Spermidine based amelioration of age-dependent memory impairment under the paradigms of enhanced autophagy and altered epigenome in *Drosophila melanogaster*.

By

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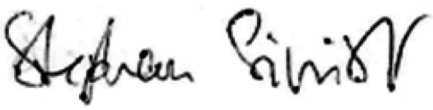
Under guidance of



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Abstract.

Aging, the process of growing old, involves gradual biological impairment of normal function probably as a result of changes made to cells and structural components which ultimately affect the organismas a whole. Among multiple biochemical correlates of aging, a decrease in endogenous polyamines has been associated with aging, although, it is still not understood if this is the cause or consequence of aging. External administration of spermidine restores the endogenous spermidine levels and promotes longevity in several model organisms including yeast, worms, flies, mice and human cell lines.A decrease in polyamine levels has been observed in memory associated structures and this could be causally associated to age associated memory impairment. Evidences from previous studies and data from the Sigrist lab suggest that spermidine administration ameliorates age-dependent memory impairment. Here, we suggest that spermidine might improve memory by preventing age-dependent accumulation of ubiquitinated proteins by upregulation of autophagy in fly heads. Also, cognitive decline with age has been attributed to epigenetic changes like changes in acetylation status of different histones. Therefore, as the second part of this study I tried to characterize *Drosophila* histone mutants in terms of longevity and learning performance. Preliminary results obtained from these assays suggest a possible role of Histone 3 locus in regulating life span and learning and memory formation in flies, whereas the flies carrying the point mutation in H3-K27 lived a normal lifespan and had memory performance just like wild-type flies.

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Last but not least I wish to avail myself of this opportunity, express a sense of gratitude and love to my friends and my beloved parents for their manual support, strength, help and for everything.

Introduction.

Aging is a universal process that involves a complex interplay between environmental and genetic factors. However, the biochemical and physiological mechanisms that determine how organisms senesce and die are still poorly understood. The fact that individual species have characteristic lifespanranging from 70-80 days in the fruit fly to 80 years for humans, but still share similar features during agingsuch as declined ability to respond to stress, increased homeostatic imbalance etc, suggests the existence of common mechanisms which determine both the rate of aging and the lifespan of an organism (Finch, 1990). Studies in m

Model organisms such as *C. elegans*, *Drosophila melanogaster* and mice have shown caloric restriction to increase the life span (Katewa and Kapahi, 2010). Caloric restriction is a dietary regimen that restricts the calorie intake, while still obtaining all necessary nutrients and vitamins, hence, different from malnutrition. Evidences provided by studies conducted in C. elegans, D. melanogaster and mice have proven particularly useful in identifying some of the mechanisms that might influence aging (Boulianne G.L., 2001, Xiang L., 2011)

Over the last two decades, some of the fundamental mechanisms underlying the aging have started to be deciphered. The first pathway shown to influence ageing in animals was the insulin/IGF-1 pathway. In fruit-flies, life expectancy is extended by more than 50% when the insulin-like receptor (*InR*) or its receptor substrate (*chico*) are mutated, or when insulin-producing cells are removed. Subsequently, it has been shown that inhibiting insulin/IGF-1 signaling resulted in phosphorylation of dFOXO, the equivalent of nematode daf-16 and mammalian FOXO3a and this activation of dFOXO led to changes in gene expression, upregulating or downregulating diverse genes that act together to increase lifespan(Hwangbo, et. al., 2004). Recently,dFOXO signaling was shown to delay the accumulation of protein aggregates (another hallmark of aging) by enhancing autophagy-dependent degradation (Demontis et. al., 2010). Autophagy involves sequestering cytoplasmic material into double membrane vesicles followed by lysosomal degradation. The pathway is highly regulated through the action of various kinases, phosphatases and GTPases (Kelekar, 2005). Autophagy is suppressed by

insulin/IGF-1 signaling (through TOR kinases) and is enhanced when animals are placed on a calorie restricted diet, suggesting upregulation of autophagy may facilitate clearance of damaged cellular particles that accumulate during cellular aging.In addition, promoting basal levels of autophagy in nervous system enhances longevity and oxidant resistance in adult *Drosophila* (Finley et. al., 2008).

By definition, aging is the result of complex interplay between genetic and epigenetic factors.Global loss of DNA methylation and hyperacetylationof particular histone residues are some of the epigenetic hallmarks of aging(Singhal. et. al., 1987,Sarg et. al. 2002). The accumulation of aberrant epigenetic marks over the lifespan drives aging-related cellular and physiological changes. Aging may be directly related to the loss of core histones H3 and H4. There is a drop in histone H3 and H4 production in late passage cells (O'Sullivanet. al., 2010) and increasing histone levels is sufficient to extend life span in yeast (Feser et. al., 2010.). Cellular senescence has been attributed to shortening of the telomeres in each cell cycle. O'Sullivan et. al., 2010 have shown that telomere shortening over successive cell divisions represent a source of chronic damage within the cells that leads to destabilization of the histone biosynthesis pathway and ultimately leading to cell death. These findings indicate that loss of epigenetic marks could be the driving factor of aging.

Polyamines and Aging.

Among the multiple biochemical correlates of ageing, a decrease in intracellular polyamines has been observed in ageing organisms (Liu et. al., 2008).The polyamines putrescine, spermidine (SPD) and spermine are a group of positively charged aliphatic amines which are able to interact with negatively charged species like DNA, RNA and proteins. They are involved in regulation of protein synthesis both at level of gene expression and translation. Involvement of these molecules in a broad array of processes, indicate a complex and an important role of polyamines in control of cellular life and death.

The polyamines putrescine, spermine and spermidine are a part of very tightly regulated polyamine metabolic pathways. There are three main sources for polyamines in

organisms: food intake, cellular synthesis and microbial synthesis in gut. Arginine, ornithine and methionine are amino acid required for polyamine synthesis. First step in this pathway is the production of ornithine from arginine. Ornithine is then decarboxylated to form putrescine, by Ornithine Decarboxylase (ODC). Parallel to putrescine production L-methionine is converted to S-Adenosyl-L-Methionine (AdoMet), which is then decarboxylated to produce DecarboxylatedAdoMet (DcAdoMet). DcAdoMet is the amino-propyl donor and is used later to produce spermidine from putrescine by spermidine synthase and spermine from spermidine by Spermine synthase(Minois et. al., 2011).

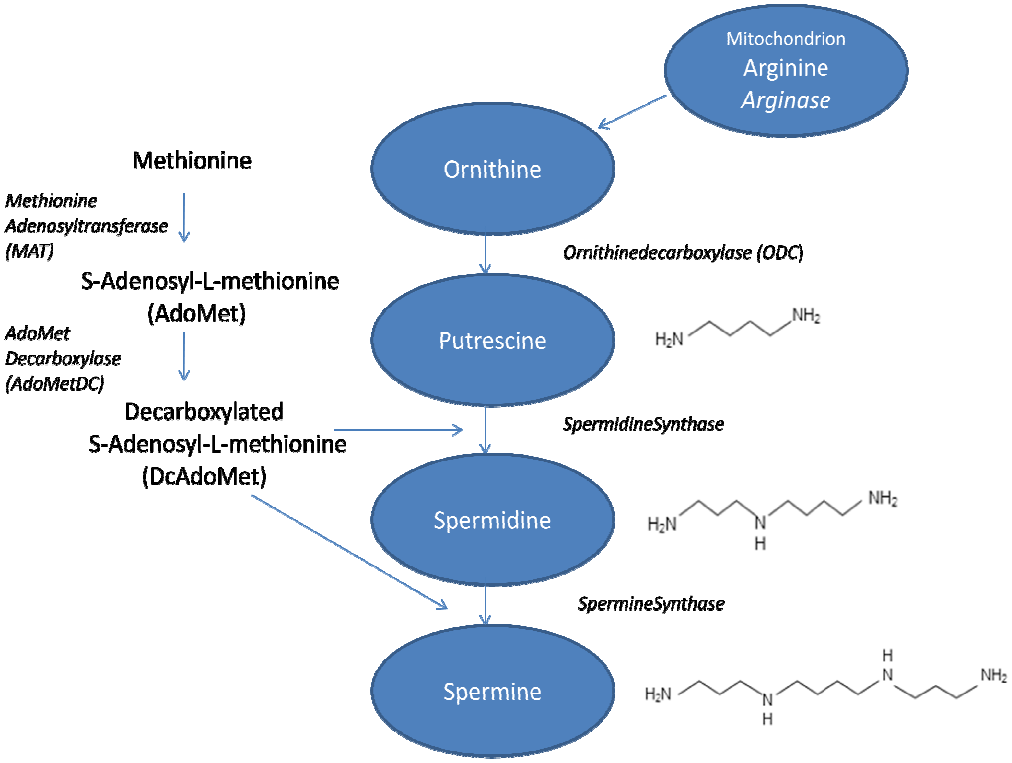


Figure 1: Polyamine synthesis.

