver line elav(x)-Gal4. Male flies are shown in **(A)**, females are shown in **(B)**. The graphs were generated based on an n=4 experiments (approximately 480 flies per sex and per genotype) and all the following p-values could be calculated using GraphPad with a Two-way ANOVA analysis with Bonferroni correction. Statistical values: For **(A)**, we could calculate p-values of less than 0,05 (Aβ-42 vs. Aβ-42 + spd at 84 hrs and for Aβ-42 + spd vs. w1118 + spd at 144 hrs), less than 0,01 (for Aβ-42 vs. w1118 at 144 hrs and for Aβ-42 + spd vs. w1118 + spd at 48 hrs) and a p-value of less than 0,001 (for Aβ-42 vs. w1118 at 48 - 132 hrs and for Aβ-42 + spd vs. w1118 + spd at 72 – 132 hrs) - the comparison between w1118 and w1118 + spd was not significant at any timepoint. For **(B)**, we could calculate the following p-values: less than 0,05 for Aβ-42 vs. Aβ-42 + spd at 108 hrs, for Aβ-42 vs. w1118 at 48 hrs and for Aβ-42 + spd vs. w1118 + spd at 144 hrs, less than 0,01 for Aβ-42 vs. Aβ-42 + spd at 84, 96 hrs and for Aβ-42 + spd vs. w1118 + spd at 72 hrs and a p-value less than 0,001 could be calculated for Aβ-42 vs. w1118 at 72 - 132 hrs and for Aβ-42 + spd vs. w1118 + spd at 84 - 132 hrs - the comparison between w1118 and w1118 + spd was not significant at any timepoint.

3.2.2 Establishing Aβ-42-transgene expression in *D. melanogaster*

To investigate the rescue phenotype of spd-treated Aβ-42 flies more closely, we performed more molecular methods on flies coming from the above mentioned stress setup. We wanted to determine an eventual change in the expression levels of the different groups of flies coming from the stress setup. Therefore, we used head extracts of Aβ-42 expressing flies that were collected under different conditions and at different timepoints to identify possible effects, spd could have on protein expression in a Western blot analysis. In order to also show the functionality of the used driver line (elavx), we took flies that did not have the transgenic construct and were crossed with our driver line (elavx>w1118) as control samples.

To determine the effect, spd has on the expression level or if the expression varies with time, we used the F1 generation flies from a normal survival setup of 20 mM manganese (Mn^{2+}) and 20 mM Mn^{2+} with 5 mM spd solution, respectively. Here we collected the flies at timepoints where we believed the effects could be visible in a Western blot analysis (at 24 h and 72 h after Mn^{2+} application).

As shown in the Western blot in Figure 14, the Aβ-42 protein band is quite faint when using a short exposure time (picture part below), therefore we also show a longer exposure time (above). We probed the membrane with the 6E10 Aβ-42-antibody, and used α-tubulin as the loading control.

There is no difference between samples from flies fed with or without spd, as we could show in the indicated box below. We had samples from the driver control (driver line elavX crossed with w1118), samples from the transgene control (Aβ-42 crossed with w1118 – undriven transgene) and samples from the pan-neuronally driven construct (elavX>Aβ-42). The controls do not show signs of Aβ-42 expression what shows that the transcription system (UAS/Gal4) is very tightly regulated.

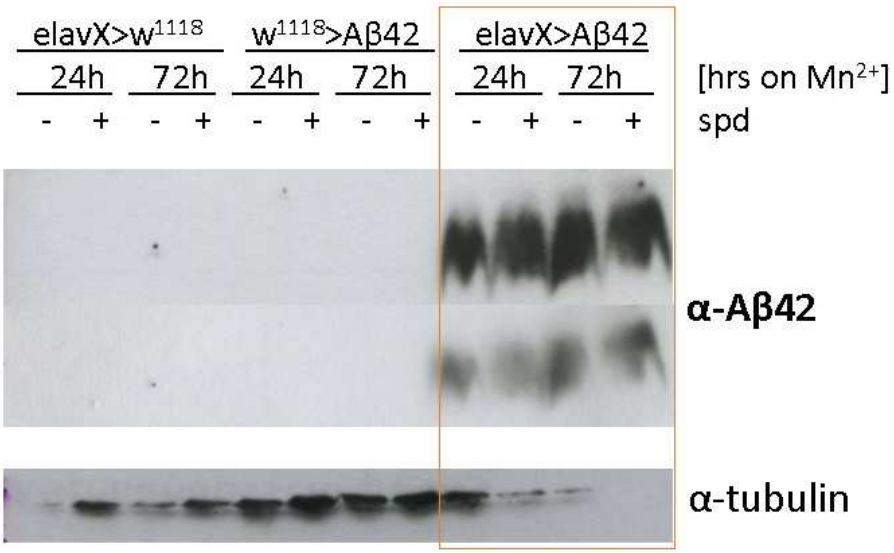

Figure 14: Successful establishment of transgenic Aβ-42-expression in *D. melanogaster*. We performed a Western blot analysis using an Aβ-42 specific antibody (6E10) to probe the obtained head extracts from flies that were collected at the indicated timepoints (with and without spermidine). Flies were immediately frozen at -80 °C before being treated according to the head extraction protocol. We used two genetic controls which are the driver control (elavX>w1118) and the transgene control (w1118<Aβ-42 –undriven construct) to compare them to the band of the 4kDa–sized Aβ-42 protein. This upper band, however, is quite faint, therefore we show two different exposure times. The lower band shows the anti α-tubulin probed loading control.

3.2.3 Administration of spermidine cannot prolong shortened lifespan of Aβ-42 expressing flies

AD, like PD, is a late onset and progressive neurodegenerative disorder that leads to severe deficiencies in several aspects of the patient's daily life. In humans, one of the symptoms, besides others, is a shortened lifespan of affected patients that get diagnosed in later stages of the disease. To address the question whether spd can ameliorate the shortening of the lifespan, we used a common setup using the fruit fly as an animal model system. Our hypothesis consisted on the presumption that spd might work as an antioxidant during the flies' life cycle, as it had a beneficial effect in the aforementioned Mn^{2+}-stress setups.

Concerning this longevity setup, we used a different approach for the administration of spd; since flies could not be kept in the carousels for 80 days, we kept them on normal fly food containing 5 mM spd.

We used F1-generation animals expressing Aβ-42 pan-neuronally (elavX>Aβ-42) and the driver control flies (elavX>w1118). Neither of them could be rescued by administration of 5 mM spd in the food, as is shown in Figure 15. The Aβ-42 expressing animals were severely

affected in their physical fitness which led to a dramatically reduced lifespan compared to the control group. Individual flies that became stuck in the food, escaped or were accidentally killed were entered as censored data.

In the graphs in Figure 15 we show a representative example figure for all of the experiments that were performed. To sum it up, the tendency was the same in every experiment, namely the premature death of the Aβ-42 expressing flies. Even with the fly food containing 5 mM spd, this demise could not be decelerated or even stopped.

