Targeted expression of the human uncoupling protein 2 (hUCP2) to adult neurons extends life span in the fly

Short Article

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Summary

The oxidative stress hypothesis of aging predicts that a reduction in the generation of mitochondrial reactive oxygen species (ROS) will decrease oxidative damage and extend life span. Increasing mitochondrial proton leak-dependent state 4 respiration by increasing mitochondrial uncoupling is an intervention postulated to decrease mitochondrial ROS production. When human UCP2 (hUCP2) is targeted to the mitochondria of adult fly neurons, we find an increase in state 4 respiration, a decrease in ROS production, a decrease in oxidative damage, heightened resistance to the free radical generator paraquat, and an extension in life span without compromising fertility or physical activity. Our results demonstrate that neuronal-specific expression of hUCP2 in adult flies decreases cellular oxidative damage and is sufficient to extend life span.

Introduction

Oxidative damage to macromolecules is thought to be one of the primary forces driving the process of aging and life span (Beckman and Ames, 1998; Sohal and Weindruch, 1996). A strong correlation between increased resistance to oxidative stress and longevity has been established in diverse organisms including *C. elegans, D. melanogaster*, and mammals (Feng et al., 2001; Finkel and Holbrook, 2000; Holzenberger et al., 2003; Lin et al., 1998; Wang et al., 2004). To date, most interventions designed to alleviate oxidative damage and extend life span have focused on either detoxification of reactive oxygen species (ROS) (Melov et al., 2000; Orr and Sohal, 1994; Parkes et al., 1998; Sun and Tower, 1999) or repair/replacement of damaged macromolecules (Chavous et al., 2001; Ruan et al., 2002). An alternative intervention is to decrease the generation of ROS at its primary source, the mitochondrial electron transport chain (ETC) where complexes I and III in association with the electronically unstable ubisemiquinone (QH•) can produce superoxide and other ROS (Brand, 2000). In *Drosophila* mitochondria, ROS generated from complex I due to reversed electron flow is sensitive to small decreases in membrane potential (Miwa et al., 2004). Increased proton leak during ETC has been postulated to decrease mitochondrial membrane potential creating a lower proton motive force leading to a reduction in QH• accumulation and ROS generation (Brand, 2000; Skulachev, 1998). These studies suggest potential targets for interventions aimed at reducing ROS production and extending life span.

Mitochondrial uncoupling proteins (UCPs) consisting of five family members are well-conserved mitochondrial carrier proteins found in all four eukaryotic kingdoms (Boss et al., 1997; Fleury et al., 1997; Gimeno et al., 1997; Heaton et al., 1978; Jarmuszkiewicz et al., 2000; Mao et al., 1999; Sanchis et al., 1998; Vidal-Puig et al., 1997). Located in the inner membrane of mitochondria, these carriers can allow protons to leak into the matrix thus disrupting the electrochemical gradient generated by the respiratory ETC. Direct consequences of increased mitochondrial uncoupling are a decrease in mitochondrial membrane potential, lowered ADP/O ratios, and an increase in respiration and metabolic rate. In addition to their shared uncoupling activities, each UCP appears to effect unique physiological outcomes, perhaps depending upon the tissue in which they are expressed. Mice deficient for UCP1, which is expressed exclusively in brown adipose tissue and is thermogenic, are cold sensitive (Enerback et al., 1997; Nicholls and Locke, 1984). The more broadly expressed UCP2 has been shown to negatively regulate insulin secretion in pancreatic β cells (Zhang et al., 2001), to play a role in parasitic infection (Arsenijevic et al., 2000), and to protect neurons against seizure and other neuronal insults (Bechmann et al., 2002; Diano et al., 2003). UCP3 with restricted expression in muscle and brown adipose tissue appears to be involved in weight control and fatty acid metabolism as targeted UCP3 expression in skeletal muscle results in hyperphagic but lean mice (Clapham et al., 2000). Moderate overexpression of both UCP2 and UCP3 under their endogenous transcriptional regulation also leads to mice with a lean phenotype (Horvath et al., 2003b). The most recently characterized family members UCP4 and UCP5 are primarily expressed in mammalian brain, but their functions remain unknown (Mao et al., 1999; Sanchis et al., 1998). Despite their diverse physiological functions, the involvement of UCPs in ROS metabolism has been demonstrated using gene knockout models and in vitro systems (Arsenijevic et al., 2000; Echtay et al., 2002; Kim-Han et al., 2001; Vidal-Puig et al., 2000). However, the notion that mitochondrial uncoupling may decrease cellular oxidative damage through a reduction in ROS production and extend life span has not been demonstrated

