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Genetically encoded redox sensor identifies the role of ROS in degenerative and mitochondrial disease pathogenesis

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Abstract

Mitochondrial dysfunction plays an important role in the pathogenesis of neurodegenerative diseases, numerous other disease states and senescence. The ability to monitor reactive oxygen species (ROS) within tissues and over time in animal model systems is of significant research value. Recently, redox-sensitive fluorescent proteins have been developed. Transgenic flies expressing genetically encoded redox-sensitive GFPs (roGFPs) targeted to the mitochondria function as a useful *in vivo* assay of mitochondrial dysfunction and ROS. We have generated transgenic flies expressing a mitochondrial-targeted roGFP2, demonstrated its responsiveness to redox changes in cultured cells and *in vivo* and utilized this protein to discover elevated ROS as a contributor to pathogenesis in a characterized neurodegeneration mutant and in a model of mitochondrial encephalomyopathy. These studies identify the role of ROS in pathogenesis associated with mitochondrial disease and demonstrate the utility of genetically encoded redox sensors in *Drosophila*.

Keywords

Drosophila melanogaster; ATP6; ATPalpha; mitochondrial dysfunction; ROS; redox sensor

Introduction

An increase in the level of reactive oxygen species (ROS) is known to contribute to the pathogenesis of several neurodegenerative diseases, including Alzheimer's, Parkinson's and Huntington's diseases, as well Amyotrophic lateral sclerosis and premature aging (Emerit et al., 2004; Halliwell, 2006). Natural antioxidant systems serve as a defense against oxidative stress. When the production of ROS overwhelms antioxidant mechanisms damage to cellular macromolecules can occur resulting in accumulation of unfolded and misfolded proteins,

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membrane instability and DNA mutations. All of these are implicated in aging/senescence and in various pathological processes resulting from neurodegenerative diseases. In addition to direct oxidation, ROS can also activate numerous signaling pathways that increase apoptosis (Allen and Tresini, 2000; Li et al., 2008; Temkin and Karin, 2007).

Since ROS are involved in a broad range of physiological and pathological processes, the measurement of ROS is critical for the investigation of the occurrence, development and outcome of diseases. Thus, numerous assays have been developed to measure ROS, each having distinct advantages and limitations. Among the most promising are genetically encoded redox sensors (Hanson et al., 2004), which have recently proven functional in mammalian cells (Dooley et al., 2004), Arabidopsis (Maughan et al., 2010; Meyer et al., 2007; Rosenwasser et al., 2010; Schwarzlander et al., 2009), and in mice (Guzman et al., 2010). Redox sensors were engineered with pairs of cysteine residues on adjacent surfaces of the fluorophore barrel structure such that excitation ratios are affected by the formation of disulfide linkages; therefore, the population of expressed roGFPs serve as a ratiometric probe of the redox status (Cannon and Remington, 2008; Dooley et al., 2004; Hanson et al., 2004). Here, we report the generation and characterization of transgenic flies expressing a genetically expressed roGFP probe to measure ROS. The principal advantages of using such an approach are: 1) the probe is genetically encoded, 2) ratiometric imaging allows one to assess redox status accurately regardless of the absolute levels of probe concentration due to expression, photobleaching or variation of tissue thickness, and 3) this method allows real time detection of redox status in different live cells and animal tissues without the permeation and preincubation of exogenous probes.

Studies of aging and various disease models in *Drosophila* would benefit from a genetically encoded means of measuring ROS within various tissues over a range of animal ages. We have generated roGFP transgenic flies bearing a mitochondrial or matrix target signal (MTS) fused to roGFP2 expressed in mitochondria (*UAS-MTSroGFP2*) and a cytosolic version (*UAS-roGFPR12*). Expression is conditional upon GAL4, allowing expression in various tissues using available GAL4 strains. We have used MTSroGFP2 to examine changes in redox status over time in several mutant and control animals. Our results demonstrate the role of progressive ROS accumulation in the pathogenesis of neurodegeneration and mitochondrial encephalomyopathy and document an increase in mitochondrial ROS observed with normal senescence of wild type animals. These data demonstrate that MTSroGFP2 is an excellent indicator for the redox status within *Drosophila*.

Materials and methods

Drosophila strains, locomotor function and pharmacology

For experiments all fly stocks were maintained on standard cornmeal–molasses agar media at 25°C. For drug treatment, groups of ~15–20 newly eclosed flies were placed in a vial containing a half circle filter paper containing the drug in DMSO vehicle. Vials and drug were changed every other day. The $ATP6^{1}$ and $ATPalpha^{DTS1}$ strains have previously been reported (Ashmore et al., 2009; Celotto et al., 2006a; Palladino et al., 2003). Locomotor function stress-sensitivity (a.k.a. bang-sensitivity) was assayed as previously(Celotto et al., 2006a; Celotto et al., 2006b; Hrizo and Palladino, 2010). The elav-Gal4 strain is from the Bloomington Drosophila stock center. Transgenes were made using the pUASTattB construct (Bischof et al., 2007). *pUASTattB-MTSroGFP2* and *pUASTattB-roGFPR12* transgenic constructs were generated using Bgl II and Not I from MTSroGFP2 and roGFPR12 clones, respectively, kindly provided by James Remington (Hanson et al., 2004). Transgenic flies were generated using standard ΦC31 integrase transgenesis with insertion into the attP18 site. *pUASTattB-roGFPR12* transgene fluorescence was dim in neurons and was not used further in these studies.

