Neuronal induction of BNIP3-mediated mitophagy slows systemic aging in Drosophila

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The effects of aging on the brain are widespread and can have dramatic implications on the overall health of an organism. Mitochondrial dysfunction is a hallmark of brain aging, but the interplay among mitochondrial quality control, neuronal aging and organismal health is not well understood. Here, we show that aging leads to a decline in mitochondrial autophagy (mitophagy) in the Drosophila brain with a concomitant increase in mitochondrial content. We find that induction of BCL2-interacting protein 3 (BNIP3), a mitochondrial outer membrane protein, in the adult nervous system induces mitophagy and prevents the accumulation of dysfunctional mitochondria in the aged brain. Importantly, neuronal induction of BNIP3-mediated mitophagy increases organismal longevity and healthspan. Furthermore, BNIP3-mediated mitophagy in the nervous system improves muscle and intestinal homeostasis in aged flies, indicating cell nonautonomous effects. Our findings identify BNIP3 as a therapeutic target to counteract brain aging and prolong overall organismal health with age.

Brain function declines with age, manifesting as impairments in learning and memory, attention, decision-making speed, sensory perception, cognitive function and motor coordination1–3. In addition, aging is the major risk factor for the development of neurodegenerative diseases, including Alzheimer’s disease and Parkinson’s disease. As the burden of age-related neurodegenerative disorders increases at an exponential rate all over the world4, there is a considerable need for a better understanding of the mechanisms of brain aging and relationship to organismal health and longevity. Examination of the molecular and cellular changes that occur during brain aging indicates significant overlap with the hallmarks of aging in other organ systems5,6. Indeed, one of the most extensively studied hallmarks of brain aging is mitochondrial dysfunction, which has also been implicated in organismal aging and neurodegenerative diseases7–9. Neurons are particularly vulnerable to mitochondrial dysfunction, given that they are postmitotic differentiated cells relying almost exclusively on oxidative phosphorylation to sustain their high energy needs. Hence, identification of interventions that could prevent the accumulation of dysfunctional mitochondria in the aging brain may provide potential approaches to counteract age-related health decline.

Autophagy, a lysosomal degradation pathway that plays essential roles in development, tissue homeostasis and disease pathogenesis8, has emerged as an important modulator of tissue and organismal aging10. In this process, cellular materials (referred to as autophagic cargo) are sequestered within double-membrane vesicles known as autophagosomes and delivered to the lysosome for degradation11. Mitochondrial autophagy (mitophagy) is a type of cargo-specific autophagy that mediates the removal of dysfunctional mitochondria12–13. Studies in diverse species have reported an age-related decline in mitophagy or mitophagy-related gene expression14–16. Moreover, studies in invertebrate models have shown that interventions facilitating mitophagy can prolong lifespan and improve tissue homeostasis during aging16–18. Thus, there is an emerging understanding that mitophagy represents a key pathway for the preservation of mitochondrial function and, hence, cell and tissue health during aging19,20. Indeed, in recent years it has been shown that mitophagy can restrain neuroinflammation21 and represents an important therapeutic target to counteract Alzheimer’s disease pathogenesis22.

The molecular mechanisms of mitophagy involve coordination of autophagy induction with mitochondrial priming for autophagic recognition23–25. BNIP3 is a BCL2 family protein with an atypical BH3 domain that primarily localizes at the mitochondrial outer membrane. It has become apparent that BNIP3 can exert multiple cellular effects involving either direct or indirect interactions with mitochondria26. Initial studies reported that BNIP3 can act as a proapoptotic protein, inducing cell death and mitochondrial dysfunction27–29. However, it has also been shown that BNIP3 can act as a potent inducer of autophagy/mitophagy, without induction of cell death, in multiple cell types29,30–33. More specifically, BNIP3 has been shown to serve as an autophagy receptor for the binding of mitochondrial priming at ATG8/LC3 on the autophagosome via its N-terminal LC3-interacting region34,35. BNIP3-mediated mitophagy has been reported to exert prosurvival effects in certain pathological conditions36,37. However, the impact of BNIP3 induction during aging on mitochondrial homeostasis and organismal health is not known.

The role of BNIP3 in mitophagy induction prompted us to determine whether BNIP3 could modulate neuronal and/or organismal aging. First, we examined whether BNIP3 can improve mitochondrial homeostasis in the aging Drosophila brain. We show that in control flies there is a striking accumulation of dysfunctional mitochondria in the aged brain. Upregulation of BNIP3 in the adult nervous system is sufficient to induce mitophagy and prevent the accumulation of dysfunctional mitochondria in an autophagy-dependent manner. In assessment of organismal aging, neuronal BNIP3-mediated mitophagy is sufficient to prolong lifespan and improve several markers of healthspan in aged flies. Interestingly, we find that neuronal BNIP3 induction improves markers of both mitochondrial homeostasis and proteostasis in aged muscle in an autophagy-dependent manner. In addition, neuronal BNIP3...
Neuronal BNIP3 induction improves mitochondrial function. With many parallels to human physiology, the fruit fly *Drosophila* is an excellent model with which to study the role of mitochondrial homeostasis in aging and lifespan determination. Here, we set out to examine the effects of BNIP3 induction on mitochondria and, accordingly, mitochondrial homeostasis in the aging fly brain. To do so, we used Gene-Switch driver lines to express a UAS-hBNIP3-HA (hereafter BNIP3) transgene created by Zhang et al. Crosses in an experiment share genetic background and developmental conditions; the inducing agent (RU486) or vehicle (ethanol) is provided in food during adulthood to drive gene expression in target tissues in a time- and dose-dependent manner. To investigate the effects of BNIP3 induction in adult neurons, we used the pan-neuronal *Elav-Gene-Switch (elavGS)* driver line. RU486-dependent transgene expression in elavGS>UAS-BNIP3 flies was validated by immunofluorescence (IF) microscopy (Extended Data Fig. 1a,b) and immunoblot analysis (Extended Data Fig. 1c).

Immunofluorescence microscopy revealed expression of BNIP3 in proximity to mitochondria in the brain, with frequent points of colocalization (Extended Data Fig. 1d). Previous studies in *Drosophila* have reported age-related alterations in mitochondrial potential and function in muscle tissue, and in mitochondria isolated from whole animals. However, little is known about potential changes to mitochondrial homeostasis in the aging fly brain. Using IF microscopy, we began by investigating mitochondrial morphology and content in aging *Drosophila* brains. Compared to brains from young adult flies, aged brains showed a striking accumulation of mitochondrial content (Fig. 1a,b). Neuronal-specific induction of BNIP3 significantly reduced brain mitochondrial content to levels similar to those detected in young animals (Fig. 1a,b), supporting a role for BNIP3 in mitochondrial homeostasis. The human brain shrinks with age in older adults; we noted an analogous reduction in the number of nuclei observed in aged *Drosophila* optic lobes (Extended Data Fig. 2a,b) and following isotropic fractionation of whole brains (Extended Data Fig. 2c,d). Remarkably, neuron-specific upregulation of BNIP3 counteracted loss of brain nuclei detected in age-matched controls (Extended Data Fig. 2a–d).

Since BNIP3 has been proposed to promote apoptosis in certain cell types and conditions, we stained brains for cleaved (activated) caspase-3 to detect cells undergoing apoptosis. Although very few cleaved caspase-3-positive cells were detected per brain when imaging entire optic lobes, there was a significant increase in aged samples compared with young controls (Extended Data Fig. 2e,f). Importantly, neuronal BNIP3 induction was associated with fewer apoptotic cells in the aged brain as detected by cleaved caspase-3 staining (Extended Data Fig. 2e,f). Cumulatively, these results suggest that neuronal BNIP3 induction can improve mitochondrial homeostasis while also being neuroprotective.