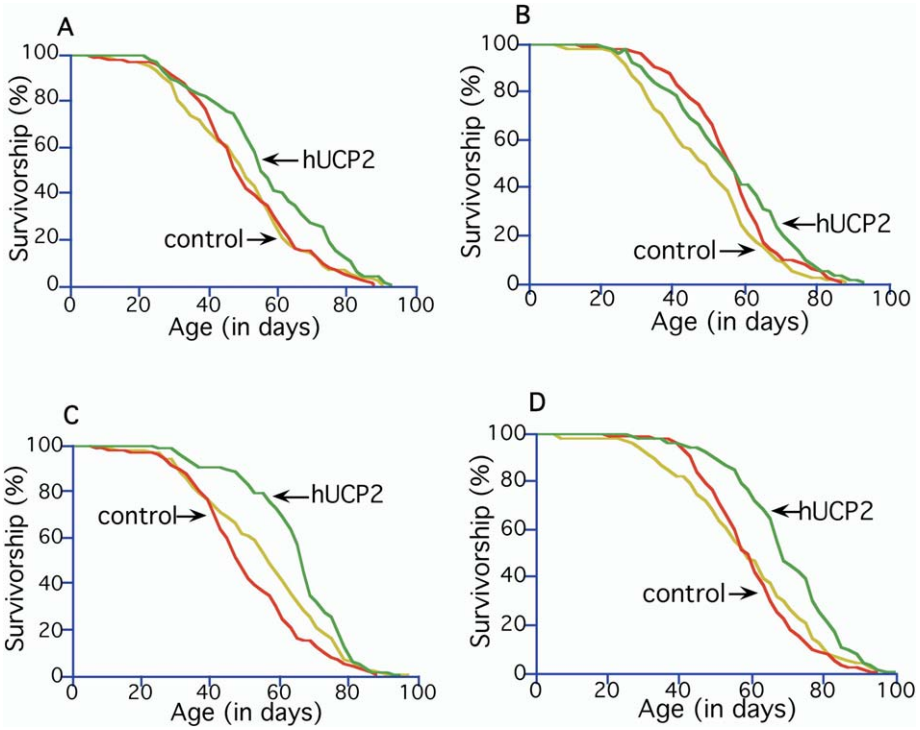

Figure 1. Adult neuronal-specific expression of hUCP2 extends life span in female and male flies

Survivorship curves for female (**A** and **C**) and male (**B** and **D**) flies are shown. Panels **A** and **B** represent one set of life span studies, Trial 1, and Panels **C** and **D** a second set of life span studies, Trial 2. Green lines are hUCP2-expressing *ELAV-GeneSwitch-Gal4/UAS-hUCP2* flies fed RU-486 containing food (200 μM); red lines are control *ELAV-GeneSwitch-Gal4/UAS-hUCP2* flies fed only diluent; and gold lines are *ELAV-GeneSwitch-Gal4/w*^{1118} flies fed RU-486 (200 μM). Trial 1: Panel **A** (females) median life spans are 55, 49, and 51 and panel **B** (male) median life spans are 57, 57, and 49; Trial 2: Panel **C** (females) median life spans are 67, 47, and 57 and panel **D** (male) median life spans are 69, 57, and 59 for hUCP2-expressing *ELAV-GeneSwitch-Gal4/UAS-hUCP2* flies fed RU-486, *ELAV-GeneSwitch-Gal4/UAS-hUCP2* flies fed diluent, and *ELAV-GeneSwitch-Gal4/w*^{1118} flies fed RU-486, respectively. Log-rank analysis shows hUCP2-expressing flies are longer lived than controls with p value < 0.0001 except for males in Trial 1 p = 0.238. Administration of RU-486 or diluent was begun on the day of eclosion and continued throughout adult life as described (Hwangbo et al., 2004). Two separate trials are shown (Trial 1 panels **A** and **B** and Trial 2 panels **C** and **D**, see Table S1). Survivorship curves with approximately 200 flies for each treatment were performed as previously described (Rogina et al., 2002).

(Brand, 2000). We set out to examine the impact of modulated mitochondrial uncoupling on the process of aging in a whole organism, *Drosophila melanogaster*.

Results and Discussion

Increased hUCP2 expression in the adult nervous system extends life span

In order to modulate mitochondrial uncoupling and assess its impact on life span, we created a transgenic fly, *UAS-hUCP2*, capable of expressing a known uncoupling protein, human UCP2, under an inducible system that permits spatial control of expression (Brand and Perrimon, 1993). We found that continuous ubiquitous expression of hUCP2 causes developmental lethality (Figure S1 available with this article online). In both flies and nematodes the nervous system has been implicated as a critical tissue in life span determination (Braeckman et al., 2001; Parkes et al., 1998), and in mammals hUCP2 protects neurons from toxic damage (Bechmann et al., 2002; Diano et al., 2003; Horvath et al., 2003a). We therefore made use of the RU-486-inducible GeneSwitch system to target hUCP2 expression to the mitochondria of neurons during adult life (Osterwalder et al., 2001). The neuronal mitochondrial-specific expression of the hUCP2 protein was confirmed by Western blot analysis and showed a 90% higher level of hUCP2 expression in females than males (Figure S2). Expression of hUCP2 in adult neurons extended life span on average 28% in females and 11% in males as compared to genetically identical flies not expressing hUCP2 (Figure 1 and Table S1). In addition, life span was extended when RU-486 was fed to *ELAV-GeneSwitch-Gal4/UAS-hUCP2* adult flies starting on day 10 (Table S1), whereas the induction of hUCP2 later than day 20 of adult life had little effect on life span (data not shown). These results suggest that an early initiation followed by continuous action of mitochondrial uncoupling is critical for life span extension.