Primary Drosophila neuronal culture

Primary neural cultures were prepared as described by O'Dowd with minor modifications (Sicaeros et al., 2007). Briefly, heads were removed from animals at 55–78h after pupation and were placed in sterile dissecting saline containing 137 mM NaCl, 5.4 mM KCl, 0.17 mM NaH_2PO_4, 0.22 mM KH_2PO_4, 33.3 mM glucose, 43.8 mM sucrose, and 9.9 mM HEPES, pH 7.4. Central brain regions were obtained and incubated in dissecting saline containing 50 U/ml papain activated by L-cysteine (1.32 mM) for 15 min at room temperature. Tissues were washed with dissecting saline and Drosophila-defined culture medium composed of Ham's F-12 DMEM (high glucose; Irvine Scientific, Santa Ana, CA) supplemented with 1 mg/ml sodium bicarbonate, 20mM HEPES, 100 μM putrescine, 30 nM sodium selenite, 20 ng/ml progesterone, 50 μg/ml insulin, 100 μg/ml transferrin, and 1 μg/ml of 20-hydroxyecdysone. Each culture was prepared from a single brain transferred to a 5 μl drop of Drosophila-defined culture medium on a ConA/laminin-coated glass coverslip. The tissue was mechanically dissociated with dissecting needles and repeated pipetting, and the cells were allowed to settle for 30 min. Culture dishes were flooded with 1.5 ml of Drosophila-defined culture medium and maintained in a 23°C humidified 5% CO2 incubator.

In vitro characterization of MTSroGFP2 in cultured neurons

The *UAS-MTSroGFP2* transgene was expressed in *Drosophila* brains using the pan neural driver *elav-GAL4*. The progeny pupa was used to generate primary neurons cultured on 10mm coverslips. On day ~4–5, coverslips were transferred to a perfusion chamber (DH35, Warner Instruments) and positioned on the movable stage of an Olympus BX50W1 epifluorescence microscope. The cells were perfused with PBS and the baseline was measured for 4 minutes before 30 minutes application of oxidizing agent H_2O_2 (at 1mM, 10mM, or 50mM concentrations). After H_2O_2 treatments, the reducing agent dithiothreitol (DTT) at 10mM was applied for 10 minutes. Images were acquired using a 60X objective at 535nm emission wavelength every minute using Hamamatsu C4742-95 with excitation wavelengths (Polychrome V) at 405nm and 488 nm for oxidized and reduced roGFP2, respectively. Analysis was performed using Simple PCI6 software (Compix Inc.) and 405nm/488nm fluorescent ratios were calculated.

In vivo adult brain redox status analysis

elav-Gal4; UAS-MTSroGFP animals were generated with and without the *ATP6* or *ATPalpha* mutations. Brains were dissected in PBS from mutant and control animals on day 3, 15 and 30. After dissection, brains were placed in mounting medium (Victor, H-1000) on a thin cover slip attached to a petri dish. An Olympus IX 81 inverted laser scanning Fluoview 1000 confocal microscope (Olympus, Tokyo, Japan) was used for imaging. Images were acquired by using a BA 510–540 emission filter for following excitation at 405 and 488 nm. The UPLSAPO 20X objective (NA 0.75, WD 0.6mm, and cover glass thickness 0.17) and Z-scan with a 10um step size were used to obtain whole brain fluorescence. At least 5 brains were imaged for each group and 4 equal sized images of neuropil area of every brain were analyzed. The 405nm/488nm fluorescence was obtained using FV10-ASW2.0 software and ImageJ (NIH) was used to subtract background and perform image analyses. Statistical analysis was performed by student's t-test.

MTSroGFP localization

Primary neural cultures were attached to glass bottom petri dishes and examined on day 5. MitoTracker Red (Invitrogen, M7521) 1 mM stock solution was diluted to the final working concentration 250 nM in neuronal culture medium DDM2. Prewarmed (23°C) staining solution containing MitoTracker Red probe was added to the culture, incubated for 30

minutes under growth conditions, cells were washed 3 times with prewarmed PBS and mounted in medium (Victor, H-1000). The cells were imaged on an Olympus IX 81 inverted laser scanning Fluoview 1000 confocal microscope (Olympus, Tokyo, Japan). The Plan Apo 60X objective (NA 1.42, WD 0.15 mm) and zoom 5.0X were used to obtain MTSroGFP2 and MitoTracker Red fluroecence images of whole neurons using 488nm excitation laser with 510–540 emission filter and 543 nm excitation laser with 555–655 emission filter, respectively.

Results

Expression of roGFP in Drosophila neurons

Redox sensing GFPs have previously been described including the roGFP2 (C48S/T65S/S147C/Q204C) variant (Cannon and Remington, 2008; Dooley et al., 2004; Hanson et al., 2004). Owing to the T65S substitution, roGFP2 has a much larger dynamic range and we selected this variant to target to the mitochondria in *Drosophila* using the pyruvate dehydrogenase MTS (mitochondria or matrix targeting signal), similar to previously described studies (Hanson et al., 2004). We generated *UAS-attB* transgenic flies expressing MTSroGFP2 in a GAL4-dependent manner.

Using *elav-Gal4*, we expressed MTSroGFP2 and examined fluorescence in cultured primary neurons. MTSroGFP2 gave bright punctate expression consistent with mitochondrial localization. To examine the specificity of the localization we used mitotracker red to examine colocalization, which was significant (Supplemental Figure 1). These data suggest that the *elav-Gal4; UAS-MTSroGFP2* will be suitable to measure neural mitochondrial ROS.

To ensure the MTSroGFP2 is dynamically reporting the redox status within *Drosophila* neurons, we performed pilot experiments using live cell imagining and pharmacologic treatments to alter cellular redox state. We used H_2O_2 followed by DTT to cause changes in redox state. The data demonstrate that MTSroGFP2 was responsive to extracellular H_2O_2 in a dose dependent manner (Figure 1A, 1B). The observed change in redox status was readily reversible with 10 mM DTT treatment. We also examined the responsiveness to redox changes using confocal microscopy and similar results were obtained (Figure 1C). These data suggest that MTSroGFP2 will likely function well as a redox sensor within *Drosophila* tissues.

