Heat Shock Protein-70 (Hsp-70) Suppresses Paraquat-Induced Neurodegeneration by Inhibiting JNK and Caspase-3 Activation in *Drosophila* Model of Parkinson's Disease

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Abstract

Parkinson's disease (PD) is one of the most common neurodegenerative disorders with limited clinical interventions. A number of epidemiological as well as case-control studies have revealed an association between pesticide exposure, especially of paraquat (PQ) and occurrence of PD. Hsp70, a molecular chaperone by function, has been shown as one of the modulators of neurological disorders. However, paucity of information regarding the protective role of Hsp70 on PQ-induced PD like symptoms led us to hypothesize that modulation of *hsp70* expression in the dopaminergic neurons would improve the health of these cells. We took advantage of *Drosophila*, which is a well-established model for neurological research and also possesses genetic tools for easy manipulation of gene expression with limited ethical concern. Over-expression of *hsp70* was found to reduce PQ-induced oxidative stress along with JNK and caspase-3 mediated dopaminergic neuronal cell death in exposed organism. Further, anti-apoptotic effect of *hsp70* was shown to confer better homeostasis in the dopaminergic neurons of PQ-exposed organism as evidenced by their improved locomotor performance and survival. The study has merit in the context of human concern since we observed protection of dopaminergic neurons in PQ-exposed organism by over-expressing a human homologue of *hsp70*, *HSPA1L*, in these cells. The effect was parallel to that observed with *Drosophila hsp70*. These findings reflect the potential therapeutic applicability of *hsp70* against PQ-induced PD like symptoms in an organism.

Introduction

Neurons, the building blocks of the nervous system, are reported to have limited regeneration capability after damage [1]. Thus, progressive loss in structure or function of neurons can result in various neurodegenerative disorders [2]. These neurodegenerative processes have been associated with a number of diseases in humans such as Parkinson's-, Alzheimer's-, Huntington's-disease, etc. Among them, Parkinson's disease (PD) has been described as the second most common progressive movement disorder [3]. It is characterized by the loss of dopaminergic neurons within the substantia nigra region of the midbrain that leads to problem in walking and difficulty in maintaining balance [4].

The multifactorial etiology of PD has been linked to aging, genetic and environmental factors [5]. However, earlier reports, including epidemiological findings [6–8] emphasized that environmental factors play major role in the pathogenesis of PD. Among the environmental factors, paraquat (PQ), a widely used herbicide, has been shown to produce PD like symptoms in exposed organisms [6,9]. This association is further supported by higher PD incidences in the population with occupational exposure to PQ [8]. Moreover, generation of oxidative stress (OS) and subsequent activation of JNK and caspase-3 mediated death of dopaminergic neurons was revealed as one of the underlying mechanisms of PQ-induced PD [7]. Since, PQ toxicity is mediated through OS, efforts have been made to diminish such negative impact by using various anti-oxidants such as superoxide dismutase (SOD), Coenzyme Q10 [10,11] etc.

Heat shock protein 70 (Hsp70), a key molecular chaperone [12], with a functional analogy to an anti-oxidant, is reported to protect cells from oxidative damage [13]. In general, heat shock proteins (HSPs) act as molecular chaperones that assist in the correct folding of nascent and stress-accumulated mis-folded proteins and prevent their aggregations [14]. Our laboratory has shown *hsp70* expression as the first-tier bio-indicator of chemical induced toxicity since this gene was found to be the first inducible gene in the organism after chemical stress [15,16]. Moreover, it has also

been reported as a negative regulator of apoptosis in an organism as it modulates apoptosis inhibiting factor (AIF), caspase-3 and others [17,18].

Besides the defensive role of Hsp70 in OS, the former is also suggested as a potential therapeutic target for the treatment of neurological diseases [12,19]. For example, protective role of Hsp70 in α-synuclein (αSN) induced toxicity was shown in different models, including *Drosophila* [19–21]. In addition, *in vitro* and *in vivo* studies have demonstrated that geldanamycin, valproic acid and celastrol induced *hsp70* expression can rescue neurotoxicity caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone [21–23]. However, these studies had limitations since the above mentioned inducers are reported to produce side effects after prolonged usage [12,24]. In this context, Samuni et al. [24] have reported hepatotoxicity after the use of geldanamycin and its analogues in rat primary hepatocytes. Considering the above, genetic manipulation of *hsp70* may be a viable option to achieve protection against chemical induced neurodegenerative disease like conditions. Except for an *in vitro* study, where *hsp70* over-expression was shown to intervene PQ-induced neurotoxicity in rat neuroblast cells [25], no *in vivo* study has been reported so far on the direct role of Hsp70 in alleviating PQ-induced PD like symptoms. Therefore, we hypothesized that over-expression of *hsp70* in the dopaminergic neurons of an organism can protect it against PQ-induced PD like symptoms.

In order to address the above, we used *Drosophila melanogaster* which is a well-established model organism for studying human neurodegenerative disorders [19,26] including PQ-induced PD [6]. Here, we over-expressed *hsp70* (both *Drosophila* and its human homologue) in the dopaminergic neurons of the flies by using a UAS/Gal4 system [27] and explored the protective role of Hsp70 against PQ-induced PD like symptoms in exposed organism.

Materials and Methods

Drosophila culture and PQ exposure

Fly stocks w^{1118}, *Df(hsp70)*, *TH-Gal4*, *Hsp70K71E* (a dominant negative mutant of *hsp70*, which results in compromised expression of endogenous *hsp70* after driving with Gal4) [28], *UAS-hsp70* (results in the over-expression of *hsp70* after driving with Gal4), and *HSPA1L* (human homologue of Hsp70) [29] were used. Flies were reared on standard *Drosophila* food [30] at $24\pm1°C$. *TH-Gal4* strain was used to modulate the expression of *hsp70* in the dopaminergic neurons of *Drosophila*. w^{1118} and *TH-Gal4>w^{1118}* were used as genetic control against *hsp70* deficient strain and strains having genetic modulation of *hsp70*, respectively.

The flies were exposed to PQ according to Girardot et al [31] with minor modifications, using 5% sucrose and 1.3% agar as the medium. Five day-old male flies were starved for 6 h on agar only and then transferred to the vials containing agar, sucrose and 10 or 20 mM PQ for 12 and 24 h. The concentration of PQ was chosen according to a previous study in *Drosophila* by Chaudhuri et al. [6]. Control flies were transferred to vials having agar and sucrose, i.e., without the test chemical. All the chemicals of highest purity were obtained from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated.

High-performance liquid chromatography (HPLC) analysis of PQ, dopamine (DA) and DOPAC

The HPLC analysis was performed using a Waters515series HPLC system (Water Milford, MA, USA). It consists of a binary pump, an on-line degasser, electro coupled and photo array detectors along with a reverse phased C-18 ODS analytical column (250 mm×4 mm; 5 μm particle size).

The level of PQ in the head of exposed *Drosophila* was estimated by following the method of Corasaniti and Nistico [32] with minor modifications. Tissue samples were homogenized in 0.1 M perchloric acid and centrifuged at 10000×g at 4°C. Supernatant from each sample was mixed with ammonium hydroxide and the content was passed through chromatographic column, prepared for ion-pair exchange and eluted with acidic methanol. The elute was evaporated to dryness and reconstituted in mobile phase consisting of 7.5 mM sodium heptanesulphonate and 0.1 M orthophosphoric acid (pH 3.0 with triethylamine) plus acetonitrile to yield a 10% (v/v) mixture. After injecting 50 μl sample in the HPLC injector, the level of PQ was estimated against a run time of 6 min (1.5 ml min^{-1} flow rate). Analysis was carried out using a Waters 2487 variable wavelength UV detector at 258 nm.

The levels of DA and DOPAC were measured by following the method of Yang and Beal [33] with minor modifications. Each sample was prepared from the dissected heads of 50–100 flies in a phosphate-buffered saline (1X PBS).The tissues were extracted with 0.1 M perchloric acid and then passed through 0.22 μm filter. Fifty microlitre extract was injected in the HPLC loop and separated on a mobile phase (0.1 M potassium phosphate, 10% methanol and 1 mM heptanesulphonic acid) with a flow rate of 1.0 ml min^{-1} against a run time of 10 min. Elutes from control and treated samples were analyzed using Waters 464 pulsed electrochemical detector against an appropriate standard.

Assay of oxidative stress (OS) parameters

To estimate PQ-induced OS, level of superoxide radical (O_2^-), SOD activity, peroxynitrite anion ($ONOO^-$) generation, and malondialdehyde (MDA) content were measured in the brain samples of control and PQ-exposed Drosophila.