We investigated the possibility that RU-486 itself may affect longevity in our fly strains by simultaneously examining *ELAV-GeneSwitch-Gal4* driver crossed to *w*^{1118} flies fed RU-486 or diluent alone. Except for the insertion of the *UAS-hUCP2* transgene, these control flies were genetically very similar to the experimental flies because the *UAS-hUCP2* strain had been backcrossed ten times to *w*^{1118}. Other studies using the same GeneSwitch system have failed to see any effect of RU-486 on life span and little effect on egg laying (Giannakou et al., 2004; Hwangbo et al., 2004). We observed that administration of RU-486 to flies of the *w*^{1118} background has an overall small impact on life span (Table S1). This discrepancy in the effect of RU-486 may be attributed to strain differences. Notwithstanding the effect of RU-486 in this genetic background, when compared to control *ELAV-GeneSwitch-Gal4/w*^{1118} flies fed with RU-486, flies expressing hUCP2 in adult neurons have a life span extension of 22% in females and 11% in males. Further evidence that the observed life span extension is caused by hUCP2 expression in adult neurons, and not the administration of RU-486 itself, is that hUCP2 expression in muscle, due to administration of RU-486 to flies carrying the inducible myosin heavy chain driver (*MHC-GeneSwitch-Gal4*) and *UAS-hUCP2*, has no positive effect on life span (data not shown) (Osterwalder et al., 2001). Although five independent transgenic lines were originally created, only the one line reported in detail produced significant levels of hUCP2 expression as determined by Western blot analysis (data not shown) and developmental lethality (Figure S1). The other four transgenic lines showed negligible expression of hUCP2, only a

5%–10% developmental lethality with ubiquitous GAL4 drivers and a 5%–10% increase in life span with RU-486 induction of the neuronal-specific GeneSwitch driver, suggesting that the level of hUCP2 expressed is important for life span extension. Concern that a genetic background effect could contribute to the life span extension seen with the hUCP2 transgenic flies is eliminated by the use of the GeneSwitch conditional system. Both the long-lived experimental and control *GeneSwitch-Gal4/UAS-hUCP2* flies are genetically identical flies, the only difference being the administration of RU-486 and the induction of hUCP2. In order to rule out the possibility that RU-486 induction of native genes at the site of insertion of the hUCP2 transgene might contribute to life span extension, the chromosomal region around the site of insertion was examined. The nearest predicted gene to the hUCP2 insertion site, within 2 Kb, is CG32835, a gene with no known function or homology to any other gene in the database. The nearest predicted gene on the other side of the hUCP2 insertion is over 30 Kb away. Examination of the RU-486-induced expression of CG32835 in *GeneSwitch-Gal4/UAS-hUCP2* flies by RT-PCR shows no expression in adult heads (Figure S3). We therefore conclude that the longevity phenotype described here is indeed the result of increased, targeted hUCP2 expression in the nervous system.

Long-lived hUCP2-expressing flies have an increase in mitochondrial uncoupling

To determine the mechanism by which neuronal-specific hUCP2 expression extends life span, we measured the physiology of mitochondria from heads of *hUCP2* long-lived flies since fly heads are greatly enriched for neuronal tissue. Mitochondria isolated from heads of hUCP2 expressing long-lived flies exhibited a 54% increase in oligomycin-insensitive respiration rates compared to control mitochondria (Figure 2A). Importantly, this increase in respiration under state 4 conditions is inhibited by the purine nucleotide GDP, further demonstrating the physiological characteristics of proton leak as the result of hUCP2 action (Figure 2A; Stuart et al., 2001a, 2001b). The increased respiration under state 4 conditions due to hUCP2 expression is associated with a decrease in the respiratory control ratio (RCR, 3.43 versus 1.48), another indicator of increased mitochondrial uncoupling (Figure 2B; Miwa et al., 2003). Mitochondria isolated from thoracic tissues from these same flies showed no significant increase in respiration in the presence of oligomycin (data not shown). This suggests that a systemic impact on life span extension may be achieved through an increase in mitochondrial uncoupling in neuronal tissue. Furthermore, administration of RU-486 to *MHC-GeneSwitch-Gal4/UAS-hUCP2* flies, while causing an increase in respiration rates under state 4 conditions in mitochondria from muscle (data not shown), does not result in life span extension. Taken together, our data demonstrate that an increase in mitochondrial uncoupling in neurons, but not muscle, is necessary and sufficient for life span extension.

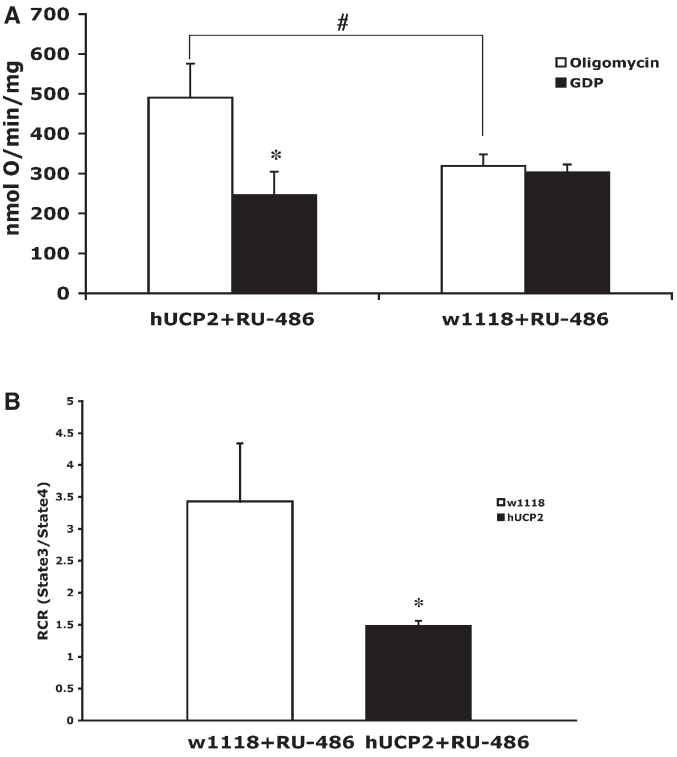

Figure 2. Neuronal expression of the hUCP2 in transgenic flies results in an increase in mitochondrial state 4 respiration rates and a lower respiratory control ratio (RCR)

