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Modulating autophagy to improve health and survival in *Drosophila melanogaster*

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AFFIDAVIT

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“Science is a way of thinking much more
than it is a body of knowledge.”

Carl E. Sagan

For my parents

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Abstract

The process of autophagy is a highly regulated form of self-digestion in cells conserved from yeast over fruit flies to humans. This intracellular self-eating process is mainly regulated by nutrient depletion, yet the exact molecular mechanisms behind this process and by which means it is correlated to various diseases is still widely unknown. One of these illnesses, in which autophagy may play a vital role, are neurodegenerative disorders, such as Alzheimer's disease (AD). Besides being the most common form of dementia with a progressive pattern of cognitive and functional impairment, this sickness is pathologically associated with extracellular plaques of amyloid-beta peptides (Aβ) and neurofibrillary tangles comprising tau proteins. Worldwide, over 46 million people suffer from this fatal ailment that is to date incurable, due to its exact pathogenesis still to a great extent being unclear. Studies suggest that the Aβ42-peptide is cause for the development and progression of AD and can be expressed in the model organism *Drosophila melanogaster,* which consequently became an established AD-model over the years.

In this work, we could confirm that not only does Aβ42 induce toxicity in fruit flies, but that this can be alleviated by administration of spermidine - a natural ubiquitous polycation. Our data confirm that this polyamine has the ability to induce autophagy and to mediate neuroprotection. However, the molecular pathway by which spermidine induces autophagy is yet to be determined. Through supplementation of myo-inositol, another natural alcohol, we could show that the positive effect of spermidine is mitigated. Not only could we demonstrate that pre-treatment with inositol lessens the protective effect of spermidine, but it also hinders spermidine to farther re-induce autophagy. This observation indicates that the Ino/IP_3-signaling pathway is somehow involved in or may even be the cause for the process of spermidine-induced autophagy and its facilitated neuroprotection.

Beyond that, in the second part of this work, we could establish that both saturated and unsaturated fatty acids and the combination of those can induce autophagy and have several beneficial properties relatively to the life- and health span, including locomotion function and stress resistance, in *Drosophila.*

Kurzzusammenfassung

Autophagie ist eine hochregulierte Form der zellulären Selbstverdauung, die sowohl u.a. in Hefe, Fruchtfliegen als auch im Menschen konserviert ist. Dieser Prozess wird hauptsächlich durch Nährstoffmangel reguliert, jedoch sind die genauen molekularen Mechanismen, die diesen Prozess leiten und mit denen er mit verschiedenen Krankheiten in Verbindung gebracht wird, noch weitgehend unbekannt. Bei neurodegenerative Erkrankungen, sowie Alzheimer scheint Autophagie doch eine entscheidende Rolle zu spielen. Alzheimer ist die häufigste Form der Demenz mit einem fortschreitenden Muster von kognitiven und funktionellen Beeinträchtigungen und ist pathologisch assoziiert mit extrazellulären Plaques aus Amyloid-beta-Peptiden (Aβ) und Neurofibrillen bestehend aus dem Tau-Protein. Weltweit leiden über 46 Millionen Menschen an dieser bis heute unheilbaren tödlichen Krankheit, da ihre genaue Pathogenese noch weitgehend ungeklärt ist. Aus Studien ist das Aβ42-Peptid als Ursache für die Entwicklung und das Fortschreiten von Alzheimer ersichtlich geworden und kann im Modellorganismus *Drosophila melanogaster* exprimiert werden, der sich somit über die Jahre zu einem etablierten Alzheimer-Modell entwickelte.

In dieser Arbeit konnten wir nachweisen, dass in Fruchtfliegen Aβ42 nicht nur Toxizität auslöst, sondern dass dies durch die Verabreichung von Spermidin, ein natürliches ubiquitäres Polykation, gelindert werden kann. Unsere Daten bestätigen, dass diese Substanz sowohl Autophagie induziert als auch neuroprotektiv wirkt. Der genaue molekulare Weg, durch den Spermidin Autophagie induziert, muss jedoch noch bestimmt werden. Durch die Supplementierung von myo-Inositol, einem natürlich vorkommenden zyklischen Alkohol, konnten wir vorweisen, dass die positive Wirkung von Spermidin gemildert wird. Es stellte sich heraus, dass die Vorbehandlung mit Inositol die schützende Wirkung von Spermidin vermindert und auch daran hindert, die Autophagie zu re-induzieren. Diese Beobachtung deutet darauf hin, dass der Ino / IP_3-Kreislauf am Prozess der Spermidin-induzierten Autophagie und seiner Neuroprotektion beteiligt ist oder sogar der Auslöser dafür sein könnte.

Ferner konnten wir in einem zweiten Teil dieser Arbeit feststellen, dass sowohl gesättigte als auch ungesättigte Fettsäuren und deren Kombinationen Autophagie induzieren können und bei *Drosophila* mehrere positive Eigenschaften in Bezug auf die Lebens- und Gesundheitsspanne, einschließlich Fortbewegungsfunktion und Stressresistenz, aufweisen.

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1. Introduction

1.1 Autophagy

Autophagy is an intracellular lysosome-mediated self-degradation process that is used to eliminate damaged organelles and protein aggregates and focuses on recycling these discarded cellular components [1]. Being present at a basal level in all mammals, it balances energy in critical times in development and in response to nutrient stress, thus being fundamental to the differentiation, development, survival and homeostasis of a cell [2, 3, 4]. There are three types of autophagy that differ in mechanisms, cargo specificity and physiological functions, namely, chaperone-mediated, micro- and macroautophagy. This master thesis focuses on the best-studied form, that is macroautophagy (henceforth referred to as autophagy), which makes use of so called autophagosomes or autophagic vacuoles (AV). These are intracellular double-membraned structures that engulf their cytoplasmic targeted materials to deliver them to the lysosome for degradation [5]. Different autophagy-related (Atg) proteins are essential for the formation of such AVs. There are five key stages in the complex process of autophagy: initiation and nucleation; Atg5-Atg12 conjugation; LC3 processing; closure; fusion with lysosome and degradation. Several different proteins, such as PI3K/ Beclin-1 and ULK1, are involved in the initiation and nucleation step. Autophagosomes arise from so-called phagophores, which in mammalian cells can be derived from different membranes and assemble at a phagophore assembly site (PAS). For the elongation and closure of the AV, the following ubiquitin-like protein conjugation systems are responsible: Atg5-Atg12 and microtubule-associated protein 1 light chain 3 (LC3). The Atg5-Atg12 conjugation system, which is formed with the help of Atg7 and Atg10, forms a complex with Atg16 that in turn functions as the ligase of the second conjugation system. In that respect, LC3 is conjugated to phosphatidylethanolamine (PE) through interaction with Atg4, Atg7 and Atg3. Hence, the soluble form of LC3 (referred to as LC3-I) is so modified to a lipidated form of LC3 (referred to as LC3-II) [6, 3]. Next to LC3-II, the Gamma-aminobutyric acid receptor-associated protein (GABARAP) is also an Atg8 (homolog of LC3 found in yeast) family protein that undergoes similar processing during autophagy and co-localizes with LC3 at both the internal and external surfaces of the autophagosome. Due to the increase of synthesis and processing of LC3 and GABARAP during autophagy, these proteins can be used as markers for

autophagy induction in cells [7]. Several different proteins act as adaptor molecules to bind to targeted structures, such as ubiquitinated proteins or protein aggregates, for degradation. Moreover, they interact with LC3 through their LC3-binding domain, thereby playing a role in selecting cargo for degradation. In this regard, the best-characterized adaptor molecule with various functions is p62 that binds to ubiquitinated protein aggregates [4]. Changes in p62 protein levels, that is the accumulation or degradation of this specific autophagic substrate, can be used to determine if autophagic vacuoles are indeed being degraded in biochemical assays, such as western blotting [7, 8].

Being a machinery that is conserved among a wide spectrum of species, including yeast, fruit flies and humans, autophagy can be activated not only by stresses, for instance starvation or pathologies, but also by treatment with pharmacological agents like rapamycin [9]. This macrolide fungicide with immunosuppressant properties is an inhibitor of mammalian target of rapamycin (mTOR) and thereby induces mTOR-dependent autophagy. This pathway is the most examined autophagy-related pathway and includes two main complexes: mTOR complex 1 (mTORC1) and 2 (mTORC2). The mTORC1 is an autophagy regulator that upon inhibition through starvation conditions or treatment with rapamycin, leads to autophagy induction via dephosphorylation-dependent activation (inhibition of mTOR-mediated phosphorylation) of Unc-51 like autophagy activating kinase 1 (ULK1) [10, 11]. The indirect autophagy inducer, mTORC2, activates the protein kinase B (also referred to as Akt), which in turn modulates autophagic activity regulated by the phosphatidylinositol-3-kinase (PI3K) pathway. The mammalian core complex of the PI3K-pathway consists of three major components including the Vps34, p150 and Beclin-1 [12]. This is a signalling cascade that interacts with mTORC1 [13] and, upon activation, converts phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3), leading to autophagy induction further downstream.

Further, from recent studies, it is established that the (de)acetylation of cytoplasmic and nuclear proteins play an essential role in the autophagy process [14]. Interestingly, natural compounds such as resveratrol and spermidine are observed to initiate mTOR-independent autophagy and cause changes in the phosphoproteome and acetylproteome [14], which is described in detail further below. In another mTOR-independent autophagy-related pathway (one of many), PIP_2 is hydrolysed to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) by phospholipase C (PLC), which in turn is activated by a G protein-coupled receptor (GPCR).

IP_3 acts as a second messenger, which binds to its receptors (IP_3R) on the endoplasmic reticulum (ER), leading to the release of Calcium ions (Ca^{2+}) stored in the ER, followed by various cellular responses [15]. Those IP_3-receptors are found to be regulators of the Beclin-1-complex, thus acting on the regulation of autophagosome formation [16]. Furthermore, IP_3 is degraded by a 5'Phosphatase and inositol polyphosphate 1-phosphatase (IPPase) to inositol monophosphate (IP_1), which in turn is hydrolysed to free inositol by inositol monophosphatase (IMPase) [15]. Studies show that intracellular levels of inositol and IP_3 can negatively regulate autophagy [17], making this so-called IP_3-pathway one of interest.

Additionally, non-canonical autophagy is yet another mode of expression of this complex process that is autophagy. Canonical autophagy is the process discussed earlier, in which various proteins, including AMPK, PIK3/Beclin-1 and all the ATG proteins, are required for the formation of the double-membraned AVs. In contrast, non-canonical autophagy is characterized by bypassing either those proteins involved in elongation and closure (Atg5, Atg7 and LC3) or those for initiation (ULK1) and nucleation (Beclin-1) [6]. One example of compounds that were found that bypass Beclin-1 and therefore stimulate non-canonical autophagy, are unsaturated fatty acids (UFAs) [18, 19]. The ability of these apparent healthier forms of fat [20] to bypass a thought to be crucial protein to the autophagy process, that is Beclin-1, is yet to be determined. Furthermore, Beclin-1 has been identified to be a crucial node for the interaction between autophagy and apoptosis [21, 22]. Defective or mutated genes regulating autophagy pathways have been implicated in an increasing number of human diseases, including neurodegenerative diseases such as Alzheimer's disease as well as various cancers [1, 2, 5, 23, 24].

1.2 Alzheimer's Disease

Alzheimer's Disease (AD) is a common age-related progressive neurodegenerative disorder that causes deterioration of nerve cells in the brain and ultimately death. This most common form of dementia accounts for estimated 46 million patients worldwide [25, 26]. Due to the cause of AD at present being poorly understood, there are no available treatments to stop or reverse the progression of this disease. However, there are two main hypotheses to its origin that are based on main pathological findings: the amyloid and tau hypothesis. Key characteristics of this disorder are the presence of plaques made of amyloid β-peptides (Aβ),

especially amyloid-β- 42 (Aβ42), and neurofibrillary tangles containing aggregated tau proteins in brain tissue [25, 27, 28, 29, 30]. Aβ42 is a short, highly self-aggregating, hydrophobic peptide generated through cleavage of the transmembrane protein, called amyloid precursor protein (APP), mediated by proteases, that are β- and γ-secretases [25, 27]. The catalytic subunit of the γ-secretase, called presenilin (PS), therefore mediates intramembrane proteolysis resulting in a C-terminal heterogeneity of the developed amyloid-peptide population. Through its imprecise cleavage, numerous Aβ species exist, yet those ending at position 40 (Aβ40) and 42 (Aβ42) are the most abundant [31]. However, the exact function of both APP and Aβ is yet to be determined. Albeit, according to the amyloid hypothesis that implies an imbalance between production and clearance of Aβ42 via autophagy, mutations in either the *APP* or *PS1* or *2* gene could be the cause for the deposition of these peptides. Being responsible for lysosome acidification and protease (γ-secretase) activation, mutations of PS1 can disrupt lysosomal functions, leading to defective clearance of AVs and consequently Aβ [32]. Following the tau hypothesis of AD, Aβ aggregation and accumulation, subsequently the toxicity of Aβ, results in the hyperphosphorylation of the major microtubule associated protein (MAP) of a mature neuron, that is tau, which in turn forms filamentous aggregates [29]. Tau is regulated by phosphorylation, which gets disrupted by Aβ and therefore resulting in intraneuronal neurofibrillary tangles [33]. As the disorder progresses, autophagy cannot compensate for the accumulation of both Aβ and tau aggregates, consequently in late AD autophagy is compromised. Nevertheless, the mechanisms that drive these changes in formation of Aβ and tau leading to autophagy impairment are still unknown at present.

1.3 Spermidine as a neuroprotective agent

Research has shown that the administration of spermidine can induce autophagy as well as consequently promote longevity in various model organisms [34, 35, 36]. This natural occurring polyamine is found in high concentrations in citrus fruits and soybeans and is an endogenous metabolite. It can be synthesized from putrescine as a ubiquitious polycation that participates in multiple known and unknown biological processes, including autophagy induction, tumor suppression, immune modulation, cardio and neuroprotection [37]. It has been reported that in aging brains, the level of polyamines is decreased [38]. However, spermidine treatment triggers

protective autophagy, which in turn enhances memory function during aging in fruit flies [39, 40]. The correlation between spermidine and autophagy induction or rather the molecular mechanism by which the polyamine induces neuronal autophagy remains at present largely unclear. Nonetheless, it is suggested that spermidine induces modifications of histone H3 by inhibiting a histone acetyltransferase (HAT), EP300, resulting in direct autophagy regulation [34, 36, 41]. Studies with fruit flies have shown that spermidine protects against α-synuclein neurotoxicity, which is a Parkinson's disease (PD)-related degenerative process [42]. Additionally, in adult mice, spermidine stimulates not only neuroprotection but also neuroregeneration, which may be useful for treatment of various neurodegenerative diseases [43]. Moreover, spermidine treatment can enhance Beclin-1-dependent autophagy influx and correspondingly stimulate the degradation of autophagy substrate p62 [34]. It is therefore tempting to speculate that spermidine-mediated induction of autophagy, as it being a crucial regulator of various age-associated pathologies, such as AD, may diminish the neurotoxic effects of Aβ42.

1.4 Myo-Inositol as autophagy inhibitor

Myo-Inositol (referred to as Inositol) is a small molecule, structurally similar to glucose (molecular formula being $C_6H_{12}O_6$). The phospholipids of this natural sugar serve as components in cell membranes and thus make inositol phospholipids key players in cellular signalling as second messengers. Inositol is either produced through a two-step-subpathway or the cyclic phosphoinositol signalling pathway [44]. The subpathway synthesises inositol through isomerization of glucose-6-phosphate (G6P) by inositol-3-phosphate synthase (IPS), followed by dephosphorylation of the resulting inositol 1-phosphate (IP_1) by inositol monophosphatase (IMPase). As described above, the cyclic synthesis incorporates the following: PIP_2 is hydrolysed by PLC to DAG and IP_3, which in turn is dephosphorylated by IMPase to inositol (via IP_2 and IP_1). Moreover, Inositol is found to block the formation of autophagophores, whereas the calcium release from the ER through the binding of IP_3 to its receptor (IP_3R) results in an inhibition of fusion of autophagophores and lysosomes. In addition, an active IP_3R interacts with Beclin-1, through which the latter is sequestered from the cytosol and not available for phagosome formation [45]. Furthermore, the inhibition of IMPase by lithium can lead to autophagy induction via depletion of intracellular levels of

inositol and IP_3 [17, 46, 47]. Inhibition of IMPase prevents recycling of inositol and inositol synthesis from inositol phosphates. It is tempting to speculate that by addition of extracellular inositol this effect may be overcome. To be more precise: if intracellular levels of inositol rise, IP_3 levels presumably rise and this may lead to autophagy being inhibited or blocked. With this in mind, it is inviting to further speculate, that by simultaneous administration of inositol and spermidine, the neuroprotection by the latter should be prevented. Accordingly, the phosphoinositol signalling pathway, specifically inositol, can regulate autophagy. Additionally, stereoisomers of inositol have been suggested to prevent aggregation of Aβ42 via micelle-formation and therefore attenuate Aβ toxicity [48]. These two aspects make this natural compound and its ability to induce an mTOR-independent pathway of autophagy induction worth further exploring.

1.5 Effects of fatty acids on health and fitness

There are both positive and negative suggestions surrounding fatty acids and their influences on the health of humans. Palmitic acid is the most common saturated fatty acid (SFA) with a 16-carbon backbone (C16:0) found in animals, plants and microorganisms. It is the major component in fats and waxes, including palm oil and body lipids, and is associated with an increased risk of cardiovascular diseases [49]. On the contrary, the most abundant fatty acid in human adipose tissue, that is oleic acid, is a monounsaturated fatty acid (UFA) associated with a decrease of low-density lipoprotein (LDL) levels [50]. This in chemical terms classified omega-9 fatty acid has a 18-carbon backbone (C18:1, *cis*-9) and is the major component of olive oil [51]. However, the trans-isomer of oleic acid, called elaidic acid (C18:1, *trans*-9), is the major trans-fat found in hydrogenated vegetable oils. It increases the activity of the plasma cholesteryl ester transfer protein (CETP) that in turn raises very low-density lipoprotein (VLDL) and lowers the high-density lipoprotein (HDL) levels [52]. LDL-particles, also called “bad cholesterol”, can transport lipid molecules into the artery walls, which attract macrophages and thus drives atherosclerosis through plaque build-ups. Their counterpart, HDL-particles, are referred to as “good cholesterol”, because they are responsible for the reverse cholesterol transport, in which they remove lipid molecules from the artery walls [20]. Both palmitic and oleic acid are major components of most often used oils, namely palm oil and olive oil. The elaidic acid is a trans-fat found in hydrogenated vegetable oils.

