Dmp53, basket and drICE gene knockdown and polyphenol gallic acid increase life span and locomotor activity in a Drosophila Parkinson’s disease model

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Abstract

Understanding the mechanism(s) by which dopaminergic (DAergic) neurons are eroded in Parkinson’s disease (PD) is critical for effective therapeutic strategies. By using the binary tyrosine hydroxylase (TH)-Gal4/UAS-X RNAi Drosophila melanogaster system, we report that Dmp53, basket and drICE gene knockdown in dopaminergic neurons prolong life span (p < 0.05; log-rank test) and locomotor activity (p < 0.05; χ² test) in D. melanogaster lines chronically exposed to (1 mM) paraquat (PQ, oxidative stress (OS) generator) compared to untreated transgenic fly lines. Likewise, knockdown flies displayed higher climbing performance than control flies. Amazingly, gallic acid (GA) significantly protected DAergic neurons, ameliorated life span, and climbing abilities in knockdown fly lines treated with PQ compared to flies treated with PQ only. Therefore, silencing specific gene(s) involved in neuronal death might constitute an excellent tool to study the response of DAergic neurons to OS stimuli. We propose that a therapy with antioxidants and selectively “switching off” death genes in DAergic neurons could provide a means for pre-clinical PD individuals to significantly ameliorate their disease condition.

Keywords: Basket, Dmp53, Drice, Drosophila, paraquat.

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Introduction

Parkinson’s disease (PD) is a common progressive neurodegenerative disorder affecting millions of people worldwide (Alves et al., 2008). This neurological condition is clinically characterized by motor disorders (Jankovic, 2008). Pathologically it is prominently characterized by progressive loss of 50-70% of dopaminergic (DAergic) neurons located in the substantia nigra, abnormal protein aggregation, oxidative stress (OS) and mitochondrial dysfunction (Zhou et al., 2008; Cuervo et al., 2010; Xie et al., 2010). Despite intense investigation, the molecular mechanism(s) of cell loss is not yet fully established for target therapeutic strategies (Dexter and Jenner, 2013). This unmet need might partially explain the failure to establish definitive anti-Parkinson drugs (Rodnitzky, 2012). Since replacement of deficient dopamine constitutes a first-line symptomatic treatment (Gazewood et al., 2013), there is an urgent need to identify the molecular components involved in the DAergic neuronal demise.

Paraquat (PQ, methyl viologen dichloride; or 1,10-dimethyl-4,40-bipyridinium dichloride) is among the most consistently associated environmental risk factors for PD (Tanner et al., 2011; Wang et al., 2011). PQ might cause neuronal deterioration via OS and mitochondrial damage (Franco et al., 2010). Because ethical and policy issues limitation in human research, most of the PQ toxic effects have been studied in in vitro and in vivo models of PD. Specifically, our research group has shown that PQ induces apoptosis - a type of programed cell death - by an OS-mediated mechanism (Jimenez-Del-Rio and Velez-Pardo, 2008). The major molecular events involve generation of O²⁻/H₂O₂, activation of the transcription factor p53, JNK (c-Jun N-terminal) kinase, mitochondria depolarization, caspase-3 activation and chromatin condensation/DNA fragmentation. Drosophila melanogaster has been used as biological tool to inquire on PD process (for a review see Muñoz-Soriano et al., 2011). Remarkably, PQ selectively destroys DAergic neurons in the fly (Chaudhuri et al., 2007), via OS and mitochondrial damage (Bonilla et al., 2006; Hosamani and Muralidhara, 2013). In agreement with this data, Vrailas-Mortimer et al. (2012) have shown that some commercially available antioxidant supplements confer significant protection to D. melanogaster against PQ and H₂O₂. Furthermore, we have recently shown that...
polyphenols [e.g., gallic acid (GA), propyl gallate (PG), epicatechin (EC)], which are well-known antioxidant and gene modulators, were able to protect, rescue and restore impaired movement activity in *Drosophila* induced by acute (Jimenez-Del-Rio et al., 2010) or chronic (Ortega-Arellano et al., 2011) PQ exposure. It has also been shown that pharmacological inhibition of JNK increased life span and locomotor activity (i.e., climbing) compared to flies treated with PQ (Jimenez-Del-Rio et al., 2008). Taken together, these findings suggest that polyphenols might modulate life span and movement capabilities in *D. melanogaster* exposed to PQ, and that JNK signalling might be involved in those effects. Since there is compelling evidence that the apoptosis pathway is conserved between *Drosophila* and mammalian cells (Oberst et al., 2008; O’Riordan et al., 2008; Mollereau, 2009), and essential signalling molecules involved in the OS response in mammalian cells, such as p53, JNK and caspase-3 are highly similar to *Drosophila Dmp53* (43%), *basket* (87%) and *drICE* (61%), respectively (Reiter et al., 2001; Chien et al., 2002), our hypothesis is that a decreased gene expression of *Dmp53, basket* and *drICE* in DAergic neurons might have a beneficial impact on flies exposed to PQ in terms of DAergic neurons survival, life span and climbing capabilities. Moreover, transgenic flies treated with antioxidants and selectively “switching off” of death genes in DAergic neurons should provide a means for pre-clinical PD individuals to significantly ameliorate their disease condition.