Several studies have investigated age related changes in ODC levels and polyamine content in experimental model organisms. Das and Kanungo, 1982 reported age related decrease in ODC activity and spermidine and spermine content in rat cortex while no

change in putrescine content was observed. Morrison et. al., 1996 indicated decreased spermine levels in occipital lobe of aged organisms while they did not observe any change in putrescine or spemidine content in this area in these organisms. Vivo et. al., 2001 demonstrated a consistent negative correlation between spermine and spermidine content and age in several areas in human basal ganglia. These evidences therefore, suggest that the ODC/polyamine system is potentially involved in the normal process of aging.

More recently, a role of polyamines in longevity has been shown, although, it remains unclear whether depletion of polyamines is cause or consequence of ageing process(Eisenberg et. al., 2009). More interestingly, they showed that spermidine administration extends the lifespan of several model organisms like yeast, worms, flies, mice and human cells and inhibits oxidative stress in ageing mice.Measurement of endogenous polyamines in these organisms confirmed that spermidine supplementation stably increased intracellular spermidine levels by about 20% compared with controls. Notably, putrescine (a polyamine inter-convertible with spermidine) was undetectable in control samples but clearly present in spermidine-fed flies indicating that spermidine was indeed taken up and metabolized byspermidine treated flies(Eisenberg et. al., 2009).

Microarray profiling of spermidinetreated yeast cells revealed transcriptional activation of several autophagy genes including atg7, atg11 and atg15 suggesting thatspermidineinduces autophagy. Spermidine effectively upregulated autophagy in *C.elegans*and *D. melanogaster*.In addition, the genetic inhibition of essential ATG genes (i.e., knockout of atg7 in yeast and flies and RNA-i mediated silencing of bec-1 in nematodes) abrogated lifespan prolonging effects of spermidine, indicating thatinduction of autophagy is certainly required for the cytoprotective and/or anti-aging effects of spermidine (Morselli et. al., 2009).

Polyamines and cognitive decline.

Cognitive decline especially in memory capacity is a normal part of aging. Although aging and cognitive decline are known occur together, there are now evidences

emergingwhich indicate that these two processes can be uncoupled at a certain molecular level. In *Drosophila,* dietary restriction promotes longevity but fails to prevent age dependent memory impairment (Buechel et. al., 2010).

Polyamines putrescine, spermidine and spermine are present at high concentrations in cerebral structures involved in learning and memory, such as amygdala and hippocampus. Polyamines have also been shown to modulate learning and memory by interacting with the polyamine-binding site at the N-methyl-D-aspartate receptor (NMDAr) (Morrison et. al 1995). Polyamine concentrations change with age and it has been suggested that region-specific changes in polyamine levels may be causally related to age-related impairments in learning and memory (Liu et. al., 2008).

Accordingly, the systemic, intra-hippocampal and intra-amygdalar administration of spermidine improves memory of mice. This is particularly relevant considering that NMDAr-associated intracellular signaling results in the activation of multiple protein targets, such as PKA, protein Kinase C (PKC), calcium calmodulin dependent protein kinase II (CaMKII) and protein kinase G (PKG), all of them playing an important role in memory consolidation pathway. The cAMP/PKA signaling pathway seems to play a role in the final phases of memory consolidation, which require protein synthesis. A role for PKA in memory has been supported by the findings that specific inhibitors impair memory and that R (AB) transgenic mouse, which express an inhibitory form of the regulatory subunit of PKA, exhibit memory deficits. CREB is a transcription factor whose phosphorylation on Ser133 by PKA causes its activation. Phosphorylated CREB levels in the hippocampus increase after inhibitory avoidance training in two peaks that correlate with the peaks of increased PKA activity (Guerra et. al., 2011).

Spermidine to the rescue of memory in aged *D. melanogaster*.

Spermidine when given to flies at optimal concentration of 5mM with food, partially restores the endogenous levels in 30-day old fly heads. Also, when spermidine fed 30 day old flies were assayed for classical olfactory avoidance tests they have a performance index as good as spermidine fed 3-day old flies unlike control group

(normal food without spermidine) where memory performance of flies drop significantly from 3-day to 30-day old (Gupta and Sigrist).

With this background the first part of the project aims to study the mechanism through which spermidine actually prevents this age dependent memory impairment (AMI) in flies. Since, accumulation of insoluble ubiquitinated protein (IUP) has been associated with neuronal aging and neurological disorders; we asked whether spermidine could influence the levels of ubiquitinated protein aggregates. In fact, we observed an age associated accumulation of ubiquitinated protein, consistent with previous studies and this accumulation largely blocked in the heads of spermidine fed old flies. The buildup of ubiquitinated proteins in turn is meant to be the result of age-associated decrease in the expression of autophagy genes in *Drosophila* neural tissues, indicating that a reduction of autophagy pathway might be responsible for the lower protein turnover(Simonsen et. al., 2008). Also, in autophagy-deficient flies there was an accumulation of ubiquitinated protein aggregates in degenerating neurons (Simonsen et. al., 2008; Juharz et. al., 2007). Similarly, genetic studies in mice have shown that suppression of neuronal autophagy can lead to the accumulation of protein aggregates and neurodegeneration (Hara et. al., 2006, Komatsu et. al., 2006). Moreover, promoting Atg8a expression in older fly brains not just drastically extended the average adult lifespan but also promoted resistance to oxidative stress. Spermidine causes upregulation of autophagy (Eisenberg et. al.,2009, Rubinsztein et. al., 2011) therefore we performed quantitative Real Time PCR (qRT-PCR) to check for changes in levels of mRNA transcripts of autophagy genes and western blots to check for the levels of Atg8a in fly heads. From qRT-PCR, we did not observe any significant change in the levels of mRNA transcripts of ATG genes in young and old flies (+/-spermidine). Notably, however, in western blots, we observed Atg8a levels to decrease with age in control group but to remain constant in heads of spermidine^{+} flies, indicating spermidine promotes autophagy in fly brains.

The second part of the project deals with characterization of *Drosophila* histone mutants in terms of longevity and memory. Histone deacetylation, a key event in epigenetic chromatin aging is associated with healthy aging in many organisms (Longo and Kennedy, 2006). Exogenous supply of spermidine to yeast or human PBMC significantly

reduces acetylation levels of specific lysine residues (Lys 9, 14 and 18) on Histone-3 (H3) by directly inhibiting histone acetyl transferase (HAT) activity (Eisenberg et. al. 2009). Hypoacetylation of histones promoted by spermidine indicates silencing of majority of genes that might be important for saving resources and for promoting longevity.

Member of polycomb silencing (PcG) and trithorax group (TrxG) are key epigenetic regulators of global transcription programs. PcG proteins influence cellular senescence as evidenced by studies in cultured mammalian cells (Schwartz and pirrotta, 2007).Polycomb silencing depend on trimethylation of Histone 3 Lysine 27 (H3K27me3) which is carried out by Polycomb Repressive Complex 2 (PRC2), one of several PcG protein complexes that collaborate to implement Polycomb silencing (Jacobs et. al. 1999).Sieboldet. al. 2010 demonstrated that mutation in E(Z), which encodes the catalytic subunit of PRC2, increases longevity and promotes resistance to oxidative stress and starvation in fruit-flies. The increased longevity and enhanced stress resistance are due to reduced H3K27me3 levels and defective polycomb silencing. Interestingly, these mutants have enhanced Odc1 expression which is an upstream regulator in the pathway of spermidine synthesis (Siebold et. al., 2010).Altered histone acetylation is associated with age-dependent memory impairment in mice (Peleg et. al, 2010). Therefore, we decided to look at the effect of H3 deletion, particularly the effect of point mutation in H3-K27 on mean lifespan. From the data, deletion of H3 locus reduces mean lifespan and leads to learning disabilities in flies. Interestingly the point mutation in H3-K27 did not have any negative effects of memory and average lifespan of these flies. Introduce histone mutants here.

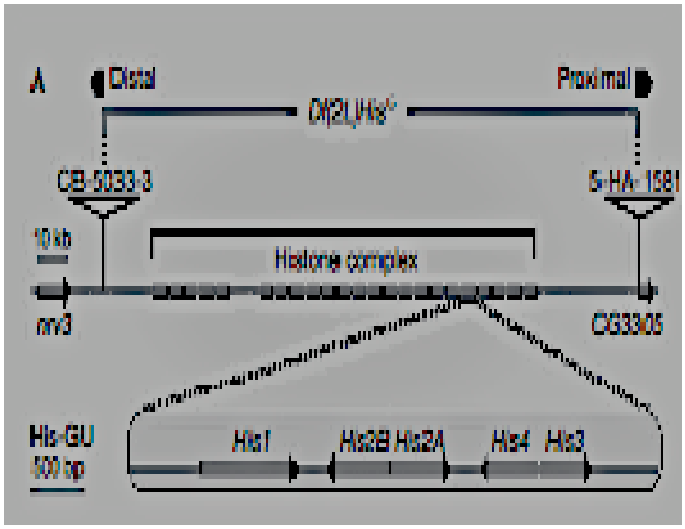
Materials and Methods.

