nature neuroscience

Restoring polyamines protects from age-induced memory impairment in an autophagy-dependent manner

Varun K Gupta[1,2], Lisa Scheunemann[1], Tobias Eisenberg[3], Sara Mertel[1], Anuradha Bhukel[1,2], Tom S Koemans[4], Jamie M Kramer[4], Karen S Y Liu[1,2], Sabrina Schroeder[3], Hendrik G Stunnenberg[5], Frank Sinner[6,7], Christoph Magnes[6], Thomas R Pieber[6,7], Shubham Dipt[8], André Fiala[8], Annette Schenck[4], Martin Schwaerzel[1], Frank Madeo[3] & Stephan J Sigrist[1,2]

Age-dependent memory impairment is known to occur in several organisms, including *Drosophila*, mouse and human. However, the fundamental cellular mechanisms that underlie these impairments are still poorly understood, effectively hampering the development of pharmacological strategies to treat the condition. Polyamines are among the substances found to decrease with age in the human brain. We found that levels of polyamines (spermidine, putrescine) decreased in aging fruit flies, concomitant with declining memory abilities. Simple spermidine feeding not only restored juvenile polyamine levels, but also suppressed age-induced memory impairment. Ornithine decarboxylase-1, the rate-limiting enzyme for *de novo* polyamine synthesis, also protected olfactory memories in aged flies when expressed specifically in Kenyon cells, which are crucial for olfactory memory formation. Spermidine-fed flies showed enhanced autophagy (a form of cellular self-digestion), and genetic deficits in the autophagic machinery prevented spermidine-mediated rescue of memory impairments. Our findings indicate that autophagy is critical for suppression of memory impairments by spermidine and that polyamines, which are endogenously present, are candidates for pharmacological intervention.

Age-induced memory impairment (AMI) is a common condition that is characterized by symptoms of cognitive decline that occur as part of the aging process. To date, molecular interventions to counteract AMI remain largely elusive, with the long lifespan of many animal models being a major limitation for studying age-related memory impairment. *Drosophila*, with its comparatively short lifespan and advanced genetics, is an ideal model system for unraveling the molecular mechanisms associated with AMI and testing putative means for preventing AMI. The age-dependent decline of (aversive) olfactory memory in *Drosophila* serves as an established model for AMI[1–4]. In this learning procedure, groups of flies are trained by presenting them with an odor that temporally coincides with the application of electric shocks and presenting a second odor without this punishment. Later on, flies can choose between the two arms of a T-maze containing either the odor that was paired with the electric shocks or the neutral odor. Learning is assessed by calculating the relative proportion of flies avoiding the punished odor. This type of associative olfactory memory is usually maintained over several hours[5].

Studies in various model organisms have implicated autophagy as a crucial regulator of the aging process[6]. Autophagy is a process of cellular self-digestion in which portions of the cytoplasm are sequestered in double- or multi-membraned vesicles (autophagosomes) and then delivered to lysosomes for bulk degradation. In *C. elegans*, increased autophagy is necessary for lifespan extension by reduced insulin-like signaling[7] and dietary restriction[8]. Similarly, increasing autophagy specifically in neurons can extend the lifespan of flies[9], whereas reducing autophagy shortens lifespan and gives rise to neurodegeneration[9,10]. Although autophagy is a key regulator of the aging process, its role in cognitive aging has not yet been addressed.

We found that the levels of simple polyamines (spermidine and putrescine) decreased in the heads of aging *Drosophila*, consistent with a decline in olfactory aversive memory in aging flies. Notably, restoration of polyamine levels by dietary spermidine supplementation suppressed AMI. Spermidine administration prevented the age-associated decrease of autophagy, and genetically induced deficits in the autophagic machinery occluded spermidine-mediated protection of AMI. In addition, we found that the effects of spermidine on memory were not a result of generically improved health, but instead reflected neuron-intrinsic regulations. These results suggest that autophagy is important for cognitive aging and that polyamines, endogenous metabolites, are candidate substances for treating AMI.

[1]Institute for Biology/Genetics, Freie Universität Berlin, Berlin, Germany. [2]NeuroCure, Charité, Berlin, Germany. [3]Institute of Molecular Biosciences, University of Graz, Graz, Austria. [4]Department of Human Genetics, Nijmegen Centre for Molecular Life Sciences, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. [5]Department of Molecular Biology, Faculty of Science, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen, Nijmegen, The Netherlands. [6]Health Institute for Biomedicine and Health Sciences, Joanneum Research Forschungs GesmBH, Graz, Austria. [7]Department of Internal Medicine, Division of Endocrinology and Metabolism, Medical University of Graz, Austria. [8]Georg-August-Universität Göttingen, Molecular Neurobiology of Behavior, Göttingen, Germany. Correspondence should be addressed to S.J.S. (stephan.sigrist@fu-berlin.de) or F.M. (frank.madeo@uni-graz).

Received 19 June; accepted 8 August; published online 1 September 2013; doi:10.1038/nn.3512

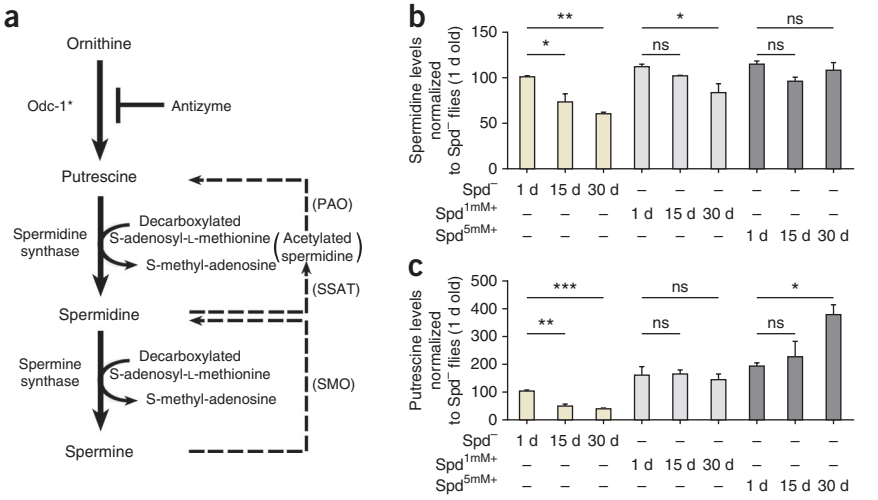


Figure 1 Spermidine feeding prevents the age-related decline of endogenous spermidine and putrescine levels. (**a**) Scheme illustrating *de novo* synthesis of polyamines from ornithine. * indicates the rate-limiting enzyme for *de novo* synthesis of polyamines. PMO, polyamine oxidase; SMO, spermine oxidase; SSAT, spermidine/spermine N(1) acetyltransferase. (**b**) Levels of spermidine in the heads of wild-type flies fed with food supplemented by 1 or 5 mM spermidine (Spd^{1mM+} or Spd^{5mM+}, respectively) or food with no spermidine (Spd^{-}). Data are shown normalized to spermidine levels of 1-d-old (1 d) Spd^{-} flies ($n = 4$ independent experiments; $F = 14.08$ for Spd^{-} flies, $F = 5.30$ for Spd^{1mM+} flies, $F = 2.62$ for Spd^{5mM+} flies; one-way-ANOVA with Bonferroni correction). 15 d, 15 d old; 30 d, 30 d old. (**c**) Levels of putrescine in the heads of aged Spd^{1mM+} or Spd^{5mM+} flies compared with Spd^{-} flies. Data are shown normalized to putrescine levels of 1-d-old Spd^{-} flies ($n = 4$ independent experiments; $F = 24.31$ for Spd– flies, $F = 0.22$ for Spd^{1mM+} flies, $F = 6.67$ for Spd^{5mM+} flies; one-way-ANOVA with Bonferroni correction). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns indicates not significant, $P > 0.05$. Data are presented as mean ± s.e.m.

RESULTS

Polyamine levels decline in aging *Drosophila* brains

A decrease in polyamine levels has been reported in aged brains of rodents and humans[11,12]. To investigate the role of polyamines in AMI, we began by measuring polyamine levels in heads of aged *Drosophila*, and analyzed the chemically related (and inter-convertible) species spermidine, putrescine and spermine (for a review, see ref. 13; **Fig. 1a**). In fact, both spermidine and putrescine levels were markedly reduced in the heads of 15-d-old flies compared with young flies (Spd^{-} 1–3 d old; Online Methods and **Fig. 1b**,**c**).

To determine whether simply feeding spermidine to flies would be sufficient to restore its expression in aged heads, we administered either 1 or 5 mM spermidine to isogenized wild-type *Drosophila* (w^{1118}) in standard fly food (Spd^{1mM+} or Spd^{5mM+}, respectively). Spermidine levels (in fly heads) were largely protected by this treatment from age-dependent decline (**Fig. 1b**). Simultaneously, putrescine levels were greater than those in normal juvenile flies (**Fig. 1c**), indicating that the cellular uptake of spermidine is accompanied by either an increased conversion into putrescine or inhibition of the endogenous conversion of putrescine to spermidine (**Fig. 1a**).