Materials and Methods

Fly stocks and culture

Wild type Canton-S and fly lines were cultured under standard conditions, as described elsewhere (Ortega-Arellano et al., 2011). The genotypes were established by standard genetics. Fly Stocks obtained from the Bloomington Stock Center (BSC) were: *TH-Gal4* (#8848), and *UAS-GFP* (#1521). *UAS-dsRNAi* (double-stranded RNA interference) lines obtained from the Vienna Drosophila RNAi stock center (VDRC) were: *UAS-drICE RNAi* (#28006), *UAS-basket RNAi* (#34138), and *UAS-Dmp53 RNAi* (#10692). Male *TH-GAL4*+/ females crossed with wild type Canton-S females to obtain heterozygous female flies (*ff1, TH-GAL4*+/; Figure 1A). Male *TH-GAL4*+/ flies were crossed with wild type Canton-S females to obtain heterozygous female flies (*ff1, TH-GAL4*+/; Figure 1B). Male *TH-GAL4*+/ flies were crossed with *UAS-X RNAi* females (Figure 1B) to obtain heterozygous female flies (*ff1, UAS-X RNAi*; *TH-GAL4*+/). Since female flies were shown to be particularly sensible to PQ (Jimenez-Del-Rio et al., 2008), the *ff1* generation was collected under brief CO2 anesthesia within 2 to 3 days after eclosion for further experiments.

Paraquat toxicity assay

The paraquat toxicity assay was performed on virgin 2- to 3-day-old *ff1* flies collected overnight and kept on regular food medium. Subsequently, 50 separated adult *ff1* flies were starved in empty vials for 3 h at 25 °C. Then, groups of five flies were placed in ten vials containing a filter paper (Bio Rad Mini Trans-Blot 1703932) saturated with 1% glucose (55.5 mM glucose, Gluc) in distilled water (dW) for 24 h. After this time, flies were starved in empty vials for 3 h at 25 °C and transferred to vials with a filter paper saturated with 200 μL (1 mM) PQ in Gluc for 15 days. Filters were changed daily. Red food dye (8 μL/mL) (Red food colour McCormick) was added to ensure homogeneity and food intake. Living flies were counted daily.
A Chi Square ($\chi^2$) test was performed to compare the proportion of percentage between independent groups. Differences were considered statistically significant at p < 0.05.

Survival test

ff1 flies were treated chronically with PQ and polyphenol (GA) as described above for 15 days. Live flies were counted daily in groups of five flies per vial. 50 flies per treatment were used. Survival curves were plotted using the Kaplan-Meier estimator. The statistical significance was calculated using the log rank test implemented in the portable IBM SPSS statistics 19 package program. The null hypothesis in all survival assays was that the exposure of genetically modified Drosophila to PQ and/or GA made no difference to the survival of the flies in the absence of these reagents. Differences were considered statistically significant at p < 0.05.