A) Flies specifically expressing hUCP2 in neurons (*ELAV-GeneSwitch-Gal4/UAS-hUCP2*) have an increase in respiration under state 4 conditions that is GDP sensitive as compared to controls (*ELAV-GeneSwitch-Gal4/w*^{1118}). A 54% increase in oligomycin-insensitive state 4 respiration was measured in heads from female *ELAV-GeneSwitch-Gal4/UAS-hUCP2* and *ELAV-GeneSwitch-Gal4/w*^{1118} flies fed with RU-486 for 10 days (open bars) (Miwa et al., 2003) (n = 5, p = 0.059, Student's t test). The inhibitory effect of the purine nucleotide GDP (0.5 mM) specific to hUCP2-induced proton leak was measured (filled bars) in the presence of oligomycin (n = 5, *p = 0.019, Student's t test). All values are presented as mean ± SEM. A p value of 0.059 between the average respiration rate of hUCP2-expressing mitochondria and control mitochondria under state 4 conditions is due to the variation in the measurements of absolute oxygen consumption rates on different days. Comparison of RCRs (see below) allowing the elimination of inter-experimental variation demonstrates a decreased RCR in hUCP2-expressing mitochondria as compared to that of control mitochondria (p = 0.042).

B) Mitochondria isolated from heads of *ELAV-GeneSwitch-Gal4/UAS-hUCP2* (hUCP2+RU-486) and *ELAV-GeneSwitch-Gal4/w*^{1118} (*w*^{1118}+RU-486) flies fed RU-486 for 10 days since eclosion were assayed for their respiratory capacities under state 3 (with ADP) and state 4 (without ADP) conditions. The standard RCRs were derived as state 3/state 4 respiratory rates. The open bar represents the average RCR for neuronal mitochondria from *ELAV-GeneSwitch-Gal4/w*^{1118} flies whereas filled bar represents the average RCR for neuronal mitochondria from *ELAV-GeneSwitch-Gal4/UAS-hUCP2* flies. The results are presented as mean ± SEM (n = 6, *p = 0.042, Student's t test). Mitochondria were isolated from approximately 200 heads in each experiment.

Increased mitochondrial uncoupling in the nervous system decreases ROS production, decreases HNE accumulation, and increases resistance to oxidative stress

We next examined the impact of increased mitochondrial uncoupling on ROS production. To do this, we measured mitochondrial hydrogen peroxide production as a direct estimate of ROS generation. As expected, mitochondria from heads and thoraces of hUCP2-expressing long-lived flies show a 40% decrease in hydrogen peroxide production as compared to controls not expressing hUCP2 (Figure 3A). A decrease in mitochondrial ROS production would lead to a decrease in oxidative damage. Indeed, we find that the amount of an aldehydic adduct 4-hydroxy-2-nonenal (HNE), a major lipid peroxidation-derived aldehyde, is 32% lower in the mitochondria from the