The ability of fatty acids to induce autophagy is strongly influenced by the length of their carbon chains. Both palmitate (salt of palmitic acid) and oleate (salt of oleic acid) trigger autophagy by inhibiting the catalytic activity of mTORC1, however through different underlying molecular mechanisms [18, 19]. Palmitate, but not oleate, induces a canonical autophagic response that requires several different complexes, such as AMPK and BECN1/PI3K complex, to be active. However, palmitate seems to have the ability to regulate autophagy in a manner that changes with cell type and with time, initially facilitating and then obstructing it [18, 19]. Oppositely, UFAs, such as oleate, induce non-canonical Beclin-1- and PI3K-independent autophagy. This response relies on the Golgi apparatus to be intact, manifested by the redistribution of LC3 to Golgi-associated vesicles [19]. In this master thesis, the ability to induce autophagy through different combinations of fatty acids (UFAs and SFAs) was to be visualized through immunoblotting.

1.6 *Drosophila* as a model organism

To further understand the complexity behind the autophagic process as a major regulatory cellular mechanism and to further establish its involvement in the pathogenesis of various neurodegenerative diseases such as AD, a suitable animal model has to be established. *Drosophila melanogaster* is a well-established model system for investigations concerning human health and diseases. Apart from being cost effective and easy to handle, a fruit fly generation takes about 10 days and results in a very large number of offspring, which accelerates the research progress and makes this genetic model system one of time efficiency. A fly genome does not possess as many redundant gene families as mammals and has homologs of approximately 70% of human disease-related genes, which makes it a unique model system for the study of multifaceted human diseases, such as AD [53, 54, 55, 56]. Additionally, the short life span of fruit flies, which is around 60 to 90 days, makes this organism suitable for studying late-onset disorders, including neurodegenerative pathologies (e.g. AD). Furthermore, every gene of *Drosophila* can be manipulated, i.e. overexpression of exogenous proteins can be achieved by transgenic strategies. The most abundantly used system to spatially control expression of target proteins is the Gal4/UAS system, where two fly lines carry either the genes of interest under the control of a yeast upstream activating sequence (UAS) or insertions of the yeast Gal4 transgene under the control of an endogenous

Drosophila promoter. Upon crossing of these transgenic fly lines, the exogenous protein of interest can be expressed in a specific location, dependent on the promoter regulating Gal4 expression [56]. Not only is *Drosophila* used as a model for human neurodegenerative diseases [56, 57], but also for autophagy studies. To this notion, the core autophagy machinery is found to be conserved in fruit flies, including various essential Atg-homologs and Ref(2)P, which is the Drosophila homolog of p62 [58, 59].

1.7 Aim of this project

To assess the neurological effect of spermidine on autophagy induction and consequently on the survival of *Drosophila*, manganese stress resistance assays were performed in this thesis. Moreover, in the same setup, it was to be established, if treatment with inositol would decrease the spermidine-mediated neuroprotection in consistency to inhibit autophagy. Simultaneous administration of inositol and spermidine should, in theory, block autophagy induction by spermidine and consequently inhibit the neuroprotective effect of spermidine.
In a starvation stress resistance assay and a lifespan assay, the effects of UFAs, SFAs and their combinations, respectively, on the survival of fruit flies was to be determined. Additionally, to assess the locomotion function of *Drosophila*, so-called climbing assays can be performed to determine the success rate of the individual flies exposed to different conditions. With this assay, the effects of palmitate, oleate, elaidic acid and their combinations, respectively, on the motoneuron functions of *Drosophila melanogaster* were to be evaluated in this study.
The main goal of this master thesis was to modulate autophagy using the model organism *Drosophila melanogaster* by administration of various substances (spermidine, inositol, UFAs and SFAs) to determine their effects on the fitness and overall health, including survival and stress resistance.

2. Materials & Methods

2.1 Flystock & Husbandry

Fruit flies from a *w*^{1118} genotype (Bloomington #3605; [60]) were used in all the experiments. They were held on standard cornmeal-molasses food (referred to as Sigrist), which was prepared as followed (1 L): 0,92 L distilled water, 4,17 g agar, 85 g molasses, 7,5 g yeast, 8,33 g soy flour, 66,67 g cornmeal, 5,25 g propionic acid (Sigma), 1,33 g methyl-4-hydroxybenzoate (Nipagin, Sigma) in 4,17 ml 95 % EtOH. The food vials were stored at 4 °C for up to 14 days. Flies were raised and maintained in an incubator with 25 °C, 70 % humidity and a 12 h:12 h light:dark cycle. Upon hatching, the flies were anesthetized for less than 5 minutes with CO_2 and sorted based on gender, only female F1 progeny were used in all experiments.

2.2 Supplementation

2.2.1 Supplementation with Spermidine

Spermidine (Sigma Aldrich) was prepared as a 1 M stock solution in sterile distilled water, aliquoted in single-use portions (500 µl) and stored at -20 °C prior to use. Spermidine (herein referred to as SPD) was mixed to liquid Sigrist in a final concentration of 4 mM at a maximum temperature of 42 °C. An *elav*^{C155}*-GAL4* genotype [61] (referred to as *elavX*) was crossed with an Abeta1-42*/TM3 Ser* (UAS) genotype [28] (referred to as AB42) or a *w*^{1118} genotype, respectively. For this, 125 female virgin *elavX* flies were mated with 30 male AB42 flies per vial, with a total of 4-5 vials. 100 female virgin *elavX* flies were mated with 30 male *w*^{1118} flies per vial with 3-4 vials in total. These parent flies were transferred to fresh vials every 3 days, forming 5 cohorts of F1 progeny in total. The first 24 hrs of the first cohort was discarded, after which every 3 days, approximately 30 female flies (1-3 days old) per vial of each of the genotypes were collected. Foregoing the first manganese stress resistance assay, the parental flies of both genotypes mated on Sigrist supplemented with or without SPD (4 mM), and their progeny was allowed to develop on the respective food. For the second survival assay, the parental flies mated on Sigrist without SPD-supplementation. Those flies that hatched on Sigrist alone, were split into two groups and aged for 14 days, preceding to manganese exposure. One of those groups aged on Sigrist, the other on Sigrist supplemented with SPD.

The progeny that hatched on Sigrist already supplemented with SPD aged (14 d) on this respective food prior to further experiments.

2.2.2 Supplementation with myo-Inositol

Myo-Inositol (Sigma-Aldrich) was mixed to liquid Sigrist to a final concentration of 5 %. An *elavX* genotype was crossed with an AB42 or a w^{1118} genotype, respectively (see 2.2.1 for further details). The first 24 hrs of the first cohort was removed, after which every 3 days, approximately 30 female flies (1-3 days old) per vial of each of the genotypes were collected and aged on the respective food for 14 days as follows: the parental flies of both genotypes mated on Sigrist supplemented with or without 5 % myo-Inositol (herein referred to as Ino), and their progeny was allowed to develop on the respective food. For the second survival assay, the parental flies mated on Sigrist without Ino-supplementation. Those flies that hatched on Sigrist alone, were split into two groups and aged for 14 days prior to manganese exposure. One of those groups aged on Sigrist, the other on Sigrist supplemented with Ino. Furthermore, the progeny that hatched on Sigrist already supplemented with Ino, aged (14 d) on this respective food preceding to further experiments.

2.2.3 Supplementation with Fatty acids

To control larval density for the survival assays, 3 to 14-day-old flies of the w^{1118} genotype were sorted into embryo cages (300 flies per cage). The embryos were harvested from apple juice agar plates, which also served as food supply that were changed every 24 hrs. These agar plates were prepared as followed (for 1 L): 700 ml sterile distilled water, 25 g agar, 300 ml apple juice, 30 g sucrose, 0,5 g methyl-4-hydroxybenzoate (Nipagin, Sigma). In addition to the agar plates, the flies were given freshly prepared yeast-paste (prepared by mixing dry baker's yeast with water in a plastic beaker and moulded with a metal spatula into a thick paste), which was smeared as a thin layer onto the mesh of the cage. After acclimation of the flies to the cages (48 hrs), a fresh apple juice agar plate was provided and the first embryos were harvested with PBS (1x) and a rounded metal spatula 24 hrs later. In total, three subsequent harvests (every 24 hrs) were made and each was placed in vials with Sigrist (2x40 µl embryos per vial). Upon hatching, the flies were sorted and the females were put into smaller food vials with Sigrist (0,8 % agar) supplemented with either FA or their combinations.

Sodium palmitate (Sigma-Aldrich; referred to as P), elaidic acid (Sigma-Aldrich; referred to as E) and sodium oleate (Sigma-Aldrich; referred to as O) served as FA in either 3,6 mM or 7,2 mM concentration, respectively. Each FA was combined with the other, leading to following combinations: palmitate and oleate (P+O; 3,6 mM each), palmitate and elaidic acid (P+E; 3,6 mM each), and oleate and elaidic acid (O+E; 3,6 mM each). These FA were each mixed thoroughly in a plastic 50 ml tube with a combination of soy flour and warm distilled water (4,125 g soy flour in 10 ml ddH_2O for 500 ml Sigrist) prior to be stirred in liquid Sigrist in the respective final concentrations at a maximum of 60 °C.

2.3 Functional Assays

2.3.1 Manganese stress resistance

After 14 days on the respective food (Sigrist, Sigrist supplemented with SPD or Sigrist supplemented with Ino, respectively), the female flies of each group were split into another two groups. One of which was stressed with manganese, while the other was stressed with SPD-supplemented manganese. The F1 progeny that developed on Sigrist but aged with SPD-supplementation, were only stressed with Mn.

Manganese (Mn) was taken from a 2 M $MnCl_2$ stock (Sigma-Aldrich) solution and mixed with a 10 % sucrose (Roth) solution (in sterile distilled water) to a final concentration of 10 mM. SPD was prepared as described above. For one group, the 10 % sucrose solution was supplemented with 4 mM SPD. Foregoing the addition of Mn, the solution of sucrose and SPD was set to pH 6,2 – 6,3 with HCl (32 %). Flies were transferred into fresh empty vials, closed by foam plugs with slits that were fitted with plastic drinking straws stuffed with a piece of filtering paper that protruded into the vial. The manganese-sucrose-solution (+/- SPD) was poured into a plastic Petri dish (30 ml per dish), which had slits for the filtering paper in the lid. The vials were then placed onto the Petri dishes upside down and the pieces of filtering paper stuffed through the lid slits, thus coming into contact with the solution. These “manganese carousels” were kept in the incubator (25 °C, 70 % humidity and 12:12 light:dark cycle) and the number of dead flies was counted every 12 hrs. Additionally, 10-20 flies of both groups (stressed with Mn +/- SPD) were put on manganese carousels for 48 hrs and then snap frozen via liquid nitrogen (Air Liquide) preceding preparations for immunoblotting. Statistical

analysis was done in GraphPad Prism 7 using the Log-rank (Mantel-Cox) test to compare the survival curves to assess the p-value.

2.3.2 Survival assay

For lifespan experiments, nine vials for each combination of FA and eight to nine vials for each FA (O 7,2 mM and E 7,2 mM had nine vials) plus eight non-FA-exposed controls were prepared. Approximately 20-30 newly eclosed female flies were collected for each condition and placed in vials with the respective supplemented food. They were transferred to fresh vials every 2-3 days and the number of dead flies was counted each time. Upon escape or mucus-caused death, the flies were censored. Data from two separate experiments were analysed using Prism (GraphPad Software) and comparison of survival was performed using log rank tests, in which each survival curve of the respective condition was compared to that of the control group.

Additionally, 10-20 flies of per condition were aged either for eight, fifteen or thirty days on the respective FA containing food and then snap frozen via liquid nitrogen (Air Liquide) preceding preparations for immunoblotting. Statistical analysis was done in GraphPad Prism 7 using the Log-rank (Mantel-Cox) test to compare the survival curves to assess the p-value.

2.3.3 Climbing assay

To assess the effect of FA on locomotor function, a climbing assay was performed. Approximately 60 flies (in four different vials) were sorted into groups and each group was fed with either combination of FA or either FA, respectively, following the survival assay experimental setup. Roughly 15 flies of each condition were placed in a self-made hard plastic climbing rack. This rack has 10 slots, in which different conditions can be evaluated simultaneously. Without anaesthesia, the flies are put between two transparent hard plastic slides that form the slots (2,5 cm in length x 0,5 cm in width), which are closed with foam plugs. After an incubation period of 20 min under red light, the rack was gently tapped three times to knock the flies to the bottom, after which they were given 30 seconds to climb up. Each trial was video captured and the number of flies that climbed up to a vertical distance of 7 cm or above after 15 seconds (success rate) was recorded. Each trial was performed three times per rack. To assess the change in fitness of flies of each condition, this assay was

repeated with the same flies once every week (starting on day 8 and ending on day 30). With Excel, a two-way-ANOVA comparison of locomotion function was performed with the mean value of data from two independent experiments. Each group was corrected for multiple comparison against the control group.

2.3.4 Starvation resistance assay

To evaluate if the effect of FA is dependent on duration of treatment, two different starvation stress resistance assays were performed. For the first experiment, the flies of the climbing assay (that is 35-day-old flies) were put in plastic vials with agar (0,1 % in sterile distilled water) and placed at 25 °C under conditions of 70 % humidity and a 12h:12h light:dark cycle. Every 12 hrs the number of deceased flies was counted. The second survival assay was performed in the same manner, yet with 14-day-old flies that aged on the respective food prior to starvation. 3-4 vials per condition holding approximately 20 female flies were used. Data were analysed using Prism (GraphPad Software). The graphs were created with the mean value of date from each vial, in which each group was compared using the Log-rank (Mantel-Cox) test and corrected for multiple comparison against the control group.

2.4 Biochemical Assays

2.4.1 Western Blot

For extraction, approximately 10 female fly heads were homogenized in SDS-Buffer (2 % SDS in ddH_2O, 1x complete and 2 mM PMSF; 2 µl per head) with pestils. After 15 min incubation at room temperature (RT), the lysates were centrifuged at 8 °C, 14000 rpm for 10 min and supernatants (debris-less FA fraction) were collected and centrifuged again with the same parameters. Lipid-less fraction was then collected and 5 x Laemmli-Buffer (10 % SDS, 50 % (v/v) glycerol, 0,1 % bromophenol blue, 10 % β-mercaptoethanol, 250 mM Tris/HCl pH 6,8) was added. Samples were boiled at 95 °C for 5 min and centrifuged at 14000 rpm for 1 min. Protein extracts (an equivalent of 4 fly heads) were separated on a 12,5 % Tris-glycine polyacrylamide gel (Hoefer), and transferred to a PVDF membrane (0,45 µm pore size, Roth ; CAPS-Buffer, 1:15 h, 220 mA).

The membranes were cut in three pieces, of which the upper and the middle part was blocked for 1-2 h with 5 % fat-free milk powder (Roth; in TBS) and the lower part with 5 % BSA (Roth; in TST). The upper parts of the membranes were blotted overnight at 4 °C with an anti-ref(2)P (1:200 in 1x TBS + 5 % milk powder; Abcam), washed three times with PBST and followed by an incubation (1 h at RT) with an anti-rabbit-antibody (1:10000 in 1x TBS + 5 % milk powder; Cell Signalling Technology). The middle membrane parts were incubated overnight at 4 °C with an anti-tubulin antibody (1:10000 in 1x TBS + 5 % milk powder; Sigma), followed by an anti-mouse-POD antibody (1:10000 in 1x TBS + 5 % milk-powder; Sigma). An anti-GABARAP (LC3) antibody (1:500 in 1x TST + 5 % BSA; MBL) functioned as primary antibody for the lower parts of the membranes, followed by an anti-rabbit antibody (1:10000 in 1x TST + 5 % BSA, Sigma). After incubation with Clarity™ Western ECL Substrate (BIO-RAD) for 3 min, immunoblots were analysed by ChemiDoc (BIO-RAD) and signal intensity was quantified using Image Lab (BIO-RAD). The ratio of LC3-II/LC3-I and the relative amounts of ubiquitous ref(2)P protein from individual samples were quantified and corrected using antibody to tubulin as loading control in the respective samples. Statistical analysis was done in Microsoft Excel using the mean value and the standard deviation of all western blot assays.

3. Results

3.1 Spermidine influences survival and autophagy in Aβ42-expressing flies

Memory loss is one of the outcomes of the pathogenesis of Alzheimer's disease. The fact that spermidine is known to induce autophagy and consequently protect from an age-induced memory impairment in *Drosophila* [40], makes this polyamine a potential candidate in fighting this disease. Previous work has proven *Drosophila* to be an established model for neurodegenerative diseases, such as AD [57]. In line with this notion, we examined the effects of SPD on the survival of Aβ42-expressing fruit flies. It was to be determined, if SPD had any effect on the Aβ-toxicity and if this effect is in correlation with its ability to induce autophagy. Moreover, it was to be ascertained whether a spatial segregation of the SPD-treatment would alter the effect of the polycation.

3.1.1 Spermidine enhances survival in Aβ42-expressing flies

In a manganese stress resistance assay, Aβ-toxicity came apparent when comparing the Control flies with the Aβ-expressing flies (Figure 1. A & B). The Control flies displayed a median overall survival of 132 hours versus 96 hours of that of Aβ-expressing flies. Both the Control flies as well as Aβ42-expressing flies lived longer when stressed with the combination of manganese and spermidine (Mn+SPD) rather than stressed with manganese (Mn) alone. The median survival of Aβ42-expressing flies was raised from 96 hours without SPD-exposure to 114 hours with supplementation. This positive effect of SPD was also seen in flies aged on SPD-added Sigrist preceding the stress assay (Figure 1. B). When focusing closely on the effect of SPD-treatment on the different genotypes, the pre-treatment with SPD (i.e. aged on SPD-supplemented Sigrist for 14 days) compared to that without shows a similar effect in the Control flies (Figure 1. D). Less viability can be observed in the Aβ42-expressing flies exposed only to Mn alone, whether they were pre-treated with SPD or not (Figure 1. C). In comparison, the survival rate of those stressed with the combination of manganese and spermidine was increased. Nevertheless, the difference in survival rates of Aβ42-expressing flies stressed with and without SPD-supplemented Mn-solution is more prominent than that of the Control flies. In other words, the treatment with SPD may affect both genotypes differently.

To sum up, spermidine seems to decrease Aβ-toxicity and subsequently enhance the survival of Aβ42-expressing flies. The pre-treatment of SPD shows no visible consequence, on the

positive effect of this compound in combination with manganese. It is apparent that the mechanisms by which SPD increases viability affects both genotypes differently.