To provide an additional marker of mitochondrial content, we examined mitochondrial DNA levels in the brains of young and aged flies. Consistent with an age-related accumulation of mitochondria, via IF microscopy we observed an increase in mtDNA in aged brains that was prevented by neuronal-specific induction of BNIP3 (Fig. 1c,d). We confirmed these changes to mtDNA levels in fly heads using quantitative PCR (qPCR) to detect cytochrome c oxidase subunit I (COI) encoded in mtDNA (Fig. 1e). To assess changes to mitochondrial function in aging brains, we examined mitochondrial membrane potential using the potentiometric dye tetramethylrhodamine, ethyl ester (TMRE). While brain mitochondria showed reduced TMRE intensity with age, neuronal induction of BNIP3 resulted in significantly greater mitochondrial membrane potential compared with age-matched controls (Fig. 1f,g). Furthermore, MitoSOX staining showed an increase in mitochondrial reactive oxygen species (ROS) in aged brains that was significantly reduced by neuronal BNIP3 induction (Fig. 1h,i). Importantly, RU486 administration and expression of a control transgene in elavGS>UAS-GFP flies had no effect on mitochondrial accumulation in aging brains (Extended Data Fig. 3a,b). Collectively, these data demonstrate that there is an increase in dysfunctional mitochondria in the aged fly brain that can be counteracted by upregulation of BNIP3 in adult neurons.

Neuronal BNIP3 induction promotes mitophagy. BNIP3 has been proposed to function as a mitophagy receptor. However, the relationships among BNIP3, mitophagy and mitochondrial function during neuronal aging are unknown. Endogenous LC3/ATG8 can be used as a marker of steady-state autophagy and for the visualization of autophagosomes. Studies in both worms and flies have reported an age-related increase in autophagosomes, reflective of a decline in autophagic flux. To explore the interplay between BNIP3 induction and autophagy, we examined the levels of endogenous ATG8a in aging brains using IF microscopy. Aged fly brains revealed an accumulation of ATG8a that was abrogated by neuronal BNIP3 induction.
neuronal BNIP3 induction (Extended Data Fig. 4a,b). To further evaluate changes to aging brain autophagy with BNIP3 induction, we used a reporter line expressing green fluorescent protein (GFP)-mCherry-ATG8a ubiquitously under the control of the endogenous ATG8a promoter50. Due to the pH-sensitive properties of GFP, ATG8a in acidic environments—autolysosomes—will display mCherry-only foci. When overexpressing BNIP3 in neurons, we detected significantly more red-only puncta compared with aged controls, indicating an increase in autolysosomes (Fig. 2a,b).

Next, to evaluate changes more specific to mitophagy, we used elavGS>UAS-BNIP3. Mitochondrial area (a.u.)

| RU486 | Young | Aged | Aged |
|-------|-------|------|------|
|       |       |      |      |

mtDNA (relative units)

| RU486 | Young | Aged | Aged |
|-------|-------|------|------|
|       |       |      |      |

TMRE intensity (a.u.)

| RU486 | Young | Aged | Aged |
|-------|-------|------|------|
|       |       |      |      |

MitoSox intensity (a.u.)

| RU486 | Young | Aged | Aged |
|-------|-------|------|------|
|       |       |      |      |

ATP5a

| RU486 | Young | Aged | Aged |
|-------|-------|------|------|
|       |       |      |      |

dsDNA

| RU486 | Young | Aged | Aged |
|-------|-------|------|------|
|       |       |      |      |

Nuclei

| RU486 | Young | Aged | Aged |
|-------|-------|------|------|
|       |       |      |      |

TMRE

| RU486 | Young | Aged | Aged |
|-------|-------|------|------|
|       |       |      |      |

MitoSox

| RU486 | Young | Aged | Aged |
|-------|-------|------|------|
|       |       |      |      |
the recently characterized mitophagy reporter line, mito-QC, that encodes a tandem GFP-mCherry fusion protein that is targeted to the outer mitochondrial membrane. With this tool, mitochondria degraded in the acidic microenvironment of lysosomes (mitolysosomes) will appear as mCherry-only puncta. In agreement with its reported role as a mitophagy receptor, upregulation of BNIP3 in neurons resulted in significantly more mitolysosomes compared with controls (Fig. 2c,d). Notably, neuronal induction of BNIP3 at midlife, for both 1 and 2 weeks (days 30–37 or 30–44), was sufficient to increase the number of detected mitolysosomes in aged brains (Extended Data Fig. 5a–d).

A key prediction of the mitophagy model is that BNIP3-mediated changes to mitochondrial homeostasis will depend on autophagy. Atg1 (autophagy-related 1, the Drosophila homolog of mammalian ULK1) is a Ser/Thr protein kinase essential in the initiation of autophagosome formation. To test whether the observed improvements to mitochondrial homeostasis associated with neuronal BNIP3 induction (Fig. 1) are dependent upon autophagy, we generated elavGS>UAS-Atg1-RNAi,UAS-BNIP3 flies. Gene induction was confirmed in brains of elavGS>UAS-Atg1-RNAi,UAS-BNIP3 flies following RU486 treatment (Extended Data Fig. 6a,b).Brains from flies with concomitant neuronal induction of Atg1-RNAi and BNIP3 showed age-related accumulation of mitochondria similar to age-matched controls (Fig. 2c,f). Furthermore, flies expressing both Atg1-RNAi and BNIP3 in neurons showed impaired mitochondrial membrane potential in aged brains (Fig. 2g,h), in contrast to observations made in flies with neuronal BNIP3 upregulation without Atg1-RNAi induction (Fig. 1g). These findings indicate an autophagy-dependent mechanism for BNIP3-induced changes to mitochondrial homeostasis in aging neurons.

**Neuronal BNIP3 induction improves healthspan and longevity.** Having observed that induction of BNIP3 in neurons improves mitochondrial homeostasis in aged brains, we decided to investigate the potential impact on organismal aging. Remarkably, adult neuronal BNIP3 induction resulted in flies with significantly longer lifespans compared with controls (Fig. 3a and Table 1). Interestingly, midlife neuronal BNIP3 induction was also sufficient to extend the maximum lifespan of flies (Extended Data Fig. 5e). Conversely, expression of BNIP3-RNAi ubiquitously or specifically in neurons in adult flies resulted in shortened lifespans compared with controls (Extended Data Fig. 8a–f). To confirm that lifespan extension was a result of BNIP3 induction, we induced mitophagy in neuronal mitochondria of aged brains (Fig. 2g,h), in contrast to controls following RU486 treatment (Extended Data Fig. 6a,b). To assess how aging health could be affected by neuronal BNIP3 induction, we tested several behavioral readouts. Throughout the course of their lifespan, RU486-treated elavGS>UAS-BNIP3 flies showed delayed reduction in locomotor activity associated with aging (Fig. 3b,c). Furthermore, these flies showed significant improvements in climbing endurance assays (Fig. 3d). No change was detected in climbing ability during aging in control flies expressing GFP in neurons (Extended Data Fig. 7c). Neuronal BNIP3 induction also conferred an increase in spontaneous daytime activity, with no detectable night-time restlessness in aged flies (Fig. 3e,f). Although some lifespan extension strategies, including dietary restriction, are associated with reduced reproductive fitness, we did not detect a change in fertility in flies with neuronal BNIP3 induction (Extended Data Fig. 7e). Overall, these data reveal that upregulation of BNIP3 in neurons prolongs not only lifespan but also several indicators of healthspan.

With changes to mitochondrial homeostasis in the brains of flies upregulating BNIP3 in neurons being dependent on Atg1 (Fig. 2e–h), we decided to test whether lifespan and healthspan improvements were also contingent on autophagy. Notably, concomitant induction of BNIP3 and Atg1-RNAi in neurons prevented the lifespan extension associated with BNIP3 induction alone (Fig. 3g). Likewise, RU486-treated elavGS>UAS-Atg1-RNAi,UAS-BNIP3 flies showed detectable improvement neither in climbing endurance assays (Fig. 3h) nor in spontaneous activity (Fig. 3i,j) compared with control flies. Hence, the ability of neuronal BNIP3 induction to prolong healthspan and extend lifespan is also dependent on autophagy.

**Neuronal induction of BNIP3 slows muscle aging.** Aging is a systemic process associated with the physiological decline of multiple organ systems. There is an emerging understanding that modulating components of neuronal aging can impact influence aging, we tested whether neuronal BNIP3 upregulation would affect feeding behavior. Using the Con-Ex feeding assay, we observed no alterations in food consumption and excretion upon neuronal BNIP3 induction (Extended Data Fig. 7d). Likewise, neuronal expression of GFP had no observable effect on feeding behavior (Extended Data Fig. 7b). To understand whether the lifespan benefits of neuronal BNIP3 expression could be repeated when targeting BNIP3 induction in other cell types, daughterless (da)GS, Act88FGS and 5966GS drivers were used to express BNIP3 ubiquitously, in muscles and in intestinal enterocytes, respectively. However, none of these other interventions increased fly lifespan (Extended Data Fig. 8a–c). Hence, we conclude that upregulation of BNIP3 specifically in neurons increases Drosophila longevity.