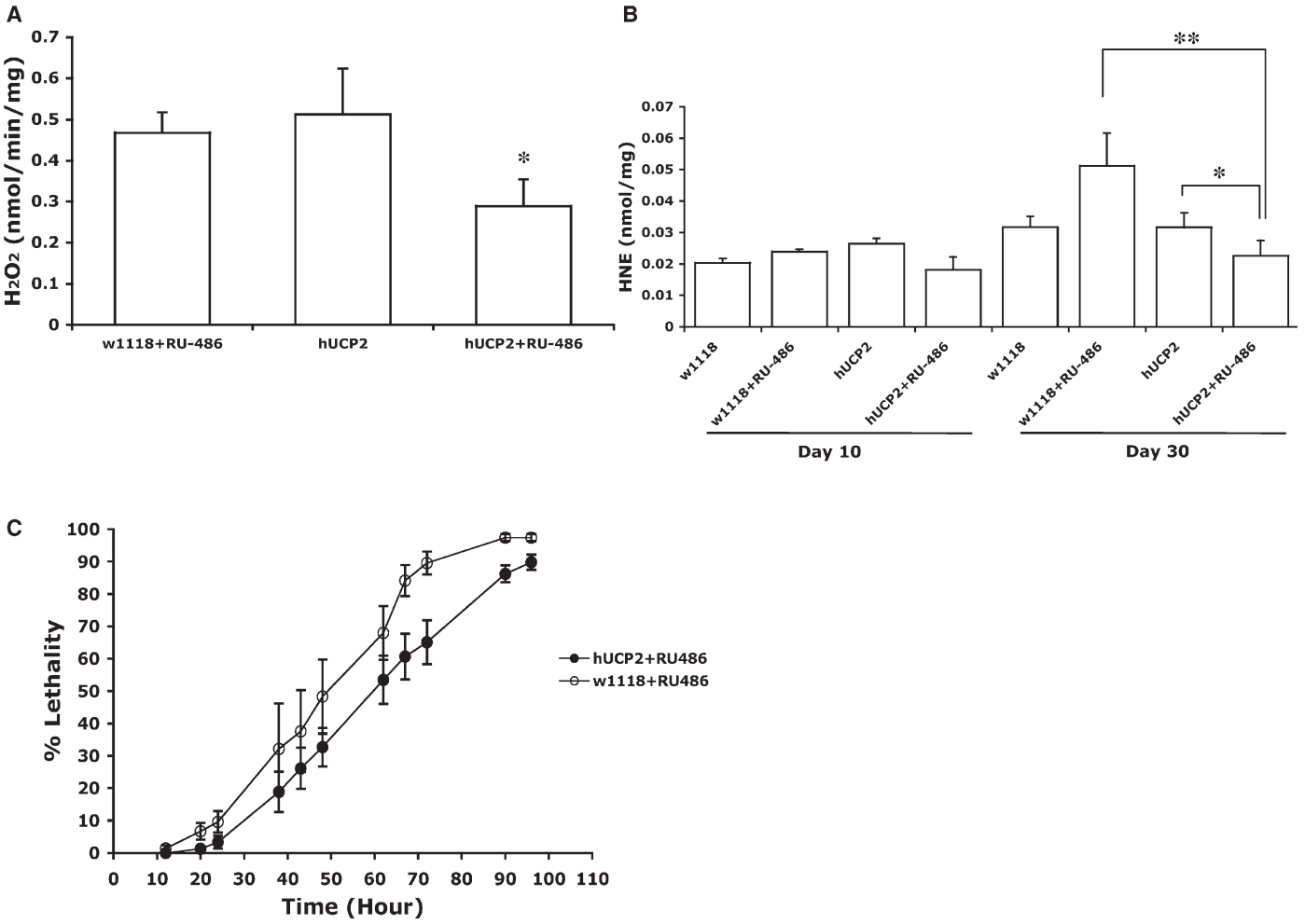

Figure 3. hUCP2-expressing flies have a decrease in H_2O_2 production, accumulate less age-associated oxidative damage, and are more resistant to a ROS generator than controls

A) A 44% decrease in mitochondrial H_2O_2 production was found in female hUCP2-expressing flies (*ELAV-GeneSwitch-Gal4/UAS-hUCP2* flies fed RU-486: hUCP2+RU-486) as compared to controls (*ELAV-GeneSwitch-Gal4/UAS-hUCP2* flies fed diluent: hUCP2; and *ELAV-GeneSwitch-Gal4/w*^{1118} flies fed RU-486: w1118+RU-486). Mitochondrial production of H_2O_2 during respiration with α-glycero-3-phosphate as substrate was measured from heads and thoraces using the Amplex Red hydrogen peroxide assay kit (Molecular Probes) (n = 4, *p = 0.0098) (Miwa et al., 2003). All values are presented as mean ± SEM. Statistically significant differences are indicated by * based on Student's t test. Results are shown for female flies. Similar results were seen with male flies.

B) Competitive ELISA assay shows that age-associated HNE accrual is decreased in adult flies specifically expressing hUCP2 in neurons (*ELAV-GeneSwitch-Gal4/UAS-hUCP2* fed RU-486; hUCP2+RU-486) as compared to controls (*ELAV-GeneSwitch-Gal4/UAS-hUCP2* fed diluent only: hUCP2, *ELAV-GeneSwitch-Gal4/w*^{1118} fed RU-486: w1118+RU-486, and *ELAV-GeneSwitch-Gal4/w*^{1118} fed diluent only: w^{1118}). HNE was measured using a competitive ELISA assay as described on isolated mitochondrial proteins from heads and thoraces of 10- or 30 day-old-flies. All values are presented as mean ± SEM. A significant decrease in HNE accumulation in 30-day-old hUCP2-expressing flies as compared to control flies is seen (n = 3, *p = 0.009, **p = 0.039). Mitochondrial extracts from approximately 50 flies were used in each assay.

C) Survival during adminstration of paraquat (20 mM paraquat in 5% sucrose) (Lin et al., 1998) shows that 10-day-old flies expressing hUCP2 in neurons (*ELAV-GeneSwitch-Gal4/UAS-hUCP2* previously fed RU-486) are more resistant than controls (*ELAV-GeneSwitch-Gal4/w*^{1118} previously fed RU-486). Closed circles represent *ELAV-GeneSwitch-Gal4/UAS-hUCP2* fed RU-486 (hUCP2+RU-486), open circles are *ELAV-GeneSwitch-Gal4/w*^{1118} fed RU-486 (w1118 + RU-486). All values are presented as mean ± SEM. Results are shown for one experiment with female flies. Similar differences were seen for males and females in three separate experiments. Each experiment included 8–10 vials with 20 flies in each vial.

head and thoraces of 30-day-old hUCP2-expressing long-lived flies as compared to controls not expressing hUCP2 (Figure 3B; Uchida and Stadtman, 1993). Consistent with a decrease in ROS production and oxidative damage, hUCP2-expressing long-lived flies also have an increased resistance to paraquat, a chemical known to generate ROS (Figure 3C). The resistance to paraquat cannot be attributed to an increase in general stress resistance that is seen in some long-lived animals (Lin et al., 1998) since the *hUCP2* long-lived animals are more sensitive than controls to other stresses such as starvation (Figure S4).

