Effects of pharmacological agents on the lifespan phenotype of Drosophila DJ-1β mutants

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Abstract

Mutations in the DJ-1 gene cause autosomal recessive, early-onset Parkinsonism. The DJ-1 protein exerts a protective role against oxidative stress damage, working as a cellular oxidative stress sensor, and it seems to regulate gene expression at different levels. In Drosophila, two DJ-1 orthologs have been identified: DJ-1α and DJ-1β. Several studies have shown that loss of DJ-1β function causes Parkinson’s disease (PD)-like phenotypes in flies such as age-dependent locomotor defects, reduced lifespan, and enhanced sensitivity to toxins that induce oxidative stress, like the herbicide paraquat. However, no dopaminergic neurodegeneration is observed. These results suggested that both locomotor and lifespan phenotypes could be either related to defects in oxidative stress response, or in dopaminergic physiology as proposed in mice models. In this study, we have employed pharmacological approaches to modify the lifespan phenotype of DJ-1β mutant flies. We have assessed the effects of chronic treatments with antiparkinsonian drugs as well as with antioxidant compounds on such phenotype finding that only antioxidants show statistically significant beneficial effects on DJ-1β mutants’ lifespan. These results strongly suggest that oxidative stress plays a causal role in the lifespan phenotype of DJ-1β mutants. Consistent with this, we find that loss of DJ-1β function results in cellular accumulation of reactive oxygen species (ROS) in adult brains, elevated levels of lipid peroxidation and an increased catalase enzymatic activity, thus indicating the existence of high oxidative stress levels in DJ-1β mutants and confirming the essential function of the DJ-1β protein in protecting the organism against oxidative insults. Our study further shows that the lifespan phenotype of DJ-1β mutant flies is amenable to pharmacological intervention, and validates Drosophila as a valuable model for testing and identifying new drugs with therapeutic potential for PD.
1. Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder. Although most PD cases are sporadic, several genes are associated with familial disease providing important insights into PD pathogenesis (Schultz, 2008). One of them is *PARK7* whose mutations cause autosomal recessive, early-onset Parkinsonism (Bonifati et al., 2003). *PARK7* (or *DJ-1*) encodes DJ-1, a small protein that was originally identified as an oncogenic factor (Nagakubo et al., 1997). However, studies in cell culture and animal models have demonstrated that the DJ-1 protein exerts a protective role against oxidative stress damage, being modified in several cysteine residues under these conditions (Canet-Aviles et al., 2004; Betarbet et al., 2006; Choi et al., 2006; Meulener et al., 2006). This modification increases with age and exposure to oxidative toxins, probably leading to DJ-1 inactivation and eventually predisposing dopaminergic (DA) neurons to stress-induced degeneration. Although the molecular function of DJ-1 is unclear, it seems that it can regulate gene expression either at the transcriptional or translational level and, interestingly, both functions are impaired by oxidation (van der Brug et al., 2008; Zhong and Xu, 2008; Blackington et al. 2009). It has been proposed a model whereby DJ-1 interacts with mRNAs and dissociates under conditions of oxidative stress, thus the apparent pleiotropic effects of DJ-1 may be related to the single function of binding to multiple transcripts (van der Brug et al., 2008).

In *Drosophila*, two *DJ-1* orthologs have been identified, *DJ-1α* and *DJ-1β*. Loss of function of these genes cause defects in oxidative stress response and locomotion as well as shortened lifespan, but only inactivation of *DJ-1α* expression via RNAi results in DA neurodegeneration (Meulener et al., 2005; Menzies et al., 2005; Park et al., 2005; Yang et al., 2005a; Lavara-Culebras and Paricio, 2007). *DJ-1β^{A286}* mutant flies were characterized in our laboratory and contain a transposable element insertion in the *DJ-1β* gene, thus encoding a truncated and
probably inactive DJ-1β protein. Despite this, they are viable and fertile but show motor impairments and a significantly shorter lifespan than wild-type flies. Moreover, they are highly sensitive to paraquat exposure (Lavara-Culebras and Paricio, 2007). Since loss of DJ-1β function does not affect DA neuron survival (Menzies et al., 2005; Meulener et al., 2005; Park et al., 2005; Lavara-Culebras and Paricio, 2007), one possibility is that the observed motor and lifespan phenotypes are due to defects in dopamine neurotransmission or DA neuron malfunction, as suggested in mice models (Chen et al., 2005; Goldberg et al., 2005).

Alternatively, they could be caused by oxidative damage. Supporting this, it was previously shown that age-dependent locomotor defects exhibited by DJ-1β mutants carrying a different allele were substantially enhanced by paraquat treatment (Park et al., 2005). Furthermore, a recent study has shown that two compounds with antioxidant and anti-inflammatory properties partially suppress DA neuron loss and locomotor dysfunction of DJ-1α RNAi flies (Faust et al., 2009). In such a scenario, we have investigated the molecular mechanisms involved in the lifespan phenotype of DJ-1β<sup>A286</sup> mutant flies, by testing the effects on this phenotype of several compounds that were added to the flies’ diet. In this study, DJ-1β mutant flies were chronically treated with representatives of the major classes of drugs used to mitigate the effects of dopaminergic neuron loss in PD patients, like L-DOPA or pergolide, and the experimental compound SK&F 38393, as well as with compounds that exhibit antioxidant activity, including vitamin C, melatonin or α-tocopherol. We find that lifespan of DJ-1β<sup>A286</sup> mutants is significantly extended after chronic treatments with antioxidant agents but it is unaffected by exposure to antiparkinsonian drugs. Although lifespan values are not restored to wild-type in the DJ-1β mutant flies, our results indicate that the lifespan phenotype is mediated, at least in part, by oxidative damage and not by defects in DA neuron physiology as previously suggested (Lavara-Culebras and Paricio, 2007). Confirming these results, we have observed an increase in several markers of oxidative stress in DJ-1β<sup>A286</sup> mutants when compared to control flies.
2. Materials and methods

2.1. Drosophila stocks and drug feeding

The stocks used in this study (y¹ w¹118, PBac{5HPw⁺}DJ-1β¹A²8⁶ and y¹, w¹118) were obtained from the Bloomington Stock Center. Flies were cultured on standard food supplemented with antiparkinsonian drugs like L-DOPA, SK&F 38393 and pergolide, or with the antioxidant compounds α-tocopherol and melatonin (purchased from Sigma) at a final concentration of 1 mM, unless otherwise stated. In non-treatment control experiments, flies were fed standard food containing the corresponding solvents recommended by the suppliers for each compound. Ascorbic acid (0.25 mg/ml) was also added to food containing L-DOPA and SK&F 38393 to prevent drug oxidation. In both cases, standard food containing 0.25 mg/ml of ascorbic acid was used as non-treatment control. L-DOPA treatment was also performed by using butylated hydroxytoluene (BHT) as antioxidant, and standard food containing 1 mM BHT was used as non-treatment control in this case. Drug and control feeding trials were carried out at 25°C from day 1-2 after eclosion through adulthood in standard vials containing 1.5 or 2 ml of medium.

2.2 Lifespan assays

Cohorts of 200 1-2-day-old DJ-1β¹A²8⁶ mutants or y, w control flies were collected and tested for each drug. Flies were separated by sex and divided in groups of 25 individuals. Those groups were put into vials with control or supplemented food. Every second or third day, flies were transferred to new vials and the number of dead flies in each vial was scored. This process was followed until all flies died, and the percentage of flies alive at each time point was graphed (Greene et al., 2003). The results were statistically tested with the Kapplan-Meier analysis with
a semiparametric log-rank test (Walker et al., 2006), and the analysis was performed with GraphPad Prism 4 software.

2.3. ROS staining of fly brains

For ROS staining of adult fly brains, 2,7-dichloroflourescein diacetate (DCFH-DA) (Molecular Probes) was used. A 1mM DCFH-DA stock solution was prepared in dimethylsulfoxide (DMSO). Brains were dissected in 1xPBS/0.1% Tween 20 and incubated with DCFH-DA (50 μM final concentration in 1xPBS) for 40 min at room temperature. After two washes with 1xPBS, brains were mounted in antifading solution (Dako) and analyzed in a fluorescence microscope (Leica).