With changes to mitochondrial homeostasis in the brains of flies upregulating BNIP3 in neurons being dependent on Atg1 (Fig. 2e–h), we decided to test whether lifespan and healthspan improvements were also contingent on autophagy. Notably, neuronal induction of BNIP3 and Atg1-RNAi in neurons prevented the lifespan extension associated with BNIP3 induction alone (Fig. 3g). Likewise, RU486-treated elavGS>UAS-Atg1-RNAi,UAS-BNIP3 flies showed detectable improvement neither in climbing endurance assays (Fig. 3h) nor in spontaneous activity (Fig. 3i,j) compared with control flies. Hence, the ability of neuronal BNIP3 induction to prolong healthspan and extend lifespan is also dependent on autophagy.
**a** elavGS>GFP-mCherry-ATG8a,UAS-BNIP3 (aged)

**b**

| elavGS>GFP-mCherry-ATG8a,UAS-BNIP3 (aged) | elavGS>GFP-mCherry-ATG8a,UAS-BNIP3 (aged) |
|-------------------------------------------|-------------------------------------------|
| ![Graph 1](image1.png) | ![Graph 2](image2.png) |

**c** elavGS>UAS-mitoQC

**d**

| elavGS>UAS-mitoQC,UAS-BNIP3 | elavGS>UAS-mitoQC,UAS-BNIP3 |
|-------------------------------|-------------------------------|
| ![Graph 3](image3.png) | ![Graph 4](image4.png) |

**e** elavGS>UAS-Atg1-RNAi,UAS-BNIP3

**f**

| elavGS>UAS-Atg1-RNAi,UAS-BNIP3 | elavGS>UAS-Atg1-RNAi,UAS-BNIP3 |
|--------------------------------|--------------------------------|
| ![Graph 5](image5.png) | ![Graph 6](image6.png) |

**g** elavGS>UAS-Atg1-RNAi,UAS-BNIP3

**h**

| elavGS>UAS-ATG1-RNAi,UAS-BNIP3 | elavGS>UAS-ATG1-RNAi,UAS-BNIP3 |
|--------------------------------|--------------------------------|
| ![Graph 7](image7.png) | ![Graph 8](image8.png) |
impaired mitophagy and the accumulation of dysfunctional mitochondria toward an elongated mitochondrial morphology linked to Drosophila aging. Previous work has revealed a midlife shift in induction of mitophagy in aged neurons could impact hallmarks of muscle systemic aging. Hence, we set out to explore whether facilitation of mitophagy in aged neurons could impact hallmarks of muscle aging. Previous work has revealed a midlife shift in Drosophila flight muscle toward an elongated mitochondrial morphology linked to impaired mitophagy and the accumulation of dysfunctional mitochondria. Remarkably, IF microscopy revealed that neuronal induction of BNIP3 resulted in smaller mitochondria in aged flight muscles (Fig. 4a,b). Induction of GFP in neurons, as a control, had no effect on mitochondrial size in aged muscle (Extended Data Fig. 9a,b). Consistent with a decrease in mitochondrial content, neuronal induction of BNIP3 reduced the amount of mtDNA detected in aged muscle (Fig. 4c,d). There is an emerging understanding that mitochondrial fission is a prerequisite for mitophagy. Interestingly, we observed that neuronal BNIP3 induction is linked to increased lifespan and healthspan in Drosophila (Fig. 3a). This effect was observed in flies with or without RU486-mediated transgene induction from day 5 onward (Fig. 3b). Moreover, RU486 induction of BNIP3 resulted in reduced spontaneous physical activity and increased climbing endurance (Fig. 3c,d). These findings suggest that mitophagy plays a crucial role in maintaining mitochondrial health and contributing to overall health and longevity in Drosophila.
to increased expression of Drp1, a Dynamin-related protein that promotes mitochondrial fission, in the thorax of middle-aged flies (Extended Data Fig. 10a). Next, we used TMRE to understand how changes in mitochondrial morphology and content in aged muscle relate to mitochondrial function. Importantly, neuronal BNIP3 upregulation significantly improved mitochondrial membrane potential in aged muscle (Fig. 4e,f).

Another major cellular hallmark of aging is the loss of protein homeostasis (proteostasis). Drosophila flight muscle accumulates aggregates of ubiquitinated proteins during aging, consistent with a decline in proteostasis. Here, we observed that neuronal BNIP3 induction significantly reduced age-associated protein aggregates in flight muscle (Fig. 4g,h). Together, these data reveal that BNIP3 induction in neurons results in cell nonautonomous changes to aging muscles in flies.

**Neuronal BNIP3 induction improves intestinal homeostasis.**

Intestinal homeostasis is essential in maintaining organismal health and longevity. In Drosophila, intestinal aging is associated with altered ISC behavior, microbial dysbiosis and loss of barrier function. Aging, the number of mitotic cells in the Drosophila midgut increases due to ISC hyperproliferation and misdifferentiation. This age-induced hyperplasia can be assayed by scoring phosphorylated histone H3 (pH3) in the intestine. To examine the impact of neuronal BNIP3 upregulation on intestinal homeostasis, we examined the numbers of pH3+ cells in young and aged intestines. Remarkably, we found that induction of BNIP3 in neurons significantly reduced pH3 counts in the posterior midgut compared with control flies (Fig. 5a,b).

Little is known about changes to enterocyte mitochondrial dynamics as organisms age. Using IF microscopy, we found that mitochondrial content significantly increased in enterocytes of the posterior midgut of aged flies compared with young controls (Fig. 5c,d). Interestingly, neuronal induction of BNIP3 resulted in reduced mitochondrial content in aged intestinal enterocytes compared with controls (Fig. 5c,d). flies expressing control GFP in neurons showed no change in enterocyte mitochondrial content compared with that in age-matched guts (Extended Data Fig. 9c,d). Consistent with findings in the thorax, we observed that neuronal BNIP3 induction also led to increased Drp1 expression in the middle-aged fly intestine (Extended Data Fig. 10b). Intestinal barrier dysfunction has emerged as a conserved characteristic of aging that has been linked to systemic inflammation, organismal health decline and mortality. To determine whether neuronal BNIP3 induction can impact the intestinal barrier, we examined intestinal integrity during aging via the ‘Smurf’ assay. Remarkably, we observed a delay in the onset of intestinal barrier dysfunction in flies upregulating BNIP3 in neurons (Fig. 5e). To determine whether improved intestinal barrier function was linked to changes in gut bacteria, we performed qPCR with universal primers to bacterial 16S rRNA to characterize alterations in microbiota dynamics in response to neuronal BNIP3 expression. However, we did not detect a significant change in gut bacterial load in aged flies with neuronal BNIP3 induction compared with controls (Extended Data Fig. 10c). Together, these findings indicate that upregulation of neuronal BNIP3 can significantly delay markers of both muscle aging and intestinal aging.

### Table 1 | Related to Fig. 3. Neuronal-specific BNIP3 induction extends lifespan

| Exp.    | RU− | RU+ | Median survival increase (%) | RU− | RU+ | Maximum survival increase (%) | P value | RU− (n) | RU+ (n) |
|---------|-----|-----|-------------------------------|-----|-----|-------------------------------|---------|---------|---------|
| Exp. 1  | 45  | 49  | 8.9                          | 70  | 79  | 12.9                         | <0.0001 | 146     | 147     |
| Exp. 2  | 40  | 45  | 12.5                         | 53  | 59  | 11.3                         | <0.0001 | 149     | 145     |
| Exp. 3  | 44  | 52  | 18.2                         | 55  | 68  | 23.6                         | <0.0001 | 153     | 125     |
| Exp. 4  | 32  | 35  | 9.4                          | 53  | 58  | 9.4                          | <0.0001 | 291     | 270     |
| Exp. 5  | 35  | 37  | 5.7                          | 58  | 63  | 8.6                          | 0.0010  | 229     | 223     |
| Exp. 6  | 35  | 39  | 11.4                         | 77  | 77  | -                            | 0.0315  | 257     | 255     |
| Exp. 7  | 35  | 44  | 25.7                         | 65  | 81  | 24.6                         | <0.0001 | 253     | 253     |

Exp., experiment (independently conducted experiment).