Validating altered redox status in mitochondria in vivo

To validate whether MTSroGFP2 was responsive to physiologically relevant mitochondrial ROS associated with dysfunction/disease states *in vivo* we examined the redox status in brains from mutants of a well-characterized gene known to encode MnSOD/SOD2 in *Drosophila* (Belton et al., 2006; Duttaroy et al., 2003; Kirby et al., 2002; Martin et al., 2009; Palladino et al., 2002; Paul et al., 2007a). In young SOD2 mutants a modest elevation in redox status is observed in heterozygotes and a marked increase is observed in trans-heterozygous animals (mutants with an established SOD2 deficiency), both compared to age-matched wild type controls (Figure 2).

Altered redox status in a neurodegenerative fly mutant

Specific mutations in the *ATPalpha* gene have previously been shown to result in progressive neuropathology. *ATPalpha* encodes the catalytic subunit of the Na^+, K^+ ATPase and specific dominant gain-of-function mutants exhibit reduced longevity and severe progressive neuropathogy (Ashmore et al., 2009; Fergestad et al., 2006; Palladino et al., 2003). We hypothesized that increased ROS might contribute to pathogenesis in

ATPalpha^{DTS1} and that ROS may be increased in an age-dependent manner. We examined the redox status of *ATPalpha*^{DTS1} and age-matched control brains as a function of age using the genetically encoded MTSroGFP2. There was no difference in redox status in young animals; however, in an age-dependent manner, mitochondrial ROS was significantly elevated in *ATPalpha*^{DTS1} animals from that of wild type animals (Figure 3A, Supplemental Figure 2A). Importantly, there was a significant and detectable increase in redox status observed over time in wild type animals.

Genetic revertants of *ATPalpha*^{DTS1} have been isolated and extensively characterized (Ashmore et al., 2009; Fergestad et al., 2006; Palladino et al., 2003; Paul et al., 2007b). The first such revertant, *ATPalpha*^{DTS1R1}, has been the focus of considerable study and the basis of the reversion is known to be a 4 base pair deletion causing a premature termination codon that prevents expression of the protein (Ashmore et al., 2009; Palladino et al., 2003). This mutation reverts the dominant gain-of-function aspects of *ATPalpha*^{DTS1}, exhibits a striking *increase* in longevity and is an ideal control for the *ATPalpha*^{DTS1} mutant. Thus, we asked whether *ATPalpha*^{DTS1R1} exhibited altered ROS using MTSroGFP2 fluorescence as an assay. Specifically, we hypothesized that ROS would be normal in *ATPalpha*^{DTS1R1} animals consistent with reversion of the gain-of-function pathogenesis of *ATPalpha*^{DTS1}. Alternatively, it is possible that ROS would be significantly lower in *ATPalpha*^{DTS1R1} animals than wild type, in part explaining the robust increase in longevity. Our results show that ROS progressively increases with time as is seen in wild type controls, is not elevated dramatically as is the case for *ATPalpha*^{DTS1}, and that ROS is not significantly decreased (Figure 3B, Supplemental Figure 2B). These data further demonstrate the utility of a genetically encoded redox sensor in the fruit fly.

Altered redox status in a fly model of mitochondrial encephalomyopathy

An endogenous mitochondrial mutation affecting *ATP6*, encoding a subunit of complex V, has been shown to model mitochondrial encephalomyopathy (Celotto et al., 2006a; Palladino, 2010). We hypothesized that increased mitochondrial ROS may be instrumental in pathogenesis of *ATP6*^{1} and may contribute to the progressive onset of disease phenotypes including locomotor impairment, reduced longevity or myodegeneration. To investigate this hypothesis we examined the redox status of *ATP6*^{1} and control brains over a time course using genetically encoded MTSroGFP2. Consistent with previous data (Figure 3A, 3B), control animals exhibited a significant increase in ROS with age (Figure 4). The redox status of *ATP6*^{1} mitochondria were not significantly changed in young animals; however, in aged animals (day 15 and 30) the redox status was significantly increased (Figure 4, Supplemental Figure 2C). ROS appeared elevated at times coinciding with the onset of phenotypes in *ATP6*^{1} mutants, suggesting that ROS may contribute significantly to pathogenesis.

Mitochondrial-targeted antioxidants improve ATP6^{1} pathogenesis

Mitochondria are a significant source of oxidative damage. Mitochondrial dysfunction is believed to trigger increased ROS production, contributing further to pathogenesis in mitochondrial encephalomyopathy and progressive neurological diseases (Addabbo et al., 2009). To determine the extent to which ROS contributes to pathogenesis, we screened a series of well-characterized mitochondrial-targeted ROS scavengers (Frantz and Wipf, 2010; Hoye et al., 2008; Jiang et al., 2007; Kagan et al., 2009; Macias et al., 2007; Wipf et al., 2005) for those that improve *ATP6*^{1} phenotypes. Initially we examined five ROS scavengers and a control XJB-5-194 with a related backbone but lacking the nitroxide ROS scavenging subunit for their ability to improve longevity in *ATP6*^{1}. We tested all six compounds in parallel in a blinded manner at 0.1 μM, 1 μM and 10 μM concentrations using identical delivery methods (Supplemental Figure 3). Only JED-E71-37 showed a significant and

reproducible increase in longevity, with 1 μM providing the most significant result (Figure 5A). Importantly, the control compound XJB-5-194 did not improve longevity. The experiment was independently replicated with 1 μM JED-E71-37 (Figure 5B). XJB-5-208 significantly altered longevity by log-rank analysis but did not significantly improve median lifespan and thus was not pursued further. We thus asked whether 1 μM JED-E71-37 would effectively improve locomotor function in *ATP6*^{1} mutants. Consistent with the modest improvement in longevity, we observed a significant improvement in locomotor function early in pathogenesis (day 13 and 17) but not later in pathogenesis (day 21). Importantly, ROS scavenger treatment did not fully ameliorate locomotor function at any time point, as wild type animals at all three time points did not paralyze in response to mechanical stress (data not shown).