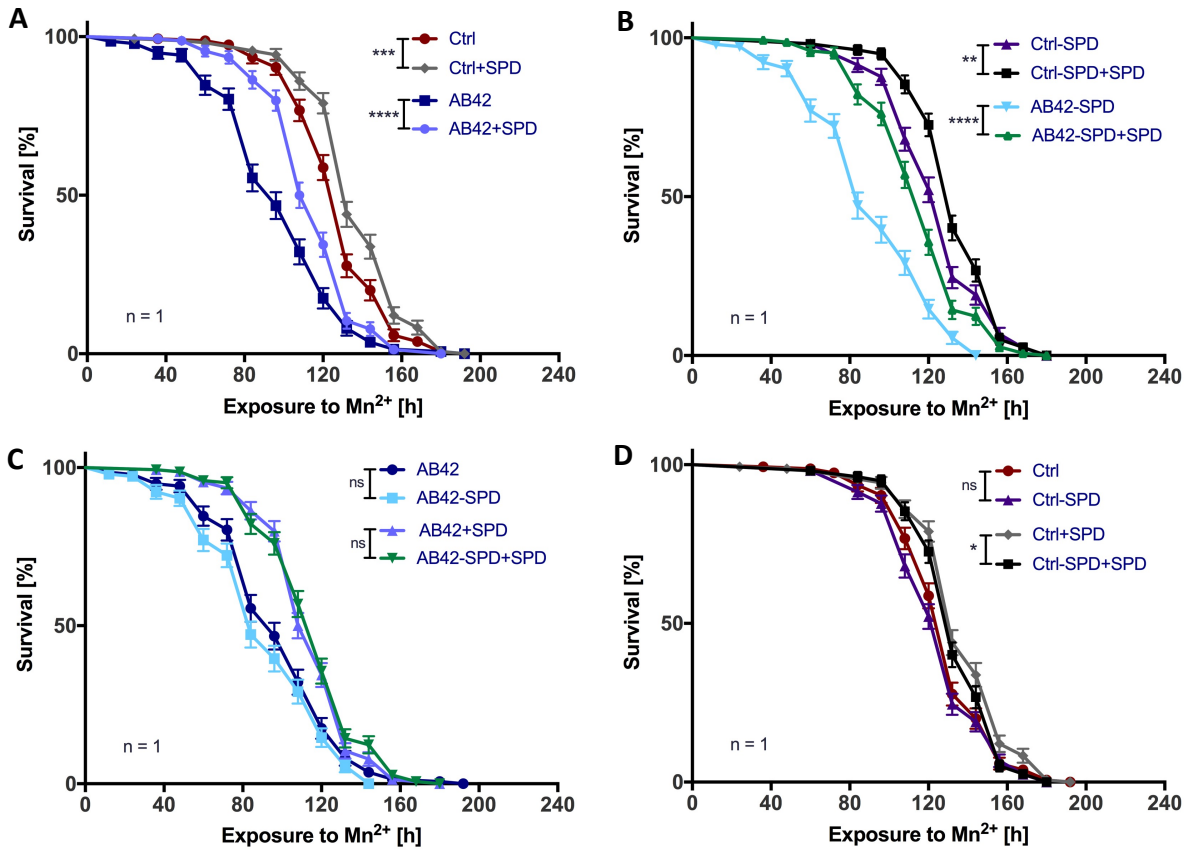

Figure 1. Spermidine decreases Aβ-toxicity and consequently increases survival of Aβ-expressing female fruit flies in a manganese stress resistance assay.

Female fruit flies of both w^{1118} (**Ctrl**) and AB42 genotypes were aged on Sigrist +/- SPD (4 mM) for 14 d preceding a manganese stress resistance assay with 10 mM $MnCl_2$ +/- SPD (4 mM) in 10% sucrose solution. Every 12 hours the number of dead flies was counted. Data are normalized means ± SE of one representative experiment. In total ten independent biological replicas were undertaken (see Figures 2, 4, 6 and supplemental data Figure S1-5) with similar tendencies, but the similarities disappear in a summary of all, due to the different survival margin of the individual experiment. Each vial contained 20-30 flies.

Asterisks indicate significance as followed: p<0,05=*; p<0,01=**; p<0,001=***; p<0,0001=****; ns >0,05

A Female flies of both w^{1118} and AB42 genotypes were aged on Sigrist foregoing a manganese stress with (**Ctrl+SPD** & **AB42+SPD**) or without (**Ctrl** & **AB42**) SPD supplementation.

B Female flies of both w^{1118} and AB42 genotypes were aged on Sigrist supplemented with SPD prior to manganese stress with (**Ctrl-SPD+SPD** & **AB42-SPD+SPD**) or without (**Ctrl-SPD** & **AB42-SPD**) SPD addition.

C Female flies of the AB42 genotype were either aged on Sigrist alone (**AB42** & **AB42+SPD**) or on Sigrist supplemented with SPD (**AB42-SPD** & **AB42-SPD+SPD**). One group was treated with Mn alone (**AB42** & **AB42-SPD**), whereas the other was treated with a combination of Mn and SPD (**AB42+SPD** & **AB42-SPD+SPD**).

D Female flies of the w^{1118} genotype were either aged on Sigrist alone (**Ctrl** & **Ctrl+SPD**) or on Sigrist supplemented with SPD (**Ctrl-SPD** & **Ctrl-SPD+SPD**). One group was treated with Mn alone (**Ctrl** & **Ctrl-SPD**), whereas the other was treated with a combination of Mn and SPD (**Ctrl+SPD** & **Ctrl-SPD+SPD**).

3.1.2 Aβ-toxicity in flies is enhanced with oversupply of spermidine

Next, we wanted to spatially split the SPD-treatment and exposure to manganese to see if pre-treatment of SPD prior to the manganese stress resistance assay would elevate its positive effect. This was done by looking at the different survival rate in flies hatching on SPD-supplemented Sigrist versus those hatching on Sigrist alone (Figure 2). With this notion, we could establish if SPD may affect the development of F1 progeny preceding hatching. Those flies that were subjected to SPD upon hatching also aged on this respective food for 14 days prior to the assay. Intriguingly, the oversupply of SPD decreases the survival rate of Aβ42-expressing flies when stressed with Mn alone to a median survival of 108:84 hours (Figure 2. A). The positive effect of SPD was only apparent in the flies hatching and aging on Sigrist alone and being subjected to the combination of Mn+SPD. At the beginning of the assay, this combination also had a positive effect on the Aβ42-expressing flies that hatched and aged on Sigrist treated with SPD, yet this effect is abolished fairly quickly. However, when comparing

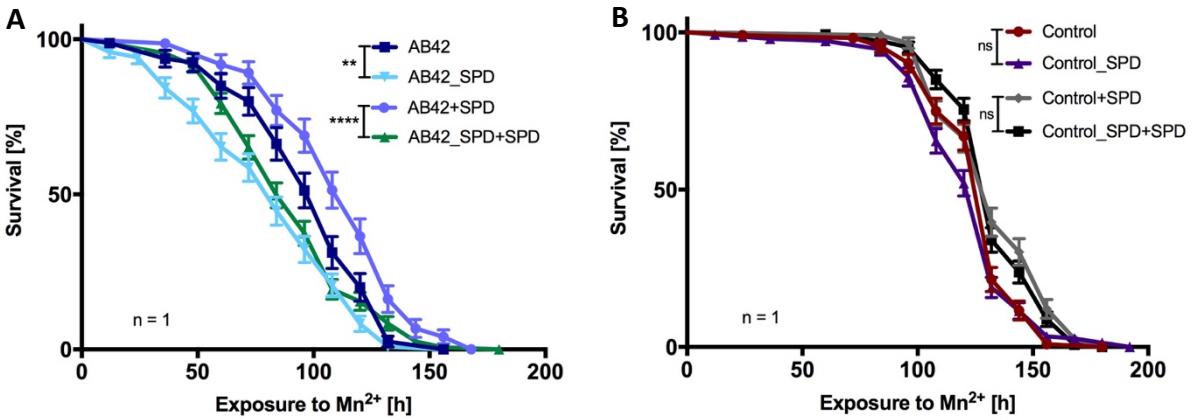

Figure 2. Supply of spermidine preceding hatching reduces survival rate of female Aβ42-expressing flies in a manganese stress resistance assay.

Female fruit flies of both (**A**) AB42 and (**B**) w^{1118} (**Control**) genotypes hatched on Sigrist +/- SPD (4 mM) and aged on the respective food for 14 d prior to manganese stress resistance assay with 10 mM $MnCl_2$ +/- SPD (4 mM) in 10% sucrose solution. Every 12 hours the number of dead flies was counted. Data are normalized means ± SE of one representative experiment. In total two independent biological replicas were done (see supplemental data Figure S4) with similar tendencies, but the similarities would disappear in a summary of both, due to the different survival margin of the individual experiments. Each vial contained 30 flies.

Asterisks indicate significance as followed: p<0,01=**; p<0,0001=****; ns >0,05

A Female Aβ42-expressing flies hatched either on Sigrist (**AB42** & **AB42+SPD**) or SPD-treated Sigrist (**AB42_SPD** & **AB42_SPD+SPD**) and aged on the respective food for 14 d foregoing being stressed with either a combination of Mn and SPD (**AB42+SPD** & **AB42_SPD+SPD**) or Mn alone (**AB42** & **AB42_SPD**).

B Female flies of the w^{1118} genotype hatched either on Sigrist (**Control** & **Control+SPD**) or SPD-treated Sigrist (**Control_SPD** & **Control_SPD+SPD**) and aged on the respective food for 14 d prior being stressed with either a combination of Mn and SPD (**Control+SPD** & **Control_SPD+SPD**) or Mn alone (**Control** & **Control_SPD**).

the Control flies there is no clear effect to be observed, whether or not they hatched and aged on SPD-added Sigrist (Figure 2. B). This fact may concur with our hypothesis that the ability of SPD to rescue Aβ42-expressing flies may be specific to their disorder.

An oversupply of spermidine (i.e. originating from and aged on SPD-treated fly food and stressed with SPD-added manganese solution) in summary seems to enhance the toxic effect of Aβ42 and thus may be lethal for Aβ42-expressing flies.

3.1.3 Spermidine-treatment leads to higher autophagy induction

Western blots were used to determine if the SPD-treatment indeed induced autophagy in our setup (Figure 3). Being an autophagic substrate associated with autophagosome degradation, p62 (or Ref(2)P, the homologue in flies) is lessened in case of autophagy induction. So, more autophagic degradation means less p62 protein levels. This coincides with the level of Ref(2)P being heightened in older fly brains, where autophagic activity is diminished [62, 63]. When comparing the ratio of autophagy induction (i.e. dividing LC3-II by LC3-I, then divided by the total amount of LC3) and the level of p62 (normalized to Tubulin) in Aβ42-expressing flies hatched and aged on Sigrist prior to being stressed with Mn, we could observe a blockage of autophagic processes (Figure 3. C). Here, the autophagy induction was lower than that of the Control flies, yet the level of p62 was nearly the same. This shows us that in the Aβ42-expressing flies used, the autophagic degradation process is indeed impaired. However, upon SPD-treatment, this problem is reversed. The flies expressing Aβ42 and stressed with the combination of Mn+SPD demonstrate higher autophagy induction as do the Control flies and in relation correspondingly lower p62-levels. When comparing the flies that were exposed to SPD-supplemented Sigrist the overall autophagy induction is higher than that of the Control flies aged on Sigrist, with one exception: the autophagy induction in the Control flies that aged on Sigrist with SPD and were stressed with Mn+SPD is decreased than that of those aged on Sigrist and stressed with Mn alone (Figure 3. B). Contrastingly to our hypothesis, the Aβ42-expressing flies that not only aged on SPD but were also stressed with Mn+SPD present a lower autophagy induction than those stressed with Mn alone. Nonetheless, the amount of p62 in those aged and stressed with SPD are nearly the same as that of the Control flies aged on SPD and stressed with Mn alone.

To conclude, the treatment with spermidine seems to induce autophagy in flies expressing the Aβ42-peptide. This ability of SPD seems to be impartial of pre-treatment yet seems to be specific for flies of the AB42 genotype.

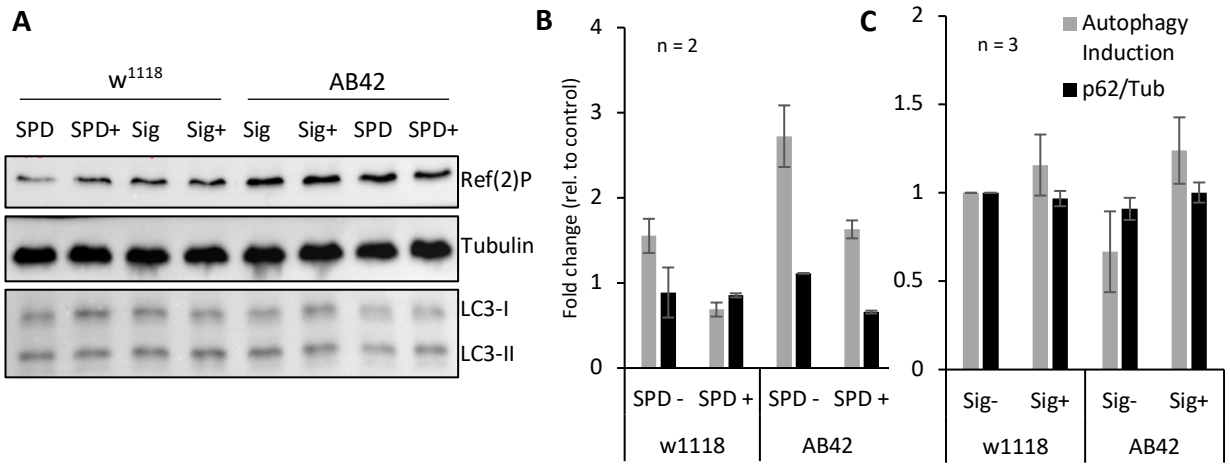

Figure 3. Spermidine induces autophagy in *Drosophila*.
Female flies of both *w*^{1118} and AB42 genotypes were aged on Sigrist food supplemented with or without SPD (4 mM) for 14 days foregoing manganese stress resistance assay with 10 mM $MnCl_2$ +/- 4 mM SPD (in 10 % sucrose solution) for 48 hrs. Preceding immunoblotting, flies were frozen with liquid nitrogen and heads extracted and homogenized with SDS-Buffer. The levels of LC3 lipidation and p62 degradation are normalized to the tubulin loading control of each sample. The autophagy induction was ascertained by dividing LC3-II by LC3-I, then the quotient is divided by the total amount of LC3. (**A**) A representative western blot is shown. (**B**) Flies of both genotypes were aged on SPD-supplemented Sigrist preceding being stressed with either a combination of Mn+SPD (**SPD +**) or Mn alone (**SPD -**). (**C**) Flies of both genotypes were aged on Sigrist prior to manganese stress with either Mn+SPD (**Sig +**) or Mn alone (**Sig -**). Data are normalized means ± SEM of two (**B**) or three (**C**) representative experiments. In total four (**B**) or nine (**C**) independent blots were performed which showed similar tendencies, but due to technical problems the level of p62/Tub was not detectable on the other blots.

3.2 Myo-Inositol impinges on the effect of spermidine

IP_3 is hydrolysed into myo-Inositol by the IMPase. So, higher amount of IP_3 leads to higher levels of inositol and vice versa[64] and thus presumably to an autophagic block. By supplementing standard fly food with additional inositol, we further wanted to intervene in this signalling pathway to establish that this Ino/IP_3-cycle may be the cause behind the SPD-mediated neuroprotection in *Drosophila*. In line with this notion, administration of Ino should not only reduce the positive effect of SPD, but SPD should not be able to induce autophagy after Ino-treatment.

3.2.1 Myo-Inositol reduces positive effect of spermidine without showing an effect on Aβ-toxicity

A manganese stress resistance assay was performed, to determine if treatment with Ino would diminish the positive effect of SPD (Figure 4). To make this observation, fruit flies were aged on Sigrist supplemented with or without Ino preceding the stress assay, in which they were either stressed with Mn with or without SPD addition. Comparing the Aβ42-expressing flies stressed with the combination of Mn+SPD with those with additional Ino-administration, the former indicated a higher survival rate (Figure 4. C). The flies expressing Aβ42 that were exposed to Ino and stressed with both Mn and SPD had a slightly higher survival rate than those stressed with Mn alone (Figure 4. B). The highest viability was observed in Aβ42-expressing flies that were aged on Sigrist and stressed with SPD-added Mn-solution. Interestingly, when looking at the effect in the w^{1118} wild type flies, those aged on Ino prior to the stress assay with manganese, show an increase in lifespan (Figure 4. A). In contrast, all other conditions have similar outcomes.

Thereafter, we wanted to identify, if Ino-treatment would affect the Aβ-toxicity in Aβ42-expressing flies (Figure 5). The Aβ triggered toxicity was visible when comparing the viability of the Aβ42-expressing flies to the Control. However, the survival rate was the similar in Aβ42-expressing flies with or without addition of Ino (Figure 5. A). Addition of SPD did not have any effect on the survival of flies expressing Aβ42 and the Control flies whether they were subjected to Ino or not (Figure 5. B).

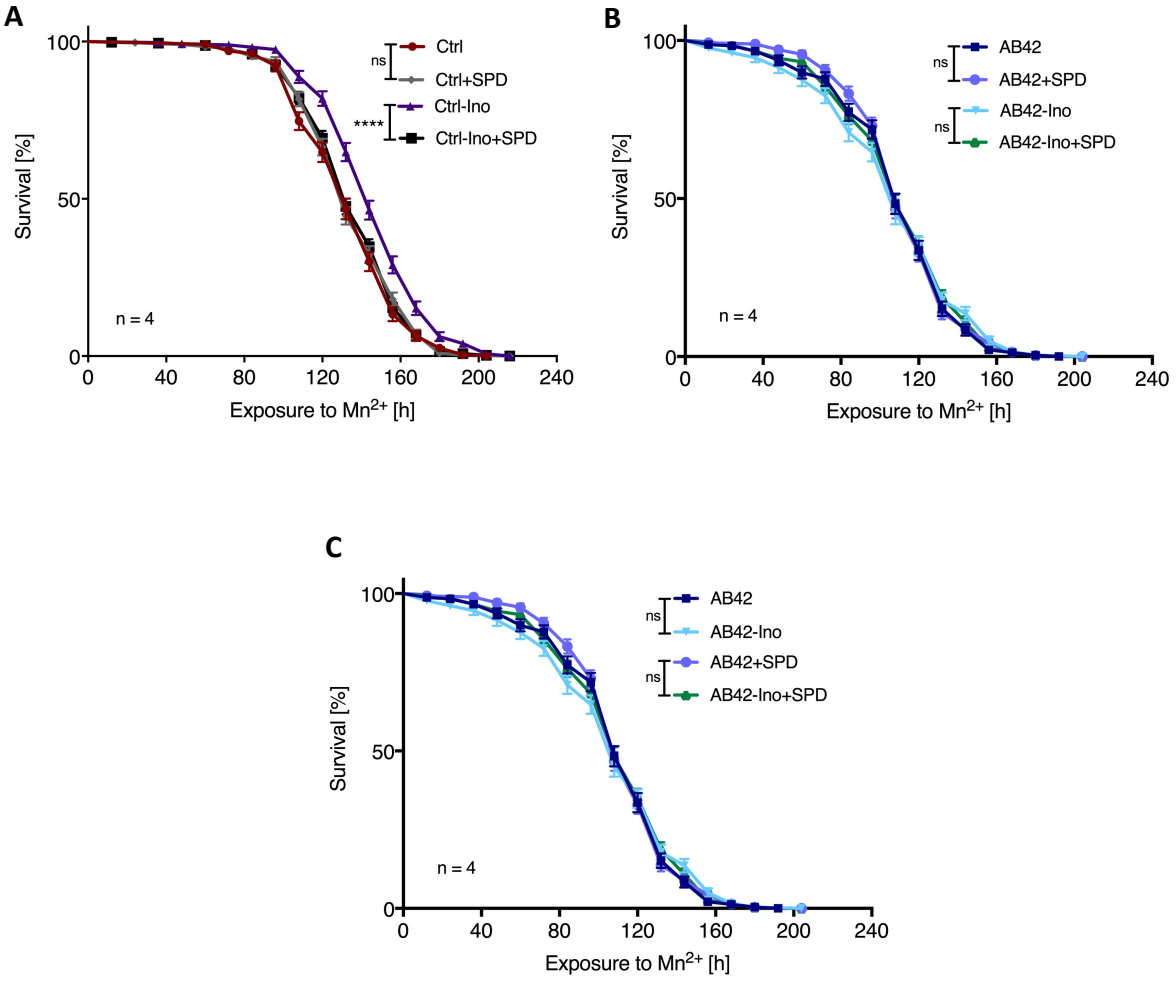

Figure 4. Simultaneous administration of myo-Inositol and spermidine reduces the positive effect of the latter in female fruit flies in a manganese stress resistance assay.