Drosophila Stocks Used:

For qRT-PCR, W1118 flies representing three different age groups, 3 day old, 15 day old and 30 day old, were used. There were two sets for each age group, one treated (treated with spermidine) and other control (fed normal food without spermidine). For western blots, 3 day old and 30 day old W1118 flies were used. Each set of

experiments involves two sets of flies, treated and control, with each set having representative flies from the above mentioned age groups. Treated groups were fed spermidine at a concentration of 5mM while control groups includes flies which were fed normal food without spermidine.

The histone gene complex of D.melanogaster is composed of 23 canonical histone gene repeat units (His-GUs), each bearing a gene array encoding the five canonical histones, H1, H2A, H2B, H3 and H4. The following figure explains their arrangement.



Following Histone Mutants were used:

$w^{-};\frac{Df(2R)HisT}{Df(2R)HisT}$: Partialdeletion of Histone locus, few histone gene units (where each unit comprises of one copy of each canonical histone, as drawn in above diagram) are deleted. However, the deleted segment still needs to be characterized.

$w^{-};\frac{Df(2R)HisC}{Cyo-ftz-LacZ}$: Deletes all 23 canonical histone gene repeat units.

$w^{-};\frac{Df(2R)HisC}{Cyo-ftz-lacZ};\frac{M\{3xHis-GU.wt\}68E}{M\{3xHis-GU.wt\}86Fb}$: In the background of complete deletion of histone locus, 6 copies of wild type histone gene units are introduced (homozygous)

$w^{-};\frac{Df(2R)HisC}{Cyo-ftz-lacZ};\frac{M\{3xHis-GU.dH3\}68E}{M\{3xHis-GU.dH3\}86Fb}$: In the background of complete deletion of histone locus, 6 copies of histone gene units with a deleted Histone H3 gene are introduced (homozygous).

$w^{-};\frac{Df(2R)HisC}{Cyo-ftz-lacZ};\frac{M\{3xHis-GU.K27R\}68E}{M\{3xHis-GU.K27R\}86Fb}$: In the background of complete deletion of all canonical histone genes, 6 copies of wildtype histone genes with H3K27 mutated to arginine (R), H3K27->R, are introduced (homozygous).

All the stocks were maintained at room temperature ~ 25°C.

Real-Time PCR:

Heads from at least 100 flies representing different ages or spermidine treatments were collected and total RNA extracted using RNeasy mini kit (Qiagen). Purity and quantity were measured by optical density. For each condition minimum of 400ng of total RNA was used for cDNA synthesis using Invitrogen Superscript III RT kit. Real time PCR were performed in 20ul reactions containing 10ul Invitrogen Fast SYBR-Green mix 2X, 4ul 1uM primers and cDNA (diluted 1:100) in 96 well optical plates. The cycling conditions for the Light Cycler 480(Roche) were 95C for 5 min, 45 cycles of 94C 30s, 55C 20s and 72C 20s. Real-time efficiencies were calculated from slopes of the standard dilution curve. RNA transcription levels were determined by direct comparison of C_T values. The relative quantities calculated by delta-delta C_T equation.

Protein Extract from Fly heads:

a.) For Checking Atg8a levels:

Flies were frozen in liquid nitrogen and then stored at -80°C. For preparing head extract, flies were put in liquid nitrogen (they should never come to room temperature), followed by vigorous shaking for 30s. The contents were then put on a chilled shallow metal plate, pre-cooled in liquid nitrogen and maintained at low temperature. Desired number of heads was collected in a 1.5 ml eppendorf tube. Heads were stored in liquid nitrogen till protein extraction starts. Tubes were briefly spin-down and solubilization buffer (2% SDS) was added (for 30 heads, 40ul SDS buffer). (Please note that different proteins of interest need different solubilization buffers). Heads were squished with a micro pistil followed by heating at 95°C for 5 min. Extract was then cooled, (avoid overcooling as SDS solidifies) and aliquots were stored at -80°C. Protein Concentration was determined using BCA Assay Kit (Thermo Fischer).

b.) For Checking IUP (Insoluble Ubiquitinated Protein) Profile:

To detect the accumulation of insoluble ubiquitinated proteins, fly heads (at least 60 per age and genotype) were homogenized in 1% Triton X, PBS containing protease inhibitors on ice. The homogenate was centrifuged (14,000*g) for 10 min (4°C) and the supernatants (Triton X soluble fraction) collected. The remaining protein pellets were washed (1% Triton X, PBS), followed by re-homogenization in 2% SDS buffer. The homogenate was then centrifuged (14,000*g) for 10 min (25°C) and the supernatants (SDS Fraction) collected. Protein aliquots were stored at -80°C

SDS Page and Western Blot:

20ug of total protein per sample was loaded and resolved on 4-20% gradient gels (Bio-Rad) for IUP (Insoluble Ubiquitinated Proteins) or 12% gels for Atg8a, followed by electro blotting to nitrocellulose membranesand western blotting. Antibodies were detected using standard ECL reagents.

Western Blotting:

Fresh Transfer Buffer was prepared. Polyacrylamide gel was freed and equilibrated in a separate buffer bowl for 10 min with orientation of gel taken care of. Blot cassette was opened with black side (cathode) below and placed in the major separation of the buffer basin. Following layers were soaked with buffer and put on the cathode side as described below: 1 Fiber Pads, 1 Whatman-Paper, PA-Gel, Nitrocellulose Membrane, 1 Whatman Paper and 1 Fiber Pad. Cassette was closed and fixed with the slide. Stirring bar was placed in the buffer tank. One ice box was placed behind the cassette and the buffer tank was filled with the transfer buffer. Blotting was done at 100V for 2 hours (or 1 hr for small proteins) in the cold room on the magnetic stirrer maintained at slow stirring, thus, avoiding bubbles. After blotting the blotting chamber was disassembled. Gels were Coomasie stained to check the transfer efficiency. Membrane was incubated with Ponceau S on the shaker for 10 min at RT followed by brief rinsing with ddH_2O and later scanning the stained membrane. Membrane was blocked with 5% milk + 0.05% Tween for 60min at RT on the rocker. Blots were incubated with Primary Antibody (in milk) overnight at 4°C on the rotation plate followed by brief rinsing 2 times 3 X 10 min

washing with PBST. Next, blots were incubated with Secondary antibody (in milk) 60 min at RT on the rocker followed by brief rinsing 2 timesand ,3 X 10 min washing with PBST. Continued with development

Development:

Appropriate pieces of copy transparency, 2 pieces for 1 membrane, were cut and membrane was placed one of them. ECL solution was mixed and added to te membrane and membrane was kept in dark for 5 min. ECL solution was decanted and second piece of transparency was placed over the membrane. The sandwich was then fixed in the developer cassette. Appropriate pieces of the x ray film was cut, one corner was marked to remember the orientation, and placed on the membrane followed by 1 minute exposure. The film was then developed (kept in developer until signal is seen, followed by brief rinsing in waster, and then 10 min in fixer). Exposure time of second development is adjusted depending on signal intensity from first exposure

Statistical analysis:

For statistical analysis, x-ray films were scanned using EPSON scanner and intensity analysis done using Image J software. The relative amounts of Atg8a and IUP proteins from individual samples were quantified and corrected using tubulin as loading control. Statistical analysis was done in Graphpad Prism software using one-way ANOVA and a Tukey post-test.

Antibodies:

Rabbit anti-GABARAP polyclonal antibody, used at 1:1000, was obtained from MBL. The rabbit Ubiquitin polyclonal antibody, used at 1:1000, was obtained from Cell Signaling Technology and mouse anti-Tubulin DM1A, used at 1:1000, was obtained from Sigma Aldrich. The secondary antibodies were horseradish peroxidase-conjugated rabbit or mouse-IgG.

Crossing Scheme to get desired genotypes for histone mutants:

Virgin Females	**X**	**Males**

$w^{-};\frac{Df(2R)HisT}{Df(2R)HisT}$	**X**	$w^{-};\frac{Df(2R)HisC}{Cyo-ftz-LacZ}$
$w^{-};\frac{Df(2R)HisT}{Df(2R)HisT}$	**X**	$w^{-};\frac{Df(2R)HisC}{Cyo-ftz-lacZ};\frac{M\{3xHis-GU.K27R\}68E}{M\{3xHis-GU.K27R\}86Fb}$
$w^{-};\frac{Df(2R)HisT}{Df(2R)HisT}$	**X**	$w^{-};\frac{Df(2R)HisC}{Df(2R)HisT};\frac{M\{3xHis-GU.wt\}68E}{M\{3xHis-GU.wt\}86Fb}$
$w^{-};\frac{Df(2R)HisT}{Df(2R)HisT}$	**X**	$w^{-};\frac{Df(2R)HisC}{Df(2R)HisT};\frac{M\{3xHis-GU.dH3\}68E}{M\{3xHis-GU.dH3\}86Fb}$

Following genotypes were collected and used for longevity and memory assays.