The improvement in phenotypes observed with antioxidant treatment demonstrates mitochondrial ROS is an important part of mitochondrial encephalomyopathy pathogenesis. The modest improvement observed suggests that ROS is a minor part of pathogenesis or that the antioxidant treatment only minimally improved ROS. To better understand the extent to which ROS contributes to mitochondrial encephalomyopathy pathogenesis, we examined ROS at days 13, 17 and 21 following chronic treatment with 1 μM JED-E71-37. The mitochondrial-targeted antioxidant was able to very significantly reduce ROS at all time points examined (Figure 6).

Discussion

Mitochondrial dysfunction and ROS have proven key to the pathogenesis of numerous disease conditions including AD, PD, HD, and ALS (Halliwell, 2006). The extent to which an altered redox status contributes to pathogenesis is actively being studied in various model systems. Although there are numerous neurodegenerative and mitochondrial disease models in *Drosophila*, assays to accurately measure ROS and mitochondrial dysfunction are limited. roGFPs have proven useful in other systems suggesting they would be valuable in *Drosophila*. We developed cytosolic and mitochondrial targeted versions and our initial studies suggest the latter is more useful, especially within neurons. MTSroGFP2 was detectable, responsive to changes in redox state in cells and was used to examine redox changes with age *in vivo.* Our data suggest that brain mitochondria *in vivo* are a much more oxidizing environment than mitochondria within cultured primary neurons (Figure 1C and 2A wild type). These data are consistent with cultured neurons being much less reliant upon OXPHOS for energy and exemplify the importance of *in vivo* studies of mitochondrial function.

Mitochondrial redox levels were examined using MTSroGFP2 in two well-characterized mutants with progressive and degenerative phenotypes. Both the *ATP6* and *ATPalpha* mutants examined showed increase in ROS *in vivo*. Importantly, ROS was not elevated in young animals but was observed to be elevated in a progressive manner in accord with phenotypes observed in these mutants. These data strongly support the assertion that a progressive increase in mitochondrial ROS may underlie the progressive nature of disease pathogenesis associated with these degenerative conditions.

Several strains of wild type flies exhibited a steady increase in redox status with age as measured by MTSroGFP2, demonstrating the importance of examining ROS over a time course and suggesting that increased mitochondrial redox status or mitochondrial dysfunction are instrumental to normal senescence. ROS has been extensively implicated in aging and previous data have led others to hypothesize that cellular and or mitochondrial dysfunction with age may lead to age-related increases in ROS (Kregel and Zhang, 2007). Although there is extensive literature to suggest ROS may underlie aging, much of the data

in support of this are based upon the notion that the damage caused by ROS may be cumulative. That said there is some evidence that ROS may increase with age within various tissues including muscle, heart, liver, and brain (Bejma and Ji, 1999; Bejma et al., 2000; Driver et al., 2000). Our data elegantly demonstrate a significant change in mitochondrial redox status with age consistent with the hypothesis that mitochondrial dysfunction and subsequent increases in ROS generation may underlie mechanisms of senescence.

ATPalpha^{DTS1} is a gain-of-function mutation in the gene encoding the Na^+/K^+ ATPase alpha subunit, previously shown to cause marked progressive neuropathology (Palladino et al., 2003). Although pathogenesis of *ATPalpha*^{DTS1} is not known, ion dyshomeostasis, altered neural signaling and elevated ROS are hypothesized to contribute to pathogenesis. Our studies using MTSroGFP2 reveal a marked elevation in redox status in an age-dependent manner. Interestingly, if we had examined only young adults we would have concluded that ROS likely did not contribute to pathogenesis, demonstrating the importance of examining adults in a time course to elucidate mechanisms of pathogenesis. It is important to note that phenotypes emerge with time and that previous histopathology revealed neuropathology only in aged animals (Fergestad et al., 2006; Palladino et al., 2003), suggesting a relationship might exist between elevated ROS and the emergence of neuropathology in *ATPalpha*^{DTS1} animals. Importantly, a revertant of the temperature-sensitive paralysis of *ATPalpha*^{DTS1} has been described and this mutant, *ATPalpha*^{DTS1R1}, does not have elevated ROS. Also, the *ATPalpha*^{DTS1R1} mutant has been shown to have a surprising ~25% increase in longevity over wild type animals (Ashmore et al., 2009). Interestingly, our data revealed that ROS is not significantly altered in *ATPalpha*^{DTS1R1}animals from controls. These data demonstrate that the gain-of-function associated with temperature-sensitive paralysis in *ATPalpha*^{DTS1}results in elevated ROS and that the surprising increase in longevity in *ATPalpha*^{DTS1R1} is associated with normal levels of ROS.

Mitochondrial encephalomyopathy is associated with mitochondrial dysfunction in oxidative phosphorylation (complex I-V) either resulting from an endogenous mitochondrial mutation or mutation of a nuclear encoded subunit of complex I – V (Leonard and Schapira, 2000a; Leonard and Schapira, 2000b). We have previously isolated an endogenous mitochondrial mutation affecting ATP6, an essential component of complex V (Celotto et al., 2006a). We have previously shown that *ATP6*^{1} causes a dramatic loss of mitochondrial ATP synthase activity (Celotto et al., 2006a; Palladino, 2010). We hypothesized that the mitochondrial dysfunction resulting from *ATP6*^{1} might cause elevated ROS that contribute to pathogenesis. Our studies using MTSroGFP2 demonstrate a progressive increase in ROS that parallels the onset of pathogenic phenotypes in *ATP6*^{1}.