Female fruit flies of both (**A**) w^{1118} (**Ctrl**) and (**B, C**) AB42 genotypes were aged on Sigrist +/- Ino (5 %) for 14 d prior to manganese stress resistance assay with 10 mM $MnCl_2$ +/- SPD (4 mM) in 10% sucrose solution. Every 12 hours the number of dead flies was counted. Data are normalized means ± SE of four independent experiments from two crossings. Each vial contained 20-30 flies.

Asterisks indicate significance as followed: p<0,0001=****; ns >0,05

A Female flies of the w^{1118} genotype were either aged on Sigrist alone (**Ctrl** & **Ctrl+SPD**) or on Sigrist supplemented with Ino (**Ctrl-Ino** & **Ctrl-Ino+SPD**). One group was stressed with Mn alone (**Ctrl** & **Ctrl-Ino**), whereas the other was stressed with a combination of Mn and SPD (**Ctrl+SPD** & **Ctrl-Ino+SPD**).

B, C Female flies expressing Aβ42 were either aged on Sigrist alone (**AB42** & **AB42+SPD**) or on Sigrist supplemented with Ino (**AB42-Ino** & **AB42-Ino+SPD**). One group was treated with Mn alone (**AB42** & **AB42-SPD**), whereas the other was treated with a combination of Mn and SPD (**AB42+SPD** & **AB42-Ino+SPD**).

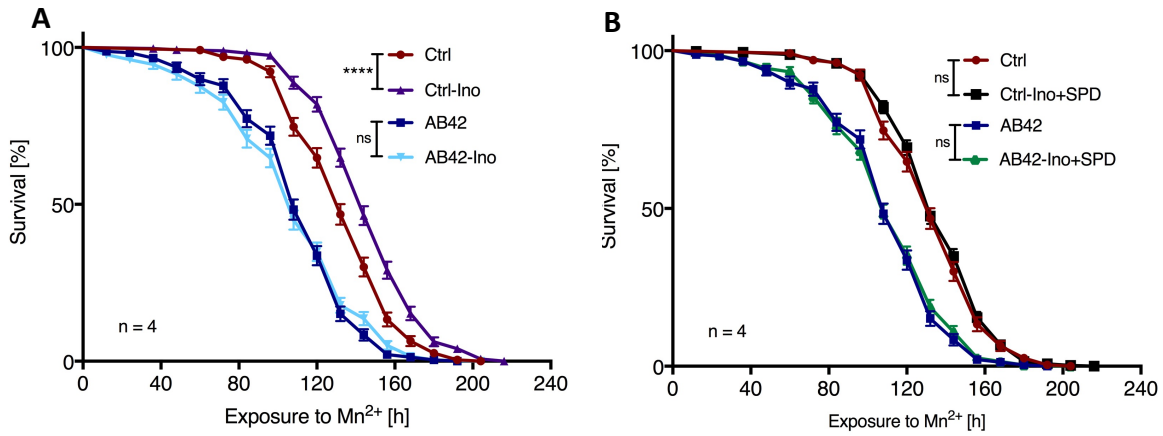

Figure 5. Myo-Inositol shows no effect on Aβ-toxicity in female fruit flies in a manganese stress resistance assay.
Female fruit flies of both w^{1118} (**Ctrl**) and AB42 genotypes were aged on Sigrist +/- Ino (5 %) for 14 d prior to manganese stress resistance assay with 10 mM $MnCl_2$ +/- SPD (4 mM) in 10% sucrose solution. Every 12 hours the number of dead flies was counted. Data are normalized means ± SE of four independent experiments from two crossings. Each vial contained 20-30 flies.
Asterisks indicate significance as followed: p<0,0001=****; ns >0,05

A Female flies of both w^{1118} and AB42 genotypes were either aged on Sigrist alone (**Ctrl** & **AB42**) or on Sigrist supplemented with Ino (**Ctrl-Ino** & **AB42-Ino**). They were then stressed with Mn.

B Female flies both w^{1118} and AB42 genotypes were either aged on Sigrist alone (**Ctrl** & **AB42**) or on Sigrist supplemented with Ino (**Ctrl-Ino+SPD** & **AB42-Ino+SPD**). One group was stressed with Mn alone (**Ctrl** & **AB42**), whereas the other was treated with a combination of Mn and SPD (**Ctrl-Ino+SPD** & **AB42-Ino+SPD**).

Furthermore, we wanted to spatially divide the Ino-treatment to see if pre-treatment of Ino prior to a manganese stress resistance assay would alter its effect. This was done by looking at the different survival rates in flies hatching on Ino-supplemented Sigrist versus those originating on Sigrist alone (Figure 6). With this notion, we could establish if Ino may affect the development of F1 progeny preceding hatching and therefore further decrease the positive effect of SPD. Those flies subjected to Ino upon hatching also aged on this respective food for 14 days preceding the assay. Interestingly, when hatching on Ino-added Sigrist and stressed with Mn alone, Aβ42-expressing flies presented a higher survival rate than those hatching on Sigrist alone (Figure 6. A). In fact, these fruit flies show the highest viability of all, even higher than those stressed with Mn+SPD. Addition of SPD to the stress-solution further decreased the survival in the Aβ42-expressing flies that were pre-treated with Ino. Comparing the effect in the Control flies, those stressed with Mn alone, survived longer than those with the combination of Mn and SPD, whether they hatched on Ino or not (Figure 6. B). Those hatched on Ino and stressed with Mn, displayed the same endurance than those hatched on

Sigrist alone. Additionally, both groups of Control flies stressed with SPD, whether hatched on Ino-added Sigrist or not, showed the same viability.

To sum up, treatment with myo-inositol seems to reduce the positive effect of spermidine without rendering the toxicity triggered by the Aβ-peptide. However, with a decrease in the number of progeny an oversupply of Ino seems to lead to higher viability. This survival effect is decreased drastically by addition of SPD. Even the Control flies with Ino-administration, yet without originating from Ino-added fly food, seem to live longer when stressed with Mn.

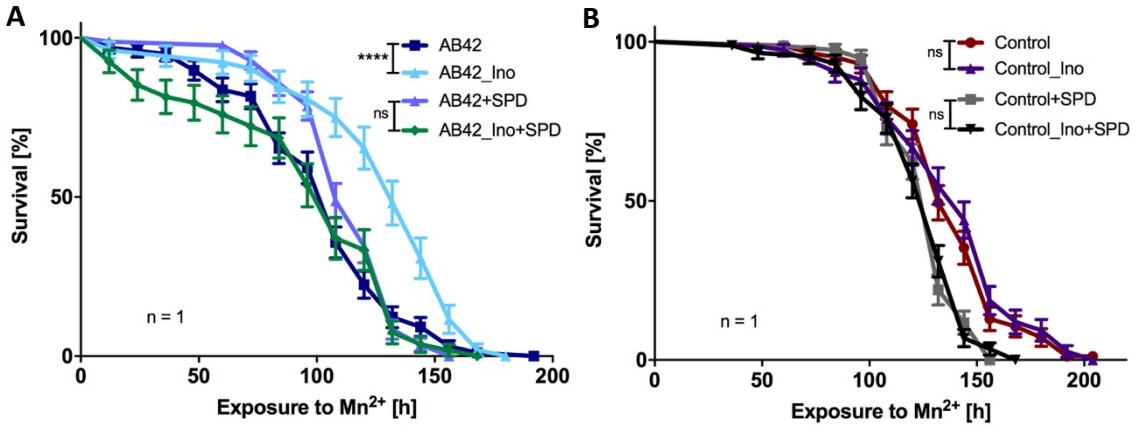

Figure 6. Hatching on myo-Inositol-supplemented Sigrist food, leads to a reduction of SPD-mediated neuroprotection in a manganese stress resistance assay with female Aβ42-expressing flies.
Female fruit flies of both (**A**) AB42 and (**B**) w^{1118} (**Control**) genotypes hatched on Sigrist +/- Ino (5 %) and aged on the respective food prior to manganese stress resistance assay with 10 mM $MnCl_2$ +/- SPD (4 mM) in 10% sucrose solution. Every 12 hours the number of dead flies was counted. Data are normalized means ± SE of one representative assessment. As a consequence of few progeny while hatching on Ino-supplemented Sigrist, this analysis was performed only twice from one crossing (see supplemental data Figure S5). Both of which displayed similar tendencies, yet due to the different survival margin of the individual experiment, a summary of both would diminish these similarities. Each vial contained 20 flies.
Asterisks indicate significance as followed: p<0,0001=****; ns >0,05

A Female Aβ42-expressing flies hatched and aged either on Sigrist alone (**AB42** & **AB42+SPD**) or on Sigrist supplemented with Ino (**AB42_Ino** & **AB42_Ino+SPD**). They were then stressed with either a combination of Mn and SPD (**AB42+SPD** & **AB42_Ino+SPD**) or Mn alone (**AB42** & **AB42_Ino**).

B Female flies of the w^{1118} genotype either hatched and aged on Sigrist alone (**Control** & **Control+SPD**) or on Sigrist supplemented with Ino (**Control_Ino** & **Control_Ino+SPD**). One group was stressed with Mn alone (**Control** & **Control_Ino**), whereas the other was treated with a combination of Mn and SPD (**Control +SPD** & **Control_Ino+SPD**).

3.2.2 Spermidine fails to induce autophagy after myo-Inositol pre-treatment

In theory, the administration of myo-Inositol should elevate the levels of IP_3 and consequently inhibit autophagy. Spermidine on the other hand, should induce autophagy. So simultaneous

treatment with both substances should not only disregard autophagy processes but pre-treatment with Ino should inhibit the induction of autophagy through farther SPD administration. To establish, if myo-Inositol does in fact intervene in SPD-induced autophagy and with it in SPD-mediated neuroprotection, immunoblotting was performed (Figure 7). The autophagy induction was ascertained by dividing LC3-II by LC3-I, then divided by the total amount of LC3 ((LC3-II/LC3-I)/(LC3-II+LC3-I)). In the Control flies (w^{1118}), an interesting effect of the Ino-treatment was observed: without SPD-added stress-solution (Ino -) the flies presented less autophagy induction than those without Ino-pre-treatment (Figure 7. B). Still, their p62 protein levels were the same as the Control. In contrast, Control flies aged on Ino-added Sigrist and stressed with both Mn and SPD had similar levels of autophagy induction and p62, which were higher than that of the Control aged on Sigrist and stressed with Mn alone. The autophagy induction in Aβ42-expressing flies was reduced with simultaneous Ino-treatment and SPD-supplemented Mn-solution. The elevation of p62 in these flies however, was double of that of their autophagy. Without being stressed with the combination of Mn and SPD, the Aβ42-expressing flies presented the same quantity of autophagy induction as the Control, yet with heightened p62 levels.

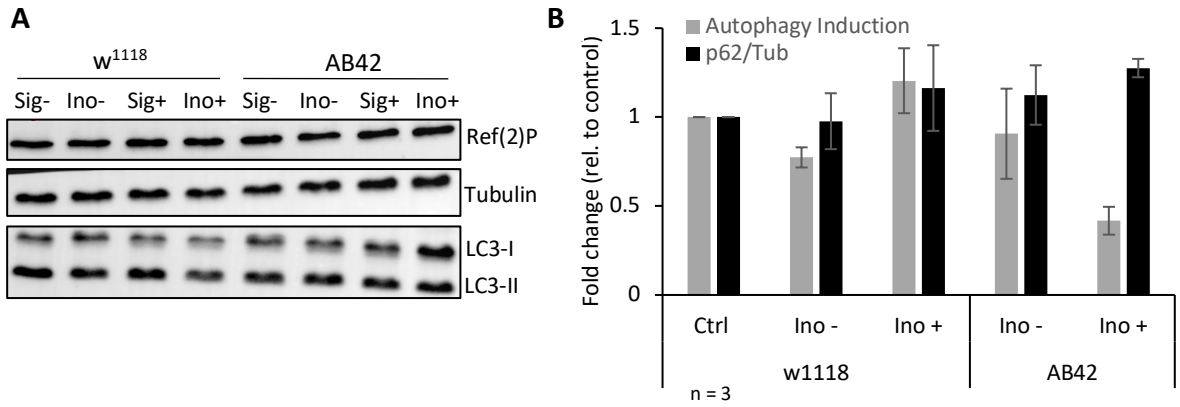

Figure 7. Simultaneous administration of inositol and spermidine reduces autophagy in *Drosophila*.
(**B**) Female flies of both w^{1118} and AB42 genotypes were aged on Ino-supplemented (5 %) Sigrist for 14 days forgoing manganese stress resistance assay with 10 mM $MnCl_2$ +/- 4 mM SPD (in 10 % sucrose solution) for 48 hrs. They were stressed with either a combination of Mn+SPD (**Ino +**) or Mn alone (**Ino -**). Flies of the w^{1111} genotype aged on Sigrist and stressed with Mn alone, functioned as a control (**Ctrl**). Data are normalized means ± SEM of three representative experiments. In total five independent blots were performed which showed similar tendencies, but due to technical problems the level of p62/Tub was not detectable in the other blots. Preceding immunoblotting, flies were frozen with liquid nitrogen and heads extracted and homogenized with SDS-Buffer. The levels of LC3 lipidation and p62 degradation are normalized to the tubulin loading control of each sample. (**A**) A representative western blot is shown. Flies aged on Sigrist and stressed with Mn (**Sig -**) or Mn+SPD (**Sig +**).

Myo-Inositol pre-treatment in summary seems to lead to a block of autophagic processes, in flies of both genotypes. Even the simultaneous administration of spermidine fails to induce autophagy after this pre-treatment.

3.3 Fatty acids affect the fitness, life- and health span of *Drosophila*

Building on previous work that showed UFAs and SFAs both induce autophagy [18, 19], we wanted to establish if a combination of those would affect this ability. Oleate served as an UFA, whereas palmitate as the SFA counterpart. Elaidic acid, being the trans-isomer of oleate, served as another UFA and was found to abolish autophagy induction by palmitate, but not by oleate in human cell lines (data not shown, unpublished data by Guido Kroemer lab). The combination of oleate and elaidic acid resulted in autophagy induction, whereas palmitate and elaidic acid resulted in autophagy suppression. In line with this notion, oleate and palmitate, respectively, should prolong fitness, life- and healthspan in *Drosophila*. Additionally, the combination of palmitate and elaidic acid should supress this positive effect, while the combination of elaidic acid and oleate should enhance it. The effect of the combination of palmitate and oleate is widely unknown.

3.3.1 Various FAs influence the lifespan of *Drosophila* differently

To determine the effects of FAs on the lifespan of *Drosophila*, an ageing experiment was performed (Figure 8). Fruit flies were maintained on Sigrist supplemented with either a combination of two FAs (Figure 8. C) or a low (Figure 8. A) or high (Figure 8. B) concentration of one specific FA. Remarkably, the flies that were retained on Sigrist alone, i.e. the Control flies, presented a lower survival rate than all others, whether supplemented with a combination or a FA itself. Those exposed to the lower concentration of FAs, show the highest survival rate with palmitate and the lowest with elaidic acid (Figure 8. A). In contrast, those subjected to the higher concentration, display the highest viability with elaidic acid and the lowest with oleate (Figure 8. B). Interestingly, when comparing the combinations of FAs, that of palmitate and oleate results in the highest endurance (Figure 8. C). Both combinations of palmitate and elaidic acid and oleate and elaidic acid, respectively, effect the lifespan equally, as the median survival of the flies exposed to these combinations is in both cases 70 days.

However, the mixture of oleate and elaidic acid seems to enhance the viability for the first 50 days compared to palmitate and elaidic acid.

To conclude, flies treated with fatty acids, whether with high or low concentrations or a combination of the three used, live longer than non-treated flies. With lower concentration, palmitate-exposed flies showed the highest viability, whereas with higher concentration elaidic acid seems to increase survival. The combinations of the three FA used seem to have an equal effect on the survival.