2.4. Measurement of lipid hydroperoxides (LPO)

Quantification of lipid peroxidation was carried out by reaction of thiobarbituric acid with the malondialdehyde (MDA) product of oxidized fatty acid breakage (Buege and Aust, 1978). 100 flies (approx. 100 mg) per genotype were homogenized in 0.6 ml of 50 mM sodium phosphate buffer, pH 6.0, 10% trichloroacetic acid (TCA) and centrifuged at a speed of 10.000 rpm for 10 min. The supernatant was divided in two aliquots, 300 μl of supernatant were mixed to 100 μl of 0.1 M EDTA and 600 μl of 1% thiobarbituric acid in 0.05 M NaOH, and then incubated at 100°C 15 min. The second aliquot (300 μl) was mixed to 0.7 ml of H2O and incubated in the same conditions as described above. This sample was used as an internal absorbance control to avoid artifacts in LPO measurement as a consequence of the different eye color of y, w control flies and DJ-1β^{A286} mutants. After cooling on ice and centrifugation at 10.000 rpm for 1 min to eliminate precipitates, malondialdehyde was measured by the
absorbance at 535 nm. The molar absorptivity of MDA (1.56 ×10^5 M⁻¹ cm⁻¹) was used to express lipid peroxidation levels as pmoles of MDA per mg of flies.

2.5. Catalase activity

Catalase activity was measured by using a protocol adapted from Jakubowski et al. (2000). Fifty flies per genotype were homogenized in 200 μl of 50 mM phosphate buffer, pH 7.0, and a mix of protease inhibitors (200 μM phenylmethylsulfonyl fluoride (PMSF), 20 μM TPcK, 200 μM pepstatin A) and assayed by adding 0.1-1 μL of sample to 50 mM phosphate buffer, pH 7.0, and 80 mM H₂O₂. Absorbance decrease was measured at 240 nm and calculations were performed using an extinction coefficient of 0.043 mM⁻¹cm⁻¹ml⁻¹. Catalase activity is expressed as μmol of H₂O₂/min/mg of protein.

3. Results and Discussion

3.1. Effects of antiparkinsonian drugs on lifespan of DJ-1β^{A286} mutant flies

*DJ-1β^{A286}* mutant flies are viable, fertile, and display age-dependent motor deficits and a significantly shorter lifespan than wild-type flies, but no DA neurodegeneration (Lavara-Culebras and Paricio, 2007). To determine whether those phenotypes are due to defects in dopamine neurotransmission or DA neuron malfunction, as suggested in mice models (Chen et al., 2005; Goldberg et al., 2005), several antiparkinsonian drugs used to mitigate the effects of DA neuron loss in PD patients were tested for their ability to modify the reduced lifespan phenotype of *DJ-1β^{A286}* mutants (Fig. 1A). The drugs used in this study were: 3,4-dihydroxy-L-phenylalanine (L-DOPA), and the dopamine agonists 2,3,4,5-tetrahydro-7,8-dihydroxy-1-
phenyl-1H-3-benzazepine (SK&F 38393) and pergolide. L-DOPA, a synthetic precursor of
dopamine biosynthesis (Radad et al., 2005), is the most effective pharmacological symptomatic
treatment of PD. It has been shown that PD patients have a reduced life expectancy, and that
treatment with L-DOPA can suppress, but not restore, that reduction in some cases (Lang and
Lozano, 1998; Dauer and Przedborski, 2003). On the other hand, both dopamine agonists
activate dopamine receptors: SK&F 38393 is the prototypical D₁ receptor agonist (Pendleton et
al., 1978), and pergolide acts preferentially on the D₂ receptor family (Standaert and Young,
1996). Some of these drugs were shown to improve motor impairments of PD transgenic models
in *Drosophila* and mice (Pendleton et al., 2002; Wakamatsu et al., 2007), and to alleviate a
motility phenotype and DA neurodegeneration in a *Drosophila* model of hereditary spastic
paraplegia (Lee et al., 2008). Moreover, pergolide was reported to protect SH-SY5Y
neuroblastoma cells from H₂O₂-induced death (Uberti et al., 2003).

To test whether L-DOPA could rescue the lifespan phenotype of *DJ-1β<sup>A286</sup>* mutants, newly-
eclosed flies were cultured on medium supplemented with 1mM L-DOPA plus ascorbic acid (or
vitamin C, a water-soluble antioxidant) to avoid oxidation of the drug, or only ascorbic acid as
control. Due to the genetic background of the *DJ-1β<sup>A286</sup>* mutants, control experiments were
performed with *y, w* mutant flies (see Materials and methods). Analyses of treated and untreated
flies of both genotypes were carried out in parallel to avoid natural fluctuations in lifespan that
occurs in *D. melanogaster* in successive generations (Izmaylov and Obukhova, 1999). The same
experiments were performed in standard food in order to test whether ascorbic acid could have
an effect by itself on flies’ lifespan. Indeed, previous studies showed that 20 mM ascorbic acid
supplementation extended the average lifespan of control *rosy*<sup>+</sup> (<i>ry</i>]<sup>+</sup>) flies, although it caused
early mortality in at a 100 mM concentration (Bahadorani et al., 2008): However, it had not
significant effect on the short lifespan of *SOD1*-deficient flies (Bahadorani et al., 2008) and was
shown to increase the toxicity of paraquat in flies at 0.43 mM (Bonilla et al., 2006). Our results
show that median and maximum lifespan values of either control or $DJ-1\beta^{A286}$ mutant flies were significantly extended after ascorbic acid supplementation (Fig. 1B, $p<0.0001$). In y, w control flies, $\sim 1.42$ mM (0.25 mg/ml) vitamin C increased median and maximum lifespan values by 17.5% and 5.1%, respectively. Similar results were obtained for $DJ-1\beta$ mutants, whose median and maximum lifespan values were increased by 19.4% and 8.4%, respectively. However, our results show that L-DOPA had no significant effect on longevity of $DJ-1\beta^{A286}$ mutants or y, w control flies, which present similar survival curves either treated or untreated with the drug (Fig. 2). One possible explanation could be the existence of a synergistic effect of ascorbic acid and L-DOPA on flies’ lifespan. To test this possibility, we have carried out lifespan analyses of $DJ-1\beta^{A286}$ mutants and y, w control flies on medium supplemented with L-DOPA but using 1 mM butylated hydroxytoluene (BHT) as alternative antioxidant additive. Preliminary results indicate that there are no significant differences in survival curves of flies cultured on medium containing BHT or BHT plus L-DOPA (data not shown), thus confirming our previous results.

Next, we analyzed the effect of SK&F 38393 on lifespan of $DJ-1\beta^{A286}$ mutants and wild-type flies using the same experimental procedure. As for L-DOPA administration, SK&F 38393 containing medium was supplemented with ascorbic acid (see Materials and methods). We found that 1mM SK&F 38393 did not significantly modify lifespan in $DJ-1\beta^{A286}$ mutant flies (Fig. 3). However, SK&F 38393 supplementation had a weak but significant effect on wild-type flies ($p<0.0005$), increasing their median and maximum lifespan values by 6.3% and 5.2%, respectively. It was previously reported that 1 mM SK&F 38393 had no effect on the climbing response of wild-type Canton S flies but almost completely reversed the deteriorating motor activity in $\alpha$-synuclein transgenic flies (Pendleton et al., 2002). However, those climbing assays were performed after 13 days of treatment with the drug, while we first detect an effect on control flies’ lifespan after more than 30 days of treatment in our experiments. Then, it is likely that SK&F 38393 supplementation could also have an effect on the motor activity of wild-type
flies. Consistent with this, other studies performed in mice showed that SK&F 38393 caused a dose-dependent increase in locomotor activity of wild-type animals (Gomez et al., 1999).

Finally, when assaying the effect of the dopamine agonist pergolide on the lifespan of DJ-1βA286 mutants and wild-type flies we found that, in contrast to previous analyses carried out in a transgenic model of PD in Drosophila (Pendleton et al., 2002), the drug was toxic at a 1mM concentration for both genotypes causing a high mortality (Supplementary Figure 1). To determine whether this toxicity was dose-dependent we repeated the lifespan analyses using 0.5 and 0.25 mM pergolide and found that the drug was still affecting viability in adult flies, thus preventing further analyses in our PD Drosophila model. Taken together, our results indicate that the reduced lifespan phenotype exhibited by the DJ-1βA286 mutants is not due to defects in DA physiology as previously suggested (Lavara-Culebras and Paricio, 2007), since the drugs used were not able to suppress that phenotype.