Reproduction and physical activity are not compromised in long-lived hUCP2-expressing flies

Delayed reproduction and decreased physical activity are two parameters known to extend life span in flies (Helfand and

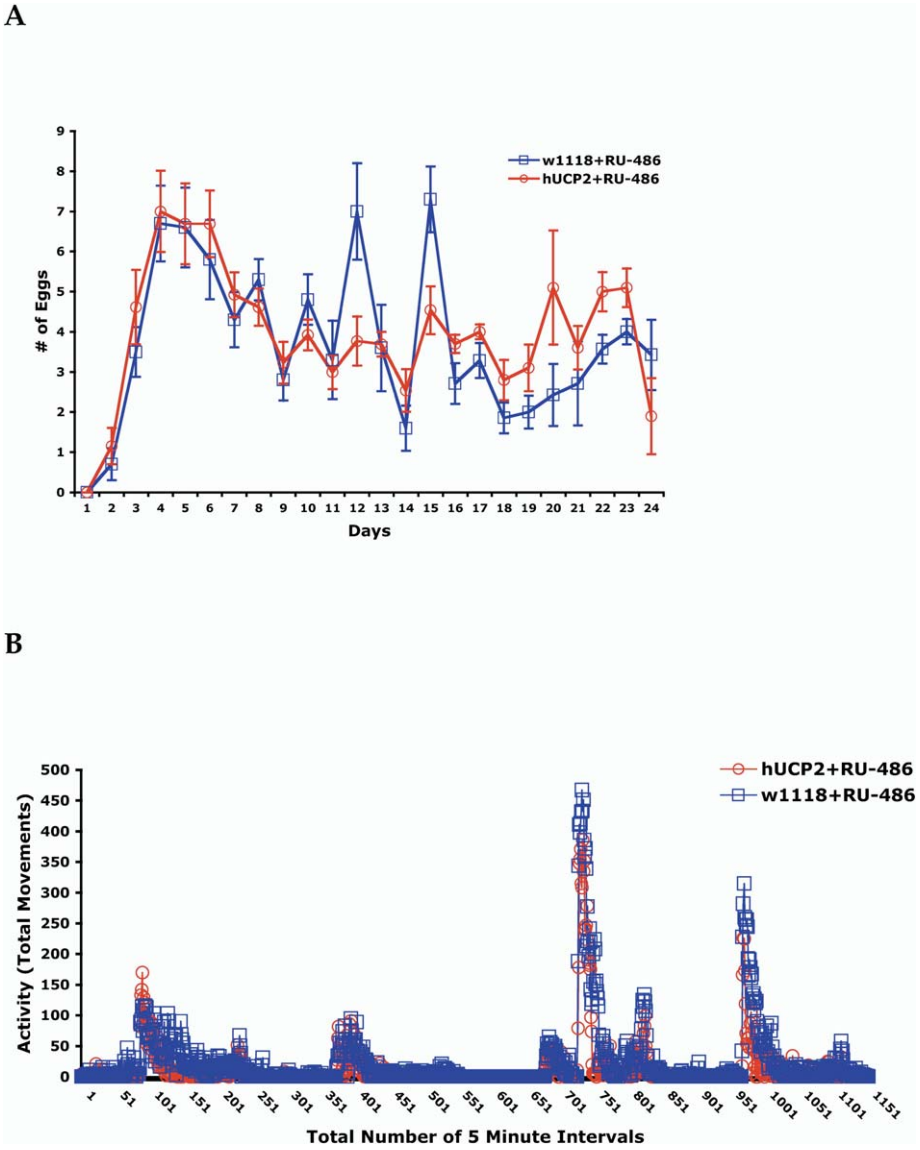

Figure 4. Female fecundity and physical activities are similar for flies expressing hUCP2 (*ELAV-GeneSwitch-Gal4/UAS-hUCP2*) and control (*ELAV-GeneSwitch-Gal4/w^{1118}*) flies fed RU-486

A) Average number of eggs per day for 20 individual females was determined from daily counts of eggs produced from single mated pairs fed RU-486 (Rogina et al., 2002). Open red circles represent egg production from *ELAV-GeneSwitch-Gal4/UAS-hUCP2* females fed RU-486 (hUCP2+RU-486), whereas open blue squares denote egg production from *ELAV-GeneSwitch-Gal4/w^{1118}* flies fed RU-486 (w1118+RU-486). All values are presented as mean ± SEM.

B) Activity of 30-day-old flies recorded with *Drosophila* Activity Monitor over a 4-day period is shown. Three independent assays, each consisting of three experimental and three control vials (20 flies/vial), were performed with comparable activity levels recorded between hUCP2-expressing flies (*ELAV-GeneSwitch-Gal4/UAS-hUCP2* fed RU-486: hUCP2+RU-486) shown in open red circles and control flies (*ELAV-GeneSwitch-Gal4/w^{1118}* fed RU-486: w1118+RU486) shown in open blue squares. Shown is a summation of total movements from a representative vial of hUCP2-expressing and control female flies. Comparable activity levels were observed between male *hUCP2* and control flies.