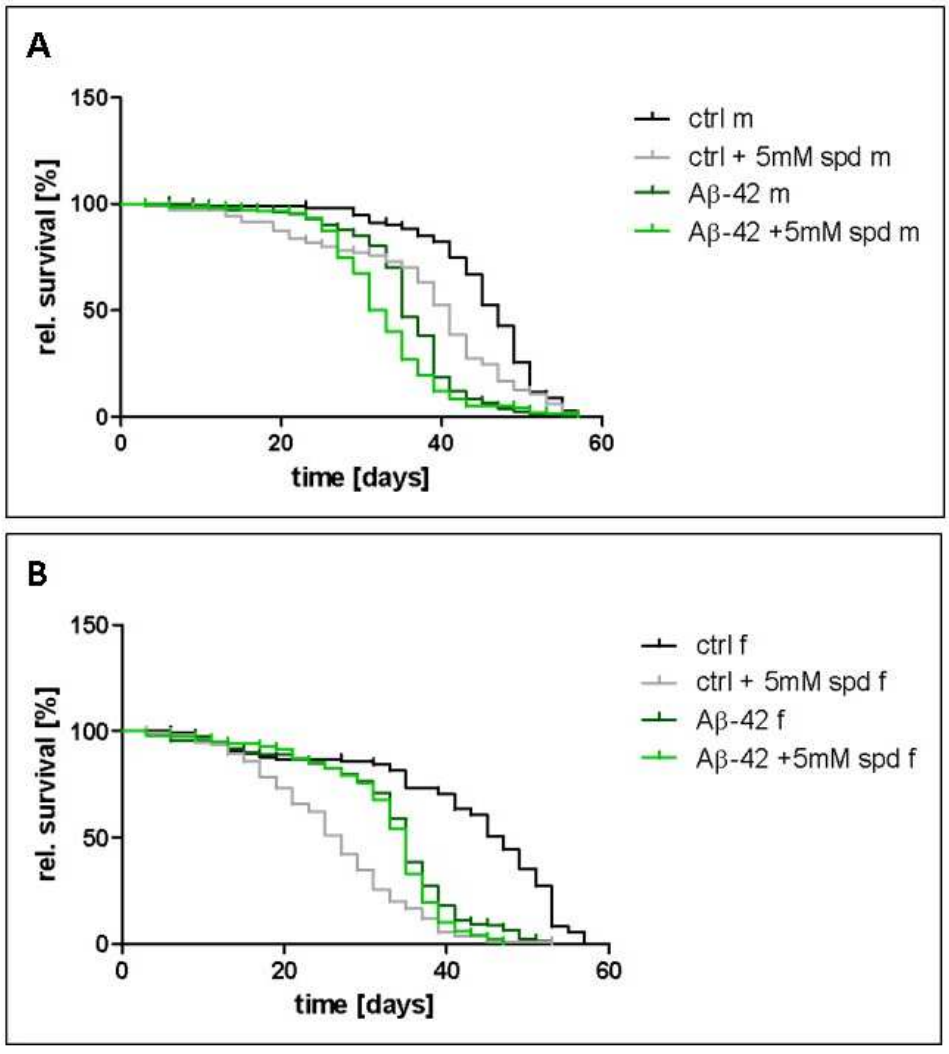

Figure 15: Spermidine (spd) cannot rescue the dramatically decreased lifespan of Aβ-42 expressing flies in the longevity setup. Young flies (born on food with or without addition of 5 mM spd) were collected one day after eclosion, let mate for 24 h, separated in males and females and put in vials containing normal fly food with or without 5 mM spd. Flies were flipped on fresh food every second day and the dead flies were counted to calculate lifespan curves. Male flies are shown in (A), females in (B).The graphs here are just a representative example of the whole experimental recurrence and are based on the data of approximately 180 flies per sex and per genotype.

Given the results of the experiments with fly food containing 5 mM spd, we carried out some more experiments with 1 mM spd in the food and we also tried to treat flies not from the beginning of their adult life but to give them 1 mM or 5 mM spd from their early middle lifespan on (day 20 after eclosion). These experiments and the consequences that can be learned from the graphs above will be talked about in the discussion section.

3.2.4 Spermidine ameliorates manganese-mediated locomotor dysfunction in Aβ-42 expressing fruit flies

Since one of the hallmarks of neurodegenerative diseases such as AD and PD is the loss of the ability control one's locomotor functions e.g. independently walk or conduct simple daily routines especially in later stages, we tried to find a scenario where we could test these locomotor difficulties in the fruitfly *D. melanogaster*.

To determine whether the effect of Aβ-42 expression on locomotion that was seen in the longevity experiments before could be restored to a normal level upon administration of spd, we used the flies' inherent property of negative geotaxis, which makes them crawl towards the top of a vertical vial. Given the results of prior experiments with the Mn^{2+}-stress setup, we used the same setting to test decline in locomotion upon Mn^{2+}-stress with or without the addition of 5 mM spd.

In these locomotion experiments, we tested the climbing ability determining the different capabilities of Aβ-42 expressing flies compared to the control group. We were able to monitor the decreasing locomotion skills of the affected transgenic flies during the time course of the experiments.

As shown in the graph below, Aβ-42 expressing flies were hardly able to overcome a height level of 7 cm (3-5 d old animals) or 4 cm (13-15 d old) within 15 seconds. Furthermore, we determined the restoration of the locomotor activity upon spd administration.

As can be seen in Figure 16, we saw a progressive decline in the locomotion over time depending on the age of the tested animals, starting with nearly 100% in the group of the young flies (after 24 h of stress) crossing the 7cm-line within 15 seconds leading to only 40% of the flies crossing the line after 72 h of stress.

With the aged flies, this decline was even more obvious (as shown in Figure 17). The flies were hardly able to climb the 4cm-line within 15 seconds, even after only 24 h of Mn^{2+}-stress. After longer exposure to Mn^{2+}, they were hardly able to move at all and only a few could cross the line.

These effects were very pronounced in female animals, but not so obvious in males. The graphs in both figures below are only showing data from female flies and are the result of 3-4 independent experiments with 160-320 flies per sex and per genotype.