Fig. 4 | Neuronal BNIP3 induction improves mitochondrial homeostasis and proteostasis in aged muscle. a. Immunostaining of indirect flight muscles from young (10-day-old) and aged (30-day-old) elavGS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward, showing mitochondrial morphology (green channel, anti-ATP5a), rhodamine phalloidin (RP, magenta channel) and nuclear DNA (blue channel, stained with DAPI). Scale bar, 10µm. Scale bar, 10µm. b. Quantification of mitochondrial size in muscle as shown in a. n = 8 biologically independent animals per condition. ***P < 0.0001; one-way ANOVA/Tukey’s multiple comparisons test. c. Immunostaining of indirect flight muscles from young (10-day-old) and aged (30-day-old) elavGS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward, showing mtDNA (green channel, anti-dsDNA) and rhodamine phalloidin (magenta channel). Scale bar, 10µm. d. Quantification of mtDNA in muscle as shown in c. n = 11 biologically independent animals per condition. ***P = 0.0009 (young versus aged RU); **P = 0.0003 (aged RU versus aged RU+); one-way ANOVA/Tukey’s multiple comparisons test. e. Staining of indirect flight muscles from young (10-day-old) and aged (30-day-old) elavGS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward, showing TMRE fluorescence. Scale bar, 10µm. f. Quantification of mitochondrial membrane potential measured by TMRE staining as shown in e. n = 6 young, n = 8 aged RU and n = 10 aged RU+ biologically independent animals, as indicated. *P = 0.0400, ***P = 0.0006; Kruskal–Wallis test/Dunn’s multiple comparisons test. g. Immunostaining of indirect flight muscles from young (10-day-old) and aged (30-day-old) elavGS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward, showing polyubiquitinated aggregates (green channel, anti-FK2) and rhodamine phalloidin (magenta channel). Scale bar, 10µm. h. Quantification of polyubiquitin (PU) aggregates in muscle as shown in g. n = 8 young, n = 9 aged RU and n = 9 aged RU+ biologically independent animals, as indicated. *P = 0.0204, **P = 0.0014; one-way ANOVA/Tukey’s multiple comparisons test. RU486 or vehicle was provided in media at a concentration of 5µg/ml in the indicated treatment groups. Data are presented as scatter plots overlaying mean values ± s.e.m.
Neuronal BNIP3 induction slows systemic aging via autophagy.

Neuronal upregulation of BNIP3 improved mitochondrial homeostasis in the brain in an autophagy-dependent manner. Here, we set out to determine whether the impact of neuronal BNIP3 induction on muscle and intestinal aging also depends on neuronal autophagy. Remarkably, Atg1-RNAi expression in neurons prevented BNIP3-associated reduction in mitochondrial size in the indirect flight muscle (Fig. 6a,b). Furthermore, mitochondria in muscles of flies with adult induction of Atg1-RNAi and BNIP3 in neurons showed significantly reduced membrane potential compared with young flies (Fig. 6c,d). Therefore, neuronal BNIP3 upregulation requires neuronal autophagy to delay the accumulation of dysfunctional mitochondria in aged muscle.

In a similar manner, we examined whether the impact of neuronal BNIP3 induction on intestinal homeostasis during aging requires autophagy. pH3 counts in the posterior midgut were significantly higher in both aged control flies and aged flies expressing Atg1-RNAi and BNIP3 in neurons compared with young controls.
In addition, we found that coexpression of Atg1-RNAi with BNIP3 in adult neurons prevented neuronal BNIP3-associated reduction in the accumulation of mitochondria in aged intestinal cells (Fig. 6g,h). Finally, we also found that the neuronal BNIP3-associated impact on intestinal barrier function during aging is autophagy dependent. Adult flies with induction of Atg1-RNAi and BNIP3 in neurons showed no difference in the number of Smurf flies compared with age-matched controls (Fig. 6i). Together, these data reveal that observed changes in cellular and physiological hallmarks of muscle and intestinal aging associated with BNIP3 induction in neurons are dependent on neuronal autophagy.

**Discussion**

Mitochondria produce ATP and are, therefore, critical for neuronal health. Mitochondrial dysfunction is one of the most well-studied cellular hallmarks of brain aging and age-onset neurodegenerative...
Decades of research in model organisms and clinical studies have revealed a decline in mitochondrial function in aged brain tissue. However, the underlying mechanisms that lead to a loss of mitochondrial activity with age are not yet understood. One potential explanation for an age-related decline in mitochondrial function would be a loss of mitochondrial content with age. However, at present, a clear understanding of how mitochondrial content changes during brain aging is missing. Using the fruit fly *Drosophila* as a model organism, we find that there is a striking increase in mitochondrial content in the aged brain. Consistent with previous studies, which have reported an age-related decline in mitochondrial respiratory function in whole flies and dissected muscle tissue, we find that mitochondria that accumulate in the aged brain show reduced mitochondrial membrane potential. Our findings were, in part, focused on a specific region of the fly brain (the optic lobe): it is beyond the scope of this study to document...
age-related changes in mitochondrial content and function in all brain regions. However, in future work, it would be interesting to examine whether there is regional or cell-type specificity in this regard. This could provide insight into the question of how neuronal mitophagy modulates systemic aging. Defects in mitophagy, a mitochondrial quality control mechanism enabling the degradation of damaged and superfluous mitochondria, have been implicated in a number of pathological contexts, including age-onset neurodegeneration. Here, we have observed that brain aging is linked to a decline in mitophagy in Drosophila. These findings lead us to conclude that a decline in mitochondrial quality control, rather than a loss of mitochondrial content, is a major factor underlying age-onset mitochondrial dysfunction in the fly brain. A logical extension of this idea is that targeting mitophagy to improve mitochondrial homeostasis in the aging brain may prove to be a viable strategy to forestall brain aging phenotypes. One potentially interesting avenue to explore would be the impact of BNIP3-mediated mitophagy on cognitive function in aged animals.

The major finding of this study is the identification of BNIP3 as a therapeutic target to counteract mitochondrial dysfunction in the aging brain and prolong healthy lifespan. BNIP3 has been reported to play roles in various cellular processes, including mitochondrial dysfunction, mitochondrial fragmentation, mitophagy and apoptosis. Hence, BNIP3 cannot be indiscriminately categorized as pro- or antiapoptotic in function. Furthermore, BNIP3 is expressed in various tissues and is regulated by several different molecules under a variety of conditions. In the present study, we find that upregulation of BNIP3 in adult neurons counteracts the accumulation of dysfunctional mitochondria in the aged brain, with no detectable induction of apoptosis in the aged brain. Interestingly, we observe that neuronal BNIP3 expression also counteracted age-related neuronal loss and brain caspase-3 activation. Hence, it would appear unlikely that a proapoptotic mechanism underlies the observed effects on mitochondrial content. Most importantly, we find that the ability of BNIP3 to prevent the accumulation of mitochondria is dependent upon the autophagy pathway. Moreover, BNIP3-mediated improvements in organismal healthspan also require autophagy. The simplest interpretation of these findings is that BNIP3 induces mitophagy to improve mitochondrial homeostasis in the aged brain, delaying organismal health decline and mortality. Interestingly, we find that neuronal upregulation of BNIP3 in middle-aged flies is sufficient to facilitate mitophagy and promote longevity. This could prove important when considering the development of interventions based upon our findings. At the same time, however, we note that the prolongevity benefits of BNIP3 upregulation may be specific to neuronal manipulations. Indeed, we failed to detect lifespan extension upon BNIP3 upregulation using ubiquitous, muscle-specific or intestine-specific manipulations. It is interesting to speculate that BNIP3 activation in non-neuronal cells may compromise tissue and organ health via proapoptotic mechanisms. This may be important to consider in the context of studies implicating BNIP3 in cardiomyocyte cell death and mitophagy in regulating tumor survival in certain conditions.

It is intriguing that neuronal BNIP3 upregulation produced alterations in cellular and physiological markers of aging in aged muscle and intestine. More specifically, neuronal BNIP3 was able to improve both mitochondrial homeostasis and proteostasis in aging muscle. In addition, neuronal BNIP3 was able to delay markers of intestinal stem cell aging and mitochondrial accumulation in the aged intestine. Finally, neuronal BNIP3 upregulation delayed the onset of intestinal barrier dysfunction in aged flies. We interpret these findings to support a model in which healthy mitochondrial function in the aging brain is essential to the maintenance of muscle and intestinal health. One potential future research direction would be to uncover the possible role of interorgan signaling in mediating these effects.