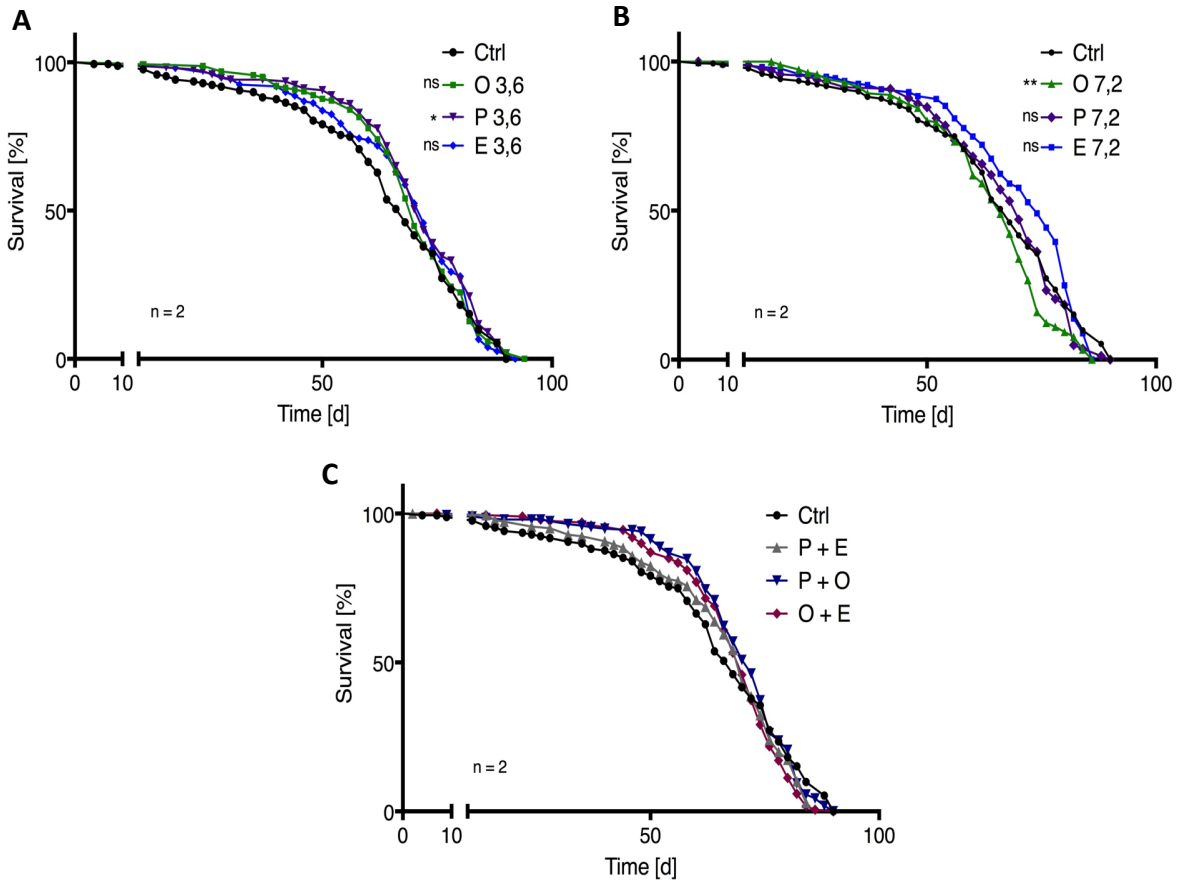

Figure 8. Effects of fatty acids on the survival of female fruit flies.
Female flies of the w^{1118} genotype were fed with Sigrist supplemented with different FA in various concentrations. Number of dead flies were counted every 2-3 days. Data are normalized means ± SE of two individual experiments with eight (**A** +**B**) or nine (**C**) vials each. Each vial contained approximately 20 flies. Asterisks indicate significance as followed: p<0,05=*; p<0,01=**; ns >0,05

A, B Flies were maintained in non-FA-exposed Sigrist (**Ctrl**) or treated with oleate (**O**) or palmitate (**P**) or elaidic acid (**E**) in either 3,6 mM (**A**) or 7,2 mM (**B**) concentration.

C Flies were maintained in non-FA-exposed Sigrist (**Ctrl**) or treated with palmitate and elaidic acid (**P+E**) or palmitate and oleate (**P+O**) or oleate and elaidic acid (**O+E**) in 3,6 mM concentration.

3.3.2 Fatty acids and their combinations affect locomotion function in *Drosophila*

Thereafter, we wanted to observe if FAs and their combinations might affect locomotion function in *Drosophila* by performing a climbing assay on a weekly basis (Figure 9). Exposure to the lower concentration of FAs, shows that both the UFAs and SFAs have a similar effect, elaidic acid being the exception (Figure 9. A). Every success rate is lower than or similar to that of the non-FA-exposed Control, except for the elaidic acid-exposed flies. Subjected to oleate, the flies manage to climb higher from d15 to d30. With palmitate, the flies reached the highest success rate on d22, with all other days resulting in similar rates than the Control. On day 8, elaidic acid-subjected flies reached the highest success rate (143%) of all treated flies. This decreased on d15, yet the fitness increased again towards day 30. Comparing the effects of these FAs from day to day, flies maintained on oleate-supplemented Sigrist failed to climb higher than the control on day 8. This also was the case for all FA-exposed flies on day 15. Flies subjected to palmitate and elaidic acid climbed higher than those exposed to oleate on day 22. Elaidic acid-treated flies were the only ones to reach a higher success rate than the Control on day 30.

Interestingly, compared to the control, flies subjected to higher concentrations of FAs display a wider variation of success rates than when treated with the lower concentration (Figure 9. B). Oleate-exposed flies accomplish to climb higher than the control on day 22. Subjected to palmitate, flies reach similar success rates than the control, with day 30 as exception, on which they climbed 40% higher, making this the highest success rate of all. Flies treated with the higher concentration of elaidic acid succeed to maintain higher success rates than the Control, except on day 15, where it drops to mimic that of the Control. Overall, oleate-treated flies present the lowest success rate on day 8, 15 and 30, whereas those exposed to elaidic acid show the highest on day 8 and d22.

Furthermore, all combinations of those FAs seem to increase the locomotion function when compared to that of the Control (Figure 9. C). The combination of palmitate and elaidic acid (P+E) lets flies exposed to it, manage to double their success rate from d15 to d30. On that point, compared to all combinations-exposed flies, P+E-treated flies achieve the highest of all success rates on day 30. The difference in success rates between each climbing is moderate to none in flies subjected to the combination of palmitate and oleate. Simultaneous administration of oleate and elaidic acid gives flies the ability to climb higher than the control

on d8 and d30. All in all, flies exposed to the simultaneous treatment of palmitate and elaidic acid achieve the highest success rate on the last two days of climbing.

To sum up, the lower concentration of fatty acids seems to have no effect on the success rate of flies, except elaidic acid. With the higher concentration however, the FA seem to increase fitness, except oleate. The combinations of the three FA used, seem to increase the locomotion function of *Drosophila*, especially palmitate combined with elaidic acid.

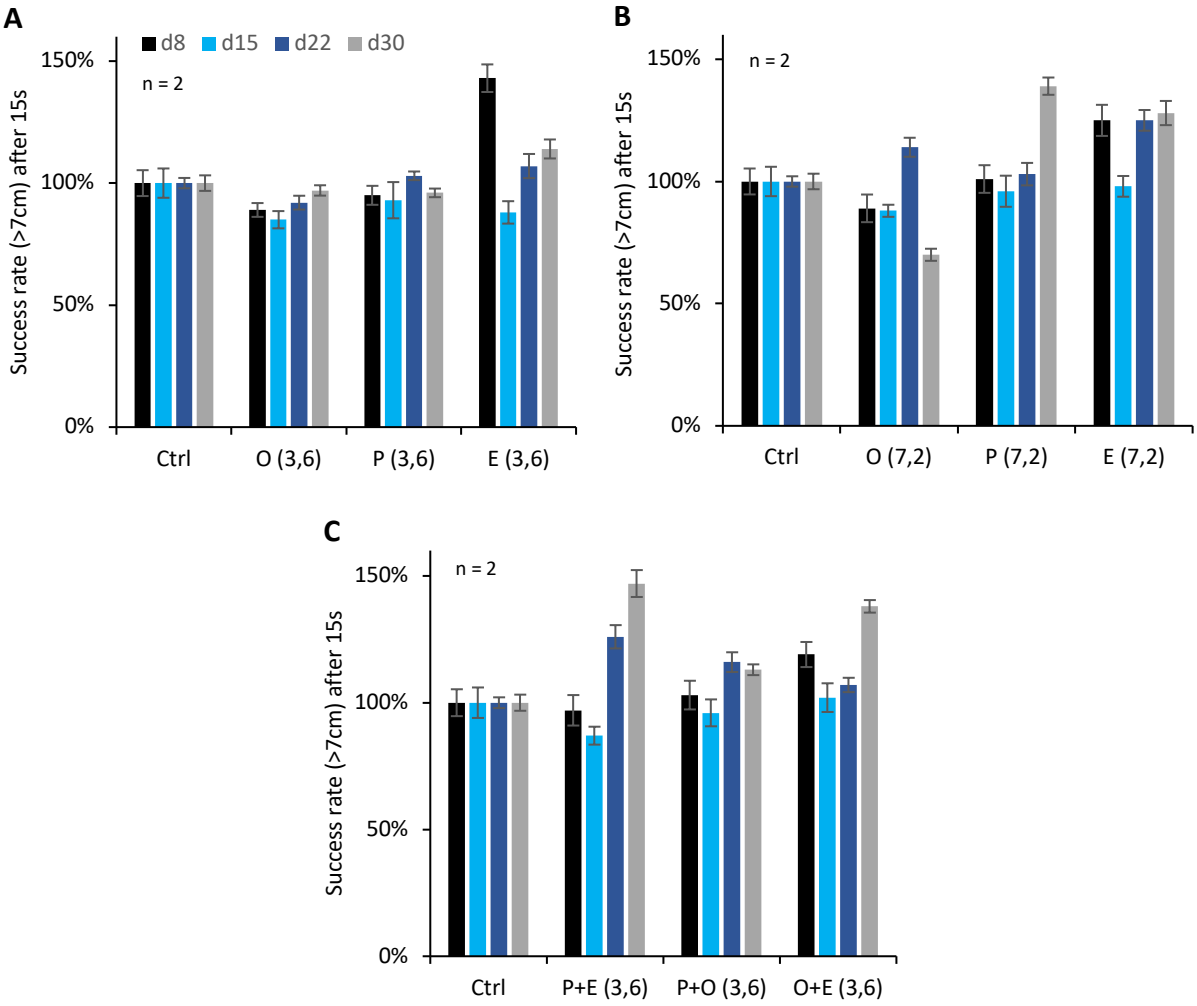

Figure 9. Effects of fatty acids on locomotion function of female fruit flies.
Female flies of the w^{1118} genotype were fed with Sigrist supplemented with different FA in various concentrations. A climbing assay was performed on a weekly basis. Flies were scored according to their climbing height (over a vertical distance of 7 cm) after 15 seconds. Data are normalized means ± SEM from two individual samplings each with eight vials. Each vial contained approximately 15 flies.

A, B Flies were maintained in non-FA-exposed Sigrist (**Ctrl**) or treated with oleate (**O**) or palmitate (**P**) or elaidic acid (**E**) in either 3,6 mM (**A**) or 7,2 mM (**B**) concentration.

C Flies were maintained in non-FA-exposed Sigrist (**Ctrl**) or treated with palmitate and elaidic acid (**P+E**) or palmitate and oleate (**P+O**) or oleate and elaidic acid (**O+E**) in 3,6 mM concentration.

3.3.3 Supplementation of palmitate and elaidic acid for 35 days leads to higher survival rates in starvation resistance assay

Following the climbing assay, the same flies were then placed into vials with water plus 1 % (w/v) agar. This led us to ascertain if the FA and their combinations affect survival in a starvation resistance assay (Figure 10). Whether the flies were treated with the lower or higher concentration of FAs, the survival rates were all alike with similar median survival: they were all higher than that of the non-FA-exposed Control flies (Figure 10. A & B). Lower concentration of oleate-treatment led to the longest survival, whereas palmitate and elaidic

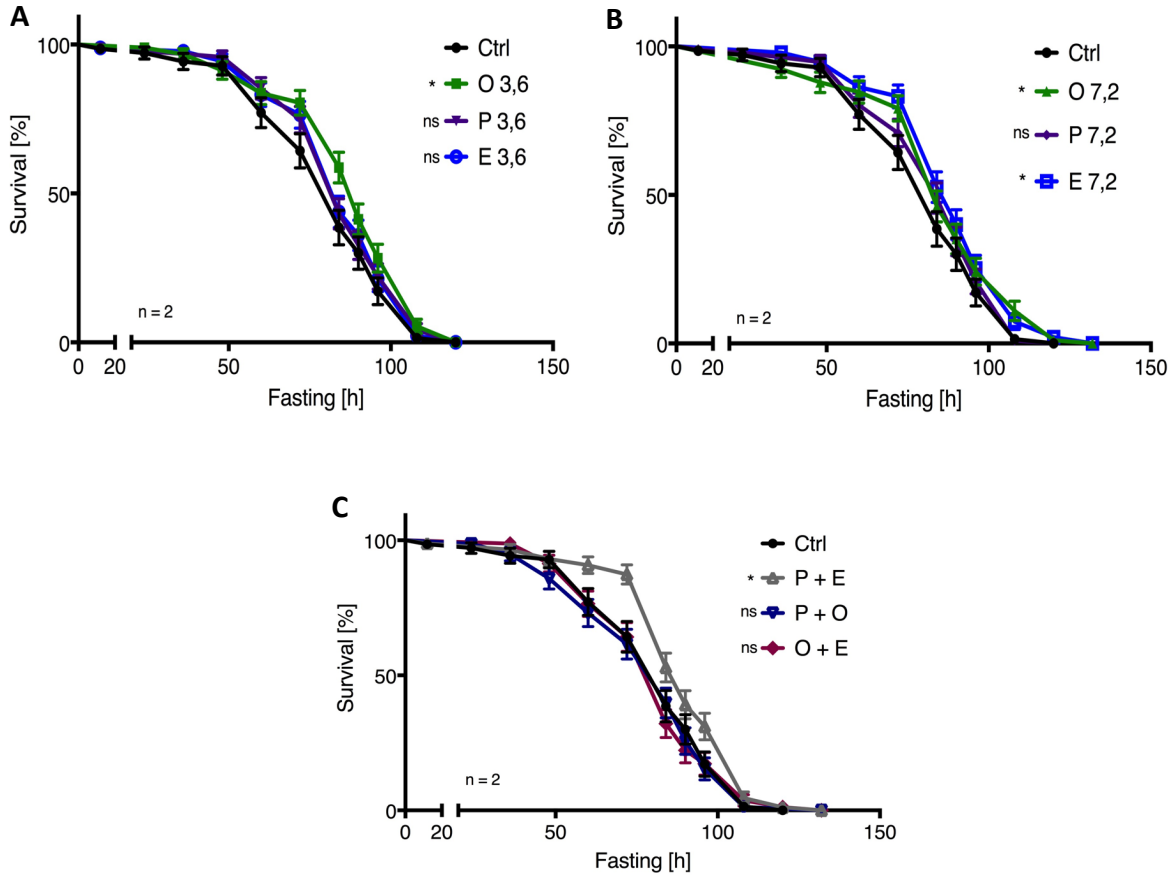

Figure 10. Supplementation of a combination of palmitate and elaidic acid (for 35 d) increases survival rate of female fruit flies in starvation resistance assay.
Female flies of the *w*^{1118} genotype were aged on Sigrist supplemented with various fatty acids with different concentrations for 35 days prior to being transferred to vials containing agar. Every 12 hours the number of dead flies was counted. Data are normalized means ± SE from two individual samplings each with four vials. Each vial contained approximately 15 flies.
Asterisks indicate significance as followed: p<0,05=*; ns >0,05

A, B Flies were aged on FA alone with either 3,6 mM (**A**) or 7,2 mM (**B**) concentration, respectively; either on oleate (**O**) or palmitate (**P**) or elaidic acid (**E**).

C Flies were aged on combinations of FA with 3,6 mM concentration; either on palmitate and elaidic acid (**P+E**) or palmitate and oleate (**P+O**) or oleate and elaidic acic (**O+E**).

acid exposure resulted in equal high survival rates (Figure 10. A). In contrast, flies subjected to the higher concentration, lived longer when exposed to elaidic acid (Figure 10. B). Intriguingly, simultaneous administration of palmitate and elaidic acid as a combination led the longest endurance, which coincides with the results of the climbing assay (Figure 10. C). Flies supplemented with the other combinations of FAs, that is O+E and P+O, presented similar survival rates than that of the control.

Whether the flies were treated with the lower or higher concentration of fatty acids for 35 days in summary the survival rates were all alike and higher than that of the non-FA-exposed control. Higher viability seems to be ascertained with lower concentration of oleate and higher concentration of elaidic acid. Simultaneous administration of palmitate and elaidic acid seems to increase survival even further in contrast to the other combinations.

3.3.4 A 14-day-treatment with fatty acids is not sufficient to increase starvation stress resistance in *Drosophila*

Next, another starvation resistance assay was performed, albeit after 14 days of FAs exposure (Figure 11). Goal of this assay was to determine if FAs and their combinations would have the same effect as after 35 days of supplementation. All flies exposed to 3,6 mM concentration of FAs and their combinations presented equally high survival rates as that of the non-FA-exposed control, except those subjected to palmitate and the combination of oleate and elaidic acid, respectively. The latter two treatments both reduced the survival (Figure 11. A & C). Additionally, all FAs with higher concentration lead to a decrease in survival rate when compared to the Control (Figure 11. B).

To conclude, a 14-day pre-treatment with fatty acids and their combinations lead to different results in this assay than after 35 days. The 14-day-treatment seems to be insufficient to increase survival in *Drosophila*. All FA given in low concentration after 14 days of treatment seem to have the same effect on survival as the non-exposed control, except palmitate and the combination of oleate and elaidic acid. Both of which seem to reduce the viability, as do all the FA in higher concentration.

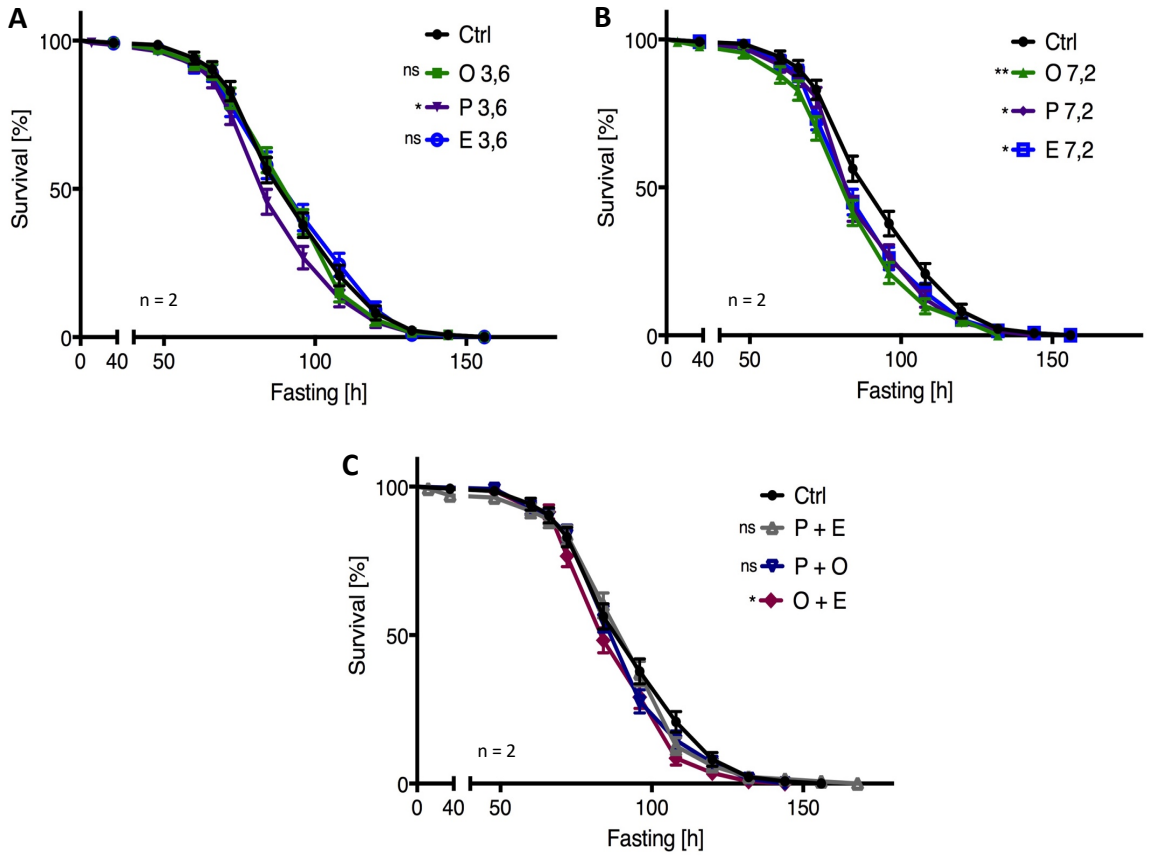

Figure 11. 14-day-treatment with FAs and their combinations is not beneficial to the survival of female flies in a starvation resistance assay.
Female flies of the w^{1118} genotype were aged on Sigrist supplemented with various fatty acids with different concentrations for 14 days prior to being transferred to vials containing agar. Every 12 hours the number of dead flies was counted. Data are normalized means ± SE from two individual samplings, each with four vials. Each vial contained approximately 20 flies.
Asterisks indicate significance as followed: p<0,05=*; p<0,01=**; ns >0,05

A, B Flies were aged on FA alone with either 3,6 mM (**A**) or 7,2 mM (**B**) concentration, respectively; either on oleate (**O**) or palmitate (**P**) or elaidic acid (**E**).