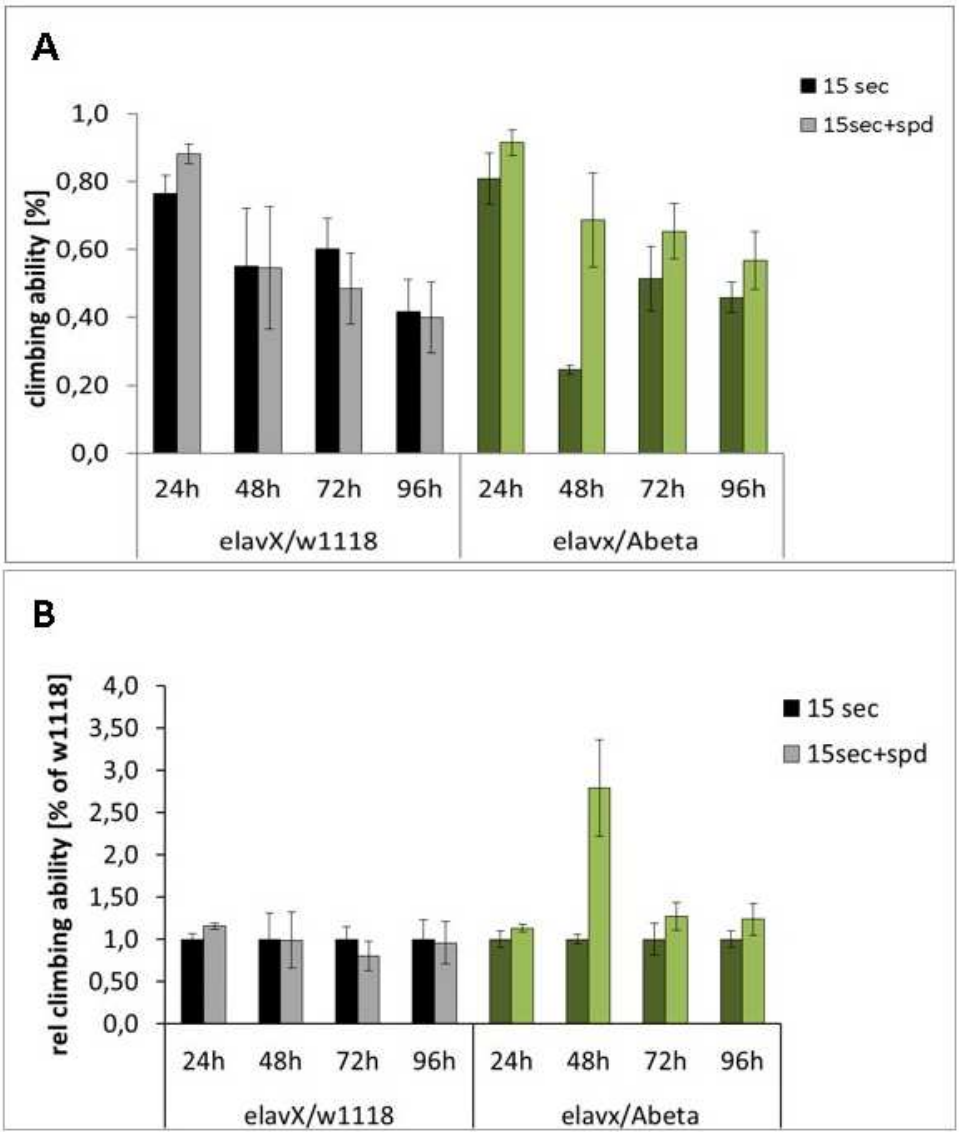

Figure 16: Restoration of climbing ability in young Aβ-42 expressing flies upon administration of 5 mM spermidine (spd). Young animals were separated 1-3 days after eclosure and were subsequently treated with a 20 mM manganese containing solution with or without addition of 5 mM spd. The flies were tested on their ability to overcome a height of 7 cm in a vertical vial at the indicated timepoints. In (A) the absolute ability to cross the 7-cm-line is shown; in (B) the relative ability is shown normalized to the 24 h value of the control flies. These graphs only show data for female flies and are the result of 3-4 independent experiments with a total number of approximately 160-320 animals and per genotype.

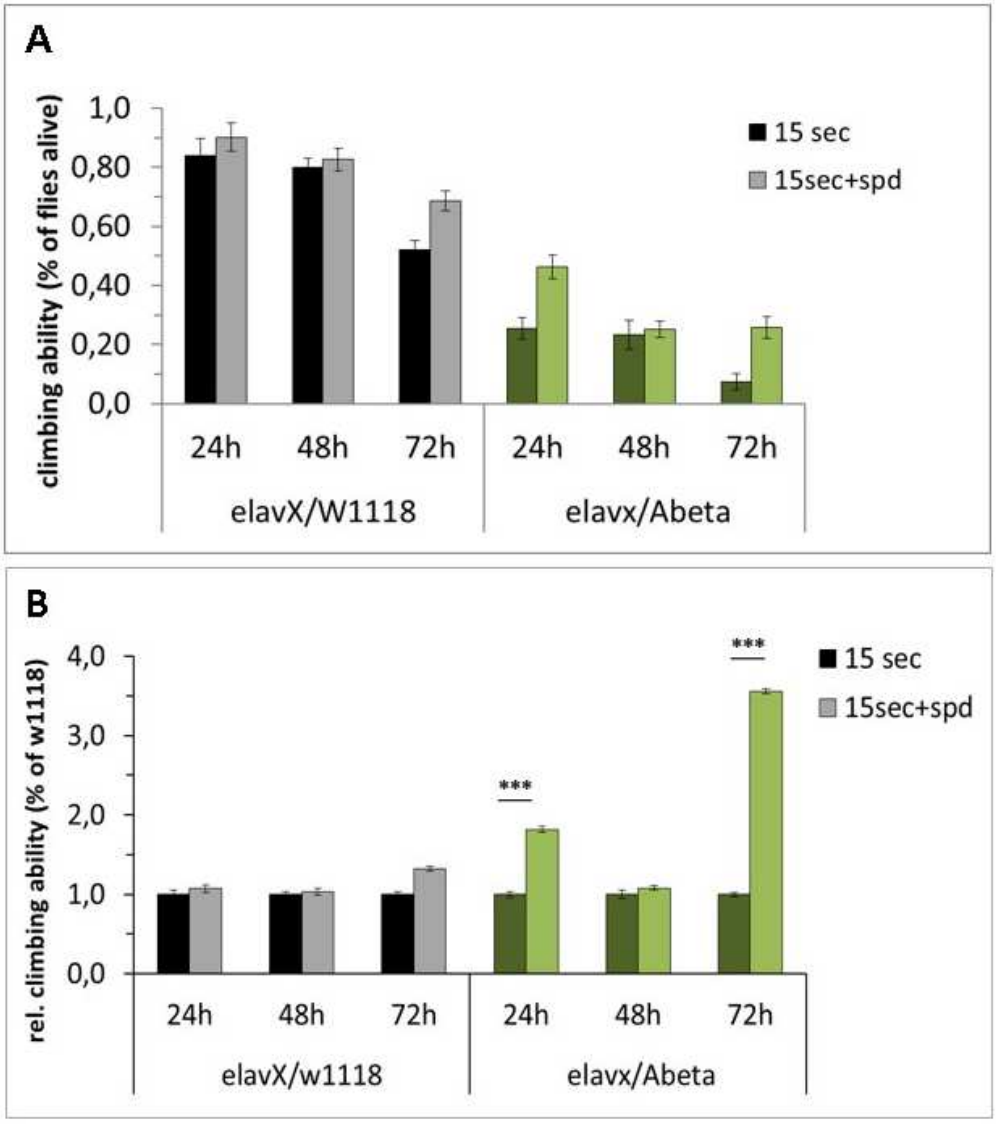

Figure 17: Significant restoration of locomotion activity in aged Aβ-42 expressing *D. melanogaster* upon treatment with 5 mM spermidine (spd). Young flies were separated 1-3 days after eclosion, let age at 25 °C for 13 days and subsequently treated with a 20 mM manganese containing solution with or without the addition of 5 mM spd. The flies were tested on their ability to overcome a height of 4 cm in a vertical vial at the indicated timepoints. In (A) the absolute ability to cross the 4-cm-line within 15 seconds is shown; in (B) the relative ability is shown normalized to the 24 h value of the control flies. In these graphs, only data for female flies is shown. The curves are the result of 3-4 independent experiments with a total number of approximately 160-320 animals per genotype. The computed P-value was less than 0,001 for the three-star significance shown here.