3.2. Effects of antioxidant compounds on lifespan of DJ-1βA286 mutant flies

It has been shown that the DJ-1 protein is involved in oxidative stress response (van der Brug et al., 2008; Zhong and Xu, 2008; Blackington et al., 2009). According to this, DJ-1βA286 mutants exhibited high sensitivity to oxidative stress induced by the herbicide paraquat (Lavara-Culebras and Paricio, 2007). We then explored the putative involvement of oxidative damage in DJ-1β-inactivation-dependent lifespan reduction by treating DJ-1βA286 mutants with compounds that exhibit antioxidant activity, including melatonin (N-acetyl-5-methoxytryptamine) and α-tocopherol. As shown above, ascorbic acid (or vitamin C) was able to significantly increase lifespan values in DJ-1βA286 mutants and control flies (see Fig. 1B). Melatonin is a hormone that can exert several functions and seems to act as antioxidant and free radical scavenger, promoting the response against oxidative injuries (Karasek, 2004; Anisimov et al., 2006), and α-
tocopherol is a vitamin E isoform that is the most powerful naturally occurring antioxidant known to date (Ricciarelli et al., 2001; Tucker and Townsend, 2005).

First, we analyzed the effect of melatonin treatment on the lifespan of DJ-1β1286 mutants and y, w control flies. Our results show that 1mM melatonin significantly increased median lifespan values by 7.5% in control flies and by 19.4% in DJ-1β mutants, although maximum lifespan values were practically unaffected in both genotypes (Fig. 4). Despite this, we found that lifespan curves of untreated and treated flies are significantly different for both genotypes ($p<0.0001$). According to these results, it was reported that chronic treatments with melatonin were able to increase lifespan values in effects after in wild-type flies (Oregon R), and also survival of flies exposed to paraquat (Izmaylov and Obukhova, 1999; Bonilla et al., 2002, 2006). Furthermore, melatonin was also shown to rescue locomotor deficits and DA neurodegeneration in a Drosophila model of sporadic PD (Coulom and Birman, 2004).

We next examined the effects of α-tocopherol on the short lifespan phenotype associated to DJ-1β loss of function. We found that this phenotype was significantly suppressed by chronic treatment of adult flies with 1mM α-tocopherol on ($p<0.0001$), as evidenced by a 11.1% and 10.3% increase in median and maximum lifespan values, respectively. However, α-tocopherol supplementation did not modify lifespan values in control animals (Figure 5A). Our results are consistent with previous studies that showed that α-tocopherol supplementation only extended lifespan or suppressed phenotypes either associated to oxidative stress conditions or to genetically compromised backgrounds, but had no visible effects on lifespan under normal laboratory conditions (Nakashima et al., 2004; Wang et al., 2006; Dias-Santagata et al., 2007; Bahadorani et al., 2008). Indeed, it was proposed that wild-type flies retain very little vitamin E from the medium, probably due to their low biological needs for this antioxidant (Draper et al., 2000). However, flies with defective oxidative stress response could retain vitamin E more efficiently than wild-type flies (Zou et al., 2007; Bahadorani et al., 2008). Then, α-tocopherol
supplementation would strengthen antioxidant defenses against oxidative injuries, and would extend lifespan in such flies. The results obtained in the present study with $DJ-1\beta^{A286}$ mutant flies, which are highly sensitive to oxidative stress (Lavara-Culebras and Paricio, 2007), would confirm this hypothesis.

Finally, we tested whether there was a sex-dependent effect of antioxidant compounds on $DJ-1\beta^{A286}$ mutants' lifespan, since previous reports showed that females have a better response to antioxidant treatments than males (Magwere et al., 2006). Confirming those observations, we found that although both sexes show a similar increase in their maximum lifespan values after $\alpha$-tocopherol supplementation (13.3% in females and 10.3% in males, Figure 5B), $DJ-1\beta^{A286}$ females exhibit a higher increase of the median lifespan value than males (26.7% and 11.1%, respectively). Similar results are obtained after melatonin treatment of $DJ-1\beta^{A286}$ mutant flies (data not shown). The sex-dependent effects of antioxidant drugs on lifespan may be explained by differences in feeding habits, in metabolic rates or in physiological differences in the response to oxidative injuries between males and females, as previously suggested (Magwere et al., 2006).

Taken together, our data show that all antioxidant compounds tested were able to significantly increase lifespan values in $DJ-1\beta^{A286}$ mutants, suggesting that this phenotype is very likely caused by deficiencies in the oxidative stress response.

3.3. Quantification of oxidative stress levels in $DJ-1\beta^{A286}$ mutant flies

To further confirm that oxidative stress plays a causative role in the lifespan phenotype of $DJ-1\beta^{A286}$ mutant flies, we decided to measure several physiological markers of oxidative stress in such flies. We first used 2,7-dichloroflourescein diacetate (DCFH-DA) staining, which is an indicator of hydroxyl-free radical levels, to analyze ROS levels in $DJ-1\beta^{A286}$ mutants’ brains.
Our results indicate that brains of 1-2-day old DJ-1βA286 flies show an increased DCFH-DA staining when compared to brains of age-matched control flies (Fig. 6A-B), thus suggesting that loss of DJ-1β function leads to an elevation of ROS levels. Similar results were obtained when analyzing fly brains after DJ-1α-inactivation in DA neurons via RNAi (Yang et al., 2006a). As indicated in the Introduction, DJ-1α is the second Drosophila DJ-1 ortholog. To further investigate whether DJ-1β is involved in cellular response to endogenous oxidative stress, we also examined DJ-1βA286 mutant flies for the extent of oxidative damage by determining lipid peroxidation (LPO) levels and catalase enzymatic activity. As shown in Fig. 6B the total amount of malondialdehyde (MDA), one of the end stage products of lipid peroxidation, in DJ-1βA286 mutants was significantly higher than in y,w control flies (p<0.05) even in 1-2-day old flies. Besides, catalase activity was also significantly increased in these mutants compared to age-matched control flies (Fig. 6C, p<0.001). The increase of these two markers of oxidative stress was also observed in flies subjected to hyperoxia treatment as an oxidative stressor (Gruenewald et al., 2009). Taken together, these results support the crucial role of oxidative stress in the lifespan phenotype of DJ-1βA286 mutant flies. It has been proposed that the human DJ-1 protein may contribute to ROS removal both as a free radical scavenger and a transcriptional/translational regulator promoting the expression of genes involved in oxidative stress defense (van der Brug et al., 2008; Zhong and Xu, 2008; Blackington et al., 2009). Our results indicate that similar functions could be proposed for the Drosophila DJ-1β protein, since defects in the oxidative stress response are evident in DJ-1βA286 mutant flies.

3.4. Conclusions

In summary, our results strongly suggest that the reduced lifespan phenotype exhibited by DJ-1βA286 mutant flies is, at least in part, due to defects in the oxidative stress response, and not in
DA physiology as proposed in DJ-1 mice models. Indeed, we show that high levels of oxidative stress are detected in DJ-1βA286 mutant flies by using different methods. Confirming this, we find that long term dietary supplementation with antioxidants is effective in significantly increasing lifespan values of DJ-1βA286 mutants. Similar doses of these drugs have been shown to suppress phenotypes associated to oxidative damage in other fly models of human diseases (i.e. Wang et al., 2006; Dias-Santagata et al., 2007). Our results also indicate that these compounds are not sufficient to completely restore lifespan values observed in control flies. This may suggest that DJ-1β loss-of-function does not just limit lifespan through oxidative damage, but possibly through introduction of other pathologies that may also be life limiting.

We also find that, while vitamin C and melatonin (both water-soluble antioxidants) are able to extend lifespan in both DJ-1β mutants and wild-type flies, the beneficial effects of α-tocopherol (lipophylic antioxidant) appear to be selective to the DJ-1β mutant flies. One explanation could be that these mutants may retain the compound more efficiently than wild-type (y, w) controls, as previously suggested for other flies with defective oxidative response (Bahadorani et al., 2008). Alternatively, this result could suggest that loss of DJ-1β function may cause oxidative damage to the membranes. Supporting this hypothesis we find a high MDA content in DJ-1β mutant flies, which is an indicator of high levels of lipid peroxidation and of free radical damage to cell membranes.