Results

The knockdown of Dmp53, basket and drICE genes in dopaminergic neurons increases life span and locomotor activity in Drosophila melanogaster exposed to paraquat

We initially wanted to evaluate life span and locomotor activity in D. melanogaster TH-Gal4$^{/+}$, (Figure 1A, control) resulting from the cross between wild type Canton S and homozygous TH-Gal4$^{+/-}$, exposed to 1 mM PQ in the experimental design (Figure 1C). As shown in Figure 2A, the proportion of surviving TH-Gal4$^{+/-}$ flies treated with PQ was significantly diminished compared to TH-Gal4$^{+/-}$ flies. Indeed, while 50% of the TH-Gal4$^{+/-}$ flies perished by day 14, the TH-Gal4$^{+/-}$ plus PQ group did so at day 5. The percentage of locomotor activity (i.e., >75% climbing performance) remained normal in TH-Gal4$^{+/-}$ flies until day 15, whereas climbing performance was drastically diminished already by day 5 when they were exposed to PQ (Figure 2B). Interestingly, knockdown flies TH-Gal4$^{+/-}$, UAS-Dmp53 RNAi$^{+/-}$ (Figure 2A,B); TH-Gal4$^{+/-}$, UAS-basket RNAi$^{+/-}$ (Figure 3A,B), and TH-Gal4$^{+/-}$, UAS-drICE RNAi$^{+/-}$ (Figure 4A,B) displayed survival percentages and climbing capabilities comparable to control flies (TH-Gal4$^{+/-}$). However, in 50% of the transgenic flies treated with PQ the percentage of survival slightly increased (by 2 days) and climbing performance moderately augmented (by 3-5 days) compared to the control group (Figures 2, 3 and 4A, B).

Gallic acid (GA) increases life span and locomotor activity in RNAi fly lines

Polyphenols have shown a high protective effect against PQ in Drosophila (Jimenez-Del-Rio et al., 2010). We, thus, investigated herein whether GA also had an effect in genetically modified flies when exposed to 1 mM PQ for
15 days. As shown in Figure 5, the presence of GA in the diet of TH-Gal4+/−, UAS-Dmp53 RNAi+/− fly lines treated with PQ increased the proportion of survival (Figure 5A) and climbing performance (Figure 5B) compared to flies treated with PQ alone. Noticeably, while 50% of the Dmp53 RNAi+/− flies treated with PQ and GA survived when scored on days 10 and 13, respectively, 50% survival in the Dmp53 RNAi+/− fly line treated with PQ alone could only be scored until day 7 and 10, respectively. Similar results were obtained with the other RNAi transgenic flies (Table 1).

Table 1 - Dmp53, bsk and drICE gene knockdown increase the life span and locomotor activity of Drosophila melanogaster chronically exposed to paraquat.