3.2.5 High staining intensity in mushroom bodies of Aβ-42 expressing flies is decreased upon spermidine treatment

AD is a progressive and late onset neurodegenerative illness that affects several fields of the patient's daily life. Besides the serious memory deficits of the patients, another hallmark of late stages of disease is the above mentioned locomotor deficiency that worsens during pathogenesis and leads to complete physical dependency on others.

As we could observe before in the negative geotaxis of the fruit fly model system that we used, the decline is obvious and very strong so that we assume a neurological cause of this phenotype. We conclude that the fast decline in locomotion has to come along with a substantial loss of unknown neuronal populations in the fly brain responsible for movement coordination.

To investigate this process in more detail we used the very simple but elaborated central nervous system of the fruit fly which also includes a specialized brain structure. We hoped to find areas in the brain which are connected to the various behaviors of the flies and are severely affected by expression of Aβ-42 showing signs of eventual damage of distinct populations of neuronal cells.

After the dissection of fly brains we stained the tissue with two different antibodies, one of them reacting against the intrinsic synaptic protein Bruchpilot (BRP) and the other one reacting against the transgenic construct Aβ-42 expressed in the flies' central nervous system via the elav(X) driver line. Flies were collected from the Mn^{2+} stress setup at the timepoints showing the most prominent results in prior experiments. We added one group of animals consisting of flies that were born on spd containing food and stressed with Mn^{2+} only to show whether the effect of spd just depends on the simultaneous application of Mn^{2+}.

We compared the staining intensities of the dissected brains scanned in a Leica SP8 microscope. In the examined brains, the staining of the mushroom body was high in animals expressing Aβ-42 protein (elavx>Aβ-42) and this staining is not seen in animals of the control group (elavx>w1118).

Non-spd born animals fed with Mn^{2+} only, showed a very high intensity in the detection channel for Aβ-42 (red) in their mushroom body areas, whereas the stained brains from flies simultaneously treated with spd showed a much lower intensity in this region. Also the brains of flies born on spd-containing food that were subsequently stressed with Mn^{2+} only, exhibit a lower staining level in this area.

3.3 Recombined flies

3.3.1 Successful establishment of a transgenic *D. melanogaster* fly line containing *ODC1* and *α*-synuclein on the same chromosome

As we could show before, spd is a powerful protectant of stress caused by manganese in a PD and AD disease model of the fruit fly *D. melanogaster*. As a consequence of this model we wanted to determine a possible effect of the intrinsic spd-generating enzyme Ornithine-Decarboxylase 1 (ODC1) on flies simultaneously expressing either the major protein of PD or AD, α-syn or Aβ-42, respectively.

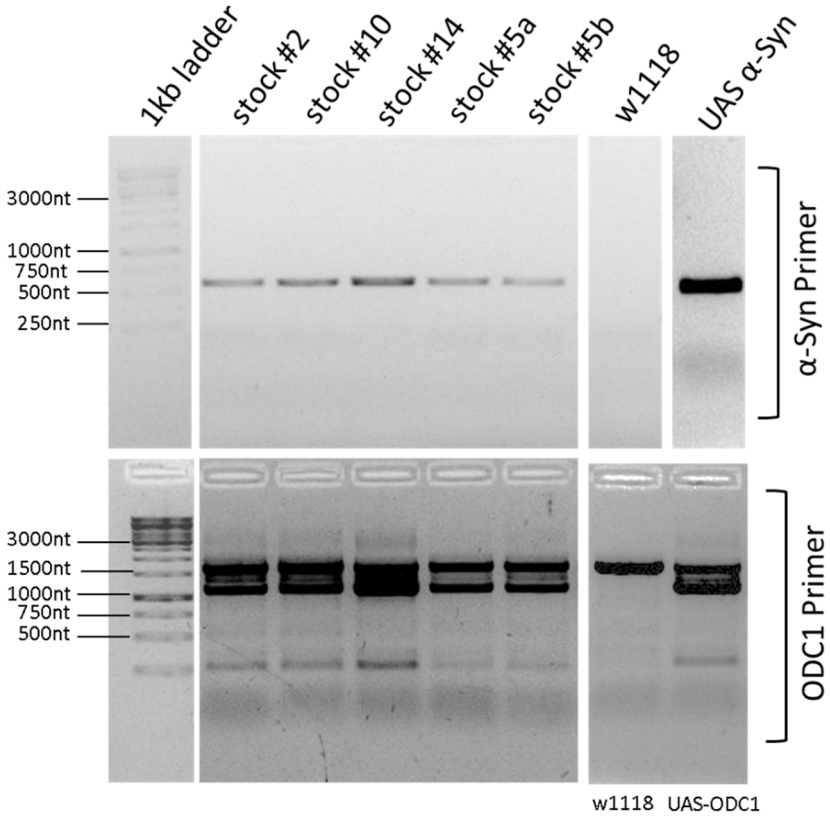

Figure 18: Verification of the concomitance of desired α-synuclein and ODC1 gene in one fly after recombination. Flies were obtained after several crosses from maternal and paternal genetic recombination during embryogenesis. The desired genes were traced by the eye color of the F1 flies after each crossing step. To confirm the successful recombination of the two desired genes, we performed a PCR with the different primer pairs and gained distinct bands for the amplified fragments (α-syn at 420 bp and ODC1 at 1274 bp).We used 7 µL of 1 kb GeneRuler DNA ladder from Fermentas as a standard in the first lane followed by the samples of the newly generated recombined flies, the negative control for α-syn (w1118) and the α-syn positive control (from the sequence verified stock) in the top part of the picture. In the lower part, after the standard, the same samples were amplified with the ODC1 primers followed by the negative control (w1118 which contains the intrinsic ODC1 of the fly which is seen as the upper band of the double-band seen in the other samples) and the positive control for the ODC1.

To address this question, we recombined the two respective genes on the same chromosome under the control of the GAL4-UAS-system. After several crosses, the flies were controlled via an appropriate PCR-program to prove the concomitance of the two desired genes. As can be seen in Figure 18 above, the obtained stocks are positive for both the UAS-*ODC1*- and the UAS-α-syn-gene. The obtained and sequence-verified flies were incorporated in the laboratory's stock collection to be used for further experiments.

3.3.2 Successful establishment of a transgenic *D. melanogaster* fly line containing *ODC1* and *Aβ-42* on the same chromosome

We assumed the same hypothesis for the AD-flies than we did for the PD- flies in the chapter before. If the intrinsic Ornithine Decarboxylase 1 (ODC1) is able to help the fly produce as much spd as it needs to be protected against the health-concerning impact of the expressed transgenic protein, we could mimic the effect of the orally administered spd given in the Mn^{2+}-stress setup.