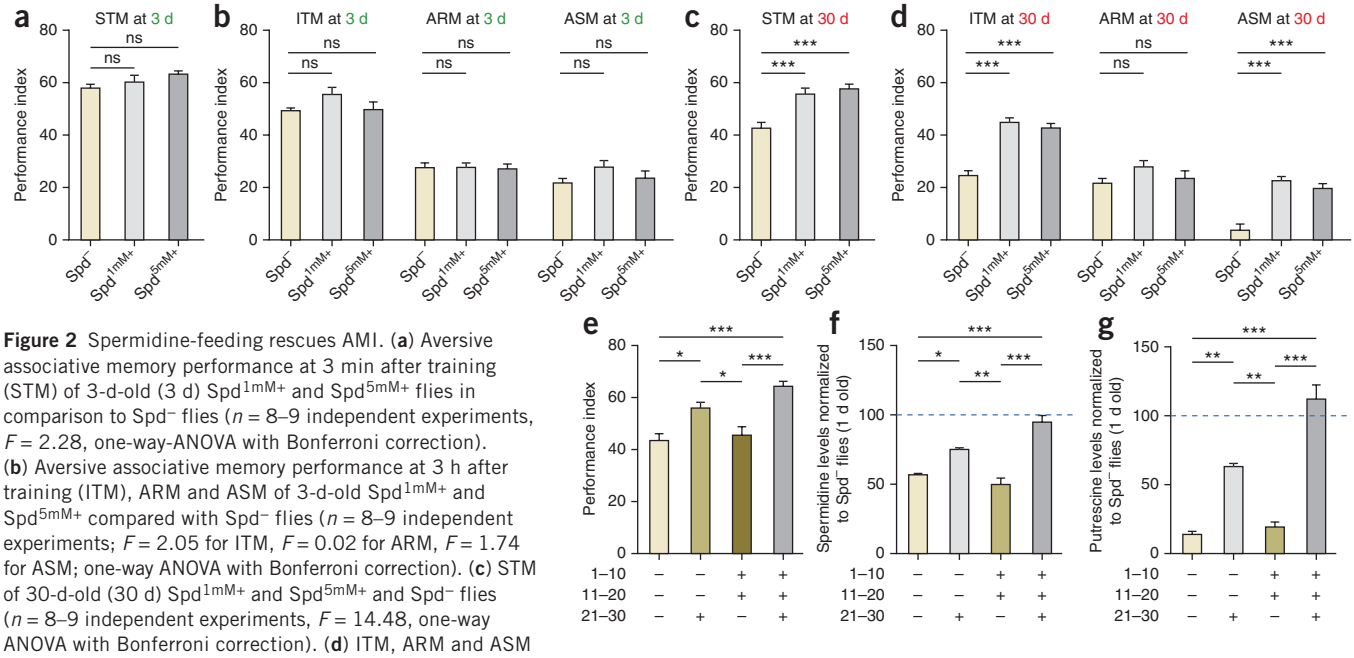


Figure 2 Spermidine-feeding rescues AMI. (**a**) Aversive associative memory performance at 3 min after training (STM) of 3-d-old (3 d) Spd^{1mM+} and Spd^{5mM+} flies in comparison to Spd^{-} flies ($n = 8$–9 independent experiments, $F = 2.28$, one-way-ANOVA with Bonferroni correction). (**b**) Aversive associative memory performance at 3 h after training (ITM), ARM and ASM of 3-d-old Spd^{1mM+} and Spd^{5mM+} compared with Spd^{-} flies ($n = 8$–9 independent experiments; $F = 2.05$ for ITM, $F = 0.02$ for ARM, $F = 1.74$ for ASM; one way ANOVA with Bonferroni correction). (**c**) STM of 30-d-old (30 d) Spd^{1mM+} and Spd^{5mM+} and Spd^{-} flies ($n = 8$–9 independent experiments, $F = 14.48$, one-way ANOVA with Bonferroni correction). (**d**) ITM, ARM and ASM of 30-d-old Spd^{1mM+} and Spd^{5mM+} compared with Spd^{-} flies ($n = 7$–9 independent experiments; $F = 43.26$ for ITM, $F = 1.74$ for ARM, $F = 26.12$ for ASM; one-way ANOVA with Bonferroni correction). (**e**) Spermidine feeding for 10 d before measuring memory was sufficient to suppress AMI in aged (30 d old) flies ($n = 9$–11 independent experiments, $F = 12.32$, one-way ANOVA with Bonferroni correction). (**f**,**g**) Spermidine feeding on days 21–30 resulted in a significant increase in the levels of spermidine and putrescine in 30-d-old flies ($n = 3$ independent experiments, $F = 32.96$ (**f**) and 64.77 (**g**), one-way ANOVA with Bonferroni correction). The feeding regime of the flies is presented beneath the graphs, with + and – indicating food with or without spermidine. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns indicates not significant, $P > 0.05$. Data are presented as mean ± s.e.m.

Table 1 Aversive olfactory avoidance and shock reactivity in aging wild-type flies with and without spermidine feeding

	Olfactory avoidance		
	OCT	MCH	Shock reactivity
w^{1118}; Spd$^-$ 3 d old	65.5 ± 4.2	63.9 ± 1.7	70.5 ± 2.0
w^{1118}; Spd^{1mM+} 3 d old	71.1 ± 3.2	63.2 ± 2.8	64.1 ± 2.9
w^{1118}; Spd^{5mM+} 3 d old	62.1 ± 2.9	62.5 ± 2.5	63.1 ± 2.4
w^{1118}; Spd$^-$ 30 d old	44.8 ± 2.7	45.2 ± 3.3	67.4 ± 1.9
w^{1118}; Spd^{1mM+} 30 d old	43.2 ± 3.8	43.0 ± 3.5	63.4 ± 3.5
w^{1118}; Spd^{5mM+} 30 d old	48.3 ± 5.5	41.8 ± 5.1	65.3 ± 1.5

All values are mean ± s.e.m.

This observation is consistent with the previously reported homeostatic control of polyamine levels[14]. On the other hand, levels of spermine declined considerably with age in both Spd$^+$ as well as Spd$^-$ flies (**Supplementary Fig. 1**).

Spermidine feeding suppresses AMI

We then asked whether this restoration of polyamine levels in aged flies could counter-act AMI. Young adult flies (3 d old) that were raised on food supplemented with spermidine (both Spd^{1mM+} or Spd^{5mM+}) showed identical olfactory short-term memory (STM; memory tested immediately after odor conditioning) and intermediate-term memory performance (ITM; memory tested 3 h after odor conditioning) when compared to isogenic controls (Spd$^-$ flies; **Fig. 2a**,**b**). Consistent with a previous report[1], we also found substantial impairment in STM that first appeared in 10 d-old flies and did not decrease any further during aging (**Supplementary Fig. 2a**). Likewise, ITM scores are also known to decline with age[1,3]. As anticipated, at 30 d of age, considerably reduced STM and ITM scores (**Fig. 2c**,**d**) were observed in control flies (Spd$^-$). In contrast, spermidine-fed (Spd$^+$) flies showed higher STM and ITM scores than Spd$^-$ flies at 30 d of age (**Fig. 2c**,**d**). In fact, the performance of 30d Spd$^+$ flies (both Spd^{1mM+} and Spd^{5mM+}) was comparable to that of young flies. In summary, simple spermidine feeding was sufficient to effectively protect both short- and intermediate-term olfactory memory from age-induced decline (**Fig. 2c**,**d**).

ITM can be dissected into anesthesia-sensitive memory (ASM) and anesthesia-resistant memory (ARM) components, which can be differentiated by distinct genetic mutants, as well as by specific pharmacological sensitivities[1,2,4,15,16]. ASM can be calculated by subtracting ARM scores, measured after amnestic cooling, from ITM. A previous study found that AMI has a strong influence on ASM, but not on ARM[1]. Consistently, we found that ARM was only slightly affected by aging, with spermidine feeding producing only a negligible effect (**Fig. 2b**,**d**). In contrast, ASM was nearly absent in control flies at 30 d of age, but was preserved in age-matched Spd$^+$ flies (**Fig. 2b**,**d**). This specific effect of spermidine in protecting ASM without affecting ARM, together with the lack of any consequential effect of spermidine feeding on memory in young flies, argues against spermidine having a general, nonspecific role in memory consolidation.

The standard conditioning procedure that we used here included application of 12 electric shocks, a potentially saturating number for memory scores[5], which might mask subtle spermidine-evoked changes in young flies. Thus, we also trained young flies under non-saturating conditions, in which we only applied two electric shocks[17]. Again, we found no difference between the memory scores of young (3 d old) adult flies raised on either normal (Spd$^-$) or spermidine supplemented food (Spd^{1mM+} or Spd^{5mM+}) (**Supplementary Fig. 2b**). This indicates that spermidine does not generally boost memory, but specifically protects aged flies from memory impairments.

Spermidine-mediated effects are specific for memory

Potentially, polyamine effects could be mediated through developmental changes, such as during critical periods in early adulthood. To address such putative developmental effects, we shifted flies between spermidine-containing and spermidine-free food. When tested on day 30, in flies fed with spermidine only between days 0 and 20 (with spermidine supplementation being withdrawn for the last 10 d before testing; **Fig. 2f**,**g**), we found that the levels of polyamines (both spermidine and putrescine) declined to levels comparable to those of controls (no spermidine supplementation for days 1–30; **Fig. 2f**,**g**), as was the memory when tested at day 30 (**Fig. 2e**). In contrast, in flies fed with spermidine for the last 10 d before testing, levels of both polyamines (spermidine and putrescine) rose (**Fig. 2f**,**g**), and memory was partially, but substantially, restored (**Fig. 2e**). The fact that restoring polyamine levels during the 10 d before testing prevented AMI rules out the idea that the effects of spermidine-feeding on suppression of AMI are a result of affected developmental processes.

Given that spermidine feeding promotes longevity[14], it might be argued that protection of memory is a byproduct of increased life expectancy and generally improved health. Thus, we asked whether spermidine feeding preserves the function of the fly nervous system in all respects or whether it rather has a more specific effect on memory. In our learning assay, we found that 30-d-old naive flies to exhibit decrease odor avoidance scores compared with 3-d-old flies, whereas the shock reactivity of these naive flies did not change (**Table 1**), consistent with previous AMI studies[1,2]. Notably, spermidine-feeding had no influence on this age-dependent decline in odor avoidance scores (**Table 1**).

Next, we wondered whether the lower memory scores in aged flies are a result of impaired processing of the olfactory information.