Rogina, 2003; Marden et al., 2003). Although the administration of RU-486 decreases egg laying in the *w^{1118}* background we found no difference in egg laying between the long-lived hUCP2-expressing *ELAV-GeneSwitch-Gal4/UAS-hUCP2* flies and the normal-lived control *ELAV-GeneSwitch-Gal4/w^{1118}* flies (Figure 4A). To determine if a tradeoff in physical activity contributes to the life span extension of the long-lived hUCP2-expressing flies, we monitored the physical activity of flies with a 24 hr *Drosophila* activity meter and found that even up to 34 days old, long-lived hUCP2-expressing flies exhibited spontaneous physical activity equal to normal-lived controls (Figure 4B). Negative geotaxis assays measuring climbing ability also showed no reduction in speed in the long-lived hUCP2-expressing flies as compared to normal-lived control flies (Figure S5; Feany and Bender, 2000). Thus, the life span extension of long-lived hUCP2-expressing flies is not a result of a decrease in reproduction or physical activity.

Components of the mitochondrial respiratory chain have been targets for interventions altering life span in model organisms. In *C. elegans*, genetic and molecular manipulation of the mitochondrial components has lead to increased resistance to ROS damage and life span extension but was associated with decreased respiration or other significant physiological tradeoffs such as retarded growth rates, lower egg production, and behavioral defects (Dillin et al., 2002; Feng et al., 2001; Larsen and Clarke, 2002; Lee et al., 2003a, 2003b). In this report we show that in *Drosophila melanogaster*, a decrease in mitochondrial ROS production, attenuation of oxidative damage, and significant life span extension can be achieved by targeting a known uncoupling protein hUCP2 to the mitochondria of adult neurons. Furthermore, the benefit of life span extension does not come at the expense of decreased mitochondrial respiration, fecundity, or physical activity. Contrary to the traditional view that increasing respiration can increase ROS production, we find that expression of hUCP2 in mitochondria of adult neurons increases state 4 mitochondrial res-

piration but decreases ROS production. This observation suggests that the type of mitochondrial respiration, state 4 versus state 3, may matter more than the rate of respiration itself with regard to ROS production and life span. In support of this is a recent study showing a strong correlation between increased uncoupling in mitochondria and increased life span as a consequence of natural variation in mice (Speakman et al., 2004). Our findings highlight the plasticity of mitochondria and suggest the intriguing possibility that genetic or pharmaceutical interventions altering mitochondrial respiration in adults could have significant positive effects on healthy life span in other metazoans.

Experimental procedures

HUCP2 transgenic and driver lines

The full-length *hUCP2* cDNA was obtained as an IMAGE clone (ID3053876) from Invitrogen, subcloned into the pUAST transformation vector, and injected into the germline of *w*; *Δ2-3/TM3* flies (Brand and Perrimon, 1993). Several F1 transformant lines were obtained and individually backcrossed to w^{1118} stock for ten generations. The *ELAV-GeneSwitch-Gal4* and *MHC-GeneSwitch-Gal4* driver lines were kindly provided by Dr. H. Keshishian (Yale University) (Osterwalder et al., 2001). The ubiquitous drivers *Tubulin-Gal4* and *Actin-Gal4* were obtained from Bloomington Stock Center. All fly stocks were maintained at 25°C on standard corn meal/yeast/sucrose/agar diet (Rogina et al., 2000).

Life span and stress resistance studies

To perform life span studies, homozygous virgin *UAS-hUCP2* and w^{1118} females were crossed to homozygous *ELAV-GeneSwitch-Gal4* and *MHC-GeneSwitch-Gal4* drivers. The progeny from these crosses were maintained on standard corn meal/yeast/sucrose/agar diet containing 200 μM RU-486 in diluent (ethanol) or diluent alone added as described (Hwangbo et al., 2004) and passed to fresh vials every other day (Rogina et al., 2000).

Both starvation and paraquat resistance assays were conducted according to Lin et al. (1998). Briefly, *ELAV-GeneSwitch-Gal4/UAS-hUCP2* and *ELAV-GeneSwitch-Gal4/*w^{1118} flies were placed in vials with RU-486 containing food immediately after eclosion for 10 days and then transferred to vials containing filter paper soaked in water (starvation assay) or a solution of 20 mM paraquat and 5% sucrose (paraquat assay) and the number of dead flies counted every 8–14 hr. Three independent experiments were performed. Each experiment used eight to ten vials with 20 males or 20 females in each vial (160–200 males and 160–200 females per experiment).

Drosophila activity meter and negative geotaxis assays

To determine spontaneous physical activity for *ELAV-GeneSwitch-Gal4/UAS-hUCP2* flies and *ELAV-GeneSwitch-Gal4/*w^{1118} control flies, a vial containing 20 adult males or females of a specific age for each genotype was placed in a *Drosophila* activity meter (TriKinetics Inc., Massachusetts) at 25°C and all movements recorded continuously under normal culturing conditions for 20 days. Flies were passed to vials with fresh food containing RU-486 every 2 days during the recordings. Three independent assays, each consisting of three experimental and three control vials (20 flies/vial), were performed.