Mitochondrial-targeted ROS scavengers are potentially an effective treatment for various mitochondrial and neurodegenerative diseases (Frantz and Wipf, 2010; Hoye et al., 2008; Kagan et al., 2009; Macias et al., 2007; Wipf et al., 2005). The finding that a mitochondrial-targeted ROS scavenger was able to improve *ATP6*^{1} phenotypes *in vivo* is promising. Furthermore, these data demonstrate ROS has an important role in pathogenesis of mitochondrial encephalomyopathy. Our data show that JED-E71-37 was able to fully ameliorate ROS at a time when locomotor function was partially improved. Furthermore, ROS was reduced at later time points but was not lowered to completely wild type levels. Consistent with the interpretation that ROS are one important aspect of pathogenesis, *ATP6*^{1} animals treated with mitochondrial-targeted ROS scavengers exhibit a modest but significantly improved longevity. It is possible that improvements in ROS observed with ROS scavenger treatment lead to the phenotypic improvements, however, it is also feasible that this complex structure could lead to phenotypic improvements by some independent mechanism of action. Five putative ROS scavengers and a control compound were examined

for efficacy *in vivo*, and one was able to significantly improve longevity and locomotor function in *ATP6*^{1} animals. These compounds have not yet been extensively tested *in vivo* and the selectivity may derive simply from better pharmacokinetic properties or lower toxicity. Alternatively, the activity of compound JED-E71-37 could be due to its improved mitochondrial delivery properties. The alkene peptide isosteres JP4-039, XJB-5-208, and their dimeric congeners JED-E71-1 and JED-E71-58 do not contain the full sequence of the lead structure XJB-5-131, which has been shown to be a highly effective mitochondrial targeting system (Jiang et al., 2007; Wipf et al., 2005). In contrast, JED-E71-37 represents a flexibly linked homodimer of XJB-5-131. Further studies will be needed to completely resolve the specific mechanism of action.

Conclusions

The data strongly support the conclusion that genetically encoded redox sensors are a valuable tool for examining mitochondrial dysfunction and ROS *in vivo*. Studies of specific *Drosophila* mutants with neurological dysfunction demonstrate their utility within neural tissues and the importance of examining ROS over a relevant time course. These studies also demonstrate that an increase in ROS is associated with normal aging, suggesting this may be integral to mechanisms of senescence. Our data support this hypothesis but further studies, possibly using an assay capable of specifically measuring mitochondrial redox status such as MTSroGFP2, will be needed to examine mitochondrial ROS over the full lifespan to better appreciate its role in the aging process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviations

ROS	reactive oxygen species
roGFP	redox-sensitive green fluorescent protein
MTS	mitochondrial or matrix target signal
DTT	dithiothreitol

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Highlights

- Genetically encoded mitochondrial redox sensors *in vivo*.
- Mitochondrial redox status increases with age suggesting a role in aging/senescense.
- Progressive mitochondrial redox changes in mitochondrial encephalopathy in vivo.
- Mitochondrial ROS associated with Na/K ATPase neurodegeneration in vivo.

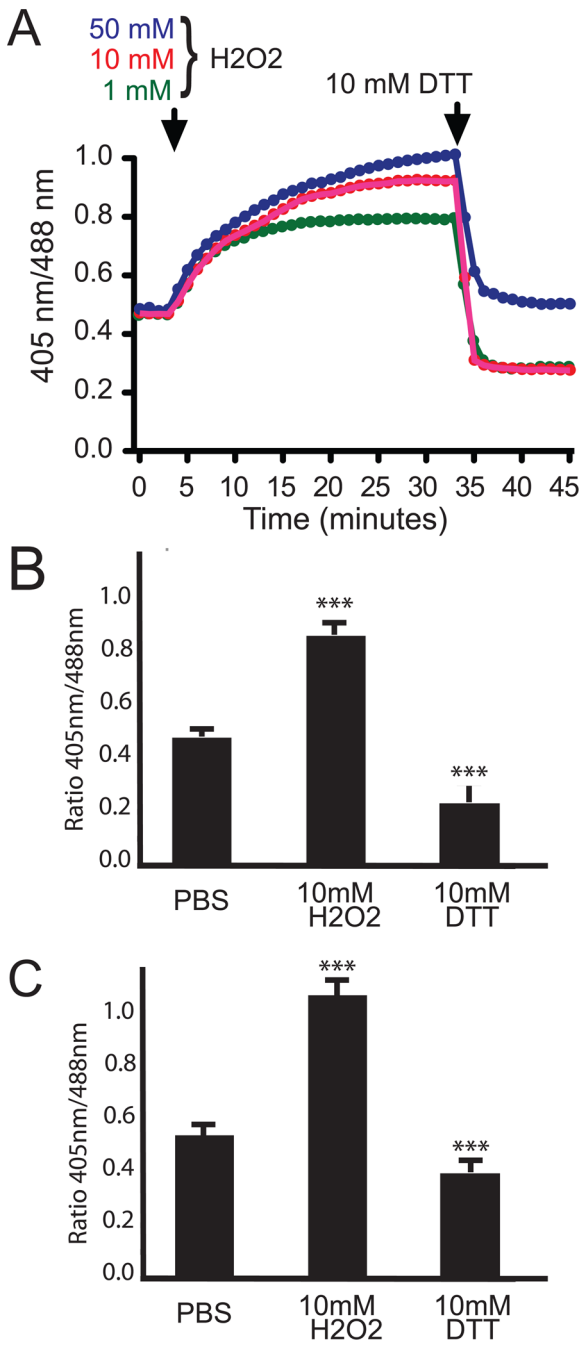

Figure 1. Dynamic change in redox-dependent fluorescence of MTSroGFP2

A) Representative time course of redox changes in primary neurons after the addition of H_2O_2 at indicated concentrations and then 10mM DTT. The increase in oxidative status observed was dose-responsive and immediately reversible upon DTT treatment. B & C) Responsiveness of MTSroGFP2 expressed within primary neurons to redox changes imaged via epifluorescence (B) or confocal microscopy (C). *** indicates a significant change (Student's t-test, $p<0.001$) from base line (PBS). For DTT treatment significance is compared to H_2O_2 levels.