C Flies were aged on combinations of FA with 3,6 mM concentration; either on palmitate and elaidic acid (**P+E**) or palmitate and oleate (**P+O**) or oleate and elaidic acic (**O+E**).

3.3.5 Fatty acid and their combinations affect autophagy in *Drosophila*

To ascertain if the combinations of FAs induce autophagy, immunoblotting was performed (Figure 12). The autophagy induction was ascertained by dividing LC3-II by LC3-I, then divided by the total amount of LC3 ((LC3-II/LC3-I)/(LC3-II+LC3-I)). The Control flies and those treated with a combination of palmitate and elaidic acid present more autophagy induction on d17 than d3 (Figure 12. B). However, the p62-protein levels of the combination are elevated. Both combinations of either oleate and elaidic acid or palmitate and oleate, respectively, induce autophagy in similar amount on both days. In addition, the p62-levels of these combinations

are also similar on both days. The autophagy induction is slightly higher in P+O than in O+E-treated flies. The highest induction of autophagy is shown in flies with supplementation of the combination of palmitate and elaidic acid on d17.

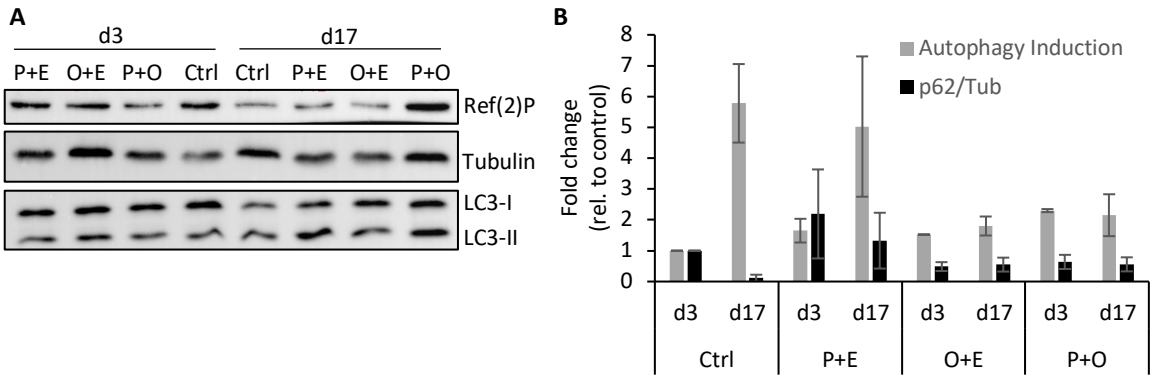

Figure 12. Combination of palmitate and elaidic acid induces autophagy in *Drosophila*.
Female flies of a *w*^{1118} genotype were aged on Sigrist food supplemented with either palmitate and elaidic acid (**P+E**), oleate and elaidic acid (**O+E**) or palmitate and oleate (**P+O**), for 3 days or 17 days, respectively. Each of the FAs was added in 3,6 mM concentration Preceding immunoblotting, flies were frozen with liquid nitrogen and heads extracted and homogenized with SDS-Buffer. The autophagy induction was ascertained as followed: (LC3-II/LC3-I)/(LC3-II+LC3-I). (**A**) A representative western blot is shown. (**B**) The levels of LC3 lipidation and p62 degradation are normalized to the tubulin loading control and to that of a non-FA-exposed control (**Ctrl**) of each sample. Data are normalized means ± SEM of two representative experiments. In total four independent blots were performed which showed similar tendencies, but due to technical problems the level of p62/Tub was not detectable on the other blots.

Moreover, by performing another immunoblot, herein with the various FAs themselves, we wanted to affirm if both SFAs and UFAs induce autophagy in *Drosophila* (Figure 13). Indeed, whether the FAs were supplemented in the lower or higher concentration, every one of them managed to induce autophagy (Figure 13. B, D, F). The overall autophagy induction was higher in the flies of day 3 (Figure 13. B) than in those of the other two days (Figure 13. D, F). Flies treated with higher concentrated elaidic acid presented the highest rate of autophagy stimulation not only on day 3 (Figure 13. B) but also, on day 17 (Figure 13. D). Even the other two FAs, when administered in the higher concentration on day 3, lead to more induction than the treatment with the lower concentration (Figure 13. B). Those subjected to oleate in the lower concentration presented an induction that was similar to their p62 protein levels. Palmitate in 3,6 mM concentration is the only FA on day 3 that resulted in a lower p62-level than that of the non-FA-exposed Control. Flies of day 17 displayed lower autophagy induction rates than that of the Control when subjected to palmitate in both concentrations and oleate

in 3,6 mM concentration, respectively (Figure 13. D). Additionally, the p62-levels of these treatments are raised and thus similar to the rate of induction and of that of the Control flies. However, this changes towards day 30, on which the flies treated with the respective FAs, all show more autophagy induction than that of the non-FA-exposed Control flies (Figure 13. F).

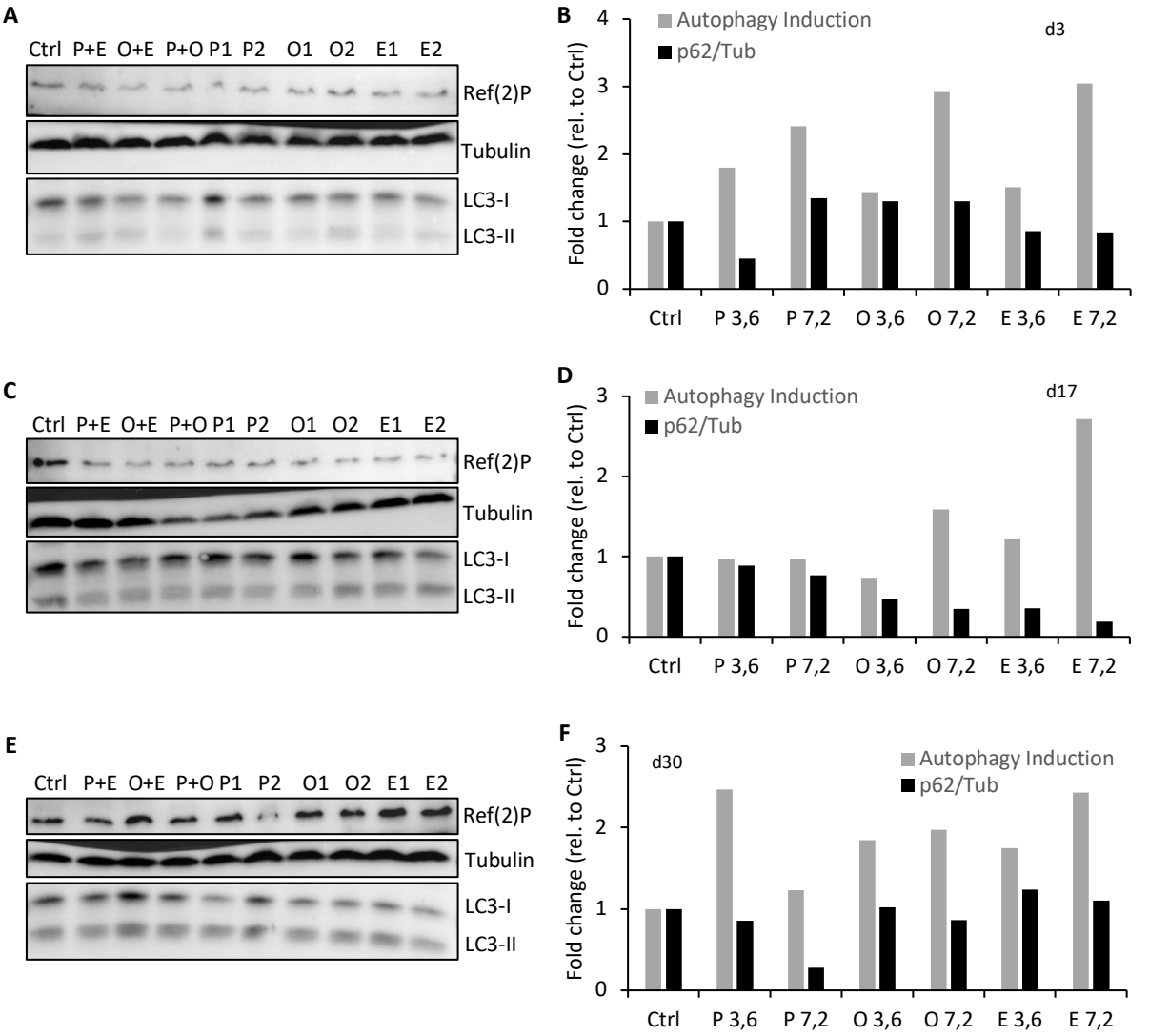

Figure 13. Saturated and unsaturated fatty acids induce autophagy in *Drosophila*.
Female flies of a w^{1118} genotype were aged on Sigrist food supplemented with either palmitate (**P**), elaidic acid (**E**) or oleate (**O**), respectively. Each of the FAs was either added in 3,6 mM (**P1; O1; E1; P3,6; O3,6; E3,6**) or 7,2 mM concentration (**P2; O2; E2; P7,2; O7,2; E7,2**). Preceding immunoblotting, flies were frozen with liquid nitrogen and heads extracted and homogenized with SDS-Buffer. Flies were maintained respective food for three (**A, B**), seventeen (**C, D**) and thirty (**E, F**) days foregoing immunoblotting. (**B, D, F**) The levels of LC3 lipidation and p62 degradation are normalized to the tubulin loading control and to that of a non-FA-exposed control (**Ctrl**) of each sample. Data are a representative analysis of one assessment. In total two independent blots were performed which showed similar tendencies, but due to technical problems the level of p62/Tub was not detectable on the other blot. (**A, C, E**) A representative western blot is shown.

The highest rate of autophagy is displayed by the flies supplemented with palmitate in the lower concentration and elaidic acid in the higher concentration. However, palmitate in the 7,2 mM concentration is the only FA on day 30 that has a lower p62 protein level as outcome of the treatment than that of the non-treated Control.

In conclusion, all fatty acids used seem to lead to an induction of autophagy. In regard to the combinations of FA, simultaneous administration of palmitate and elaidic acid seems to induce autophagy the most. Overall, all FA-combinations show more autophagy induction of day 17 rather than day 3. Also, both saturated and unsaturated fatty acids seem to induce autophagy when given in the higher concentration rather than lower concentration. Elaidic acid, whether given in low or high concentration, is the only FA that induces autophagy on all three days of immunoblotting.

4. Discussion

Alzheimer's disease (AD) is at present an incurable fatal neurodegenerative disease that is the most common form of dementia. Millions of people around the world have to fight this progressive disorder with only symptomatic treatment available. The underlying pathological mechanisms behind this illness are still poorly understood.

Spermidine is a ubiquitinous polycation that is found in various foods, such as cheese and soy beans. It is established that this substance induces autophagy and consequently can ameliorate age-induced memory loss. Being a natural component, no side effects are known to this day, yet its various beneficial properties have been implicated in several studies. However, the exact molecular mechanisms or pathways by which spermidine can induce autophagy are not clear.

Myo-Inositol is a natural sugar with its polyphosphates being polyol precursors in second messenger systems in the cell. Through various studies, it was discovered that high intracellular levels of inositol and IP_3 can negatively regulate autophagy [46]. In line with this notion, the Ino/IP_3-pathway may be the key to the mechanism behind spermidine-induced autophagy.

Fatty acids, being it saturated or unsaturated, have a bad reputation through their involvement in cardiovascular diseases. However, studies uncovered that both SFAs and UFAs can induce autophagy in different ways, which should lead to benefits in relation to life- and health span.

The development of sophisticated models to research the exact effects of aforementioned substances is fundamental. Drosophila melanogaster is an established model for neurodegenerative diseases and for performing various life- and health span assays, including the evaluation of fitness and stress resistance.

4.1 Spermidine mediates neuroprotection

In the various assays performed, Aβ-toxicity became apparent, which asserts the effectiveness of our model used. When interpreting the results of the manganese stress resistance assay, both genotypes lived longer when stressed with spermidine-added manganese-solution, indicating that spermidine enhances survival. Aβ42-expressing flies lived longer with the spermidine-treatment than without (Figure 1). This implicated that

spermidine mediates neuroprotection via reducing Aβ triggered toxicity, which in turn strengthens our hypothesis. Yet, for its ability to take effect, spermidine apparently needs to be administered in a stress situation. Backing this assumption up, Aβ42-expressing flies aged on spermidine-added Sigrist food only showed an increase in survival when stressed with the combination of manganese and spermidine (Figure 1). Nonetheless, the difference in survival rates between the flies stressed with spermidine-added manganese-solution or manganese alone is evidently higher in Aβ42-expressing flies than in flies of the w^{1118} wild type flies. Thus, the rescuing effect of spermidine may be specific to the Aβ42 genotype or to flies with a malfunctioning autophagy machinery.

4.2 Oversupply of spermidine ameliorates Aβ-toxicity

Through the spatial and timely division of the distribution of spermidine, it revealed that spermidine somehow effects the embryonic development of the fly offspring. Namely, it further decreases the lifespan of Aβ42-expressing flies if hatched on spermidine-exposed Sigrist (Figure 2). The additional stress with the combination of manganese and spermidine does seem to mitigate this negative side-effect of early spermidine-pre-treatment, yet not to a full extent. In line with this notion, it's tempting to assume that the supply of spermidine in an early stage of development or the oversupply of this substance may ameliorate Aβ triggered toxicity. In another study, it has been shown that high concentrations of SPD lead to a decrease in survival, which would support our observations [34]. Due to the fact that SPD is produced by cells itself, it must have a vital role for the organism and therefore must be highly regulated. Thus, meddling in this regulation by oversupplying the natural compound, is evidently bad for the organism and its survival. Here, it seemed again that spermidine does exert a different reaction in both genotypes, being that the flies of the w^{1118} genotype presented no difference in endurance, whether with spermidine-pre-treatment or without or even whether stressed farther with spermidine-added manganese solution or without. Nonetheless, if the effect of early pre-treatment of spermidine is specific to Aβ induced neurotoxicity being enhanced, it would make sense that the flies not expressing Aβ42 would not show any outcome at all.

4.3 Spermidine induces autophagy

The evidence of Aβ42-expressing flies showing less autophagy induction than the control, while both maintained on Sigrist food (Figure 3), indicates that the autophagic machinery of the former, is indeed somehow impaired. In addition, the p62 protein levels of these flies were has high as that of the control, further implicating an autophagic block in Aβ42-expressing flies. This supports the theory of Aβ42 being the cause for autophagy obstruction and therefore may lead to brain plaques and nerve deterioration in Alzheimer's disease. As a result of increased autophagy induction in Aβ42-expressing flies upon spermidine-treatment, this substance seems to reverse or mitigate the aforementioned. This seems to coincide with the result of the manganese stress resistance assay and correlates with our hypothesis that spermidine-induced autophagy may be the reason for its ability to facilitate neuroprotection. However, the immunoblotting of the flies of the Aβ42 genotype that aged on spermidine-added Sigrist food show the exact opposite of the corresponding outcome of the survival assay. Here, those aged and stressed with administration of spermidine show a lower autophagy induction than those stressed with manganese alone. Yet, if looking at the p62-levels of the latter, which are higher than that of the control, it suggests that even though the autophagy rate is higher as previously mentioned, it has a lesser throughput than that of flies with both spermidine-treatments. In the control flies, the autophagy induction seems to be largely unaffected whether with or without spermidine exposure, which further concurs with the assumption that spermidine utilizes various actions in the two different genotypes.

4.4 Simultaneous administration of myo-Inositol mitigates spermidine-mediated neuroprotection without effecting Aβ induced toxicity

Spermidine leads to an increase of lifespan in Aβ42-expressing flies. This effect is mitigated through a treatment with myo-Inositol. We could confirm that with inositol-exposure, spermidine had only a moderate to no effect on the endurance of the Aβ42-expressing flies (Figure 4, 5). Supporting our hypothesis, this observation implies that with inositol administration and the consequent increase of IP_3-levels lead to the demise of the rescuing effect of spermidine. The increase of lifespan in w^{1118} wild type flies upon treatment with inositol and farther being stressed with manganese alone, could be due to the lack of

autophagy dysfunction in those flies. The Ino/IP_3-signaling pathway may negatively regulate autophagy in organisms with an already impaired autophagy machinery (i.e Aβ42-expressing flies). All studies that established this pathway influences autophagy have been performed with organisms with autophagy impairment, including in humans who suffer from bipolar disorder, rather than looking at the effect on those with an intact autophagy process. Another assumption is that organisms need inositol for diverse functions in cells, thus through supplementation with inositol the lack of it in the standard fly food may be redressed, leading to an increase in survival consequently to pre-treatment with inositol.

When looking at Aβ42-induced toxicity, our data shows that subjection to inositol has no effect, positive or negative, on this cytotoxity (Figure 5). Flies of the Aβ42 genotype presented with the same survival rate, whether treated with inositol or not. These two groups both had equally decreased lifespans compared to that of the control flies, evidently affiliated with the toxicity triggered by Aβ42. With addition of spermidine to the pre-treatment of inositol, the cytotoxicity was slightly reduced. However, this effect was so minimal compared to the outcome of spermidine-supplementation without prior inositol-administration.

Furthermore, our data demonstrates that through the spatial and timely divided inositol-supplementation, it influences the embryonic development of the *Drosophila* offspring (Figure 6). It has to be mentioned that both genotypes of flies hatching on inositol-added Sigrist food had a markedly lower outcome of F1 progeny than those originating on only Sigrist. Therefore, if early administration of inositol may favour the survival of the fittest, a significant increase in manganese stress resistance of this progeny flies may be one of the consequences. This assumption is backed by the Aβ42-expressing flies managing to acquire the highest viability when originating on inositol-added Sigrist and being farther stressed with manganese. The addition of spermidine to these pre-treated flies, decreased their lifespan, nearly to be comparable with the Aβ42-expressing flies hatched on Sigrist and stressed with manganese alone. Even in the w^{1118} wild type flies those pre-treated with inositol and stressed with manganese displayed an increase in lifespan, compared to those stressed with both manganese and spermidine. So, to say, inositol in high concentrations seems to be detrimental in embryonic development, which is worsened in mutated flies by addition of spermidine.