**Methods**

**Fly stocks.** The fly strain UAS-hBNIP3-HA was kindly provided by Z. Zhang (Central South University, Changsha, Hunan, China); Elav–Gene-Switch (ElavGS) was provided by H. Keshishian (Yale University, New Haven, CT, USA); daughterless–Gene-Switch (dauGS) was provided by H. Tri colore (Université Paris Diderot–Paris 7, Paris, France); 5966–Gene-Switch (5966GS) was provided by H. Jasper (Genentech, San Francisco, CA, USA); Actin88F–Gene-Switch (Act88FGS) was provided by F. Demontis (St. Jude Children’s Research Hospital, Memphis, TN, USA); GFP-mCherry-ATG8a was provided by E. Baehrlecke (University of Massachusetts Medical School, Worcester, MA, USA); and UAS-mito-QC was provided by A. J. Whitworth (University of Cambridge, UK). The UAS-Agt1-RNAi (16133) line was received from the Vienna Drosophila RNAi Center.

**Fly husbandry and lifespan analysis.** Flies were maintained in vials containing cornmeal medium (1% agar, 3% yeast, 1.9% sucrose, 3.8% dextrose, 9.1% cornmeal, 1.1% acid mix and 4.5% methylparaben, all concentrations given in w/v). Flies were collected under light nitrogen-induced anesthesia and housed at a density of 30 female flies per vial. All flies were kept in a humidified, temperature-controlled incubator with a 12/12-h dark/light cycle at 25 °C. RU486 was dissolved in ethanol and administered in media as indicated while preparing food. Flies were flipped to fresh vials every 2–3 days and scored for death.

**Immunostaining and image analysis.*** For brain and muscle immunostaining, flies were fixed in 0.5% formaldehyde in PBS for 20 min at room temperature. After fixation, hematoxylin and brains were dissected and fixed again for 5 min.
For gut immunostaining, intact adult guts were dissected and fixed for 30 min in 4% formaldehyde in PBS, dehydrated for 5 min in each of 50%, 75%, 87.5%, and 100% methanol and rehydrated for 5 min in each of 0%, 25%, and 12.5% methanol in 0.2% Triton X-100 in PBS (PBST). Samples were then rinsed three times for 10 min with PBST and blocked in 3% bovine serum albumin (BSA) in PBST (PBST-BSA) for 1 h. Primary antibodies were diluted in PBST-BSA and incubated overnight at 4°C. Primary antibodies used were: mouse-anti-ATPS5a 1:250 (no. 15H4AC, abcam), rabbit-anti-HA 1:250 (no. 3724, Cell Signaling), rabbit-anti-hBNIP1 1:400 (no. D7U1T, Cell Signaling), mouse-anti-FK2 1:250 (no. BML-PW8810-0500, ENZO), rabbit-anti-atg8a 1:250 (ref. 13), mouse-anti-dsDNA 1:250 (no. ab27156, abcam), rabbit-anti-cleaved caspase-3 1:400 (no. D175, Cell Signaling). Triplicate groups (10 flies) was then tallied and quantified.

Consumption–excretion assays were performed on spontaneous activity per fly during a 12/12-h dark/light cycle. Triplicate groups (10 flies) was then tallied and quantified.

Intestinal integrity assays were performed as previously described66. ImageJ software.

Flies were anesthetized and dissected in cold Drosophila Schneider’s medium (DSM). Brain and hemi thoraces were incubated in TMRE staining solution (100 nM TMRE (no. T669, Thermo Fisher Scientific) in DSM) for 12 min at room temperature. After staining, samples were rinsed once in wash solution (25 nM TMRE in DSM) for 30 s before being mounted in wash solution. Images were acquired immediately using a Zeiss LSM780 confocal microscope and mitochondrial membrane potential was measured using Mito-QC and Mito-SM staining.

Images were taken with a Zeiss LSM780 or LSM880 confocal microscope and analyzed using ImageJ software to measure intensity, puncta, mitochondrial area and aggregate sizes. Images of mitochondria in gut were taken from the posterior midgut regions. The number of PH3+ cells was counted in whole midgut, defined from R1 to R5.

TMRE staining. Flies were anesthetized and dissected in cold Drosophila Schneider’s medium (DSM). Brain and hemithoraces were incubated in TMRE staining solution (100 nM TMRE (no. T669, Thermo Fisher Scientific) in DSM) for 12 min at room temperature. After staining, samples were rinsed once in wash solution (25 nM TMRE in DSM) for 30 s before being mounted in wash solution. Images were acquired immediately using a Zeiss LSM780 confocal microscope and mitochondrial membrane potential was measured using Mito-QC and Mito-SM staining.

GFP-mCherry-ATG8a-tandem and Mito-QC staining. Flies were anesthetized and dissected in cold DSM, and brains were mounted in DSM solution. Images were acquired immediately on a Zeiss LSM780 or LSM880 confocal microscope and autolysosomes or mitolysosomes (mCherry-only foci) were quantified using ImageJ software.

GFP-mCherry-ATG8a-tandem and Mito-QC staining. Flies were anesthetized and dissected in cold DSM, and brains were mounted in DSM solution. Images were acquired immediately on a Zeiss LSM780 or LSM880 confocal microscope and autolysosomes or mitolysosomes (mCherry-only foci) were quantified using ImageJ software.

Smurf assay. Intestinal integrity assays were performed as previously described66. Flies were aged to the indicated time points with standard RU– or RU+ media and autolysosomes or mitolysosomes (mCherry-only foci) were quantified using ImageJ software.

mtDNA measurement. Total cellular DNA from ten heads was prepared by homogenization in 10 mM Tris-HCl pH 8.6, 1 mM EDTA, 0.1% Triton X-100 and 10 μg/ml proteinase K. Following 60-min incubation at 37°C, proteinase K was heat-inactivated at 95°C for 5 min. mtDNA was quantified relative to nuclear DNA by the ratio of amplons of COI to amplons of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in quantitative real-time PCR. Primer sequences: COI, GAATTGAGACATCTTGGACC and GCACAACTAAATTTCCATCAATCC; GAPDH, GAGGAATAACGAGCTAAGGTGTC and AATGGGGTCCTGGCTGAAGTCC.

Climbing activity assay. In negative geotaxis assays, flies were gently tapped to the bottom of 10-cm vials. After 10 s, the number of flies that climbed above 5 cm was recorded. For forced climbing assays, 100 adult female flies from each treatment group were placed in 200-ml glass cylinders. The cylinders were tapped rapidly and the numbers of flies in the upper, middle and lower thirds were recorded. For checking internal (consumed) dye, flies were pre-homogenized in 150 μl of solution from the PowerSoil bead tube using a motor pestle. The homogenate was then transferred to the bead tube and the manufacturer’s protocol was followed. PCR was performed with PowerUp SYBR Green Master mix (Applied Biosystems) on a CFX96 Real Time PCR system. Cycling conditions were as follows: 95°C for 10 min, 95°C for 15 s then 60°C for 60 s, cycled 40 times. Equalized amplitudes of GAPDH were used as a normalization reference. Biolog CFX Mx Real Time PCR system was used to collect and analyze quantitative real-time PCR data. Primer sequences used were as follows:

GAPDH, GGCGTGAAGATGGGTTGACAG and TGAAGAGCGAAAAGGTAGGC; CAGTAGC; DRPI, ATTI1GTITCTGACGGATC and GAACCTTCTGGGGAGCTTC.

cqPCR for 16S ribosomal RNA gene. Genomic DNA was extracted from samples using PowerSoil DNA isolation kit (MoBio). All flies were surface sterilized, as previously described, before sample preparation. To ensure consistent homogenization, ten flies were pre-homogenized in 150 μl of solution from the PowerSoil bead tube using a motor pestle. The homogenate was then transferred to the bead tube and the manufacturer’s protocol was followed. PCR was performed with PowerUp SYBR Green Master Mix (no. A25777, Applied Biosystems) on a CFX96 Real Time PCR system. Cycling conditions were as follows: 95°C for 10 min, 95°C for 15 s then 60°C for 60 s, cycled 40 times. 16S gene expression values were normalized to the value of the loading control gene Act5C. Primer sequences were: Act5C, TGGGCAAGAGGATCA and ACCACCTCTTCTACCTTC.