Generation of superoxide (O_2^-) in the brain tissue of control and PQ-exposed groups was measured by flowcytometry using dihydroethidium dye (DHE; Invitrogen, USA) as described previously [34]. In brief, single-cell suspension was prepared from the dissected brain using collagenase (0.5 mg/ml) [30]. The cell suspension was incubated with DHE (10 μM) for 1 h in the dark at $24\pm1°C$. After incubation, the cell suspension was washed with 1X PBS and finally, re-suspended in 1X PBS for analysis. The quantity of DHE oxidized to its fluorescent form, 2-hydroxyethidium (HE), was measured. HE fluorescence was quantified at an excitation/emission wavelength of 535/617 nm, using a Becton Dickinson flowcytometer (BD Biosciences, New Jersey, USA). O_2^- level was measured using the mean fluorescence intensity of HE in each sample.

Superoxide dismutase (SOD) (superoxide: superoxide oxidoreductase EC 1.15.1.1) activity in the brain tissue of control and exposed organism was determined biochemically following the method described earlier [35] with minor modifications [15]. The reaction mixture was prepared as given in table 1.The reaction was started and stopped by using 780 μM reduced nicotinamide adenine dinucleotide (NADH) and glacial acetic acid (GAA) respectively. Finally, the coloured end product was extracted using n-butanol. Enzyme concentration required for 50% inhibition of chromogen production (optical density at 560 nm) was considered as one unit of enzyme activity. The results were expressed as specific activity in units min^{-1} mg^{-1} protein.

Peroxynitrite ($ONOO^-$) production in Drosophila brain was monitored using dihydrorhodamine 123 (DHR 123; Cayman Chemical, MI, USA) as described previously [36] using a Becton Dickinson flowcytometer. The conversion of non-fluorescent DHR 123 to rhodamine, a fluorescent product after its oxidation with $ONOO^-$, was measured at an excitation/emission wavelength of 500/536 nm. Briefly, single cell suspension from control

Table 1. Composition of reaction mixture for assaying SOD activity.

	Sodium pyrophosphate buffer (0.052M; pH 8.3)	Doubled distilled water	10% brain homogenate (w/v)	Nitroblue tetrazolium (300 μM)	Phenazine methosulphate (186 μM)	n-butanol
Blank	-	-	-	-	-	4.0 ml
Standard	1.2 ml	1.2 ml	-	300 μl	100 μl	4.0 ml
Sample	1.2 ml	1.175 ml	25 μl	300 μl	100 μl	4.0 ml

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and exposed groups was incubated with 20 μM DHR 123 for 10 min at $24\pm1°C$. Thereafter, the cells were washed with 1X PBS and suspended in the same buffer before analysis. The mean fluorescence intensity was used as a measure of $ONOO^-$ generation.

Generation of malondialdehyde (MDA), an intermediate during lipid peroxidation (LPO), was measured using tetraethoxypropane as an external standard [37]. The assay mixture was prepared using 25 μl of brain homogenate, 10% SDS, 20% acetic acid (pH 3.5) and 0.8% thiobarbituric acid (TBA). The mixture was kept in boiling water (100°C) for 1 h. Finally, the coloured end product was extracted using n-butanol. MDA content was expressed in terms of n moles MDA formed mg^{-1} protein after measuring the absorbance at 532 nm.

Protein content in brain sample of control and PQ-exposed groups were estimated by the method of Lowry et al [38], using bovine serum albumin (BSA) as the standard in range of 50–1000 μg/ml .

Western blot analysis

Brain tissues from control and PQ-exposed flies were homogenized in a buffer (50 mM Tris-HCl; pH 7.5, 1 mM EGTA, 0.5 M NaCl, 1% Triton X-100, 1 mM DTT with protease inhibitors) [39]. Each tissue homogenate was centrifuged at $10000\times g$ for 5 min at 4°C. The supernatant was used for protein estimation and western blotting. Quantification of protein in each sample was performed using Bradford's reagent in order to ensure equal loading. Protein samples (40 μg/lane) from control and exposed groups were separated by a 12.5% linear sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 15 mA. After electrophoresis, polypeptides were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech Limited, Buckinghamshire, England) using a semi-dry transfer method (TE70 PWR, Amersham Biosciences, NJ, USA). The membrane was incubated with TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) containing 5% non-fat milk for blocking non-specific binding of antibodies. After blocking, the membrane was probed with primary antibodies. Anti-Phospho-JNK, anti-cleaved caspase-3 (1:1000; Cell Signaling Technology, MA, USA), anti-JNK (1:500; Santa Cruz, TX, USA),

Drosophila Hsp70 monoclonal antibody (7Fb; 1:250) and anti-β tubulin (1:500; Developmental Studies Hybridoma Bank, IA, USA) were used as the primary antibodies. For immuno-detection, Horse Radish Peroxidase conjugated secondary antibodies (1:2000) were used. Finally, proteins were visualized using Femato reagent (Thermo Fisher Scientific, Rockford, USA) on a GelDoc (Bio-Rad, CA, USA).

Assay of DEVDase (caspase 3-like) activity

The assay was performed according to the manufacturer's protocol (Bio Vision Inc. CA, USA). Briefly, the supernatant from 10% brain homogenate of control and PQ-exposed groups was mixed with chilled cell lysis buffer, 2X reaction buffer and 200 μM substrate. The reaction mixture was incubated at 37°C for 1.5 h and absorbance of colorimetric reaction was read at 405 nm on a Cintra 20 ultraviolet spectrophotometer (GBC Scientific Equipment, Melbourne, Australia). The DEVDase activity was calculated in terms of μmol pNA released min^{-1} mg^{-1} protein.

### Immunofluorescent analysis of dopaminergic neurons

Adult fly brains from control and PQ-exposed groups were fixed with cold 4% paraformaldehyde and dissected for immunostaining [40]. Brain tissues were washed thrice in PBST (1X PBS + 0.1% Triton X 100) and incubated overnight at 4°C in blocking solution [1X PBST + 5% heat inactivated fetal bovine serum (FBS)]. The tissues were then incubated over-night with primary antibody at 4°C. Rabbit anti-*Drosophila* Tyrosine Hydroxylase (1:200; DTH) and Cy-3 conjugated goat anti-rabbit (1:400) were used as the primary and secondary antibody, respectively. After staining, brain tissues were mounted in Vectashield mounting media (Vector Laboratories, CA, USA). Tissues were visualized under a Leica TCS-SPE confocal microscope (Nussloch, Germany) and images were captured at 200X. The numbers of DTH-positive neurons within each of the major dopaminergic neuron clusters were determined by visual examination of individual confocal Z-series images. Finally, the images were processed using Adobe Photoshop 7.0 software.

Table 2. PQ estimation in the head of different fly strains after 24 h exposure.

	w^{1118}	Df(hsp70)	TH-Gal4>w^{1118}	TH-Gal4>UAS-hsp70	TH-Gal4>HSP70K71E	TH-Gal4>HSPA1L
0 mM	ND	ND	ND	ND	ND	ND
10 mM	1.76±0.23	1.85±0.21	1.69±0.25	1.72±0.18 2.68±	1.75±0.22	1.67±0.15
20 mM	2.64±0.25	2.77±0.18	2.98±0.14	0.26	2.86±0.24	2.74± 0.21