| Line                  | Noxious/antioxidant | Treatment | Concentration (mM) | Survival (50%) | K-M, p | Climbing (50%) | χ², p |
|-----------------------|---------------------|-----------|--------------------|----------------|--------|----------------|-------|
| TH-Gal4+/−            | PQ                  | 1         | 0                  | 14 ± 0.6       | 1 vs. 2, p < 0.005 | >15    | 1 vs. 2, p < 0.05 |
|                       | PQ                  | 2         | 1                  | 5 ± 0.3        | 2 vs. 4, p < 0.005   | 5      | 2 vs. 4, p < 0.05   |
|                       | GA                  | 3         | 0.1+1              | 15             | 3 vs. 4, p < 0.005   | >15    | 3 vs. 4, p < 0.005   |
|                       | GA + PQ             | 4         | 0.1+1              | 11 ± 0.4       | 4 vs. 12 16, n.s.    | 13     | 4 vs. 12 16, n.s.    |
| UAS-Dmp53(RNAi)+/−    | PQ                  | 5         | 0                  | 15 ± 0.4       | 1 vs. 5, n.s.         | >15    | 1 vs. 5, n.s.         |
| TH-Gal4+/−            | PQ                  | 6         | 1                  | 7 ± 0.4        | 2 vs. 6, p < 0.005   | 10     | 2 vs. 6, p < 0.005   |
|                       | GA                  | 7         | 0.1                | 15             | 6 vs. 8, p < 0.005   | >15    | 6 vs. 8, p < 0.005   |
|                       | GA + PQ             | 8         | 0.1+1              | 10 ± 0.5       | 7 vs. 8, p < 0.005   | 13     | 7 vs. 8, p < 0.005   |
| UAS- bsk(RNAi)+/−     | PQ                  | 9         | 0                  | 15 ± 0.4       | 1 vs. 9, n.s.         | >15    | 1 vs. 9, n.s.         |
| TH-Gal4+/−            | PQ                  | 10        | 1                  | 7 ± 0.5        | 2 vs. 10, p < 0.005  | 9      | 2 vs. 10, p < 0.005  |
|                       | GA                  | 11        | 0.1                | 15             | 10 vs. 12, p < 0.005 | >15    | 10 vs. 12, p < 0.005 |
|                       | GA + PQ             | 12        | 0.1+1              | 10 ± 0.5       | 11 vs. 12, p < 0.005 | 13     | 11 vs. 12, p < 0.005 |
| UAS-drICE (RNAi)+/−   | PQ                  | 13        | 0                  | 15             | 1 vs. 13, n.s.        | >15    | 1 vs. 13, n.s.        |
| TH-Gal4+/−            | PQ                  | 14        | 1                  | 7 ± 0.5        | 2 vs. 14, p < 0.005  | 8      | 2 vs. 14, p < 0.005  |
|                       | GA                  | 15        | 0.1                | 15             | 14 vs. 16, p < 0.005 | >15    | 14 vs. 16, p < 0.005 |
|                       | GA + PQ             | 16        | 0.1+1              | 11 ± 0.4       | 15 vs. 16, p < 0.005 | 13     | 15 vs. 16, p < 0.005 |

*a*represents number of days at which 50% of total flies have been killed.

*b*represents number of days at which 50% of climbing ability is impaired.

Abbreviations: Paraquat, PQ; Gallic Acid, GA; K-M, Kaplan-Meier test; n.s., no significance; χ², Chi-square test.
melanogaster lines chronically exposed to PQ compared to controls. These findings comply with the notion that altered gene function, either by mutation or knockdown can modulate the susceptibility to a known environmental PD risk factor such as PQ (Goldman et al., 2012). However, the PQ toxic effect was dependent on the genetic background of the exposed flies. One possible explanation for this finding is that while some DAergic neurons can cope with a rise in OS, others are more vulnerable (Wang and Michaelis, 2010). Because of such selective vulnerability, these neurons are usually the first to exhibit cell death and functional decline (i.e. climbing performance).

A second possibility is that some mutated (Goldman et al., 2012) or experimentally knocked down gene(s) may confer resistance to DAergic neurons against PQ-driven OS, thereby increasing life span and locomotor activity. Our data support the latter hypothesis. In fact, Dmp53, basket and drICE RNAi described herein in an in vivo system appears to mirror the pharmacological inhibition of p53, JNK, caspase-3 and cell survival previously seen in vitro (Jimenez-Del-Rio and Velez-Pardo, 2008). Therefore, these data suggest that PQ induces a molecular mechanism of cell death in DAergic neurons that is similar in Drosophila melanogaster, mice (Peng et al., 2004) and primary human cells (Jimenez-Del-Rio and Velez-Pardo, 2008, 2012).