Universal primers for the 16S ribosomal RNA gene were against variable regions 1 (V1F) and 2 (V2R), as previously reported.

Isotopic fractionation. Protocol was adapted from refs. 78, 80. Flies were fixed in 3.7% formaldehyde in PBS for 20 min at room temperature. After fixation, brains were dissected and fixed for 1.5 h at 4°C. Samples were then rinsed three times in 50% ethanol each with PBS at 4°C, blocked in 3% BSA and blocked in a secondary antibody anti-rat-488 1:500 (Invitrogen) for 48 h at 4°C on a nutator. Samples were washed three times for 10 min each with PBST at room temperature. Brains were then incubated with secondary goat-anti-rat-488 1:500 (Invitrogen) for 48 h at 4°C on a nutator. Following incubation with secondary antibodies, To-Pro-3 DNA 1:500 (no. T3605, Thermo Fisher Scientific) was added to samples for 30 min. Tissues were then washed three times for 10 min each with PBST at room temperature. Brains from the indicated treatments were pooled in groups of three and transferred to a solution containing 40 mM sodium citrate and 1% triton X-100. Samples were heated for 10 min at 60°C and centrifuged at 1,000g for 5 min before removal of the dissociation buffer. Brains were treated with 20 μl of 20% PBS. Homogenates were mixed thoroughly by pipette for 30 s to further promote dissociation of nuclei, before imaging at 20x using a Zeiss LSM 780 Imager M2 and Zen 2009 (Carl Zeiss).

Immunoblot assay. Samples were collected as indicated and lysates separated by SDS–polyacrylamide gel electrophoresis using standard protocols. Membranes were probed with antibodies or antisera against anti-actin (1:15,000 dilution, Sigma), Amersham ECL Prime Western Blotting Detection Reagent (GE Life Sciences) was used to visualize the presence of horseradish peroxidase, with the chemiluminescent signal recorded using Syngene Pxi Western Blot Imager. Image analysis was done via ImageJ software.

Statistics. GraphPad Prism 9 (GraphPad Software) was used to perform statistical analysis and graphical display of data. Significance is expressed as P values as determined by two-tailed, unpaired, parametric or nonparametric tests as indicated in figure legends. When comparing two groups, unpaired t-tests were used when data met criteria for parametric analysis, with Mann–Whitney tests used for nonparametric analysis. To compare more than two groups when parametric tests were appropriate, one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test was performed. To compare more than two groups sampled from a Gaussian distribution without assuming equal variances, Welch and Brown–Forsythe ANOVAAs were used. When comparing more than two groups when data did not meet requirements for parametric tests, Kruskal–Wallis tests were used in PBST (PBST) with Dunn’s multiple comparisons post hoc tests were used. When performing grouped analyses with multiple comparisons, two-way ANOVA with Sidák’s multiple comparisons test were performed. Bar graphs depict...
mean ± s.e.m. The number (n) of biological samples used in each experiment can be found in figure legends. Log-rank (Mantel–Cox) tests were used to compare survival curves. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications.4–6 Blinding was performed when possible, specifically when conducting microscopy for TMRE, MitoSox, ATG8a-tandem and mito-QC. Blinding was not always possible during experimental setup due to the need to carefully document fly genotypes when generating crosses, or to track groups assigned RU486 versus vehicle throughout lifespans. All experiments were conducted under the same conditions, and control and experimental samples were treated equally and in parallel to exclude bias. Additionally, all images were taken in the same location and depth in each tissue type and on the same day in a given experiment. Parents of experimental flies were randomly grouped into mating vials with ten virgin females to seven males. Upon eclosion, experimental flies were randomly assigned to mating bottles (ten vials per bottle) for 3 days. These bottles were then sorted into vials containing 30 mated females each before evenly distributing them, assigned randomly into treatment and control groups. No animals or data points were excluded from analyses. The difference between two groups was defined as statistically significant for the following P-values: *P < 0.05, **P < 0.01, ***P < 0.001 (and nonsignificant when P > 0.05).

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All data generated or analyzed during this study are included in the figures and text, with representative images accompanying quantified results where applicable, unless otherwise noted. Further information is available from the corresponding author upon reasonable request.