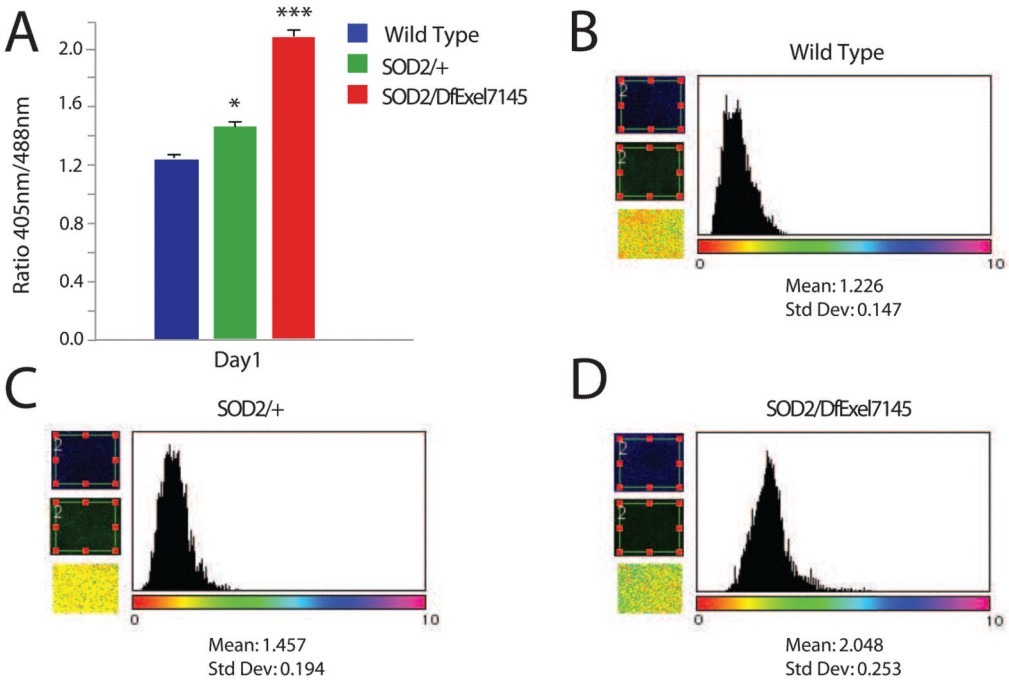

Figure 2. Increased redox status in SOD2 mutant flies

MTSroGFP2 fluorescence was examined in brains of SOD2/DfExl7145 mutants, heterozygotes (SOD2/+) and wild type controls. A) The SOD2 mutant flies exhibited an elevated redox status from age-matched controls (Student's T test, * is $p<0.05$, *** is $p<0.001$, n= 5 for each genotype). B-D) Ratio image calculations and histograms were obtained using ImageJ for each genotype indicated. Representative histograms are provided.