As mentioned above we recombined two genes on the same chromosome in one fly. Again after some crossing rounds, the flies were controlled via a matched PCR-program to prove the presence of the two desired genes. As shown in Figure 19 below, we got some positive stocks (for both the UAS-ODC1- and the UAS-Aβ-42-gene) which were incorporated in the laboratory's stock collection (after sequence-verification) to be used in further experiments.

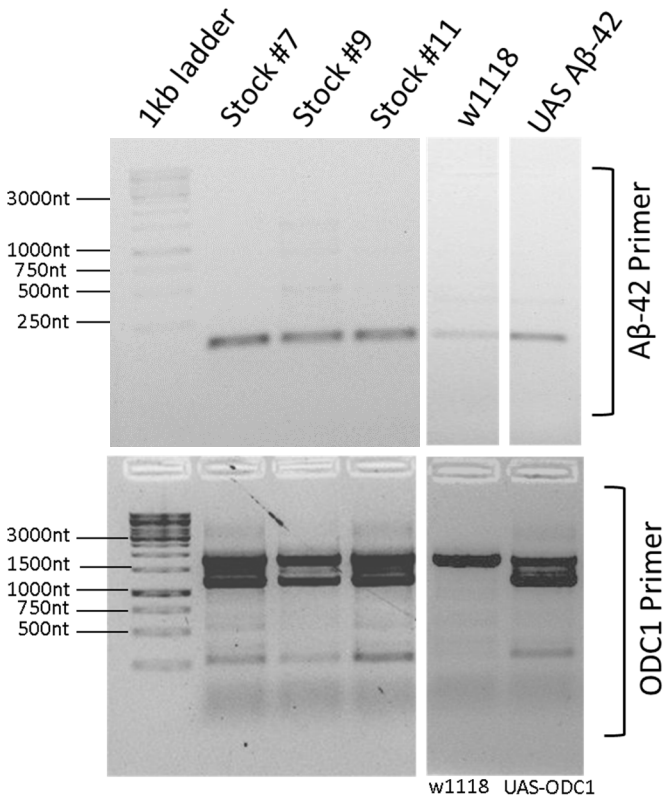

Figure 19: Verification of the coexistence of desired Aβ-42 and ODC1 gene in one fly after recombination. Flies were obtained after several crosses from maternal and paternal genetic recombination during embryogenesis. The desired genes were traced by the eye color of the F1 flies after each crossing step. To confirm the successful recombination of the two desired genes, we performed a PCR with the different primer pairs and gained distinct bands for the amplified fragments (Aβ-42 at 96 bp and ODC1 at 1274 bp). We used 7 µL of 1 kb GeneRuler DNA ladder from Fermentas as a standard in the first lane followed by the samples of our freshly generated flies, the negative control w1118 and the Aβ-42 positive control (from the sequence verified stock) in the top part of the picture. In the lower part, after the standard, the same samples were amplified with the ODC1 primers followed by the negative control (w1118 which contains the intrinsic ODC1 of the fly which is seen as the upper band of the double-band seen in the other samples) and the positive control for the ODC1.

3.3.3 Recombined flies do not show the same survival rate in the manganese stress setup than the flies treated with spermidine

Since we were able to generate flies with a third chromosome containing both the disease-causing Aβ-42 or α-syn gene and the intrinsic, but now overexpressed ODC1 gene, we tested them in the same Mn^{2+}-stress setup as the other animals. This previous setup revealed a beneficial effect of spd on the animals expressing either Aβ-42 or α-syn, so we thought introducing higher amounts of the intrinsic rate limiting enzyme ODC1 would simulated these effect under the same stress conditions.

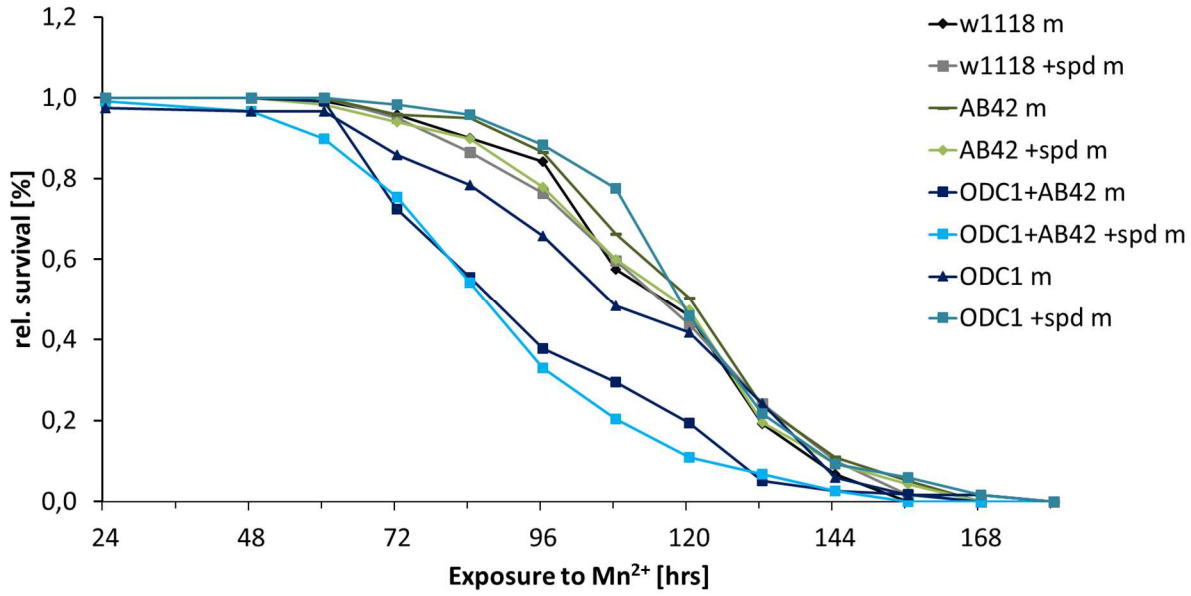

Figure 20: Flies with recombined chromosome containing ODC1 gene die faster in the manganese (Mn^{2+}) stress setup. Young wild type, Aβ-42-expressing, recombined (ODC1 and Aβ42 expressing) and ODC1 expressing flies (2-5 days) were kept on 10% sucrose supplemented with 20 mM Mn^{2+} with and without addition of 5 mM spermidine and survival was determined at indicated time points over a period of 5-7 days. Expression of Aβ-42, ODC1 and the recombination of these genes was achieved by using the UAS/Gal4 system and the pan-neuronal driver line elav(x)-Gal4. This graph was generated based on the data of one preliminary experiment with approximately 120 flies per sex and per genotype.