Data represent mean ± SD of three identical experiments made in triplicates; values were represented in μM. ND: not detected.
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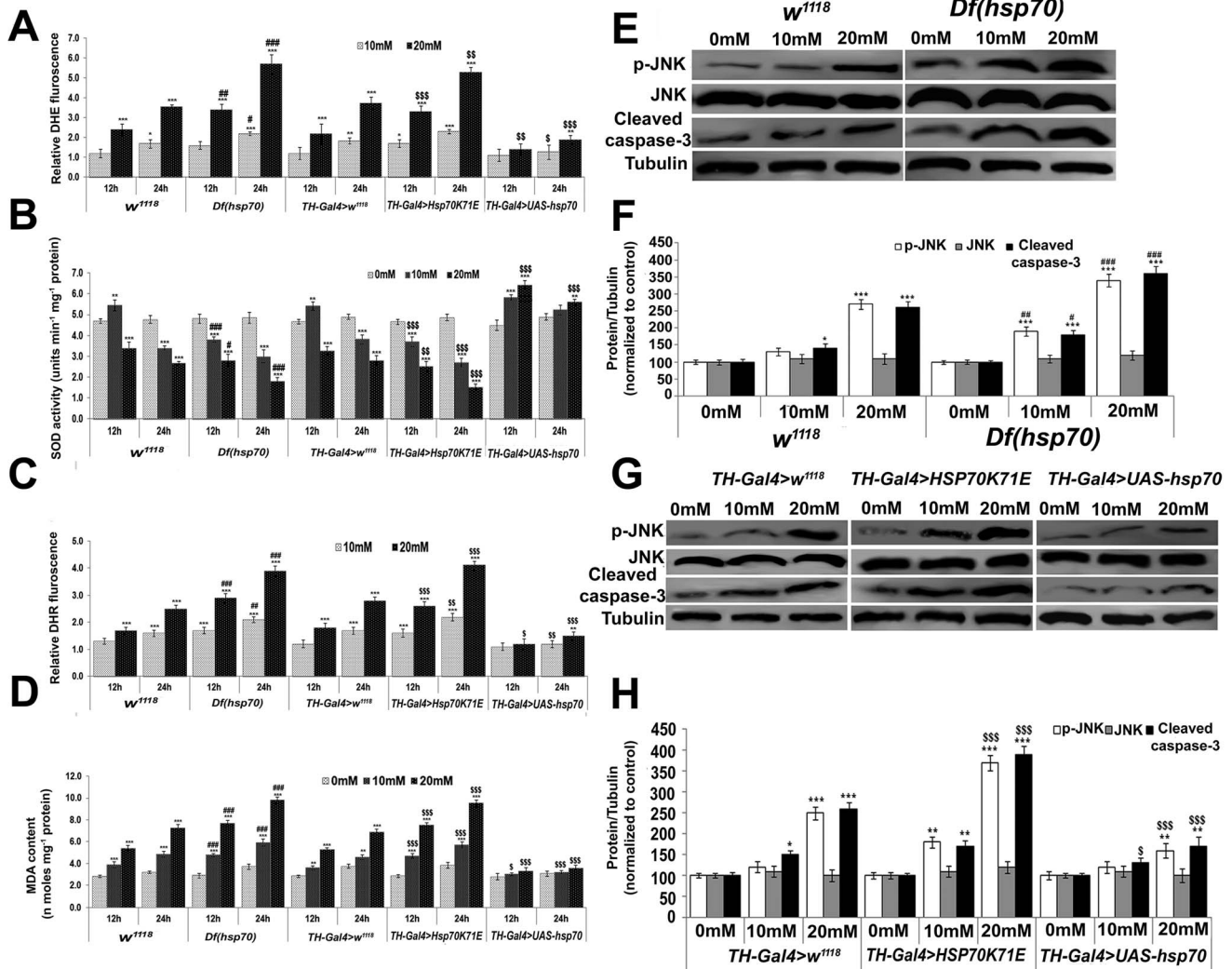
Figure 1. hsp70 over-expression in the dopaminergic neurons of Drosophila protect them from PQ-induced OS. (A) Time and dose dependent increase in superoxide (O_2^-) in the brain of PQ-exposed w^{1118}, Df(hsp70), TH-Gal4>w^{1118} and TH-Gal4>Hsp70K71E flies and less generation of O_2^- in exposed TH-Gal4>UAS-hsp70 flies as compared to TH-Gal4>w^{1118}. (B) Decreased SOD activity in the brain of PQ-exposed w^{1118}, Df(hsp70), TH-Gal4>w^{1118} and TH-Gal4>Hsp70K71E flies and an increased enzyme activity in exposed TH-Gal4>UAS-hsp70 flies. (C) Graphical representation of peroxynitrite ($ONOO^-$) generation using DHR 123 staining. $ONOO^-$ generation was also found to be less like O_2^- generation in PQ-exposed TH-Gal4>UAS-hsp70 flies. (D) Histogram depicting the extent of lipid peroxidation as determined by MDA content in w^{1118}, Df(hsp70), TH-Gal4>Hsp70K71E and TH-Gal4>UAS-hsp70 flies. (E) Representative immuno-blot images of p-JNK, JNK and cleaved caspase-3 levels in the protein samples from brain tissues of control and PQ-exposed w^{1118} and Df(hsp70) flies. (F) Densitometry analysis of data normalized against loading control tubulin. (G) Representative immuno-blotting images of TH-Gal4>w^{1118}, TH-Gal4>HSP70K71E and TH-Gal4>UAS-hsp70 flies. (H) Densitometry analysis of data normalized against loading control tubulin. Values are mean ± SD (n = 3). Significance were ascribed as *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs. control; $^\$p<0.05$, $^{\$\$}p<0.01$ and $^{\$\$\$}p<0.001$ vs. TH-Gal4>w^{1118}; #$p<0.05$, ##$p<0.01$ and ###$p<0.001$ vs. w^{1118}.
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Climbing assay

The locomotor performance of control and PQ-exposed flies was performed based on the negative geotaxis behavior, as described previously [41,42] with minor modifications. In brief, 20 flies in a vertical plastic tube (18 cm×2 cm) were gently tapped. After 30 sec, flies those climbed over the 15 cm distance (n_{top}) and those remaining below (n_{bot}) that mark were scored separately. Ten trials were performed each at one min interval and three independent experiments were carried out for each genotype. For each experiment, using the formulae $[1/2[(n_{tot}+n_{top}-n_{bot})/n_{tot}]$, a performance index (PI) was calculated from the values obtained. Results were expressed as mean ± SD of the scores obtained from three independent experiments.