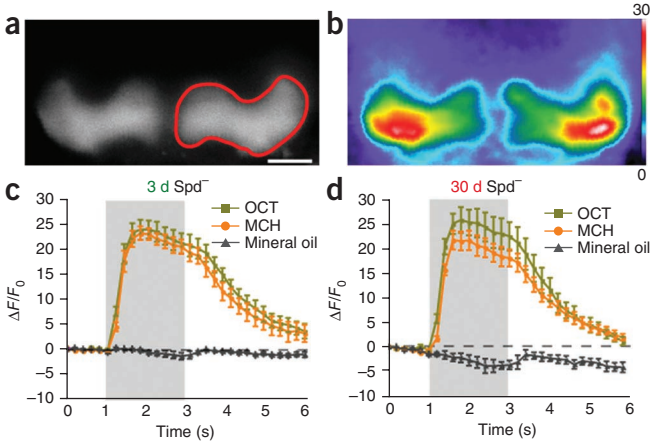


Figure 3 Ca^{2+} imaging in the Kenyon cells in response to odors in aging wild-type flies. (**a**) Expression of GCaMP3.0 in the mushroom body of an individual fly, focused on the horizontal lobes. The red line indicates a region of interest used to determine changes in fluorescence emission. Scale bar represents 50 µm. (**b**) False color–coded image of Ca^{2+} activity in the horizontal mushroom body lobes shown in **a**. Warm colors indicate high levels, cold colors indicate low levels or no Ca^{2+} activity. The numbers indicate changes in fluorescence ΔF (%). (**c**,**d**) Time course of Ca^{2+} increase in horizontal mushroom body lobes of 3-d-old and 30-d-old Spd$^-$ flies evoked by the odors 4-methyl-cyclohexanol (MCH) or 3-octanol (OCT) in comparison with the diluent, mineral oil (n = 5 flies, non-parametric Mann-Whitney U test found no substantial difference between the Ca^{2+} increase of 3-d-old and 30-d-old Spd$^-$ flies evoked by the odors). The gray bars indicate the duration of the odor stimuli. Data are presented as mean ± s.e.m.

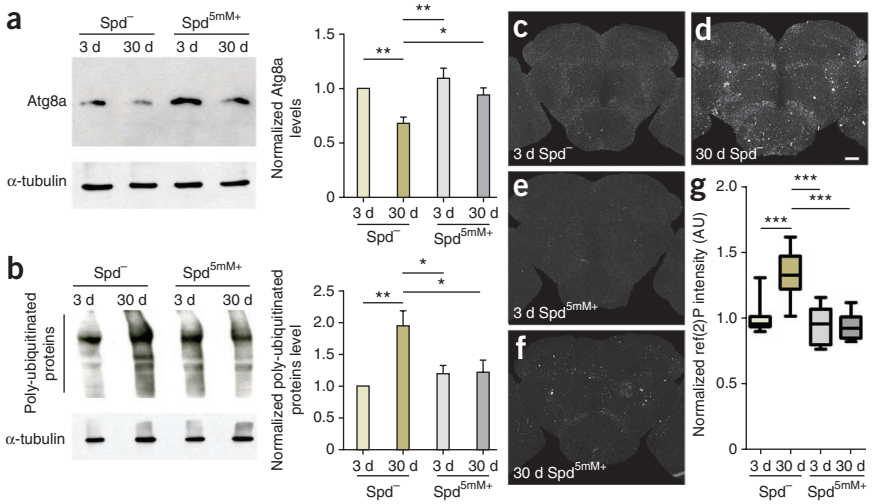


Figure 4 Spermidine feeding induces autophagy in the *Drosophila* brain. (**a**) Quantification of Atg8a proteins levels from head extracts normalized to α-tubulin ($n = 17$ or 8–9 technical replicates from two independent biological aging replicates for all data, $F = 7.52$, one-way-ANOVA with Tukey post-test). The full-length blot is shown in **Supplementary Figure 9a**. (**b**) Western blot analysis of poly-ubiquitinated proteins (SDS-soluble protein fraction) from head extracts from Spd⁻ (3 and 30 d old) flies compared with Spd^{5mM+} (3 and 30 d old) flies. Right, quantification of poly-ubiquitinated proteins levels normalized to α-tubulin ($n = 10$ or 5 technical replicates from two independent biological aging replicates for all data, $F = 6.32$, one-way ANOVA with Tukey post-test). The full-length blot is shown in **Supplementary Figure 9b**. (**c**–**f**) Adult brains of 3-d-old and 30-d-old Spd⁻ flies, as well as 3-d-old and 30-d-old Spd^{5mM+} flies immunostained for ref(2)P. Scale bar represents 25 μm. (**g**) Quantification of ref(2)P intensity in the central brain region normalized to 3-d-old Spd⁻ ($n = 12$–13 independent brains for all data, $F = 26.58$, one-way ANOVA with Bonferroni correction). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; $P > 0.05$. Data are presented as mean ± s.e.m.

We measured changes in intracellular Ca^{2+} in the horizontal lobes of mushroom body in response to the two odors used in the learning assay using a fusion of the mushroom-specific enhancer mb247 to the Ca^{2+} indicator GCaMP3.0. We found no differences in the amplitude or time course of signals recorded in young (3 d old) and aged (30 d old) flies (**Fig. 3**). Thus, it seems AMI is not caused by generic changes in odor information processing or in the intrinsic excitability of Kenyon cells. However, learning-associated Ca^{2+} transients in the dorsal paired medial neurons were recently found to decrease with aging[3].

We also found an age-induced decrease in locomotor functions of flies, which was not rescued in 30-d-old flies by spermidine treatment (**Supplementary Fig. 3**). These results argue against a genuinely generic effect on nervous system function, but rather indicate that higher polyamine levels specifically influence associative olfactory learning abilities in aging flies.

Spermidine-mediated protection requires efficient autophagy

What might be the mechanistic underpinnings of spermidine-mediated protection from AMI? Spermidine administration induces autophagy in several model organisms[14] and autophagy is crucially important for spermidine-mediated promotion of lifespan in yeast, *C. elegans* and *Drosophila*[14]. Notably, reduced basal autophagy in the nervous system of mice and flies has been shown to cause neurodegeneration[9,18–20]. In addition, the expression of several key genes in the autophagic pathway has been reported to decline with aging in the brain of humans and flies, potentially increasing neuronal vulnerability to the toxic effects of protein aggregates[6,9].

Thus, we investigated whether autophagy might be critical for spermidine-mediated protection from AMI. To address this, we first assayed the levels of Atg8a (a widely used marker for autophagy) in western blots from fly head extracts[21]. Consistent with a previous report[9], Atg8a protein levels were considerably reduced in heads from 30-d-old Spd⁻ flies compared with 3-d-old Spd⁻ flies (**Fig. 4a**). Spermidine administration, however, blocked this age-related decline in Atg8a protein levels effectively (as seen by protein levels in the heads from Spd^{5mM+} flies; **Fig. 4a**). Suppression of autophagy has been associated with the accumulation of ubiquitinated protein aggregates[9,10,22]. In fact, we observed that the age-associated increase in the amount of poly-ubiquitinated proteins was largely blocked in heads of spermidine-treated *Drosophila* (Spd^{5mM+}; **Fig. 4b**).

We next asked whether these ubiquitinated proteins were really being degraded by autophagy. The p62 family of proteins is closely associated with protein inclusions containing ubiquitin, as well as with key components of the autophagy pathway, thereby mediating autophagic clearance of ubiquitinated proteins[19,23]. In addition, ref(2)P, a *Drosophila* homolog of p62, was recently reported to accumulate with ubiquitinated neural protein aggregates in aged wild-type flies and autophagy mutants[22]. For a nervous system–specific readout, we stained brains from flies of different ages with antibody to ref(2)P. As expected, ref(2)P levels increased with age, and spermidine administration suppressed this age-dependent increase (**Fig. 4c–g**). Thus, spermidine administration seems to prevent the accumulation of ubiquitinated proteins, most likely as a direct consequence of enhancing autophagy in aged *Drosophila* brains.

We then tested whether autophagy is functionally required for spermidine-mediated protection from AMI. Both *Atg7* and *Atg8* are essential for autophagy in *Drosophila*[9,10,24–26]. Given that *Atg7*$^{-/-}$ and *Atg8a*$^{-/-}$ flies have a mean lifespan of only 30 d (ref. 10), we decided to test memory in both mutants at 20 d of age (**Fig. 5**). We found that *Atg7*$^{-/-}$ flies (**Fig. 5a,c**) showed reduced memory scores at a young age (3 d of age; **Fig. 5a**), which further declined relative to controls at later time points (20 d of age; **Fig. 5c**). Notably, the memory-promoting effects of spermidine on STM were eliminated in *Atg7*$^{-/-}$ flies (**Fig. 5a,c**). We also tested the role of Atg8a using the hypomorphic allele *Atg8a*^{EP362} of the X-chromosomal *Atg8a*. Female *Atg8a*$^{-/-}$ flies also showed a reduced memory performance at both young (3 d old) and old age (20 d old), even with spermidine administration (**Fig. 5b,d**), indicating that spermidine failed to mediate AMI protection in *Atg8a*$^{-/-}$ flies. Thus, the integrity of the autophagy system seems to be required for spermidine-mediated protection from AMI.

Spermidine causes genome-wide transcriptional changes

Taken together, our results indicate that spermidine administration results in nervous system–specific regulations that lead to the suppression of AMI, with the upregulation of autophagy being an essential component. However, spermidine has also been shown to cause major changes in the transcriptional status of yeast and cultivated human cells[14,27]. In addition, spermidine feeding in flies was recently