What we could find was that the ODC1 seemed to have an effect on the animals but just with the help of externally added spd, as can be seen in Figure 20. We also tested the flies that were expressing the harmful Aβ-42 protein at the same time, detecting that overexpression of ODC1 does not help the diseased animals surviving the Mn^{2+}-stress better. The curve of the animals bearing the recombined chromosome with Aβ-42 and ODC1 on it was even declining faster indicating that these flies were dying more quickly – even faster than the animals that were just expressing Aβ-42.

4. Discussion

4.1 α-synuclein

4.1.1 Spermidine rescues manganese-induced death of α-synuclein-expressing flies

As there are manifold environmental and genetic factors contributing to the pathogenic conditions of Parkinson's disease (PD), we wanted to focus on the ones that can be influenced by human actions and appropriated use of specific drugs. Given that we were working in an animal model system, we had to mimic the effects of Parkinsonism (that are normally caused by specific metabolic stress factors in humans), in the fruit flies we were using. These stress conditions can be realized under different settings in the model system using various substances to aggravate the health conditions of α-syn expressing flies approaching similar symptoms as than the ones that are observed in humans.

In these experiments we wanted to show the beneficial effect of spd on flies that are kept under stress conditions. It is known that the most commonly used substance to induce morbus Parkinson in flies is Paraquat, although, we did not use that substance as it is poisonous to humans and the environment causing severe health problems (including lung, heart and liver damage[61]). Instead we used Mn^{2+} as a substance causing neurological alterations in flies (and humans)[62].

What we could find out was, that flies that were born on standard fly food and were acutely stressed with 20 mM Mn^{2+} only, were not able to survive that stress for more than some days. As we tried to ameliorate these symptoms, the flies did not really live longer but seemed to die at later timepoints. We also tested flies that were born and raised on 5mM spd-containing fly food and did not have any spd in the Mn^{2+}-stress treatment afterwards. They did not react a lot different to that stress treatment, than flies that did not have spd in the food before but were treated with spd simultaneously to Mn^{2+}.

Given that there were different sex-specific results for males and females in the survival curves, we only showed the coherent connected data in the results-part before (Figure 5, Figure 6). Here we can say that the overall tendency of the survival curves for both sexes is the same, namely towards a specific spd-rescue of the α-syn expressing flies compared to the control flies. The different behavior of the sexes in this setup needs to be evaluated further, but we are can say that spd is able to rescue both sexes, even if both react a bit differently.

After several experiments with different driver lines (elavX-Gal4 and nSyB-Gal4) in young and aged flies we saw that we needed to decide which driver line to take for which purposes. We saw, that the results in younger flies were more consistent and reproducible with the elavX-Gal4 driver line and in aged flies we had better performances with the nSyB-Gal4 driver line. Given that these two lines have a slightly different expression pattern in the developmental stages of the flies, we chose elavX-Gal4 for younger flies, as the expression of this line is not consistent when the flies get older than 2 weeks. For the aged flies we had to use a driver line that was being expressed over a longer period of time and also after the eclosion of the adult animals – nSyB-Gal4 is thought to be expressed longer in these stages, the expression also continues in the aged animals .

To find out whether the effect of spd-treatment has to do with an alteration of the expression level of the α-syn-protein, we directed the studies towards Western Blot analysis of the proteome.

4.1.2 Establishing α-synuclein expression in transgenic flies

As spd can inhibit Histone-Acetyltransferases (HATs) and therefore leads to hypoacetylation of histones which influences the protein expression of distinct regions of the genome, cells can age in a healthy manner avoiding oxidative stress[43]. Hence, we collected flies to extract the proteins from their central nervous system (CNS) in order to determine possible alterations in α-syn gene expression and oxidative damage statistically via computational analysis of performed Western Blots.

The expression of α-syn in the transgenic flies could be proven via those Western Blots, but the alteration of protein levels was difficult to determine. There is preliminary data for a slight difference concerning the protein amount in the cells, but the datasets are not yet validated.

We also had minor problems with the extraction of the proteins from the flies' CNS, as we were not able to detect any bands on the Western Blot membrane after the first trials. So we decided to use another blotting membrane-material changing from Nitrocellulose-membranes to PVDF-membranes, even changing the pore-size from 0, 45 µm to a smaller size of 0, 2 µm in order to avoid the losing of any protein molecules because of too large pore-sizes and too small proteins. After several trials with different lysis buffers and extraction methods we were able to obtain the western blot shown in 3.1.2. Given the hypothetical change in α-syn level in the cells upon spd-administration, this might lead to general changes inside the cells

approaching a healthy way of cellular aging. Therefore we also tested the animals upon their overall lifespan – and if this could be prolonged.

4.1.3 Spermidine does not prolong the lifespan of *D. melanogaster*

In the longevity experiments with our flies we wanted to show the potential influence of spd on the average lifespan of 40-60 days. We tried to determine if the average lifetime of a population can be changed upon the simple administration of the natural substance spd via the fly food. As already mentioned before (see 3.1.3) we could not find a definite answer to that question as several rounds of experiments brought controversial results. Some of the obtained datasets looked quite promising but their eventual outcome was being influenced by different batches of fly food and seasonal changes.

We observed that the flies are an extremely sensitive model organism, which makes it relatively difficult to obtain reproducible longevity results – at least in our case. Some collections of flies just lived longer than the others and we also saw the phenomenon of early hatching flies living longer/better than the later hatched ones which makes a big difference in a lifespan analysis. Even though, we synchronized the age of the flies by taking just one-day old animals, we still found a difference in the outcome of the experiments.

For the α-syn expressing flies, the applied concentration of 5 mM spd in the fly food seemed to not fulfill our expectations completely concerning a prolonged lifespan of the treated flies. The next experiments are planned to be conducted with a concentration of 1 mM spd in the fly food, that might change the outcome of the longevity test, as 1 mM spd might be sufficient over a long period of time and 5 mM might have been too much as a long term (lifelong) treatment.

As PD is known to not only influence only the lifespan and the survival, but also the locomotor abilities (as rigidity and tremor are the hallmarks in humans), we set up an experimental procedure to directly address this question.

4.1.4 Ability to overcome a certain height in negative geotaxis is enhanced upon spermidine-treatment

In the locomotion test of the transgenic α-syn expressing flies, we could directly test the climbing ability of the flies and get statistically utilizable data.

Here, we could show that the Mn^{2+} treatment in combination with spd-administration had a big influence on this aspect of daily flies' life. Flies that were tested under acute stress conditions showed severe perturbances in their locomotion ability.

In the previous setups, the age-variation of the flies did not matter a lot, here in contrast, we had to adjust the initial level of 7 cm for young flies (3-5 d old) to a height of 4 cm for the aged flies (13-15 d old). The young flies started with an initial value of around 90 % of animals to overcome the 7 cm and were declining in their ability to climb to about less than 70 % (in the w1118 control flies), but the α-syn expressing flies started at just 75 % ability to overcome 7 cm and were declining to about less than 50 % after several hours of Mn^{2+} stress. Hence we had to adjust the height for aged flies, because they were performing so poorly already at the first timepoint (not even 50 % could overcome the 7 cm level) in that same setup so we decided to lower the height to 4 cm to get acceptable results (starting at around 70%). We stayed at these height levels for the different age-groups also in the experiments with the Aβ-42 expressing flies (mimicking AD).