Paraquat resistance test

Male Drosophila were separated at the pupal stage and after emergence, the flies were fed normal food for five days. Four hundred flies were starved for 6 h on 1.3% agar to ensure non-existence of food particles in their digestive tract and also to save them from dehydration. After fasting, 20 flies were kept in each vial containing filter papers soaked with 200 μl solution consisting of 5% sucrose and 20 mM PQ, while control flies received 5% sucrose only. Solutions were renewed every alternate day till the last fly died. Scoring of dead flies was performed after every 12 h.
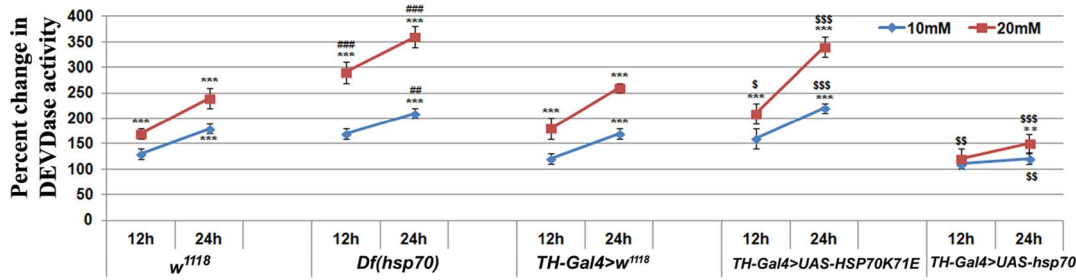
Figure 2. DEVDase (caspase 3-like) activity in the brain of PQ-exposed *Drosophila*. Graphical representation of DEVDase activity in the brain homogenate of *Drosophila* after their exposure to PQ for 12 and 24 h; A significant decrease in enzyme activity was observed in the brain of PQ-exposed *hsp70* over-expressing strain. Data are mean ± SD (n = 3). Significance ascribed as **$p<0.01$ and ***$p<0.001$ vs. control; $^{\$}p<0.05$; $^{\$\$}p<0.01$ and $^{\$\$\$}p<0.001$ vs. *TH-Gal4>w^{1118}*; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs. *w^{1118}*.
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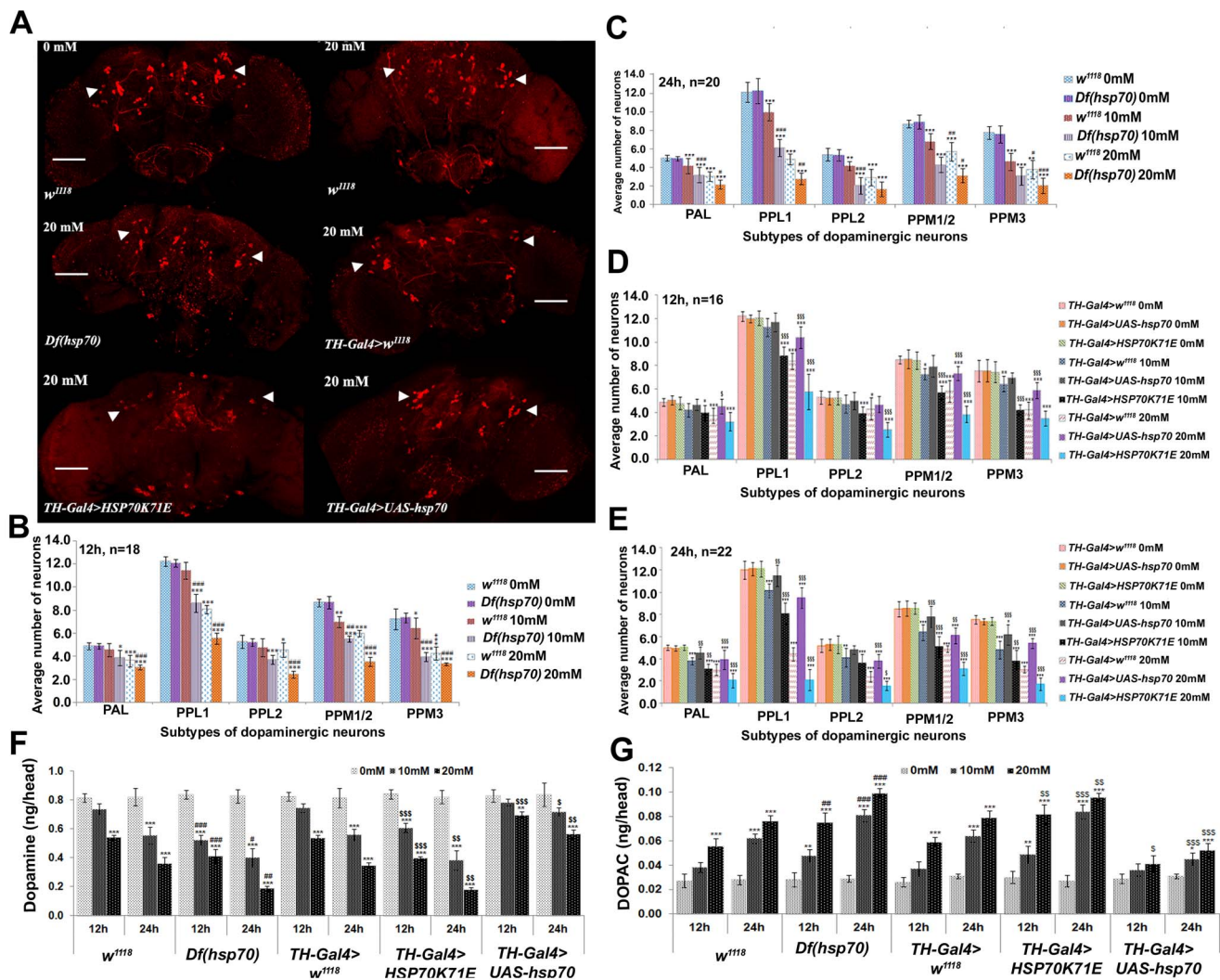
Figure 3. *hsp70* over-expression in the dopaminergic neurons of *Drosophila* protects them from PQ-induced neuronal degeneration.
(A) Representative confocal images of brains of control *w^{1118}* and 20 mM PQ-exposed *w^{1118}*, *Df(hsp70)*, *TH-Gal4>w^{1118}*, *TH-Gal4>HSP70K71E* and *TH-Gal4>UAS-hsp70* fly for 24 h after anti-DTH staining. Quantitative representation of the average number of neurons in the dopaminergic clusters of adult flies that were exposed to PQ (B–D) for 12 and (C–E) 24 h. Graphical representation of (F) DA and (G) DOPAC levels in PQ-exposed *w^{1118}*, *Df(hsp70)*, *TH-Gal4>w^{1118}*, *TH-Gal4>HSP70K71E* and *TH-Gal4>UAS-hsp70* flies. Arrow head indicates PPL1 cluster. Significance ascribed as *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs. control; $^{\$}p<0.05$, $^{\$\$}p<0.01$ and $^{\$\$\$}p<0.001$ vs. *TH-Gal4>w^{1118}*; $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs. *w^{1118}*. Bar = 20 μm
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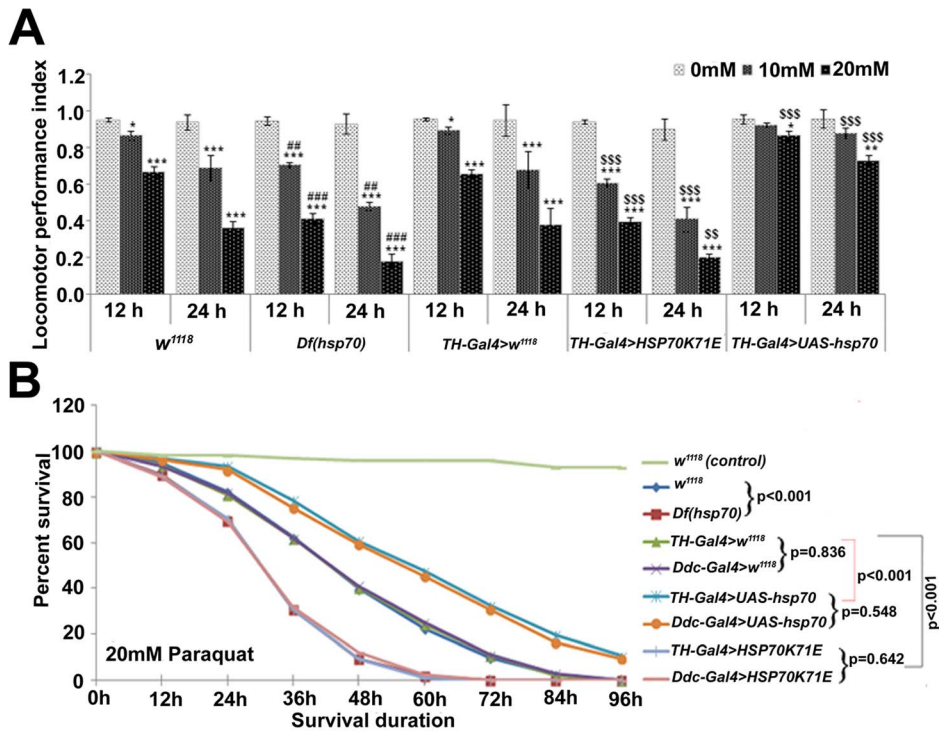
Figure 4. *hsp70* over-expression protects PQ-exposed flies from impaired locomotor performance and poor survival. (A) Graphical representation of the negative geotaxis assay performed in PQ-exposed *w1118*, *Df(hsp70)*, *TH-Gal4>w1118*, *TH-Gal4>HSP70K71E* and *TH-Gal4>UAS-hsp70* flies after their exposure to PQ. (B) Survival curve representing pooled data of five independent replicate experiments. Values are mean ± SD (n = 5). Significance ascribed as *p<0.05, **p<0.01 and ***p<0.001 vs. control; $$p<0.01 and $$$p<0.001 vs. *TH-Gal4>w1118*; ##p<0.01 and ###p< 0.001 vs. *w1118*.
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Statistical analysis

Analysis of all data was performed in a Prism software (GraphPad version 5.0, San Diego, CA, USA) using two-way analysis of variance (ANOVA) followed by Bonferani's test for multiple comparisons. After ascertaining the homogeneity of variance and normality of data, $p<0.05$ was considered as statistically significant. Pearson's correlation was calculated and then linear regression analysis was carried out. Using Kaplan–Meier's analysis with stratified log rank test by SPSS software version 13.0 (SPSS Inc., Chicago, USA), survival was assessed.

Results

Recovery of PQ from the exposed Drosophila

We detected ~2.68–2.98 μM PQ in the head of flies exposed to 20 mM PQ for 24 h. However, in the head of control flies, PQ level was beyond the limit of detection (Table 2). An insignificant difference was observed in the amount of PQ recovered from each genotype at both the exposure concentrations (10 and 20 mM) (Table 2).

hsp70 over-expression diminished PQ-induced OS along with JNK and caspase-3 activation

Prior to experiments, stocks were validated by RT-PCR using gene specific primers (Figure S1). We observed significantly less (~1.8 fold) generation of O_2^- in the brain of PQ-exposed flies (20 mM for 24 h) that over-expressed hsp70 in their dopaminergic neurons (*TH-Gal4>UAS-hsp70*) as compared to similarly exposed *TH-Gal4>w1118* flies (Figure 1A). Conversely, similar exposure to Df(Hsp70) and TH-Gal4>HSP70K71E flies resulted

in ~5.7 and ~5.3 fold increase in O_2^- generation respectively as compared to respective controls (Figure 1A). Further, significantly decreased SOD activity was observed in the brain of 20 mM PQ-exposed *w1118*, Df(Hsp70), TH-Gal4>HSP70K71E and TH-Gal4>w1118 flies for 24 h, while similarly exposed *TH-Gal4>UAS-hsp70* flies showed significantly increased SOD activity (Figure 1B). Concomitant with increased SOD activity and less generation of O_2^- in 20 mM PQ-exposed TH-Gal4>UAS-hsp70 flies, significantly lower $ONOO^-$ generation (~1.3 fold) was observed in their brain as compared to respective TH-Gal4>w1118 (Figure 1C). However, a time- and concentration-dependent increase in $ONOO^-$ generation was observed in the brain of PQ-exposed w1118, Df(Hsp70), TH-Gal4>HSP70K71E and TH-Gal4>w1118 flies in comparison to respective controls (Figure 1C). We also observed significantly lower (~0.7 fold) MDA content in exposed TH-Gal4>UAS-hsp70 flies as compared to TH-Gal4> w1118 flies (Figure 1D). Conversely, significantly higher MDA content was observed in the brain of PQ-exposed w1118, Df(Hsp70) TH-Gal4>HSP70K71E and TH-Gal4>w1118 flies in comparison to their respective controls (Figure 1D).