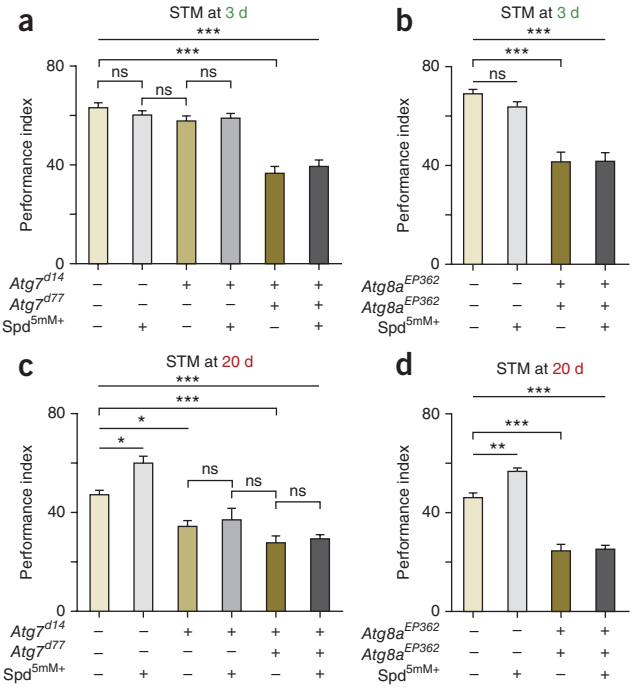
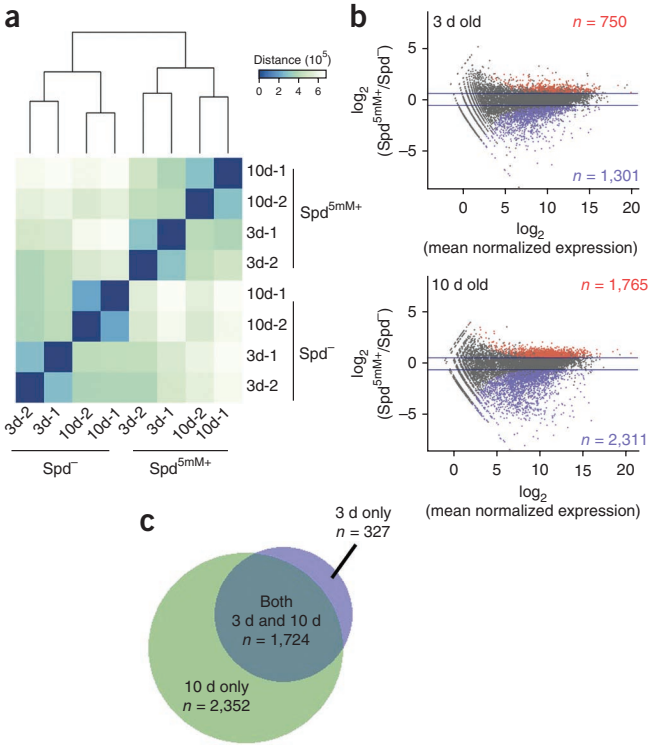


Figure 5 Autophagy is required for spermidine's effects on AMI. (**a**) Aversive associative memory performance 3 min after training (STM) was markedly reduced in 3-d-old $Atg7^{-/-}$ flies ($Atg7^{d14}/Atg7^{d77}$) compared with $Atg7^{-/+}$ flies ($Atg7^{d14}/CG5335^{d30}$) and wild-type flies ($Atg7^{+/+}$) ($n = 8$–11 independent experiments, $F = 25.82$, one-way ANOVA with Bonferroni correction). The $CG5335^{d30}$ line harbors an $Atg7^{+}$ chromosome related to $Atg7^{d14}$ and $Atg7^{d77}$ that serves as a genetic background control. (**b**) Olfactory learning was disrupted in 3-d-old female flies homozygous for *Atg8a* hypomorph ($Atg8a^{-/-}$ or $Atg8a^{EP362}/Atg8a^{EP362}$) ($n = 8$–9 independent experiments, $F = 22.8$, one-way ANOVA with Bonferroni correction). (**c**) STM is severely impaired in 20-d-old $Atg7^{-/-}$ ($Atg7^{d14}/Atg7^{d77}$) flies and 20-d-old $Atg7^{-/+}$ heterozygous ($Atg7^{d14}/CG5335^{d30}$) when compared to age-matched wild-type flies ($n = 8$–9 independent experiments, $F = 18.02$, one-way ANOVA with Bonferroni correction). (**d**) Similarly, STM was markedly decreased in 20-d-old $Atg8a^{-/-}$ ($Atg8a^{EP362}/Atg8a^{EP362}$) mutant female flies compared with control flies ($Atg8a^{+/+}$) without any effect by spermidine feeding ($n = 8$–9 independent experiments, $F = 68.43$, one-way ANOVA with Bonferroni correction). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns indicates not significant, $P > 0.05$. Data are presented as mean ± s.e.m. The aversive olfactory avoidance and shock reactivity of different mutant are shown in **Supplementary Table 5**.

shown to protect from stress in both autophagy-dependent and autophagy-independent pathways[28].

To explore the possibility that transcriptional modulation might be involved in spermidine-mediated suppression of AMI, we performed next generation mRNA sequencing (RNA-seq) in duplicate on head extracts prepared from 30-d-old Spd^{5mM+} flies and compared them with extracts from 30-d-old Spd^{-} flies. RNA-seq, with its base pair–precise resolution allows quantitative global mapping of transcribed regions at superior levels of sensitivity and accuracy. Under our stringent conditions of analysis, only a few genes were found to be consistently either upregulated or downregulated in 30-d-old heads from spermidine-treated flies when compared with age-matched controls (data not shown).



However, we reasoned that if transcriptional reprogramming is causally involved in the protective effects of polyamines, then it might precede, or at least be concomitant with, its effects on memory. In another experiment, we analyzed the kinetics of polyamine decline and memory. A substantial decline of both polyamines (spermidine and putrescine), together with memory, was observed by 10 d of age, which only slightly decreased further by 15 d (**Supplementary Fig. 4**).

We then carried out RNA-seq on head extracts prepared from 3-d-old and 10-d-old Spd^{5mM+} flies and compared them with age-matched Spd^{-} flies. Sequenced reads were then aligned to the *Drosophila* genome with high stringency, allowing only one mismatch per read, and an average of 96% of aligned reads mapped to exons (**Supplementary Table 1**). Thereafter, the number of reads mapping to each gene was quantified and normalized for library size using DESeq[29]. Hierarchical clustering of the normalized reads revealed a high degree of consistency between the biological replicates, clearly showing that spermidine-treated samples clustered away from untreated samples (**Fig. 6a**). Using an established statistical method based on the negative binomial distribution of sequenced reads[29] (**Supplementary Fig. 5**), we then identified large global changes in gene expression induced by spermidine feeding, with 2,051 genes and 4,076 genes being modulated by a factor of 1.5-fold ($P < 0.05$) in the heads of 3-d-old and 10-d-old flies. respectively (**Fig. 6b** and **Supplementary Table 2**). Notably, 84% of the genes that were differentially regulated after 3 d were also altered at 10 d of age (**Fig. 6c**).

Figure 6 Spermidine feeding induces widespread transcriptional changes in fly heads during aging. (**a**) Dendrogram and heat map illustrating Euclidean distances between gene expression profiles from 3-d-old and 10-d-old fly heads with or without spermidine feeding. (**b**) Differential expression analysis examining the effect of spermidine feeding on gene expression in heads of 3-d-old and 10-d-old flies using DESeq. Red and blue dots indicate a significant up- and down-regulation, respectively (fold change > 1.5; adjusted $P < 0.05$). The numbers of differentially regulated genes is indicated. Dark blue lines represent a 1.5-fold change. (**c**) Venn diagram indicating the overlap in differentially expressed genes at 3 and 10 d of age under spermidine treatment. The raw sequence data for this figure are available in **Supplementary Table 3**.

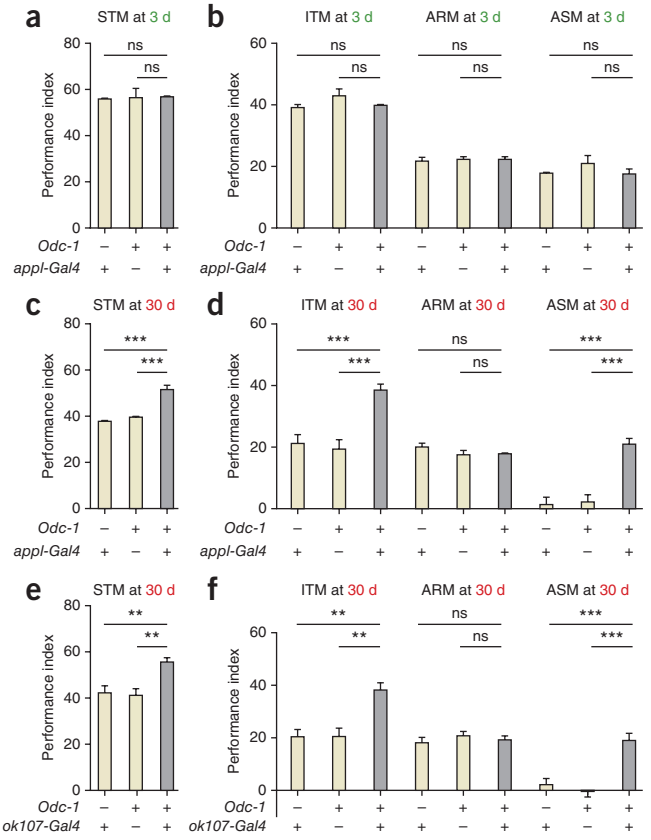


Figure 7 Brain-specific expression of Odc-1 is sufficient to suppress AMI. (**a**) Aversive associative memory performance in 3 min after training (STM) in 3-d-old female flies with pan-neuronal expression of *UAS–Odc-1* ($n = 7$ independent experiments, $F = 0.04$, one-way ANOVA with Bonferroni correction). (**b**) Aversive associative memory performance in 3 h after training (ITM), ARM and ASM of 3-d-old *appl*-driven *UAS–Odc-1* female flies compared with control flies ($n = 7$ independent experiments; $F = 1.95$ for ITM, $F = 0.10$ for ARM, $F = 1.05$ for ASM; one-way ANOVA with Bonferroni correction). (**c**) Pan-neuronal expression of the *UAS–Odc-1* in a wild-type background suppressed AMI in 30-d-old female flies ($n = 7$ independent experiments, $F = 21.35$, one-way-ANOVA with Bonferroni correction). (**d**) ITM, ARM and ASM of 30-d-old female flies with pan-neuronal expression of *UAS–Odc-1* compared with their genetic controls ($n = 7$–8 independent experiments; $F = 17.49$ for ITM, $F = 1.58$ for ARM, $F = 24.61$ for ASM; one-way ANOVA with Bonferroni correction). (**e**) Expressing Odc-1 in just the mushroom body was sufficient to protect from STM decline in 30-d-old flies ($n = 9$–12 independent experiments, $F = 9.25$, one-way ANOVA with Bonferroni correction). (**f**) ITM, ARM and ASM of 30-d-old flies expressing mushroom body–specific *Odc-1* compared with their genetic controls ($n = 8$–9 independent experiments; $F = 11.73$ for ITM, $F = 0.50$ for ARM, $F = 16.6$ for ASM; one-way ANOVA with Bonferroni correction). $^{**}P < 0.01$, $^{***}P < 0.001$; ns indicates not significant, $P > 0.05$. Data are presented as mean ± s.e.m.