4.5 Myo-inositol inhibits spermidine-induced autophagy

We could confirm that spermidine induces autophagy in our model organism used (Figure 3). In addition, we could ascertain that with administration of inositol the autophagy is blocked in both genotypes of flies (Figure 7). The Aβ42-expressing flies with inositol-supplementation displayed an autophagy induction similar to that of the non-inositol-exposed control flies. Yet, through their p62 protein levels being higher than that of the control, the impairment of autophagy through inositol and Aβ42 itself is indicated. To further clarify, if there is an intact autophagy machinery, no accumulation of p62 protein levels is present, due to a greater extent of the degradation process of autophagosomes. Moreover, our data demonstrates that Aβ42-expressing flies exposed to both inositol and farther spermidine display twice as much amount of p62 than its autophagy rate, suggesting that through the inositol administration, there is too much intracellular IP_3 for the precise regulation of autophagy through spermidine to take effect. Thus, spermidine can't induce autophagy after pre-treatment with inositol, which coincides with the results of the manganese stress resistance assay. This signifies that inositol does play a role in and may even be the cause of the mechanisms by which spermidine induces autophagy and facilitates neuroprotection.

4.6 Treatment of fatty acids and their combinations benefits different aspects of life- and health span, including fitness and stress resistance

Through various experiments, we could see that the treatment of the used fatty acids and their combinations act differently in relation to the assay on the life- and health span, including locomotion function and stress resistance, of *Drosophila*. To sum up, every fatty acid and every one of their combinations increased the lifespan in an ageing assay (Table. 1). This can be a consequence of the offering of fatty acids, as the standard fly food used is quite fat-free. So, with the addition of fatty acids various deficiencies the flies might have are overcome. Every lipid used either improved or had the tendency to improve the locomotion function of *Drosophila*, except oleate in both concentrations that decreased the fitness. Inspite of our hypothesis, the simultaeonous administration of palmitate and ealidic acid turned out to not only increase the lifespan and survival, but also improve the locomotion function. In contrast, the combination of oleate and elaidic acid however, had no impact on the survival. The increase in lifespan of the flies subjected to fatty acids in the starvation assay

after 35 days of exposure, may be through the fatting of these flies and therefore they endured longer than the non-fatty-acid-exposed control. Largely, the elaidic acid showed the most positive effects of all fatty acids, whether in administered in high or low concentration. It has to be mentioned that while mixing the higher concentrations of the fatty acids in Sigrist food, not everything of the lipid was emulsified. Overall, we could show that the supplementation of these fatty acids and their combination led to various beneficial properties, despite most of their bad reputations.

Table 1. A summary of the results from administering various fatty acids and their combinations in *Drosophila*.
Four different assays were performed with female w^{1118} wild type flies, in which various fatty acids in different concentrations and their combinations, respectively were supplemented. (**P+E**) combination of palmitate and elaidic acid, both in 3,6 mM concentration. (**O+E**) combination of oleate and elaidic acid, both in 3,6 mM concentration. (**P+O**) combination of palmitate and oleate, both in 3,6 mM concentration. (**P**) palmitate, (**O**) oleate and (**E**) elaidic acid were given in either 3,6 mM or 7,2 mM concentration.

	Starvation Resistance Assay (35 d)	**Starvation Resistance Assay (14 d)**	**Ageing**	**Climbing Assay**
P+E	Highest survival	No effect	Increases lifespan	Improves fitness
O+E	No impact	Reduces survival	Increases lifespan	Improves fitness
P+O	No impact	No effect	increases lifespan	Tendency to improve fitness
P 3,6	Increases survival	Reduces survival	increases lifespan	No improvement
P 7,2	Increases survival	Reduces survival	Increases lifespan	Tendency to improve fitness
O 3,6	Highest survival	No effect	Increases lifespan	Decreases fitness
O 7,2	Increases survival	Reduces survival	Increases lifespan	Tendency to decrease fitness
E 3,6	Increases survival	No effect	Increases lifespan	Improves fitness
E 7,2	Highest survival	Reduces survival	Highest lifespan	Improves fitness

4.7 Both saturated and unsaturated fatty acids induce autophagy

Overall, our data shows that both saturated and unsaturated fatty acids induce autophagy. Though, it varies from the different days the immunoblots were performed. Contrary to our hypothesis, every combination of fatty acids managed to induce autophagy. However, the combinations of oleate and elaidic acid and that of palmitate and oleate, respectively,

presented the induction of autophagy in a consistent manner from day 3 to day 17. Mainly all FA and their combinations either induced or showed the tendency to induce autophagy in an earlier point in time (i.e. d3). The induction of autophagy later in time (i.e. on day 17 or d30), displays predominantly no to moderate throughput, when comparing the amount of p62 protein levels to that of autophagy induced. This may indicate that in an aging organism, autophagy is impaired in a way that apparently no further supplementation with fatty acid or combination of the former can mitigate.

5. Conclusion

To sum up, this work identified that spermidine does mediate neuroprotection and therefore may be the key to fighting nerve deterioration in neurodegenerative illnesses, such as Alzheimer's disease. As it undoubtedly induces autophagy, it is safe to assume that there is a correlation between this ability and the aforementioned. Furthermore, as it seems, spermidine increases the autophagy-activation competence in stress situations, such as through starvation (i.e. through manganese) but does not enhance the basal autophagy level. Due to its molecular mechanisms still being widely unclear, further research surrounding this polycation in relation to its ability to induce autophagy, and therefore its role in the process of aging and in fighting diseases, must be undertaken. The exact pathway with which spermidine induces autophagy could be the answer to a lot of questions. Moreover, our data implicates the Ino/IP_3-signaling pathway to be a factor in spermidine-induced autophagy. Not only does inositol reduce the protective effect of spermidine, it also inhibits the re-induction of autophagy by the polyamine. It is vital that further subsequent experiments are performed to enhance our understanding of this correlation and how this may help solve the issues with autophagy dysfunction in incurable diseases.

Our data showed that the fatty acids and their combinations used have both positive and negative properties in relation of life- and health span, including locomotion function and stress resistance, in *Drosophila*.

6. Supplemental Data

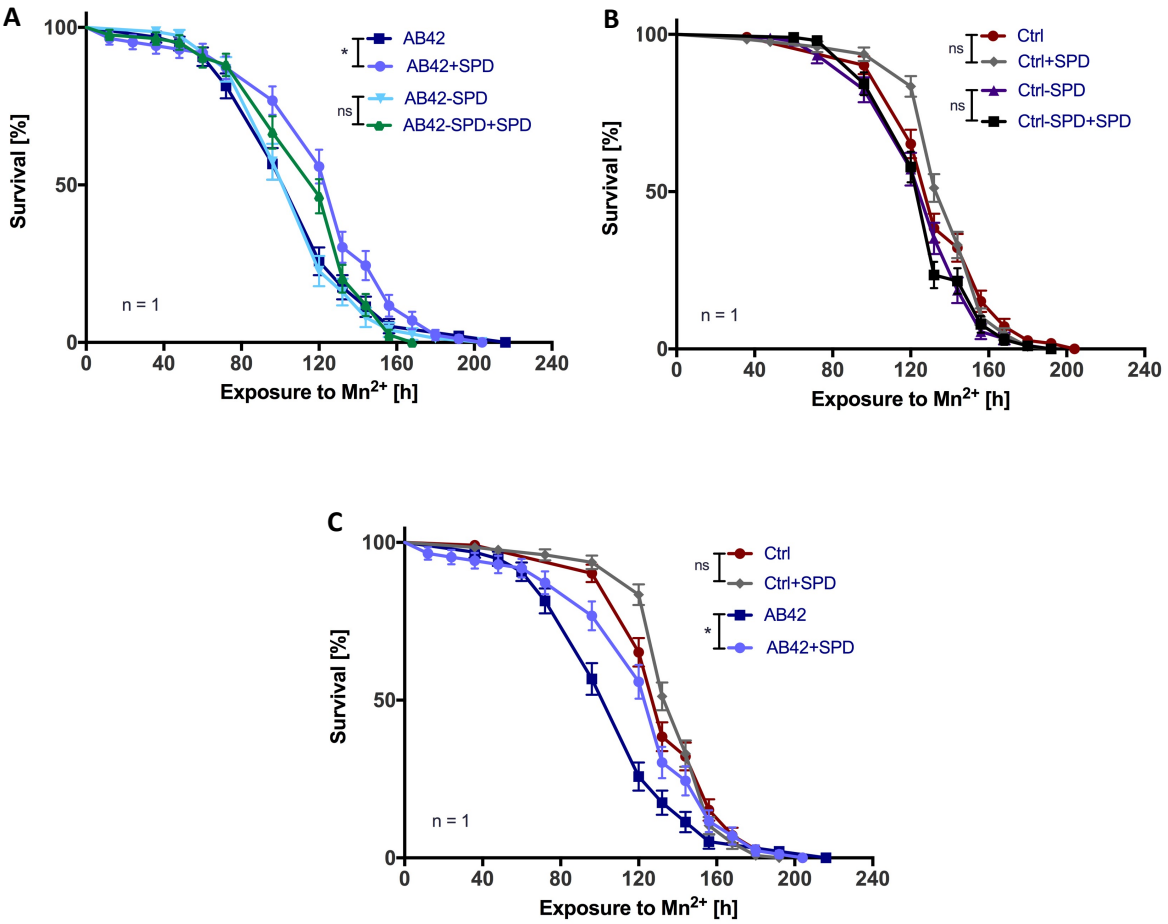

Figure S1. Effects of spermidine on Aβ-toxicity and the survival of Aβ-expressing female fruit flies in a manganese stress resistance assay.

Female fruit flies of both w^{1118} (**Ctrl**) and AB42 genotypes were aged on Sigrist +/- SPD (4 mM) for 14 d preceding a manganese stress resistance assay with 10 mM $MnCl_2$ +/- SPD (4 mM) in 10% sucrose solution. Every 12 hours the number of dead flies was counted. Data are normalized means ± SE of one representative experiment. In total ten independent biological replicas were undertaken (see Figures 1-2, 4,6 and S1,3-5) with similar tendencies, but the similarities disappear in a summary of all, due to the different survival margin of the individual experiment. Each vial contained 20-30 flies.

A Female flies of the AB42 genotype were either aged on Sigrist alone (**AB42** & **AB42+SPD**) or on Sigrist supplemented with SPD (**AB42-SPD** & **AB42-SPD+SPD**). One group was treated with Mn alone (**AB42** & **AB42-SPD**), whereas the other was treated with a combination of Mn and SPD (**AB42+SPD** & **AB42-SPD+SPD**).

B Female flies of the w^{1118} genotype were either aged on Sigrist alone (**Ctrl** & **Ctrl+SPD**) or on Sigrist supplemented with SPD (**Ctrl-SPD** & **Ctrl-SPD+SPD**). One group was treated with Mn alone (**Ctrl** & **Ctrl-SPD**), whereas the other was treated with a combination of Mn and SPD (**Ctrl+SPD** & **Ctrl-SPD+SPD**).

C Female flies of both w^{1118} and AB42 genotypes were aged on Sigrist foregoing a manganese stress with (**Ctrl+SPD** & **AB42+SPD**) or without (**Ctrl** & **AB42**) SPD supplementation.

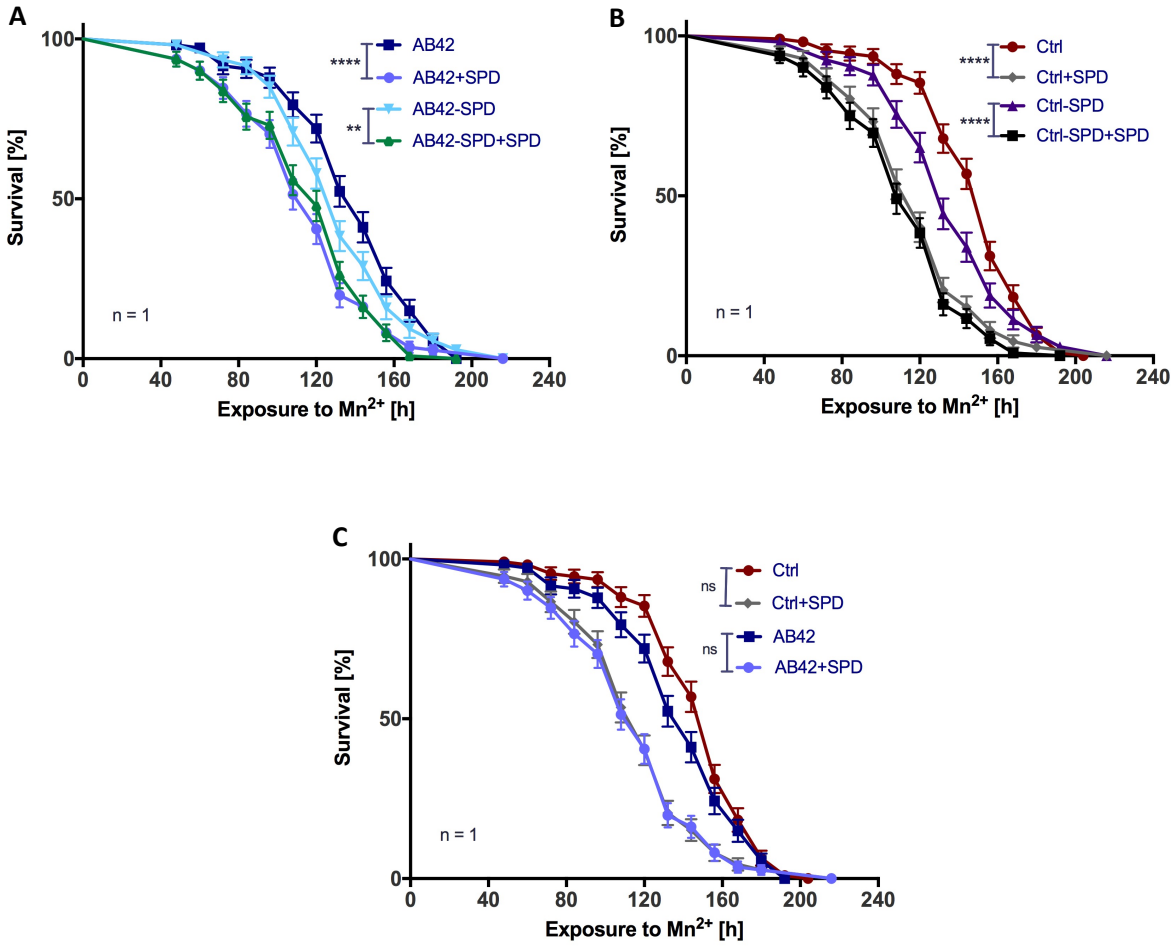

Figure S2. Effects of spermidine on Aβ-toxicity and the survival of Aβ-expressing female fruit flies in a manganese stress resistance assay.

Female fruit flies of both w^{1118} (**Ctrl**) and AB42 genotypes were aged on Sigrist +/- SPD (4 mM) for 14 d preceding a manganese stress resistance assay with 10 mM $MnCl_2$ +/- SPD (4 mM) in 10% sucrose solution. Every 12 hours the number of dead flies was counted. Data are normalized means ± SE of one representative experiment. In total ten independent biological replicas were undertaken (see Figures 1-2, 4,6 and S1,3-5) with similar tendencies, but the similarities disappear in a summary of all, due to the different survival margin of the individual experiment. Each vial contained 20-30 flies.

A Female flies of the AB42 genotype were either aged on Sigrist alone (**AB42** & **AB42+SPD**) or on Sigrist supplemented with SPD (**AB42-SPD** & **AB42-SPD+SPD**). One group was treated with Mn alone (**AB42** & **AB42-SPD**), whereas the other was treated with a combination of Mn and SPD (**AB42+SPD** & **AB42-SPD+SPD**).

B Female flies of the w^{1118} genotype were either aged on Sigrist alone (**Ctrl** & **Ctrl+SPD**) or on Sigrist supplemented with SPD (**Ctrl-SPD** & **Ctrl-SPD+SPD**). One group was treated with Mn alone (**Ctrl** & **Ctrl-SPD**), whereas the other was treated with a combination of Mn and SPD (**Ctrl+SPD** & **Ctrl-SPD+SPD**).

C Female flies of both w^{1118} and AB42 genotypes were aged on Sigrist foregoing a manganese stress with (**Ctrl+SPD** & **AB42+SPD**) or without (**Ctrl** & **AB42**) SPD supplementation.

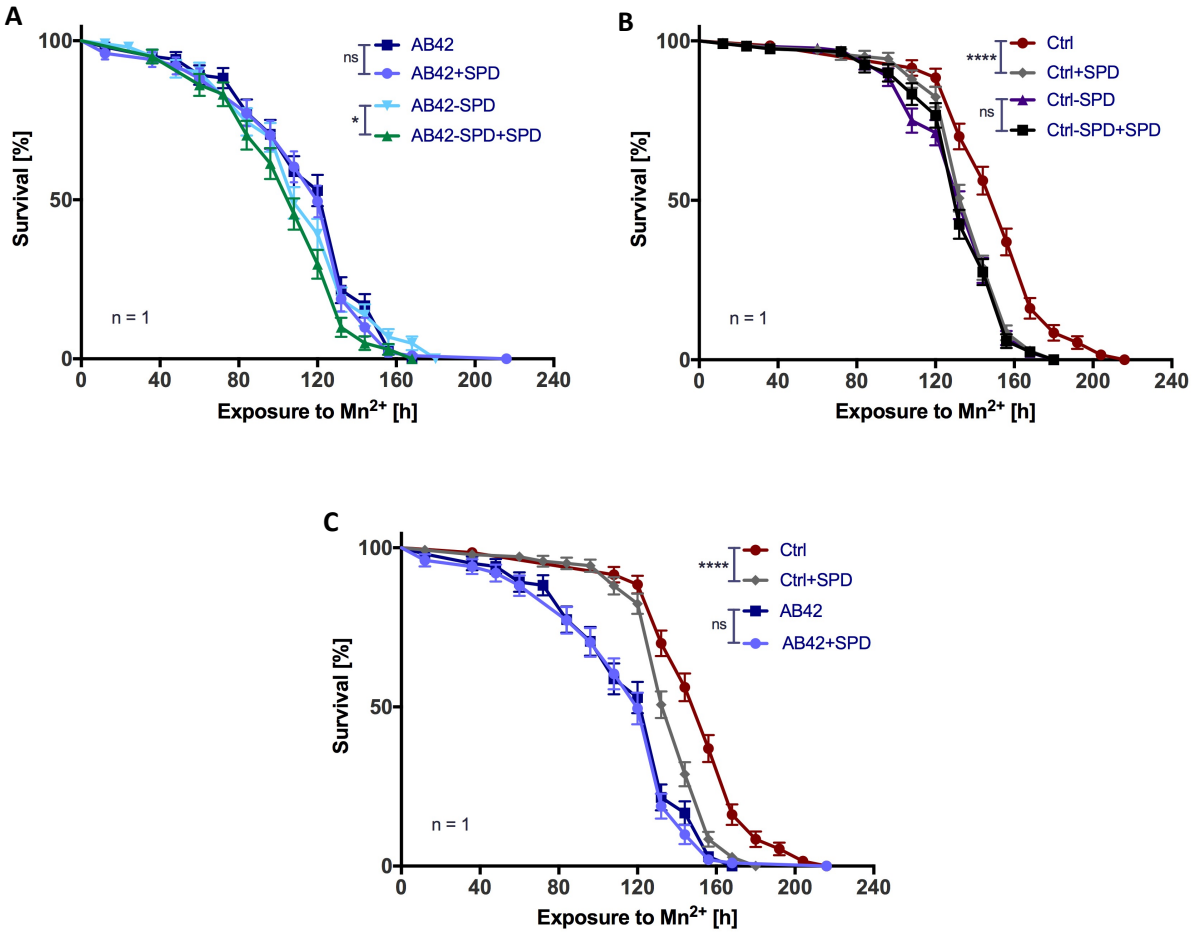

Figure S3. Effects of spermidine on Aβ-toxicity and the survival of Aβ-expressing female fruit flies in a manganese stress resistance assay.