Along with reduced OS in exposed TH-Gal4>UAS-hsp70 flies, the levels of p-JNK and cleaved caspase-3 were found to be significantly lower in the brain of these organisms as compared to respective TH-Gal4>w1118 (Figure 1G–H). A similar trend was also observed for DEVDase activity (Figure 2). Unlike the above, we observed significantly higher levels of p-JNK and cleaved caspase-3 along with increased DEVDase activity in the brain of PQ-exposed w1118, Df(Hsp70) and TH-Gal4>HSP70K71E flies (Figure 1E–H; Figure 2) in comparison to respective controls.
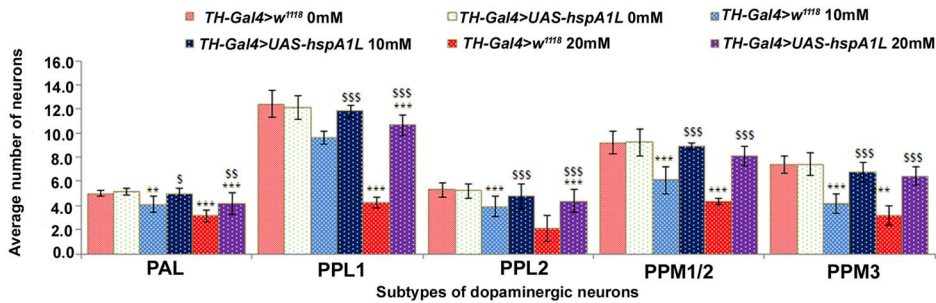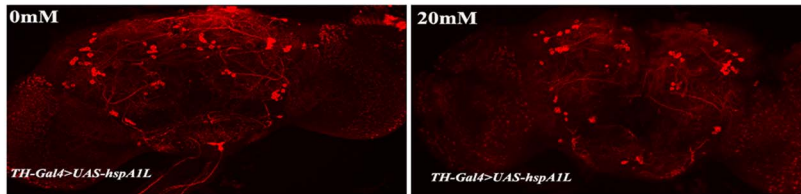
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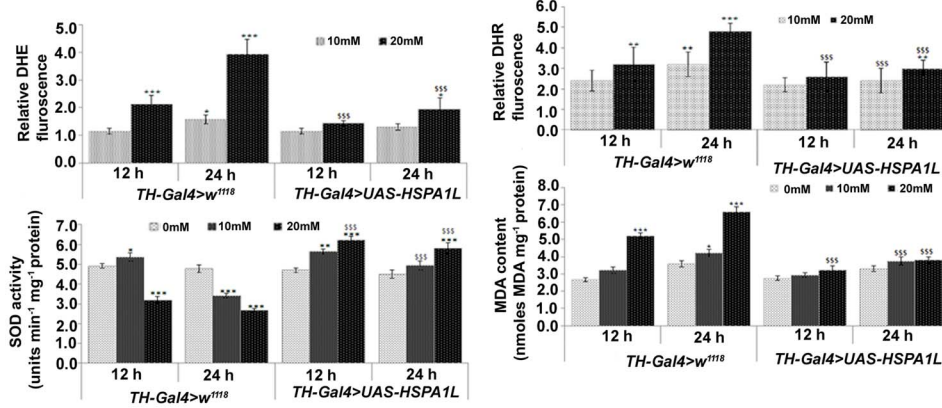
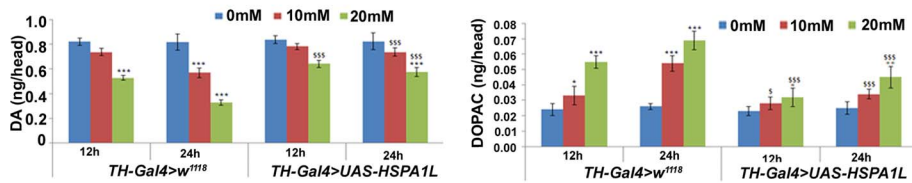## Activation of JNK and caspase-3
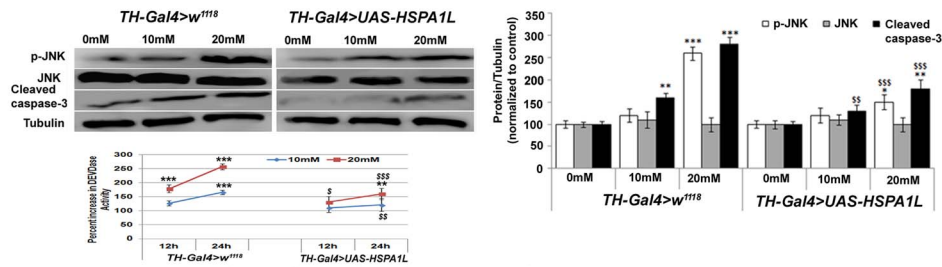
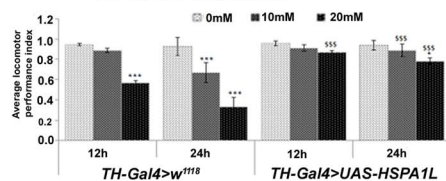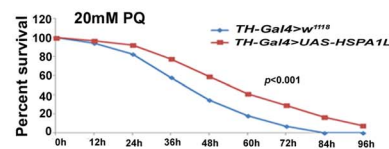## Imaging and counting of dopaminergic

Level of dopamine (DA) and DOPAC

Locomotor behaviour

Resistance behaviour

Figure 5. Over-expression of a human homologue of *hsp70* in the dopaminergic neurons of *Drosophila* provided them resistance against PQ-induced PD like symptoms. Like PQ-exposed *TH-Gal4>UAS-hsp70* flies, exposed *TH-Gal4>UAS-HSPA1L* flies also exhibited reduced levels of OS as evidenced by less O_2^- generation, improved SOD activity, less formation of $ONOO^-$ and non-significant increase in MDA content. JNK phosphorylation and cleaved caspase-3 level was significantly less in the brain of PQ-exposed *TH-Gal4>UAS-HSPA1L* flies in comparison to Gal4 control, as represented by immune-blotting images and densitometry analysis. Dopaminergic neuronal health depicted by representative confocal image of anti-DTH stained brain of control and PQ-exposed *TH-Gal4>UAS-HSPA1L* flies and graphical representation of dopaminergic neurons in different clusters (n = 22). In the brain of PQ-exposed *TH-Gal4>UAS-HSPA1L* flies, less decline in DA and lower level of DOPAC was observed. Improved locomotor performance and survival advantage were also observed in these flies. Values are mean ± SD (n = 3). Significance ascribed as *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs. control; $p<0.05$, $^{$$}p<0.01$ and $^{$$$}p<0.001$ vs. *TH-Gal4>w^{1118}*.
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hsp70 over-expression protects dopaminergic neuronal degeneration

Concurrent with lower OS and less JNK activation observed in the brain of PQ-exposed *hsp70* over-expressing flies, we also observed significantly less neurodegeneration in these organisms (Figure 3A; 3D–E). However, an extensive loss of dopaminergic neurons occurred in PQ-exposed *Df(Hsp70)* and *TH-Gal4>HSP70K71E* flies (Figure 3A–E) as compared to respective *w^{1118}* and *TH-Gal4>w^{1118}* flies. In addition, we observed a non-significant difference in the dopaminergic neuronal degeneration among PQ-exposed Gal4- or UAS- alone flies (Figure S2). After analysis, PPL1 cluster was found to be the most sensitive cluster as compared to others, which is parallel to a previous report [6]. In this context, *w^{1118}* flies that were exposed to 20 mM PQ for 24 h, exhibited ~61% neuronal degeneration in the PPL1 cluster while in similarly exposed *Df(hsp70)* and *TH-Gal4>HSP70K71E* flies, neurodegeneration was ~77 and ~83% respectively (Figure 3C; 3E). In PQ-exposed *TH-Gal4>UAS-hsp70* flies, only ~22% neurodegeneration was observed in their PPL1 cluster (Figure 3E). The observed protection of dopaminergic neurons in PQ-exposed *hsp70* over-expressing flies was further supported by significantly less decline in DA and lowered DOPAC levels as against respective *TH-Gal4>w^{1118}* flies (Figure 3F–G). However, flies with a *hsp70* deficiency [*Df(hsp70)*] or diminished Hsp70 level [*TH-Gal4>HSP70K71E*] showed further loss of DA and higher DOPAC level as compared to similarly exposed *w^{1118}* and *TH-Gal4>w^{1118}* flies (Figure 3F–G).