To provide insights into the function of the spermidine-regulated brain transcriptome, we subjected all of the genes found to be either up- or downregulated by spermidine feeding to gene ontology enrichment analysis[30] (**Supplementary Table 3**). The genes that were modulated by spermidine feeding in both 3-d-old and 10-d-old flies showed strong enrichment for GO terms, such as response to starvation, response to oxidative stress, phagocytosis and cellular homeostasis (**Supplementary Table 3.3**). This is consistent with the idea that general protective effects are mediated by spermidine administration. In addition, we also found over 2,000 transcriptional changes that were specific for 10-d-old flies (**Supplementary Tables 2.1** and **2.2**). On one hand, the GO term 'aging' was considerably enriched in 10-d-old Spd^{5mM+} flies, indicating that age-protective gene functions are induced when polyamine levels are remain high during the onset of aging. On the other hand, transcriptional changes that are specific for neuronal genes appear to have a role in Spd^{5mM+} flies, with 'neuron differentiation' and 'neuron development' being the top two enriched GO terms (**Supplementary Table 3.2**). In addition, several genes (14 in total) with the GO annotation 'learning' were found to be specifically upregulated in 10-d-old Spd^{5mM+} flies when compared with untreated flies (**Supplementary Tables 2.2** and **3.2**). Notably, several of the learning-related genes (along with other genes) that were modulated by spermidine feeding were subsequently analyzed and validated by quantitative reverse-transcription real-time PCR (**Supplementary Fig. 6**). Thus, learning-associated processes operating downstream of odor information processing could be a part of the polyamine-mediated protection of memory, indicating that brain-specific manipulation of polyamine synthesis might be sufficient for protecting against AMI.

Mushroom body–specific Odc-1 expression protects from AMI

To determine whether nervous system–specific manipulations of polyamine levels are sufficient to protect against AMI, we genetically manipulated polyamine synthesis in specific brain regions. Ornithine decarboxylase-1 (Odc-1), which is highly conserved across evolution, is the rate-limiting enzyme for the *de novo* synthesis of polyamines (**Fig. 1a**); its activity is tightly regulated at all steps, starting from its initial synthesis continuing to its degradation[31]. We used the neuron-specific *appl-Gal4* driver to express *Odc-1* in the nervous system (*appl>Odc-1*) and found that *appl>Odc-1* flies were effectively protected from AMI in both the STM and ITM assays (**Fig. 7a–d**). In fact, *appl>Odc-1* flies showed almost identical test scores to aged flies in which AMI was suppressed by spermidine feeding (**Fig. 2c,d**). This finding suggests that promoting polyamine synthesis specifically in the nervous system is sufficient to suppress AMI.

Kenyon cells, which are neurons comprising the mushroom body of *Drosophila* brains, are known to be important for forming associative olfactory memories[15,32]. The re-expression of the memory gene *rutabaga* in Kenyon cells alone is sufficient to rescue the severe learning deficits of *rutabaga* mutant flies[33]. We expressed Odc-1 in mushroom body Kenyon cells (using *ok107-Gal4*) and found no effect on the memory scores (both STM and ITM) of young *ok107>Odc-1* flies (3 d old) when compared with age-matched controls (**Supplementary Fig. 7**). Notably, aged *ok107>Odc-1* flies (30 d old) exhibited considerably higher STM and ITM scores when compared with genetic controls (**Fig. 7e,f**). Thus, promoting polyamine synthesis in a neuron population representing only about 2% of the *Drosophila* brain (Kenyon cells) was sufficient to protect from AMI. This finding confirms once more that polyamine restoration does not execute its effects on AMI via systemic regulations or generally improved health of the organism.

DISCUSSION

Aging is a multi-facet process that entails a decline of cognitive functions such as learning and memory. The proportion of older adults in our population is expected to grow rapidly over the next two decades. It is therefore increasingly important to advance research efforts for elucidating the mechanisms associated with cognitive aging to develop effective interventions and preventative therapies. We sought to understand the fundamental mechanisms of AMI.

Polyamines (putrescine, spermidine and spermine; **Fig. 1a**) are among the substances that have been reported to decline with age. Putrescine shows an age-related decline in the CA1 region of hippocampus and the dentate gyrus region in rodents[34], and the levels of spermidine and spermine have been shown to decrease with increasing age in rats[11]. Notably, levels of spermidine and spermine in basal ganglia also decrease with age in humans, suggesting that these polyamines are involved in white matter changes during aging[12]. We found that the levels of all three polyamines (putrescine, spermidine and spermine) declined in the heads of aged flies relative to young flies (**Fig. 1b**,**c**). Although the decline of polyamines might be regarded as an established biochemical correlate of aging, the causal relationship to age-related deficits in cognitive functions has not been established. Simple dietary supplementation of spermidine allowed us to restore polyamine levels in the heads of aged flies (both spermidine and putrescine) to those seen in juveniles. This simple procedure was sufficient to effectively protect both short- and intermediate-term olfactory memory from age-induced decline.

The effects of spermidine to the protection of memories were specific in several regards. First, spermidine feeding had no effect on memory in young flies, either in terms of short or intermediate-term components, arguing against the possibilty that spermidine might function as a general memory enhancer (**Fig. 2a**,**b**). Second, ITM has two components, with aging strongly affecting ASM, but not ARM[1]. We found that spermidine feeding had only a negligible effect on ARM; instead, spermidine administration specifically prevented the age-related decline of ASM (**Fig. 2b**,**d**). Third, polyamine restoration appeared to specifically suppress AMIs, as olfactory avoidance scores of naive flies (**Table 1**) and locomotion activity (**Supplementary Fig. 3**) declined with age in both Spd^{+} flies and age-matched Spd^{-} flies. Fourth, in flies fed with spermidine for the last 10 d before testing (Spd^{-}, 1–20 + Spd^{5mM+}, 21–30), polyamine levels (spermidine and putrescine) increased (**Fig. 2f**,**g**), and memory was considerably restored (**Fig. 2e**), indicating that AMI suppression by spermidine administration is not a result of altered development. Furthermore, expressing Odc-1 in just Kenyon cells was sufficient to ameliorate AMI (**Fig. 7e**,**f**). These findings indicate that spermidine-mediated suppression of AMI is not executed via its effects on systemic regulations or a generally improved health of the organism, but rather result from an intrinsic regulation of a small fraction of neurons.

The formation of memory requires dynamic changes in the neurons, including synapse formation and synaptic plasticity, steered by regulated protein synthesis and equally important protein degradation. In fact, the execution of effective quality control over proteins appears to be important for neurons to maintain proper neuronal physiology and functioning[35]. The process of autophagy is an important route for removing misfolded proteins and damaged organelles from cells via lysosomal-mediated bulk degradation[20]. Spermidine has been shown to operate as a natural inducer of autophagy in various model systems, including yeast, *C. elegans*, *Drosophila* and mice[14,36]. In fact, we found that spermidine feeding alleviated the age-induced dysfunction of autophagic machinery in flies, thereby preventing the accumulation of poly-ubiquitinated proteins and ref(2)P (**Fig. 4**).

What might be the mechanism by which spermidine administration prevents the decline of autophagy in aged *Drosophila*? Spermidine treatment induces autophagy in enucleated cells within a few hours as effectively as the well-known autophagy inducer rapamycin[27], arguing for fast, post-transcriptional regulation. On the other hand, using RNA-seq, we found that positive regulators of autophagy (such as *Atg1a*)[37,38] were upregulated by spermidine feeding (**Supplementary Fig. 6** and **Supplementary Table 2**). This is consistent with the transcriptional induction of autophagy genes that has been observed in spermidine-treated yeast[14]. Thus, it appears likely that polyamine restoration is associated with an integrated autophagy response on both short-term post-transcriptional and long-term transcriptional levels.

Several different studies in model organisms have suggested that autophagy is a crucial regulator of the age-associated pathologies[6,20]. Loss of autophagy has been shown to trigger neurodegeneration in mice[39], and autophagy-mediated clearance of TDP-43–positive inclusions has been reported to rescue learning impairment associated with a mouse model of neurodegenerative diseases involving TDP-43 proteinopathies[36]. Similar to mice, reducing autophagy shortens lifespan and results in neurodegeneration in flies[9,10]. We found that the autophagic machinery was critical for amelioration of AMI, a *per se* nonpathological process, by spermidine feeding (**Fig. 4**).

The expression of Atg8a specifically in fly brains has been reported to prevent accumulation of ubiquitinated and oxidized proteins and to increase average adult lifespan[9]. Nevertheless, we found that overexpression of Atg8a with *appl-Gal4* ($atg8^{EP362}$ / *appl-Gal4*; **Supplementary Fig. 8**) had no effect on AMI, whereas spermidine feeding protected from AMI in this background. Thus, suppression of AMI seems to not be a byproduct of increased lifespan. Consistently, dietary restriction has been reported to increase the lifespan of *Drosophila*, but fails to protect cognitive functions with age[40]. In conclusion, spermidine feeding might trigger an integrated protection response, of which autophagy induction is one, but not the only, crucial component. In other words, several parallel pathways might be influenced by spermidine treatment that are, as a whole, responsible for suppression of AMI. Notably, both autophagy-dependent and autophagy-independent pathways were recently shown to be required for stress resistance by spermidine feeding in *Drosophila*[28].

In addition, we found a broad transcriptional reprogramming in fly heads under spermidine treatment, with numerous GO terms related to neuron development and differentiation being highly enriched (**Supplementary Table 3.2**). Several genes that have been reported to function in memory were among those upregulated in response to spermidine treatment (**Supplementary Tables 2** and **3**). The adenylate cyclase *rutabaga* is essential for ASM formation[41], and we found *rut* to be upregulated in Spd^{5mM+} flies (**Supplementary Table 2**). Furthermore, we found that a number of genes with conserved, central roles in memory and learning processes (*stau*, *mnb, Nmdar2, DopR2* and *Oamb*)[42–46] were transcriptionally upregulated in Spd^{5mM+} flies (**Supplementary Fig. 6** and **Supplementary Table 2**). In fact, it should be noted that the loss of spermine synthase is associated with changes in brain morphology and intellectual disability in humans (Synder-Robinson syndrome[47]). Similarly, among the most markedly downregulated genes following spermidine restoration was *Glaz*, the *Drosophila* homolog of Apolipoprotein D (ApoD; **Supplementary Table 2**). ApoD is one of the most upregulated proteins in the aged mammalian brain, and its increasing expression strongly correlates with aging-associated cognitive disorders[48].