Similar beneficial effects of antioxidant compounds on several mutant backgrounds and Drosophila models of human diseases have been reported. Indeed, α-tocopherol supplementation has been shown to suppress several phenotypes found in Drosophila models of neurodegenerative diseases like taupathies (Dias-Santagata et al., 2007), PD (Wang et al., 2006), or pantothenate-kinase-associated neurodegeneration (PKAN, Yang et al., 2005b), and to extend lifespan of flies with deficiencies in oxidative stress response (Bahadorani et al., 2008), although it could not rescue the lethal phenotype of a Drosophila model of Huntington’s disease.
(Bahadorani and Hilliker, 2008). Moreover, vitamin E was also effective in suppressing phenotypes found in mouse models of human tauopathy (Nakashima et al., 2004) or Down syndrome (Lockrow et al., 2009), counterparting the increased oxidative stress observed in those models. Regarding this, vitamin E has proved efficacious in reducing markers of oxidative stress (Pratico et al., 1998; Hong et al., 2004), restoring endogenous antioxidant enzyme functions to normal levels in the mouse model of Down syndrome (Lockrow et al., 2009) and in rodent models of oxidative stress (Zaidi and Banu, 2004). Although these effects can be achieved through its antioxidant function, the non-antioxidant properties of α-tocopherol in modulating gene expression may also play a role (Ricciarelli et al., 2001; 2007; Tucker and Townsend, 2005). Then, it can be proposed that α-tocopherol could suppress the lifespan phenotype of DJ-1βA286 mutant flies based on its antioxidant function, eliminating ROS and then preventing DJ-1β oxidation, but also affecting either some signaling pathways that regulate DJ-1β oxidation or genes important to fly survival whose expression may be compromised by lack of DJ-1β function.

Our present study further shows that the lifespan phenotype of DJ-1βA286 mutant flies is amenable to pharmacological intervention since manipulation of antioxidant defence mechanisms with several compounds can modify such phenotype. Interestingly, a recent study has shown that treatments with minocycline and celestrol, two drugs with antioxidant and anti-inflammatory activities, are able to attenuate DA neuron loss and reduction of brain dopamine levels as well as locomotor defects associated to DJ-1α-inactivation via RNAi in flies (Faust et al., 2009). Treatments with both drugs partially suppress those phenotypes and have no significant effect on DA neuron number, dopamine levels or climbing activity in control flies. Since DJ-1α is the second ortholog of the human DJ-1 gene in Drosophila, these findings emphasize the role of oxidative stress in DJ-1 pathogenesis and support the use of this organism as a model to elucidate the molecular mechanisms underlying PD. Thus, Drosophila DJ-1
mutants could serve as a good model for pharmacological rescue studies of PD, enabling efficient screening of compounds able to suppress PD-like phenotypes. This could lead to the identification of novel factors involved in DJ-1-related PD pathogenesis and compounds to prevent oxidative stress occurring during PD in humans.

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**References**


**Figure legends**

**Figure 1.** Survival curves of *y, w* control flies and *DJ-1β^A286* mutants either untreated and after chronic treatment with vitamin C. (A) Comparison of survival curves of *y, w* control flies (black squares) and *DJ-1β^A286* mutants cultured in standard fly food. *DJ-1β^A286* mutant flies have a significantly shorter lifespan than *y, w* control flies as determined by using Kaplan-Meier log-rank statistical test (*p*<0.0001). (B) Survival curves of *y, w* control flies and *DJ-1β^A286* mutants after chronic treatment with 0.25 mg/ml (~1.42 mM) vitamin C. Flies of both genotypes treated with ascorbic acid (open triangles) show significant increase of their lifespan values. The median and maximum lifespan values are as follows: *y, w* untreated 40 and 67.5 days; *y, w* treated with ascorbic acid 47 and 71 days; *DJ-1β^A286* untreated 36 and 53.5 days; *DJ-1β^A286* treated 43 and 58 days. The significance of the difference between survival curves of treated and untreated flies was analyzed using the Kaplan-Meier log-rank statistical test (*p*<0.0001 in both cases).

**Figure 2.** Survival curves of *y, w* control flies and *DJ-1β^A286* mutants after chronic treatment with 1mM L-DOPA. The median and maximum lifespan values are as follows: *y, w* untreated or treated with L-DOPA 43 and 71 days; *DJ-1β^A286* untreated 45 and 58 days; *DJ-1β^A286* treated 47 and 59 days. For both genotypes, there are no significant differences between untreated (black squares) and L-DOPA treated flies (open triangles) as determined by using the Kaplan-Meier log-rank statistical test.

**Figure 3.** Survival curves of *y, w* control flies and *DJ-1β^A286* mutants after chronic treatment with 1mM SK&F 38393. The median and maximum lifespan values are as follows: *y, w* untreated 47 and 67.5 days; *y, w* treated with SK&F 38393 50 and 71 days; *DJ-1β^A286* untreated
36 and 53.5 days; $DJ-I\beta^{A286}$ treated 38 and 55.5 days. Treated $y, w$ control flies (open triangles) show a mild but significant increase of their lifespan values when compared to untreated $y, w$ flies (black squares) as determined by using Kaplan-Meier log-rank statistical test ($p<0.0005$). In contrast, SK&F 38393 treated and untreated $DJ-I\beta^{A286}$ mutants do not show significant differences.

**Figure 4.** Survival curves of $y, w$ control flies and $DJ-I\beta^{A286}$ mutants after chronic treatment with 1mM melatonin. The median and maximum lifespan values are as follows: $y, w$ untreated 40 and 62.5 days; $y, w$ treated with melatonin 43 and 64 days; $DJ-I\beta^{A286}$ untreated 36 and 57 days; $DJ-I\beta^{A286}$ treated 43 and 55.5 days. Melatonin treatment results in a mild but statistically significant increase of their lifespan values (especially of median lifespan) in both genotypes. The significance of the difference between survival curves of treated and untreated flies was analyzed using the Kaplan-Meier log-rank statistical test ($p<0.0001$ for both genotypes).

**Figure 5.** Survival curves of $y, w$ control flies and $DJ-I\beta^{A286}$ mutants after chronic treatment with 1mM $\alpha$-tocopherol. (A) Comparison of survival curves of control and $DJ-I\beta^{A286}$ flies after chronic treatment with 1mM $\alpha$-tocopherol. The median and maximum lifespan values are as follows: $y, w$ untreated 40 and 68.5 days; $y, w$ treated with $\alpha$-tocopherol 40 and 61.5 days; $DJ-I\beta^{A286}$ untreated 36 and 48.5 days; $DJ-I\beta^{A286}$ treated 40 and 53.5 days. $\alpha$-tocopherol supplementation results in a significant increase of $DJ-I\beta^{A286}$ mutants lifespan values as determined by using Kaplan-Meier log-rank statistical test ($p<0.0001$). In contrast, $y, w$ flies do not show significant changes in their lifespan values. (B) Survival curves of male and female $DJ-I\beta^{A286}$ mutant flies after chronic treatment with 1mM $\alpha$-tocopherol. The median and maximum lifespan values are as follows: $DJ-I\beta^{A286}$ males untreated 36 and 48.5 days; $DJ-I\beta^{A286}$ males treated 40 and 53.4 days; $DJ-I\beta^{A286}$ females untreated 30 and 44 days; $DJ-I\beta^{A286}$ females
treated 38 and 50 days. In both sexes, α-tocopherol supplementation significantly extends lifespan as determined by using Kaplan-Meier log-rank statistical test ($p<0.0001$ in both cases). However, females show higher increase of the lifespan values than males.