Improved locomotor performance and survival benefit in PQ-exposed *hsp70* over-expressing *Drosophila*

To examine the behavioral response as a measure of PQ-induced PD like symptoms in exposed Drosophila, we monitored locomotor performance of flies that were exposed to PQ. Nearly 64% of PQ-exposed w^{1118} flies (20 mM PQ for 24 h) failed to cross the 15 cm mark within 30 sec while ~85% of exposed Df(hsp70) flies met the same fate (Figure 4A). The latter group of flies even failed to cross the stipulated height after 90 sec. Conversely, improved locomotor behavior was observed in exposed hsp70 over-expressing flies, where only ~31% of them failed to cross the above mark in stipulated time.

The benefit of over-expressing hsp70 in the dopaminergic neurons in exposed organism could be weighed by the survival advantage gained by these flies (~33 and ~11% fly survived after 72 and 96 h of 20 mM PQ exposure respectively) as against respective TH-Gal4>w^{1118} flies (only ~11% survived after 72 h exposure period while none after 96 h) (Figure 4B).

Targeted expression of *HSPA1L* in dopaminergic neurons protects the organism from PQ-induced PD like symptoms

To examine whether over-expression of a human homologue of hsp70 (HSPA1L) in the dopaminergic neurons of PQ-exposed flies can resist PD like symptoms in a way similar to that observed in Drosophila hsp70 over-expressing strain, we investigated the above endpoints. PQ-exposed TH-Gal4>UAS-HSPA1L flies exhibited significantly less generation of reactive species (O_2^-, $ONOO^-$), increased SOD activity, improved dopaminergic neuronal health and locomotor performance, less activation of apoptotic signalling along with their improved survival in comparison to similarly exposed TH-Gal4>w^{1118} (Figure 5).

Discussion

Considering the anti-apoptotic function of Hsp70 and association of PQ exposure with PD, the results of the present study provide evidences for the first time on the protective role of Hsp70 against PQ-induced PD like symptoms in exposed Drosophila. The protective mechanism of Hsp70 against PQ-induced neuro-degeneration was observed in terms of reduced OS, stalled activation of JNK and caspase-3 mediated dopaminergic neuronal apoptosis in exposed organism.

A non-significant difference in the amount of PQ recovered from different genotypes of exposed Drosophila suggests a nearly equivalent feeding pattern of all tested strains. The amount of PQ recovered supports the underlying mechanism of poor absorption and localization of the PQ in Drosophila brain similar to mammalian models [43,44].

The adverse effect of PQ is reported to be associated with free radical generation [11] especially superoxide radical (O_2^-), which is dismutated to hydrogen peroxide (H_2O_2) by an anti-oxidant defence enzyme, SOD, in the cellular system [45]. Since an

Table 3. Correlation among ROS generation and % survival in PQ-exposed *Drosophila*.

	% survival	O_2^-	$ONOO^-$
% survival	1		
O_2^-	−0.99681**	1	
$ONOO^-$	−0.99378**	0.985479**	1

Negative correlation was observed between generation of ROS ($O_2^-/ONOO^-$) and percent survival in different strains of *Drosophila* exposed to 20 mM PQ for 24 h. n = 4 degree of freedom; **$p<0.01$.
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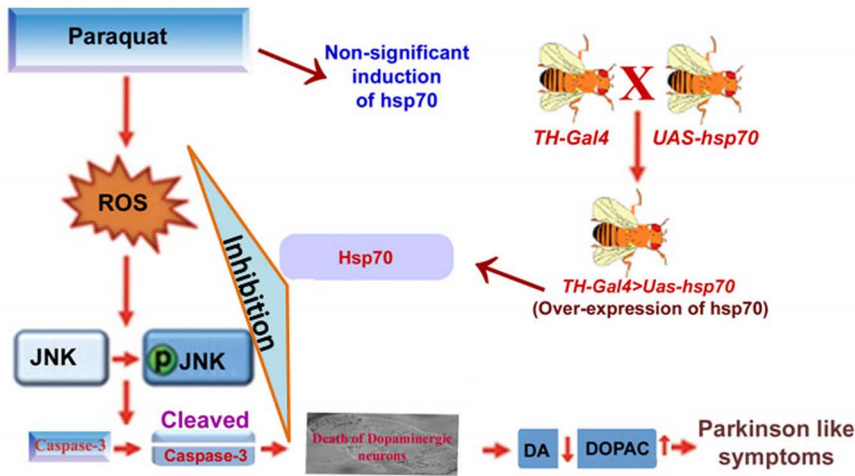
Figure 6. Schematic representation of the neuro-protective role of Hsp70 against PQ-induced PD like symptoms. PQ-induced ROS generation led to the activation of JNK and caspase-3, which eventually caused death of the dopaminergic neurons and induces PD like symptoms in the exposed organism. The observed PD like symptoms was resisted by over-expression of one of the key chaperons, Hsp70, in the dopaminergic neurons of *Drosophila*. Hsp70 inhibits PQ-induced activation of JNK and caspase-3 mediated neuronal cell death, thereby providing protection to the organism.
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imbalance between reactive oxygen species (ROS) generation and anti-oxidant defence leads to OS, decreased SOD activity and increased O_2^- generation can enhance OS in PQ-exposed organism. A negative co-relation drawn between hsp70 expression and O_2^- generation while a positive co-relation drawn between SOD activity and hsp70 expression indicates the possible protective role of Hsp70 against PQ-induced OS. Interestingly, we observed decreased SOD activity in the brain of PQ-exposed w^{1118}, Df(hsp70) and TH-Gal4>HSP70K71E flies along with generation of higher level of O_2^-. Since O_2^- is a substrate of SOD, higher level of O_2^- may lead to increased SOD activity in w^{1118}, Df(hsp70) and TH-Gal4>HSP70K71E flies. Instead, decreased SOD activity was observed in these organisms which is explained in the following: The reaction between O_2^- and nitric oxide (NO) generates peroxynitrite ($ONOO^-$) anion in a cell causing nitration of SOD which can diminish its activity [36,46]. Further, reciprocal relation observed between $ONOO^-$ generation and SOD activity in the brain of PQ-exposed organism provides a plausible explanation for the decreased SOD activity in these organisms. Since LPO is considered as one of the mechanisms of PQ toxicity [47], decreased LPO in the brain of PQ-exposed hsp70 over-expressing flies suggests the protective role of Hsp70 against PQ-induced OS. Our observation finds support from an earlier study wherein lifelong over-expression of hsp70 in the skeletal muscle of mice provided them protection against age-related OS through the proper functioning of anti-oxidant system [48].

PQ-induced OS has been shown to be linked with neuronal cell death by JNK phosphorylation and caspase-3 activation [7] which was supported by our observations in PQ-exposed w^{1118} and Df(hsp70) flies. However, diminished JNK phosphorylation and caspase-3 activation observed in PQ-exposed hsp70 over-expressing flies is in agreement with the anti-apoptotic role of Hsp70 [49,50]. Since JNK activation and apoptosis are considered as one of the principal mechanisms of PQ-induced neurodegeneration [7], blockage of these events by hsp70 over-expression suggests better survival of neuronal cells against PQ exposure. Given that hsp70 was found to be non-significantly induced in the brain of PQ-exposed w^{1118} flies (Figure S3), over-expression of hsp70 in the

brain of exposed organism is likely to counteract PQ-induced cell death by virtue of its anti-apoptotic and anti-oxidative properties.

The observed protection achieved in hsp70 over-expressing flies against OS and JNK activation was confirmed by improved dopaminergic neuronal health in these organisms. Dopaminergic neuronal cell death has been considered as the pathological hallmark of PD [51] which is manifested by a decline in DA and an elevation of DOPAC level. In this context, increased $ONOO^-$ generation in exposed organism may cause nitration of TH, a key enzyme in DA synthesis [52] which may inactivate it suggesting a negative role of the anion on enzymatic activity of TH. In addition, increased $ONOO^-$ generation was in parallel to depleted DA content along with degeneration of dopaminergic neurons in PQ-exposed w^{1118}, Df(hsp70) and TH-Gal4> HSP70K71E flies. Reversal of the above findings in hsp70 over-expressing flies established a neuro-protective role of Hsp70 against PQ insult in exposed organism.