With regard to the specific transcriptional modulation resulting from enhanced spermidine levels, spermidine treatment in yeast modulates the activity of histone acetyl-transferases (HATs, such as Iki3p and Sas3p). This suggests that spermidine might actually affect epigenetic regulation, allowing (among other regulation) the induction of autophagy-relevant transcripts[14]. Notably, altered histone acetylation has been associated with age-dependent memory impairment and neurodegeneration in mice[49,50].

Thus far, only a few substances have been found that protect from age-dependent memory decline, all of which are biologically

exogenous compounds that can result in adverse side effects. Spermidine, however, is an endogenous metabolite and has the potential to become a high-value candidate for the treatment of age-induced dementia in humans.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. The raw sequence data obtained by RNA-seq are available at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under series accession number GSE38998.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We would like to thank T. Neufeld (University of Minnesota), L. Luo (Stanford University) and the Bloomington Stock Center for fly stocks, and S. Gaumer (University Versailles) for ref(2)P antibody. We are also grateful to M.G. Holt and B. Gerber for critically reading the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft to S.J.S. (Exc257, FOR1363), as well as A6/SFB 958 and DynAge Focus Area (Freie Universität Berlin) to S.J.S., the European Union (FP7 Gencodys HEALTH-241995) to H.G.S. and A.S., a VIDI grant from the Netherlands Organization for Scientific Research (917-96-346) to A.S., the BMBF (BCCNII, grant number 01GQ1005A) to A.F., and the Emmy Noether Program to M.S. F.M. is grateful to the FWF for grants LIPOTOX, P23490, P24381 and I1000 (DACH). T.E. is recipient of an APART fellowship of the Austrian Academy of Sciences.

AUTHOR CONTRIBUTIONS

V.K.G., F.M. and S.J.S. designed the study. V.K.G., L.S., T.E., C.M., S.M., T.S.K., J.M.K., A.B., S.D., K.S.Y.L., S.S. and C.M. performed the experiments. V.K.G., L.S., T.E., T.S.K., J.M.K., A.B., K.S.Y.L., S.S., S.D., C.M., F.M. and S.J.S. analyzed the data. F.S., M.S., T.R.P., A.F. and H.G.S. provided protocols, reagents and advice. All of the authors commented on the manuscript. V.K.G., A.S., F.M. and S.J.S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

1. Tamura, T. *et al.* Aging specifically impairs amnesiac-dependent memory in *Drosophila*. *Neuron* **40**, 1003–1011 (2003).
2. Yamazaki, D. *et al.* The *Drosophila* DCO mutation suppresses age-related memory impairment without affecting lifespan. *Nat. Neurosci.* **10**, 478–484 (2007).
3. Tonoki, A. & Davis, R.L. Aging impairs intermediate-term behavioral memory by disrupting the dorsal paired medial neuron memory trace. *Proc. Natl. Acad. Sci. USA* **109**, 6319–6324 (2012).
4. Saitoe, M., Horiuchi, J., Tamura, T. & Ito, N. *Drosophila* as a novel animal model for studying the genetics of age-related memory impairment. *Rev. Neurosci.* **16**, 137–149 (2005).
5. Tully, T. & Quinn, W.G. Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J. Comp. Physiol. A* **157**, 263–277 (1985).
6. Bishop, N.A., Lu, T. & Yankner, B.A. Neural mechanisms of ageing and cognitive decline. *Nature* **464**, 529–535 (2010).
7. Meléndez, A. *et al.* Autophagy genes are essential for dauer development and life-span extension in *C. elegans*. *Science* **301**, 1387–1391 (2003).
8. Hansen, M. *et al.* A role for autophagy in the extension of lifespan by dietary restriction in *C. elegans*. *PLoS Genet.* **4**, e24 (2008).
9. Simonsen, A. *et al.* Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult *Drosophila*. *Autophagy* **4**, 176–184 (2008).
10. Juhász, G., Erdi, B., Sass, M. & Neufeld, T.P. Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in *Drosophila*. *Genes Dev.* **21**, 3061–3066 (2007).
11. Das, R. & Kanungo, M.S. Activity and modulation of ornithine decarboxylase and concentrations of polyamines in various tissues of rats as a function of age. *Exp. Gerontol.* **17**, 95–103 (1982).
12. Vivó, M. *et al.* Polyamines in the basal ganglia of human brain. Influence of aging and degenerative movement disorders. *Neurosci. Lett.* **304**, 107–111 (2001).
13. Minois, N., Carmona-Gutierrez, D. & Madeo, F. Polyamines in aging and disease. *Aging (Albany, NY)* **3**, 716–732 (2011).
14. Eisenberg, T. *et al.* Induction of autophagy by spermidine promotes longevity. *Nat. Cell Biol.* **11**, 1305–1314 (2009).
15. Heisenberg, M. Mushroom body memoir: from maps to models. *Nat. Rev. Neurosci.* **4**, 266–275 (2003).
16. Quinn, W.G. & Dudai, Y. Memory phases in *Drosophila*. *Nature* **262**, 576–577 (1976).
17. Liu, X. & Davis, R.L. The GABAergic anterior paired lateral neuron suppresses and is suppressed by olfactory learning. *Nat. Neurosci.* **12**, 53–59 (2009).
18. Hara, T. *et al.* Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* **441**, 885–889 (2006).
19. Komatsu, M. *et al.* Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell* **131**, 1149–1163 (2007).
20. Rubinsztein, D.C., Marino, G. & Kroemer, G. Autophagy and aging. *Cell* **146**, 682–695 (2011).
21. Klionsky, D.J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* **4**, 151–175 (2008).
22. Bartlett, B.J. *et al.* p62, Ref(2)P and ubiquitinated proteins are conserved markers of neuronal aging, aggregate formation and progressive autophagic defects. *Autophagy* **7**, 572–583 (2011).
23. Pankiv, S. *et al.* p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J. Biol. Chem.* **282**, 24131–24145 (2007).
24. Mizushima, N. *et al.* A protein conjugation system essential for autophagy. *Nature* **395**, 395–398 (1998).
25. Nakatogawa, H., Ichimura, Y. & Ohsumi, Y. Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. *Cell* **130**, 165–178 (2007).
26. Xie, Z., Nair, U. & Klionsky, D.J. Atg8 controls phagophore expansion during autophagosome formation. *Mol. Biol. Cell* **19**, 3290–3298 (2008).
27. Morselli, E. *et al.* Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome. *J. Cell Biol.* **192**, 615–629 (2011).
28. Minois, N. *et al.* Spermidine promotes stress resistance in *Drosophila melanogaster* through autophagy-dependent and -independent pathways. *Cell Death Dis.* **3**, e401 (2012).
29. Anders, S. & Huber, W. Differential expression analysis for sequence count data. *Genome Biol.* **11**, R106 (2010).
30. Huang, D.W., Sherman, B.T. & Lempicki, R.A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**, 44–57 (2009).
31. Pegg, A.E. Regulation of ornithine decarboxylase. *J. Biol. Chem.* **281**, 14529–14532 (2006).
32. Davis, R.L. Olfactory memory formation in *Drosophila*: from molecular to systems neuroscience. *Annu. Rev. Neurosci.* **28**, 275–302 (2005).
33. McGuire, S.E., Le, P.T., Osborn, A.J., Matsumoto, K. & Davis, R.L. Spatiotemporal rescue of memory dysfunction in *Drosophila*. *Science* **302**, 1765–1768 (2003).
34. Liu, P., Gupta, N., Jing, Y. & Zhang, H. Age-related changes in polyamines in memory-associated brain structures in rats. *Neuroscience* **155**, 789–796 (2008).
35. Lee, J.A. Neuronal autophagy: a housekeeper or a fighter in neuronal cell survival? *Exp. Neurobiol.* **21**, 1–8 (2012).
36. Wang, I.F. *et al.* Autophagy activators rescue and alleviate pathogenesis of a mouse model with proteinopathies of the TAR DNA-binding protein 43. *Proc. Natl. Acad. Sci. USA* **109**, 15024–15029 (2012).
37. Chang, Y.Y. & Neufeld, T.P. An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. *Mol. Biol. Cell* **20**, 2004–2014 (2009).
38. Demontis, F. & Perrimon, N. FOXO/4E-BP signaling in *Drosophila* muscles regulates organism-wide proteostasis during aging. *Cell* **143**, 813–825 (2010).
39. Komatsu, M. *et al.* Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* **441**, 880–884 (2006).
40. Burger, J.M., Buechel, S.D. & Kawecki, T.J. Dietary restriction affects lifespan but not cognitive aging in *Drosophila melanogaster*. *Aging Cell* **9**, 327–335 (2010).
41. Isabel, G., Pascual, A. & Preat, T. Exclusive consolidated memory phases in *Drosophila*. *Science* **304**, 1024–1027 (2004).
42. Dubnau, J. *et al.* The staufen/pumilio pathway is involved in *Drosophila* long-term memory. *Curr. Biol.* **13**, 286–296 (2003).
43. Heisenberg, M., Borst, A., Wagner, S. & Byers, D. *Drosophila* mushroom body mutants are deficient in olfactory learning. *J. Neurogenet.* **2**, 1–30 (1985).
44. Keleman, K. *et al.* Dopamine neurons modulate pheromone responses in *Drosophila* courtship learning. *Nature* **489**, 145–149 (2012).
45. Xia, S. *et al.* NMDA receptors mediate olfactory learning and memory in *Drosophila*. *Curr. Biol.* **15**, 603–615 (2005).
46. Zhou, C. *et al.* Molecular genetic analysis of sexual rejection: roles of octopamine and its receptor OAMB in *Drosophila* courtship conditioning. *J. Neurosci.* **32**, 14281–14287 (2012).
47. Schwartz, C.E., Wang, X., Stevenson, R.E. & Pegg, A.E. Spermine synthase deficiency resulting in X-linked intellectual disability (Snyder-Robinson syndrome). *Methods Mol. Biol.* **720**, 437–445 (2011).
48. Elliott, D.A., Weickert, C.S. & Garner, B. Apolipoproteins in the brain: implications for neurological and psychiatric disorders. *Clin. Lipidol.* **51**, 555–573 (2010).
49. Peleg, S. *et al.* Altered histone acetylation is associated with age-dependent memory impairment in mice. *Science* **328**, 753–756 (2010).
50. Fischer, A., Sananbenesi, F., Wang, X., Dobbin, M. & Tsai, L.H. Recovery of learning and memory is associated with chromatin remodeling. *Nature* **447**, 178–182 (2007).