Negative geotaxis assays were performed to compare the climbing ability of hUCP2-expressing *ELAV-GeneSwitch-Gal4/UAS-hUCP2* and *ELAV-GeneSwitch-Gal4/*w^{1118} control flies as described (Feany and Bender, 2000). Briefly, upon eclosion, 20 adult male or female flies were placed into a vial with food containing RU-486 food and negative geotaxis assays were conducted every day. Three trials for each group of 20 flies were performed where flies were gently tapped to the bottom of a cylinder made with two empty food vials taped end-to-end and allowed to climb. The number of flies reaching above a 50% mark of the total cylinder length in 1 min was recorded and the average calculated.

Female fecundity

Female fecundity was determined from daily counts of eggs produced by 20 individual females in single mating pairs of either *ELAV-GeneSwitch-Gal4/UAS-hUCP2* or *ELAV-GeneSwitch-Gal4/*w^{1118} flies fed with RU-486 containing yeast/sucrose/agar food. The flies were passed to new vials every day and the number of eggs laid was counted and recorded for the first 24 days of the adult life (Marden et al., 2003).

Mitochondrial respiration

To obtain mitochondria from heads only, 10-day-old flies (*ELAV-GeneSwitch-Gal4/UAS-hUCP2* or *ELAV-GeneSwitch-Gal4/*w^{1118}) living on RU-486 containing food since the time of eclosion were collected on ice, heads removed and kept on ice for less than 30 min before isolation of mitochondria. Isolation of mitochondria from heads was performed as described (Miwa et al., 2003).

Following measurement of total mitochondrial protein, respiration of freshly isolated mitochondria was determined in a Clark-type oxygen electrode at 25°C (Rank Brothers Ltd., United Kingdom). Mitochondria were suspended at a protein concentration of 150 μg/ml in electrode buffer containing 20 mM α-glycero-3-phosphate as substrate as described (Miwa et al., 2003). ADP (1 mM), oligomycin (1 μg/ml), and GDP (0.5 mM) were added sequentially to record state 3, state 4, and GDP-sensitive mitochondrial respiration, respectively. The substrate α-glycero-3-phosphate, ADP, and GDP were dissolved in water and oligomycin was dissolved in ethanol before adding to reactions. All chemicals were purchased from Sigma.

Mitochondrial hydrogen peroxide measurements

Hydrogen peroxide production of mitochondria isolated from heads and thoraces of 10-day-old flies (*ELAV-GeneSwitch-Gal4/UAS-hUCP2* or *ELAV-GeneSwitch-Gal4/*w^{1118}) with or without continual RU-486 feeding from the time of eclosion was measured under normal respiration conditions (see above) using the Amplex Red kit (Molecular Probes). Mitochondrial hydrogen peroxide production was determined using a spectrophotometer at absorption maxima of 563 nm.

Measurement of 4-hydroxy-2-nonenal (HNE) levels in mitochondrial proteins

A competitive ELISA assay was used to measure HNE associated with mitochondrial proteins from heads and thoraces of 10- and 30-day-old flies (*ELAV-GeneSwitch-Gal4/UAS-hUCP2* or *ELAV-GeneSwitch-Gal4/*w^{1118}) with or without continual RU-486 feeding from the time of eclosion. We modified previously reported ELISA assays for HNE (Uchida et al., 1995) (Alpha Diagnostics, Inc. and MDBio, Inc.) using a commercially available rabbit polyclonal anti-4-HNE antibody (Alpha Diagnostic) and HNE conjugated to GAPDH (Uchida and Stadtman, 1993). Mitochondrial proteins were extracted from dissected tissues following the procedure described by Yan and Sohal (Boss et al., 1998), and protein lysates were sonicated in the presence of 5 mM BHT. To perform competitive ELISA assays, mitochondrial protein extracts were mixed with the anti-4-HNE antibody before adding to a microtiter plate that had been coated with purified HNE-conjugated GAPDH protein (GAPDH and HNE from Calbiochem). Plates were incubated for 1 hr at room temperature and washed extensively with wash buffer. A secondary antibody conjugated to HRP (1:5000) was added to the wells and plates were incubated for 1 hr at room temperature, washed with wash buffer, and the detection buffer TMB (Alpha Diagnostic International, Inc) was added. The plates were read at $O.D._{450}$ using a microplate reader (Bio-Rad). Standard HNE curves in these assays were established using dilutions of purified HNE-GAPDH mixed with the anti-HNE antibody. The final amount of HNE accumulation was calculated based upon one HNE moiety conjugated to each molecule of GAPDH (Uchida and Stadtman, 1993).

Western blot analysis

To detect neuronal, mitochondrial expression of hUCP2 protein in *ELAV-GeneSwitch-Gal4/UAS-hUCP2* flies, 100 μg of mitochondrial protein from either heads or bodies was isolated from adults fed RU-486 food from eclosion for 10 days and compared to genetically identical flies from the same cohort that were not fed RU-486. The mitochondrial extract was resolved on a 12% SDS-PAGE and transferred onto a PVDF membrane. Western blot analysis with an anti-hUCP2 antibody at 1:1000 (N-19, Santa Cruz) and an anti-cytochrome oxidase antibody at 1:1000 (DA5, Molecular Probes) was performed (Fridell et al., 2004).

Statistical analysis

Statistical analysis for independent life span trials was performed using log-rank test (StatView). Results for all other assays were analyzed using paired Student's t test.

Supplemental data

Supplemental data include five figures and one table and are available with this article online at http://www.cellmetabolism.org/cgi/content/full/1/2/145/DC1/.

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