**Figure 6.** Oxidative stress markers in $y, w$ control flies and $DJ-I\beta^{A286}$ mutants. (A-B) DCFH-DA staining of whole-mount adult brains from 1-2-day-old $y, w$ and $DJ-I\beta^{A286}$ flies. Weak signals in A represent a combination of weak ROS staining and autofluorescence of adult fly brains. Note that loss of $DJ-I\beta$ function leads to an elevation of ROS levels (compare A with B). (C) Increase in lipid peroxidation (LPO) product malondialdehyde (MDA) in 1-2-day old $DJ-I\beta^{A286}$ mutants compared to age-matched $y, w$ control flies ($p<0.05$). (D) Catalase (CAT) enzymatic activity is also increased in 1-2-day old $DJ-I\beta^{A286}$ mutants compared to age-matched $y, w$ control flies ($p<0.001$). In C and D data are expressed as mean and standard deviation of replicate determinations for 4 and 3 samples, respectively.
Effects of pharmacological agents on the lifespan phenotype of Drosophila DJ-1β mutants

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Abstract

Mutations in the DJ-1 gene cause autosomal recessive, early-onset Parkinsonism. The DJ-1 protein exerts a protective role against oxidative stress damage, working as a cellular oxidative stress sensor, and it seems to regulate gene expression at different levels. In Drosophila, two DJ-1 orthologs have been identified: DJ-1α and DJ-1β. Several studies have shown that loss of DJ-1β function causes Parkinson’s disease (PD)-like phenotypes in flies such as age-dependent locomotor defects, reduced lifespan, and enhanced sensitivity to toxins that induce oxidative stress, like the herbicide paraquat. However, no dopaminergic neurodegeneration is observed. These results suggested that both locomotor and lifespan phenotypes could be either related to defects in oxidative stress response, or in dopaminergic physiology as proposed in mice models. In this study, we have employed pharmacological approaches to modify the lifespan phenotype of DJ-1β mutant flies. We have assessed the effects of chronic treatments with antiparkinsonian drugs as well as with antioxidant compounds on such phenotype finding that only antioxidants show statistically significant beneficial effects on DJ-1β mutants’ lifespan. These results strongly suggest that oxidative stress plays a causal role in the lifespan phenotype of DJ-1β mutants. Consistent with this, we find that loss of DJ-1β function results in cellular accumulation of reactive oxygen species (ROS) in adult brains, elevated levels of lipid peroxidation and an increased catalase enzymatic activity, thus indicating the existence of high oxidative stress levels in DJ-1β mutants and confirming the essential function of the DJ-1β protein in protecting the organism against oxidative insults. Our study further shows that the lifespan phenotype of DJ-1β mutant flies is amenable to pharmacological intervention, and validates Drosophila as a valuable model for testing and identifying new drugs with therapeutic potential for PD.
1. Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder. Although most PD cases are sporadic, several genes are associated with familial disease providing important insights into PD pathogenesis (Schultz, 2008). One of them is PARK7 whose mutations cause autosomal recessive, early-onset Parkinsonism (Bonifati et al., 2003). PARK7 (or DJ-1) encodes DJ-1, a small protein that was originally identified as an oncogenic factor (Nagakubo et al., 1997). However, studies in cell culture and animal models have demonstrated that the DJ-1 protein exerts a protective role against oxidative stress damage, being modified in several cysteine residues under these conditions (Canet-Aviles et al., 2004; Betarbet et al., 2006; Choi et al., 2006; Meulener et al., 2006). This modification increases with age and exposure to oxidative toxins, probably leading to DJ-1 inactivation and eventually predisposing dopaminergic (DA) neurons to stress-induced degeneration. Although the molecular function of DJ-1 is unclear, it seems that it can regulate gene expression either at the transcriptional or translational level and, interestingly, both functions are impaired by oxidation (van der Brug et al., 2008; Zhong and Xu, 2008; Blackington et al. 2009). It has been proposed a model whereby DJ-1 interacts with mRNAs and dissociates under conditions of oxidative stress, thus the apparent pleiotropic effects of DJ-1 may be related to the single function of binding to multiple transcripts (van der Brug et al., 2008).

In Drosophila, two DJ-1 orthologs have been identified, DJ-1α and DJ-1β. Loss of function of these genes cause defects in oxidative stress response and locomotion as well as shortened lifespan, but only inactivation of DJ-1α expression via RNAi results in DA neurodegeneration (Meulener et al., 2005; Menzies et al., 2005; Park et al., 2005; Yang et al., 2005a; Lavara-Culebras and Paricio, 2007). DJ-1β_{A286} mutant flies were characterized in our laboratory and contain a transposable element insertion in the DJ-1β gene, thus encoding a truncated and
probably inactive DJ-1β protein. Despite this, they are viable and fertile but show motor impairments and a significantly shorter lifespan than wild-type flies. Moreover, they are highly sensitive to paraquat exposure (Lavara-Culebras and Paricio, 2007). Since loss of DJ-1β function does not affect DA neuron survival (Menzies et al., 2005; Meulener et al., 2005; Park et al., 2005; Lavara-Culebras and Paricio, 2007), one possibility is that the observed motor and lifespan phenotypes are due to defects in dopamine neurotransmission or DA neuron malfunction, as suggested in mice models (Chen et al., 2005; Goldberg et al., 2005). Alternatively, they could be caused by oxidative damage. Supporting this, it was previously shown that age-dependent locomotor defects exhibited by DJ-1β mutants carrying a different allele were substantially enhanced by paraquat treatment (Park et al., 2005). Furthermore, a recent study has shown that two compounds with antioxidant and anti-inflammatory properties partially suppress DA neuron loss and locomotor dysfunction of DJ-1α RNAi flies (Faust et al., 2009). In such a scenario, we have investigated the molecular mechanisms involved in the lifespan phenotype of DJ-1βA286 mutant flies, by testing the effects on this phenotype of several compounds that were added to the flies’ diet. In this study, DJ-1β mutant flies were chronically treated with representatives of the major classes of drugs used to mitigate the effects of dopaminergic neuron loss in PD patients, like L-DOPA or pergolide, and the experimental compound SK&F 38393, as well as with compounds that exhibit antioxidant activity, including vitamin C, melatonin or α-tocopherol. We find that lifespan of DJ-1βA286 mutants is significantly extended after chronic treatments with antioxidant agents but it is unaffected by exposure to antiparkinsonian drugs. Although lifespan values are not restored to wild-type in the DJ-1β mutant flies, our results indicate that the lifespan phenotype is mediated, at least in part, by oxidative damage and not by defects in DA neuron physiology as previously suggested (Lavara-Culebras and Paricio, 2007). Confirming these results, we have observed an increase in several markers of oxidative stress in DJ-1βA286 mutants when compared to control flies.
2. Materials and methods

2.1. Drosophila stocks and drug feeding

The stocks used in this study (y¹ w¹1118, PBac[5HPw¹]DJ-1β¹²⁸⁶ and y¹, w¹1118) were obtained from the Bloomington Stock Center. Flies were cultured on standard food supplemented with antiparkinsonian drugs like L-DOPA, SK&F 38393 and pergolide, or with the antioxidant compounds α-tocopherol and melatonin (purchased from Sigma) at a final concentration of 1 mM, unless otherwise stated. In non-treatment control experiments, flies were fed standard food containing the corresponding solvents recommended by the suppliers for each compound. Ascorbic acid (0.25 mg/ml) was also added to food containing L-DOPA and SK&F 38393 to prevent drug oxidation. In both cases, standard food containing 0.25 mg/ml of ascorbic acid was used as non-treatment control. L-DOPA treatment was also performed by using butylated hydroxytoluene (BHT) as antioxidant, and standard food containing 1 mM BHT was used as non-treatment control in this case. Drug and control feeding trials were carried out at 25°C from day 1-2 after eclosion through adulthood in standard vials containing 1.5 or 2 ml of medium.

2.2 Lifespan assays

Cohorts of 200 1-2-day-old DJ-1β¹²⁸⁶ mutants or y, w control flies were collected and tested for each drug. Flies were separated by sex and divided in groups of 25 individuals. Those groups were put into vials with control or supplemented food. Every second or third day, flies were transferred to new vials and the number of dead flies in each vial was scored. This process was followed until all flies died, and the percentage of flies alive at each time point was graphed (Greene et al., 2003). The results were statistically tested with the Kapplan-Meier analysis with
a semiparametric log-rank test (Walker et al., 2006), and the analysis was performed with
GraphPad Prism 4 software.

2.3. ROS staining of fly brains

For ROS staining of adult fly brains, 2,7-dichloroflourescein diacetate (DCFH-DA)
(Molecular Probes) was used. A 1mM DCFH-DA stock solution was prepared in
dimethylsulfoxide (DMSO). Brains were dissected in 1xPBS/0.1% Tween 20 and incubated
with DCFH-DA (50 μM final concentration in 1xPBS) for 40 min at room temperature. After
two washes with 1xPBS, brains were mounted in antifading solution (Dako) and analyzed in a
fluorescence microscope (Leica).