ONLINE METHODS

Fly stocks and rearing conditions. All fly strains were reared under standard laboratory conditions (25 °C with 12-h:12-h light:dark cycle). Flies from an isogenized w^{1118} strain were used as the wild-type control. $Atg7^{d14}$, $Atg7^{d77}$ and $CG5335^{d30}$ flies were kindly provided by T. Neufeld[10] (University of Minnesota). These chromosomes were obtained from imprecise P-element using $Atg7^{EY10058}$ and $Atg7^{d06996}$ in the *Atg7* locus; $Atg7^{d14}$ and $Atg7^{d77}$ delete different exonic region of *Atg7*, thus trans-allellic combination of both creates an *Atg7* null mutant situation. $CG5335^{d30}$ only deletes an intronic region in the *Atg7* locus (not disturbing *Atg7* function) without affecting any exon or *Atg7* expression level, and serves as a background matched control here.

$Atg8a^{EP362}$ allele containing a P-element insertion in the *Atg8a* locus in the w^{1118} background was obtained from the Bloomington *Drosophila* Stock Center, and *appl-Gal4*, $appl^{d(AG1a)}$ was a kind gift from L. Luo (Stanford University).

UAS–Odc-1 was constructed based on an *Odc-1* cDNA clone (GH13851) obtained from the Berkeley *Drosophila* Genome Project. One fragment was obtained via PCR using 5′-CACCATGGCGGCCGCTACCCCTGAAAT-3′ and 5′- CTATATAGCTTGGAAGTACAGGGTCTTGGGG-3′ primers and then ligated into pENTR-dTOPO (Invitrogen) according to the manufacturer's instructions. Finally, pENTR-dTOPO-*Odc-1* was recombined with pUAST-attB-rfa (kindly provided by C. Klämbt, University of Münster) using the Gateway System (Invitrogen) to yield pUAST-attB-*Odc-1*. After confirmation by double-strand sequencing, transgenic flies were generated using the PhiC31 system with defined landing sites in the *Drosophila* genome was used[51,52]. For behavior experiments, transgenic *UAS–Odc-1* flies were out-crossed to our wild-type w^{1118} control for ten generations.

Spermidine (Sigma Aldrich) was prepared as a 2 M stock solution in sterile distilled water, aliquoted in single-use portions and stored at −20 °C. Fly food was prepared according to Bloomington media recipe (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/media-recipes.htm) with minor modification, which we refer to as normal food. After food had cooled down to 40 °C, spermidine was added to normal food to a final concentration of 1 mM or 5 mM, and called Spd^{1mM+} or Spd^{5mM+}, respectively. For all experiments, parental flies mated on either normal or Spd^{+} (Spd^{1mM+} or Spd^{5mM+}) food, and their progeny was allowed to develop on the respective food. Flies used in all experiments were F1 progeny. For aging, the flies were collected once a day, as a results specific age indicated will be age in days ± 24 h. For time-shift experiments (**Fig. 2e**,**f**), the F1 progeny was allowed to age on one kind of food (normal or Spd^{5mM+}) and then subsequently put on the other-kind of food (Spd^{5mM+} or normal) for specified time of time (for instance, days 1–20 on normal food and days 21–30 on Spd^{5mM+} food or vice-versa).

Extraction of polyamines for liquid chromatography–mass spectroscopy (LC-MS) measurements. Spd^{-} and Spd^{+} (both Spd^{1mM+} or Spd^{5mM+}) flies were quick frozen in liquid nitrogen and stored at −80 °C until further processing. Subsequently, heads were collected after vortexing by mechanical separation. Extraction of polyamines from fly heads was performed according to the freeze-thaw method described previously[14,53] with slight modifications. Briefly, about 20–30 mg of fly heads were semi-homogenized using Ultra-Turrax (IKA Laboratory Equipment) and polyamines extracted with 600 µl 5% trichloroacetic acid (vol/vol) by three repeated freeze-thaw cycles. After extraction, ammonium formate (0.4 M final concentration) was added to supernatants and stored at −80 °C until polyamine measurements were performed using LC-MS.

Polyamine measurements using LC-MS. Polyamines were determined according to the method described previously[54]. Fly head extracts were diluted 1:20 in water and isotopically labeled internal standards for spermidine ($^{13}C_4$-spermidine) and putrescine ($^{2}H_8$-putrescine) were added. $^{13}C_4$-spermidine was also used for internal standardization of spermine. Calibration standards were prepared by spiking extraction buffer with specific concentrations of spermidine, putrescine, spermine and internal standards. Polyamines were derivatized to carbamyl-derivatives[54] by adding 125 µl of 1 M carbonate buffer (pH 9), 800 µl of water and 20 µl of isobutyl chloroformate to 100 µl of sample or calibration standard containing internal standards. All analysis were carried out on an Ultimate 3000 System (Dionex, LCPackings) coupled to a Quantum TSQ Ultra AM (Thermo Scientific) using an electrospray ion source. The system was controlled by Xcalibur Software 2.0. The stationary phase was a Kinetex 2.6-µm C18 100-A 50-mm × 2.1-mm column (Phenomenex). 250 µl of the derivatized samples were loaded on to an online-SPE column (Strata X, Phenomenex) using eluent A (flow rate = 1.5 ml min^{-1}). After 2 min, online-SPE was switched to the analytical column and the polyamines were eluted and separated on the analytical column within 4 min using isocratic conditions (80% eluent B, flow rate 250 µl min^{-1}). Polyamines were detected in multiple reaction monitoring mode using following transitions: Spermidine (*m*/*z* 446 -> 298V), putrescine (*m*/*z* 289 -> 115), spermine (*m*/*z* 603 -> 155), $^{13}C_4$-spermidine (*m*/*z* 450 -> 302), $^{2}H_8$-putrescine (*m*/*z* 297 -> 123).

Behavioral assays. Standard single-cycle olfactory associative memory was performed as previously described[1,5] with minor modifications. Briefly, about 60–80 flies received one training session, during which they were exposed sequentially to one odor (conditioned stimulus, CS^{+}, 3-octanol or 4-methyl-cyclohexanol) paired with electric shock (unconditioned stimulus) and then to a second odor (CS^{-}, 4-methyl-cyclohexanol or 3-octanol) without the unconditioned stimulus for 60 s with a 30-s rest interval between each odor presentation. For STM (memory tested immediately after odor conditioning), the conditioned odor avoidance was tested immediately after training. During testing, flies were exposed simultaneously to the CS^{+} and CS^{-} in a T maze for 30 s. The flies were then trapped in either T maze arm, anesthetized and counted. From this distribution, a performance index was calculated as the number of flies avoiding the shocked odor minus the number avoiding the non-shocked odor divided by the total number of flies, multiplied by 100. A 50:50 distribution (no learning) yielded a performance index of zero and a 0:100 distribution away from the CS^{+} yielded a performance index of 100. A final performance index was calculated by the average of both reciprocal indices for the two odors.

For ITM, flies were trained as described above, but tested 3 h after training. As a component of ITM, ARM was separated from ASM by cold-amnestic treatment, during which the trained flies were anesthetized 90 s on ice at 30 min before testing. In the end, ASM was calculated by subtracting the performance index of ARM from that of ITM for each training session on the same day, respectively.

Experiments were double blinded with respect to genotype and treatment and differently aged groups of various genotypes were measured side by side in randomized order.

Immunohistochemistry, confocal imaging and quantification. Adult brains were dissected in HL3 on ice and immediately fixed in cold 4% paraformaldehyde (vol/vol) for 20 min at 20–30 °C. After fixation, the samples were then incubated in 1% PBT (phosphate-buffered saline (PBS) containing 1% Triton X-100, vol/vol) for 20 min and pre-incubated in 0.3% PBT (PBS containing 0.3% Triton X-100) with 10% normal goat serum (vol/vol) for 2 h at 20–30 °C. For primary antibody treatment, samples were incubated in 0.3% PBT containing 5% normal goat serum and the primary antibodies for 48 h at 20–30 °C. After primary antibody incubation, brains were washed in 0.3% PBT, four times for 30 min at 20–30 °C, and then overnight at 4 °C. All samples were then incubated in 0.3% PBT with 5% normal goat serum containing the secondary antibodies for 24 h at 20–30 °C. Brains were washed four times for 30 min at 20–30 °C, then overnight at 4 °C. Brains were finally mounted in Vectashield overnight before confocal scanning (Vector Laboratories). Antibodies were used at the following dilutions: rabbit antibody to ref(2)P (1:100)[55] and Cy3-conjugated antibody to rabbit (1:400, Jackson ImmunoResearch, 111-165-006).

Image stacks of specimens were imaged on a Leica TCS SP5 confocal microscope (Leica Microsystems) using a 20×, 0.7 NA oil objective for whole-brain imaging with voxel size of 361 × 361 × 200 nm. Images were quantified using ImageJ software (http://rsbweb.nih.gov/ij/). Briefly, confocal stacks were merged into a single plane by using the maximum projection function. Subsequently, region of central brain was manually selected (using the free-hand function) and fluorescence intensity arbitrary units were measured and normalized to the area of the central brain for each brain.