Desired Genotype	Representation
$w^{-};\frac{Df(2R)HisC}{Df(2R)HisT}$	T/C
$w^{-};\frac{Df(2R)HisC}{Df(2R)HisT};\frac{M\{3xHis-GU.K27R\}68E}{M\{3xHis-GU.K27R\}86Fb}$	K27R
$w^{-};\frac{Df(2R)HisC}{Df(2R)HisT};\frac{M\{3xHis-GU.wt\}68E}{M\{3xHis-GU.wt\}86Fb}$	wt
$w^{-};\frac{Df(2R)HisC}{Df(2R)HisT};\frac{M\{3xHis-GU.dH3\}68E}{M\{3xHis-GU.dH3\}86Fb}$	dH3

Longevity Assays:

Longevity assays were performed as previously done by Burger, et. al. 2010. Longevity assays were done in three different groups: 1.) Equal number of Males and Females kept together, 2.) Only Males, 3.) Only females. Temperature was maintained at 25°C. Following mating and sexing, 1-day old males and females were transferred, at least in groups of 20, to 40-mlfly vial containing 2-3 ml of food of the appropriate diet. The number of dead flies was scored every day. Food was changed every second day. Flies

that escaped or were stuck in the food but were still alive were treated as censored observations. The bottles were rotated throughout the incubator to negate the location effect on fly death. For all the longevity experiments, Canton-S was used as control genotype. The percentage of flies remaining alive at a given time point, days for life span, was calculated from the total starting number of flies for a particular genotype.

Olfactory conditioning assays and learning memory:

Protocol for olfactory aversive conditioning was adopted from Quinn and Tully, 1985, in which flies were first conditioned to associate an odor with aversive mechanical shock and then tested for the choice between this and another odor in a T-maze.

The conditioning and test procedures were carried out on groups of 20-40 flies (mixture of males and females). On the day of assay, each group of flies were transferred without anesthesia to a 10-ml test tube without food and exposed to one conditioning cycle. Each conditioning cycle consisted of 60s exposure to one odor accompanied by 12 bouts of mechanical shock at 5s intervals followed by 30s of humidified air, 60s exposure to another odor without shock and completed by 60s of humidified air. Octanol and MCH (methylcyclohexanol) dissolved in paraffin oil were used as odorants. Octanol was used at a dilution of 1:150 and MCH was used at a dilution of 1:500.

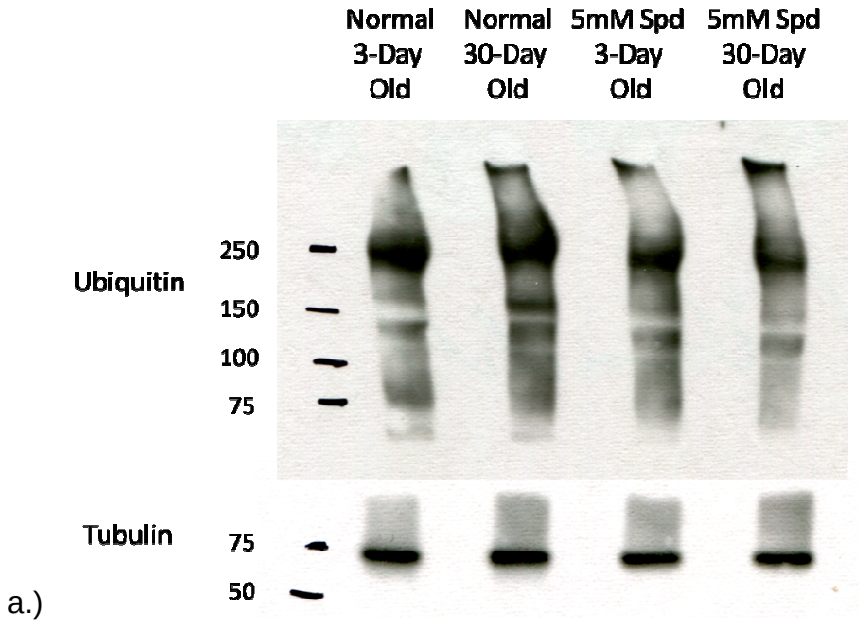
After 60s from the end of conditioning (60s), the flies were tested for choice between the two odors. The flies were then transferred to the central chamber of a T-maze. From that moment, they were given 30s to choose between the odors. The flies choosing each arm of the T-maze (i.e. each odor) were subsequently counted and proportions of flies choosing octanol vs. MCH were calculated. Flies that remained in the central chamber of the T-maze were excluded from this calculation, thus differences in locomotor performance did not bias the learning results. The replicate unit in the learning assay consisted of two groups, one conditioned to avoid Octanol and other conditioned to avoid MCH. A value of the memory score was calculated as the difference between the first and the second group in the proportion of flies choosing Octanol. The memory scores were analyzed using ANOVA.

Results and Discussion.

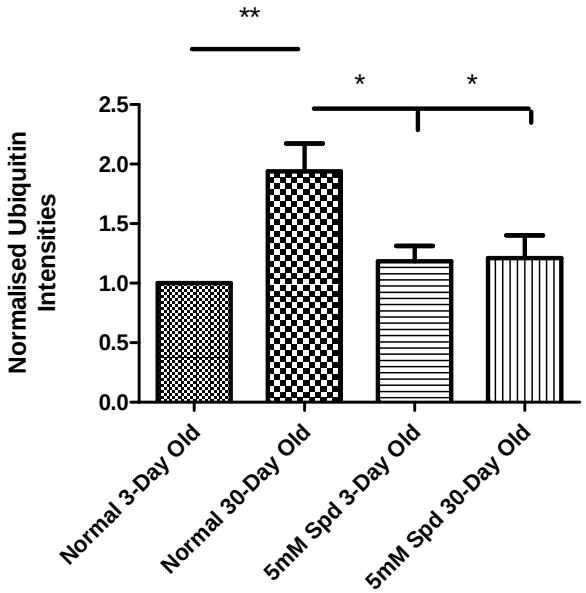
Part 1: Spermidine and autophagy:

Results:

Age dependent changes in Insoluble Ubiquitinated Protein (IUP) levels (+/- spermidine). Figure 1**:** a.) Change in levels of ubiquitinated proteins with age and spermidine treatment in fly heads. 3-day old flies and 30-day old flies represent the two different age groups. Tubulin is used as loading control. b.) Normalized Ubiquitin intensities plotted for each group. Data is expressed as mean and SEM calculated from replicates from two different ageing experiments (Number of blots = 12, Number of independent cohorts = 2, *p<0.05; **p<0.01 versus normal 3-day old control values, One-way Analysis of Variance with a Tukey post-test)



a.)



b.)

To analyze the effect of spermidine on ubiquitinated protein levels, 3 day and 30 day old flies were treated with spermidine and then compared to ubiquinated protein levels of 3 day and 30 day old control flies. Heads were isolated and subsequently the total protein was isolated in sequential detergents, 1% Triton-X-100 (in PBS) followed by re-solubilization in 2% SDS and further used for western blot and probed with anti-ubiquitin antibody. Tubulin was used as loading control. IUP levels in normal 3 day old were set as reference. As shown in the figure 1a and quantified in figure 1b, the levels of insoluble ubiquitinated proteins are low in normal 3-day old flies. There is around 100% increase in accumulation of ubiquitinated proteins with age. Spermidine-fed 3-day old and 30-day old flies have significantly lower levels of ubiquitinated protein aggregates compared to control 30 day old flies.

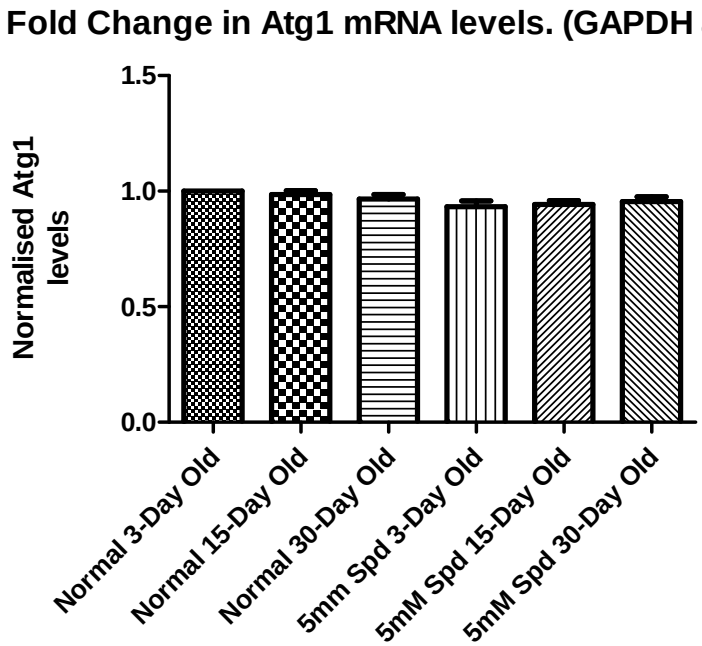
RT-PCR for Autophagy genes expression.