This conclusion is strongly supported by several observations. First, the Drosophila genome contains all the genes encoding canonical mitochondrial (Miwa et al., 2003; Oberst et al., 2008) and cell death proteins (Shi, 2004; O’Riordan et al., 2008; Steller, 2008), suggesting that they function in a similar manner as in mammalian cells (see O’Riordan et al., 2008). Indeed, the Drosophila Apaf-1 related killer (Dark) assembles in an apoptosome that functions within the intrinsic cell death pathway similar to that seen in mammals (Yuan et al., 2011). During apoptosis, the initiator caspase Dronc (ortholog of the mammalian pro-caspase-9) is activated by the Dark
Interestingly, it has been shown that a Dmp53/JNK (basket)-dependent feedback amplification loop is essential for the apoptotic response to stress in *Drosophila* (Shlekov and Morata, 2012). It is therefore reasonable to think that PQ might be able to trigger activation of Dmp53, basket and drICE via O$_2^·/H_2O_2$ signaling and cell death (Steller, 2008) in a similar fashion as proposed in mammalian cells (Jimenez-Del-Rio and Velez-Pardo, 2012). In accordance with this view, several reports have shown that PQ induces activation of JNK associated with OS and cell death *in vitro* and *in vivo* in mammalian DAergic neurons (Chun et al., 2001; Peng et al., 2004; Klintworth et al., 2007; Ramachandiran et al., 2007; Choi et al., 2010; Niso-Santano et al., 2010). But these observations stand in contradiction with others that have shown that overexpression of JNK (Wang et al., 2003; Inamdar et al., 2012) or overexpression of JNK target genes, such as human peroxiredoxin II (hPrxII) and Jafrac1 (a *Drosophila* homolog of hPrxII) (Lee et al., 2009) confer protection against PQ-induced toxicity in DAergic neurons of the fly. The reason for these contradictory observations is not yet known. Therefore, further research will be required to clarify this issue.

Finally, PQ induces PD-like clinical features in *Drosophila*, including resting tremor, bradykinesis, and postural instability with or without neuronal damage. A similar PD-like phenotype can be generated by pharmacological inhibition of the tyrosine hydroxylase enzyme with α-MT (alpha-methyl-tyrosine). In these experiments no neuronal damage was observed, but functional impairment (climbing performance) was evident (Bonilla-Ramirez et al., 2011).

In conclusion, these data suggest that the knockdown of specific gene(s) in the *Drosophila* brain may provide basic information about the mechanism(s) of cell death/survival and clinical behavior in human PD.

Polyphenols have been postulated as potential neuroprotectant molecules in neurodegenerative disorders including PD (Albarracin et al., 2012). In agreement with others (Long et al., 2009; Peng et al., 2011) and our previous investigations (Jimenez-Del-Rio et al., 2010; Ortega-Arellano et al., 2011), we found that GA prolongs life span and climbing activity in wild type or knocked down *D. melanogaster*. These data suggest that GA (and probably other polyphenols) may protect DAergic neurons independently of the genetic background of the fly, either via direct interaction with ROS, enzymes, receptors and/or transcription factors (Fraga et al., 2010), or through antioxidant gene regulatory mechanisms (Kim et al., 1997; Peng et al., 2011). Our findings suggest that GA can protect DAergic neurons against PQ. Consequently, this polyphenol is capable of modifying the life span and locomotor capabilities of flies exposed to OS stimuli.

In summary, induced RNAi of specific pro-apoptotic genes in DAergic neurons in *D. melanogaster* increased survival and climbing performance under PQ treatment.
Since polyphenols such as GA displayed antioxidant capacity, they may certainly contribute to develop nutritional strategies oriented towards preventing PD. Our data suggest that pharmacologically targeted proteins or knockdown of critical death signaling genes, such p53, JNK and caspase-3 in DAergic neurons, together with antioxidant exposure, may retard neural deterioration or neuronal loss, thereby, restoring or prolonging locomotor activity in PD patients. In addition, our in vivo data on flies link directly to in vitro work done on OS-induced cell death in mammalian cells (Shi, 2004; O’Riordan et al., 2008; Steller, 2008; Jimenez-Del-Rio and Velez-Pardo, 2012). Understanding the mechanism(s) by which dopaminergic neurons are eroded in PD is critical for the development of effective therapeutic strategies.

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