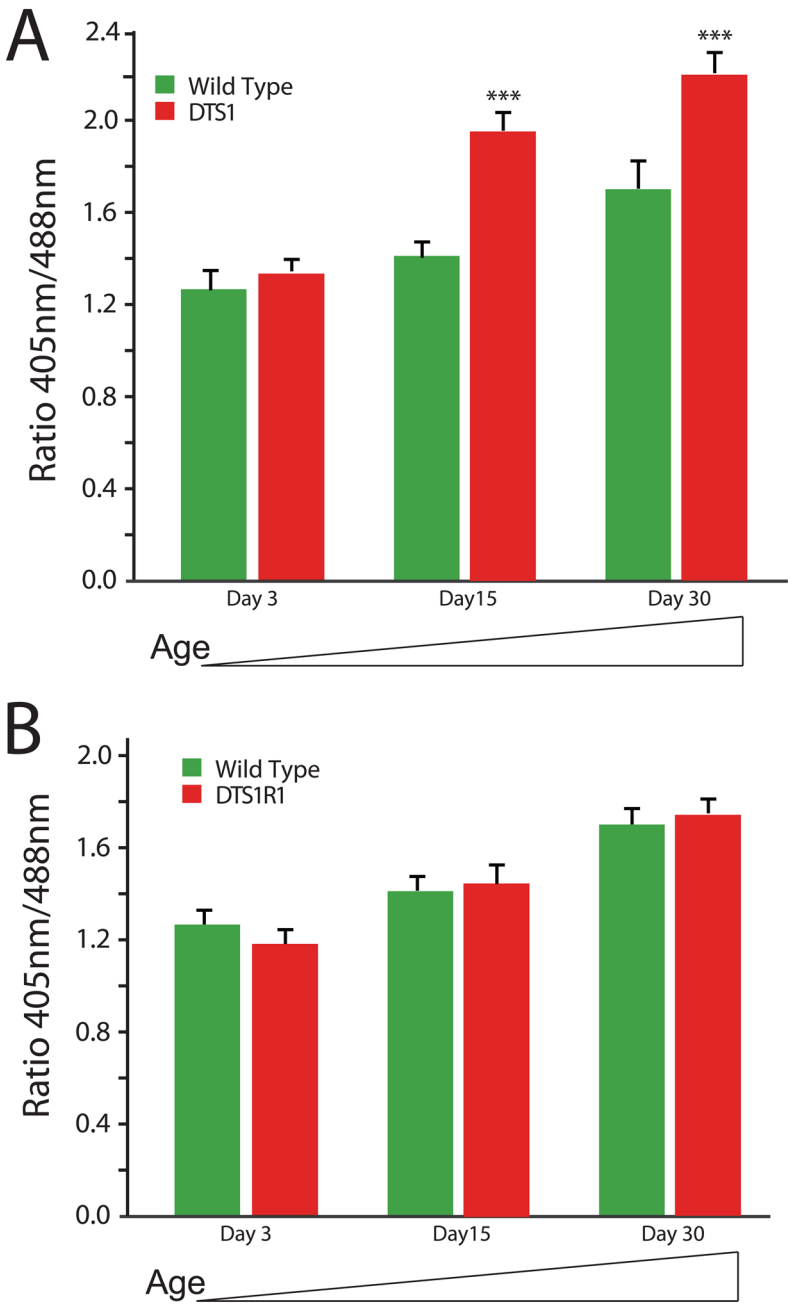

Figure 3. Progressive redox status in aged *ATPalpha* mutant flies

A) Both wild type and *ATPalpha*^{DTS1} mutant flies exhibit an age-dependent increase in neural ROS as measured using MTSroGFP2. At day 3, there is no difference between mutant and control brains (Student's t-test, P>0.05). At day 15 and day 30 the ROS levels are significant increased in *ATPalpha*^{DTS1} over age-matched control animals P<0.001(***). Wild type at day 30 are also increased from day 3 wild type (p<0.05). B) Wild type and *ATPalpha*^{DTS1R1} mutant flies exhibit an age-dependent increase in neural ROS as measured using MTSroGFP2. At all time points examined there is no difference between mutant and control brains (Student's t-test, P>0.05). Wild type at day 30 are also increased from day 3 wild type (p<0.05).

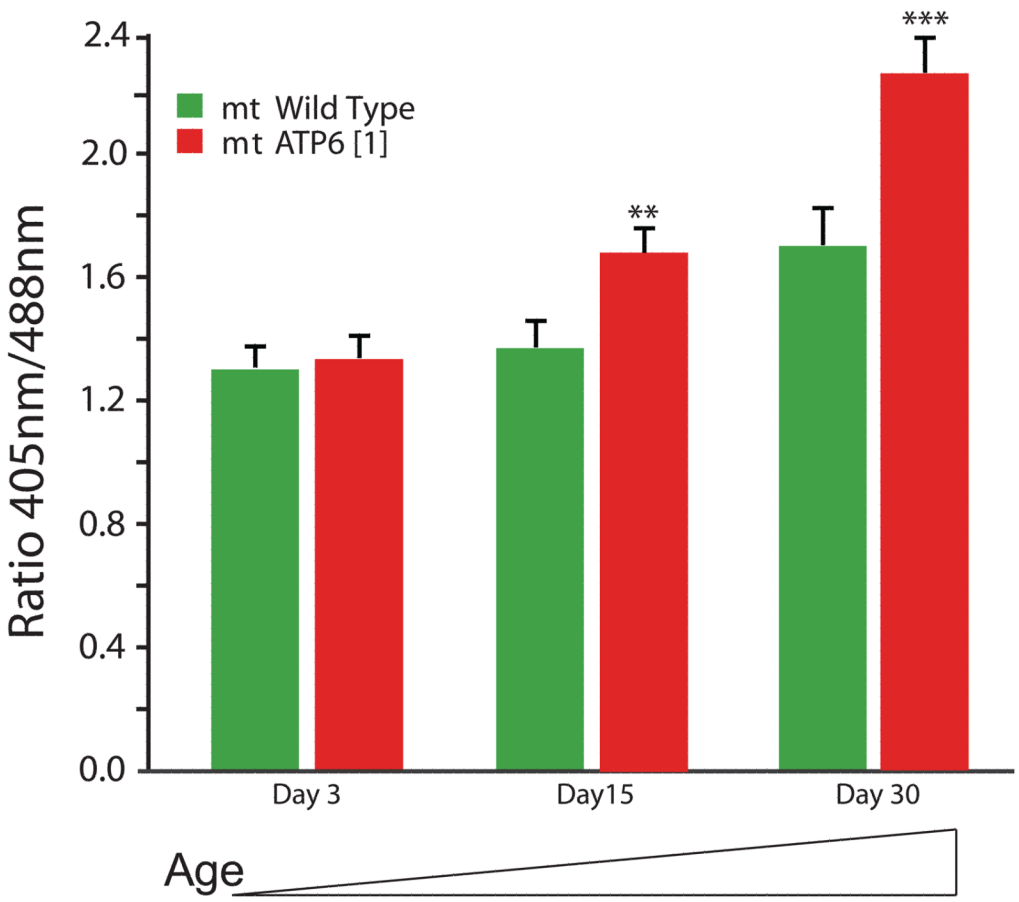

Figure 4. Progressive altered redox status in aged *ATP6*^{1} mutant flies

With age (day 15 and 30) *ATP6*^{1} mutants exhibit increased ROS compared to age-matched control animals that was not evident in young mutants. ** is p <0.01 and *** is p <0.001, Student's t-test. Wild type at day 30 are also increased from day 3 wild type (Student's t-test, p<0.05).