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Author contributions
E.T.S. and D.W. conceived the project. E.T.S. and J.-H.P. performed experiments. E.T.S. and J.-H.P. analyzed the data and prepared figures. E.T.S., J.-H.P. and D.W. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | RU486 induces BNIP3 expression in the brain of elavGS>UAS-BNIP3 flies. (a) Immunostaining of brains from 10-day-old elavGS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward, showing BNIP3 expression level (green channel, anti-HA) and nuclear DNA (blue channel, stained with DAPI). Scale bar is 50 µm. (b) Quantification of BNIP3 expression level in brain as shown in (a). n = 10 biologically independent animals per condition. ***p<0.0001; unpaired t test. Data are presented as scatter plots overlaying mean values +/- SEM. (c) Western blot detection of BNIP3 transgene induction in the heads of day 14 elavGS>UAS-BNIP3 flies with or without 9 days of RU486 treatment. n = 5 biological replicates per condition with 10 flies pooled per replicate. (d) Immunostaining of brains from young (10-day-old) elavGS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward, showing mitochondrial morphology (green channel, anti-ATP5a) and BNIP3 (red channel). Arrows and outlines indicate sites of colocalization. Scale bar is 3 µm. RU486 was provided in the media at a concentration of 5 µg ml⁻¹. Images are representative of 5 samples treated with RU and 6 samples provided vehicle.
Extended Data Fig. 2 | Neuronal BNIP3 induction prevents loss of neurons in aged brains. (a) TOPRO staining of nuclei in brains from young (10-day-old) and aged (30-day-old) elavGS\textsuperscript{>}UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward. Scale bar is 5 µm.
(b) Quantification of nuclei per 350 µm\textsuperscript{2} of optic lobe as shown in (a). n = 12 young, 12 aged RU- and 15 aged RU+ biologically independent animals per condition, as indicated. *p=0.0219, **p=0.0097; one-way ANOVA/Tukey’s multiple comparisons test.
(c) Immunostaining of brains from young (10-day-old) and aged (30-day-old) elavGS\textsuperscript{>}UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward, showing cleaved (activated) caspase-3 (green channel) and nuclear DNA (blue channel, stained with To-Pro-3). Scale bar is 5 µm.
(d) Quantification of cleaved caspase-3 in one optic lobe per fly as shown in (a). n = 8 young, 9 aged RU- and 10 aged RU+ biologically independent animals per condition, as indicated. *p=0.0140 (young vs. aged RU-), *p=0.0119 (aged RU- vs. aged RU+); one-way ANOVA/Tukey’s multiple comparisons test.
(e) Immunostaining of nuclei isolated via isotropic fractionation from brains of young (10-day-old) and aged (30-day-old) elavGS\textsuperscript{>}UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward, showing ELAV (green channel) and DNA (blue channel, stained with To-Pro-3). Scale bar is 50 µm.
(f) Quantification of neuronal (elav+) nuclei isolated from brains via isotropic fractionation as shown in (e). n = 6 biological replicates per condition with 3 pooled brains per replicate. *p=0.0263, **p=0.0098; one-way ANOVA/Tukey’s multiple comparisons test. Data are presented as scatter plots overlaying mean values +/- SEM.
Extended Data Fig. 3 | RU486 treatment in control flies has no effect on mitochondria homeostasis in aged brains. (a) Immunostaining of brains from young (10-day-old) and aged (30-day-old) elavGS>UAS-GFP flies with or without RU486-mediated transgene induction from day 5 onward, showing mitochondria morphology (red channel, anti-ATP5a) and nuclear DNA (blue channel, stained with DAPI). Scale bar is 5 µm. (b) Quantification of mitochondria area in brain as shown in (a). n = 8 biologically independent animals per condition. *p=0.0327 (both), non-significant (n.s.); Kruskal-Wallis test/Dunn’s multiple comparisons test. RU486 was provided in the media at a concentration of 5 µg ml⁻¹. Data are presented as scatter plots overlaying mean values +/- SEM.
Extended Data Fig. 4 | Neuronal specific BNIP3 induction reduces ATG8 levels in aged brains. (a) Immunostaining of brains from young (10-day-old) and aged (30-day-old) elavGS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward, showing ATG8a levels (red channel, anti-ATG8a). Scale bar is 5 µm. (b) Quantification of Atg8a levels in brain as shown in (a). n = 7 young, 6 aged RU- and 8 aged RU+ biologically independent animals, as indicated. *p=0.0446 (young vs. aged RU-), *p=0.0169 (aged RU- vs. aged RU+); Kruskal-Wallis test/Dunn’s multiple comparisons test. RU486 was provided in the media at a concentration of 5 µg/ml. Data are presented as scatter plots overlaying mean values +/− SEM.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 Midlife neuronal induction of BNIP3 induces mitophagy and extends lifespan. (a and c) mito-QC of brains from 37-day-old (a) and 44-day-old (c) flies. Genotypes analyzed were elavGS/UAS-mito-QC, UAS-lacZ; as a control, and elavGS/UAS-mito-QC, UAS-BNIP3. RU486-mediated transgenes were induced from day 30 to day 37 (a) or from day 30 to day 44 (c). Images shown of merged GFP and mCherry along with punctate mCherry-only foci (from merged images where GFP has been quenched; mitolysosomes). Scale bar is 5 µm. (b and d) Quantification of the number of mitolysosomes per 500 µm² brain area and average size (µm²) as shown in (a) and (c) at day 37 (b) and day 44 (d). (b) n = 7 control and 11 BNIP3+ biologically independent animals. *p = 0.0102, **p = 0.0089; unpaired t tests. (d) n = 8 biologically independent animals per condition. *p = 0.0115, **p = 0.0053; Mann-Whitney test (#), unpaired t tests (size). (e) Survival curves of elavGS/UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 30 onward. The shaded area indicates the duration of BNIP3 induction. ***p = 0.0010; log-rank test; n = 150 RU- and 147 RU+ biologically independent animals. RU486 was provided in the media at a concentration of 25 µg/ml. Data are presented as scatter plots overlaying mean values +/- SEM.
Extended Data Fig. 6 | RU486 induces BNIP3 expression in the brain of elavGS>UAS-ATG1-RNAi,UAS-BNIP3 flies. (a) Immunostaining of brains from 10-day-old elavGS>UAS-Atg1RNAi,UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward, showing BNIP3 expression level (green channel, anti-HA) and nuclear DNA (blue channel, stained with DAPI). Scale bar is 50 μm. (b) Quantification of BNIP3 expression level in brain as shown in (a). n = 20 RU- and 15 RU+ biologically independent animals. ***p=0.0008; unpaired t test. RU486 was provided in the media at a concentration of 5 μg/ml. Data are presented as scatter plots overlaying mean values +/- SEM.
Extended Data Fig. 7 | RU486 treatment in control flies has no effect on lifespan and healthspan, and neuronal-specific BNIP3 induction does not alter food consumption or fecundity. (a) Survival curve of elavGS>UAS-GFP flies with or without RU486-mediated transgene induction from day 5 onward. non-significant (n.s.); log-rank test. n = 228 RU- and 218 RU+ biologically independent animals. (b) Con-Ex feeding assay of 10-day-old elavGS>UAS-GFP flies with or without RU486-mediated transgene induction from day 5 onward. n = 6 vials of 10 flies per condition. non-significant (n.s.); unpaired t test. (c) Climbing index as a measure of endurance of 30-day-old elavGS>UAS-GFP flies with or without RU486-mediated transgene induction from day 5 onward. n = 8 biological replicates with 100 flies per replicate. non-significant (n.s.); unpaired t test. RU486 was provided in the media at a concentration of 5 µg ml⁻¹. Data are presented as mean values ± SEM. (d) Con-Ex feeding assay of 10-day-old elavGS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward. n = 6 vials of 10 flies per condition. non-significant (n.s.); unpaired t test. (e) Fecundity of 37-day-old elavGS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward. n = 6 vials of 30 RU- biologically independent animals and 5 vials of 30 RU+ biologically independent animals with values normalized per fly, as indicated. non-significant (n.s.); unpaired t test. RU486 was provided in the media at a concentration of 5 µg ml⁻¹. Data are presented as scatter plots overlaying mean values ± SEM unless otherwise indicated.
Extended Data Fig. 8 | Ubiquitous, gut- or muscle-specific BNIP3 induction shortens lifespan. (a) Survival curves of daGS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward. ***p=0.0006 (RU0 vs. RU5) and ***p<0.0001 (RU0 vs. each other RU dose), (n.s.) non-significant; log-rank test; n = 208 RU-, 200 RU5, 204 RU10, 198 RU25 and 201 RU50 biologically independent animals. (b) Survival curves of 5966GS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward. ***p<0.0001 (RU0 vs. each RU dose); log-rank test; n = 201 RU-, 194 RU5, 197 RU10, 199 RU25 and 204 RU50 biologically independent animals. (c) Survival curves of Act88FGS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward. ***p<0.0001 (RU0 vs. each RU dose); log-rank test; n = 239 RU-, 473 RU5, 233 RU10, 234 RU25 and 238 RU50 biologically independent animals. (d) Survival curves of daGS>UAS-BNIP3-RNAi flies with or without RU486-mediated transgene induction from day 5 onward. ***p=0.0006; log-rank test; n = 209 RU- and 233 RU+ biologically independent animals. (e) Survival curves of daGS>UAS-BNIP3-RNAi flies with or without RU486-mediated transgene induction from day 30 onward. ***p<0.001; log-rank test; n = 209 RU- and 202 RU+ biologically independent animals. (f) Survival curves of elavGS>UAS-BNIP3-RNAi flies with or without RU486-mediated transgene induction from day 5 onward. *p=0.0203; log-rank test; n = 165 RU- and 180 RU+ biologically independent animals.
Extended Data Fig. 9 | RU486 treatment in control flies has no effect on mitochondria homeostasis in aged muscle and gut. (a) Immunostaining of indirect flight muscles from young (10-day-old) and aged (30-day-old) elavGS>UAS-GFP flies with or without RU486-mediated transgene induction from day 5 onward, showing mitochondria morphology (red channel, anti-ATP5a), muscles (magenta channel, stained with phalloidin/F-Actin) and nuclear DNA (blue channel, stained with DAPI). Scale bar is 10 μm. (b) Quantification of mitochondria area in muscles as shown in (a). n = 6 biologically independent animals per condition. **p=0.0020, ***p=0.0001, non-significant (n.s.); one-way ANOVA/Tukey’s multiple comparisons test. (c) Immunostaining of guts from young (10-day-old) and aged (30-day-old) elavGS>UAS-GFP flies with or without RU486-mediated transgene induction from day 5 onward, showing mitochondria morphology (red channel, anti-ATP5a) and nuclear DNA (blue channel, stained with DAPI). Scale bar is 5 μm. (d) Quantification of mitochondria area in guts as shown in (c). n = 21 biologically independent replicates per condition. *p=0.0131, **p=0.0017; one-way ANOVA/Tukey’s multiple comparisons test. RU486 was provided in the media at a concentration of 5 μg ml⁻¹. Data are presented as scatter plots overlaying mean values ± SEM.
Extended Data Fig. 10 | Neuronal-specific BNIP3 upregulation induces midlife Drp1 expression in the thorax and gut but does not alter microbial dynamics in the gut. (a) qPCR analyses of Drp1 mRNA levels relative to GAPDH in the thorax on days 10, 30 and 44 in elavGS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward. n = 5 biological replicates with 5 dissected thoraxes pooled per replicate. **p=0.0023; two-way ANOVA/Šídák’s multiple comparisons test. (b) qPCR analyses of Drp1 mRNA levels relative to GAPDH in dissected guts on days 10, 30 and 44 in elavGS>UAS-BNIP3 flies with or without RU486-mediated transgene induction from day 5 onward. n = 5 biological replicates with 5 dissected guts pooled per replicate. ***p<0.0001; two-way ANOVA/Šídák’s multiple comparisons test. RU486 was provided in the media at a concentration of 5 µg ml⁻¹. Data are presented as scatter plots overlaying mean values +/− SEM. (c) Bacterial levels assayed by qPCR of 16S rRNA gene in surface sterilized, elavGS>UAS-BNIP3 flies with or without 5 µg ml⁻¹ RU486 treatment from day 5 post-eclosion onwards. n.s.; not significant, two-way ANOVA/Šídák’s multiple comparisons test; n = 3 replicates of ten flies pooled per condition. RU486 was provided in the media at a concentration of 5 µg ml⁻¹. Data are presented as scatter plots overlaying mean values +/− SEM.
Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐ A description of all covariates tested

☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

Give P values as exact values whenever suitable.