Less degeneration of dopaminergic neurons and better locomotor performance in PQ-exposed hsp70 over-expressing flies observed in this study substantiate the earlier observed co-relation between dopaminergic neuronal degeneration and loco-motor performance in different models [6,53]. Thus, from both gain- as well as loss-of-function data, it is apparent that Hsp70 can rescue PQ-induced neurodegeneration by modulating OS induced activation of JNK and caspase-3 mediated cell death. The observed protection was further substantiated by improved survival of exposed hsp70 over-expressing organism. Two Gal4 lines (Ddc and TH) were used for manipulation of gene expression in dopaminergic neurons of Drosophila [54,55]. We observed almost similar trend in the survival of PQ-exposed hsp70 over-expressing flies (TH-Gal4>UAS-hsp70 and Ddc-Gal4>UAS-hsp70) as compared to respective Gal4 control and w^{1118} flies suggesting Gal4 independent effect of Hsp70 (Figure 4B). Our observation finds support from a previous study by Azad et al. [56], wherein survival advantage against hypoxia was demonstrated by over-expressing hsp70 in the brain (c739-Gal4) and lymph gland (hand-Gal4) of flies. In addition, less generation of free radicals in the brain of PQ-exposed hsp70 over-expressing flies and a negative co-relation drawn between free radical

generation and survival (Table 3) suggest that improved survival of these organisms was due to lower generation of reactive species. This is a consistent observation which is supported by an earlier report, wherein survival of the organism was shown to be affected by free radicals [57].

The study assumes significance in context of human relevance since a comparable protection against PD like symptoms in PQ-exposed organism was observed using both Drosophila hsp70 and its human homologue (HSPA1L) suggesting the therapeutic potential of Hsp70 in neurodegenerative disorders caused by environmental chemicals.

Conclusions

The study demonstrated that hsp70 over-expression (both Drosophila and its human homologue) in the dopaminergic neurons of Drosophila protects the organisms from PQ-induced PD like symptoms as evidenced by improved dopaminergic neuronal health, better locomotor performance and improved survival. PQ-induced adversities, mainly governed by the generation of reactive species (O_2^- and $ONOO^-$), was found to be responsible for the phosphorylation of JNK and activation of caspase-3 mediated dopaminergic neuronal cell death in exposed organism (Figure 6). In conclusion, we have presented the possible mechanism of protection against PQ-induced PD like symptoms in exposed organism using a key molecular chaperone, Hsp70. Further, the study recommends the use of Drosophila as an in vivo model for the evaluation of potential therapeutic targets against environmental chemicals induced disease like condition with minimum ethical concern.

Supporting Information

Figure S1 **hsp70 expression in the brain of Drosophila.** For fly stock validation genotypically, total RNA was isolated from the brain of five-day-old flies using TRI reagent (Ambion, Austin, TX, USA), chloroform, isopropanol and ethanol following the manufacturer's instructions. The isolated RNA was used for cDNA synthesis using Revert Aid H Minus first strand cDNA synthesis kit (Fermentas MD, USA) according to the manufacturer's protocol. Each reaction mixture consisted of total RNA, 0.5 μg/μl oligo (dT)$_{18}$ primer, 5x reaction buffer, 20 U Ribolock ribonuclease inhibitor, 10.0 mM dNTP mixture, 200 U Revert Aid H Moloney Murine Leukemia Virus reverse transcriptase (M-MuLV RT) and DEPC water to make a final volume of 20 μl. The cDNA was amplified by PCR on a thermocycler (Eppendorf, Hamburg, Germany) using gene specific primers (Table S1). PCR reaction mixture (total 25 μl) consisted of 1X Taq buffer, 1.5 mM MgCl$_2$, 0.20 mM dNTPs mixture, 0.40 μM each of forward and reverse primer, 1U Taq DNA polymerase (Fermentas Life Sciences, MD, USA), 2 μl cDNA and milli-Q water. The amplicons were separated on an 1.5% agarose gel containing ethidium bromide at 5 V/cm and visualized with a VERSA DOC

Imaging System Model 1000 (Bio-Rad, CA, USA). The intensity of the band was quantified by Quantity One software (Bio-Rad, CA, USA). Each experiment was carried out thrice with three independent biological replicates. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Representative agarose gel picture showing the expression of hsp70 (A). Lanes 1-7 (L-R): Marker; no template control (NTC); w^{1118}; Df(hsp70); TH-Gal4>w^{1118}; TH-Gal4>UAS-hsp70 and TH-Gal4> HSP70K71E. (B) Agarose gel picture showing expression of HSPA1L. Lanes 1-4 (L-R): Marker; NTC; TH-Gal4>w^{1118} and TH-Gal4>UAS-HSPA1L.
(TIF)

Figure S2 A non-significant difference in the dopaminergic neuronal degeneration between Gal4 and UAS construct bearing flies that were exposed to PQ. Confocal images and neuronal cell counts in the PPL1 cluster of Drosophila exposed to 20 mM PQ for 24 h, as revealed by anti-DTH staining. Magnification: 400X.
(TIF)

Figure S3 **PQ exposure to w^{1118} flies resulted in non-significant induction of hsp70 in their brain.** Hsp70 level as revealed by immune-blotting of protein samples prepared from the brain tissues of PQ-exposed Drosophila. For positive control (PC), flies were given temperature shock (37°C) for 1 h in a moistened glass vial. Flies were then allowed to recover at 25°C for 30 min before the sample preparation. A non-significant difference ($p > 0.05$) in the level of Hsp70 was observed in the flies that were exposed to 20 mM PQ for 24 h as compared to unexposed flies.
(TIF)

Table S1 **Genes and their primer sequences used for PCR amplification.**
(DOC)

Acknowledgments

Author Contributions

Conceived and designed the experiments: AKS AKT MZA DKC. Performed the experiments: AKS PP HSC DKC. Analyzed the data: AKS PP DKP DKC. Contributed reagents/materials/analysis tools: AKS DKC. Wrote the paper: AKS DKC.

References

1. Horner PJ, Gage FH (2000) Regenerating the damaged central nervous system. Nature 407: 963–970.
2. Brown RC, Lockwood AH, Sonawane BR (2005) Neurodegenerative diseases: an overview of environmental risk factors. Environ Health Perspect 113: 1250–1256.
3. Dorsey ER, Constantinescu R, Thompson JP, Biglan KM, Holloway RG, et al. (2007) Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. Neurology 68: 384–386.
4. Forno LS (1996) Neuropathology of Parkinson's disease. J Neuropathol Exp Neurol 55: 259–272.
5. Marsden CD (1990) Parkinson's disease. Lancet 335: 948–952.
6. Chaudhuri A, Bowling K, Funderburk C, Lawal H, Inamdar A, et al. (2007) Interaction of genetic and environmental factors in a Drosophila parkinsonism model. J Neurosci 27: 2457–2467.
7. Peng J, Mao XO, Stevenson FF, Hsu M, Andersen JK (2004) The herbicide paraquat induces dopaminergic nigral apoptosis through sustained activation of the JNK pathway. J Biol Chem 279: 32626–32632.
8. Tanner CM, Kamel F, Ross GW, Hoppin JA, Goldman SM, et al. (2012) Rotenone, paraquat, and Parkinson's disease. Environ Health Perspect 119: 866–872.
9. Berry C, La Vecchia C, Nicotera P (2010) Paraquat and Parkinson's disease. Cell Death Differ 17: 1115–1125.

10. Peng J, Stevenson FF, Doctrow SR, Andersen JK (2005) Superoxide dismutase/catalase mimetics are neuroprotective against selective paraquat-mediated dopaminergic neuron death in the substantial nigra: implications for Parkinson disease. J Biol Chem 280: 29194–29198.

11. Somayajulu-Nitu M, Sandhu JK, Cohen J, Sikorska M, Sridhar TS, et al. (2009) Paraquat induces oxidative stress, neuronal loss in substantia nigra region and parkinsonism in adult rats: neuroprotection and amelioration of symptoms by water-soluble formulation of coenzyme Q10. BMC Neurosci 10: 88.

12. Witt SN (2009) Hsp70 molecular chaperones and Parkinson's disease. Biopolymers 93: 218–228.

13. Kalmar B, Greensmith L (2009) Induction of heat shock proteins for protection against oxidative stress. Adv Drug Deliv Rev 61: 310–318.

14. Chen S, Brown IR (2007) Translocation of constitutively expressed heat shock protein Hsc70 to synapse-enriched areas of the cerebral cortex after hyperthermic stress. J Neurosci Res 85: 402–409.