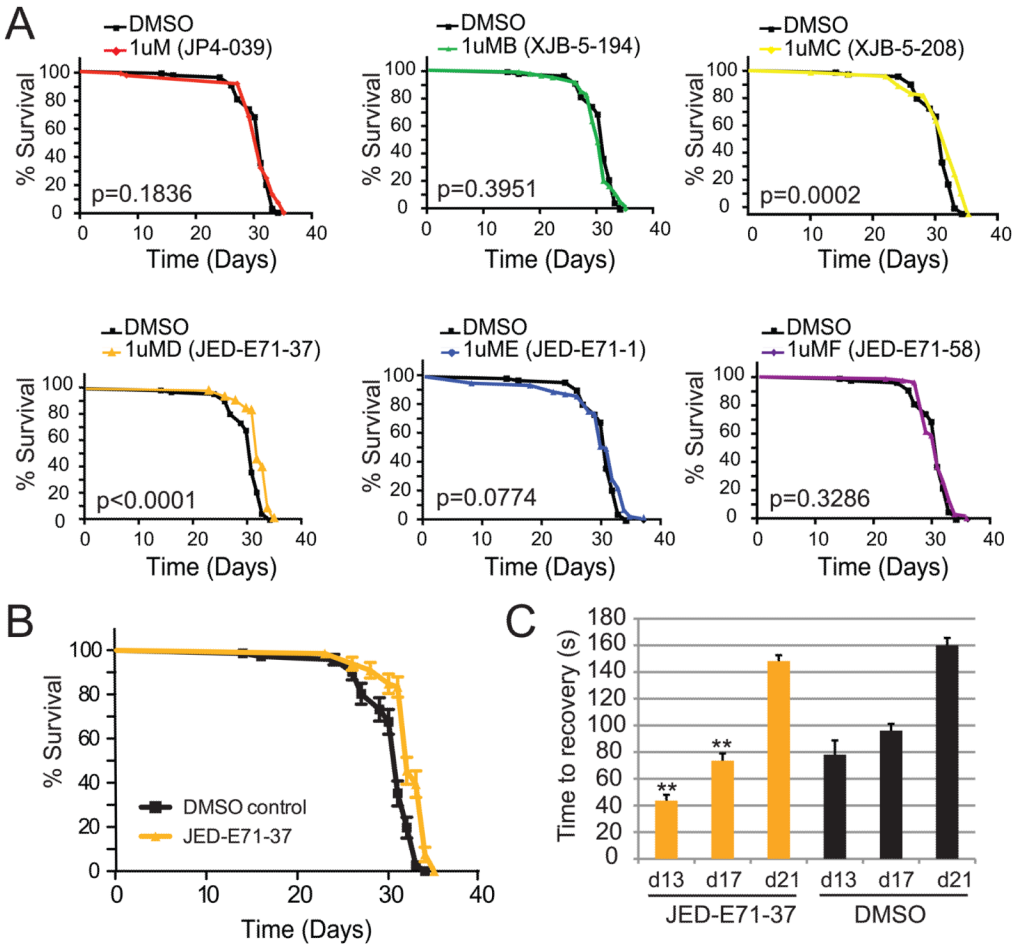

Figure 5. Mitochondrial targeted antioxidants improve *ATP6[1]* animals

A) Five mitochondrial-targeted ROS scavengers and a control (XJB-5-194) were tested for efficacy at improving longevity in *ATP6[1]* animals. Only JED-E71-37 significantly improved longevity. B) Treatment with JED-E71-37 results in a statistically significant and reproducible increase in lifespan. Shown are the combined independent replicates of JED-E71-37 and DMSO control treatments (log-rank test, $p < 0.0001$). C) Treatment with JED-E71-37 improves stress-sensitive locomotor function at day 13 and 17 but not significantly at day 21. ** $p<0.01$, student's t-test.

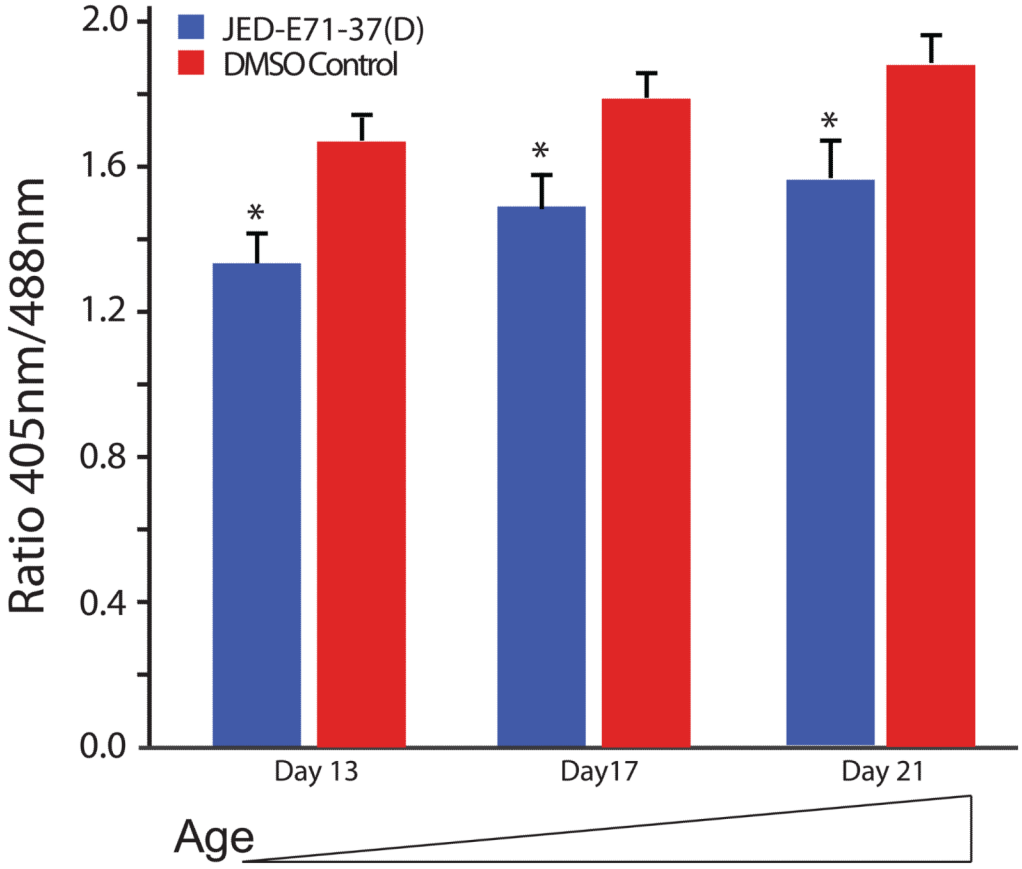

Figure 6. The mitochondrial-targeted antioxidant JED-E71-37 significantly reduces *ATP6*^{1} ROS

ATP6^{1} mutant flies chronically treated with JED-E71-37 have significantly reduced mitochondrial oxidative stress at all time points examined relative to age-matched vehicle only treated *ATP6*^{1} animals (*p<0.05).