To check for the change in transcriptional levels of autophagy genes with age, quantitative real-time PCR were done for three different age groups (1-day old, 15-day old and 30-day old) control and compared to spermidine treatment. There are several autophagy genes, but we did qRT-PCR for Atg1, Atg2, Atg5, Atg7 and Atg8, using SYBR Green as age-associated changes have been documented for these before. Actin5C and GAPDH were used as endogenous controls.Efficiency plots were drawn to

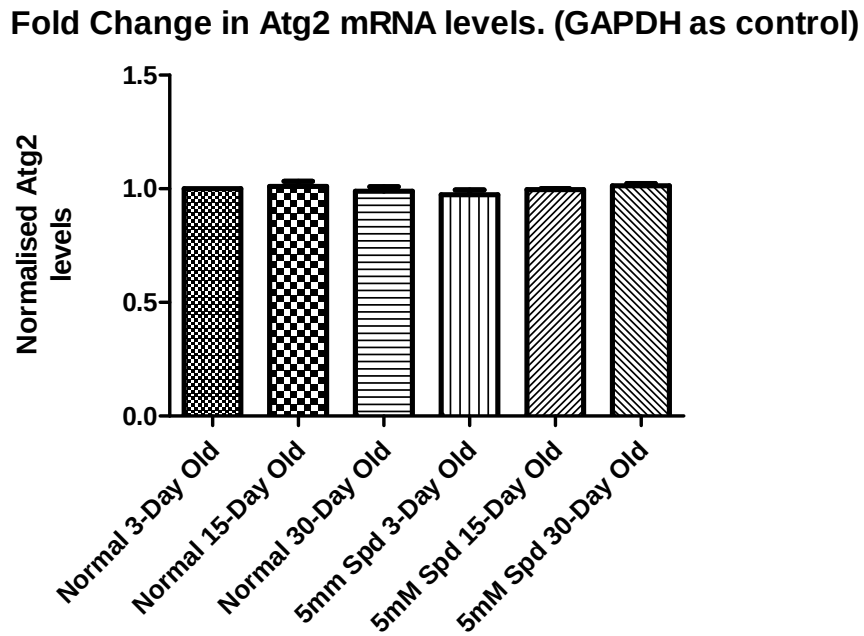
compare the PCR amplification efficiency between gene of interest and control. Efficiency curves for Atg5 and Atg7 couldnotbe plotted due to their failure to amplify. Below mentioned are the efficiency values for genes used for RT-PCR.

Gene	Efficiency values.
atg1	98%
atg2	91%
atg8	80%
Actin5C	94%
GAPDH	95%

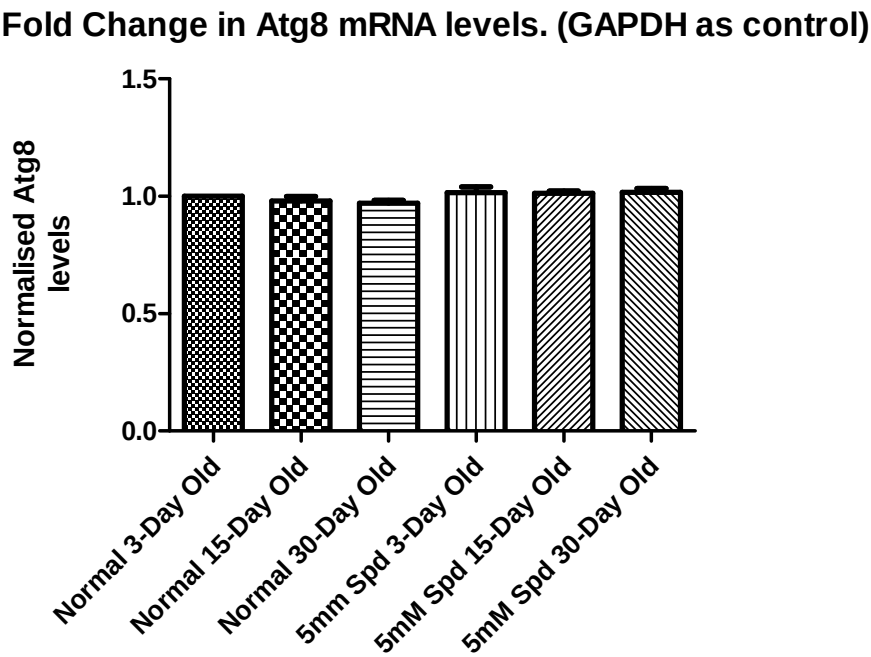
Figure 2: Fold change mRNA expression levels of a.) Atg1 b.) Atg2 and c.) Atg8 with age and spermidine treatment. (GAPDH used as endogenous control)



a.)



b.)

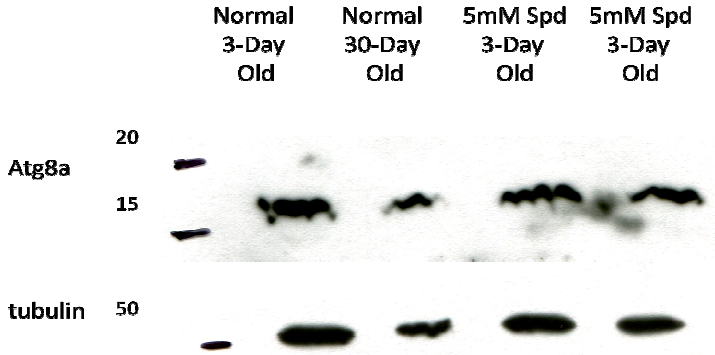


c.)

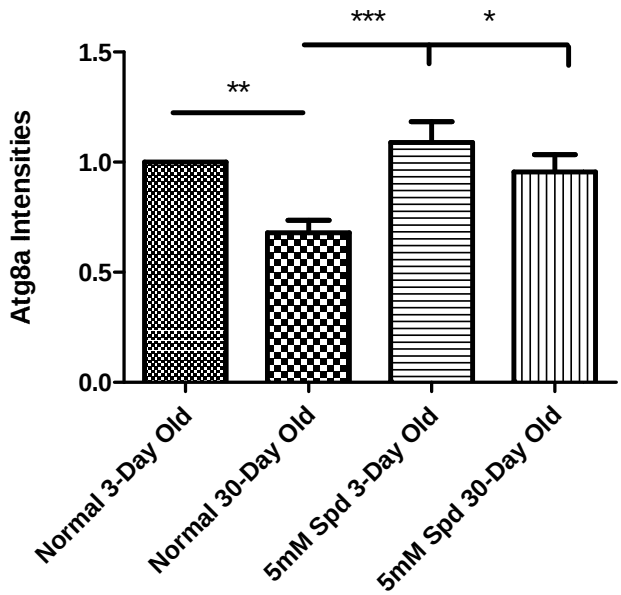
From the RT-PCR data shown in figure 2, we did not observe any noticeable change in mRNA levels of autophagy genes Atg1, Atg2 and Atg8 with age (comparing normal 3-

day old, normal 15 day old and normal 30 day old. Also, Spermidine treatment did not have any effect on the gene expression levels of these genes.

Figure 3: Age Dependent changes in Atg8a protein levels (+/-spermidine): a.) Changes in protein expression levels of Atg8a with age(in 3-day old and 30-day old flies) in control and spermidine treated samples. Tubulin used as loading control. b.) Normalized Atg8a intensities plotted for each group. Data is expressed as mean and SEM calculated from replicates from two different ageing experiments. (Number of blots = 17, Number of cohorts = 2, *p<0.05, **p<0.01, ***p<0.001 versus normal 3-day old control values, One-way Analysis of Variance with a Tukey post-test).



a.)



a.)

To analyze the effect of spermidine on Atg8a protein levels, 3 day and 30 day old flies were treated with spermidine and then compared to Atg8a protein levels of 3 day and 30 day old untreated flies. Heads were isolated and the total protein was isolated in 2% SDS which was further used for western blot. The blots were probed with anti Atg8a antibody (also known as GABARAP antibody) with tubulin as loading control. Atg8a is a small protein between 20kDa and 15kDa and in mammals the fast moving lipidated form is meant to be associated with autophagosomes. As shown in figure 3a and 3b, Atg8a levels are higher in heads of normal 3-day old flies. Whereas we see a 30% drop in autophagy levels in heads of normal 30-day old. Spermidine treatment on the other hand is able to restore the Atg8a protein levels in 30 day old flies in contrast with normal 30 day old flies.

Discussion:

Continuous turnover of intracellular proteins is essential for the maintenance of cellular homeostasis as well as for the regulation of multiple cellular functions. Proteins are usually tagged for selective destruction by covalent attachment of Ubiquitin, a small, compact protein that is highly conserved. The accumulation of ubiquitinated protein aggregates due to aberrant protein folding or due tosuppression of protein degradation has been observed in many human neurodegenerative diseases as well as in aged neural tissues. We therefore, tried to reproduce the already known age dependent accumulation of IUP in fly heads and asked if spermidine has any effect on this IUP profile. For this, the fly heads were processed in sequential detergent dilutions thus allowing extraction of proteins based on their solubility properties in non-ionic (Triton-X) and ionic (SDS) detergents. Ubiquitinated proteins accumulate in SDS fraction. From the western blots of SDS soluble fraction, normal 3-day old flies exhibit low IUP levels while normal 30 -day old flies show a significant accumulation of ubiquitinated proteins (Figure 1). This is consistent with previous findings (Nezis et. al., 2008, Bartlett et. al., 2011). IUP levels seem to be more or less constant in spermidine fed3-day and Spermidine fed 30-day old flies (Figure 1b). Spermidine, therefore, prevents age-dependent accumulation of proteins aggregates.