As the locomotion center of the flies is located in the CNS of the flies, we had the idea to also examine the fly brains at the same timepoints that we used for the locomotion test in a morphological approach in order to determine the level of protein staining.

4.1.5 Staining intensity of wholemount brains of α-synuclein-flies is not significantly changed upon spermidine administration

Since PD's main symptoms consist of the locomotion difficulties and the finding of α-syn containing aggregates in the cell bodies of dopaminergic neurons, we tried to find these stained regions in the responsible areas of the fly brains. In these brain scans we were able to detect a defined staining pattern of α-syn within the cells but we were not able to distinguish between eventual aggregates and just higher intensity because of possible higher expression of the protein in the affected cell bodies.

After all, the method revealed a higher staining intensity in the mushroom bodies and in the cell body-regions, where the used driver line, even if it was supposed to have a uniform pan-neuronal expression, seemed to have local maximas.

Nevertheless we were interested in the differences between the indicated timepoints of the Mn^{2+} stress setup and the possible influence of the spd treatment. We could find, that the administration of spd seemed to lead to a lower relative staining intensity of α-syn in the

whole fly brain. The housekeeping-gene Bruchpilot that we used as a control could not be used for comparison purposes, as the intensity changes within aging of the flies[64].

With these results we can conduct further experiments coming to a significantly valuable number of stained brains so we can test the hypothesis of spd reducing the amount of α-syn in the fly brain – either by influencing the expression itself or by inducing autophagy via activation of ATG-genes. To address this hypothesis, we are currently developing a ref2p/p62 antibody that should be used as an autophagy-induction indicator in the cells, as ref2p/p62 is a key regulator upon autophagy induction[63]. We are also currently working on analyses concerning the loss of dopaminergic neurons upon α-syn overexpression – using an anti-TH antibody that stains dopaminergic neurons (already used but statistically not yet evalutated – work in progress).

4.2 Amyloid-β-42 (Aβ-42)

4.2.1 Spermidine rescues Aβ-42 expressing flies in a manganese-dependent survival setup

Given that AD patients have a reduced cellular oxidative stress resistance capacity, we wanted to find out if this fact can be ameliorated in our fly model by administration of spd in a Mn^{2+}-stress setup. Some theories suggest that spd enhances autophagy and thus gives the cells the possibility to better cope with stress. In this setup we used the Mn^{2+} stress to mimic intracellular reactive oxygen species generation which leads to various damages within the cell including oxidation of proteins and thus leading to higher toxicity of Aβ-42 species.

Here, we could find that flies respond to spd administration positively showing better survival upon Mn^{2+}-stress. We followed the experiments for approximately 8-9 days with countings of dead flies every 24 hours for the first 2 days and then every 12 hours for the following days every day at the same time to synchronize the experiments and make them comparable to the others. As we could find out spd seems to enhance the stress resistance capacity in fruit flies but the pathways remain obscure.

4.2.2 Establishing Aβ-42-transgene expression in *D. melanogaster*

To determine, whether the survival was due to a reduced Aβ-42 protein amount in the fly heads, we conducted a Western blot analysis where we tried to show a dosage-dependent change in the amount of protein detected on the membrane.

Therefore we treated the flies for 24 h or 72 h with Mn^{2+} only or Mn^{2+} and spd simultaneously in the normal survival setup. We found that there was a discrepancy between our hypothesis and the actual outcome on the Western blot, namely were we not able to state, that spd might help to reduce the amount of protein found in the cell and this protein reduction can also be detected on the blot. The reasons for this divergence between theory and reality might be manifold. We think that here we might have to separate expression and protein detection. On one hand, continuous expression leads to a lot of protein which can then be made visible on the membrane, on the other hand, even if the expression is already stopped, the half-life of the protein matters a lot in detecting it in a subsequent Western blot. What we can see very well in the blot is the clear absence of the transgenic protein in the control groups; the flies, which had a non-driven construct (elavX>w1118 and Aβ-42>w1118) did not show any detectable traces of the desired proteins (neither APPL, nor Aβ-42).

We could also not prove the tested hypothesis that spd might alter the amount of Aβ-42-protein in the cell during stress-conditions. Our theory was that during oxidative stress (Mn^{2+}) the cells might need more antioxidant substances (such as spd) to repair or prevent cellular damage. In this case, we thought that upon spd-treatment, the amount of detectable Aβ-42 in the cell might be lower than without spd. To prove that assumption we need to establish a very sensitive assay in the future because it was not possible to see any changes in the actual Western blot analysis.

What makes the Western blot a bit difficult here is the fact that the protein cannot easily be separated from the big bulk of other proteins in a normal SDS-page, due to its size. α-syn itself is very small and is said to forms aggregates in the cell, which might be one reason for the difficult extraction. We needed to perform the separation in a 20% gel, not a precast gradient gel and be very careful not to lose the last band of the marker (10 kD) during the separation. After several attempts we were able to find the best extraction-buffer-separation combination to finally even detect the bands visible in Figure 14.

The band itself is rather dispersed what may be due to the small size of the protein and the difficult separation. Because of the correlation between Aβ-protein amount in the neurons and patho-phenotype, we also wanted to determine the lifespan and the possible effects spd could have on that aspect of the life of the animals.

4.2.3 Administration of spermidine cannot prolong shortened lifespan of Aβ-42 expressing flies

Since patients with diagnosed AD have a remarkably reduced lifespan when the disease is manifest[64], we addressed this question by determining the middle lifespan of our flies expressing Aβ-42 protein in their central nervous system. We could find out that flies that were growing on spd-containing fly food were developing faster and had bigger larvae at the same stages where the flies developing on normal fly food had smaller larvae. We think this was due to the important role spd plays in the fertilization process in several model organisms, which could also be true for *D. melanogaster*[65]. Additionally we found that cohorts of flies hatching from earlier laid eggs were fitter and tend to live longer than flies hatching from eggs that were laid later in the experiment. These earlier cohorts also had slightly different survival curves during the longevity timecourse, but this phenotype needs further investigations to be validated.

Additionally, we let the flies mate before separating them to use in the experiments, which seems to have an influence on their stress levels. Mated females seemed to be able to cope better with stress when separated from males than unmated females. Unmated females seemed to die earlier when kept in big bottles (populated with ca. 150 animals) prior to dividing them into portions of 20 flies (and putting them into small vials) and starting the longevity experiment.

As mentioned in 3.2.3, we could show that spermidine administrations tends to kill flies prematurely before they reach their average life span. We were able to show the possible beneficial effect of spermidine in one single experiment where we obtained a different distribution towards spd-rescue in the survival curves. In 3.2.3 we show that spermidine can rescue the Aβ-42 expressing flies even to a higher extent than the control flies indicating a specific rescue for “ill” animals. Given this encouraging data, we were keen on determining the effect spermidine has on the locomotion abilities of the treated animals in climbing experiments.