Ca^{2+} imaging using GCaMP3.0. For Ca^{2+} imaging, GCaMP3.0 (ref. 56) was homozygously expressed under direct control of the *mb247* enhancer. 3-d-old or 30-d-old female flies were briefly anesthetized on ice and immobilized in a small chamber under thin sticky tape. A small window was cut through the sticky tape and the cuticle of the head capsule using a splint of a razor blade. Trachea were carefully removed and the brain was covered with Ringer's solution[57] (which contains 5 mM HEPES, 130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$,

pH = 7.3). Optical imaging was performed using a fluorescence microscope (Zeiss Axioscope 2 FS) equipped with a xenon lamp (Lambda DG-4, Shutter Instrument), a 14 bit CCD camera (Coolsnap HQ, Photometrics), a 20× water-immersion objective and a GFP filter set. Image acquisition was controlled using the software Metafluor (Visitron Systems, Puchheim). Images were acquired at a frame rate of 5 Hz, an illumination time of 80 ms and an excitation wavelength of 488 nm. Odors (4-methyl-cyclohexanol or 3-octanol, diluted 1:100 or 1:150, respectively, in mineral oil, or pure mineral oil) were applied to the flies' antennae for 2 s each using a custom-built olfactometer at an air flow rate of 1 l min^{-1}. Four to five odor stimulations were applied to each individual fly. Acquired images were aligned using the ImageJ plugin TurboReg and a customized Java script. Fluorescence emission was determined within a region of interest covering the horizontal mushroom body lobes, and background fluorescence determined in a region of interest outside the labeled structure was subtracted. Changes in fluorescence emission were calculated as $\Delta F/F_0$ where F is the fluorescence measured at each time point and F_0 the baseline fluorescence as the average of five frames before odor onset. The $\Delta F/F_0$ values of the four to five stimulations were averaged for each fly. For creating false color–coded images, five frames covering the peak of the increase in fluorescence were averaged and the average of five, preceding odor onset was subtracted.

Transcriptional profiling and Gene Ontology (GO) analysis. Spd^- and Spd^{5mM+} flies of different ages (3 and 10 d) were flash frozen in liquid nitrogen and heads were collected after vortexing by mechanical separation. Total RNA was extracted from approximately 100 heads per sample using the RNeasy Lipid tissue Mini kit (Qiagen). Biological duplicates were performed for all conditions. mRNA was purified using the Oligotex kit (Quiagen) and was reverse transcribed into a cDNA using the Superscript III Reverse Transcriptase (Invitrogen). Second strand synthesis was performed using the *E. coli* and T4 DNA polymerases (New England Biolabs). DNA end repair was performed on 9–20 ng of double stranded cDNA followed by ligation of Illumina sequencing adaptors and size selection for 300 bp. Fragments were amplified linearly (14 PCR cycles), as validated by quantitative real-time PCR (qPCR), and sample quality was assessed using the 2100 Agilent Bioanalyzer. Cluster generation and sequencing-by-synthesis (36 bp) was performed using the Illumina Genome Analyzer IIx according to standard protocols of the manufacturer, with the exception of one sample (Spd^-, 3 day, rep 2), which was sequenced on an Illumina HiSeq 2000. The image files generated by the Genome Analyzer IIx/Illumina HiSeq 2000 were processed to extract DNA sequence data. The raw sequence data from this study are available at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under series accession number GSE38998. From the GAIIx, we obtained between 28 and 37 million reads, while the HiSeq produced 66 million reads (**Supplementary Table 2**). Sequenced reads were aligned to the *Drosophila* genome (NCBI build 5) using the Illumina Analysis Pipeline and the ELAND alignment software allowing one mismatch. Only tags that uniquely aligned to the genome were considered for further analysis and total alignment efficiency was between 70% and 82% (**Supplementary Table 1**), with the majority of unaligned reads mapping to repetitive sequences. The number of aligned reads mapping to gene coding sequences was counted using the python script htseq-count (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html) with gene annotations extracted from the file *Drosophila*_melanogaster.BDGP5.70.gtf, available at http://www.ensembl.org. In all samples, over 95% of aligned reads mapped to exons and over 92% mapped unambiguously to one exon (**Supplementary Table 1**). The unambiguously mapped reads (ranging from 19–22 million reads for samples sequenced with the Illimina GAIIx and 45 million reads for the sample sequenced with the Illumina HiSeq) were used for further analysis of differential gene expression with the DESeq software[29]. The number of reads per gene was normalized using DESeq and hierarchical clustering on Euclidean distances was performed using the normalized values, revealing a high degree of similarity between biological replicates (**Fig. 5a**). DESeq uses variability between biological replicates in all conditions to estimate a dispersion value for each gene, which is essential for determining the statistical significance of differential expression. Notably, we observed a high degree of correlation between gene expression level (normalized number of reads) and dispersion, with genes having a low number of reads exhibiting higher dispersion (**Fig. 5b**). We then used DESeq to identify genes that were differentially expressed in response to spermidine based on the negative binomial distribution (adjusted $P < 0.05$, fold change > 1.5; **Fig. 5b**). Overlap of differentially expressed genes (**Fig. 5b**) was determined and visualized as a Venn diagram using BioVenn[58], and GO analysis was performed using DAVID[30]. The full data set of differentially expressed transcripts and GO-terms are provided in **Supplementary Tables 2** and **3**, respectively.

qPCR. For validation of differential expression, qPCR was performed using the GO-TaqQPCR master mix (Promega) and the 7900HT Fast Real Time PCR system (Applied Biosystems) according to the manufacturer's instructions. qPCR was performed using dilutions of sequenced cDNA libraries from heads of 10-d-old spermidine-treated and untreated flies. Gapdh1 and 14-3-3 epsilon were used as reference genes for normalization and calculation of fold change differences between spermidine-treated and untreated samples. Subsequently, the Ct values of spermidine-treated samples were subtracted from that of control samples, resulting in $-\Delta C'T$ and the relative concentrations were calculated as $2^{-\Delta C'T}$. All primers were tested for amplification efficiency according to standard methods (**Supplementary Table 4**).

Protein extraction, SDS-PAGE and western analysis. To detect poly-ubiquitinated proteins (PUPs), female fly heads (at least 60 of different age groups) were homogenized in 1% Triton X-100 (PBS containing protease inhibitors) on ice, the homogenates were centrifuged (12,000*g*) for 10 min (4 °C) and the supernatant (Triton X-100–soluble fraction) was collected. The remaining protein pellets were extracted with 2% SDS buffer (SDS fraction). In the case of Atg8a, the fly heads of females were homogenized in a 2% SDS buffer containing protease inhibitors. Protein concentrations were determined using BCA Assay Kit (Thermo Fischer) and 10 µg of total protein per sample was loaded and resolved on 4–20% gradient gels (BioRad) or 12% gels for PUP and Atg8a, respectively, followed by electroblotting to nitrocellulose membranes (Millipore). Subsequently, blots were probed with monoclonal mouse antibody to tubulin (1:1,000, Sigma Aldrich), which served as loading control, together with polyclonal rabbit antibody to ubiquitin (1:1,000, Cell Signaling Technology) and polyclonal rabbit antibody to GABARAP (detects ATG8a, used 1:1,000, MBL) as previously described[9]. Immunoblots were scanned and intensity analysis was done using ImageJ software (using the gels and measurement function). The relative amounts of the PUP and Atg8a proteins from individual samples were quantified and corrected using antibody to tubulin as loading control. Statistical analysis was done in Microsoft Excel and GraphPad Prism software using one-way ANOVA and a Tukey post-test.

Climbing assay. Locomotor function of fruit flies was assessed using the climbing assay as previously reported[59] with some slight modifications. Briefly, flies were previously sex-separated under mild CO_2 anesthesia at days 1 and 28. After recovery for 48 h (that is 3-d-old and 30-d-old flies), 10 flies were placed in a plastic vial, given 30 s to climb up. Each trial was video captured, and at the end of each trial the number of flies that climbed up to a vertical distance of 7 cm or above was recorded. Each trial was performed six times.

Statistics. Data were analyzed with Prism (GraphPad Software). No statistical methods were used to pre-determine sample sizes, but our sample sizes are similar to those reported in previous publications[1,3,9,14,60]. For the behavioral studies and polyamine estimation, the data were collected with the investigator blind to the genotypes, treatment and age of genotypes. There was no blinding in the other experiments. The data were collected and processed side by side in randomized order for all experiments. Data distribution was assumed to be normal but this was not formally tested. For comparison of more than two groups one-way ANOVA was used with either Bonferroni correction (except for **Fig. 4a**,**b** for which one-way ANOVA with Tukey correction was used. To compare two groups, non-parametric Mann-Whitney *U* tests were used (in case of **Fig. 3**).

51. Bateman, J.R., Lee, A.M. & Wu, C.T. Site-specific transformation of *Drosophila* via phiC31 integrase-mediated cassette exchange. *Genetics* **173**, 769–777 (2006).
52. Groth, A.C., Fish, M., Nusse, R. & Calos, M.P. Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31. *Genetics* **166**, 1775–1782 (2004).

doi:10.1038/nn.3512

53. Minocha, R., Shortle, W.C., Long, S.L. & Minocha, S.C. A rapid and reliable procedure for extraction of cellular polyamines and inorganic ions from plant tissues. *J. Plant Growth Regul.* **13**, 187–193 (1994).
54. Byun, J.A. *et al.* Analysis of polyamines as carbamoyl derivatives in urine and serum by liquid chromatography-tandem mass spectrometry. *Biomed. Chromatogr.* **22**, 73–80 (2008).
55. Nezis, I.P. *et al.* Ref(2)P, the *Drosophila melanogaster* homologue of mammalian p62, is required for the formation of protein aggregates in adult brain. *J. Cell Biol.* **180**, 1065–1071 (2008).
56. Tian, L. *et al.* Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat. Methods* **6**, 875–881 (2009).
57. Estes, P.S. *et al.* Traffic of dynamin within individual *Drosophila* synaptic boutons relative to compartment-specific markers. *J. Neurosci.* **16**, 5443–5456 (1996).
58. Hulsen, T., de Vlieg, J. & Alkema, W. BioVenn: a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. *BMC Genomics* **9**, 488 (2008).
59. Feany, M.B. & Bender, W.W. A *Drosophila* model of Parkinson's disease. *Nature* **404**, 394–398 (2000).
60. Zarnack, K. *et al.* Direct competition between hnRNP C and U2AF65 protects the transcriptome from the exonization of Alu elements. *Cell* **152**, 453–466 (2013).