2.4. Measurement of lipid hydroperoxides (LPO)

Quantification of lipid peroxidation was carried out by reaction of thiobarbituric acid with
the malondialdehyde (MDA) product of oxidized fatty acid breakage (Buege and Aust, 1978).
100 flies (approx. 100 mg) per genotype were homogenized in 0.6 ml of 50 mM sodium
phosphate buffer, pH 6.0, 10% trichloroacetic acid (TCA) and centrifuged at a speed of 10.000
rpm for 10 min. The supernatant was divided in two aliquots, 300 μl of supernatant were mixed
to 100 μl of 0.1 M EDTA and 600 μl of 1% thiobarbituric acid in 0.05 M NaOH, and then
incubated at 100ºC 15 min. The second aliquot (300 μl) was mixed to 0.7 ml of H₂O and
incubated in the same conditions as described above. This sample was used as an internal
absorbance control to avoid artifacts in LPO measurement as a consequence of the different eye
color of y, w control flies and DJ-1βA286 mutants. After cooling on ice and centrifugation at
10.000 rpm for 1 min to eliminate precipitates, malondialdehyde was measured by the
absorbance at 535 nm. The molar absorptivity of MDA \((1.56 \times 10^5 \text{M}^{-1} \text{cm}^{-1})\) was used to express lipid peroxidation levels as pmoles of MDA per mg of flies.

2.5. Catalase activity

Catalase activity was measured by using a protocol adapted from Jakubowski et al. (2000). Fifty flies per genotype were homogenized in 200 μl of 50 mM phosphate buffer, pH 7.0, and a mix of protease inhibitors (200 μM phenylmethylsulfonyl fluoride (PMSF), 20 μM TPcK, 200 μM pepstatin A) and assayed by adding 0.1-1 μL of sample to 50 mM phosphate buffer, pH 7.0, and 80 mM H₂O₂. Absorbance decrease was measured at 240 nm and calculations were performed using an extinction coefficient of 0.043 mM⁻¹cm⁻¹ml⁻¹. Catalase activity is expressed as μmol of H₂O₂/min/mg of protein.

3. Results and Discussion

3.1. Effects of antiparkinsonian drugs on lifespan of DJ-1β⁴²₈₆ mutant flies

*DJ-1β⁴²₈₆* mutant flies are viable, fertile, and display age-dependent motor deficits and a significantly shorter lifespan than wild-type flies, but no DA neurodegeneration (Lavara-Culebras and Paricio, 2007). To determine whether those phenotypes are due to defects in dopamine neurotransmission or DA neuron malfunction, as suggested in mice models (Chen et al., 2005; Goldberg et al., 2005), several antiparkinsonian drugs used to mitigate the effects of DA neuron loss in PD patients were tested for their ability to modify the reduced lifespan phenotype of *DJ-1β⁴²₈₆* mutants (Fig. 1A). The drugs used in this study were: 3,4-dihydroxy-L-phenylalanine (L-DOPA), and the dopamine agonists 2,3,4,5-tetrahydro-7,8-dihydroxy-1-
phenyl-1H-3-benzazepine (SK&F 38393) and pergolide. L-DOPA, a synthetic precursor of
dopamine biosynthesis (Radad et al., 2005), is the most effective pharmacological symptomatic
treatment of PD. It has been shown that PD patients have a reduced life expectancy, and that
treatment with L-DOPA can suppress, but not restore, that reduction in some cases (Lang and Lozano, 1998; Dauer and Przedborski, 2003). On the other hand, both dopamine agonists
activate dopamine receptors: SK&F 38393 is the prototypical D₁ receptor agonist (Pendleton et
al., 1978), and pergolide acts preferentially on the D₂ receptor family (Standaert and Young,
1996). Some of these drugs were shown to improve motor impairments of PD transgenic models
in Drosophila and mice (Pendleton et al., 2002; Wakamatsu et al., 2007), and to alleviate a
motility phenotype and DA neurodegeneration in a Drosophila model of hereditary spastic
paraplegia (Lee et al., 2008). Moreover, pergolide was reported to protect SH-SY5Y
neuroblastoma cells from H₂O₂-induced death (Uberti et al., 2003).

To test whether L-DOPA could rescue the lifespan phenotype of DJ-1β^{A286} mutants, newly-
eclosed flies were cultured on medium supplemented with 1mM L-DOPA plus ascorbic acid (or
vitamin C, a water-soluble antioxidant) to avoid oxidation of the drug, or only ascorbic acid as
control. Due to the genetic background of the DJ-1β^{A286} mutants, control experiments were
performed with y, w mutant flies (see Materials and methods). Analyses of treated and untreated
flies of both genotypes were carried out in parallel to avoid natural fluctuations in lifespan that
occurs in D. melanogaster in successive generations (Izmaylov and Obukhova, 1999). The same
experiments were performed in standard food in order to test whether ascorbic acid could have
an effect by itself on flies’ lifespan. Indeed, previous studies showed that 20 mM ascorbic acid
supplementation extended the average lifespan of control rosy^{+5} (ry^{+5}) flies, although it caused
early mortality in at a 100 mM concentration (Bahadorani et al., 2008): However, it had not
significant effect on the short lifespan of SOD1-deficient flies (Bahadorani et al., 2008) and was
shown to increase the toxicity of paraquat in flies at 0.43 mM (Bonilla et al., 2006). Our results
show that median and maximum lifespan values of either control or $DJ-1\beta^{A286}$ mutant flies were significantly extended after ascorbic acid supplementation (Fig. 1B, $p<0.0001$). In y, w control flies, $\sim 1.42$ mM (0.25 mg/ml) vitamin C increased median and maximum lifespan values by 17.5% and 5.1%, respectively. Similar results were obtained for $DJ-1\beta$ mutants, whose median and maximum lifespan values were increased by 19.4% and 8.4%, respectively. However, our results show that L-DOPA had no significant effect on longevity of $DJ-1\beta^{A286}$ mutants or y, w control flies, which present similar survival curves either treated or untreated with the drug (Fig. 2). One possible explanation could be the existence of a synergistic effect of ascorbic acid and L-DOPA on flies’ lifespan. To test this possibility, we have carried out lifespan analyses of $DJ-1\beta^{A286}$ mutants and y, w control flies on medium supplemented with L-DOPA but using 1 mM butylated hydroxytoluene (BHT) as alternative antioxidant additive. Preliminary results indicate that there are no significant differences in survival curves of flies cultured on medium containing BHT or BHT plus L-DOPA (data not shown), thus confirming our previous results.

Next, we analyzed the effect of SK&F 38393 on lifespan of $DJ-1\beta^{A286}$ mutants and wild-type flies using the same experimental procedure. As for L-DOPA administration, SK&F 38393 containing medium was supplemented with ascorbic acid (see Materials and methods). We found that 1mM SK&F 38393 did not significantly modify lifespan in $DJ-1\beta^{A286}$ mutant flies (Fig. 3). However, SK&F 38393 supplementation had a weak but significant effect on wild-type flies ($p<0.0005$), increasing their median and maximum lifespan values by 6.3% and 5.2%, respectively. It was previously reported that 1 mM SK&F 38393 had no effect on the climbing response of wild-type Canton S flies but almost completely reversed the deteriorating motor activity in $\alpha$-synuclein transgenic flies (Pendleton et al., 2002). However, those climbing assays were performed after 13 days of treatment with the drug, while we first detect an effect on control flies’ lifespan after more than 30 days of treatment in our experiments. Then, it is likely that SK&F 38393 supplementation could also have an effect on the motor activity of wild-type
flies. Consistent with this, other studies performed in mice showed that SK&F 38393 caused a dose-dependent increase in locomotor activity of wild-type animals (Gomeza et al., 1999).

Finally, when assaying the effect of the dopamine agonist pergolide on the lifespan of DJ-1βA286 mutants and wild-type flies we found that, in contrast to previous analyses carried out in a transgenic model of PD in Drosophila (Pendleton et al., 2002), the drug was toxic at a 1mM concentration for both genotypes causing a high mortality (Supplementary Figure 1). To determine whether this toxicity was dose-dependent we repeated the lifespan analyses using 0.5 and 0.25 mM pergolide and found that the drug was still affecting viability in adult flies, thus preventing further analyses in our PD Drosophila model. Taken together, our results indicate that the reduced lifespan phenotype exhibited by the DJ-1βA286 mutants is not due to defects in DA physiology as previously suggested (Lavara-Culebras and Paricio, 2007), since the drugs used were not able to suppress that phenotype.