4.2.4 Spermidine ameliorates manganese-mediated locomotor dysfunction in Aβ-42 expressing fruit flies

As the longevity experiments did not show any consistent results, we wanted to examine another aspect of the simulated disease in these animals, namely their ability to perform normal locomotion skills e.g. crawling, climbing, and flying. Thus we tested the flies under

acute stress conditions in the Mn^{2+} stress setup. Animals that were solely treated with Mn^{2+} were highly affected in their ability to climb the indicated vertical distance of some centimeters in the plastic testing vial. In this setup we had to adjust the climbing height depending on the age of the flies and their treatment.

We saw that young flies, even if they were stressed, started with a higher percentage on the first timepoint of the experiment than the aged flies. So we had to adjust the height of the vertical climbing distance from 7 cm (for the young flies) to 4 cm for the aged flies to start with an acceptable percentage of around 70-80 % in the group of the aged flies.

We also had to perform the whole experiment in a dark room that was just lit with red light, otherwise the flies would start to climb to the upper side of the vial just because of light coming from above which would result in positive phototaxis and not negative geotaxis.

We supposed that these locomotion difficulties might have an organic cause and we decided to investigate this more closely, so we dissected whole fly brains to find the areas of higher staining intensities indicating a higher protein amount of Aβ-42.

4.2.5 High staining intensity in mushroom bodies of Aβ-42 expressing flies is decreased upon spermidine treatment

As mentioned before, the locomotion difficulties observed in the flies expressing Aβ-42 protein were very obvious and so we decided to investigate this phenotype more intensively. We dissected whole fly brains and stained them with the appropriated antibody for Aβ-42 and Bruchpilot (BRP), a housekeeping neuronal protein ubiquitous in the presynaptic terminals of the flies. At first we wanted to use the BRP-signal as a reference background signal, but this signal is also changing with the age of the flies and within the scanned brain tissue[66]. Therefore we were just able to quantify the staining intensities as relative staining units determining the relative change of the Aβ-42 signal related to the BRP-signal.

We found that the mushroom bodies could be seen very clearly and brightly stained in our samples, which might be due to the used driver line elavX which generally has a higher expression pattern in certain regions of the fly brain even if it is said to be a pan-neuronal driver line. As this is a naturally occurring phenomenon, we did not give that much attention to it.

Since we were able to study the effects of transgenic expression of Aβ-42 and α-syn in different experimental setups under the influence of spd-administration, we were interested in a possible combination of the morbid genetic condition and a possible intrinsic counterpart.

ODC1, which is the intrinsic rate-limiting enzyme for the synthesis of polyamins (as spermidine and its precursors) in the fruit fly and has a homolog in humans, was the reasonable choice for a new set of experiments with flies that carry both the morbid and the beneficial genes on one chromosome under the same promoter.

4.3 Recombined flies

4.3.1 Successful establishment of a transgenic *D. melanogaster* fly line containing *ODC1* and *α*-synuclein or ODC1 and Aβ-42 on the same chromosome

As mentioned before we were interested in the effect, the combination of the morbid and the beneficial genes in one single fly might have on the survival of the animals. So we started the procedure of the recombination of the gene in one fly. Since the desired genes were on the same chromosomes in different animals we needed to apply the method of recombination and could not use the combination method. With the combination method, we would not have been able to get the two genes under the same promoter, what we could achieve with the recombination. As it is known, in a recombination event, parts of the chromosome can be exchanged with parts that are flanked with a homologous region. Here, we exchanged parts of the third chromosome of Ornithine Decarboxylase 1 (ODC1) containing flies with the Aβ-42 or the α-syn locus, resulting in flies carrying both genes on the same chromosome under the same promoter.

During the recombination process we encountered some minor problems. One of these problems had to do with the collection of the virgin flies at each step of the crossing scheme. As we were doing that kind of genetic experiment for the first time, we had to figure out which were the desired virgins we had to collect, figuring out which flies carried the right markers.

We were always having 3-5 approaches of each crossing step to ensure the desired outcome. We always had single crosses (one male and one female), which means that these two flies had to mate, which is not always simple because of the specific mating behavior. The mating procedure between male and female fly always has to follow the right procedure, before the female is able to lie fertilized eggs[67]. This could be one of the reasons why at least one of the

vials was never populated with eggs and larvae; other causes include changes in air humidity, temperature or different food batches.

As the recombination of the genes was successful (see 3.3.1 and 3.3.2), we decided to test these flies in one of our experimental setups choosing the one that seemed to yield the most reproducible data – the Mn^{2+}-stress setup.

4.3.2 Recombined flies do not show the same survival rate in the manganese stress setup than the flies treated with spermidine

Since we tested the flies with the recombined chromosome in the same stress setup as the other animals, we wanted to determine the effect of an intrinsic overexpression of the rate-limiting enzyme ODC1. Here, we tested the flies that had one copy of the pathogenic Aβ-42 or the α-syn gene on the same chromosome than the ODC1. We were interested in a possible mimicry of the spermidine-effect that was observed before in the oral administration-condition under Mn^{2+}-stress.

What we could find here, did not fulfill our expectations of an effective mimicry of the spermidine-effect that was seen before. We thought that the ODC1-overexpression might help the flies to produce more spermidine intrinsically with the higher amount of ODC1 in the cells. But the influence of the pathogenic Aβ-42 protein seemed to be stronger than we thought and ODC1 was not able to counteract on that effect, not even improving the devastating conditions of these flies. Our hypothesis to that observations is that either the amount of ODC1 enzyme or/and the intrinsic production of spermidine is not sufficient (either because of short ODC1-half-life or because of unknown other reasons) or the production of spermidine could even be too high so that it has even deleterious effects on the animals and hence is not able to counteract the Aβ-42 patho-phenotype.

6. Outlook

Given the dramatic impact, spermidine has on the fruit fly model in so many measurable aspects of this organism, we suggest further investigations. Still, there are some characteristic hallmarks of AD and PD that we did not tested yet in this model organism, including the most specific feature of Alzheimer's – progressive memory loss. Data of Iijima et al.[38, 39] show a very obvious and strong phenotype, we are on the way to test our specific Aβ-42 expressing flies in a very similar setup.

Nevertheless, we could show that the administration of certain amounts of spermidine have beneficial effects in several experimental setups in our model. Given the results in several experiments, that give us new insights in different regulatory mechanisms, the data for the longevity setup encourage us to work on an optimization of the established setup. We were interested in a relative change of a lifespan extension with different concentrations of spermidine, as we think that the concentration of 5 mM that we use in the stress setup might not show the right outcome in the animals that should survive for a longer period of time. As mentioned before, there are studies that show a controversial effect of spermidine when applied in certain developmental stages or over a longer period of time.

As the obtained data are missing some crosslinking details, we consider this study as still ongoing and not finished up to now. The acquired material gives a huge insight in the organismal effects, spermidine has on fruit flies, but the mechanistic aspects need to be investigated in further detail. We present a stimulating perception of spermidine administration changing many aspects of AD and PD that might even be applicable to human patients, but the underlying molecular mechanisms need to be clarified to make further steps towards testing the substance in vertebrate model organisms, such as the mouse model.

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