Proteins tagged with Ubiquitin can either be degraded by proteosomal machinery or can be processed via autophagy pathway, depending on p62 binding. P62/Ref(2)P family of proteins has been shown to act like an adaptor molecule between protein inclusions containing Ubiquitin as well as key components of the autophagy pathways resulting in the autophagic clearance of cytoplasmic protein bodies or sequestosomes. Moreover, Ref(2)P, Drosophila homologue of mammalian p62, was recently shown to be a major component of protein aggregates that are formed when proteosomal and autophagy activity diminishes in aged brain of flies (Nezis et. al., 2008). Earlier studies in mouse models have indicated that autophagy is crucial to prevent this accumulation of ubiquitinated proteins (Rubinzstein, 2006). Also, promoting Atg8a expression in older fly brains drastically not just extended the average adult lifespan but also promoted resistance to oxidative stress. Evidences from Eisenberg et. al., 2009 suggest that induction of autophagy is indeed required for lifespan-prolonging and cyto-protective effects of Spermidine. Therefore, next we asked if there is any change in the levels of autophagy in the heads of spermidine-fed flies. For this, we first performed quantitative Real-Time PCR to determine the changes at the level of ATG gene expression. Finley et. al showed an age-dependent decrease in mRNA transcript levels of ATG genes in wild-type flies. Surprisingly, we did not observe any change in the expression levels of autophagy genes between normal 3-day old and normal 30-day old flies (Figure 2). This discrepancy noted could be attributed to the fact that protein extraction was done from a mixed population of males and females whereas in previous studies, people have specifically used female heads for qRT-PCR and western blots. Ageing is sex specific and hence, it is preferable to use one sex for these quantitative studies. One of the future experiment therefore, focuses on exploring changes in levels of gene expression of autophagy genes with age upon spermidine administration in female heads only.For current work, the next thing that we wanted to see was if there is any change at protein level of autophagy regulatory proteins.

Antibody probings were done for Atg8a because there are models which indicate that under the conditions of high autophagy demand or when Atg8a gene expression is suppressed, Atg8a has the potential to become the rate limiting member of the pathway.(Simonsen et. al., 2008).Flies express two conserved members of Atg8/LC3

family, Atg8a and Atg8b, with Atg8a being predominantly expressed. Antibody probing was done for Atg8a in protein extracts prepared in 2%SDS from fly heads. From the blots, we observe a significant age dependent reduction in atg8a protein levels, which can be inversely correlated to IUP accumulation. Notably, the protein levels for Atg8a were restored in head extracts of flies fed with spermidine (Figure 3b). Thus, spermidine prevents the age dependent accumulation of protein aggregates in brain in an autophagy dependent pathway. This might be one of the ways by which spermidine might be acting to prevent cognitive decline.

Western blot data itself is unable to establish the causal relationhip between clearance of ubiquitinated proteins and activation of autophagy pathway convincingly. One experiment to strongly establish this causal relationship would be to look at the p62/Ref(2)P protein expression profile in spermidine treated flies and control flies. Study done by Bartlett et. al. 2010 validates p627/Ref(2)P as a conserved marker of neuronal aging and aggregate formation. We know that Ref(2)P labeled ubiquitinated proteins are cleared up by autophagy. Also, It has been shown before that Ref(2)P levels increase with age (Bartlett et. al. 2010). Along with current western blot data, if we see a decrease in Ref(2)P levels in spermidine treated 30-day old flies, then this might provide a stronger support for the statement that spermidine in fact prevents age dependent accumulation of misfolded proteins by upregulation of autophagy.

We don’t observe any change in the mRNA levels of autophagy genes whereas we see an increase in levels of Atg8 protein levels. This discrepancy in levels of Atg8 protein and not mRNA could be due to the protocol that we followed. Previous studies concentrated on looking at both mRNA profile and protein profile mainly in female heads, whereas we tried to quantitate the mRNA levels in mixed population of males and females. However, for western blots we focused on protein extracted from female heads only. So, one of the future experiments focuses on quantitating mRNA levels with age (+/- Spermidine).

Future work addresses the question that how in detail spermidine influences autophagy. For this, RNA sequencing is being performed currently to find out the transcriptional

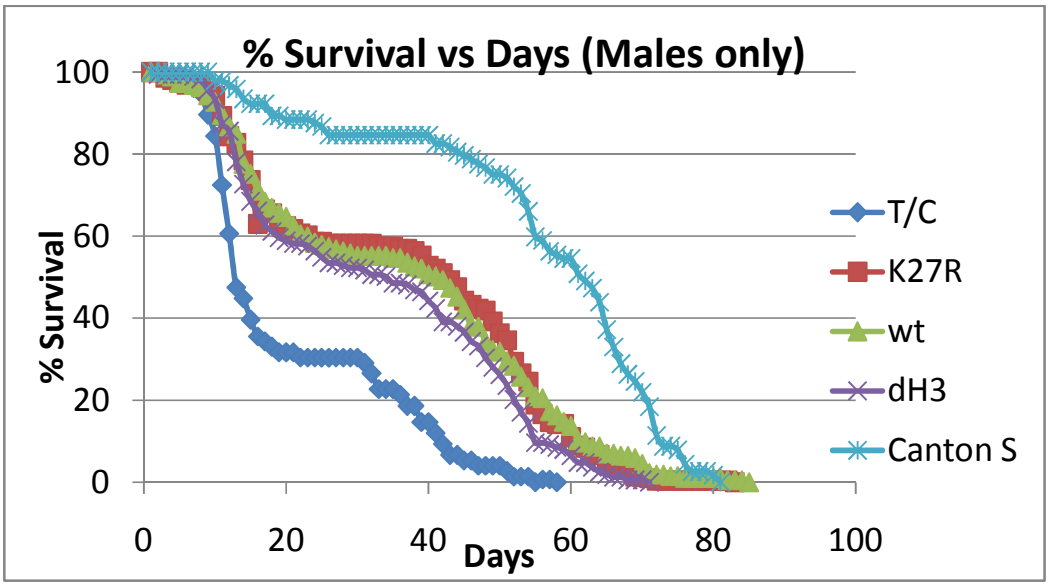
targets of spermidine. Besides this, detailed proteomic study would be performed to find out the direct protein targets of spermidine.

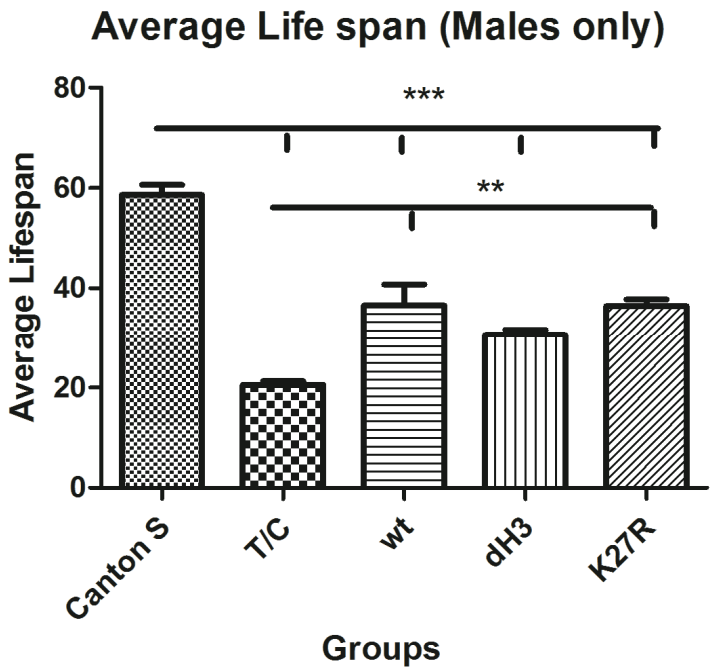
Part 2: Characterization of *Drosophila melanogaster* Histone mutants

Results:

Figure 4: Longevity (Males Only): a.) Survival curve for histone mutants and control group. On the y-axis is the survival probability (Survival probability is defined as the probabilities of surviving to an age given the flies were alive after 24-36 hours of eclosion. b.) Mean lifespan plotted for each group. Data is plotted as Average age and SEM. (N~5, **p<0.01, ***p<0.001 versus Canton S control value, One-way ANOVA with a Bon Feroni post test)

a.)