3.2. Effects of antioxidant compounds on lifespan of DJ-1βA286 mutant flies

It has been shown that the DJ-1 protein is involved in oxidative stress response (van der Brug et al., 2008; Zhong and Xu, 2008; Blackington et al., 2009). According to this, DJ-1βA286 mutants exhibited high sensitivity to oxidative stress induced by the herbicide paraquat (Lavara-Culebras and Paricio, 2007). We then explored the putative involvement of oxidative damage in DJ-1β-inactivation-dependent lifespan reduction by treating DJ-1βA286 mutants with compounds that exhibit antioxidant activity, including melatonin (N-acetyl-5-methoxytryptamine) and α-tocopherol. As shown above, ascorbic acid (or vitamin C) was able to significantly increase lifespan values in DJ-1βA286 mutants and control flies (see Fig. 1B). Melatonin is a hormone that can exert several functions and seems to act as antioxidant and free radical scavenger, promoting the response against oxidative injuries (Karasek, 2004; Anisimov et al., 2006), and α-
tocopherol is a vitamin E isoform that is the most powerful naturally occurring antioxidant known to date (Ricciarelli et al., 2001; Tucker and Townsend, 2005).

First, we analyzed the effect of melatonin treatment on the lifespan of $DJ-1^{\beta_1286}$ mutants and $y, w$ control flies. Our results show that 1mM melatonin significantly increased median lifespan values by 7.5% in control flies and by 19.4% in $DJ-1^{\beta}$ mutants, although maximum lifespan values were practically unaffected in both genotypes (Fig. 4). Despite this, we found that lifespan curves of untreated and treated flies are significantly different for both genotypes ($p<0.0001$). According to these results, it was reported that chronic treatments with melatonin were able to increase lifespan values in effects after in wild-type flies (Oregon R), and also survival of flies exposed to paraquat (Izmaylov and Obukhova, 1999; Bonilla et al., 2002, 2006). Furthermore, melatonin was also shown to rescue locomotor deficits and DA neurodegeneration in a *Drosophila* model of sporadic PD (Coulom and Birman, 2004).

We next examined the effects of $\alpha$-tocopherol on the short lifespan phenotype associated to $DJ-1^{\beta}$ loss of function. We found that this phenotype was significantly suppressed by chronic treatment of adult flies with 1mM $\alpha$-tocopherol on ($p<0.0001$), as evidenced by a 11.1% and 10.3% increase in median and maximum lifespan values, respectively. However, $\alpha$-tocopherol supplementation did not modify lifespan values in control animals (Figure 5A). Our results are consistent with previous studies that showed that $\alpha$-tocopherol supplementation only extended lifespan or suppressed phenotypes either associated to oxidative stress conditions or to genetically compromised backgrounds, but had no visible effects on lifespan under normal laboratory conditions (Nakashima et al., 2004; Wang et al., 2006; Dias-Santagata et al., 2007; Bahadorani et al., 2008). Indeed, it was proposed that wild-type flies retain very little vitamin E from the medium, probably due to their low biological needs for this antioxidant (Draper et al., 2000). However, flies with defective oxidative stress response could retain vitamin E more efficiently than wild-type flies (Zou et al., 2007; Bahadorani et al., 2008). Then, $\alpha$-tocopherol
supplementation would strengthen antioxidant defenses against oxidative injuries, and would extend lifespan in such flies. The results obtained in the present study with DJ-1βA286 mutant flies, which are highly sensitive to oxidative stress (Lavara-Culebras and Paricio, 2007), would confirm this hypothesis.

Finally, we tested whether there was a sex-dependent effect of antioxidant compounds on DJ-1βA286 mutants’ lifespan, since previous reports showed that females have a better response to antioxidant treatments than males (Magwere et al., 2006). Confirming those observations, we found that although both sexes show a similar increase in their maximum lifespan values after α-tocopherol supplementation (13.3% in females and 10.3% in males, Figure 5B), DJ-1βA286 females exhibit a higher increase of the median lifespan value than males (26.7% and 11.1%, respectively). Similar results are obtained after melatonin treatment of DJ-1βA286 mutant flies (data not shown). The sex-dependent effects of antioxidant drugs on lifespan may be explained by differences in feeding habits, in metabolic rates or in physiological differences in the response to oxidative injuries between males and females, as previously suggested (Magwere et al., 2006).

Taken together, our data show that all antioxidant compounds tested were able to significantly increase lifespan values in DJ-1βA286 mutants, suggesting that this phenotype is very likely caused by deficiencies in the oxidative stress response.

3.3. Quantification of oxidative stress levels in DJ-1βA286 mutant flies

To further confirm that oxidative stress plays a causative role in the lifespan phenotype of DJ-1βA286 mutant flies, we decided to measure several physiological markers of oxidative stress in such flies. We first used 2,7-dichloroflorescein diacetate (DCFH-DA) staining, which is an indicator of hydroxyl-free radical levels, to analyze ROS levels in DJ-1βA286 mutants’ brains.
Our results indicate that brains of 1-2-day old $DJ-1\beta^{A286}$ flies show an increased DCFH-DA staining when compared to brains of age-matched control flies (Fig. 6A-B), thus suggesting that loss of $DJ-1\beta$ function leads to an elevation of ROS levels. Similar results were obtained when analyzing fly brains after $DJ-1\alpha$-inactivation in DA neurons via RNAi (Yang et al., 2006a). As indicated in the Introduction, $DJ-1\alpha$ is the second Drosophila $DJ-1$ ortholog. To further investigate whether DJ-1$\beta$ is involved in cellular response to endogenous oxidative stress, we also examined $DJ-1\beta^{A286}$ mutant flies for the extent of oxidative damage by determining lipid peroxidation (LPO) levels and catalase enzymatic activity. As shown in Fig. 6B the total amount of malondialdehyde (MDA), one of the end stage products of lipid peroxidation, in $DJ-1\beta^{A286}$ mutants was significantly higher than in $y,w$ control flies ($p<0.05$) even in 1-2-day old flies. Besides, catalase activity was also significantly increased in these mutants compared to age-matched control flies (Fig. 6C, $p<0.001$). The increase of these two markers of oxidative stress was also observed in flies subjected to hyperoxia treatment as an oxidative stressor (Gruenewald et al., 2009). Taken together, these results support the crucial role of oxidative stress in the lifespan phenotype of $DJ-1\beta^{A286}$ mutant flies. It has been proposed that the human DJ-1 protein may contribute to ROS removal both as a free radical scavenger and a transcriptional/translational regulator promoting the expression of genes involved in oxidative stress defense (van der Brug et al., 2008; Zhong and Xu, 2008; Blackington et al., 2009). Our results indicate that similar functions could be proposed for the Drosophila DJ-1$\beta$ protein, since defects in the oxidative stress response are evident in $DJ-1\beta^{A286}$ mutant flies.

3.4. Conclusions

In summary, our results strongly suggest that the reduced lifespan phenotype exhibited by $DJ-1\beta^{A286}$ mutant flies is, at least in part, due to defects in the oxidative stress response, and not in
DA physiology as proposed in DJ-1 mice models. Indeed, we show that high levels of oxidative stress are detected in DJ-1β<sup>A286</sup> mutant flies by using different methods. Confirming this, we find that long term dietary supplementation with antioxidants is effective in significantly increasing lifespan values of DJ-1β<sup>A286</sup> mutants. Similar doses of these drugs have been shown to suppress phenotypes associated to oxidative damage in other fly models of human diseases (i.e. Wang et al., 2006; Dias-Santagata et al., 2007). Our results also indicate that these compounds are not sufficient to completely restore lifespan values observed in control flies. This may suggest that DJ-1β loss-of-function does not just limit lifespan through oxidative damage, but possibly through introduction of other pathologies that may also be life limiting. We also find that, while vitamin C and melatonin (both water-soluble antioxidants) are able to extend lifespan in both DJ-1β mutants and wild-type flies, the beneficial effects of α-tocopherol (lipophylic antioxidant) appear to be selective to the DJ-1β mutant flies. One explanation could be that these mutants may retain the compound more efficiently than wild-type (y, w) controls, as previously suggested for other flies with defective oxidative response (Bahadorani et al., 2008). Alternatively, this result could suggest that loss of DJ-1β function may cause oxidative damage to the membranes. Supporting this hypothesis we find a high MDA content in DJ-1β mutant flies, which is an indicator of high levels of lipid peroxidation and of free radical damage to cell membranes.