15. Gupta SC, Siddhu HR, Saxena DK, Chowdhuri DK (2005) Hazardous effect of organophosphate compound, dichlorvos in transgenic Drosophila melanogaster (hsp70-lacZ): induction of hsp70, anti-oxidant enzymes and inhibition of acetylcholinesterase. Biochim Biophys Acta 1725: 81–92.

16. Mukhopadhyay I, Saxena DK, Chowdhuri DK (2003) Hazardous effects of effluent from the chrome plating industry: 70 kDa heat shock protein expression as a marker of cellular damage in transgenic Drosophila melanogaster (hsp70-lacZ). Environ Health Perspect 111: 1926–1932.

17. Mosser DD, Caron AW, Bourget L, Denis-Larose C, Massie B (1997) Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis. Mol Cell Biol 17: 5317–5327.

18. Ravagnan L, Gurbuxani S, Susin SA, Maisse C, Daugas E, et al. (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. Nat Cell Biol 3: 839–843.

19. Auluck PK, Chan HY, Trojanowski JQ, Lee VM, Bonini NM (2002) Chaperone suppression of alpha-synuclein toxicity in a Drosophila model for Parkinson's disease. Science 295: 865–868.

20. Flower TR, Chesnokova LS, Froelich CA, Dixon C, Witt SN (2005) Heat shock prevents alpha-synuclein-induced apoptosis in a yeast model of Parkinson's disease. J Mol Biol 351: 1081–1100.

21. Shen HY, He JC, Wang Y, Huang QY, Chen JF (2005) Geldanamycin induces heat shock protein 70 and protects against MPTP-induced dopaminergic neurotoxicity in mice. J Biol Chem 280: 39962–39969.

22. Cleren C, Calingasan NY, Chen J, Beal MF (2005) Celastrol protects against MPTP- and 3-nitropropionic acid-induced neurotoxicity. J Neurochem 94: 995–1004.

23. Pan T, Li X, Xie W, Jankovic J, Le W (2005) Valproic acid-mediated Hsp70 induction and anti-apoptotic neuroprotection in SH-SY5Y cells. FEBS Lett 579: 6716–6720.

24. Samuni Y, Ishii H, Hyodo F, Samuni U, Krishna MC, et al. (2010) Reactive oxygen species mediate hepatotoxicity induced by the Hsp90 inhibitor geldanamycin and its analogs. Free Radic Biol Med 48: 1559–1563.

25. Donaire V, Niso M, Moran JM, Garcia L, Gonzalez-Polo RA, et al. (2005) Heat shock proteins protect both MPP(+) and paraquat neurotoxicity. Brain Res Bull 67: 509–514.

26. Chan HY, Warrick JM, Gray-Board GL, Paulson HL, Bonini NM (2000) Mechanisms of chaperone suppression of polyglutamine disease: selectivity, synergy and modulation of protein solubility in Drosophila. Hum Mol Genet 9: 2811–2820.

27. Duffy JB (2002) GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis 34: 1–15.

28. Li LB, Xu K, Bonini NM (2007) Suppression of polyglutamine toxicity by the yeast Sup35 prion domain in Drosophila. J Biol Chem 282: 37694–37701.

29. Warrick JM, Morabito LM, Bilen J, Gordesky-Gold B, Faust LZ, et al. (2005) Ataxin-3 suppresses polyglutamine neurodegeneration in Drosophila by a ubiquitin-associated mechanism. Mol Cell 18: 37–48.

30. Singh MP, Ram KR, Mishra M, Shrivastava M, Saxena DK, et al. (2010) Effects of co-exposure of benzene, toluene and xylene to Drosophila melanogaster: alteration in hsp70, hsp60, hsp83, hsp26, ROS generation and oxidative stress markers. Chemosphere 79: 577–587.

31. Girardot F, Monnier V, Tricoire H (2004) Genome wide analysis of common and specific stress responses in adult drosophila melanogaster. BMC Genomics 5: 74.

32. Corasaniti MT, Nistico G (1993) Determination of paraquat in rat brain by high-performance liquid chromatography. J Chromatogr 643: 419–425.

33. Yang L, Beal MF (2011) Determination of neurotransmitter levels in models of Parkinson's disease by HPLC-ECD. Methods in Molecular Biology: Clifton, NJ. 793. pp. 401–415.

34. Jordan J, Galindo MF, Tornero D, Benavides A, Gonzalez C, et al. (2002) Superoxide anions mediate veratridine-induced cytochrome c release and caspase activity in bovine chromaffin cells. Br J Pharmacol 137: 993–1000.

35. Nishikimi M, Appaji N, Yagi K (1972) The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun 46: 849–854.

36. Lim SY, Jang JH, Na HK, Lu SC, Rahman I, et al. (2004) 15-Deoxy-Delta12,14-prostaglandin J(2) protects against nitrosative PC12 cell death through up-regulation of intracellular glutathione synthesis. J Biol Chem 279: 46263–46270.

37. Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95: 351–358.

38. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.

39. Liu Z, Wang X, Yu Y, Li X, Wang T, et al. (2008) A Drosophila model for LRRK2-linked parkinsonism. Proc Natl Acad Sci U S A 105: 2693–2698.

40. Wang Z, Ferdousy F, Lawal H, Huang Z, Daigle JG, et al. (2011) Catecholamines up integrates dopamine synthesis and synaptic trafficking. J Neurochem 119: 1294–1305.

41. Coulom H, Birman S (2004) Chronic exposure to rotenone models sporadic Parkinson's disease in Drosophila melanogaster. J Neurosci 24: 10993–10998.

42. Du G, Liu X, Chen X, Song M, Yan Y, et al. (2010) Drosophila histone deacetylase 6 protects dopaminergic neurons against {alpha}-synuclein toxicity by promoting inclusion formation. Mol Biol Cell 21: 2128–2137.

43. Daniel JW, Gage JC (1966) Absorption and excretion of diquat and paraquat in rats. Br J Ind Med 23: 133–136.

44. Merritt TJS, Douglas L, Rzezniczaka TZ, Wattersonb JH (2011) Rapid and simple analysis of paraquat in tissue homogenate by ultra-high performance liquid chromatography. Anal Methods 3: 1428–1432.

45. Fridovich I (1997) Superoxide anion radical (O2-.), superoxide dismutases, and related matters. J Biol Chem 272: 18515–18517.

46. Demicheli V, Quijano C, Alvarez B, Radi R (2007) Inactivation and nitration of human superoxide dismutase (SOD) by fluxes of nitric oxide and superoxide. Free Radic Biol Med 42: 1359–1368.

47. Bus JS, Aust SD, Gibson JE (1976) Paraquat toxicity: proposed mechanism of action involving lipid peroxidation. Environ Health Perspect 16: 139–146.

48. Broome CS, Kayani AC, Palomero J, Dillmann WH, Mestril R, et al. (2006) Effect of lifelong overexpression of HSP70 in skeletal muscle on age-related oxidative stress and adaptation after nondamaging contractile activity. FASEB J 20: 1549–1551.

49. Gabai VL, Meriin AB, Yaglom JA, Volloch VZ, Sherman MY (1998) Role of Hsp70 in regulation of stress-kinase JNK: implications in apoptosis and aging. FEBS Lett 438: 1–4.

50. Lee JS, Lee JJ, Seo JS (2005) HSP70 deficiency results in activation of c-Jun N-terminal Kinase, extracellular signal-regulated kinase, and caspase-3 in hyperosmolarity-induced apoptosis. J Biol Chem 280: 6634–6641.

51. Fearnley JM, Lees AJ (1991) Ageing and Parkinson's disease: substantia nigra regional selectivity. Brain 114: 2283–2301.

52. Dinis-Oliveira RJ, Remiao F, Carmo H, Duarte JA, Navarro AS, et al. (2006) Paraquat exposure as an etiological factor of Parkinson's disease. Neurotoxicology 27: 1110–1122.

53. McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, et al. (2002) Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. Neurobiol Dis 10: 119–127.

54. Feany MB, Bender WW (2000) A Drosophila model of Parkinson's disease. Nature 404: 394–398.

55. Friggi-Grelin F, Coulom H, Meller M, Gomez D, Hirsh J, et al. (2003) Targeted gene expression in Drosophila dopaminergic cells using regulatory sequences from tyrosine hydroxylase. J Neurobiol 54: 618–627.

56. Azad P, Ryu J, Haddad GG (2011) Distinct Role Of Hsp70 In Drosophila Hemocytes During Severe Hypoxia. Free Radic Biol Med 51: 530–538.

57. Kregel KC, Zhang HJ (2007) An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. Am J Physiol Regul Integr Comp Physiol 292: R18–36.