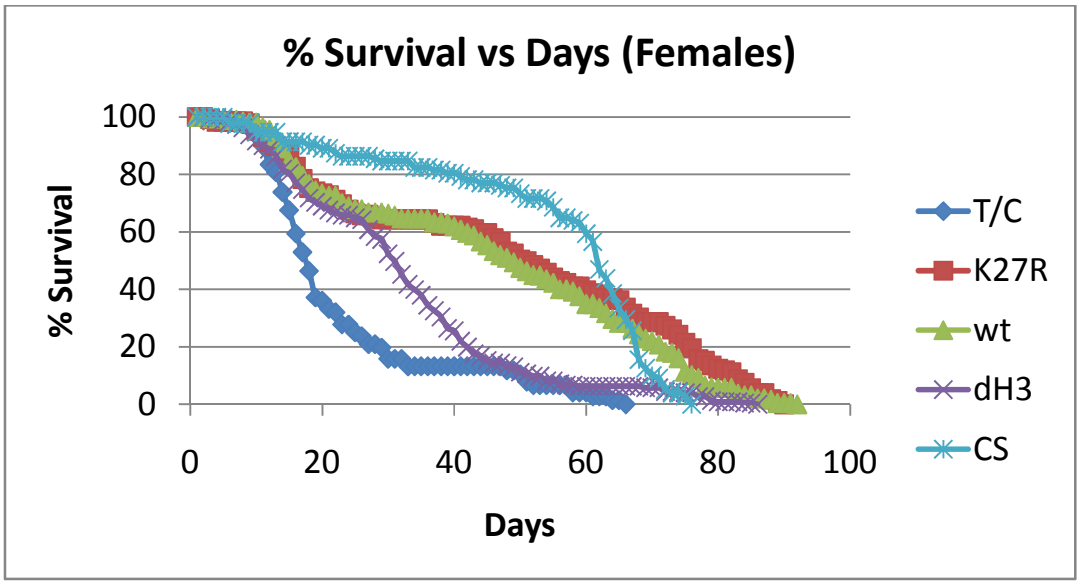


b.)

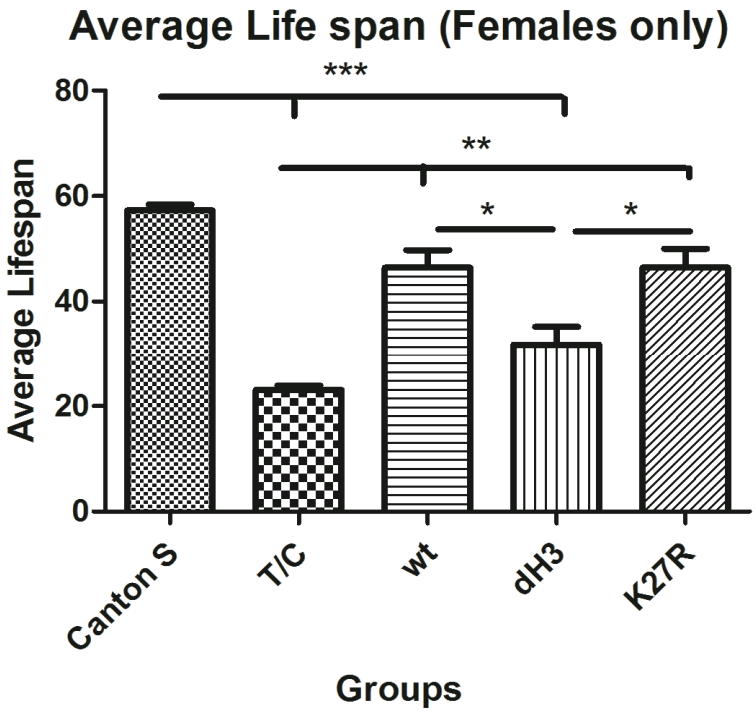
From the above figure 4b, we noticed that all the male histone mutants live a significantly shorter life span compared to Canton S males, particularly mutant with partial deletion of histone locus living for the shortest time (around 20 days). Interestingly, mutant with 6 copies of histone 3 deleted lived as long life span as mutant with 6 copies of wild type histone gene units replaced. Substituting 6 copies of wild type histone gene units could partially rescue the normal life span in these mutants. Also, replacing 6 copies of point mutated H3-K27->R could partially rescue the longevity in these mutants. The flies did not show any other morphological defect or defect in locomotion.

Figure 5: Longevity (females only): a.) Survival curve for histone mutants and control group.b.)Mean lifespan plotted for each group. Data is plotted as Average age and SEM. (N~5, **p<0.01, ***p<0.001 versus Canton S control value, One-way ANOVA with a Bon Feroni post test)

a.)



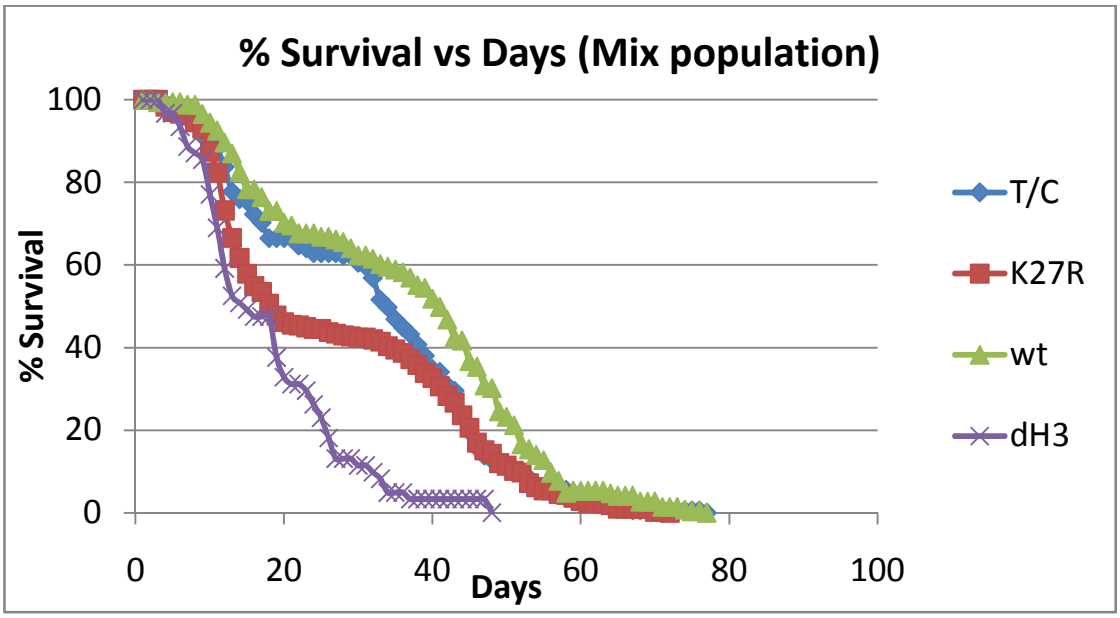
b.)

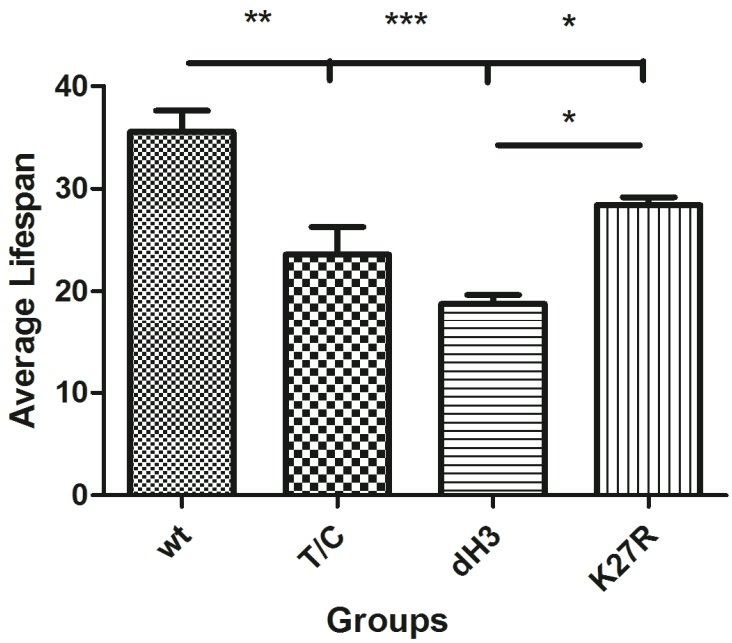


From figure 5a and 5b, we can say that in females also the partial deletion of histone locus results in a short lifespan. However, in females, deletion of Histone 3 locus in females has a drastic effect on lifespan which could be completely recued by substitution of six wild-type H3 units as well 6 point mutated H3K27->R in the mutants. Unlike male histone mutants, female histone mutants with 6 wild type copies substituted and 6 wild type copies with H3-K27->R substituted live a normal life span compared to control females. Though we don't see any significant difference between mean age of CS females, wt females and H3-K27R females from the raw data we can see that Canton S females live for a pretty much longer tome than the mutant females.

Figure 6: Longevity (Mix Population): a.) Survival curve for histone mutants and control group. b.) Mean lifespan plotted for each group. Data is plotted as Average age and SEM. (N~5, **p<0.01, ***p<0.001 versus Canton S control value, One-way ANOVA with a Bon Feroni post test)

a.)





b.)