Similar beneficial effects of antioxidant compounds on several mutant backgrounds and <i>Drosophila</i> models of human diseases have been reported. Indeed, α-tocopherol supplementation has been shown to suppress several phenotypes found in <i>Drosophila</i> models of neurodegenerative diseases like taupathies (Dias-Santagata et al., 2007), PD (Wang et al., 2006), or pantothenate-kinase-associated neurodegeneration (PKAN, Yang et al., 2005b), and to extend lifespan of flies with deficiencies in oxidative stress response (Bahadorani et al., 2008), although it could not rescue the lethal phenotype of a <i>Drosophila</i> model of Huntington’s disease.
(Bahadorani and Hilliker, 2008). Moreover, vitamin E was also effective in suppressing phenotypes found in mouse models of human tauopathy (Nakashima et al., 2004) or Down syndrome (Lockrow et al., 2009), counterpointing the increased oxidative stress observed in those models. Regarding this, vitamin E has proved efficacious in reducing markers of oxidative stress (Pratico et al., 1998; Hong et al., 2004), restoring endogenous antioxidant enzyme functions to normal levels in the mouse model of Down syndrome (Lockrow et al., 2009) and in rodent models of oxidative stress (Zaidi and Banu, 2004). Although these effects can be achieved through its antioxidant function, the non-antioxidant properties of α-tocopherol in modulating gene expression may also play a role (Ricciarelli et al., 2001; 2007; Tucker and Townsend, 2005). Then, it can be proposed that α-tocopherol could suppress the lifespan phenotype of DJ-1βA286 mutant flies based on its antioxidant function, eliminating ROS and then preventing DJ-1β oxidation, but also affecting either some signaling pathways that regulate DJ-1β oxidation or genes important to fly survival whose expression may be compromised by lack of DJ-1β function.

Our present study further shows that the lifespan phenotype of DJ-1βA286 mutant flies is amenable to pharmacological intervention since manipulation of antioxidant defence mechanisms with several compounds can modify such phenotype. Interestingly, a recent study has shown that treatments with minocycline and celestrol, two drugs with antioxidant and anti-inflammatory activities, are able to attenuate DA neuron loss and reduction of brain dopamine levels as well as locomotor defects associated to DJ-1α-inactivation via RNAi in flies (Faust et al., 2009). Treatments with both drugs partially suppress those phenotypes and have no significant effect on DA neuron number, dopamine levels or climbing activity in control flies. Since DJ-1α is the second ortholog of the human DJ-1 gene in Drosophila, these findings emphasize the role of oxidative stress in DJ-1 pathogenesis and support the use of this organism as a model to elucidate the molecular mechanisms underlying PD. Thus, Drosophila DJ-1
mutants could serve as a good model for pharmacological rescue studies of PD, enabling efficient screening of compounds able to suppress PD-like phenotypes. This could lead to the identification of novel factors involved in \textit{DJ-1}-related PD pathogenesis and compounds to prevent oxidative stress occurring during PD in humans.

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Figure 1. Survival curves of y, w control flies and DJ-1βA286 mutants either untreated and after chronic treatment with vitamin C. (A) Comparison of survival curves of y, w control flies (black squares) and DJ-1βA286 mutants cultured in standard fly food. DJ-1βA286 mutant flies have a significantly shorter lifespan than y, w control flies as determined by using Kaplan-Meier log-rank statistical test (p<0.0001). (B) Survival curves of y, w control flies and DJ-1βA286 mutants after chronic treatment with 0.25 mg/ml (~ 1.42 mM) vitamin C. Flies of both genotypes treated with ascorbic acid (open triangles) show significant increase of their lifespan values. The median and maximum lifespan values are as follows: y, w untreated 40 and 67.5 days; y, w treated with ascorbic acid 47 and 71 days; DJ-1βA286 untreated 36 and 53.5 days; DJ-1βA286 treated 43 and 58 days. The significance of the difference between survival curves of treated and untreated flies was analyzed using the Kaplan-Meier log-rank statistical test (p<0.0001 in both cases).

Figure 2. Survival curves of y, w control flies and DJ-1βA286 mutants after chronic treatment with 1mM L-DOPA. The median and maximum lifespan values are as follows: y, w untreated or treated with L-DOPA 43 and 71 days; DJ-1βA286 untreated 45 and 58 days; DJ-1βA286 treated 47 and 59 days. For both genotypes, there are not significant differences between untreated (black squares) and L-DOPA treated flies (open triangles) as determined by using the Kaplan-Meier log-rank statistical test.

Figure 3. Survival curves of y, w control flies and DJ-1βA286 mutants after chronic treatment with 1mM SK&F 38393. The median and maximum lifespan values are as follows: y, w untreated 47 and 67.5 days; y, w treated with SK&F 38393 50 and 71 days; DJ-1βA286 untreated
36 and 53.5 days; \textit{DJ-1}\textsubscript{A286} treated 38 and 55.5 days. Treated \textit{y, w} control flies (open triangles) show a mild but significant increase of their lifespan values when compared to untreated \textit{y, w} flies (black squares) as determined by using Kaplan-Meier log-rank statistical test (p<0.0005). In contrast, SK&F 38393 treated and untreated \textit{DJ-1}\textsubscript{A286} mutants do not show significant differences.

**Figure 4.** Survival curves of \textit{y, w} control flies and \textit{DJ-1}\textsubscript{A286} mutants after chronic treatment with 1mM melatonin. The median and maximum lifespan values are as follows: \textit{y, w} untreated 40 and 62.5 days; \textit{y, w} treated with melatonin 43 and 64 days; \textit{DJ-1}\textsubscript{A286} untreated 36 and 57 days; \textit{DJ-1}\textsubscript{A286} treated 43 and 55.5 days. Melatonin treatment results in a mild but statistically significant increase of their lifespan values (especially of median lifespan) in both genotypes. The significance of the difference between survival curves of treated and untreated flies was analyzed using the Kaplan-Meier log-rank statistical test (\(p<0.0001\) for both genotypes).

**Figure 5.** Survival curves of \textit{y, w} control flies and \textit{DJ-1}\textsubscript{A286} mutants after chronic treatment with 1mM \(\alpha\)-tocopherol. (A) Comparison of survival curves of control and \textit{DJ-1}\textsubscript{A286} flies after chronic treatment with 1mM \(\alpha\)-tocopherol. The median and maximum lifespan values are as follows: \textit{y, w} untreated 40 and 68.5 days; \textit{y, w} treated with \(\alpha\)-tocopherol 40 and 61.5 days; \textit{DJ-1}\textsubscript{A286} untreated 36 and 48.5 days; \textit{DJ-1}\textsubscript{A286} treated 40 and 53.5 days. \(\alpha\)-tocopherol supplementation results in a significant increase of \textit{DJ-1}\textsubscript{A286} mutants lifespan values as determined by using Kaplan-Meier log-rank statistical test (\(p<0.0001\)). In contrast, \textit{y, w} flies do not show significant changes in their lifespan values. (B) Survival curves of male and female \textit{DJ-1}\textsubscript{A286} mutant flies after chronic treatment with 1mM \(\alpha\)-tocopherol. The median and maximum lifespan values are as follows: \textit{DJ-1}\textsubscript{A286} males untreated 36 and 48.5 days; \textit{DJ-1}\textsubscript{A286} males treated 40 and 53.4 days; \textit{DJ-1}\textsubscript{A286} females untreated 30 and 44 days; \textit{DJ-1}\textsubscript{A286} females
treated 38 and 50 days. In both sexes, α-tocopherol supplementation significantly extends lifespan as determined by using Kaplan-Meier log-rank statistical test ($p<0.0001$ in both cases). However, females show higher increase of the lifespan values than males.

**Figure 6.** Oxidative stress markers in y, w control flies and $DJ-I\beta^{A286}$ mutants. (A-B) DCFH-DA staining of whole-mount adult brains from 1-2-day-old y, w and $DJ-I\beta^{A286}$ flies. Weak signals in A represent a combination of weak ROS staining and autofluorescence of adult fly brains. Note that loss of $DJ-I\beta$ function leads to an elevation of ROS levels (compare A with B). (C) Increase in lipid peroxidation (LPO) product malondialdehyde (MDA) in 1-2-day old $DJ-I\beta^{A286}$ mutants compared to age-matched y, w control flies ($p<0.05$). (D) Catalase (CAT) enzymatic activity is also increased in 1-2-day old $DJ-I\beta^{A286}$ mutants compared to age-matched y, w control flies ($p<0.001$). In C and D data are expressed as mean and standard deviation of replicate determinations for 4 and 3 samples, respectively.
Supplementary Figure 1 legend

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