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Fluorescent image acquisition: Zeiss ZEN software (version 2.6, Carl Zeiss)
qRT-PCR: BioRad CFX manager version 3.1 (Bio-Rad Laboratories)
Target number and size: ImageJ (version 1.53c)

Data analysis

Excel 2016 and GraphPad Prism 9 were used for general data analysis.
Images were analyzed using ImageJ 1.53c.
Additional details are provided in the materials and methods section.

For manuscripts utilizing custom algorithms or software that are not central to the research but are yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. Github). See the Nature Research guidelines for submitting code & software for further information.

Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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- A description of any restrictions on data availability

All data generated or analyzed during this study are included in the figures and text with representative images accompanying quantified results where applicable unless otherwise noted. Further information is available from the corresponding author upon reasonable request.
Field-specific reporting

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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose these points even when the disclosure is negative.

Sample size
No statistical methods were used to pre-determine sample sizes. Sample sizes used in this study are similar to those reported in previous publications (Uigherait et al., 2014, Cell Reports; Rana et al., 2017, Nat Commun; Aparicio et al., 2019, Cell Reports).

Data exclusions
No data were excluded from the analyses.

Replication
All data presented were from independent biological replicates or independent experiments. All attempts at replication were successful. For lifespan assays, there were no attempts to replicate negative results, i.e. shortened lifespans upon BNP3 overexpression ubiquitously or in the muscle or gut. When lifespan extensions were observed, results were confirmed in at least one independent experiment. Our extended data table includes seven independent experiments, all of which found that neuronal specific BNP3 induction resulted in prolonged lifespan.

Randomization
Experimental and control flies were maintained under the same conditions and allocated to treatments/group randomly. Steps were taken to avoid batch effects.

Blinding
Blinding was not always possible during experimental setup given that investigators needed to carefully document the genotypes of flies when generating crosses or to track assigned groups being maintained on RU vs. vehicle throughout lifespan. Blinding was performed when possible, specifically when conducting microscopy for TMRE, MitoSox, ATG8a-tandem, and mitoQC. All experiments were conducted under the same conditions, and control and experimental samples were treated equally and in parallel to exclude bias. Additionally, all images were taken in the same location and depth in each tissue type.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a                             | n/a     |
| [x] Antibodies                  | [x] ChiP-seq |
| [x] Eukaryotic cell lines       | [ ] Flow cytometry |
| [x] Palaeontology and archaeology| [ ] MRI-based neuroimaging |
| [x] Animals and other organisms |         |
| [x] Human research participants |         |
| [x] Clinical data               |         |
| [x] Dual use research of concern|         |

Antibodies

Antibodies used
- mouse anti-ATPSa (15H4C4, abcam)
- rabbit anti-HA (3724, Cell Signaling)
- rabbit anti-bBNP3 (D7U1T Cell Signaling)
- mouse anti-FK2 (BML-PW8810-0500, ENZO)
- rabbit anti-atg8a (home made, Rana et al., 2017)
- mouse anti-dsDNA (ab27156, abcam)
- rabbit anti-PH3 (06-570, Millipore)
- rabbit anti-cleaved caspase-3 (Asp175, Cell Signaling)
- rat anti-elav (7F8A10, DSHB)
- rabbit or mouse AlexaFluor-488 (A-11001 or A-11008, Thermo Fisher Scientific)
- rabbit or mouse AlexaFluor-568 (A-11031 or A-11036, Thermo Fisher Scientific)
- phallolin AlexaFluor-568 (A12380, Thermo Fisher Scientific)

Validation
All antibodies were used in accordance to the manufacturer guidelines.

mouse anti-ATPSa (15H4C4, abcam) was validated by the manufacturer and in the following publications:
Bawa S et al., Elife [2020] 9:e52358.
Chen PL et al., Nat Commun [2020] 11(1):2592.
Aparicio R et al., Cell Rep (2019) 28(4):1029-1040.e5.

rabbit anti-HA [3724, Cell/Signaling] was validated by the manufacturer and the following publications:
Exposito-Alonso D et al., Elife (2020) 9:e57000.
Samposi MM et al., PLoS Genet (2020) 16(8):e1009003.

rabbit-anti-hNIP3 (07U1T, Cell Signaling) was validated by the manufacturer and the following publications:
Chooong C-J et al., Autophagy (2021) 17(10):2962–2974.
Labuschagne C et al., Cell Metabolism (2019) 30(4):720-734.e5.

mouse anti-FK2 (BML-PW8810-0500, ENZO) was validated by the manufacturer and the following publications:
Tamao K et al., Am J Pathol (2008) 173(6):1806-17.
Aparicio R et al., Cell Rep (2019) 28(4):1029-1040.e5.

rabbit anti-atg8Aa (home made, Rana et al., 2017) was validated by the following publications:
Rana A et al., Nat Commun (2017) 8(1):448.
Aparicio R et al., Cell Rep (2019) 28(4):1029-1040.e5.

mouse anti-dsDNA (ab27156, abcam) was validated by the manufacturer and the following publications:
Hu Q et al., Cell Rep (2020) 30:1235-1245.e4.
Aparicio R et al., Cell Rep (2019) 28(4):1029-1040.e5.

rabbit anti-PH3 (06-570, Millipore) was validated by the manufacturer and the following publications:
Choi NH et al., Aging Cell (2008) 7(3):318-34.
Szuperkó M et al., Elife (2018) 7:e33220.

rabbit anti-cleaved caspase-3 [Asp175, Cell Signaling] was validated by the manufacturer and the following publications:
Mihl M et al., PLOS Biology (2018) 16(7):e2005796
Liou NF et al., Nature Communications [2018] 9(1):2232

rat anti-elav (7E8A10, DSH8) was validated by the manufacturer and the following publications:
Raji J et al., PLOS One (2021) 16(5):e0250381
Peng JJ et al., Elife (2019) 8:e47372

rabbit or mouse AlexaFluor-488 (A-11001 or A-11008, Thermo Fisher Scientific) were validated by the manufacturer and the following publications:
Yun HY et al., PloS Biol (2019) 17(7):e3000967.
Foggetti A et al., Cell Rep (2019) 27(13):3725-3732.e5.

rabbit or mouse AlexaFluor-568 (A-11031 or A-11036, Thermo Fisher Scientific) were validated by the manufacturer and the following publications:
Schips TG et al., Nat Commun (2019) 10(1):76.
Namiki S et al., Elife (2018) 7:e34272.
Sapmaz A et al., Nat Commun (2019) 10(1):1454.
Yip SH etc., Cell Rep (2019) 26(7):1787-1799.e5.

phalloidin AlexaFluor-568 (A12380, Thermo Fisher Scientific) was validated by the manufacturer and the following publications:
Helfand BT et al., J Cell Biol (2002) 157:795-806.
Meary F et al., J Biol Chem (2007) 282:14226-14237

Animals and other organisms

Policy information about studies involving animals. ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Drosophila melanogaster. Female flies were used in experiments and maintained throughout their entire lifespan unless samples were collected at the days specified in corresponding figure legends, specifically on days 10, 30, or 51 post eclosion. Crosses generated and used in this study were elavG > UAS-BNIP3; elavG > GFP; mCherry-ATG8a, UAS-BNIP3; elavG > UAS-mito-QC, UAS-lacZ; elavG > UAS-mito-QC; UAS-BNIP3; elavG > UAS-Atg1RNAi; UAS-BNIP3; elavG > UAS-GFP; daG > UAS-BNIP3; ActBtl > GUS > UAS-BNIP3; daG > UAS-BNIP3-RNAi; and elavG > UAS-BNIP3-RNAi.

Wild animals
Our study did not involve any wild animals.

Field-collected samples
Our study did not involve any field-collected samples.

Ethics oversight
No ethical approval was required.

Note that full information on the approval of the study protocol must also be provided in the manuscript.