Female fruit flies of both w^{1118} (**Ctrl**) and AB42 genotypes were aged on Sigrist +/- SPD (4 mM) for 14 d preceding a manganese stress resistance assay with 10 mM $MnCl_2$ +/- SPD (4 mM) in 10% sucrose solution. Every 12 hours the number of dead flies was counted. Data are normalized means ± SE of one representative experiment. In total ten independent biological replicas were undertaken (see Figures 1-2, 4,6 and S1,3-5) with similar tendencies, but the similarities disappear in a summary of all, due to the different survival margin of the individual experiment. Each vial contained 20-30 flies.

A Female flies of the AB42 genotype were either aged on Sigrist alone (**AB42** & **AB42+SPD**) or on Sigrist supplemented with SPD (**AB42-SPD** & **AB42-SPD+SPD**). One group was treated with Mn alone (**AB42** & **AB42-SPD**), whereas the other was treated with a combination of Mn and SPD (**AB42+SPD** & **AB42-SPD+SPD**).

B Female flies of the w^{1118} genotype were either aged on Sigrist alone (**Ctrl** & **Ctrl+SPD**) or on Sigrist supplemented with SPD (**Ctrl-SPD** & **Ctrl-SPD+SPD**). One group was treated with Mn alone (**Ctrl** & **Ctrl-SPD**), whereas the other was treated with a combination of Mn and SPD (**Ctrl+SPD** & **Ctrl-SPD+SPD**).

C Female flies of both w^{1118} and AB42 genotypes were aged on Sigrist foregoing a manganese stress with (**Ctrl+SPD** & **AB42+SPD**) or without (**Ctrl** & **AB42**) SPD supplementation.

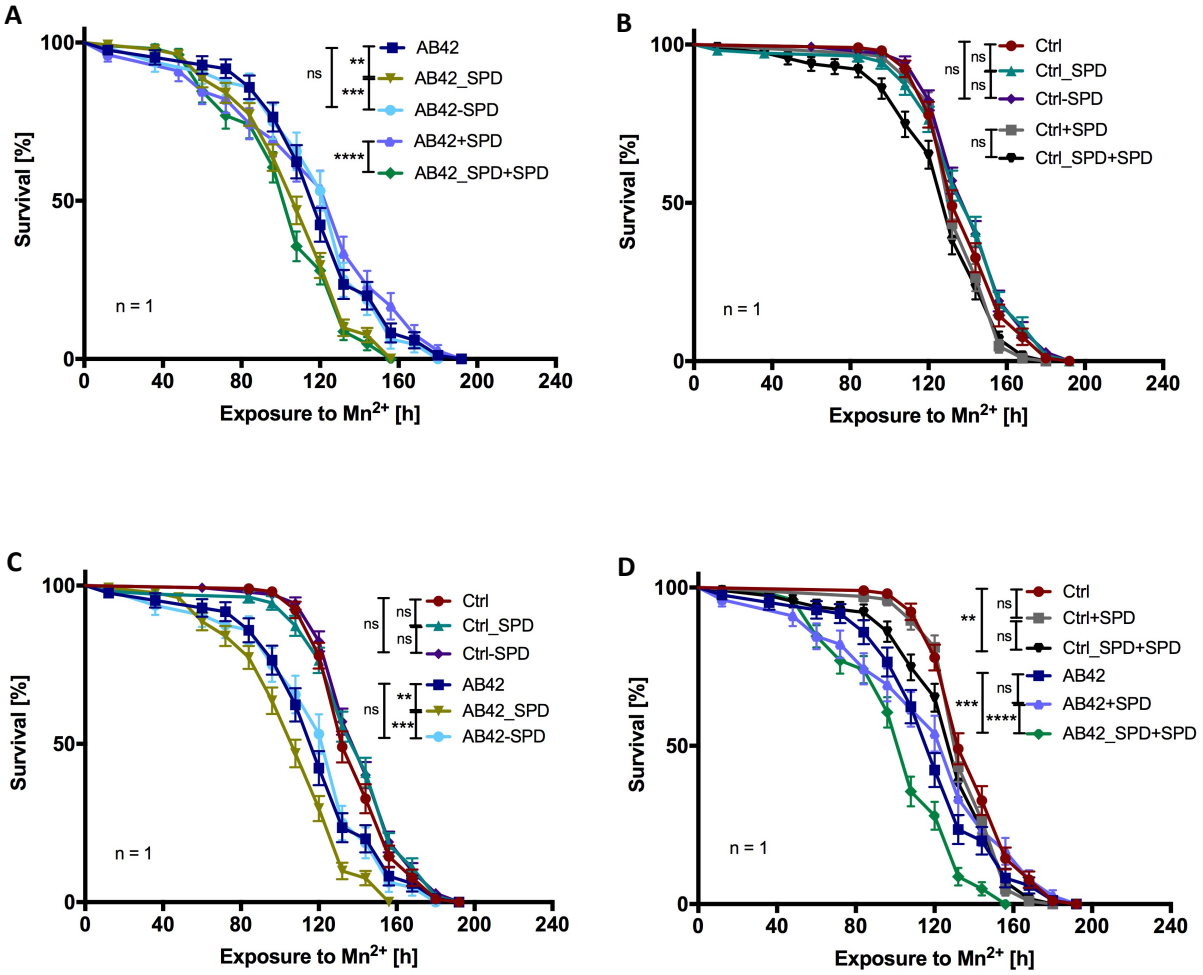

Figure S4. Effects of spermidine supply preceding hatching on the survival rate of female Aβ42-expressing flies in a manganese stress resistance assay.

Female fruit flies of both (**A**) AB42 and (**B**) w^{1118} (**Ctrl**) genotypes hatched on Sigrist +/- SPD (4 mM) and aged on the respective food for 14 d prior to manganese stress resistance assay with 10 mM $MnCl_2$ +/- SPD (4 mM) in 10% sucrose solution. Every 12 hours the number of dead flies was counted. Data are normalized means ± SE of one representative experiment. In total two independent biological replicas were done (see Figure 2) with similar tendencies, but the similarities would disappear in a summary of both, due to the different survival margin of the individual experiments. Each vial contained 30 flies.

A Female Aβ42-expressing flies hatched either on Sigrist (**AB42, AB42+SPD & AB42-SPD**) or SPD-treated Sigrist (**AB42_SPD** & **AB42_SPD+SPD**) and aged on the respective food (**AB42-SPD** aged on SPD-treated Sigrist) for 14 d foregoing being stressed with either a combination of Mn and SPD (**AB42+SPD** & **AB42_SPD+SPD**) or Mn alone (**AB42, AB42_SPD & AB42-SPD**).

B Female flies of the w^{1118} genotype hatched either on Sigrist (**Ctrl, Ctrl+SPD & Ctrl-SPD**) or SPD-treated Sigrist (**Ctrl_SPD** & **Ctrl_SPD+SPD**) and aged on the respective food (**Ctrl-SPD** aged on SPD-treated Sigrist) for 14 d prior being stressed with either a combination of Mn and SPD (**Ctrl+SPD** & **Ctrl_SPD+SPD**) or Mn alone (**Ctrl, Ctrl_SPD & Ctrl-SPD**).

C Female of both genotypes either hatched on Sigrist (**Ctrl, Ctrl-SPD, AB42 & AB42-SPD**) or SPD-treated Sigrist (**Ctrl_SPD & AB42_SPD**) and aged on the respective food (**Ctrl-SPD & AB42-SPD** aged on SPD-treated Sigrist) for 14 d prior being stressed with Mn.

D Female of both genotypes either hatched on Sigrist (**Ctrl, Ctrl+SPD, AB42 & AB42+SPD**) or SPD-treated Sigrist (**Ctrl_SPD+SPD & AB42_SPD+SPD**) and aged on the respective food for 14 d prior being stressed with Mn (**Ctrl & AB42**) or a combination of Mn and SPD (**Ctrl+SPD, Ctrl_SPD+SPD, AB42+SPD & AB42_SPD+SPD**).

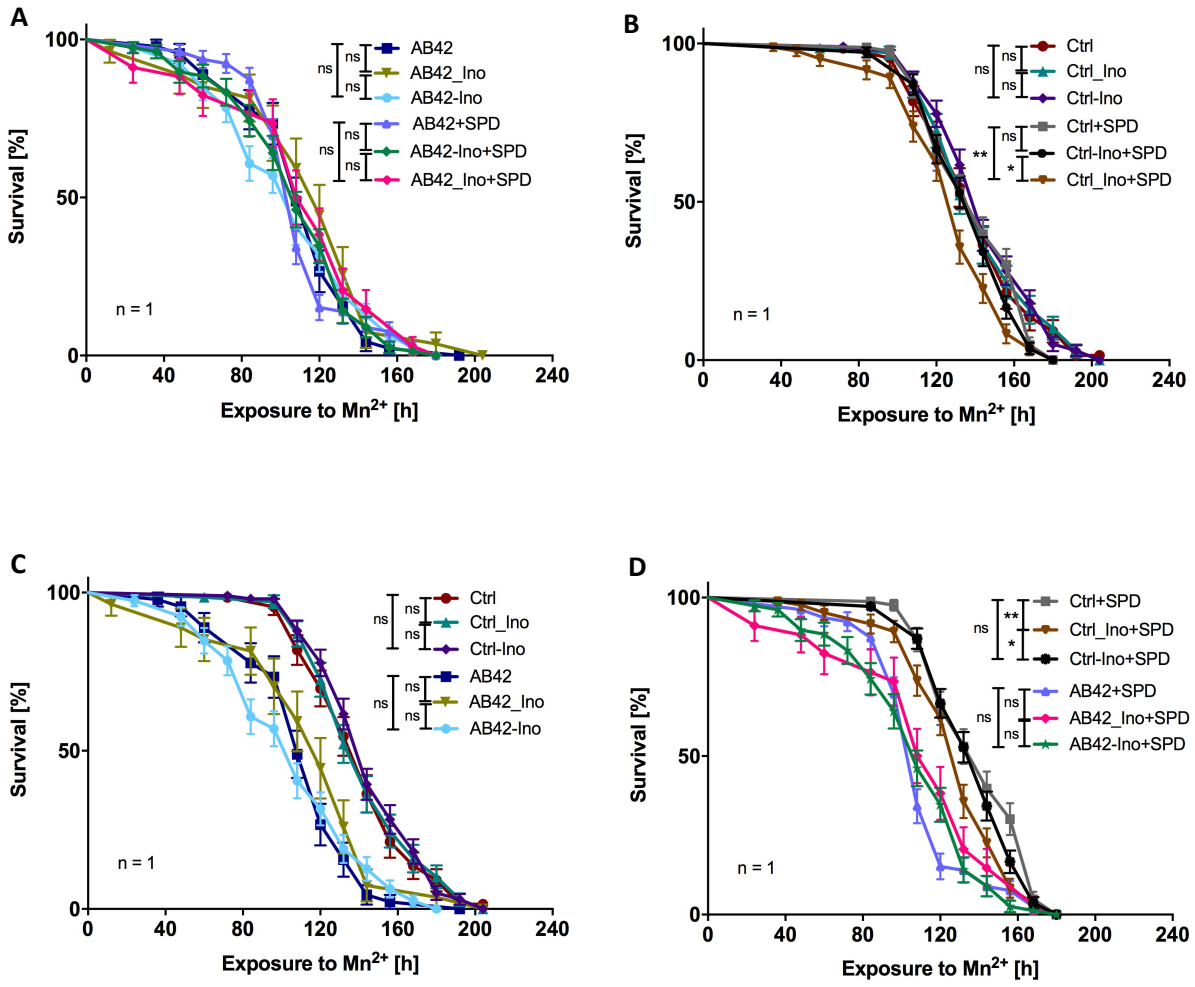

Figure S5. Effects of hatching on myo-Inositol-supplemented Sigrist food on the SPD-mediated neuroprotection in a manganese stress resistance assay with female Aβ42-expressing flies.

Female fruit flies of both (**A**) AB42 and (**B**) w^{1118} (**Ctrl**) genotypes hatched on Sigrist +/- Ino (5 %) and aged on the respective food prior to manganese stress resistance assay with 10 mM $MnCl_2$ +/- SPD (4 mM) in 10% sucrose solution. Every 12 hours the number of dead flies was counted. Data are normalized means ± SE of one representative assessment. As a consequence of few progeny while hatching on Ino-supplemented Sigrist, this analysis was performed only twice from one crossing (see Figure 6). Both of which displayed similar tendencies, yet due to the different survival margin of the individual experiment, a summary of both would diminish these similarities. Each vial contained 20 flies.

A Female Aβ42-expressing flies hatched either on Sigrist (**AB42, AB42+SPD, AB42-Ino** & **AB42-Ino+SPD**) or Ino-treated Sigrist (**AB42_Ino** & **AB42_Ino+SPD**) and aged on the respective food (**AB42-Ino** & **AB42-Ino+SPD** aged on Ino-treated Sigrist) for 14 d foregoing being stressed with either a combination of Mn and SPD (**AB42+SPD, AB42_Ino+SPD** & **AB42-Ino+SPD**) or Mn alone (**AB42, AB42_Ino** & **AB42-Ino**).

B Female flies of the w^{1118} genotype hatched either on Sigrist (**Ctrl, Ctrl+SPD, Ctrl-Ino** & **Ctrl-Ino+SPD**) or Ino-treated Sigrist (**Ctrl_Ino** & **Ctrl_Ino+SPD**) and aged on the respective food (**Ctrl-Ino** & **Ctrl-Ino+SPD** aged on Ino-treated Sigrist) for 14 d prior being stressed with either a combination of Mn and SPD (**Ctrl+SPD, Ctrl_Ino+SPD** & **Ctrl-Ino+SPD**) or Mn alone (**Ctrl, Ctrl_Ino** & **Ctrl-Ino**).

C Female of both genotypes either hatched on Sigrist (**Ctrl, Ctrl-Ino, AB42** & **AB42-Ino**) or Ino-treated Sigrist (**Ctrl_Ino** & **AB42_Ino**) and aged on the respective food (**Ctrl-Ino** & **AB42-Ino** aged on Ino-treated Sigrist) for 14 d prior being stressed with Mn.

D Female of both genotypes either hatched on Sigrist (**Ctrl-Ino+SPD, Ctrl+SPD, AB42-Ino+SPD & AB42+SPD**) or Ino-treated Sigrist (**Ctrl_Ino+SPD & AB42_Ino+SPD**) and aged on the respective food (**Ctrl-Ino+SPD** & **AB42-Ino+SPD** aged on Ino-treated Sigrist) for 14 d prior being stressed with a combination of Mn and SPD.

7. Abbreviations

AB42	*Aβ 1-42/TM3 Ser (UAS) – Drosophila* genotype
AD	Alzheimer's Disease
Akt	Protein Kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated kinase
APP	Amyloid precursor protein
Atg	Autophagy related gene
AV	Autophagic vacuoles
Aβ	Amyloid-β-peptides
Aβ42	Amyloid-β-42
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
CAPS	N-Cyclohexyl-3-Aminopropanesulfanic acid
CETP	Plasma cholesteryl ester transfer protein
cm	Centimetre
CO_2	Carbon dioxide
Ctrl	Control; w^{1118} genotype
d	days
DAG	Diacylglycerol
ddH_2O	Double distilled water
E	Elaidic acid
elavX	$elav^{C155}$-*GAL4 – Drosophila* genotype
ER	Endoplasmic reticulum
EtOH	Ethanol
FA(s)	Fatty acid(s)
g	gram
G6P	Glucose-6-phosphate
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GPCR	G-protein coupled receptor
HAT(s)	Histone acetyltransferase(s)

HCl	Hydrogen chloride
HDL	High-density lipoprotein
hrs/h	Hours
IMPase	Inositol monophosphatase
Ino	Myo-Inositol
IP_1	Inositol monophosphate
IP_2	Inositol 4,5-biphosphate
IP_3	Inositol 1,4,5-triphosphate
IP_6	Inositol hexaphosphate
IP_3R	Inositol 1,4,5-triphosphate receptor
IPPase	Inositol polyphosphate 1-phosphatase
IPS	Inositol 3-phosphate synthase
L	Litre
LC3	Microtubule-associated protein 1 light chain 3
LC3-I	Soluble form of LC3
LC3-II	Lipidated form of LC3
LDL	Low-density lipoprotein
M	Molar
mA	Milliampere
MAP	Microtubule associated protein
ml	Millilitre
mM	Millimolar
Mn	Manganese
$MnCl_2$	Manganese chloride
mTOR	Mammalian target of rapamycin
mTORC 1 (or 2)	mTOR complex 1 or 2
ns	Not significant
O	Oleate
O+E	Combination of oleate and elaidic acid
P	Palmitate
P+E	Combination of palmitate and elaidic acid
P+O	Combination of palmitate and oleate

PAS	Phagophore assembly site
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with tween
PD	Parkinson's disease
PE	Phosphatidylethanolamine
PI3K	Phosphatidylinositol-3-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PS (1 or 2)	Presinilin (1 or 2)
PVDF	Polyvinyliden fluoride
rpm	Rotations per minute
RT	Room temperature
s	Seconds
SDS	Sodium dodecyl sulfate
SE	Standard error
SEM	Standard error of the mean
SFA(s)	Saturated fatty acid(s)
Sigrist	resembles standard cornmeal-molasses food
SPD	Spermidine
STD	Standard deviation
TBS	Tris-buffered saline
Tris	Tris(Hydroxymethyl)aminomethane
TST	Tris-buffered saline with tween
UAS	Upstream activating sequence (yeast)
UFA(s)	Unsaturated fatty acid(s)
ULK1	Unc-51 like autophagy activating kinase 1
VLDL	Very low-density lipoprotein
µL	Microlitre
µM	Micromolar

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