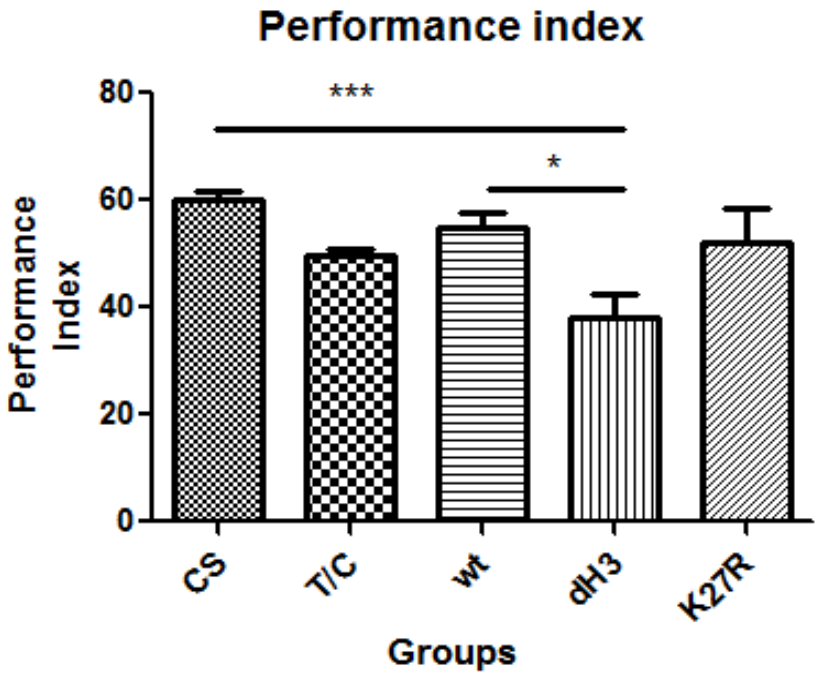
When males and females were aged together in one culture vial, mutants with Histone 3 locus missing live a drastically reduced lifespan. Mutants following partial deletion of histone locus also live a shorter lifespan. The longevity phenotype is partially restored in mutants when 6 wild type copies with H3K27->R are replaced back. The normal lifespan is completely restored when 6 wild type copies are replaced back.

	Males	Females	Males-Females together
Canton-S	58.63	57.31	
T/C	20.6	23.07	23.57
wt	36.56	46.41	35.54
dH3	30.63	31.85	18.79
K27R	36.46	46.46	28.41

Tabular representation of average life of control and histone mutants.

Effect of Histone deficiencies on Short-term Memory:

Figure 7: Plotted are the mean performance index and SEM of 3-5 day old flies from each group. (N = 8, *p<0.05, ***p<0.001, One-Way ANOVA with Bon Feroni post test)



Looking at the data in figure 7, we can say that the mutants with H3 deletion show a learning deficit when tested for 3 minute memory. The memory however is restored with substitution of six copies of wild type H3 in these mutants. Also, substitution of six copies of wild type histone locus with point mutated H3-K27->R results in complete rescue of memory deficits in these mutants.

Discussions:

As mentioned earlier, aging is a complex process involving changes in genetically and epigenetically programmed processes. Therefore, as second part of my project I tried to explore the link between epigenetics, longevity and memory. Acetylation and methylation of histones has been regarded as an important process during physiological aging of various organisms. In this respect, genetic and pharmacological manipulation

of histone acetylation status has been repeatedly linked to influence cellular and organismal lifespan (Morselli et. al., 2009, Bjedov et. al., 2010).

Previous studies indicate that aging correlates with brain region specific changes of gene expression. It is however, not well understood how aging affects gene expression and if those changes are causally linked to memory impairment. Recent datasuggests that histone acetylation might play an important role in orchestrating gene expression program initiated by memory consolidation. Administration of HDAC inhibitors that shift the balance of H4K12 acetylation is able to reinstate learning induced gene expression and memory function in 16-month old mice (Peleg et. al., 2010)

Despite the fundamental role of canonical histones, there was no experimental system for higher eukaryotes until nowto address the questions directly at the level of histone structure. So far, studies concerning histone function have been done through modification of activity of histone methyl transferases (DNMTs) or histone acetyl transferases (HATs) or histone deacetylases (HDAC's) which target specific residue and specific post translational modification. Günesdoganet. al., 2010 developed an experimental system for model organism *Drosophila melanogaster* that can serve as a tool to directly assess histone functions by molecular genetics together with transgene dependent rescue.

Drosophila provides a suitable system to study histone functions unlike other genetically accessible eukaryotic model systems in which histone genes are distributed throughout the genome. Drosophila is composed of 23 canonical histone gene repeat units (His-GUs) each bearing a gene array encoding the 5 canonical histones (H1, H2A, H2B, H3 and H4). Mutant Df(2R)HisC, with all histone genes removed from genome, was generated using *DrosDel*system (Ryder et. al.). Embryonic lethality of homozygous HisC mutants allows validation of transgene-based rescue. The functional replacement of endogenous histones by histone transgene offers a unique opportunity to directly address the relevance of specific histone modification sites in a multicellular organism.

Hence, I started the second part of my project with characterization of histone mutants in terms of longevity and memory. Crosses were set to obtainfollowing four

genotypes:1.) Partial deletion of histone locus (T/C), 2.) In the background of histone deletion 6 copies of wild-type histone gene units replaced back (wt), 3.) In the background of histone deletion 6 copies of wild-type histone gene units without histone 3 substituted (dH3) and 4.) In the background of histone deletion 6 copies of wild type histone gene units with H3-K27 mutated to arginine(R) replaced back (H3-K27->R).

H3-Lys27 is an important residue because it can either be methylated or acetylated depending upon gene being silent or active. As mentioned before in introduction, trimethylation of Histone 3 lysine 27 (H3K27me3) is essential for the establishment and maintenance of polycomb silencing and is brought about by polycomb repressive complex 2 (PRC2). Long-lived E(z) mutants have reduced H3K27me3 levels. Interestingly, these mutants also have enhanced Odc1 expression, which is an upstream regulator in the pathway of spermidine synthesis (Sieboldet. al., 2010). H3K27me3 is a putative regulator of Odc1 expression and in absence of H3K27me3, elevated levels of Odc1 are observed which leads to enhanced longevity and increased resistance to oxidative stress and starvation. Hence, we tried to look at the effect of H3K27 defect on longevity and memory of *D.melanogaster*.

Longevity experiments were conducted in three different sets, males only, females only and males-females together. Although partial deletion of histone locus resulted in short lifespan, interestingly mutantscarrying deletion of histone locus showed a sex specific effect. When males and females were kept in separation we observed that in males, a deletion of H3 resulted in no significant difference in lifespan of these mutant compared to wt while in females the deficiency led to a smaller lifespan. Also, when males and females were aged together, we observed a drastic reduction in lifespan of the dH3 mutants, with mean age even less than T/C mutants. This longevity phenotype could be rescued by replacing 6 copies of wild type histone 3 in the mutant background. Interestingly, substituting 6 copies of point mutated H3K27->R could rescue the life span in these female mutants although not equal to life span of Canton S females. When tested separately we don’t see any difference between average life span of wt and H3K27R mutants, however, when males and females were allowed to age together, we see a significant difference between mean age of these two groups with wt having a

longer mean life span than H3-K27->R. From this observation we can say that complex genetic and environmental interactions are going here in this case which collectively decide the mean life of these mutants.

Next, we tried to explore the link between histone deficiency and learning and memory performance. For this, flies were trained with classical associative learning assays and then tested for 3-min memory (STM). From memory data, we found flies mutant for only histone 3 locus showed significantly lower performance index. If deletion of histone-3 is responsible for memory deficits then, replacing wild-type copies of H3 should restore it. In fact, we found replacement of six copies of wild-type H3 could rescue the memory defects. In addition, we observed that replacement of six-copies of point-mutated H3 (H3-K27R) also resulted in rescue of memory impairment. Surprisingly, we did not observe any notable memory defects in T/C mutants where few histone gene units are deleted, for definition of histone gene unit refer to material and method section, which also has H3 removed along with other histone genes. From these observations, we can say that complex epigenetic interactions are involved in memory formation.

From the preliminary data obtained from longevity and memory assays we know that a deletion of H3 locusgene results in shorter lifespan,in a sex specific manner. Also, deletion of H3 results in memory deficits in flies. Interestingly, these defects could be partially rescued by substituting 6 wildtype copies of histone 3 suggesting a possible role of H3 in maintaining life span and in memory formation. Also, substitution of 6 copies of point-mutated H3-K27->R could partially rescue longevity and completely restore memory in these mutants. As mentioned before, trimethylation at H3-K27 has a genesilencing effect. Mutation of lysine 27 to arginine removes this repressing effect and results in expression of genes which were silenced by H3K27me3, one of them being *Odc1,* an upstream regulator in polyamine synthesis pathway.Enhanced Odc1 expression in H3-K27->R mutants promotesspermidine synthesis which could give rise to longevity and memory phenotypes we observe.H3-K27 can be both methylated and acetylated. K27 mutated to R can still undergo mono- and di-methylation which might therefore partially rescue these phenotypes.

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